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PULSE RADIOLYSIS OF PROTEINS AS A TOOL TO DETERMINE THE PK(A) OF CERTAIN HISTIDINES ON MODIFIED AND UNMODIFIED PROTEINS AND THE PULSE RADIOLYSIS OF DISULFIDES

The Ohio State University

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THE PKₐ OF CERTAIN HISTIDINES
ON MODIFIED AND UNMODIFIED PROTEINS AND
THE PULSE RADIOLYSIS OF DISULFIDES

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate School
of The Ohio State University

By
Jerald Paul Steiner

The Ohio State University
1983

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PUBLICATIONS


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LIST OF ABBREVIATIONS AND SYMBOLS

Abs  absorbance
BAEE  benzoylarginine ethyl ester
BTEE  benzoyl-L-tryosine ethyl ester
CO₂⁻  carbon dioxide radical
DMSO  dimethyl sulfoxide
DTNB  5,5'-dithiobis(2-nitrobenzoic acid)
DTPA  dithiopropionic acid
DTT  dithiothreitol
ε  extinction coefficient in M⁻¹ cm⁻¹
ε-aq  solvated electron in water
EDA  ethylenediamine
λ  wavelength in nm
λ_max  position of peak maximum in nm
Mₚ  molecular weight
NTCI  N-trans-cinnamoylimidazole
OD  optical density
•OH  hydroxyl radical
ox-DTT  oxidized dithiothreitol
PAPMA  p-aminophenyl mercuric acetate
PMSF  phenylmethanesulfonyl fluoride
PNPA  p-nitrophenyl acetate
RNase  ribonuclease A
RSSR⁻  disulfide radical anion
RSSRH  protonated disulfide radical
t-BuOH  tert-BuOH
THF  tetrahydrofuran
I. INTRODUCTION

In the study of mechanisms and intermediates in biochemical systems where reaction times are very short, two techniques are often used: relaxation methods and rapid mixing. Pulse radiolysis\(^1\) provides a means for monitoring fast reactions such as electron transport and free radical intermediates in photosyntheses and enzymatic oxidation of substrates. In addition, pulse radiolysis has been used to investigate the initial chemical effects of ionizing radiation on biological compounds and the relationship between protein function and protein structure.

Pulse radiolysis is the electron impact analogue of flash photolysis that allows the introduction of a short burst of high-energy electrons into the system under study. Following irradiation, free radicals, ions, and excited molecules are formed at yields depending on the system under study and with lifetimes varying from picoseconds to seconds. Most of the transients formed from the pulse radiolysis of biological systems have lifetimes in the microsecond to the millisecond time range.

Many detection techniques have been used successfully to study the short-lived transients formed by pulse radiolysis.
radiolysis these include spectrophotometry (vacuum ultraviolet, ultraviolet, visible, and infrared spectroscopy) as well as electron spin resonance and solution conductivity. Near-ultraviolet spectroscopy and visible spectroscopy were used exclusively to obtain the data presented in this dissertation. Under appropriate conditions, the identity of transient species formed by pulse radiolysis can be established by measuring the transient absorption spectra and the kinetics of the transients and comparing them to spectra and kinetics reported in the literature.\(^2\)

In pulse radiolysis of dilute solutions containing proteins, the energy is transferred to the solvent, namely, water. The energy transferred to water is distributed through chemical reactions to form the primary products:\(^1\text{-}^4\)

\[
\text{H}_2\text{O} \rightarrow e^{-}\text{aq}, \cdot\text{H}, \cdot\text{OH}, \text{H}_2, \text{H}_2\text{O}_2, \text{H}_3\text{O}^+ \quad (1)
\]

The first step in the formation of these primary radicals (\(e^{-}\text{aq}, \cdot\text{OH}, \text{and} \cdot\text{H}\)) involves the ionization of water.

\[
\text{H}_2\text{O} \rightarrow \text{H}_2\text{O}^+ + e^- \quad (2)
\]

During this process the electron becomes thermalized and hydrated, yielding the hydrated electron.

\[
e^- \rightarrow e^-_t \xrightarrow{\text{H}_2\text{O}} e^{-}\text{aq} \quad (3)
\]
The unstable water ion radical reacts with a water molecule to yield the hydroxyl radical and the hydronium ion.

\[ \text{H}_2\text{O}^+ + \text{H}_2\text{O} \rightarrow \text{H}_3\text{O}^+ + \cdot \text{OH} \quad (4) \]

The hydrogen atom results from many reactions; one is the reaction of the thermalized electron with the hydronium ion produced in reaction 4.5

\[ \text{e}^- \cdot + \text{H}_3\text{O}^+ \rightarrow \cdot \text{H} + \text{H}_2\text{O} \quad (5) \]

The primary products (and their yields) are the hydrated electron (e^-aq, G = 2.8), atomic hydrogen (\( \cdot \text{H}, G = 0.55 \)), the hydroxyl radical (\( \cdot \text{OH}, G = 2.9 \)), molecular hydrogen (\( \text{H}_2, G = 0.45 \)), hydrogen peroxide (\( \text{H}_2\text{O}_2, G = 0.75 \)), and hydronium ion (\( \text{H}_3\text{O}^+, G = 2.8 \)) where G is the number of atoms or molecules produced per 100 eV absorbed by the solution.1-4

While a mixture of reactive radicals is produced by the pulse, where e^-aq and \( \cdot \text{OH} \) predominate, one radical can be obtained in major abundance over the others by chemical interconversion and selective scavenging. The yield of the hydroxyl radical can be doubled (G = 5.5) and the hydroxyl radical can be made the major reactive radical by converting e^-aq to \( \cdot \text{OH} \) in water solutions saturated (22 mM) with \( \text{N}_2\text{O} \):

\[ \text{e}^-\text{aq} + \text{N}_2\text{O} + \text{H}_2\text{O} \quad 8.7 \times 10^9 \text{ M}^{-1} \text{ s}^{-1} \rightarrow \text{N}_2 + \cdot \text{OH} + \text{OH}^- \quad (6) \]
however, it is difficult to convert or scavenge atomic hydrogen. Consequently, $^{\cdot} \text{OH}$ accounts for 90% of all reactive radicals and $^{\cdot} \text{H}$ accounts for the remaining 10%.\textsuperscript{1-4}

The solvated electron can be made the major radical by selectively removing $^{\cdot} \text{OH}$ with tert-butyl alcohol, thus leaving $e^- \text{aq}$ ($G = 2.8$) as the predominant reducing species and some $^{\cdot} \text{H}$ ($G = 0.55$).

\[ ^{\cdot} \text{OH} + \text{HOC(CH}_3\text{)}_3 \rightarrow 10^9 \text{ M}^{-1} \text{s}^{-1} \rightarrow \text{H}_2\text{O} + \text{HOC(CH}_2\text{)}(\text{CH}_3)_2 \] (7)

The tert-butyl alcohol radical is less reactive than the $^{\cdot} \text{OH}$ and is effectively stable over the time range of most pulse radiolysis experiments.\textsuperscript{2}

Primary radicals can also be used to make secondary radicals. Irradiation of formate-containing solutions that have been saturated with CO\textsubscript{2} yields the CO\textsubscript{2}\textsuperscript{-} radical.\textsuperscript{4}

\[ e^- \text{aq} + \text{CO}_2 \rightarrow 7.6 \times 10^9 \text{ M}^{-1} \text{s}^{-1} \rightarrow \text{CO}_2^- \cdot \] (8)

\[ ^{\cdot} \text{OH} + \text{HCO}_2^- \rightarrow 2.5 \times 10^9 \text{ M}^{-1} \text{s}^{-1} \rightarrow \text{H}_2\text{O} + \text{CO}_2^- \cdot \] (9)

\[ ^{\cdot} \text{H} + \text{HCO}_2^- \rightarrow 4 \times 10^8 \text{ M}^{-1} \text{s}^{-1} \rightarrow \text{H}_2 + \text{CO}_2^- \cdot \] (10)

The CO\textsubscript{2}\textsuperscript{-} radical can also be obtained when CO\textsubscript{2} is replaced by N\textsubscript{2}O. In this case, within 1 $\mu$s after the pulse, all of the $e^- \text{aq}$ is converted to $^{\cdot} \text{OH}$ (equation 6), which in turn reacts with HCO\textsubscript{2}\textsuperscript{-} to form the CO\textsubscript{2}\textsuperscript{-} radical.
Ethanol quite easily scavenges for •OH:

\[ •\text{OH} + \text{CH}_3\text{CH}_2\text{OH} \rightarrow \text{H}_2\text{O} + \text{CH}_3\text{CHOH} \]  

(11)

but unlike the tert-butyl radical, the ethanol radical is more reactive and can be used as a secondary radical.

The solvated electron\(^1,2,4\) is a very potent reducing agent (with a redox potential of -2.91 V). It has a strong absorbance in the visible region (\(\varepsilon = 18,500 \text{ M}^{-1} \text{ cm}^{-1}, \lambda_{\text{max}} = 720 \text{ nm}\)), and consequently, many rate constants have been compiled for the reactivity of e\(^{-}\)aq with inorganic and organic compounds. The hydroxyl radical\(^1-4\) is a powerful oxidant (redox potential of ca. 3 V), which absorbs weakly in the ultraviolet and is therefore difficult to monitor directly. Nonetheless, as for solvated electron, many rate constants have been compiled for the reaction of •OH with numerous compounds. Further discussion concerning the reactivity of •OH as well as the reactivity of secondary radicals on proteins and prosthetic groups will not be presented since this is not relevant to this dissertation. Such material as well as additional material pertaining to the reactivity of e\(^{-}\)aq with enzymes can be found in some selective review articles: Klapper and Faraggi (1979), Bielski and Gebucki (1977), Adams and Wardman (1977), Shafferman and Stein (1975), and Adams et al. (1972).\(^2-4,6,7\)

The hydrated electron reacts readily with amino acids, their derivatives, and their analogues. It is most reactive
with the disulfide bridge, the sulfhydryl group, the histidyl group, the peptide bond carbonyl, and aromatic amino acid chains.

Cystine\textsuperscript{2-4} reacts with a high rate constant with e-aq, resulting in the disulfide radical anion, RSSR\textsuperscript{-}, which absorbs very strongly in the visible range:

\[
e^{-}\text{aq} + \text{RSSR} \overset{(2-30) \times 10^9 \text{ M}^{-1} \text{s}^{-1}}{\longrightarrow} \text{RSSR}^- \quad (12)
\]

(\(\epsilon = 9000 \text{ M}^{-1} \text{ cm}^{-1}\)) with a band centered at 410 nm.\textsuperscript{2} The high extinction coefficient, the band position, and the high reactivity of RSSR make the disulfide radical anion very easy to identify in enzymes (see Figure 1 for a pictorial representation). The extinction coefficient reported here for cystine and for other absorbing species is based on three assumptions. First, 100% of e-aq produced reacted with precursor to yield the absorbing species. Second, the amount of e-aq produced is known accurately. Finally, only one electron reacts with each precursor to yield the absorbing species. Usually these assumptions are correct.

The sulfhydryl\textsuperscript{2,3} group reacts with e-aq almost as rapidly as cystine under the same conditions; however, the proposed products have low extinction coefficients:

\[
e^{-}\text{aq} + \text{RSH} \overset{}{\longrightarrow} \text{R}^+ + \text{SH} \quad (13)
\]
(ε = 700 and 900 M$^{-1}$ cm$^{-1}$ for products of thioglycolic acid and thiolacetic acid, respectively), and therefore direct spectral evidence for reaction 13 in enzymes would be difficult to see. Nevertheless, the magnitude of the rate constant in reaction 10 makes this reaction important in proteins with free sulfhydryl groups.

The reductive deamination$^2,8$ seen with amino acids and small peptides may also occur in proteins and enzymes. On the basis of steady-state radiolysis of diglycine and N- (chloroacetyl)glycine, it has been proposed that the hydrated electron attaches initially to the carboxyl group, 1, which is followed by deamination.

$$\text{e}^{-}\text{aq} + \text{H}_3\text{NCHC-R'} \rightarrow (1-3) \times 10^8 \text{ M}^{-1} \text{ s}^{-1} + \text{R} \cdot \text{O}^-$$

$$\text{H}_3\text{NCHC-R'} \rightarrow \text{H}_3\text{NCH}_2\text{C-R'} \quad (14)$$

$$\text{H}_3\text{NCHC-R'} \rightarrow \text{NH}_3 + \text{CHC-R'} \quad (15)$$

Faraggi and Tal$^8$ reported that at low pH the deamination product (2) prevails, which has a $\lambda_{\max}$ near 430 nm and an $\varepsilon = 1100 \text{ M}^{-1} \text{ cm}^{-1}$. At high pH where the amino group is not protonated, a new species appears below 300 nm with an extinction coefficient of about 2000 M$^{-1}$ cm$^{-1}$. This is assumed to be the carbonyl electron adduct (1). They also reported that the second-order rate constant increased linearly with the chain length of polyglycine and
polyalanine and that the yield of the deaminated radical decreased with chain length and the $\lambda_{\text{max}}$ shifted toward the blue with increasing chain length. These observations suggest that deamination would be difficult to detect spectrally and the carbonyl electron adduct (1) may prevail in enzymes because of their size and complexity.

The yield of the deaminated radical is dependent upon the concentration of the oligopeptide, increasing with higher concentrations. Faraggi and Tal\(^8\) have proposed that the observed concentration dependence is consistent with dimerization of the oligopeptide and that the electron once attached can be transferred from one chain across to the other via the hydrogen bonds that hold them together. If correct, such a mechanism for electron transport has important implications with respect to the reaction of e\(^{aq}\) and enzymes.

The reactions of hydrated electrons with aromatic side chains of amino acids have been studied but not characterized as well as the reaction of the hydrated electron with disulfides or carbonyls. The hydrated electron reacts moderately rapidly (compared to disulfides and thiols under the same conditions) with tryptophan,\(^2\) yielding a transient that

\[
e^{-aq} + \text{Trp} \quad (2-5) \times 10^8 \text{ M}^{-1} \text{s}^{-1} \rightarrow \text{Trp}^- \quad (16)
\]
absorbs at 340 nm with an extinction coefficient of 4000 M\(^{-1}\) cm\(^{-1}\).

Tyrosine\(^2\) reacts with e\(^-\)aq at about the same rate constant but yields a transient at 355 nm with a lower extinction coefficient, 1500 M\(^{-1}\) cm\(^{-1}\).

\[
\text{e}^-\text{aq} + \begin{array}{c}
\begin{array}{c}
\text{OH} \\
\end{array}
\end{array} 
\begin{array}{c}
\begin{array}{c}
\text{R} \\
\end{array}
\end{array} 
\xrightarrow{3 \times 10^8 \text{ M}^{-1} \text{s}^{-1}} \text{Tyr}^-. \quad (17)
\]

Finally, phenylalanine\(^2\) reacts relatively slowly with the hydrated electron compared to the other two aromatic amino acids under the same conditions, yielding a transient that absorbs at 312 nm for benzene and phenylalanine with a reported\(^2\) extinction coefficient of 2500-3550 M\(^{-1}\) cm\(^{-1}\) and at 325 nm for N-acetylphenylalanine with a reported extinction coefficient of 1200 M\(^{-1}\) cm\(^{-1}\).

The positively charged imidazolium group reacts rapidly with the hydrated electron\(^2,9,10\)

\[
\text{e}^-\text{aq} + \begin{array}{c}
\begin{array}{c}
\text{R} \\
\end{array}
\end{array} \begin{array}{c}
\begin{array}{c}
\text{R} \text{H} \text{N} \text{N} \text{H} \\
\end{array}
\end{array} 
\xrightarrow{4 \times 10^9 \text{ M}^{-1} \text{s}^{-1}} \begin{array}{c}
\begin{array}{c}
\text{R} \\
\end{array}
\end{array} \begin{array}{c}
\begin{array}{c}
\text{R} \text{H} \text{N} \text{N} \text{H} \\
\end{array}
\end{array} \quad (19)
\]
The transient absorbs strongly with one band centered near 360 nm ($\epsilon = 2000 \text{ M}^{-1} \text{ cm}^{-1}$) and a second near 290 nm ($\epsilon \text{ ca. 5000 M}^{-1} \text{ cm}^{-1}$). The uncharged imidazole group reacts much more slowly than protonated imidazole with the hydrated electron under the same conditions, $k = 1.4 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$.

![Reaction Equation]

Because of this large reactivity difference, the yield of the electron-imidazole adduct is pH dependent, being greatest at low pH. This allows for determination of the imidazole pK$_a$ even in the presence of other potentially reactive sites: 6.8 for Gly-His, for example. The pH dependence of the 360-nm band has also been used to determine the pK$_a$s of histidine side chains in RNase A, $\alpha$-chymotrypsin, and trypsin. A single pK$_a$ of 5.9 was observed with RNase A,$^{10}$ which was very close to the pK$_a$ values of the two histidines located at the active site of the protein.$^{11}$ Estimated pK$_a$s of 4.3 and 4.2 were obtained for trypsin$^{10}$ and $\alpha$-chymotrypsin,$^{9,10}$ respectively. These values are close to the pK$_a$ obtained by NMR$^{12}$ and IR$^{13}$ for the active site on trypsin and other closely related serine proteases.$^{14}$ However, other NMR experiments$^{15,16}$ as well as repetition of past experiments$^{17,18}$ with serine proteases have shown that the pK$_a$ for the active site histidine is
closer to 7. It is not clear whether the $pK_a$ obtained by using pulse radiolysis reflects the actual $pK_a$ of the active site histidine or of another imidazole in the molecule.

What may be of greater significance is the observation that not all protein histidyl groups react with $e^{-aq}$ to form an observable 360-nm band. RNase A\textsuperscript{10} has four histidines, yet only one $pK_a$ was observed. Trypsin\textsuperscript{9} and $\alpha$-chymotrypsin\textsuperscript{10} have six and five histidines, respectively, yet no normal $pK_a$ was observed: only an imidazole with an apparent low $pK$. Lysozyme\textsuperscript{10} and $\alpha$-lactalbumin\textsuperscript{2} both contain histidine, but no 360-nm band was observed with either protein. The fact that the single histidine of lysozyme is known to be at the surface of the enzyme\textsuperscript{10} and the fact that no 360-nm band was observed suggest that accessibility to solvent cannot alone explain the apparent lack of reactivity. Finally and most importantly, no 360-nm band was observed with enzymatically "inactive" precursors of trypsin and chymotrypsin, namely, trypsinogen\textsuperscript{9} and chymotrypsinogen,\textsuperscript{10} even though the structures about the active site histidines differ only slightly between the zymogen and fully active enzyme. Clearly, not all imidazoles can form the electron adduct.

The primary intent of this dissertation was to show that the 360-nm band observed in serine proteases does indeed belong to the active site imidazole. This was done by chemically modifying groups at the active site that are
necessary for enzyme activity and comparing the pulse radiolysis spectra obtained after the solvated electron reacted with the native enzyme and the chemically modified enzyme.

Approximately one-fourth of this dissertation contains material concerning the effect of solvent on the spectra and kinetics of the product formed from the one-electron reduction of several disulfides.
Figure 1. Block representation relating the wavelength range in which the reduced species has been reported to absorb with the reported extinction coefficient.
II. EXPERIMENTAL

A general discussion of the technique of pulse radiolysis can be found elsewhere. The following section pertains to the experimental details relevant to this study.

The Linear Accelerator (Linac). A Varian V-7715A linear accelerator was used as the high-energy electron source. It could be operated in the energy range of 2-6 MeV for pulses that were 40-1400 ns in duration. In this study the Linac was routinely operated in the 3-4-MeV range with pulse lengths ranging from 100 to 500 ns in duration. Pulses yielded 1-3 rads or 1-22 µM in solvated electron depending on the pulse length and the cell used. A typical time profile of a 300-ns pulse is shown in Figure 2. The rise and decay time for the rectangular wave form pulses was approximately 10 ns and was independent of the pulse width.

Pulses that are below 100 ns in duration provide poor spectra since the extinction coefficients of the charged and uncharged radicals from the pulse radiolysis of proteins are small (700-4000 M⁻¹ cm⁻¹). Pulses that are longer than 500 ns in duration would necessitate the use of higher concentrations of enzyme to ensure that 80-100% of the e-aq reacts with a single protein molecule.
The electron source of the linear accelerator was a thoriated tungsten cathode, heated by electron bombardment from a tungsten filament. During bombardment, the electron cloud that formed about the cathode was shaped and steered into an acceleration cavity. Once in this cavity, the electrons were accelerated along the 1.5-m length of the cavity by using microwaves, focused by using a system of quadrapoles, and injected into the sample cell with a beam diameter of about 2.3 cm at the point of incidence. For a single pulse, the electron beam was directed at the sample
cell for various lengths of time through the use of electro-
static inflector plates that controlled the position of the
electron beam and therefore the length of time the electron
beam hit the sample cell.

The stability of the dose from pulse to pulse was
usually within 10% over the 1-2-h period required to take a
complete spectrum. Since there was no method available to
normalize the pulse to pulse variation in taking a spectrum,
the spectrum point reproducibility was dependent on the
reproducibility of the pulse for that day.

Further information on the electronic design and the
spatial dimensions of the linear accelerator can be found in
the "Varian Linear Accelerator Model V-7715/V-7716 Instruc-
tion Manual."

Optics and Detection. The optics used for the moni-
toring of the transient species are shown in Figure 3. The
analyzing light source used in the pulse radiolysis experi-
ments was an Osram high-pressure xenon arc lamp (type
XB0450). A Corning-type 0-53 filter with a cutoff point at
320 nm and a lamp shutter was used to prevent photolysis
occurring in the reaction cell. Mirrors were used to pass
the light through the cell twice, thus doubling the path
length and therefore doubling the sensitivity of the detec-
tion system to changes in concentration of the absorbing
species. The desired wavelength was selected by using a
Bausch and Lomb monochromator (33-86-07) that contained a
1200 grooves/mm grating, with a 7.4 nm/mm reciprocal dispersion, and had a range of 200-700 nm. The entrance and exit slits were usually set at 1.25 and 0.7 mm, respectively, giving a band-pass of 5.18 nm.

The optical absorption at the desired wavelength was monitored by using one of three photomultipliers. The first of these photomultipliers was the RCA-7200 "slow" detector. It had an S-19 spectral response with a 10-90% rise time of 80 ns. This rise time was essentially the rise time of the Nexus signal conditioning amplifier that was coupled to the photomultiplier. The 7200 detector was used when the half-life of the reaction being monitored was greater than 0.5 µs and less than 20 µs. The proper signal intensity was
achieved by varying the detector voltage (350-650 V) and/or varying the widths of the monochromator slits. Usually no more than 500 V was applied to the 7200 detector because larger voltages resulted in excessive noise.

The second photomultiplier used was the RCA-1P28 "fast" detector that had an S-5 spectral response and an approximate 10-90% rise time of less than 10 ns. To get a sufficient amount of current output from the detector, it was necessary to flash the xenon arc lamp. This was done by charging a capacitor and then discharging the capacitor into the lamp in synchrony with the electron pulse. A profile of the lamp flash is shown in Figure 4. The flashing of the lamp would increase the intensity of the light 25-40 times depending on the wavelength being monitored. The constant light intensity plateau of the flash was the limiting factor with this detector, making the longest total scan time that could be used about 20 μs or 2 μs/division maximum sweep time. Unlike the 7200 "slow" detector mentioned earlier, the 1P-28 "fast" detector input voltage was never changed from 1000 V. The proper signal intensity was usually achieved by varying the charging voltage to the capacitor that flashes the lamp and sometimes varying the width of the monochromator slits.

The third, and most used, detector was the "modified" 1P-28 "very slow" detector. Like the 1P-28 "fast" detector, the "modified" 1P-28 detector used an RCA-1P-28
Figure 4. Example of the lamp flash profile. Taken with the 7200 "slow" detector at 420 nm. The top portion of the lamp flash is essentially flat for 20 μs.

photomultiplier with an S-5 spectral response. Unlike the 1P-28 "fast" detector, the signal was not increased by flashing the lamp but was increased by increasing the terminating resistance before the signal current reached the data storage device.

In light of equation 21, flashing the lamp increases

\[ V = IR \]  \hspace{1cm} (21)  

the current (I) and therefore increases the signal voltage (V). The signal voltage (V) is what is stored as data. Since the flat portion of the lamp flash is only 50 μs long,
the signal voltage \((V)\) is increased by increasing the termination resistance \((R)\) in the "very slow" detection system and not by flashing the lamp. However, increasing the terminating resistance \((R)\) increases the time required for the voltage to change. Consequently, the "modified" 1P-28 detector cannot be used at any sweep rates faster than 1 \(\mu s/\text{division}\). Even at the lowest setting of 1 kohm, some "electronic" rounding of the trace is noticeable in the first 0.5 \(\mu s\) after the pulse (Figure 5). Increasing the resistance increases the "electronic" rounding of the trace.

Great care was taken in interpreting traces with sweep rates of 1-10 \(\mu s\). The pulse radiolysis of proteins produces transients with very long lifetimes that are often monitored with very slow sweep rates (100-500 \(\mu s/\text{division}\)); no "electronic" rounding is noticeable even at the maximum resistance setting of 10 kohm.

With the "very slow" detector, the proper signal intensity was achieved by varying the detector voltage (350-750 \(V\)) and by varying the end terminator resistance box (1, 2, 5, and 10 kohm). The entrance and exit slits were usually set at 1.25 and 0.7 mm, respectively. In all cases the detector signal output was recorded as voltage on a TEKtronix 7912 AD programmable digitizer. The digitizer replaced the old trace capturing system, an oscilloscope and Polaroid film.
Figure 5. Example of oscilloscope traces showing how the "very slow" detector responds with varying resistance. Traces were taken at 465 nm by using an air-saturated KSCN (5 mM) solution in the sample cell.

In a typical experiment where a spectrum was the desired product, a trace at a given wavelength was captured on the 7912 AD programmable digitizer. The captured trace was read into the 4051 TEKtronix microcomputer as 512 voltage values. Those voltage values were processed and stored on magnetic tape along with the appropriate constants such as digitizer horizontal and vertical score factors, dial wavelength, and the initial lamp intensity before the pulse \((I_0)\). The data were processed to reduce the amount of space they would ordinarily occupy on magnetic tape. The stored
trace can be recalled for kinetic analysis or for the construction of a spectrum.

After the trace was stored on tape, six OD values at that wavelength at six predetermined times were automatically converted to six absorbance values (Figure 6) and then plotted on a TEKtronix 4662 plotter as OD versus corrected wavelength. When the six absorbance values at six times at one wavelength are combined with six absorbance values at six times at other wavelengths, a time-dependent spectrum is produced (see Figure 7, for example). The advantage of a time-dependent spectrum is 2-fold. First, the construction of a spectrum from noisy oscilloscope traces is made easier since a spectrum constructed where time = T1 is often quite similar in shape to the spectrum constructed where time = T2. "Bad" points in the spectrum constructed where time = T1 would appear when the spectrum is compared to the spectrum constructed where time = T2. Second, two species with different $\lambda_{\text{max}}$ and widely different kinetics are often readily apparent in a time-dependent spectrum, and kinetics for the two respective species can subsequently be obtained. An extreme example is Figure 7, where A decays to form B.

The construction of the spectrum in real time has three advantages. First, a "bad" pulse that is difficult to spot on a trace can often be spotted after it has been plotted and then another trace taken. Second, unexpected features in a spectrum can be checked by taking additional traces
Figure 6. Example trace where six voltage values were taken at six times.

Figure 7. Example where two absorbing species can be seen in a time-dependent spectrum. Here B decays faster than A.
around the wavelength of interest. Without this real time spectra capability, the entire spectrum would have to be repeated to check the unexplained features. Finally, the volume of solution used to obtain a spectrum can be reduced since duplicate traces are not often necessary. Traces that result from "bad" pulses can be redone. In addition, "noisy" traces could be numerically smoothed by using a weighted or unweighted smoothing routine before the trace is used to construct a spectrum.

**Kinetic Analysis.** The type of kinetics and the kinetic rate constants were determined by linearizing the data according to a first- or second-order rate equation and comparing their respective fits. The equation used for first order is

\[
\ln (A - A_\infty) = \ln (A_0 - A_\infty) - kt
\]  

(22)

and for second order is

\[
\frac{1}{A - A_\infty} = kt + \frac{1}{A_0 - A_\infty}
\]

(23)

where \(A\) = absorbance at any time \(t\), \(A_0\) = absorbance at \(t = 0\), \(A_\infty\) = absorbance at \(t = \infty\) (any residual absorbance), and \(k\) = rate constant. The derivation of these simple rate equation can be found in any good introductory physical chemistry book. Routinely, both first- and second-order type plots (\(\ln (A - A_\infty)\) versus \(t\) and \(1/(A - A_\infty)\) versus \(t\) were
done on the same data to ensure that the data truly fit one order and not the other (Figure 8). To get second-order rate units, i.e., M\(^{-1}\) s\(^{-1}\), for the rate (k) obtained in the second-order type plot, the rate must be multiplied by the extinction coefficient and the path length (i.e., 3.33 \times 10^5 x 4 \text{ cm} \times 9200 \text{ M}^{-1} \text{ cm}^{-1}). For first-order plots the plotted rate (k) would also be the first-order rate constant since first-order rate constants have units of s\(^{-1}\) and no concentration terms.

When the kinetics appear to be complex, i.e., two first orders, two second orders, or one first and one second order, a nonlinear curve fitting routine was used. One fitting routine translated from a Fortran program called "GRIDSEARCH" uses "brute force" to obtain the best fit. The program varies one parameter (such as the rate constant) while holding the other parameters constant and compares the calculated data point to the actual data point. When a minimum (based on \(\chi^2\)) is obtained, the next parameter is varied while holding the others constant (including the last parameter) until a minimum is again reached. This process is repeated until a minimum change occurs between iterations. At that point when the best fit has been obtained, the parameters are displayed along with a plot of residuals (i.e., calculated data minus real data, quantity squared), a table of comparisons between calculated data and data, and a \(\chi^2\) for the fit.
Figure 8. Typical kinetic plot where the trace at the left has been converted to absorbance and plotted as first order (negative slope) and second order (positive slope). Comparing the fit and correlation coefficient of the two types of plots shows that the data reflect a second-order decay. The arrows on the trace on the left indicate the following: $I_0 = I_0$ level before the pulse = 0.777 V; $T_0$ = time zero; the data used for kinetic analysis start at $T_1$ and end at $T_2$; $R_0$ = residual that translates to absorbance at "infinite" time = 0.005748 absorbance unit.
The second fitting routine called "MARQUART" is far more elaborate and faster than "GRIDSEARCH" where not just one parameter but all the parameters are varied at once. The output from "MARQUART" is the same as the output from "GRIDSEARCH".

Cell and Solution Delivering System. The reaction cell system used for the titration of proteins is shown in Figure 9. Only two flow-through cells were used. One was a 1 cm path length stock cell from Precision Cell, Inc., No. 72, with dimensions 3 x 7 x 10 mm (0.21 mL path length volume) (Figure 10). The optical windows were composed of high-purity quartz (Quarasil) to reduce radiation bluing. The entire cell was enclosed in a protective copper housing that could withstand much physical abuse. A hole was drilled at a right angle to the light path to allow the high-energy electrons to enter the cell.

The second cell (Figure 10) had a 2-cm path length and was custom made by Thermal American Fused Quartz Co. Its optical windows were also composed of high-purity quartz. The cell dimensions were 3 x 5 x 20 mm and had a 0.3 mL path length volume.

The reservoir contained a rubber septum to allow addition of acid or base for pH adjustment. The pH electrode was held in a sleeve that was designed to hold a thermometer (Ace Glass Co.). The upper arm of the reservoir was attached to a glass syringe. The syringe held argon that was
used to push the solution into the cell. A second larger and unjacketed reservoir was used for larger solutions and for solutions not needing cooling or heating. A syringe was used in place of the reservoir when titration and/or cooling of the system was not necessary.

To keep unirradiated solution loss to a minimum, the Teflon tubing leading from the stopcock to the bottom of the cell was 0.05 mm in diameter. The "T" stopcock was the smallest available (Ace) and made of Teflon. The cell exit tubing was about 3 times larger in diameter to allow bubbles to escape and to make it easier to see when the cell was filled.

The "Y" stopcock that was connected between the argon pressure source and the cell exit tubing served to police the back-pressure to the cell. To empty the cell, the "Y" stopcock was positioned to give pressure to the cell. The Teflon stopcock was positioned to allow the irradiated solution to pass out of the cell, past the reservoir, and down the waste tubing into a beaker. To fill the cell, the "Y" stopcock was positioned so that the cell exit tubing was opened to the air. The Teflon stopcock was positioned so that when pressure was applied to the syringe, solution would flow from the reservoir to the cell. Then both stopcocks were closed. Passing the solution in one direction through the cell was also possible with this system but was impractical for titration work. In addition, this
Figure 9. Diagram of the reaction cell system used for the titration of proteins.

Figure 10. Diagram of the two flow-through cells used in pulse radiolysis experiments. The figure at the left is a representation of the copper housing used to enclose the small 1 cm path length cell shown in the center. The representation at the right is of the 2-cm cell.
back-flushing method ensures that the irradiated solution was completely removed before the fresh solution entered the cell.

Dosimetry. Dosimetry was done in the same cell used for the experiment. In the case of protein work, the dose was determined at several different pulse lengths before the protein was prepared for pulse radiolysis. Then the protein was prepared to a concentration such that the concentration of electrons would be 10-15% of the protein concentration. This procedure would usually mean that 85-95% of the electrons would react with the protein.

The dosimetry solution was 5 mM KSCN in oxygen-saturated water. The reaction is

\[ \cdot \text{OH} + \cdot \text{SCN} \rightarrow \cdot \text{SCN} + \cdot \text{OH} \]  \hspace{1cm} (24)

\[ \cdot \text{SCN} + \cdot \text{SCN} \rightarrow (\text{SCN})_2^- \]  \hspace{1cm} (25)

where oxygen was used to scavenge for e\textsuperscript{-aq}.

\[ \text{O}_2 + e\textsuperscript{-aq} \rightarrow \text{O}_2^- \]  \hspace{1cm} (26)

The maximum OD was calculated by extrapolating the decay curve for the SCN dimer back to time zero (Figure 11) and converting percent transmission to absorbance by knowing that \( I_0 = 800 \text{ mV} \):

\[
\% T = \frac{I}{I_0} = \frac{800 - 360}{800} = 0.550 = 0.550
\]  \hspace{1cm} (27)

\[ \text{OD} = \log \left(\frac{1}{T}\right) \approx 0.260 \]  \hspace{1cm} (28)
Since the $G$ values for $e^{-aq}$ and $\cdot OH$ are the same, $[e^{-aq}]$ is equal to $[\cdot OH]$ and $[\cdot OH]$ is equal to $[(SCN)_{2}^{-\cdot}]$. The dose was calculated by placing this OD into the following equation based on Beer's law:

$$\ [e^{-aq}] = (OH) = [(SCN)_{2}^{-\cdot}] = \frac{OD}{C} \cdot \ell \quad (29)$$

where $\varepsilon_{465} = 7600 \text{ M}^{-1} \text{ cm}^{-1}$ and $\ell$ = path length in cm, yielding $[e^{-aq}] = 17 \times 10^{-6} \text{ M}$.

**Sample Preparation for Pulse Radiolysis.** Five times distilled, demineralized water was the solvent used when
Experimental 32

Water was required as the solvent. This water was prepared by first redistilling commercial doubly distilled demineralized water in the presence of potassium dichromate, phosphoric acid (1 g each per 500 mL), and oxygen. Then the water was distilled in the presence of potassium permanganate, sodium hydroxide (1 g each per 500 mL), and oxygen. Finally, the water was redistilled again. The distillation with dichromate and permanganate in the presence of oxygen was used to remove any organic contaminants by oxidizing them to a nonvolatile form. Many organic contaminants can react with $\text{e}^{\text{-aq}}$ and/or $\cdot\text{OH}$, therefore reducing the amount of $\text{e}^{\text{-aq}}$ and/or $\cdot\text{OH}$ reacting with the protein. The resistivity of this water was usually 1.2 Mohm and better.

Proteins were prepared in water from a demineralizer, and the buffer was changed to 5 times distilled water for radiolysis. This was done since the output from the still used to prepare water for pulse radiolysis was too low to support the large volumes of water required for the preparation of the proteins. The resistivity of the water from the demineralizer was usually 0.9 Mohm.

Ethanol was the purest available and used with no further purification. All other organic solvents were refluxed with LiAlH$_4$ and distilled under argon or nitrogen. tert-Butyl alcohol was carefully recrystallized 4 times by freezing to remove water. Imidazole was recrystallized from
toluene and dried under vacuum. All other chemicals except for the proteins were used without further purification.

The Pulse Radiolysis of Proteins with e⁻aq. The pulse radiolysis of a protein was routinely done in 10 mM that was 0.1 M in tert-butyl alcohol and argon saturated. Usually proteins were prepared in 10 mM phosphate buffer (and NaCl) and then made the proper pH and degassed by slowly bubbling argon through it for 30 min. After the solution was degassed, the pH was always within 0.05 pH unit of the pH determined before degassing.

The tert-butyl alcohol was used to scavenge for ·OH, and argon was used to remove O₂ since O₂ is highly reactive toward the electron. This would leave e⁻aq (and H⁺) to react with the protein (in argon-saturated solutions there is 4.6 times more e⁻aq than H⁺).

The buffers used in the pulse radiolysis of papain and thiosubtilisin were 10 mM in NaCl as well as 10 mM in phosphate to increase the ionic strength of the buffer because the proteins were passed through a Sephadex G-25 column before use. The buffer used in the pulse radiolysis of subtilisin was 60 mM in NaCl and 10 mM in phosphate because this was the buffer used to purify the protein. The buffer used in the pulse radiolysis of asparaginase was also 50 mM in NaCl because NaCl was reported to help stabilize the enzyme. Since α-chymotrypsin froths when bubbled, the buffer was bubbled with argon before the protein was added and
then slowly bubbled after tert-butyl alcohol was added and the solution adjusted to the proper pH. Asparaginase also froths when bubbled so it (ca. 20 mL) was dialyzed against 1000 mL of degassed buffer overnight.

The Pulse Radiolysis of Proteins with \( \text{CO}_2^\cdot \). When \( \text{CO}_2^\cdot \) was the reactive radical used, 0.1 M sodium formate and \( \text{N}_2\text{O} \) were used instead of 0.1 M tert-butyl alcohol and argon. This would yield only the \( \text{CO}_2^\cdot \) radical anion to react with the protein.

Titration Method. The single-point titration method was used to obtain the titration curves for subtilisin and thiosubtilisin and oxidized DTT. First, the monochromator was set at the desired wavelength (360 or 390 nm) and the solution adjusted to the pH at which the titration was to start and degassed. Then two to four traces were taken of the species absorbing at the desired wavelength. The pH of the solution was then increased or decreased and two to four more traces were taken. The smallest amount of degassed dilute HCl (0.25 M, 0.12 M) or dilute NaOH (0.25 M, 0.12 M) in 10 mM phosphate was used to adjust the solution pH, thus reducing the dilution of the enzyme solution. In addition, the experiment was designed so that the [e-aq] was less than 8% of the [enzyme]. Having such a high concentration of enzyme relative to [e-aq] reduces the effect of dilution on the yield observed at the desired wavelength. Indeed, the dilution effect was small as evidenced by the minor change
of concentration as determined by spectroscopy, of enzyme concentration before titration, and of enzyme concentration after spectroscopy. Often the change in absorbance, which reflected the change in concentration from dilution, was within the uncertainty of the spectrophotometer and in the pipetting used to make the measurements since the same concentration was obtained for the enzyme in the sample before and after the titration. A majority of the spectra reported in this dissertation are plotted as single points (at one time after the pulse) at each wavelength. Actually, each point represents the average of two or more points at its respective wavelength.
PROTEASES

Proteases comprise a group of enzymes that have been well studied and well characterized. Out of this class of enzymes, the serine proteases have held a considerable interest among the scientific community, largely because of the nature of the active site and because serine proteases are found in many and seemingly unrelated systems. The active site triad of the serine proteases, Asp-His-Ser, has been found in animal digestive tracts, in blood, in venom, and in plants. The following is an examination of some serine proteases and the very similar cystine proteases using pulse radiolysis.
Chymotrypsins

BACKGROUND

α-Chymotrypsin is a digestive enzyme that hydrolyzes proteins in the small intestine. It will preferentially hydrolyze the peptide bond on the carboxyl side of the aromatic side chains tyrosine, tryptophan, and phenylalanine. It is synthesized in the pancreas as chymotrypsinogen, its inactive precursor. α-Chymotrypsin is prepared commercially by the method of Yapel et al. or Kunitz, which involves multiple recrystallizations of the zymogen followed by activation with trypsin and more recrystallization.

α-Chymotrypsin consists of three polypeptide chains connected by two interchain disulfide bonds. The molecule has a molecular weight of 25,000 and is comprised of 241 amino acid residues. The molecule contains 30 amino acids that may be monitored by pulse radiolysis, 2 histidines, 10 half-cystines, 8 tryptophans, 6 phenylalanines, 4 tyrosines, and no free cysteine. The molecule is ellipsoid with all charged groups located on the surface, leaving most of the interior hydrophobic. The chains tend to be fully extended and run parallel to one another, making the peptide groups available for hydrogen bonding. Parts of the molecule have a secondary structure that resembles an antiparallel pleated sheet. The molecule has no cleft on its surface; however, the active site does lie in the vicinity of open regions in
the molecule where the structure is not closely packed and is comprised of a serine, a histidine, and an aspartic acid.\textsuperscript{22-24} The hydroxyl group of serine-195 is in close proximity to the imidazole group of histidine-57, which in turn is in very close proximity to the carboxylic acid of aspartic acid-102.\textsuperscript{23,24}

Much work has been done on \(\alpha\)-chymotrypsin, making it one of the most thoroughly studied enzymes. However, the controversy surrounding the mechanism of catalytic action involving the active site triad Asp-His-Ser is still present. Ever since Blow\textsuperscript{25} postulated a "charge-relay" mechanism for \(\alpha\)-chymotrypsin based on X-ray data that showed Asp-102, His-57, and Ser-195 aligned nicely to act in a concerted fashion in peptide hydrolysis, many groups have examined the mechanism of action at the active site of \(\alpha\)-chymotrypsin as well as other proteins that contain a similar triad.

If the charge-relay mechanism is to account for the catalytic activity of the enzyme, it is necessary that the mechanism be energetically reasonable. The difficulty with the concerted mechanism lies in the fact that the active site imidazole must have a much lower pK\textsubscript{a} than normal (pK\textsubscript{a} = 4.5). Indeed, such a low pK\textsubscript{a} assignment to imidazole has been made by Hunkapiller et al.,\textsuperscript{14} who prepared \(\alpha\)-lytic protease (a member of the serine protease family) enriched in \(^{13}\text{C}\) at the C(2) position of its single histidine. From
the NMR spectrum they assigned a pKₐ of <4 to the histidine and a pKₐ of 6.75 to the carboxyl of the adjacent aspartic acid. Using IR, Koepppe and Stroud²⁶ supported the results obtained by Hunkapiller et al.¹⁴ when they reported a pKₐ of 6.8 for an acid that they assigned to the aspartyl carboxyl. Markley and Porubcan²⁷ reported that the NMR resonance of the proton on C(2) of the imidazole ring of the histidine in the catalytic triad in porcine trypsin varied with changing pH in such a manner as to correspond to an acid of pKₐ = 4.5. Using pulse radiolysis, Faraggi et al.¹⁰ reported pKₐs of 4.2 and 4.3 for an imidazole in α-chymotrypsin and trypsin, respectively. Further support for the assignment of a low pKₐ to the histidyl group has been supplied by study of model systems²⁹ and theoretical calculations.³⁰

On the other hand, Robillard and Shulman¹⁵,³¹ reported that low-field ¹H NMR for trypsin, chymotrypsin, subtilisin, and α-lytic protease could be assigned to protons in hydrogen bonds between the histidyl and aspartyl residues of the catalytic triads of these enzymes. The pH dependencies of these resonances gave a pKₐ of about 7.5 and were assigned to the active site imidazole and not to the active site aspartyl carboxylate. Bachovchin and Roberts¹⁶ prepared α-lytic protease enriched with ¹⁵N in the imidazolium ring in its single histidine. They determined a pKₐ of 7.0 for the histidyl residue by monitoring the changes of its ¹⁵N chemical shifts by ¹⁵N NMR. Bachovchin et al.¹⁷ also repeated
the experiment originally performed by Hunkapiller et al.\textsuperscript{14} with a more powerful NMR. They showed that the coupling constant in the $^{13}$C-enriched imidazole in $\alpha$-lytic protease does change with pH, yielding a pK\textsubscript{a} of 7. They concluded that the original Hunkapiller et al. experiment must be wrong. Finally, Markley et al.\textsuperscript{18} retracted the low pK\textsubscript{a} of 4.5 reported 2 years earlier.\textsuperscript{27} They succeeded in titrating the C(2) proton of His-57 in chymotrypsin by proton NMR and got a pK\textsubscript{a} of 6.78 in D\textsubscript{2}O.

The large amount of NMR data that suggests a normal pK\textsubscript{a} for the active site imidazole in serine proteases, the retraction of the NMR data indicating a low pK\textsubscript{a} for the active site imidazole, and the assignment change in the pK\textsubscript{a} of the active site imidazole in $\alpha$-lytic protease from <4 to 7 after the experiment was repeated on a more powerful NMR leaves the IR results, the pulse radiolysis results, and some theoretical calculations with the low pK\textsubscript{a} assignment for the active site imidazole in serine proteases. This makes it imperative to ensure that the pK\textsubscript{a} derived by using the pulse radiolysis technique does indeed belong to the active site of chymotrypsin.
METHODS

Purification of Native α-Chymotrypsin To Remove Peptide Fragments. A total of 1.45 g (by weight) of 3 times crystallized α-chymotrypsin (Sigma) was dissolved in a minimum amount of pH 4 water (12 mL) and applied to a 5 x 23 cm (452 cm³) Sephadex G-25 column that had been equilibrated with the pH 4 water. The eluant was pulled through the column and a UV monitor system at a flow rate of 7 mL/min with a peristaltic pump. After the first peak was collected in a graduated cylinder (1.15 g (OD) in 100 mL) and concentrated by ultrafiltration to 15.5 mL in a 142 mm diameter Millipore concentrator that was fitted with an Amicon PM-10 membrane, it was frozen with liquid nitrogen and lyophilized. This purified α-chymotrypsin was stored frozen until needed.

Preparation of PMS-chymotrypsin. A total of 1.53 g (by weight) of 3 times crystallized α-chymotrypsin (Sigma) was dissolved in 20 mL of 0.1 M Tris (tris(hydroxymethyl)-aminomethane) buffer, pH 8.0, that was 0.03 M in CaCl₂ to help stabilize the enzyme. About 1.8 mL (2-fold molar excess) of a phenylmethanesulfonyl fluoride stock solution (13 mg of PMSF/mL of 1,4-dioxane) was added over the space of 1 min to the moderately stirred buffer containing enzyme. The last few drops of the PMSF in dioxane leave the solution cloudy. This is presumably undissolved PMSF. The blocking of the active site serine was determined to be complete when
the rate of turnover using the substrate \textit{N-}trans-cinnamoyl-imidazole was 1\% or less of the rate determined before the addition of PMSF. Care must be taken to ensure the buffer does not contribute to the rate. \textit{p}-Nitrophenyl acetate and the \(\alpha\)-chymotrypsin-specific benzoyltryosine ethyl ester (BTEE) can also be used to follow loss of enzyme activity with equal or better sensitivity. The \textit{N-}trans-cinnamoyl assay was run at 345 nm with 100 \(\mu\)L of substrate in CH\(_3\)CN and 100 \(\mu\)L of enzyme solution in 25 mM phosphate buffer, pH 7.0, that was 75 mM in NaCl.

\textbf{Purification of PMS-chymotrypsin.} After the blocking was complete, which took 15-60 min, the solution was centrifuged to remove insolubles and applied at a flow rate of 4 mL/min to a 5 x 23 cm Sephadex G-25 column that was equilibrated with pH 4 water. This one step removed the blocking agent PMSF, exchanged the buffer to a more lyophilizable form, and removed the low molecular weight peptides found to be present in commercial \(\alpha\)-chymotrypsin. The first peak was
collected and concentrated (853 mg (OD)/30 mL) by ultrafiltration using a Millipore 142 mm diameter concentrator fitted with an Amicon PM-10 membrane. Finally, the PMS-chymotrypsin was frozen with dry ice-methanol, lyophilized, and stored frozen until needed.

Preparation of N-Methylchymotrypsin. A total of 2.69 g (by weight) of 3 times crystallized α-chymotrypsin (Sigma) was dissolved in 70 mL of Tris buffer, pH 8.0, that was 0.02 M in CaCl₂. About 3.5 mL of stock methyl p-nitrobenzenesulfonate (MNBS, 174 µmol/mL of methanol) was added over a time period of 3 min to the moderately stirred enzyme solution. Rate assays of the now opaque solution were taken at regular intervals using the substrate N-trans-cinnamoylimadazole (NTCI). When the rate of NTCI disappearance reached 50% of the initial rate (about 20 min), a 2-fold molar excess of PMSF in dioxane was added to the slowly stirred solution. About 30 min later, when the rate of NTCI disappearance had reached 1% or less of the initial rate, all 74 mL of enzyme solution was applied to a 5 x 23 cm G-25 column that was equilibrated with 25 mM acetate, pH 5.0, that was 0.2 M in KCl (the equilibration buffer for the next step) at a flow rate of 7 mL/min to remove the excess PMSF and MNBS. In addition, this step also removed most of the peptides present in this commercial form. The first peak, which contained 2.04 g in 180 mL, was concentrated down to about 35 mL by using ultrafiltration.
Purification of N-Methylchymotrypsin. The concentrate was applied to a 5 x 24 cm α-chymotrypsin affinity column (or ovomucoid column) that had been equilibrated with 2 L of 25 mM acetate, pH 5.0, buffer that was 0.2 M in KCl (Figure 12). After about 1 L of buffer had passed through the column at 3.8 mL/min, the first peak containing 1.464 mg (OD)

\[
\begin{align*}
\alpha-CT &+ O=S=O \xrightarrow{pH 8} Me-CT + O=S=O \\
\text{active} & \quad \text{MNBS} \quad \text{N\textdegree}_3\text{-methyl-} \quad \text{NBS} \\
\alpha\text{-chymotrypsin} & \quad \text{chymotrypsin}
\end{align*}
\]
of PMS-chymotrypsin and perhaps some N-methyl-PMS-chymotrypsin emerged. Later, 419 mg (OD) of N-methylchymotrypsin emerged after the eluting buffer was changed to pH 3 water. Both peaks were concentrated by ultrafiltration, lyophilized, and stored frozen for later use.

Preparation of Anhydrochymotrypsin.28 This procedure for the preparation of anhydrochymotrypsin is very similar to the procedure for the preparation and purification of PMS-chymotrypsin. The purification of crude anhydrochymotrypsin uses the same ovomucoid column that was used to purify the methylchymotrypsin; i.e., both are retained by the column.

Preparation of PMS-chymotrypsin.28,32 A total of 1.74 g (by weight) of 3 times crystallized α-chymotrypsin (Sigma) was dissolved in 72 mL (15% of the G-25 volume) of 0.1 M Tris buffer, pH 8.0, that was 0.1 M in NaCl, 0.12 M in CaCl₂, and 13% methanol (v/v). At 5-min intervals, 0.1 mL of PMSF in p-dioxane (18 mg/mL) was added to a moderately stirred buffer containing enzyme. When the activity fell to less than 1% of the original activity (ca. 1.5 h) by using the N-trans-cinnamoyl assay, the enzyme buffer was exchanged and the low molecular weight species removed by using a 5 x 23 cm Sephadex G-25 column equilibrated with pH 4 water. The first peak was concentrated by ultrafiltration using an Amicon PM-10 membrane, quickly frozen, and lyophilized.
Preparation of Crude Anhydrochymotrypsin. All of the PMS-chymotrypsin was dissolved in 200 mL of ice-cold 0.1 M KOH and allowed to incubate (no stirring) for 1 h at 3 °C. Since PMS-chymotrypsin has been reported to desulfonate to active α-chymotrypsin at high pH, the incubation mixture was made 0.05 M in Tris, pH 8.0, and concentrated to about 100 mL, and 0.1 mL of PMSF (9.9 mg/mL of dioxane) was added to block any active α-chymotrypsin.

Since anhydrochymotrypsin and native α-chymotrypsin are retained by the column, PMSF was used to modify the unwanted native α-chymotrypsin to PMS-chymotrypsin, which is not retained by the column. PMSF was added after concentrating and not before because it was not known how compatible PMSF and the concentrating membrane were. Concentration was necessary to reduce the 200 mL to a volume that could be successfully applied to a 5 x 23 cm Sephadex G-25 column.
Purification of Crude Anhydrochymotrypsin. All of the crude anhydrochymotrypsin was dissolved in 15 mL of 25 mM acetate, pH 5.0, buffer that was 0.2 M in KCl and applied to the 5 x 24 ovomucoid column that had been equilibrated with 2 L of the above buffer (Figure 13). After about 1 L of buffer had passed through the column at 7.5 mL/min, the first peak containing 1328 total OD units emerged. Later, 425 mg (OD) of anhydrochymotrypsin emerged after the eluting buffer was changed to pH 3 water. The second peak was concentrated by ultrafiltration, lyophilized, and stored frozen.

![Figure 13. Purification of anhydrochymotrypsin. The crude anhydrochymotrypsin collected from the Sephadex G-25 column was applied to a 5 x 24 cm affinity column to which turkey ovomucoid had been covalently attached. The buffer was changed from 25 mM acetate (0.2 M KCl), pH 5.0, to 1 mM HCl at the arrow. The column flow rate was 7.5 mL/min. The second peak contained 425 mg of anhydrochymotrypsin.](image-url)
After the buffer was exchanged over a 5 x 23 cm Sephadex G-25 column equilibrated with pH 4 water, the first peak was concentrated, lyophilized, and stored frozen for further use.

Preparation of \( \delta \)-Chymotrypsin.\(^{32} \) This rapid \( \delta \) activation of chymotrypsinogen A at 5 °C is complete and results in a mixture of predominantly \( \delta \)-chymotrypsin and some \( \pi \)-chymotrypsin. However, care must be taken because autolysis inevitably converts the mixture to \( \gamma \)-chymotrypsin and \( \alpha \)-chymotrypsin where the Tyr\(_{146}\)-Thr\(_{147}\) bond has been cleaved. In addition to the complications of stopping the activation before formation of \( \gamma \)-chymotrypsin, the enzyme prepared by rapid activation is ordinarily contaminated with 2-3% trypsin and its degradation products. In this preparation, acetyltrypsin, not trypsin, is used and a batch chromatographic step is used that is reported to eliminate the activating enzyme from the preparation.

To remove any unacetylated trypsin that may adhere to the chromatographic material in the chromatographic step, 100 mg (by weight) of acetyltrypsin (Sigma lot no. 82F-8095, 15,400 BAEE units/mg) was added to 10 mL of deionized water that contained 4 drops of 1 M CaCl\(_2\). This gave 88 mg based on OD \((E^1%,(280 \text{ nm}) = 14.3 \text{ absorbance units})\), which was incubated with 8 cm\(^3\) of Whatman CM-52 that had been pre-equilibrated with 50 mM phosphate, pH 6.2. After 15 min the suspension was centrifuged for 8 min and the supernatant
collected. The pellet was washed with 5 mL of the same buffer and centrifuged. The pellet was discarded and the two supernatants were combined, yielding 71 mg of acetyltriptysin in 16.6 mL.

To activate chymotrypsinogen A, 2 g (by weight) (1.84 g (OD)) of 6 times crystallized and lyophilized chymotrypsinogen A (Sigma lot no. 111F-8055, 0.43 BTEE unit/mg) was placed in a beaker that was located in an ice-water bath to which 89 mL of ice-cold 0.1 M Tris buffer, pH 7.5, and 1 mL of 1 M CaCl₂ were added. After the protein dissolved, the solution has a specific activity of 0.35 BTEE unit/mg of protein (OD). Then 71 mg of acetyltriptysin in 50 mM phosphate buffer, pH 6.2, was added and the pH increased to 7.5 with 0.5 M NaOH. The temperature was maintained at 5 °C and the pH was maintained at 7.5 (with minor addition of 0.5 M NaOH) for the 90 min required for activation. Enzyme assays using BTEE were used to follow the activation. The activation started out rapidly. At 30 min the solution had a specific activity of 70 units/mg. At 60 min the specific activity had almost reached a maximum (100 units/mg). At 90 min the solution had a specific activity of 104 units/mg, and the pH was then lowered to 4 with cold 1 M HCl. An approximately 40-cm³ dry cake of Whatman CM-52 that had been equilibrated with 50 mM phosphate, pH 6.2, was added to the solution and the solution stirred at 5 °C for 20 min. The pH of the solution increased to 5.22. Then the cellulose
chymotrypsins 50

was collected by suction and washed twice with 60 mL of 50 mM phosphate buffer, pH 6.2, stirring the mixture for 5 min during each wash. The initial solution and the two washes were saved and later checked for total OD. The enzyme was eluted by stirring the cellulose with 60 mL of 80 mM phosphate for 10 min at 5 °C. The eluant was collected by suction. Elution was carried out 3 more times with 60 mL of 80 mM phosphate. The eluants were pooled and the pH was adjusted to 3 with 1 M HCl. The enzyme was concentrated to 32 mL by ultrafiltration and applied to a 5 x 23 cm Sephadex G-25 column that had been equilibrated with pH 3 water. The protein peak was collected at 19 mL/min and concentrated by ultrafiltration to 27 mL. The concentrate that contained 848 mg (OD) and had a specific activity of 81 BTEE units/mg was quick frozen and lyophilized. The lyophilized enzyme also had a specific activity of 81 units/mg.

Since the wash contained at least 900 mg of chymotrypsin based on OD, 50 cm³ of pre-equilibrated Whatman CM-52 was suspended in the wash and the procedure repeated. The second batch yielded 350 mg that had the same specific activity, 81 units/mg.

**Preparation of the Ovomucoid Column.** The ovomucoid column was prepared according to Moehler and Whitaker. In their preparation turkey ovomucoid was covalently attached by cyanogen bromide activation to Sepharose 4B according to the procedure of Ryan and Feeney, except the coupling step
was performed at pH 7.5 instead of pH 9.0 because more total activity was found at the lower pH. Since less protein coupled at pH 7.5 than at pH 9, the maximum suggested value of 30 g of CNBr to 100 cm$^3$ of gel to 1 g of ovomucoid was the minimum used. To increase the yield even more, the ovomucoid was dissolved in 50 mL of coupling buffer to 100 cm$^3$ of gel cake instead of 100 mL of coupling buffer.

The modified procedure is as follows. A 100-cm$^3$ cake of Sepharose 4B was washed with 0.5-1 L of water and placed into a 600-mL beaker that contained a pH electrode, a thermometer, 100 mL of water, and a stir bar. The beaker was centered in an ice bath with a 50-mL buret containing 8 N NaOH positioned above the 600-mL beaker. About 2 L of ice-cold 0.1 M phosphate buffer, pH 7.5, ice, a very large Buchner funnel with suction flask, and 1 g of turkey ovomucoid (from Sigma) dissolved in 50 mL of the same buffer should be within easy reach. All of this must be situated in a good hood.

Thirty grams (or more) of CNBr was rapidly weighed in the hood and dissolved in a minimum amount of dimethyl sulfoxide (DMSO). This step is very important since it eliminates the occurrence of undissolved CNBr chunks staying with the gel and ending up in the coupling step. CNBr can lower the pH of the coupling buffer and therefore decrease the total amount of protein bound. After about 5 min the 30 g of CNBr had completely dissolved in about 20 mL of DMSO.
As the dissolved CNBr was added to the rapidly stirred gel, finely divided CNBr precipitated out of solution at the point of entry and then redissolved. The CNBr was added over a span of 3 min, taking great care in keeping the pH 11 and in keeping the temperature below 30 °C. Care should be taken to ensure that the temperature of the gel prior to adding CNBr is no lower than 15 °C and no higher than 25 °C since the reaction rate can start out too slow or proceed too quickly if the initial temperature is outside this range. As the temperature rises during the activation, more ice should be added to the ice bath to keep the temperature below 30 °C. As a last resort ice can be added directly to the reaction mixture to keep the reaction from running wild. Since activation liberates HBr, NaOH must be constantly supplied to the unbuffered reaction mixture to maintain a pH of 11 so the hydroxyl anion of the gel can continue to attack the CNBr.

The reaction should be complete in 15 min, and this is indicated by the decrease in the rate of H+ liberation. At this point the mixture was cooled to 1 °C by adding ice directly to the mixture and then rapidly washing the activated gel for 2 min or less with 2 L of ice-cold 0.1 M phosphate, pH 7.5, buffer. When the washing was completed, the bottom of the funnel was capped and the buffer containing ovomucoid was added quickly to the gel cake located in the funnel. Immediately the slurry was gently stirred.
and quantitatively transferred to an Erlenmeyer by using the minimum amount of buffer to effect transfer. The Erlenmeyer was rocked in the cold for 12-24 h to allow complete coupling. The 2 L of buffer is necessary to remove any remaining CNBr and to equilibrate the gel. Capping the funnel and adding the ovomucoid to the gel rather than adding the gel to the ovomucoid significantly increase the amount of protein coupled.

Prior to pouring of the column, the gel should be collected by suction and washed with several liters of column equilibration buffer.

The capacity of the column was checked, and the elution characteristics of the column were verified by using a small amount of column material and an excess amount of α-chymotrypsin (Figure 14). The column material had a capacity of 1 mg of α-chymotrypsin/cm³ of gel. The column behaved as expected where a second peak emerged from the column after the column buffer was changed from 25 mM acetate (0.2 M KCl), pH 5.0, to 1 mM HCl. This column has affinity for α-chymotrypsin, N-methylchymotrypsin, and anhydrochymotrypsin but no affinity for PMS-chymotrypsin.34

Rate Assays for Chymotrypsin Activity. **N-trans-Cinnamoylimidazole (NTCI).**36 This assay was performed in a 1-cm quartz cuvette of 3.5-mL capacity that was held at 25 °C in a thermostated compartment. Exactly 3.00 mL of 25 mM acetate, pH 7.0, that was 75 mM in NaCl plus 0.100 mL of
A total of 21 mg of α-chymotrypsin was applied to a small column that contained 4.32 cm$^3$ of gel material. The second peak contained 4.03 mg of α-chymotrypsin and was eluted by changing the column buffer from 25 mM acetate (0.2 M KCl), pH 5, buffer to 1 mM HCl at the arrow.

NTCI stock solution (10 mM in water) was allowed to come to thermal equilibrium, after which any nonenzymatic hydrolysis was recorded as a decrease in absorbance at 345 nm with the recorder set at 1 cm/min and 0.1 absorbance unit full scale. Then 1-3 μg of chymotrypsin in 0.100 mL or less was mixed into the cuvette, and the linear decrease in absorbance was recorded for 5-10 min. The activity, per milliliter in the sample, which is defined as 1 unit of activity = 1 μmol of NTCI loss/min, can be calculated as

$$\text{units/mL} = \frac{\Delta A/(c \cdot \ell)}{\text{cm} \times \text{rate}} \times \frac{\text{total volume in cuvette (L)}}{\text{volume of sample added to cuvette (mL)}}$$

(33)
where \( \Delta A \) = change of absorbance over the time measured, \( \varepsilon = 2.89 \times 10^3 \text{ M}^{-1} \text{s}^{-1} \), \( l = 1 \text{ cm} \), cm = number of centimeters in which \( \Delta A \) was measured on the chart recorder, rate = chart recorder speed = 1 cm/min, total volume in cuvette = 3.00 mL of buffer + 0.100 mL of NTCI + sample volume, volume of sample added to cuvette = 0.100 mL or less, and units/mL = activity of the sample.

Benzoyl-L-tyrosine Ethyl Ester (BTEE). This assay appears to be the industry standard for reporting chymotrypsin activity. Before the assay was performed, the BTEE stock solution (15.7 mg of BTEE plus 30 mL of methanol plus water to make 50 mL) was mixed with an equal volume of assay stock buffer (0.10 M Tris-HCl, 0.10 M CaCl\(_2\), pH 7.8) and allowed to sit for 30 min at 30 °C. This step is a modification of the procedure outlined by Wilcox\(^{32}\) where he states that 1.5 mL of BTEE stock solution and 1.5 mL of assay stock solution should be mixed in the cuvette just prior to assay. This procedure was not followed because gas bubbles would adhere to the cuvette and interfere with the assay. The assay was performed as follows. Exactly 3.00 mL of assay buffer was placed in a 1-cm quartz cuvette (3.5-mL capacity) that was held at 30 °C in a thermostated compartment. After 5 min 1-3 \( \mu \text{g} \) of chymotrypsin in 0.100 mL or less was mixed and the linear increase in the absorbance was followed at 256 nm for 6 min. The chart recorder was set at 1 cm/min and 0.100 full scale. The activity was calculated as
\[
\text{units} = \frac{\Delta A/(\epsilon \cdot l)}{\text{mL}} \times \frac{\text{total volume in cuvette (L)}}{\text{cm} \times \text{rate} \times \text{volume of sample added to cuvette (mL)}}
\] (34)

where \(\Delta A\) = change of absorbance over the time measured, \(\epsilon = 964 \text{ M}^{-1} \text{ s}^{-1}\), \(l = 1 \text{ cm}\), \(cm = \) number of centimeters in which \(\Delta A\) was measured on the chart recorder, \(\text{rate} = \) chart recorder speed = 1 cm/min, \(\text{total volume in cuvette} = 3.00 \text{ mL of buffer} + \text{volume of sample and substrate used, volume of sample added to cuvette} = 0.100 \text{ mL or less, and units/mL} = \text{activity of the sample.}\)
RESULTS

Confirmation of the α-Chymotrypsin Spectrum. The spectrum resulting from the reaction between e-aq and α-chymotrypsin at pH 4.8 (Figure 15) has a major peak centered at 410 nm and a much smaller peak centered at 360 nm. These spectral features correspond well to features reported by Faraggi et al.\textsuperscript{10} where the 410-nm band was assigned to the disulfide radical anion (RSSR\textsuperscript{-}) and the 360-nm band was assigned to the imidazolium radical (Im\textsuperscript{+}). Even at 0.6 pH unit above the pK\textsubscript{a} of 4.2 reported by Faraggi et al.,\textsuperscript{10} the 360-nm imidazolium band is well defined.

![Figure 15. α-Chymotrypsin spectrum taken at pH 4.8. The major peak centered at 410 nm belongs to RSSR\textsuperscript{-}. The smaller peak centered at 365 nm belongs to the imidazolium adduct. The spectrum was taken in 10 mM phosphate that was 0.1 M in tert-butyl alcohol, pH 4.80, and saturated with argon, where [en\textsubscript{z}] = 0.14 mM and [e-aq] = 13 μM. The X represents a spectrum taken 25 us after the end of a 300-ns pulse. The + represents a spectrum taken 0.100 ms after the end of the pulse, o = 0.3 ms, * = 0.5 ms, and x = 1.6 ms. Each point at its respective time and wavelength represents the average of two or more points.](image-url)
PMS-chymotrypsin is an inactive form of α-chymotrypsin where the active site serine is covalently attached to the sulfur on PMS (Figure 16). The spectrum resulting from the e-aq reacting with this form of α-chymotrypsin at pH 4.6 has the 410-nm RSSR⁻ peak but no peak at 360 nm (Figure 17). Taking spectra at an even lower pH would actually result in
reduction of the overall spectrum amplitude because of increased competition between $H^+$ and protein for $e^-aq$:

$$H^+ + e^-aq \xrightarrow{2 \times 10^{10} \text{ M}^{-1} \text{s}^{-1}} H^-$$  \hspace{1cm} (35)

$\text{N-Methylchymotrypsin}$ is also an inactive form of $\alpha$-chymotrypsin where the $N(3)$ position on the active site imidazole has been methylated (Figure 18). When the spectrum resulting from the $e^-aq$ attachment to this form of $\alpha$-chymotrypsin at pH 4.6 (Figure 19) is compared to the spectrum for active $\alpha$-chymotrypsin taken at pH 4.8 (Figure 17), no 360-nm band is apparent--only the 410-nm RSSR$^-$ band.

Imidazole and $\text{N-Methylimidazole}$. Since the spectrum for the $e^-aq$ attachment to $\text{N-methylimidazole}$ has never been reported, it is not known whether the loss of the 360-nm band is due to a change in the spectrum because of the $\text{N-methylimidazole}$ absorbance spectrum being different from the imidazolium spectrum or something else. When the spectrum resulting from the $e^-aq$ attachment to $\text{N-methylimidazole}$ (Figure 20) is compared to the spectrum resulting from the $e^-aq$ attachment to imidazole (Figure 21), both spectra have

![Figure 18. Active site of N-methylchymotrypsin.](image)
two peaks. However, the positions of the two peaks in the N-methylimidazole spectra (Figure 20) seem to be shifted about 5-8 nm to longer wavelength relative to the position of the comparable peaks in the imidazole spectrum (Figure 21). Because the two spectra differ only slightly, the absence of the 360-nm band in the N-methylchymotrypsin spectrum cannot be due to a wavelength shift of the 360-nm band from the methylation of the active site imidazole. The lowering of the pKₐ below 4.2 upon methylation of the active site imidazole would not be expected since N-methylimidazole has a pKₐ that is shifted 0.25 pH unit up from the normal pH of 7 for imidazole to 7.25 for N-methylimidazole.
Figure 20. N-Methylimidazole spectrum at pH 5.65. Note the large peak centered at 305 nm and the shoulder around 360 nm. The conditions used were as follows: \([N\text{-methylimidazole}] = 25 \text{ mM}, [\text{aq-en}] = 20 \mu\text{M}, 10 \text{ mM} \text{ phosphate}, 0.1 \text{ M tert-butyl alcohol}, \) and argon saturated. \(X = 1 \mu\text{s}, \circ = 10 \mu\text{s}, \bigcirc = 30 \mu\text{s}, \) and 
\(\otimes = 80 \mu\text{s} \) after the end of a 500-ns pulse.

Figure 21. Imidazole spectrum taken at pH 5.43. Note the large peak centered at 290 nm and the smaller peak centered at 360 nm. The conditions used were as follows: \([\text{imidazole}] = 25 \text{ mM}, [\text{aq-en}] = 20 \mu\text{M}, 10 \text{ mM phosphate, 0.1 M tert-butyl alcohol, and argon saturated}. \(X = 1 \mu\text{s}, \circ = 10 \mu\text{s}, \bigcirc = 30 \mu\text{s, and } \otimes = 80 \mu\text{s after the end of a 500-ns pulse.}
Anhydrochymotrypsin is an inactive form of α-chymotrypsin where two hydrogens and an oxygen have been eliminated from the active site serine, resulting in dehydroalanine (Figure 22). Like the spectra obtained for the other forms of inactive α-chymotrypsin, no 360-nm band was apparent—only the 410-nm band belonging to RSSR⁻ (Figure 23).

Figure 22. Active site of anhydrochymotrypsin.

Figure 23. Anhydrochymotrypsin spectrum taken at pH 4.64. Note the absence of the 360-nm imidazolium band. The conditions used were as follows: [enz] = 0.20 mM, [εag] = 12.6 μM, 10 mM phosphate, 0.1 M tert-butyl alcohol, and argon saturated. X = 25 μs, * = 0.1 ms, ø = 0.3 ms, * = 0.5 ms, ø = 0.8 ms, and x = 1.6 ms after the end of a 300-ns pulse.
δ-Chymotrypsin. The pH of 4.2 estimated by Faraggi et al.\textsuperscript{10} for the 360-nm band in α-chymotrypsin using pulse radiolysis is very close to the pH at which α-chymotrypsin dimerization is reported to be maximized.\textsuperscript{32,37-39} In addition, PMS-chymotrypsin and N-methylchymotrypsin are reported to reduce dimerization but not eliminate it completely.\textsuperscript{39} Nothing has been reported concerning whether anhydrochymotrypsin dimerizes. Dimerization is reported to involve the electrostatic interaction between the active site imidazole and the terminal carboxyl of tyrosine-146. However, dimerization cannot occur with δ-chymotrypsin, a precursor form of α-chymotrypsin that has 130% of α-chymotrypsin activity, because the carboxylate of tyrosine-146 is covalently bonded to threonine-147 and not free to help the dimerization process.\textsuperscript{39}

The spectrum resulting from the e\textsuperscript{−aq} reacting with δ-chymotrypsin at pH 4.6 shows both the 410-nm RSSR\textsuperscript{−} band and the 360-nm imidazolium band (Figure 24). This result unambiguously shows that the pH dependence observed for the imidazolium band is not due to dimerization of chymotrypsin or its blocked form.

This region displays a pH dependence where the pH dependence is below 5 (Figure 25). The yield at 358 nm levels off below pH 5.5 (solid line) and is due to proton successfully competing with δ-chymotrypsin for e\textsuperscript{−aq} (equation 35). This competition can be accounted for by comparing the rate of
e⁻aq reacting with δ-chymotrypsin and H⁺ to the rate of e⁻aq reacting with H⁺ in the buffer at pHs below 5.5 and then calculating the fraction of e⁻aq reacting with protein from \((k_p - k_b)/k_b\) where \(k_p\) and \(k_b\) are the pseudo-first-order decay constants for e⁻aq in protein and blank solutions, respectively. When the loss of e⁻aq is accounted for, the curve continues to increase and is represented by the dashed line. Notice that the error bars become larger as the extrapolation is continued to lower pHs.

![Graph](image-url)

**Figure 24.** δ-Chymotrypsin spectrum at pH 4.64. Note both the 360-nm imidazolium band and 410-nm BSSR⁻ band are present. The conditions used were as follows: [enz] = 0.16 mM, [e⁻aq] = 15.4 μM, 10 mM phosphate, 0.1 M tert-butyl alcohol, and argon saturated. X = 50 μs, * = 0.1 ms, o = 0.2 ms, * = 0.3 ms, and * = 0.8 ms after the end of a 400-ns pulse.
Figure 25. Yield at 350 nm versus pH for trypsin at $t = 20$ ms. The yield at 350 nm levels off below pH 5.5 (solid line). When the yield loss is accounted for (see the text), the curve continues to increase (dashed line).
Papain

BACKGROUND

Papain is the principal protein component from the papaya latex of *Carica papaya*. It is a protease that is used to prevent "chill hazes" in beer, to prevent wool shrinkage, and to tenderize meat.\(^4^0\) It specifically hydrolyzes the peptide bond of L-arginine and/or L-lysine in peptides and proteins as well as esters that contain one of these side chains.

The procedure for the isolation of crystalline papain by Kimmel and Smith is still the procedure most widely used today.\(^4^1\) It is well-known that the enzyme prepared in this way has variable activity and this variable activity depends on the thiol content of the enzyme. Papain prepared by this method contains three forms of the enzyme that cocrystallize: nonactivatable papain, activatable papain, and active papain. Papain whose active site thiol has been oxidized to a sulfinic acid is called nonactivatable papain. Activatable papain has a mixed disulfide bond at the active site with free cysteine used in the isolation procedure. Activatable papain may be activated by thiols, sulfide, sulfite, cyanide, or sodium borohydride. Papain activity is enhanced when heavy metal complexing agents such as EDTA are present.\(^4^0,4^2\) Active papain has a single free thiol group.
Papain is a single peptide chain of 212 amino acid residues with a molecular weight of 23,420 daltons.\textsuperscript{43,44} The molecule contains 37 amino acids that may be "seen" by pulse radiolysis: 2 histidines, 6 half-cystines, 4 phenylalanines, 19 tyrosines, 5 tryptophans, and 1 free cysteine. The protein is folded into two distinct domains that are divided by a deep cleft. The active site lies at the surface of this cleft and consists of the only free cysteine, a histidine, and an asparagine. The imidazole group of histidine-159 is in close proximity to the thiol group of cysteine-25 and is attached to the opposite wall of the cleft. The imidazole group of histidine-159 is partially embedded in a hydrophobic region and is hydrogen bonded to asparagine-175.\textsuperscript{40,45} This system of three residues, Cys-His-Asn, is quite similar to the system of three residues found in the catalytic site of serine proteases: Ser-His-Asp.

Some work has been done to characterize the active site. From the studies of several substrates, the bimolecular acylation constant $k_{cat}/K_m$ has a bell-shaped pH dependence with $pK_a$'s of about 4.2 and 8.2.\textsuperscript{40,46-49} The deacylation step has an apparent $pK_a$ around 4.\textsuperscript{40,50} Fluorescence intensity for the active enzyme shows a pH dependence with a $pK_a$ of about 8.6 and a $pK_a$ of 4.1 when a mixed disulfide is formed with the active site thiol.\textsuperscript{40,51-54} A similar $pK_a$ shift has been seen with NMR.\textsuperscript{55,56} In fact, this shift has been assigned to the active site imidazole\textsuperscript{54-57} where the
active site imidazole has a pK_a of around 4 when the active site thiol is blocked and a pK_a of 8.5 when the active site thiol is free (Figure 26).

block:

\[
\text{(Asn)} \quad (\text{His}) \quad (\text{Cys})
\]

\[
\text{C} = \text{O} \quad \text{H-N} + \text{N-H} \quad \text{S-S} \quad \text{pK}_{\text{His}} = 4
\]

unblock:

\[
\text{(Asn)} \quad (\text{His}) \quad (\text{Cys})
\]

\[
\text{C} = \text{O} \quad \text{H-N} + \text{N-H} \quad \text{H-S} \quad \text{pK}_{\text{His}} = 8.5
\]

Figure 26. Active site of blocked and unblocked papain.
METHODS

Currently there are three recognized methods for the isolation of fully activatable papain: affinity chromatography, activated thiol chromatography, and mercury affinity chromatography. The first method\textsuperscript{58,59} involves the attachment of the tetrapeptide glycyglycyl-(o-benzyl-L-tyrosyl)-L-arginine to a Sepharose column. Active papain binds well to this tetrapeptide whereas the nonactivatable oxidized papain does not. The column separates active from nonactivatable papain when the ionic strength of the eluant is reduced.

The second method\textsuperscript{60,61} takes advantage of the observation that 2,2'-dipyridyl disulfide reacts rapidly and stoichiometrically with the active site thiol of papain at low pH even in the presence of other thiols. This papain-2,2'-dipyridyl disulfide specificity was used in developing a method of covalent chromatography in which a column of Sepharose-glutathione 2-pyridyl disulfide selectively forms a mixed disulfide bond with the active site thiol of papain with the concomitant release of 2-thiopyridone. After the nonactivatable papain has been washed through the column, active papain can be eluted with a small molecular weight thiol.

The third method\textsuperscript{51-53} takes advantage of papain's single and very reactive thiol group and the tight binding constant between thiols and mercury ion. When a mixture of
active and nonactive papain is passed through a column in which ρ-aminophenyl mercuric acetate has been covalently attached to Sepharose, active papain is retained. Fully active papain can be displaced from the column as mercuric papain when a solution of HgCl$_2$ or mercuric acetate is used. The mercuric papain is stable for years and can be fully activated as required.

Since 40-50% of commercial papain is nonactivatable papain, it had to be purified. Choosing affinity chromatography would involve the synthesis and subsequent attachment of the tetrapeptide to the column support. In light of the difficulty in synthesizing the peptide, the relatively low capacity of the column, the gram quantities of papain needed, and the loss of column capacity with repeated use that I experienced with a small amount of purchased material, this affinity column material was not used.

Covalent chromatography using glutathione 2-pyridyl disulfide as the active ligand was not used because the results were not anywhere near what was reported.$^{60,61}$

The mercury affinity column was chosen because the column was easy and relatively inexpensive to prepare, the column had a very high capacity (6.7 mg/cm$^3$), and the papain could be eluted off the column as the fully activatable Hg-papain and stored for later use.
Purification of Commercial Papain.41,52,59 This was initiated by activating 1 g (by weight) of 2 times crystallized papain (Sigma) in 900 mL of activating buffer (10 mM pyrophosphate, 10 mM Na₂SO₃, 20 mM EDTA, 20 mM 2-mercaptopethanol, 2 mM DTT, pH 8.2) at room temperature and under slow stirring. After 1 h the solution was clear. The solution was made 65% saturated in (NH₄)₂SO₄ with the slow addition of solid (NH₄)₂SO₄. After the solid (NH₄)₂SO₄ had dissolved, the milky solution was placed in an ice-water bath for 30 min and then centrifuged (10,000 rpm) in the cold for 25 min. The protein was collected as the precipitate, washed with the column equilibration buffer (50 mM acetate, 1 mM EDTA, 10 mM Na₂SO₃, 0.1 M KCl, pH 5.0) that was 70% saturated in (NH₄)₂SO₄, and centrifuged again for 25 min. The protein precipitate was dissolved in the column equilibrating buffer (ca. 729 mg (OD) in 190 mL) and applied at a flow rate of 3.7 mL/min to a 2.5 x 12.5 cm mercury column that had previously been equilibrated with the equilibrating buffer (Figure 27). After the sample was applied, the column was washed with 50 mM sodium acetate, 200 mM KCl, pH 5, and 10% EtOH (v/v).

Since the capacity of the column was not exceeded, the peak emerging during the wash had no activity. Papain emerged after the addition (arrow) of the elution buffer (50 mM sodium acetate, 0.5 M NaCl, 2 mM mercury acetate, 10% ethanol (v/v), pH 5.0). This third peak contained 357 mg of
inactive mercury papain that had a total of 350 units of activity after activation (see Rate Assay for Papain Activity). This represents 49% of the total OD applied. Ethanol was used instead of DMSO as suggested by Sluyterman and Widjens\textsuperscript{51} because DMSO was not compatible with the Amicon PM-10 membrane used to concentrate papain.

Since mercury papain is not very soluble, it was concentrated at room temperature in a Millipore concentrator with an Amicon PM-10 membrane. The milk-white concentrate was stored at 4 °C in the elution buffer until activated for pulse radiolysis.
Activation of Hg-Blocked Papain. A total of 1.8 L of 5 times distilled demineralized water was carefully made 0.01 M in phosphate, adjusted to the desired pH, and degassed with argon for 4-6 h. On the day of the experiment, this buffer was placed in the column reservoir that was configured for more argon bubbling and bubbled for an additional 2-3 h (Figure 28). The desired amount of mercury papain was transferred to a 15-mL centrifuge tube and made 36 mM in 2-mercaptoethanol and 10 mM in EDTA. Activation was continued for 1 h at room temperature with moderate rocking. During this hour, roughly half of the degassed buffer was used to equilibrate a 5 x 24 cm Sephadex G-25 column. At the end of the activation period the mixture was centrifuged if not clear and applied to the degassed column at a flow rate of 19 mL/min. To ensure maximum separation between the protein peak and activator peak, the volume applied was always 5% or less of the total column volume. The column's progress was monitored at 280 nm by using a flow cell. The first peak was collected under argon in a Millipore concentrator that was carefully rinsed with the eluting buffer. The protein was concentrated, the concentration checked spectroscopically ($\varepsilon^{1%}(280 \text{ nm}) = 24.7$ absorbance units), 23,000 daltons), and the protein diluted to the desired concentration (0.08-0.5 mM) for pulse radiolysis. The entire procedure to remove the activator was done anaerobically to reduce the formation of oxidized papain 73
papain (papain-$S-S$-papain and papain-$SO_3H$). When a papain preparation was checked, it showed that 96% of the papain was active when Ellman's$^{62}$ Reagent (DTNB) was used.
Preparation of the Mercury Affinity Column. $p$-Aminophenyl mercuric acetate (PAPMA) was covalently coupled to cyanogen bromide activated Sepharose 4B (Figure 29):51-53

![Chemical structure diagram]

Figure 29. Diagram of the preparation of mercury Sepharose.

This column has a very short spacer arm between the gel support and the Hg$^+$ yet works very well in separating thiol compounds from non-thiol compounds.

$$R-SH + Z + \text{Hg}^+X \rightarrow \text{Hg-SR} + Z + \text{HX} \quad (36)$$

Elution can be effected by HgCl$_2$ or mercuric acetate:

$$\text{Hg-SR} + \text{HgCl}_2 \rightarrow \text{HgCl} + \text{RSHgCl} \quad (37)$$
Elution can also be brought about by introducing a second thiol compound such as 2-mercaptoethanol or cysteine:

\[ \text{Hg-SR} + \text{R'SH} \rightarrow \text{Hg-SR'} + \text{RSH} \]  (38)

The correct selection of buffer when using the Hg column is critical. Mercury(II) is compatible with only a few common buffers. An acetate, pH 5, buffer system was chosen for papain. A citrate, pH 6.2, buffer system was chosen for thiosubtilisin since subtilisin is reported to be unstable below pH 5.5. Buffers with higher pKₐs are usable; however, nucleophilic attack at the isothiourea linkage and subsequent loss of ligand have been reported.⁵³

Sluyterman and Wijdenes⁵¹ suggest using 10% DMSO or 10% CH₃CN (v/v) in the preparation of all buffers to make the protein more soluble and to reduce hydrophobic interactions between the protein and the column material. Since DMSO is not compatible with the Amicon PM-10 membrane, ethanol was substituted for DMSO.

The preparation of the Hg column was as follows⁵¹-⁵³ (see Preparation of the Ovomucoid Column also). A 100-cm³ cake of Sepharose 4B was washed with 0.5-1 L of water and placed into a 600-mL beaker that contained a pH electrode, a thermometer, 100 mL of water, and a stir bar. The beaker was centered in an ice bath with a 50-mL buret containing 8 N NaOH positioned above the 600-mL beaker. About 2 L of ice-cold 0.1 M bicarbonate buffer, pH 9.0, ice, a very large
Buchner funnel with suction flask, and 10 g of PAPMA/40 mL of DMSO in 160 mL of the above buffer should be within easy reach. All of this must be situated in a good hood.

Twenty-five grams (or more) of CNBr was rapidly weighed in the hood and ground into a fine powder. This must be done carefully since CNBr is so poisonous. The danger can be significantly reduced by dissolving CNBr directly into CH$_3$CN without grinding. This step would also eliminate the possibilities of undissolved CNBr chunks staying with the gel and ending up in the coupling step. CNBr can lower the pH of the coupling buffer and therefore decrease the total amount of ligand bound.

The solid CNBr was added all at once to the rapidly stirred slurry. The pH was maintained at pH 11 with the 8 N NaOH and the temperature was maintained at 25 °C by adding ice to the ice-water bath. Care should be taken to ensure that the temperature of the gel prior to the addition of CNBr is no lower than 15 °C and no higher than 25 °C since the reaction rate can start out too slow or proceed too quickly if the initial temperature is outside this range. If necessary, ice can be added directly to the reaction mixture to keep the reaction from progressing too rapidly.

After 15 min there should be a marked decrease in the rate of H$^+$ liberation. This indicates the reaction is complete. At this point the mixture was cooled to 1 °C by adding ice directly to the mixture and then rapidly washing
the activated gel in 2 min or less with the 2 L of ice-cold 0.1 M bicarbonate buffer, pH 7.5. When the washing was completed, the bottom of the funnel was capped and the buffer containing PAPMA was added quickly to the gel cake located in the funnel. Immediately the slurry was gently stirred and quantitatively transferred to an Erlenmeyer flask. The Erlenmeyer was rocked in the cold for 48 h to allow complete coupling. Then 1 mL of 2-aminoethanol was added and the flask rocked for 4 more h to block any remaining coupling sites.

Prior to pouring of the column, the gel was washed 4 times with 20% DMSO in water. Once the column was poured, the column was washed with 500 more mL of 20% DMSO in water.

The capacity of the column was checked by equilibrating the column with 0.1 M phosphate, pH 8.0, followed by introducing the same buffer that was 2.5 mM in DTNB and 10 mM in Na₂SO₃ to the column. The yellow column was washed with the first phosphate buffer until the effluent was colorless and then washed with 50 mM acetate at pH 5.0 to remove all phosphate. The yellow color on the column was eluted with the 50 mM acetate buffer, pH 5.0, that was 2 mM in HgCl₂. In order to liberate the free thiophenol anion, the eluant was made 20 mM in 2-mercaptoethanol and 20 mM in Tris and pH 8.0. The capacity of the column in micromoles per milliliter of gel was calculated by knowing the extinction coefficient for the anion at 412 nm is 13,600 M⁻¹ cm⁻¹.
If the total capacity of the column is checked, the 2-aminoethanol step is not usually necessary. If the column capacity is checked by using a known aliquot of packed gel, the 2-aminoethanol step is necessary since protein can bind irreversibly. The total column capacity check was used since the capacity of the column is found and the remaining coupling sites are blocked at the same time. The yield on this preparation was only 2.9 μmol/mL of gel. For 100 cm$^3$ of gel and if only 10% capacity for papain is assumed, this column should have a maximum capacity for papain of 670 mg.

Rate Assay for Papain Activity. Benzoyl-L-arginine Ethyl Ester (BAEE). Before papain could be assayed, it usually required activation since it usually existed in the Hg-blocked form or as a mixed disulfide. Activation was initiated by mixing an equal volume of assay activating buffer (50 mM acetate, 36 mM 2-mercaptoethanol, 9 mM EDTA, pH 4.43) and enzyme, which were then allowed to incubate at room temperature for 10-15 min. The enzyme concentration was routinely about 0.7 mg/mL. When the activity of a solution that contained a higher enzyme concentration was to be measured, the enzyme was diluted to 0.7 mg/mL with the appropriate buffer. While the papain was being activated, 3.00 mL of assay buffer (50 mM acetate, pH 5.0) plus 0.100
mL of BAEE stock solution (53 mg/10 mL of H₂O, made fresh daily) was placed in a 1 cm path length quartz cuvette and allowed to equilibrate to 20 °C. Then 0.100 mL of the now active papain solution was added to the cuvette, and the rate was followed at 255 nm by using a 0.1 absorbance range and 1 cm/min chart speed on the chart recorder. The increase in absorbance at 255 nm is linear for 10-20 min for pure papain, and there is virtually no non-enzymatic hydrolysis to correct for.

A unit of activity is defined as the amount of micromoles of product formed per minute. The number of units per milliliter in the sample was calculated as

\[
\text{units/mL} = \frac{\Delta A / (\varepsilon \ell)}{\text{cm/chart speed}} \times \frac{\text{vol. in cuvette (L)}}{\text{vol. of sample used (mL)}}
\]

(40)

where \(\Delta A = 0.0287 = \text{(number of blocks)} \times \text{(chart range)}/(100 \text{ blocks})\), \(\ell = 1 \text{ cm}, \varepsilon = 1204 \text{ M}^{-1} \text{ cm}^{-1}, \text{cm} = 10 \text{ cm moved}, \text{chart range} = 0.1 \text{ OD full scale}, \text{chart speed} = 1 \text{ cm/min}, \text{vol. of sample used} = 0.100 \text{ mL}, \text{vol. in cuvette} = 3.00 \text{ mL of buffer} + 0.100 \text{ mL of BAEE} + 0.100 \text{ mL of enzyme} = 0.0032 \text{ L total, and units/mL} = 0.0763.

28.7 blocks

\[\begin{array}{c}
\text{10 cm moved} \\
\end{array}\]

\[\begin{array}{c}
\text{28.7 blocks} \\
\end{array}\]
The specific activity for pure papain obtained with this assay was routinely 1.03-1.09 units/mg when the extinction coefficient of \( \varepsilon^{1%} = 24.7 \) absorbance units at 280 nm was used to determine the concentration.

Determining the Number of Free Thiol Groups in Papain. 5,5'-Dithiobis(2-nitrobenzoic acid) (DTNB). Since papain has only one free and very active thiol group, DTNB can be used with very good results in determining the concentration of papain. For the assay, 0.025 mL of DTNB pale yellow stock solution (11 mM DTNB in water) plus 3.00 mL of 0.1 M phosphate, pH 8, buffer was placed into a 1 cm path length cuvette and the spectrophotometer zeroed at 400 nm. Then, 0.100 mL of enzyme solution was mixed into the cuvette. Volumes other than 0.100 mL of enzyme can be used; however, the concentration of enzyme in the cuvette should not exceed the concentration of DTNB in the cuvette (0.088 mM). After 15 min, the absorbance reached a maximum of 0.066 absorbance unit when a solution prepared for radiolysis was used. The concentration of free thiol and therefore the number of active sites in papain were calculated as follows: Knowing \( \varepsilon = 13,600 \text{ M}^{-1} \text{ cm}^{-1} \) at pH 7.5 and 400 nm

\[
\frac{0.066}{13,600} \times \frac{3.125}{0.1} = 0.152 \text{ mM} \quad (41)
\]
When $\varepsilon_{1\%}(280 \text{ nm}) = 14.7$ absorbance units was used for papain, the amount of enzyme in the sample was calculated to be 0.157 mM, showing that papain had 97% of the expected sulfhydryl content.
RESULTS

The spectra of active papain consistently show a 410-nm band and a 355-nm band from low pH (5.3) to high pH (7.9) (Figures 30-32). The 410-nm band can readily be assigned to the disulfide radical anion (RSSR•), and the 355-nm band is characteristic of the imidazolium radical (ImH•). Since the amplitude of the imidazole band remains constant over the pH range (Figure 33) studied and since the imidazole band is still present at pH 7.92 (Figure 32), at least one of the two histidines in papain must still be protonated at this high pH. Solubility problems would not allow a spectrum to be taken at a higher pH. This problem has been reported by others.56,57 However, the data do suggest that the pKₐ for this imidazole band is above pH 7.9.

In Figure 30 there appears to be a slight shoulder at 390 nm that is even more apparent in Figure 31 but absent in Figure 32. Such a feature has never been reported before in a protein, which is understandable considering its very close proximity to the disulfide at 410 nm. Indeed, the imidazole band in papain was never reported until now.63 After reviewing the literature,93-100 it appears that a disulfide radical anion RSSR•• or a protonated disulfide radical RSSRH• could be responsible for the shoulder at 390 nm. The identity of this 390-nm shoulder will be explored later under the section entitled DISULFIDES.
Figure 30. Papain spectrum taken at pH 5.57. Note the 410-nm RSSR$^-$ band and the 355-nm imidazolium band. There also appears to be an absorbance around 385 nm. The conditions were as follows: [enz] = 0.10 mM, [e$^-_{aq}$] = 9.9 μM, 10 mM phosphate, 10 mM NaCl, 0.1 M tert-butyl alcohol, and argon saturated. $X = 25$ μs, $α = 0.3$ ms, and $α = 1.6$ ms after the end of a 250-ns pulse.

Figure 31. Papain spectrum taken at pH 7.10. The 355-nm imidazolium band and the RSSR$^-$ band are present. There is also a third band centered at 390 nm that is very close to the 410-nm RSSR$^-$ band. The conditions used were as follows: [enz] = 0.10 mM, [e$^-_{aq}$] = 9.5 μM, 10 mM phosphate, 10 mM NaCl, 0.1 M tert-butyl alcohol, and argon saturated. $X = 25$ μs, $α = 0.3$ ms, and $α = 1.6$ ms after the end of a 300-ns pulse.
Figure 32. Papain spectrum taken at pH 7.92. The 355-nm imidazolium band is still there. The 410-nm band is quite broad compared to that of Figures 26 and 28 and shows no fine detail in the 385-nm region. The conditions used were as follows: \([\text{enz}] = 0.07 \text{ mM}, [\text{eaq}] = 6.0 \mu\text{M}, 10 \text{ mM phosphate, 10 mM NaCl, 0.1 M tert-buty alcohol, and argon saturated}. X = 25 \mu\text{s}, \theta = 0.3 \text{ ms, and } \tau = 1.6 \text{ ms after the end of a 200-ns pulse.}

Figure 33. Effect of pH on the yield at 355 nm. This was done by using the single-point titration method outlined under EXPERIMENTAL. The conditions used were as follows: \([\text{enz}] = 0.14 \text{ mM}, [\text{eaq}] = 9 \mu\text{M}, 10 \text{ mM phosphate, 10 mM NaCl, 0.1 M tert-buty alcohol, and argon saturated. Taken at 0.3 ms after the end of a 300-ns pulse.}
The fact that an imidazole in papain remains protonated even at pH 8 is in itself interesting but the fact that this imidazole behaves differently with increasing pH when a similar experiment was performed on the blocked enzyme (CH$_3$-papain) is even more interesting (Faraggi and Klapper$^{64}$).

At low pH (Figure 34) both the 410-nm disulfide radical anion (RSSR$^{-}\cdot$) peak and the 360-nm imidazolium (ImH$^{+}$) band are present. At high pH (Figure 35) only the 410-nm RSSR$^{-}\cdot$ peak is apparent. The absorbance as a function of pH (Figure 36) suggests a pK$_{a}$ of 5.6. No band in the 390-nm region is apparent.

This shift in the pK$_{a}$ from pH 5.5 to above pH 8 when the thiol methyl group is removed from papain-SSCH$_3$ to form active papain correlates well to pK$_{a}$s derived by other methods that were described in the introduction for papain.
Figure 34. CH₃S-papain spectrum taken at pH 5.0. Both the 410-nm RSSR⁻ band and the 355-nm imidazolium band are easily seen. The conditions were as follows: [enz] = 95 µM, [e⁻aq] = 4.0 µM, 1 mM phosphate, 0.1 M tert-butyl alcohol, and argon saturated. Taken at 8 us after the pulse.

Figure 35. CH₃S-papain spectrum taken at pH 6.5. Only the 410-nm RSSR⁻ band is apparent. The conditions used were as follows: [enz] = 95 µM, [e⁻aq] = 4.9 µM, 1 mM phosphate, 0.1 M tert-butyl alcohol, and argon saturated. Taken at 8 us after the pulse.
**Figure 36.** Absorbance as a function of pH for CH₃S-papain. The results can be analyzed in terms of a single titration curve with a pKₐ of 5.63 ± 0.02 (see Appendix A for derivation) that is represented as the solid line. The conditions were as follows: \([\text{enz}] = 0.095-0.10\text{ mM}, [\text{e}^-\text{aq}] = 4.0-6.0\text{ mM}, 1\text{ mM phosphate, 0.1 M tert-butyl alcohol, and argon saturated. Taken at 8 us after the end of the pulse. The one-point titration method was used to obtain these data (see EXPERIMENTAL).}\)
**Subtilisins**

**BACKGROUND**

Subtilisin BPN' (or NOVO) is a bacterial protease that was first isolated by B. Hagihara. As a protease it is not specific toward any particular amino acid side chain. Subtilisin is not completely stable at any pH value, and preparations usually contain 10-40% autolysis products. Subtilisin is most stable at pH 7 and in the cold. Unfortunately, pH 7 is also the pH where subtilisin proteolytic activity is at its maximum.

Subtilisin BPN' is a single peptide chain of 275 amino acid residues with a molecular weight of 27,532. The molecule contains 22 amino acid residues that may be "seen" by pulse radiolysis: 6 histidines, 3 phenylalanines, 10 tyrosines, 3 tryptophans, and no cystine or cysteine. The protein is folded into three parts, resulting in a molecule that is close to sperical where the core is composed almost entirely of hydrophobic amino acid side chains. The molecule contains approximately 30% distorted α helices, some of which are parallel to each other, and the molecule also contains some peptide chain segments that form a twisted parallel chain pleated sheet. The active site is located in a shallow groove at the junction of the three folded parts. The active site serine-221 is in close proximity to the imidazole group of histidine-64, which is
in turn in close proximity to the carboxylic acid of aspar-
tic acid-32.67,68

The bacterial protease subtilisin Carlsberg (Mr = 27,287) was first isolated by Guntelberg and Ottensen.69
Its primary structure differs in only 84 out of the 275 amino acid residues found in the BPN' enzyme and in one deletion at position number 56. The secondary and tertiary structure of the Carlsberg enzyme is very close to the structure observed in the BPN' enzyme. The Carlsberg enzyme has 23 amino acid residues that may be seen by pulse radiolysis: 5 histidines, 13 tyrosines, 4 phenylalanines, and 1 tryptophan. The molecule has 1 less histidine, 3 more tyrosines, 1 more phenylalanine, and 2 less tryptophans. Like subtilisin BPN', subtilisin Carlsberg has no cystine or cysteine.67

Some work has been done on the active site of subtili-
sin. Results out of Bender's70-72 laboratory have shown that the pH profile for the acylation (K_cat) of subtilisin using p-nitrophenyl esters had a pK_a of about 7.2. When Omar et al.73 examined the low-field portion of the 360-MHz 1H NMR spectrum of PMS-subtilisin in D_2O as a function of pH, they reported four titratable resonances in the NMR spectral region that contains the histidyl C(2)-H resonances. They showed that these four resonances had uncorrected pK_a values ranging from 5.4 to 6.4, and they postulated that the two remaining histidyl residues gave rise to very broad
resonances that could not be used for determining pKₐs. Similarly, Jordan et al.⁷⁴ reported the histidyl C(2)-H resonances titrated with a pKₐ of 7 ± 0.5.

These experiments seem to leave no doubt that the active site imidazole has a normal pKₐ of around 7. But what is more interesting are some preliminary experiments done on the synthetic enzyme, thiosubtilisin, where the serine -OH has been replaced by a -SH. On the basis of the ¹H NMR resonance assigned to the C(1)-H of the active site histidyl, Jordan and co-workers⁷⁴,⁷⁵ reported that the active site imidazole may have a pKₐ above 8.4 and that modifying the active site changes the pKₐ from about 7 to above 8.4.
METHODS

Purification of Commercial Subtilisin BPN'. There are three published procedures for the purification of subtilisin BPN'. The first involves affinity chromatography where the cost of materials and the high salt concentration necessary to remove BPN' from the ligand make this technique undesirable. The second involves molecular separation on Sephadex G-25 or Sephadex G-50. Although this is a relatively fast technique, it only removes the smaller peptides present in all commercial BPN' preparations. I have found that the third technique, ion-exchange chromatography, gives almost 90% higher specific activity than gel filtration over Sephadex G-50.

Chromatography of Subtilisin BPN'. The procedure for the purification of subtilisin BPN' was taken from Polgar and Bender and modified. Instead of using carboxymethyl-cellulose as the separation medium, carboxymethyl-Sephadex C-50 was used with a stepwise elution profile with NaCl instead of a linear phosphate gradient.

One gram of subtilisin BPN' (608 mg based on $E^{1%} = 1.17$ absorbance units) in 20 mL was applied to a 5 x 28 cm water-jacketed column of Sephadex C-50 equilibrated with 10 mM phosphate-20 mM NaCl, pH 7.0, buffer that had been made with 5 times distilled, demineralized water (Figure 37). The flow rate was set at 7 mL/min, and both column and entering buffers were maintained at 3 °C. After the third
peak emerged, the buffer ionic strength was increased to 80 mM by making it 60 mM in NaCl. The fourth peak was collected in ice, separated into aliquots of 140 mg (based on OD) each, and frozen with dry ice (355 mg in 770 mL: 58% based on OD; 36% based on weight). Care must be taken in using the correct ionic strength since ionic strengths above 80 mM will release a fifth peak that has no activity.

On the day of the experiment, the frozen BPN' solution was thawed in warm water and quickly concentrated by ultrafiltration with an Amicon PM 10, 10K molecular weight cutoff
filter at 4 °C and 40 psi with slight agitation. After the
concentrate is collected, the concentration was determined
by spectroscopy and the solution diluted to the desired
concentration of 0.4 mM (11 mg/mL) for pulse radiolysis.

Because of the instability of BPN' preparations, chromo-
matography was done as quickly as possible at conditions
where the enzyme is most stable: 4 °C and pH 7. The eluant
must be collected quickly and stored frozen. I have found
that storage in the dilute form maintains a higher activity
than storage as a concentrate and concentration just prior
to radiolysis over an Amicon PM 10, 10K molecular weight
cutoff filter removes some of the small molecular weight
peptides that form even in the frozen state. I customarily
use BPN' within 4 days because loss of activity can exceed
20% if stored for more than that time. Five times distilled
water was used because the elution buffer was the radiolysis
buffer. Using other water would necessitate buffer exchange
over Sephadex G-25, which would mean loss of more activity
due to the extra time required for chromatography and recon-
centration.

Before pulse radiolysis was performed on BPN', the
solution was made 0.1 M in tert-butyl alcohol and degassed
in a syringe with prepurified argon for 20 min. The solu-
tion was then placed in a jacketed reservoir that was fitted
with a glass combination pH electrode and maintained at 4
°C. Transfer of the solution to the 1 cm path length cell
(0.3-mL volume) was done by the argon pressure system described under EXPERIMENTAL. The concentrations used were 0.4 mM enzyme, 10 mM phosphate, 60 mM NaCl, and 0.1 M tert-butyl alcohol.

In all cases, a 500-ns pulse yielding about 17 μM plus 5% electrons per pulse was used to obtain both spectra and the pH curve. The pH curve was obtained by varying the pH and taking several data traced at 360 nm for each pH. A spectrum was obtained by taking a data trace at varying wavelengths at a single pH. In all cases a fresh solution was used for each data trace, because the absorbance would be consistently higher if the solution in the cell were given a second pulse of electrons.

Purification of Commercial Subtilisin Carlsberg. The similarity between the enzymes allows the procedure for the purification of BPN\(^1\) to be extended to the purification of the Carlsberg enzyme. Figure 38 is an example of an elution profile obtained when 1 g of subtilisin Carlsberg (811 mg based on OD, ε\(^{1%}\) = 1.043 absorbance units) in 20 mL was applied to a 5 x 28 cm water-jacketed column of Sephadex C-50. In this case, the buffer ionic strength was increased to 80 mM. After the fourth peak emerged, the fourth peak was collected.

The conditions for the pulse radiolysis of subtilisin Carlsberg were exactly the same as those used with subtilisin BPN\(^1\). The same assay for activity used for
subtilisin BPN' was suitable for determining the activity of subtilisin Carlsberg, except the final specific activity was found to be lower (as expected). A typical specific activity for the pool collected after chromatography was 950 units/mg. A typical specific activity for subtilisin Carlsberg before application to the Sephadex C-50 column was 300-400 units/mg.

**Preparation of Thiosubtilisin BPN',67,70,78,79** The first step in the preparation of BPN'-SH involves the preparation of PMS-BPN'. Instead of purifying the active BPN' over Sephadex C-50, PMS-BPN' was prepared first and then
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purified in the inactive form. The conditions used for the purification over the Sephadex C-50 column were exactly the same as those used for active BPN' except the column was run at room temperature.

One gram (509 mg (OD)) of BPN' was dissolved into 23 mL of 0.1 M phosphate buffer, pH 7.5, which is the optimum pH for BPN' activity. Then 9 mg in 0.7 mL of dioxane was added very slowly to the slowly stirred solution containing BPN'. The blocking was allowed to occur for 40 min at which time the solution was tested and showed no activity with the PNPA assay.

The crude PMS-BPN' was applied to a 5 x 15 cm Sephadex C-50 column (Figure 39) that had been equilibrated with 0.01 M phosphate, pH 7.0, that was 0.2 M in NaCl. After the third peak emerged, the buffer salt concentration was increased to 0.06 M (arrow). The PMS-BPN' peak was collected and concentrated by ultrafiltration. Then the concentrate was diluted with 100 mL of 0.1 M imidazole buffer, pH 7.0, and concentrated again. This was repeated 3 more times and had the same effect as dialyzing against the same buffer but was faster. Once the phosphate was removed and the protein concentrated (334 mg/14 mL) to 20-25 mg/mL, the solution was made 0.1 M in CaCl$_2$ and 0.1 g/mL in sodium thioacetate. Then the mixture was gently swirled to dissolve the NaSCOCH$_3$ and CaCl$_2$ and placed in a water bath that was set for 39 °C. After 19-20 h the mixture was centrifuged and applied to a 5
Figure 39. Elution profile of PMS-subtilisin BPN' off a Sephadex G-50 column. The conditions were the same as those outlined in Figure 37 except the column was run at room temperature. The salt concentration of the elution buffer was changed from 20 to 60 mM at the arrow.

x 23 cm Sephadex G-25 column to remove the low molecular weight thiols and peptide fragments that developed during the incubation. The protein peak (250 mg (OD)) was immediately applied to a 2.5 x 6 cm Hg column that had been equilibrated with 1 mM EDTA, 10 mM Na$_2$SO$_3$, 50 mM citrate, and 100 mM NaCl, pH 6.2 (Figure 40). After the sample was added, the column was washed with 50 mM citrate and 0.2 M NaCl, pH 6.2, until the OD had decreased to below 0.01. At this point the elution buffer was applied (50 mM citrate, 0.5 M NaCl, 2 mM mercuric acetate, 10% ethanol (v/v), pH
Figure 40. Elution profile of thiosubtilisin BPN' off a Hg column. The column buffer was changed from 50 mM citrate and 0.2 M NaCl, pH 6.2, to 50 mM citrate, 0.5 M NaCl, 2 mM mercuric acetate, and 10% ethanol (v/v), pH 6.2, at the arrow. Hg-blocked thiosubtilisin was eluted as peak number 2.

6.2; at the arrow), thus releasing the thiosubtilisin in the Hg-blocked form (205 mg in 245 mL). The Hg-S-BPN' was concentrated by ultrafiltration and stored in the cold for later.

Before pulse radiolysis, the Hg-blocked thiosubtilisin was activated by introducing a 100 times molar excess of BME and allowing the Hg$^{2+}$ to transfer from BPN'-SHgX to the small molecular weight mercaptan. Then the mercaptan as well as the buffer was removed from the protein by using the same procedure that was used to remove the activator from
papain, a degassed Sephadex G-25 column equilibrated with the desired buffer made with 5 times distilled water.

**Rate Assay for Subtilisin Activity.** The BPN' activity assay used the p-nitrophenyl acetate assay as outlined by Polgar and Bender. In a typical assay, 100 mL of PNPA stock solution (36-41 mM PNPA in CH₃CN) was added to 3.00 mL of assay buffer (0.1 M phosphate, pH 7.5) and the nonenzymatic liberation of phenol anion was followed for a few minutes as an increase in absorbance at 400 nm. Then 10-100 μL of BPN' solution was added, and the faster enzymatic hydrolysis of PNPA was followed for about 5-6 min. The enzymatic rate is calculated after adjusting for the nonenzymatic hydrolysis of PNPA.

\[
\text{units/mL} = \frac{\Delta A/(\epsilon \cdot \lambda)}{\text{cm} \times \text{rate}} \times \frac{\text{total volume in cuvette (L)}}{\text{vol. of sample added to cuvette (mL)}}
\]

(42)

where \(\Delta A\) = change of absorbance over the time measured, \(\epsilon = 18,300 \text{ M}^{-1} \text{ cm}^{-1}\), \(\lambda = 1 \text{ cm}\), \(\text{cm} = \text{number of centimeters in which } \Delta A\text{ was measured on the chart recorder}\), \(\text{rate} = \text{chart recorder speed } = 4 \text{ cm/min}\), \(\text{volume of sample added to cuvette } = 0.100 \text{ mL or less}\), \(\text{unit/mL} = \text{activity of sample}\), and \(\text{total volume in cuvette } = 3.00 \text{ mL of buffer + volume of sample and substrate used}\). One unit of BPN' activity is defined as 1 nmol of p-nitrophenol anion formed per min per mL in 10 mM phosphate buffer, pH 7.5, at 22°C. The number of
milligrams is derived from knowing that 1 mg/mL = 1.17 absorbance units with a path length of 1 cm.

A typical specific activity for the pool collected after chromatography was 1300 units/mg. Typical specific activity for commercially available BPN before application to the Sephadex C-50 column was 300-400 units/mg.
RESULTS

The spectrum resulting from the reaction of e-aq with subtilisin BPN' is shown in Figure 41. The most apparent feature in the spectrum obtained at pH 5.6 is the peak centered at 350 nm. This feature is close to where the imidazolium adduct is reported to absorb in proteins (355-365 nm) but slightly lower in wavelength. Bisby et al. postulated that this peak contained the absorbances due to both tyrosine and imidazolium adducts since the rise was still there at pH 8. When spectra taken at pHs 7.40 and 7.83 (Figures 42 and 43) are compared to the spectrum obtained at 5.6 (Figure 41), it is quite apparent that the magnitude of the 350-nm peak relative to background is less. The absorbance of this peak as a function of pH gives rise to a curve that is shown to have a pK_\text{a} of 6.3 when a nonlinear fitting routine is used (Figure 44).

When the spectrum for the e-aq attachment to inactive PMS-subtilisin BPN' (Figure 45) is compared to the spectrum for the e-aq attachment to active subtilisin BPN' (Figure 41) obtained at the same low pH, the magnitude of the peak is much less--much like that of the peak observed in the spectrum for active subtilisin at high pH (Figure 43). This is strong evidence that the 350-nm peak does belong to the active site imidazole and this active site imidazole has a pK_\text{a} of 6.3 under the conditions where these data were taken.
Figure 41. Spectrum resulting from the e⁻aq reacting with subtilisin BPN' at pH 5.64. There is no RSRS⁻ band in the 410-nm region because subtilisin BPN' has no disulfides. The peak centered at 345-350 nm is probably a combination of imidazole, tyrosine, and tryptophan. The spectrum was taken in 10 mM phosphate that was 60 mM in NaCl, 0.1 M in tert-butyl alcohol, pH 5.64, and saturated with argon. \([\text{enz}] = 0.14 \text{ mM} \) and \([\text{e}^-\text{aq}] = 11.7 \mu \text{M}\). The X represents a spectrum taken at 10 μs after the end of the pulse. The + represents a spectrum taken at 50 μs after the end of a 400-ns pulse, 0 = 0.1 ms, * = 0.3 ms, # = 0.5 ms, and $\ast$ = 0.8 ms.

Figure 42. Spectrum of subtilisin BPN' at pH 7.40. Notice that the 345-350-nm peak has been reduced to a rise. The conditions used to take the spectrum were as follows: \([\text{enz}] = 0.13 \text{ mM} \), \([\text{e}^-\text{aq}] = 17.7 \mu \text{M} \), 10 mM phosphate, 60 mM NaCl, 0.1 M tert-butyl alcohol, and argon saturated. \(+ = 50 \mu \text{s} \), 0 = 0.1 ms, and $\ast = 0.3 \text{ ms}$ after the end of a 400-ns pulse.
Figure 43. Spectrum of subtilisin BPN' at pH 7.03. The 345-350-nm peak is still there. The conditions used were as follows: [enz] = 0.16 mM, [eaq] = 16 μM, 10 mM phosphate, 60 mM NaCl, 0.1 M tert-butyl alcohol, and argon saturated. X = 10 μs, + = 50 μs, 0 = 0.1 ms, and * = 0.6 ms after the end of the pulse.

Figure 44. Absorbance at 350 nm as a function of pH for subtilisin BPN'. The results can be analyzed in terms of a single titration curve with a pK_a of 6.34 ± 0.64 (see Appendix A for derivation) that is represented as the solid line. The conditions were as follows: [enz] = 0.40 mM, [eaq] = 20 μM, 10 mM phosphate, 60 mM NaCl, 0.1 M tert-butyl alcohol, and argon saturated. Taken at 0.5 ms after the end of a 500-ns pulse. The one-point titration method was used to obtain these data (see EXPERIMENTAL).
Figure 45. Spectrum of PMS-subtilisin BPN' at pH 5.50. Note that the 345-350-nm peak is just a rise when compared to the peak obtained for active subtilisin BPN' at pH 5.64 (Figure 41). The conditions used were as follows: [enz] = 0.15 mM, [a'-aq] = 14 μM, 10 mM phosphate, 0.1 M tert-butyl alcohol, and argon saturated. X = 10 μs, + = 50 μs, o = 0.1 ms, and 0 = 0.8 ms after the end of a 400-ns pulse.
The spectrum obtained for subtilisin Carlsberg at low pH (Figure 46) is similar to the spectra obtained for subtilisin BPN' at low pH (Figure 41). Like BPN', the Carlsberg enzyme has no disulfide and hence no 410-nm band. It does have a peak at 350 nm whose magnitude is reduced at higher pHs (Figure 47). This peak also titrates with a $pK_a$ of 6.3 (Figure 48).

Boric acid is reported to coordinate with the active site of subtilisin and mimic the proposed transition-state intermediate and force the imidazole to remain protonated even at a pH that is well above the $pK_a$ for the active site imidazole.\textsuperscript{15,74,75,80} Boric acid was also shown not to react with e$_{-aq}$ or at least very slowly since it has been used to buffer solutions for radiolysis. Yet at low pH, only a small rise could been seen in a spectrum of subtilisin Carlsberg that contained 0.3 M boric acid (Figure 49).

Increasing the pH from 5.5 to 7.8 does little to the spectrum: the small rise is still apparent (Figure 50). The spectrum at low pH for subtilisin BPN' in the presence of boric acid also shows a small rise (Figure 51). The relative magnitude of the rise at 350 nm relative to background in all three spectra (Figures 49-51) compared quite well to the rise observed with spectra obtained for subtilisin BPN' or Carlsberg at high pH. This suggests that the rise is due to the tyrosine-electron adduct and that the
Figure 46. Spectrum of subtilisin Carlsberg at pH 5.50.
Like subtilisin BPM, subtilisin Carlsberg has no disulfides
and hence no band in the 410-nm region and has a band at
345-350 nm. The conditions used were as follows: [enz] =
0.16 mM, [e-aq] = 15.4 pH, 10 mM phosphate, 60 mM NaCl, 0.1
M tert-butyl alcohol, and argon saturated. X = 10 us, *=
50 μs, α = 0.1 ms, and γ = 0.8 ms after the end of the
pulse.

Figure 47. Spectrum of subtilisin Carlsberg at pH 7.90.
Notice how small the 345-350-nm peak is compared to that of
a similar spectrum at a lower pH (Figure 46). The condi­
tions used were as follows: [enz] = 0.16 mM, [e-aq] = 13.4
pH, 10 mM phosphate, 60 mM NaCl, 0.1 M tert-butyl alcohol,
and argon saturated. *= 50 μs, α = 0.1 ms, and γ = 0.8 ms
after the end of the pulse.
Figure 48. Absorbance at 350 nm as a function of pH for subtilisin Carlsberg. The results can be analyzed in terms of a single titration curve with a pKₐ of 6.28 ± 0.35 (see Appendix A for derivation) that is represented as the solid line. The conditions used were as follows: [enz] = 0.40 mM, [e⁻aq] = 36 μM, 10 mM phosphate, 50 mM NaCl, 0.1 M tert-butyl alcohol, and argon saturated. Taken at 0.1 ms after the end of a 500-ns pulse. The one-point titration method was used to obtain these data (see EXPERIMENTAL).
Figure 49. Spectrum of subtilisin Carlsberg at pH 5.50 in the presence of B(OH)₃. Boric acid appears to reduce the 345-350-nm band from the peak observed in the absence of B(OH)₃ (Figure 48) to a rise. The conditions used were as follows: [enz] = 0.25 mM, [e-aq] = 18.6 μM, 10 mM phosphate, 60 mM NaCl, 0.3 M tert-butyl alcohol, 0.3 M B(OH)₃, and argon saturated. X = 10 μs, + = 50 μs, o = 0.1 ms, and ◆ = 0.8 ms after the end of the pulse.

Figure 50. Spectrum of subtilisin Carlsberg at pH 7.80 in the presence of B(OH)₃. The conditions used were as follows: [enz] = 0.16 mM, [e-aq] = 15.4 μM, 10 mM phosphate, 60 mM NaCl, 0.1 M tert-butyl alcohol, 0.3 M B(OH)₃, and argon saturated. + = 50 μs, o = 0.1 ms, and ◆ = 0.8 ms after the end of the pulse.
Figure 51. Spectrum of subtilisin BPN' at pH 5.50 in the presence of $\beta(\text{OH})_3$. The conditions used were as follows: 

- $[\text{enz}] = 0.15$ mM, $[\alpha\text{-aq}] = 14$ mM, 10 mM phosphate, 60 mM NaCl, 0.1 M tert-butyl alcohol, 0.3 M $\beta(\text{OH})_3$, and argon saturated.
- $\times = 10$ ms, $+ = 50$ ms, $\circ = 0.1$ ms, and $\square = 0.5$ ms after the end of the pulse.
boric acid inhibits the formation of the electron-imidazole adduct that has been observed in spectra at low pH where no borate was present.

The CO$_2^-$ radical has been reported to be very effective in reducing disulfides, somewhat effective in reducing imidazoles at very, very low pH, and ineffective in reducing other amino acid residues. Figure 52 is a spectrum taken at pH 5.6 under conditions where the CO$_2^-$ radical was the reducing species. As expected, there is no band in the imidazolium region, only a shoulder. The fact that there is any absorbance at all is surprising.

The chemical modification of the -OH to a -SH on subtilisin BPN' resulted in a spectrum (Figures 53 and 54) that differed little from the spectrum obtained for native subtilisin BPN' (Figure 41) and native subtilisin Carlsberg (Figure 46) at pH 5.5. Like the Carlsberg and the BPN' enzymes, the magnitude of the 350-nm band decreases relative to background with increasing pH (Figures 53-56), and like the Carlsberg and BPN' enzymes a rise is still apparent in the spectrum at pH 8.18 (Figure 56). The yield of the 350-nm band with pH (Figure 57), behaves exactly like that of the Carlsberg and BPN' enzymes (Figures 48 and 44) with a pK$_a$ of 6.4.
Figure 52. Spectrum from the reduction of subtilisin BPN’ by CO$_2^-$ at pH 5.61. Notice that there is no peak and no real rise in the 345-350-nm region of the spectrum, only a shoulder. The conditions used were as follows: [enz] = 0.15 mM, [e$^{-aq}$] = 12.6 μM, 10 mM phosphate, 60 mM NaCl, 0.1 M sodium formate, and argon saturated. X = 10 μs, + = 50 μs, and o = 0.1 ms after the end of a 300-ns pulse.

Figure 53. Spectrum of thiosubtilisin BPN’ at pH 5.47. Note that the spectrum shape is quite similar to the spectrum shape seen for both subtilisin BPN’ (Figure 42) and subtilisin Carlsberg (Figure 46) at the same low pH. The conditions used were as follows: [enz] = 0.20 mM, [e$^{-aq}$] = 6.7 pH, 10 mM phosphate, 10 mM NaCl, 0.1 M tert-butyl alcohol, and argon saturated. X = 25 μs, + = 0.3 ms, o = 0.3 ms, and + = 0.5 ms after the end of a 200-ns pulse.
Figure 54. Spectrum of thiosubtilisin BPN' at pH 5.97. Note that the band at 345-350 nm is reduced relative to that of the spectrum taken at pH 5.47 (Figure 53). The conditions used were as follows: [enz] = 0.20 mM, [e-aq] = 10 mM, 10 mM phosphate, 10 mM NaCl, 0.1 M tert-butyl alcohol, and argon saturated. X = 25 μs, ++ = 0.1 ms, a = 0.3 ms, and s = 1.6 ms after the end of a 400-ns pulse.

Figure 55. Spectrum of thiosubtilisin BPN' at pH 7.44. Note that the band at 345-350 nm is now a rise. The conditions used were as follows: [enz] = 0.20 mM, [e-aq] = 10 mM, 10 mM phosphate, 10 mM NaCl, 0.1 M tert-butyl alcohol, and argon saturated. ++ = 0.1 ms, a = 0.3 ms, * = 0.5 ms, and s = 1.6 ms after the end of a 300-ns pulse.
Figure 56. Spectrum of thiosubtilisin BPN' at pH 0.18. The conditions used were as follows: [enz] = 0.22 mM, [e^aq] = 22 μM, 10 mM phosphate, 10 mM NaCl, 0.1 M tert-butyl alcohol, and argon saturated. + = 0.1 ms, o = 0.3 ms, and • = 1.6 ms after the end of a 500-ns pulse.

Figure 57. Absorbance at 350 nm as a function of pH for thiosubtilisin BPN'. The results can be analyzed in terms of a single titration curve with a pK_a of 6.36 ± 0.32 (see Appendix A for derivation) that is represented as the solid line. The conditions used were as follows: [enz] = 0.30 mM, [e^aq] = 25 μM, 10 mM phosphate, 60 mM NaCl, 0.1 M tert-butyl alcohol, and argon saturated. Taken at 0.1 ms after the end of a 500-ns pulse. The one-point titration method was used to obtain these data (see EXPERIMENTAL).
Discussion

All of the proteins studied have one thing in common: they all contain the three functional groups necessary to fit the criteria proposed by Blow et al. for a "charge relay," namely, an Asp-His-Ser for subtilisin and α-chymotrypsin and an Asp-His-Cys for papain and thiosubtilisin BPN'.

When Blow et al. first reported a buried aspartic acid in α-chymotrypsin using X-ray diffraction, they suggested that its role in catalysis was to increase the nucleophilicity of serine-94 by effectively shifting the equilibrium position of the histidine and serine protons toward the aspartic acid. This would effectively increase the negative charge on the serine hydroxyl, i.e., the "charge relay system" (Figure 58).

![Figure 58. "Charge relay" proposed by Blow et al.](image)

This report initiated a storm of experiments and mechanistic proposals to substantiate or refute the Blow et al. proposal.)
Further refinement of the α-chymotrypsin X-ray coordinates showed that there is no strong hydrogen bond between the Y-hydroxyl of serine and the N(3) of histidine in what is called the "resting state" of the enzyme.\(^{81}\) The nitrogen-oxygen distance was found to be too long and at an improper angle for optimal hydrogen bond formation. Instead, there is a water molecule (or sulfate molecule from crystallization for X-ray) bound to the serine hydroxyl and a second water molecule coordinated to the N(3) nitrogen of histidine-57. However, X-ray data continue to show that there is a hydrogen bond between the buried aspartic acid-132 and the N(1) nitrogen of histidine-57. Thus, the proposed "charge relay" is not complete in the resting state of the enzyme. But, there are X-ray and NMR data that do suggest that the "charge relay" does exist after the active site undergoes a conformational change, resulting from enzyme interacting with substrate.

α-Chymotrypsin. In light of the X-ray data, it would first appear that chemical modification of the active site serine would not have any effect on the active site imidazole pK\(_a\). Actually, a subtle conformational change has been shown to occur when a chymotrypsin-PMS-chymotrypsin (serine blocked) X-ray difference map was made.\(^{23,81}\) A conformational change at the active site has been shown to occur when the inactive zymogen is activated to chymotrypsin.\(^{82}\) And a subtle conformational change at the active site is
reported to occur when chymotrypsin is made inactive by methylating the N(3) position of the active site histidine. These reports would give some credibility to the theory that the loss of the 360-nm imidazolium band seen in the pulse radiolysis of inactive forms of α-chymotrypsin is due to the loss of the mechanism by which the solvated electron migrates to the active site imidazole as a result of the active site undergoing a conformational change. No X-ray work on anhydrochymotrypsin has been reported, but it is not unreasonable to expect some conformational change to occur when this inactive form of α-chymotrypsin is made.

A second possible reason why no 360-nm band is observed in any of the blocked forms of α-chymotrypsin as well as the zymogen is based on the water molecules that have been reported to be hydrogen bonded to the active site serine hydroxyl and the N(3) nitrogen of the active site histidine. X-ray data have shown that methylation of the active site imidazole displaces the water molecule normally hydrogen bonded to this site. The bulky PMS group displaces the water molecule that is normally hydrogen bonded to the serine hydroxyl and may even disrupt the hydrogen bond between the active site imidazole and its water molecule. α-Chymotrypsinogen has no water molecules at the active site, and finally, anhydrochymotrypsin has no serine for water to coordinate to. Perhaps one or both of these water molecules are required for proper migration of the
solvated electron to the active site imidazole. Displacement of such a water molecule would result in no 360-nm imidazolium band.

Finally, a third possibility why PMS-chymotrypsin and PMS-chymotrypsin alone has no 360-nm band could be that PMS can also absorb electrons. It is possible that PMS captures the solvated electron before it reaches the imidazole or, in the more unlikely case, once the imidazole receives the electron it immediately transfers the electron to PMS, thus yielding no 360-nm band. The methyl group as well as the dehydroalanine group in anhydrochymotrypsin would not be expected to react with the solvated electron to any competitive degree.

What is clear is that no titratable 360-nm imidazolium band can be seen when a pulse radiolysis spectrum is taken of any of the inactive forms of α-chymotrypsin.

Papain. The pulse radiolysis results obtained for papain nicely parallel the results obtained by other techniques. A \( pK_a \) of 5.5 was obtained for CH\(_3\)S-papain, which shifted to above pH 8 when a similar experiment was done on fully active (+95%) papain (Figures 36 and 33). The pI point of 9.6 combined with the high concentration of papain required for pulse radiolysis made working at pHs higher than pH 8 impossible; thus, the \( pK_a \) for the native enzyme could not be obtained.
The pKₐ of 5.5 obtained by pulse radiolysis for the blocked enzyme is a little high when compared to the pKₐ of 4.0 ± 0.5 obtained by other techniques. However, the ionic strength used with pulse radiolysis was about 5 times lower than the ionic strength used by others and the problem in accurately compensating for solvated electron loss due to reaction with the proton below pH 5 could

$$\text{H}^+ + e^-_{aq} \rightarrow \text{H}^\cdot$$

both be contributing factors to the difference in the pKₐs. The close proximity of the potentially reactive disulfide in the form of the blocking groups, -SCH₃, could also contribute to the difference in the pKₐs.

Subtilisin. The subtilisin data are quite interesting. First of all, the imidazole peak is not in the correct place (Figures 41 and 46, and this peak, although reduced in amplitude, is still present (Figures 43 and 47) well above the apparent pKₐ of 6.3 (Figures 44 and 48) observed for these enzymes when pulse radiolysis is used. Bisby et al. made the same observation and postulated that the peak is most likely due to a combination of histidine, tyrosine, and tryptophan. This is a quite plausible explanation considering histidine absorbs at 360 nm, tryptophan absorbs at 345 nm, and tyrosine absorbs at 355 nm and both enzymes contain these residues (see Figure 1). In fact, Bisby et al. have shown that in both subtilisin Carlsberg and
subtilisin BPN' at least one tyrosine and one tryptophan are solvent accessible.

The fact that the modification of the active site serine to a cysteine with subtilisin BPN' resulted in no change from the apparent pKₐ of 6.3 (Figures 44, 48, and 57) observed with both native subtilisins was surprising since the pulse radiolysis results obtained from the blocking experiments on both α-chymotrypsin and papain have resulted in changes in the yield in the imidazole region of the spectrum, a shift in the pKₐ, or loss of the imidazole band altogether. Furthermore, a shift in the pKₐ to a high pH was expected based on some preliminary NMR reports. The fact that introduction of a PMS group to the active site serine of subtilisin BPN' resulted in reduction of the imidazole portion of the spectrum taken at the same low pH alleviated some of the concerns that the imidazole seen by pulse radiolysis was not the active site imidazole. Further support for the pH dependence of the imidazole region belonging to the active site imidazole comes from subtilisin data taken where a high concentration of boric acid was present.

Boric acid has been used by NMR spectroscopists to study both α-chymotrypsin and subtilisin.15,74,75,80 It is believed to coordinate at the active site of these enzymes and mimic the proposed tetrahedral intermediate (Figure 59).
Figure 59. Boric acid mimicking proposed transition-state intermediate.

In the case of subtilisin, the boric acid is reported to force the active site imidazole to be protonated even at pH 8.18,74,75,92 Yet like the results obtained with PMS-subtilisin BPN', both subtilisin BPN' and subtilisin Carlsberg appear to have no imidazole contribution to the 350-nm peak at low pH (Figures 49 and 51). The relative magnitude of the peak is about the same at low pH as at high pH.

It is difficult to understand why this would occur. Boric acid has no affect on the electron decay and therefore would not compete with the imidazole for the electron. The conformational change that has been reported to occur in subtilisin82 (as well as other serine proteases)23,81,82 during turnover could contribute to this observation. The possibilities used to explain the observed loss of the imidazole band with α-chymotrypsin could also be used to explain the PMS and boric acid results: perturbation of the active site structure and expulsion of water molecules normally located in the active site during the "resting stage" of the enzyme.
The pKₐs. The pKₐ for papain has already been discussed. The pKₐ for the subtilisin is well within the range for pulse radiolysis. However, the low pKₐ reported by Faraggi et al.⁹,¹⁰ for α-chymotrypsin in light of the general view held now that the active site imidazole pKₐ is around 7 and my results with blocked α-chymotrypsin showing that the imidazole that Faraggi et al.⁹,¹⁰ titrated was the active site imidazole is still disturbing.

What if the pKₐ of 4.2 for α-chymotrypsin reported by Faraggi et al.⁹,¹⁰ is not the pKₐ for the active site imidazole but reflects the pKₐ for the adjacent aspartic acid that is reported to participate in a tight bond to the active site imidazole? The pKₐ agrees quite well with the pKₐ given to the active site aspartate by groups using NMR.¹⁷,⁸¹ The current hypothesis is that perhaps the active site imidazole does not have a sufficient positive charge to attract the solvated electron until the aspartic acid for which it shares a proton becomes protonated. Below pH 4 the imidazole becomes fully protonated and can accept solvated electrons. At higher pHs, the imidazole does not have a sufficient positive charge to attract e⁻aq and therefore cannot be seen. The introduction of blocking groups could result in perturbation of the active site that ultimately results in the lowering of the pKₐ of Asp and/or displacement of water located at the active site that are necessary
for electron migration to the imidazole. What is clear is the fact that the electron can "see" the active site (Figure 60).

Figure 60. Diagram representing the hypothesis that the pK$_a$ reported for the active site imidazole of chymotrypsin may actually reflect the pK$_a$ for the active site aspartic acid. Part A represents the active site above the reported active site histidyl pK$_a$ of 7. Part B represents the active site at an intermediate pH where the active site imidazole is partially protonated and cannot be "seen" by the solvated electron. Part C represents the active site where the aspartic acid has become protonated.
IV. DISULFIDES

BACKGROUND

The most distinctive feature in transient optical spectra resulting from the pulse radiolysis (e-aq) of most proteins is a peak in the region of 400-420 nm. This peak has been attributed to the disulfide radical anion (RSSR-) that occurs when e-aq attaches to a disulfide in the protein. However, the yields of RSSR- vary from protein to protein even when the number of disulfides present in each protein molecule is taken into account. The pulse radiolysis spectra of porcine RNase at low pH show no absorbance due to RSSR-. Only 40% of the electrons reacting with α-chymotrypsin reside on the disulfide in the protein,9,10 25% with papain,63 and 60% with trypsin.63 It appears that the tertiary structure of a protein plays a role in the yield of RSSR- in the molecule.2 Save for the variation in RSSR- yields, very little is known about the role of protein tertiary structure in the scavenging and migration of e-aq generated by pulse radiolysis. However, the mechanism and action of radiolysis on the individual
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amino acid functionalities in proteins are fairly well understood from the large amounts of data collected on the radiolysis of amino acids and simple molecules in aqueous solutions. Consequently, much of the pulse radiolysis work that has been done on simple disulfides can be and often is used as a basis to explain spectral features observed in more complex molecules such as proteins.

It appears that the rate at which \( e^-_{aq} \) attaches to disulfides depends on the charge on the molecule near the disulfide, which in turn is dependent on pH. When the pH is raised from 6.2 to 12.1, the second-order rate constant for the attachment of \( e^-_{aq} \) to cystine decreases from \( 1.5 \times 10^{10} \) to \( 0.5 \times 10^{10} \) M\(^{-1}\) s\(^{-1}\) (Table 1).\(^93\)

Table 1. Rate Constants of \( e^-_{aq} \) with Some Disulfides\(^93\)

<table>
<thead>
<tr>
<th>disulfide</th>
<th>( k(e^-_{aq} + RSSR), M^{-1} \text{s}^{-1} )</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>cystine ([-\text{SCH}_2\text{CH(NH}_3^+\text{)COOH}_2])</td>
<td>(1.5 \times 10^{10})</td>
<td>6.4</td>
</tr>
<tr>
<td></td>
<td>(0.5 \times 10^{10})</td>
<td>12.1</td>
</tr>
<tr>
<td>penicillamine disulfide ([-\text{SC(CH}_3)_2\text{CH(NH}_3^+\text{)COOH}_2])</td>
<td>(1.2 \times 10^{10})</td>
<td>7.0</td>
</tr>
<tr>
<td></td>
<td>(0.35 \times 10^{10})</td>
<td>12.4</td>
</tr>
<tr>
<td>(\beta,\beta')-dithiodipropionic acid ([-\text{SCH}_2\text{CH}_2\text{COOH}_2])</td>
<td>(0.44 \times 10^{10})</td>
<td>6.4</td>
</tr>
<tr>
<td></td>
<td>(0.43 \times 10^{10})</td>
<td>10.8</td>
</tr>
</tbody>
</table>

A similar reduction in the rate constant occurs with penicillamine disulfide. However, dithiodipropionic acid

...
(DTDPA) shows no reduction in rate. Apparently the decrease in the rate constant reflects the loss of the positive charge as the pH is raised above the pKₐ for the amine. The rate constant reported for the two amine-containing disulfides at high pH (where the amine is unprotonated) is close to the rate reported for DTDPA at neutral pH. DTDPA does not contain an amine.

The charged environment around RSSR can also play a role in the rate for the disappearance of RSSR⁻.

\[ RSSR⁻ → RS⁻ + RS⁻ \]  \hspace{1cm} (44)

Cystamine and cystine dimethyl ester RSSR⁻'s decay faster at high pH than at neutral pH (Table 2).93

Table 2. Rate Constants for the Decay of Some Disulfides93

<table>
<thead>
<tr>
<th>disulfide</th>
<th>pH</th>
<th>k, s⁻¹</th>
<th>pKₐ of NH₃⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>cystamine disulfide ([-\text{SCH₂CH₂NH₃⁺}]₂)</td>
<td>7.0</td>
<td>(0.35 \times 10^6)</td>
<td>8.82, (X 10^6)</td>
</tr>
<tr>
<td></td>
<td>12.0</td>
<td>(1.25 \times 10^6)</td>
<td>9.16</td>
</tr>
<tr>
<td>cystine dimethyl ester disulfide ([-\text{SCH₂CH(NH₃⁺)COOCH₃]}₂)</td>
<td>6.5</td>
<td>(0.20 \times 10^6)</td>
<td>6.9</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>(0.60 \times 10^6)</td>
<td></td>
</tr>
<tr>
<td>glutathione disulfide (\text{HOOCCH(NH₃⁺)CH₂}) (\text{HOOCCH₂NHCHCHNC≡O})\ \text{CH₂-S⁻J₂})</td>
<td>7.0</td>
<td>(0.20 \times 10^6)</td>
<td>8.57, (X 10^6)</td>
</tr>
<tr>
<td></td>
<td>12.0</td>
<td>(0.20 \times 10^6)</td>
<td>9.54</td>
</tr>
</tbody>
</table>

It appears that the loss of the proton from the amino group destabilizes RSSR⁻, resulting in a shorter lifetime.
However, the decay rate for glutathione RSSR\(^-\) does not change after the amino group is deprotonated. Apparently the amines on glutathione RSSR are too far from the disulfide to affect the decay rate of RSSR\(^-\). The products from the decay of RSSR\(^-\) (equation 44) have a very low extinction coefficient in the region from 300 to 600 nm. The thiolate anion absorbs below 300 nm and the thyl radical has a band centered near 330 nm with an extinction coefficient of about 300 M\(^-1\) cm\(^{-1}\).93

The \(\lambda_{\text{max}}\) for simple disulfide radical anions varies quite widely from 400 nm for dithiodiacetic acid to 450 nm for penicillamine disulfide at neutral pH (Table 3). The dimer of cysteine, cystine, has a \(\lambda_{\text{max}}\) at 420 nm. The extinction coefficient is about \(9 \times 10^3\) M\(^-1\) cm\(^{-1}\) for all three disulfides.93

Table 3. Spectral Maxima for Some Simple Disulfides\(^{93}\)

<table>
<thead>
<tr>
<th>disulfide</th>
<th>(\lambda_{\text{max}}, \text{nm})</th>
<th>(\varepsilon, \text{M}^{-1}) cm(^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>dithiodiacetic acid ([\text{-SCH}_2\text{COOH}]_2)</td>
<td>400</td>
<td>(9.5 \times 10^3)</td>
</tr>
<tr>
<td>penicillamine disulfide</td>
<td>450</td>
<td>(&gt;8.8 \times 10^3)</td>
</tr>
<tr>
<td>cystine</td>
<td>420</td>
<td>(7.3 \times 10^3)</td>
</tr>
</tbody>
</table>

The \(\lambda_{\text{max}}\) for all disulfides remains invariant with pH except for cyclic disulfides.93,94 As the pH is decreased
in the case of lipoic acid (thiotic acid), the $\lambda_{\text{max}}$ shifts from 405 to 385 nm, where the species absorbing at 385 nm has been attributed to a protonated disulfide radical anion ($\text{RSSRH}^-$)\textsuperscript{93,94} (Figure 61).

\[
\begin{align*}
\text{RSSR}^- & + \text{H}^+ \rightarrow \\
\text{RSSR}^\cdot
\end{align*}
\]

![Figure 61. Two possible forms of the protonated disulfide radical anion of lipoate.](image)

A $pK_a$ of 5.4\textsuperscript{93} and a $pK_a$ of 5.8\textsuperscript{94} have been assigned to this transition between the protonated and unprotonated disulfide radical (equation 45).

However, there are conflicting reports concerning the pulse radiolysis of the six-membered ring cyclic disulfide, oxidized dithiothreitol (ox-DTT). Redpath\textsuperscript{95} reports a $\lambda_{\text{max}}$ at 390 nm, no blue shift with decreasing pH, and a large reduction in the extinction coefficient at 390 nm with decreasing pH that gives a $pK_a$ of 5.0. Chan et al.\textsuperscript{96} report a $\lambda_{\text{max}}$ at 405 nm, a 20-nm blue shift with decreasing pH, and a large reduction in the extinction coefficient at 405 nm with decreasing pH that gives a $pK_a$ of 5.5. The blue shift reported by Chan et al. nicely parallels the blue shift
observed with lipoic acid; however, the extinction coefficient at pH 1 (250 M⁻¹ cm⁻¹) is 27 times lower than the extinction coefficient reported for lipoic acid at the same pH (6900 M⁻¹ cm⁻¹). Redpath's report more nearly falls in line with the results reported for linear disulfides—no blue shift with decreasing pH and a very small extinction coefficient at low pH. Both authors agree that the ox-DTT radical anion decays by second-order kinetics and has a lifetime in the hundreds of microseconds at neutral pH, which is comparable to that of the lipoic acid anion radical at the same concentration.

A blue shift as a function of pH has never been reported in any linear disulfide. In the case of glutathione disulfides, the loss of absorbance as a function of decreasing pH has an apparent pKₐ of 5.2 but with no associated blue shift.97,98 Shafferman postulates that RSSR⁻ is rapidly protonated to RSSHR, which in turn is rapidly cleaved to form RS· and RSH (equation 46). Thus, RSSRH has not been seen.

\[
\text{RSSR}^- + \text{H}^+ \rightarrow [\text{RSSHR}^-] \rightarrow \text{RS}^+ + \text{RSH} \quad \text{(46)}
\]

Linear disulfides decay rapidly by first-order kinetics (5 \times 10⁻⁷ s⁻¹), and their half-life decreases dramatically as the pH is decreased below neutral pH. The half-life for the same concentration of lipoic acid anion also decreased with decreasing pH but through second-order kinetics and not
first-order kinetics. It is generally agreed that the cyclic structure of lipoic acid hinders the usual first-order dissociation observed with linear RSSR\(^-\), leaving lipoic acid RSSR\(^-\) to decay through disproportionation (Figure 62). This stabilization by the ring forces the lipoic acid radical to have a lifetime of several hundred microseconds instead of the few microseconds observed with linear disulfides. Stabilization of RSSR\(^-\) by the ring may stabilize RSSHR\(^+\) as well.

Disulfides in Proteins. A review of the literature reveals that the \(\lambda_{\text{max}}\) attributed to a disulfide in proteins can range from 405 to 425 nm depending on the pH and the protein (Table 4). In addition, the decay for the 415-nm disulfide peak in papain has been described as being complex and not simply first or second order corresponding to the type of decay rates observed with small linear or cyclic disulfides. Considering that papain has three disulfides.

\[
\begin{align*}
\text{RSSR} \quad &\xrightarrow{\text{cleavage}} \quad \text{RS}^- + \text{RS}^-
\end{align*}
\]

\[
\begin{align*}
\text{RSSR} \quad &\xrightarrow{2\text{H}^+} \quad \text{ASH SR} + \text{RSSR}
\end{align*}
\]

Figure 62. Diagram showing why cyclic disulfide radical anions cannot undergo first-order decay.
and considering that $\lambda_{\text{max}}$ for RSSR$^-$ and decay kinetics are dependent on adjacent charge groups and the geometric structure of the disulfide, there is no reason why proteins with more than one disulfide cannot have complex decay kinetics in the RSSR$^-$ region of the spectrum and multiple disulfide $\lambda_{\text{max}}$.

Table 4. $\lambda_{\text{max}}$ for Disulfide-Containing Proteins

<table>
<thead>
<tr>
<th>Protein</th>
<th>$\lambda_{\text{max}}$, nm</th>
<th>pH</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha$-chymotrypsin</td>
<td>405</td>
<td>5.0</td>
<td>10</td>
</tr>
<tr>
<td>RNase</td>
<td>410</td>
<td>8.5</td>
<td>10</td>
</tr>
<tr>
<td>Trypsin</td>
<td>400 405</td>
<td>4.4</td>
<td>10</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>425 420</td>
<td></td>
<td>99 89</td>
</tr>
<tr>
<td>Papain</td>
<td>415</td>
<td></td>
<td>63</td>
</tr>
</tbody>
</table>

It is well-known that protein molecules contain what are often characterized as hydrophilic and hydrophobic regions. A disulfide in a hydrophilic environment may have a different $\lambda_{\text{max}}$ and decay kinetics than a disulfide in a hydrophobic region. Adjacent charge groups appear to affect the $\lambda_{\text{max}}$ and decay kinetics of disulfide radicals; however, nothing is known about the effect that solvent has on the $\lambda_{\text{max}}$ and decay kinetics of disulfide radicals.
METHODS

Pentadistilled demineralized water was used when water was the solvent (see page 32). Buffers were 10 mM in phosphate and 0.1 M tert-butyl alcohol in pentadistilled water. The purest ethylenediamine was refluxed with a small amount of LiAlH₄ for 4-6 h and distilled under argon, taking the middle fraction. The same procedure was used to prepare tetrahydrofuran (THF). Absolute ethanol was the purest grade available and was used without further purification. Alkaline ethanol was made by saturating absolute ethanol with dried NaOH. Acidic ethanol was made by adding a very small amount of concentrated HCl to give a final concentration of 0.1-1 mM HCl. Lipoic acid and lipoamide were obtained from Sigma Biochemical Co. Oxidized dithiothreitol (ox-DTT) and dithiodipropionic acid (DTPA) were obtained from Aldrich Chemical Co. All were used without further purification.

The solutions were degassed in foil-wrapped syringes with argon for 30 min just prior to pulse radiolysis. Because the analyzing light increased the yield of the disulfide radical (anion), presumably through a photolyzation reaction, a minimum amount analyzing light was routinely and reproducably used to take data. This resulted in a significant improvement in reducing the scatter on each
spectrum. The previous solution was routinely used to adjust the initial light intensity ($I_0$) before the fresh solution was introduced into the cell. The fresh solution never saw the analyzing light until the trace data were taken. In all the spectra reported, each point at each respective time and wavelength represents the average of two or more absorption values.

When water was the solvent, tert-butyl alcohol was added to scavenge for the oxidizing species, $\cdot$OH. Since the identity of the oxidizing species in the other solvents used (i.e., EDA, THF, and ethanol) are not known (except for ethanol) and would be difficult to scavenge for anyway, the oxidizing species were not dealt with. In addition, the oxidizing species ($\cdot$OH) is not reported to react with disulfides and by analogy the oxidizing species generated in the other solvents would not be expected to do so either.
RESULTS

Effect of Solvent on the $\lambda_{\text{max}}$ and Decay Kinetics of Various Disulfides. Lipoic Acid. The $\lambda_{\text{max}}$ in the spectra resulting from the one-electron reduction of lipoic acid shows a blue shift from 406 to 384 nm when the pH is decreased from 8.05 to 4.60 (Figure 63). This shift has been reported by others and has been attributed to the protonation of the disulfide radical anion (equation 47).

$$\text{RSSR} + H^+ \rightarrow \text{RSSR}^\cdot \quad (47)$$

The blue shift in the $\lambda_{\text{max}}$ as a function of decreasing pH is complemented by a change in the second-order decay rate from $2k = 0.77 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$ at pH 8.05 to $2k = 6.1 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$ at pH 4.60. Figure 64 in an example of the
Figure 63. Lipoic acid in water. The $\lambda_{\text{max}}$ in the spectra resulting from the one-electron reduction of lipoate shows a blue shift from 406 to 384 nm when the pH is decreased from 8.05 to 4.60. The conditions used were as follows: $x = 5$ mM lipoic acid, 20 $\mu$M $e^aq$, 10 mM phosphate, 1.5 M tert-butyl alcohol, pH 4.60, 5 $\mu$s after the pulse, and argon saturated. $o = 2$ mM lipoic acid, 20 $\mu$M $e^aq$, 10 mM phosphate, 0.1 M tert-butyl alcohol, pH 8.05, 10 $\mu$s after the pulse, and argon saturated.

Figure 64. Example decay kinetic analysis of the 406-nm absorbance after the one-electron reduction of lipoate at pH 8.0. The rate constant ($7.5 \times 10^6$ M$^{-1}$ s$^{-1}$) was derived by multiplying the slope from the second-order plot by the extinction coefficient (9200 M$^{-1}$ cm$^{-1}$) and the path length (2 cm). The conditions were the same as those used in Figure 63.
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kinetics observed for the decay of the lipoate radical anion at pH 8.0. The decay constant obtained at pH 8.0 compares favorably to the decay constant reported by Faraggi et al.\textsuperscript{94} (see Table 5) and to the decay constant reported by Hoffman and Hayon\textsuperscript{93} in the same pH range.

Table 5. Comparison of Lipoic Acid Decay Rates with Those Reported in the Literature\textsuperscript{(a)}

<table>
<thead>
<tr>
<th>pH</th>
<th>this work</th>
<th>Hoffman and Hayon</th>
<th>Faraggi et al.</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.60</td>
<td>61</td>
<td>40</td>
<td>34</td>
</tr>
<tr>
<td>8.05</td>
<td>7.7</td>
<td>7</td>
<td>5</td>
</tr>
</tbody>
</table>

\textsuperscript{(a)}Multiply number by $1 \times 10^8 \text{ M}^{-1} \text{s}^{-1}$ to get the second-order rate constant, $2k$.

The decay constant observed at low pH compares favorably with both literature reports\textsuperscript{93,94} after the observed rate ($61 \times 10^8 \text{ M}^{-1} \text{s}^{-1}$) is adjusted by using the extinction coefficient reported by Hoffman and Hayon\textsuperscript{93} and Faraggi et al.\textsuperscript{94} (6900 and 7000 M$^{-1}$ cm$^{-1}$, respectively) for RSSRH at low pH instead of the extinction coefficient of 9200 M$^{-1}$ cm$^{-1}$ that I used, i.e., $61 \times 10^8 \text{ M}^{-1} \text{s}^{-1} \times 6900/9200 = 45 \times 10^8 \text{ M}^{-1} \text{s}^{-1}$.

The reason why the extinction coefficient of 9200 M$^{-1}$ cm$^{-1}$ was used to report the rate constant obtained at low pH is 2-fold. First, it is difficult to assign a proper extinction coefficient between the two pHs where the high and low extinction coefficients were reported because of the dramatic change in the extinction coefficient close to the
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pKₐ for this transition. Second, since the extinction coefficient is not known for these disulfides in the organic solvents discussed below, a single extinction coefficient was chosen to allow for comparisons.

Ethanol has a lower dielectric constant (25) than water (78) and therefore would mimic the less polar environment that a disulfide in a protein might experience. However, ethanol is polar enough to allow the acid-base characteristics observed for lipoate in water to occur in ethanol. When ethanol is changed from alkaline conditions to acidic conditions, the λ_max also shifts to the blue (Figure 65). If it is assumed that the extinction coefficient used in water is valid in ethanol (9200 M⁻¹ cm⁻¹), the decay rates for the radical change from 1.7 x 10⁹ M⁻¹ s⁻¹ in acidic ethanol (absolute ethanol made 0.1-1 mM in HCl) to 0.087 M⁻¹ s⁻¹ in alkaline ethanol (absolute ethanol saturated with dried NaOH). Figure 66 is an example of the type of kinetics that were obtained when lipoate was in acidic ethanol.

The 330-nm band that is present in the spectrum of lipoic acid in pH 8 water is apparent in the spectrum taken of lipoic acid in ethanol and even more apparent in the spectrum taken of lipoic acid in alkaline ethanol (Figure 65).

Ethylenediamine (EDA) has a lower dielectric constant (11) than ethanol (25) and therefore would offer an even less polar environment than ethanol. EDA is also a base and
Figure 65. Lipoic acid in ethanol. The $\lambda_{\text{max}}$ shifts from 405 to 385 nm when alkaline ethanol is made acidic. The conditions used were as follows: $\circ = 1.8$ mM lipoic acid in alkaline ethanol, $T = 100$ us. $\star = 2.1$ mM lipoic acid in ethanol, $T = 2.5$ us. $\times = 3.3$ mM lipoic acid in acidic ethanol, $T = 2$ us. The dose in water was 14 $\mu$M for all of the curves.

Figure 66. Example decay kinetic analysis of the 389-nm absorbance after the one-electron reduction of lipoic acid in acidic ethanol. The rate constant ($1.8 \times 10^8$ M$^{-1}$ s$^{-1}$) was derived by multiplying the slope from the second-order plot by the extinction coefficient ($9200$ M$^{-1}$ cm$^{-1}$) and the path length (2 cm). The conditions were the same as those used in Figure 65.
the spectrum done on lipoate reflects this fact (Figure 67). The $\lambda_{\text{max}}$, centered at 417 nm, is shifted about 12 nm to the red relative to lipoate in alkaline water and relative to lipoate in alkaline ethanol (Figures 63, 65, and 67). The small peak that was centered at 330 nm when lipoate was in alkaline ethanol or water (Figures 63 and 64) is also shifted to the red and is centered at 345 nm when lipoate is in ethylenediamine (Figure 67). The decay constant is more than 2.5 times smaller than the decay constant observed for the lipoate radical anion in alkaline ethanol and more than 23 times smaller than the decay constant observed for the lipoate radical anion at pH 8.05. (The slow decay rate is not surprising since ethylenediamine is a base, and from the results from the pH studies of lipoate in water, the lipoate radical anion is more stable under alkaline conditions. What is surprising is the 10-20-nm shift to the red in the $\lambda_{\text{max}}$ when the solvent is changed from alkaline water or alkaline ethanol to ethylenediamine.)

Tetrahydrofuran (THF) is the least polar solvent tried (7). The $\lambda_{\text{max}}$ appears to be centered at around 395 nm (Figure 68). If it is assumed that the extinction coefficient for lipoic acid radical is the same in THF as in water, the decay constant for lipoic acid radical in THF of $5.1 \times 10^9 \text{ M}^{-1} \text{ cm}^{-1}$ compares to the decay constant for lipoic acid radical in low-pH water.
Figure 67. Time-dependent spectrum of lipoic acid in EDA. The \( \lambda_{\text{max}} \) is at 417 nm and note the smaller peak at 345 nm. The conditions used were as follows: 2.67 mM lipoic acid, 5.9 uM \( \text{e}^- \text{aq} \) dose in water, and argon saturated. \( x = 50 \mu s \), \( + = 0.5 \mu s \), \( o = 1 \mu s \), \( * = 2 \mu s \), and \( \bullet = 3 \mu s \) after a 500-ns pulse.

Figure 68. Time-dependent spectrum of lipoic acid in THF. The \( \lambda_{\text{max}} \) is at 395 nm. The conditions used were as follows: 2 mM lipoic acid, 5.9 uM \( \text{e}^- \text{aq} \) dose in water, and argon saturated. \( x = 1 \mu s \), \( + = 20 \mu s \), and \( o = 120 \mu s \) after a 700-ns pulse.
Lipoamide. One major concern was that the observed shift of $\lambda_{\text{max}}$ in water, the apparent change of the extinction coefficient, and the change in the decay constant with varying pH all had a pK\textsubscript{a} that was very close to the pK\textsubscript{a} of 4.7 for the carboxylic acid on lipoate. And it was quite possible that the observed changes with varying pH do not reflect the protonation of RSSR\textsuperscript{-} but reflect the protonation of the carboxylate on lipoate instead. One way to find out was to perform similar experiments on lipoamide, which is the structural analogue of lipoate but not acidic, that were performed on lipoate.

\[ \text{Figure 69. Structure of lipoamide.} \]

The spectrum for the single electron reduction of lipoamide in water could not be obtained in exactly the same manner used to obtain the spectra for lipoic acid because of the low solubility of the amide in water. To get the RSSR\textsuperscript{-} spectra in water, lipoamide was first dissolved in a small amount of pure ethanol and then added to the 5 times distilled water that was 10 mM in phosphate. This same trick was tried with tert-butyl alcohol but failed, presumably, because lipoamide was more soluble in ethanol than
tert-butyl alcohol. The final concentration of ethanol was 1.2 M. Ethanol, like tert-butyl alcohol, will scavenge for

\[ \text{CH}_3\text{CH}_2\text{OH} + \cdot\text{OH} \rightarrow \text{CH}_3\dot{\text{CHOH}} + \text{H}_2\text{O} \quad (11) \]

\( \cdot\text{OH} \); however, the resulting ethanol radical is much more reactive than the tert-butyl alcohol radical and will reduce lipoic acid\(^{125} \) (I also saw this).

\[ \text{CH}_3\text{CHOH} + \text{S-S} \rightarrow \text{S-S} + \text{CH}_3\text{CHO} + \text{H}^+ \quad (49) \]

This reaction was put to use by converting e\(^{-}\text{aq} \) to \( \cdot\text{OH} \) by degassing the solution with \( \text{N}_2\text{O} \) (equation 6) and using the ethanol radical to generate the disulfide radical anion.

The spectra obtained for lipoamide in water are very similar to the spectra obtained for lipoic acid, their \( \lambda_{\text{max}} \) values undergo a blue shift with decreasing pH. The \( \lambda_{\text{max}} \) for lipoamide shifts from 397 to 385 when the pH is changed from 12.20 to 3.30 (Figure 70). This shift is accompanied by an increase in the rate from \( 0.71 \times 10^9 \text{ M}^{-1}\text{s}^{-1} \) to \( 3.1 \times 10^9 \text{ M}^{-1}\text{s}^{-1} \) when the pH is changed from 12.20 to 3.30.

A similar shift in the \( \lambda_{\text{max}} \) was observed when the solvent was changed from alkaline ethanol to acidic ethanol (Figure 71). This shift was also accompanied by an increase in the rate from \( 0.095 \times 10^9 \text{ M}^{-1}\text{s}^{-1} \) in alkaline ethanol to \( 1.7 \times 10^9 \text{ M}^{-1}\text{s}^{-1} \) in acidic ethanol (see Figure 72 for an
Figure 70. Lipoyamide in water. The $\lambda_{max}$ shifts from 397 to 385 nm when the pH is changed from 12.2 to 3.3. The condition used were as follows: 2.5 mM lipoyamide, 1.2 M ethanol, and 10 mM phosphate. $o = 25$ us and $x = 10$ us after a 500-ns pulse.

Figure 71. Lipoyamide in ethanol. The $\lambda_{max}$ for ASSR shifts from 400 to 386 nm when the solvent was changed from alkaline ethanol to acidic ethanol. There is a smaller peak centered at 330 nm in the spectrum of lipoyamide in alkaline ethanol. The conditions used were as follows: $o = 1.0$ mM lipoyamide in alkaline ethanol and $x = 2.6$ mM lipoyamide in acidic ethanol. Both spectra were taken 50 us after a 500-ns pulse that gave a dose of 22 $\mu$J e$^{-}\text{aq}$ in water. Both were argon saturated.
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Figure 72. Example decay kinetics of the absorbance at 405 nm after the one-electron reduction of lipooamide in alkaline ethanol. The conditions were the same as those used in Figure 71.

Figure 73. Time-dependent spectra of lipooamide in EDA. The RSSR⁺ λmax is centered at about 418 nm and note the peak centered at about 364 nm. The conditions used were as follows: 2.5 mM lipooamide, 17.5 μM e−aq dose in water, and argon saturated. x = 50 ns, τ = 0.5 ms, v = 1 ms, τ = 1.5 ms, and s = 2 ms after a 350-ns pulse.
example of the kinetics). Like the lipoate spectrum in alkaline ethanol, there is a smaller peak centered at 330 nm. Any doubts concerning the method used to obtain the lipoamide spectra in water should be eased since a similar shift was obtained when ethanol was used as the solvent.

This time when the solvent was changed to ethylenediamine, the $\lambda_{\text{max}}$ shifted about 15-20 nm to the red (Figure 73) relative to the $\lambda_{\text{max}}$ observed with lipoamide in alkaline water (Figure 70) or alkaline ethanol (Figure 71). The decay constant ($0.055 \times 10^9 \text{ M}^{-1} \text{s}^{-1}$) is also smaller than the decay constant observed for lipoamide in alkaline ethanol ($0.095 \times 10^9 \text{ M}^{-1} \text{s}^{-1}$) and much smaller than the decay constant observed for lipoamide in alkaline water ($0.71 \times 10^9 \text{ M}^{-1} \text{s}^{-1}$). The $\text{RS}\cdot$ peak is centered at 344 nm (Figure 73) and is red shifted about 14 nm from the $\text{RS}\cdot$ peak observed at 330 nm when alkaline ethanol was the solvent (Figure 71).

Like lipoic acid, in THF the $\lambda_{\text{max}}$ for lipoamide is centered around 387 nm (Figure 74) and the decay constant is $6.9 \times 10^9 \text{ M}^{-1} \text{s}^{-1}$ (see Figure 75 for an example) compared to $5.1 \times 10^9 \text{ M}^{-1} \text{s}^{-1}$ for lipoic acid.

Oxidized Dithiothreitol or Oxidized Cleland's Reagent. The spectrum resulting from the one-electron reduction of oxidized dithiothreitol (ox-DTT) at pH 9.30 has a $\lambda_{\text{max}}$ at 390 nm (Figure 76). No blue shift with decreasing pH was noted, just a reduction in the yield, resulting in a very
Figure 74. Time-dependent spectrum of lipoamide in THF. The \( \lambda_{\text{max}} \) is centered at about 387 nm. The conditions used were as follows: 3 mM lipoamide, 18.7 \( \mu \)M eaq dose in water, and argon saturated. \( e = 10 \) us, \( \tau = 20 \) us, \( \tau = 30 \) us, and \( x = 60 \) us after a 500-ns pulse.

Figure 75. Example decay kinetics of the absorbance at 389 nm of the one-electron reduction of lipoamide in THF. The conditions were the same as those used in Figure 74.
Figure 76. ox-DTT in water. The \( \lambda_{\text{max}} \) does not shift when the pH is reduced from 9.3 to 3.3; only a reduction of yield is apparent. The conditions used were as follows: \( o = 10 \) ns after a 500-ns pulse, 5 mM ox-DTT, 13 \( \mu \)M e\textsuperscript{aq}, 10 mM phosphate, 1.17 M t-BuOH, pH 9.3, and argon saturated. \( x = 0.25 \) ns after a 200-ns pulse, 5 mM ox-DTT, 13 \( \mu \)M e\textsuperscript{aq}, 10 mM phosphate, 1.5 M t-BuOH, pH 3.3, and argon saturated.

Figure 77. ox-DTT titration. The yield at 390 nm and 10 us as a function of pH can be analyzed in terms of a single titration curve with a pK\(_a\) of 4.97 \pm 0.24 (see Appendix A for function). The conditions used were as follows: 5 mM ox-DTT, 17.1 \( \mu \)M e\textsuperscript{aq}, 1.5 M tert-butyl alcohol, 500-ns pulse, and argon saturated.
broad peak at that was still centered at around 390 nm at pH 3.30.

The absorbance as a function of pH (Figure 77) has a pKₐ of 4.93 but cannot reflect a yield curve since at the high concentration (5 mM) used, no solvated electron will be lost to H⁺ (equation 35) until pH 4. This pKₐ compares very well to the pKₐ of 5.2 reported by Redpath² and is close to the pKₐ of 5.5 reported by Chan et al.⁶ Both groups obtained their results by the same method: they oxidized dehydodithiothreitol with ·OH instead of reducing ox-DTT with e⁻aq. They proposed the following scheme to explain the resulting RSSR⁻ spectrum:

\[
\begin{align*}
\text{HO} &\quad \text{OH} \\
\text{S} &\quad \text{S} \\
\text{H} &\quad - \\
\text{H} &\quad \text{H} \\
\end{align*}
\quad + \quad \cdot \text{OH} \quad \rightarrow \quad \begin{align*}
\text{HO} &\quad \text{OH} \\
\text{S} &\quad \text{S} \\
\cdot &\quad - \\
\cdot &\quad \text{H₂O} \quad (50)
\end{align*}
\]

\[
\begin{align*}
\text{HO} &\quad \text{OH} \\
\text{S} &\quad \text{S} \\
\cdot &\quad - \\
\end{align*}
\quad \rightarrow \quad \begin{align*}
\text{HO} &\quad \text{OH} \\
\text{S} &\quad \text{S} \\
\cdot &\quad - \\
\end{align*}
\quad (51)
\]

Redpath also reduced oxidized dithiothreitol with e⁻aq and reported that the spectrum was exactly the same as the spectrum obtained from the oxidation of dehydodithiothreitol after the difference in e⁻aq and ·OH yields were taken into account. Chan et al.⁶ reported a blue shift with decreasing pH and a λ_max of 405 nm at high pH; however, neither of these were noted in any of the spectra obtained
for ox-DTT in water. What was observed was that the $\lambda_{\text{max}}$ remains invariant at 390 nm from low to high pH (Figure 76), exactly what was reported by Hoffman and Hayon.93

Like the five-membered cyclic disulfides, decreasing the pH from 9.3 to 5.1 did increase the decay constant from $1.6 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$ to $9.2 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$. This change is not as big as that observed with the first two disulfides, probably because the pH of 5.1 at which the decay constant was obtained is very close to the pK$_a$ of 5 (Figure 77) for the protonation of this disulfide and therefore the decay constant would be expected to be larger at an even lower pH.

The spectrum resulting from the one-electron reduction of ox-DTT in alkaline ethanol has a $\lambda_{\text{max}}$ of about 396 nm (Figure 78). This peak is slightly shifted toward the red relative to the peak observed for RSSR$^-$ in water (Figure 76) and has a decay constant ($3.6 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$; see Figure 79, for example) that is not far from the decay constants for the RSSR$^-$ in water. Unfortunately, I did not continue the spectrum down below 360 nm so the presence of the 330-nm band cannot be confirmed. However, the spectrum obtained for ox-DTT in ethanol (Figure 80) does contain a small band. This band centered at 320 nm is a little lower than the 330-nm band observed for lipoate in ethanol (Figure 65). Since no pH dependence for $\lambda_{\text{max}}$ was noted in water, a spectrum of ox-DTT in acidic ethanol was not tried.
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Figure 78. Time-dependent spectrum of ox-DTT in alkaline ethanol. The λ_{max} for RSSR^- is centered at 396 nm. The conditions used were as follows: 2.5 mM ox-DTT, 16 μM e^-aq dose in water, and argon saturated. X = 1 μs, • = 51 μs, ○ = 0.10 ms, □ = 0.2 ms, ■ = 0.3 ms, and x = 0.4 ms after a 500-ns pulse.

Figure 79. Example decay kinetics for the absorbance at 399 nm after the one-electron reduction of ox-DTT in alkaline ethanol. The conditions were the same as those used in Figure 78.
Figure 80. Time-dependent spectrum of ox-DTT in ethanol. The RSSR⁻ λ_{max} is centered at 394 nm and note the smaller peak centered at 320 nm. The conditions used were as follows: 1.89 mM ox-DTT, 16.1 μM e⁻aq dose in water, and argon saturated. + = 2.5 us, o = 5 us, * = 100 us, ■ = 200 us, and x = 400 us after a 500-ns pulse.

Figure 81. Time-dependent spectrum of ox-DTT in EDA. The RSSR⁻ λ_{max} is centered at 400 nm and note the smaller peak centered at about 340 nm. The conditions used were as follows: 2.5 mM ox-DTT, 17 μM e⁻aq dose in water, and argon saturated. X = 2 us, + = 10 us, o = 20 us, * = 30 us, ■ = 40 us, and x = 80 us after a 500-ns pulse.
When the solvent was changed to ethylenediamine, the $\lambda_{\text{max}}$ shifted to 400 nm (Figure 81) and the decay constant ($4.1 \times 10^9 \text{ M}^{-1} \text{s}^{-1}$) was not too different from the values reported in the other two solvents (i.e., $3.6 \times 10^9$ and $1.6 \times 10^9 \text{ M}^{-1} \text{s}^{-1}$).

The spectrum resulting from the one-electron reduction of ox-DTT in THF has a $\lambda_{\text{max}}$ centered at about 403 nm (Figure 82). The reduced species decays very rapidly with a decay constant of $2k = 260 \times 10^9 \text{ M}^{-1} \text{s}^{-1}$. This decay constant is too large and may reflect the fact that the extinction coefficient for RSSR$^-$ is not the same in water as in THF.

Dithiopropionic Acid (DTPA). The spectrum resulting from the one-electron reduction of DTPA at pH 7.30 has a $\lambda_{\text{max}}$ of 420 nm (Figure 83). This is the same $\lambda_{\text{max}}$ that Hoffman and Hayon\textsuperscript{93} reported for the same compound. They also reported that the $\lambda_{\text{max}}$ was invariant with pH. The spectrum resulting from the one-electron reduction of DTPA at pH 3.30 substantiates their second observation, that $\lambda_{\text{max}}$ was still 420 nm (Figure 83). Even though this disulfide decays by first-order kinetics, the decay constant increases with decreasing pH, i.e., $2.0 \times 10^6 \text{ s}^{-1}$ to $110 \times 10^6 \text{ s}^{-1}$.

When the solvent was changed to alkaline ethanol, the first thing noticeable is the broad rise at long time that increases from 480 to 330 nm (Figure 84). When a difference spectrum is constructed where the absorbances obtained at the fastest five times are subtracted from the
Figure 82. Time-dependent spectrum of ox-DTT in THF. The RSSR$^{-}$ $\lambda_{\text{max}}$ is centered at about 403 nm. The conditions used were as follows: 5 mM ox-DTT, 11.7 $\mu$M $e^{-}$aq dose in water, and argon saturated. $x = 1$ $\mu$s, $+$ = 1.5 $\mu$s, $o = 2$ $\mu$s, $*$ = 3 $\mu$s, $* =$ 4 $\mu$s, and $x =$ 8 $\mu$s after a 200-ns pulse.

Figure 83. DTPA in water. The $\lambda_{\text{max}}$ did not change from 420 nm when the pH was reduced from 7.3 to 3.3; just a reduction in amplitude was seen. The conditions used were as follows: 5 mM DTPA, 6 $\mu$M $e^{-}$aq, 10 mM phosphate, 1.6 M t-BuOH, and argon saturated. $o = 0.1$ $\mu$s (pH 7.3) and $x = 0.15$ $\mu$s (pH 3.3) after the pulse.
Figure 84. Time-dependent spectrum of DTPA in alkaline ethanol. Notice the rise starts at 350 nm and increases down to 340 nm. The conditions used were as follows: 7 mM DTPA, 18 μM eaq dose in water, and argon saturated. + = 1 μs, o = 1.5 μs, * = 2.0 μs, * = 2.5 μs, and x = 3.0 μs after a 500-ns pulse.

Figure 85. Time-dependent difference spectrum of DTPA in alkaline ethanol. The λmax is centered at 420 nm. The conditions were the same as those used in Figure 84. The absorbance values at + = 1 μs and o = 1.5 μs were subtracted from the absorbances taken at 3 μs after the pulse.
longest time (Figure 85), the $\lambda_{\text{max}}$ did not change from the $\lambda_{\text{max}}$ of 420 nm observed in water. This increase in background with decreasing wavelength most likely reflects the formation of the $\cdot$SH radical, which is reported to occur very rapidly with linear disulfides. The decay constant taken at 420 nm ($7 \times 10^6$ s$^{-1}$) compares quite well to the decay constant observed for DTPA at pH 7.30 ($2.0 \times 10^6$ s$^{-1}$).

A difference spectrum for DTPA in EDA (Figure 86) had to be made because, like the spectrum observed for DTPA in alkaline ethanol, this spectrum also had a large background absorbance starting at 350 nm and increasing with decreasing wavelength (Figure 87). The $\lambda_{\text{max}}$ for DTPA shifts from a $\lambda_{\text{max}}$ of 420 nm seen in the spectrum taken in water and in the difference spectrum taken in alkaline ethanol to a $\lambda_{\text{max}}$ of 435 nm (Figure 86) in the difference spectrum taken in EDA. As expected, the decay rate ($0.46 \times 10^6$ s$^{-1}$) is a bit smaller in value compared to values obtained for DTPA in alkaline ethanol and pH 7.0 water ($1.7 \times 10^6$ s$^{-1}$ and $2.0 \times 10^6$ s$^{-1}$, respectively).

Finally, the decay constant for DTPA in THF is $4.0 \times 10^6$ s$^{-1}$ (see Figure 88 for example kinetics) and the $\lambda_{\text{max}}$ for the single-electron reduction of DTPA in THF is 428 nm (Figure 89). There appears to be a slight rise occurring below 360 nm, but a difference spectrum of DTPA in THF was not made.
Figure 86. Time-dependent difference spectrum of DTPA in EDA. The $\lambda_{\text{max}}$ is centered at about 435 nm. The conditions used were as follows: 2.5 mM DTPA, 17 $\mu$M e-saq dose in water, and argon saturated. $X = 0.1$ us, $* = 1$ us, $@ = 2$ us, $\# = 3$ us, and $* = 4$ us after a 500-ns pulse. All absorbances were subtracted from the absorbances taken at 7 us.

Figure 87. Time-dependent spectrum of DTPA in EDA. Notice the background rise that increases from 380 to 320 nm. The conditions were the same as those used in Figure 86.
Figure 88. Example decay kinetic analysis of the 420-nm absorbance after one-electron reduction of DTPA in EDA. Notice that the data fit first-order kinetics better than second-order kinetics. The conditions were the same as those used in Figure 87.

Figure 89. Time-dependent spectrum of DTPA in THF. The $\lambda_{\text{max}}$ is centered at about 420 nm. The conditions used were as follows: 5 mM DTPA, 0.5 \text{mM} \text{H}^+ \text{aq} dose in water, and argon saturated. $X = 0.17 \text{us}$, $+ = 0.15 \text{us}$, $o = 0.2 \text{us}$, $* = 0.25 \text{us}$, and $x = 0.35 \text{us}$ after a 100-ns pulse.
DISCUSSION

The spectrum for the solvated electron in ethanol, EDA, and THF has been reported, so it was not surprising to see the disulfide radical in these solvents. The $\lambda_{\text{max}}$ for the solvated electron in ethanol$^{102}$ is reported to be centered at 700 nm ($\epsilon = 15,000$ M$^{-1}$ cm$^{-1}$), 1350 nm ($\epsilon = 20,000$ M$^{-1}$ cm$^{-1}$) in EDA,$^{102-104}$ and 2100 nm ($\epsilon = 40,000$ M$^{-1}$ cm$^{-1}$) in THF.$^{102-104}$ The solvent dielectric constant as well as the class of solvent (alcohol, amine, hydrocarbon, etc.) is reported to play a role in the $\lambda_{\text{max}}$ position of these and other solvents.$^{104}$

The $\lambda_{\text{max}}$ shift observed for both five-membered cyclic disulfides (lipoate and lipoamide) in water parallels the shift observed in ethanol when their solutions were changed from alkaline to acidic conditions (Table 6). The shift observed with lipoamide in either solvent reaffirms my contention and the proposals found in earlier reports$^{93,94}$ that the shift observed for lipoic acid in water reflects the protonation of the disulfide radical anion and not the protonation of the $\delta$-carboxylic acid on lipoic acid; lipoamide has no carboxylic acid. Unlike the results obtained for the two five-membered cyclic disulfides, no shift in $\lambda_{\text{max}}$ was noted with either six-membered cyclic disulfides, ox-DTT, or linear DTPA when the solutions were changed from alkaline to acidic pH (Table 6). This was expected.$^{93,94}$ The protonated disulfide had never been reported with
### Table 6. \( \lambda_{\text{max}} \) and Decay Rates for Four Disulfides in Various Solvents (Decay Rate in Parentheses)

<table>
<thead>
<tr>
<th>Disulfide</th>
<th>Water low pH</th>
<th>Water high pH</th>
<th>Ethanol acidic (k)</th>
<th>Ethanol alkaline (l)</th>
<th>EDA (k)</th>
<th>THF (k)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dithiopropionic acid (a)</td>
<td>420(c) (110)</td>
<td>420(d) (2.0)</td>
<td>---</td>
<td>420 (1.7)</td>
<td>435</td>
<td>428</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>(0.46)</td>
<td></td>
<td>(4.0)</td>
</tr>
<tr>
<td>Oxidized dithiothreitol (b)</td>
<td>390(e) (9.2)</td>
<td>390(e) (1.6)</td>
<td>---</td>
<td>390 (3.6)</td>
<td>400</td>
<td>403</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(4.1)</td>
<td></td>
<td>(200)</td>
</tr>
<tr>
<td>Lipoic acid (b)</td>
<td>386(g) (0.1)</td>
<td>400(h) (0.77)</td>
<td>385 (1.7)</td>
<td>405 (0.087)</td>
<td>417</td>
<td>395</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(0.032)</td>
<td></td>
<td>(5.1)</td>
</tr>
<tr>
<td>Lipomide (b)</td>
<td>385(i) (3.1)</td>
<td>397(j) (0.71)</td>
<td>385 (1.7)</td>
<td>400 (0.095)</td>
<td>410</td>
<td>397</td>
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<tr>
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<td></td>
<td></td>
<td>(0.055)</td>
<td></td>
<td>(6.9)</td>
</tr>
</tbody>
</table>

(a) Multiply number by \( 1 \times 10^6 \) s\(^{-1} \) to get the first order rate constant. (b) Multiply number by \( 1 \times 10^8 \) M\(^{-1}\) s\(^{-1} \) to get the second-order rate constant, 2k. The extinction coefficient used to obtain the second-order rate constant was 9200 M\(^{-1}\) cm\(^{-1} \). (c) pH 3.30. (d) pH 7.30. (e) pH 3.30. (f) pH 9.30. (g) pH 4.60. (h) pH 8.06. (i) pH 3.30. (j) pH 12.20. (k) 0.1 M HCl. (l) Saturated with dried NaOH.
linear disulfides\textsuperscript{93,97,98,100} or the six-membered cyclic disulfide ox-DTT.\textsuperscript{95} The Chan et al.\textsuperscript{96} report is the exception. In all four disulfides studied, the disulide radical is more stable under alkaline conditions than under acidic conditions (Table 6), and this stability is reflected in a decrease in the decay constant upon going from acidic conditions to alkaline conditions.

One interesting sidelight is the appearance of a smaller band in cyclic disulfides that is about 60 nm to the blue relative to the major RSSR\textsuperscript{-} peak when the solvent is alkaline water, EDA, ethanol, or alkaline ethanol (Figures 63, 65, 67, 71, 73, 75, and 81). A much broader absorbance can be seen in this region with the linear disulfide, DTPA (Figures 84 and 87). This band has been reported after the one-electron reduction of linear disulfides or after hydrogen abstraction of a thiol by \(\cdot\text{OH}\).\textsuperscript{93} A rise in this region has also been reported after the one electron reduction of ox-DTT.\textsuperscript{95} What is interesting about this band is that in cyclic disulfides the disulfide radical anion band and the smaller band centered around 330 nm appear to decay at about the same rate and through second-order kinetics (see Figure 90 for an example). Since the extinction coefficient of RSSR\textsuperscript{-} in these solvents is not really known and since the extinction coefficient of RS\textsuperscript{•} for these disulfides is not known, simply comparing their second-order kinetics cannot be done because an extinction coefficient must be known in
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Figure 90. Example decay kinetics observed at 327 and 399 nm for the one-electron reduction of lipoamide in alkaline ethanol. Notice the absorbance of both peaks (Figure 71) decays by second-order kinetics. The conditions were the same as those used in Figure 71.
Figure 91. Comparison of the loss of absorbance per unit of time of "RSSR" with the loss of absorbance per unit of time of "RS." for ox-DTT, lipoamide, and lipoate in EDA, alkaline ethanol, and ethanol (see Appendix D for details). 

a = ox-DTT in EDA (slope = 0.663; r = 0.9986), 
b = ox-DTT in ethanol (slope = 0.505; r = 0.9667), 
c = lipoate in EDA (slope = 0.469; r = 0.998), 
d = lipoate in alkaline ethanol (slope = 0.3523; r = 0.9979), 
e = lipoamide in EDA (slope = 0.4120; r = 0.9990), and 
f = lipoamide in alkaline ethanol (slope = 0.4060; r = 0.9998) where r = correlation coefficient. The data were taken from the same traces used to construct the spectra of these species reported earlier.
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each case. However, when the change in absorbance for RSSR⁻ per unit of time is plotted against the change in absorbance for RS⁻ per the same unit of time, the plots are linear (Figure 91). Nonlinearity to one side of the least-squares line or the other would reflect a difference between the two rates. (This was seen when two rates that are known to be different are compared in this way). Linearity might reflect two things: the species identified as RSSR⁻ and RS⁻ are the same species or RSSR and RS are related by a fast equilibrium, thus giving the appearance that RSSR⁻ and RS⁻ are the same species (see Appendix D for the derivation).

The fast equilibrium is a very attractive concept in light of the nature of the cyclic disulfide and can be visualized as follows:

\[
\text{RSSR}^- \xrightarrow{2H^+} \text{RSSR} \xrightarrow{\text{rapid equilibration}} \text{RSSR}^- + \text{RSH HSR} \\
\text{RS}^- \xrightarrow{\text{very slow}} \text{SR RS SR RS}^- \\
\]

Figure 92. Possible reason why the 330-nm and "RSSR⁻" bands appear to decay by the same rate.

This theory encompasses the idea that RSSR⁻ still decays quite rapidly by first-order kinetics like linear disulfide radical anions but is rapidly re-formed because of the close proximity of the thiol anion and the thiyl radical forced on
them by the cyclic structure of these disulfides. This equilibrium is rapid and allows these cyclic disulfides to have a much longer lifetime than linear disulfides, thereby forcing RSSR⁻ to decay through disproportionation. This would also mean that if RS⁺ "SR decays, it must decay much more slowly than the disproportionation of RSSR⁻."

If the equilibrium idea is correct and if the ratio of the yield at 330 nm to the yield at 400 nm reflects the equilibrium constant, then this equilibrium constant would be expected to change with temperature. In addition, as an indirect proof for the existence of such an equilibrium, the pKₐ derived from the yield at 330 nm should be equal to the pKₐ for the yield at 400 nm. Positive results from both of these experiments would give this theory a significant boost.

In all four cases, changing the solvent from alkaline water or alkaline ethanol to EDA resulted in a red shift in the λ_max from 12 to 30 nm. However, changing the solvent from alkaline water or alkaline ethanol to THF resulted in only a small red shift in the λ_max of about 5 nm in the linear disulfide and the six-membered cyclic, whereas a 10-nm blue shift was noted for the two five-membered cyclic disulfides. The blue shift seen in EDA and the red-blue shift seen in THF may be caused by three factors: dielectric constant, alkalinity of the solvent, and the geometric structure of the disulfide. Since EDA is a base, one would
expect the disulfide radical to exist as an anion and consequently have a high $\lambda_{\text{max}}$ relative to that of alkaline solvents. This is reflected in the long lifetimes of the transients relative to the shorter lifetimes seen in alkaline solvents. The low dielectric constant for EDA (11) relative to that of water (78) may also contribute a few more nanometers to the red shift. Such a shift with decreasing dielectric constant is not unprecedented since a red shift was observed in the $\lambda_{\text{max}}$ for the solvated electron when the solvent was changed from water to organic solvents.102-104

What is clear is that the $\lambda_{\text{max}}$ for a disulfide radical anion does not shift to the blue when the solvent is changed to a less polar and more hydrophobic environment. This strongly suggests that any bands found in proteins between 385 and 400 nm, such as the shoulder seen in the one-electron reduction of papain (Figures 31 and 32), are not due to a disulfide in a hydrophobic environment but most likely reflect a disulfide radical anion that has been protonated or a disulfide radical anion that is structurally similar to the six-membered cyclic disulfide ox-DTT.
disulfides in proteins

Disulfides in Proteins

METHODS

Asparaginase. Approximately 400 mg of L-asparaginase EC-2 suitable for human injection was kindly supplied by Merck Sharpe & Dohme. Discontinuous gel electrophoresis was performed on a sample using the procedure (glycine, pH 8.3, buffer, 7% polyacrylamide) described by Ornstein and Davis.\textsuperscript{116} Electrophoresis showed two distinct bands that have been seen before (Figure 93),\textsuperscript{107-110,116} where the larger band and the smaller band have been ascribed to be a monomer-dimer aggregate.\textsuperscript{110} The aggregate represents about 10\% of the total amount of asparaginase and is reported to have the same activity as the monomer.\textsuperscript{110} Therefore, the enzyme was used as supplied after the mannitol was removed by dialysis. The dialysis buffer was 50 mM NaCl and 10 mM phosphate, pH 6.9.

Figure 93. Drawing representing the 5.5 x 8 cm disc gel electrophoretic pattern seen after electrophoresis was performed on 60 μg of asparaginase.

The specific activity of asparaginase increased from 14.2 to 15.3 units after dialysis using the asparaginase
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assay (see Asparaginase Assay) and the extinction coefficient of $\varepsilon_{1%} = 7.1$ for asparaginase. After dialysis, the solution containing asparaginase was diluted up into two equal parts. One was made 0.1% in NaN$_3$ and stored in the refrigerator. The other was quickly frozen with dry ice-acetone and stored frozen. (This was done because the machine went down for almost 2 months and I was not sure which method was the best.) After 1 month the specific activity had dropped down to 14.2 and 13.6 units from the sample taken from the refrigerator and the freezer, respectively. The frozen material was used to obtain the pH 6.94 data and has a specific activity of 12-13 units before radiolysis. After radiolysis the material had no activity. Since it is difficult to understand how a small amount of radiation could destroy all the activity, the stability of asparaginase in 0.1 M tert-butyl alcohol and/or 10 mM phosphate (the conditions used for pulse radiolysis) was checked by using the asparaginase assay and the frozen material. Asparaginase appears to be stable in 0.1 M tert-butyl alcohol but is not stable when degassed gently. Therefore, the pH 6.94 spectrum and the disulfide decay kinetics are of inactive asparaginase. After 2 months the frozen material had no activity before it was used for pulse radiolysis.

RNase. This was purchased from Worthington Biochemical Co. as the salt-free powder and was used without purification.
Papain. Papain was purified and activated as described under the subsection entitled Papain under PROTEASES.

Asparaginase Assay. The asparaginase assay was taken from Bryant and Carpenter\textsuperscript{118} where the loss of the amide bond is monitored as asparaginase catalyzes the conversion of Asn to Asp and NH\textsubscript{3}. The assay was done as follows. Exactly 3.00 mL of assay buffer (50 mM boric acid, 9.2 mM L-asparagine, pH 8.25) was placed in a cuvette and allowed to come to thermal equilibrium of 30°C. After 10-15 min 50-100 µL of solution containing asparaginase (0.17-0.4 mg/mL) was placed in the cuvette and mixed. The loss of amide was monitored as a decrease in absorbance at 215 nm (4 cm/min, 1 absorbance unit full scale). One unit corresponds to the number of absorbance units at 215 nm per minute, i.e., ΔAbs/min. No extinction coefficient was used. The purpose of the assay was to monitor any change in the specific activity while working with the enzyme. The specific activity was defined as the number of units of activity per milligram of protein, i.e., units/mg. The number of milligrams was derived from the solution absorbance by knowing ε\textsuperscript{1%} = 7.1 at 278 nm.\textsuperscript{117} The specific activity of my sample had a high of 16 units/mg.
RESULTS AND DISCUSSION

The decay of transients formed from the pulse radiolysis of proteins can best be described as complex. This is understandable since studies of individual amino acids have shown that virtually all transients generated after the one-electron reduction of amino acids absorb in the same region (see Figure 1). Consequently, most of the spectra from these transients overlap, thereby complicating the decay kinetics. The disulfide radical anion may be the best candidate to study the decay rate of a transient in a protein because its extinction coefficient is almost 2 times higher than the next closest extinction coefficient for an amino acid transient (see Figure 1) and the percentage of solvated electron residing on disulfides is higher than that of any other protein functional group except for histidine.\textsuperscript{2-4} However, there is a further complication, and this complication is reflected in papain because the RSSR\textsuperscript{-} decay kinetics are complicated (Figure 94) and cannot be fit to first or second order. Indeed, Clement et al.\textsuperscript{23} commented that the disulfide radical anion decay kinetics in papain are complicated.

Papain. Since RSSR\textsuperscript{-} decay and spectral characteristics are influenced by adjacent charged groups, type of solvent, and type of disulfide (see BACKGROUND and METHODS for Simple Disulfides), it is not unreasonable to expect one or more of the three disulfides in papain to exist in a different
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Figure 94. First- and second-order kinetic analysis of the 405- and 387-nm absorbances after reduction of papain by e^-aq at pH 7.1. The conditions were the same as those in Figure 31.

environment and that upon reduction these transients have different spectral and kinetic properties from one another. Such spectral differences may be reflected in the spectra obtained for papain (Figures 30 and 31), where a shoulder at 385 nm in the spectrum taken at pH 5.57 (Figure 30) develops into a peak when the pH is changed to 7.1 (Figure 31). The kinetics of the two peaks are complicated (Figure 94) and yet appear to decay at about the same rate, which is most easily visualized by the equal change in the absorption spectrum with time (Figure 31). A plot of ΔAbs/Δt of the 385-nm absorbing species versus the 405-nm absorbance is linear (Figure 95), which would be expected since the two maxima appear only 20 nm apart, suggesting that the spectral bands are well overlapped. The three spectra for papain
Figure 95. A absorbance/\Delta t plot for the 905-nm peak (x axis) and the 309-nm peak (y axis). The linearity suggests that the two absorbances are decaying at the same rate. The conditions were the same as those used in Figure 31.

(Figures 31-33) are the best representatives of a series of papain spectra that show this shoulder at 385-390 nm.

The CO_2^- radical is reported to reduce some disulfides in proteins and not others.\(^3,89,90\) In the hope of using the selectivity of the CO_2^- radical, the spectrum of papain reduced by CO_2^- was tried with good results (Figure 96). The fact that the 385-nm band is absent and the fact that the disulfide maximum in the CO_2^--papain spectrum seems to line up with the 405-nm band of the e^-aq plus papain spectrum suggest that the 385-nm band represents a disulfide that is somehow different from the disulfide(s) absorbing at 405 nm. And what was even more exciting was that the RSSR^- decay appeared to fit first-order kinetics far better than second-order decay kinetics (Figure 97). The first part of
Figure 96. Comparison of the e^-aq-reduced spectrum of papain with the COO^- reduced spectrum of papain at pH 7.1. Notice how the peak in the COO^- papain spectrum (o) appears to line up with the right bump of the e^-aq-papain spectrum (x). Also, notice that the e^-aq-papain spectrum (o) has the 355-nm imidazolium band and the COO^-papain spectrum (x) does not. The spectra have been normalized to where [e^-aq] = [COO^-] = 9.5 mM. Both spectra were taken in 10 mM phosphate and 10 mM NaCl where t = 300 μs. In the e^-aq-papain spectrum, 0.1 M tert-butyl alcohol and argon were used. In the COO^-papain spectrum, 0.1 M formate and H2O were used.

The trace used to obtain the kinetics (Figure 98) appears to flatten out. This feature was present in all traces used to obtain the decay kinetics at this wavelength (409 nm) and at wavelengths around it. When this portion of the curve is excluded from the kinetic analysis by using range B instead of range A (see Figure 98), the first-order fit is better (Figure 98) with a rate of 123 s^-1.

From traces taken on a different day and on a different preparation of papain, the decay also looks closer to first order than second order (Figure 99) and the beginning of the
Figure 97. First- and second-order decay kinetics for RSSR after the reduction of papain by CO\textsuperscript{2−} at pH 7.1, whole curve analysis. Notice that the data fit first order better than second order. Also, notice the divergences from the best-fit line in the upper left corner and the smaller divergence at the lower right corner. The conditions are the same as those used in Figure 95 for the CO\textsuperscript{2−}-papain spectrum.

Figure 98. First- and second-order kinetic analysis for RSSR decay after the reduction of papain by CO\textsuperscript{2−} at pH 7.1, latter curve analysis. The data indicated as A in the trace were used in Figure 97. The data indicated as B were used for the kinetic analysis as shown on the right. This resulted in a better first-order fit than that in Figure 97.
trace (Figure 99) does not flatten out as much as seen in Figure 98. The time-dependent data also appear to diverge from the least-squares line to a first-order decay at the beginning of the trace (Figure 99). When the faster portion of the trace is removed from the data analysis (Figure 99, range B), the first-order fit is better (Figure 100) with a rate (137 s\(^{-1}\)) that is not too different from the rate (124 s\(^{-1}\)) reported above. The above rate constants are derived from data taken from only the first two half-lives. Additional half-lives could not be obtained because these data were already taken at the longest sweep of 1 ms/division available on the TEKtronix digitizer.

The inability to go to longer sweep rate to increase the number of half-lives analyzed and the slight deviation of the first-order fit make the type of RSSR\(^{-}\) decay in papain unclear. H\(^{\bullet}\), another reducing agent that may also display some of the same selectivity that was demonstrated by the CO\(_2\)^\(-\) radical, was not attempted since Clement et al.\(^{63}\) reported no disulfide band when papain was reduced by H\(^{\bullet}\).

Asparaginase. L-Asparaginase was the only protein we could find that we could get in sufficient quantities and that has a single disulfide.\(^{107}\) L-Asparaginase EC-2 from \textit{Escherichia coli} B catalyzes the conversion of L-asparaginase to L-aspartic acid and ammonia:\(^{107}\)

\[
\text{Asn} \longrightarrow \text{Asp} + \text{NH}_3 \quad (52)
\]
Figure 99. First- and second-order kinetic analysis for RSSR⁺ decay after the reduction of papain by CO₂⁻ at pH 7.1, whole curve analysis. Notice that the data fit first order better than second order. The divergence at both the upper left and lower right of the fit is still there. The entire trace (right, dashed line A) was used for the kinetic analysis. The conditions used were as follows: [eni] = 0.26 mM, [CO₂⁻] = 26 mM, 10 mM phosphate, 0.1 M formate, and N₂O saturated.

Figure 100. First- and second-order kinetic analysis for RSSR⁺ decay after the reduction of papain by CO₂⁻ at pH 7.1, latter curve analysis. The data used for kinetic analysis are represented by B in Figure 99. The conditions were the same as those used in Figure 99.
The enzyme is a tetramer that has a molecular weight of 130,000. Each monomer has an identical sequence and has one disulfide per monomer with no free cysteine. Results from nondissociative electrophoresis and sedimentation equilibrium have shown that the molecule will exist in aggregates of two to four tetramers per aggregate; unfortunately, this may have caused problems as will be seen shortly.

The spectrum resulting from the reaction of e-aq with asparaginase at pH 6.94 yielded the 410-nm disulfide band and a 355-nm imidazolium band (Figure 101). The 355-nm band was not unexpected since there is evidence that an imidazole is present at the active site, and this enzyme may have a "charge-relay" type of active site since there may also be a serine at the active site. Unfortunately, the disulfide decay was still complex (Figure 102). Indeed, when the first part of the curve is analyzed (Figure 102, right), the data appear to fit second-order kinetics ($k_2 = 1 \times 10^9 \text{ M}^{-1} \text{s}^{-1}$) (Figure 103, left), and if the latter part of the curve is analyzed, the data appear to fit first-order kinetics ($k_1 = 428 \text{ s}^{-1}$) (Figure 103, right). The data do not seem to fit concurrent first- and second-order kinetics when nonlinear fitting is used (see Appendix C for derivation of equation) (Figure 104). Additional attempts to fit these data to concurrent first-order decays and
Figure 101. Time-dependent spectrum resulting from e–aq reacting with asparaginase at pH 6.9. Notice both the 360-nm imidazole band and the 410-nm disulfide band. The conditions used were as follows: [enz] = 67 μM, [e–aq] = 10 μM phosphate, 50 mM NaCl, 0.1 M tert-butyl alcohol, and argon saturated. X = 20 μs, + = 0.1 ms, o = 0.3 ms, * = 0.5 ms, e = 0.8 ms, and y = 0.8 ms after the end of the pulse. This spectrum, like most of the following spectra, consists of points that represent the sum of two or more data points per wavelength per time.

Figure 102. First- and second-order kinetic analysis for R55R– decay after the reduction of asparaginase by e–aq at pH 6.9, whole curve analysis. The data do not fit either first or second order. The conditions used were the same as those in Figure 101.
Figure 103. First- and second-order kinetic analysis for RSSR* decay after the reduction of asparaginase by e-aq at pH 6.9, first-portion and latter-portion analysis. (Left) The first part of the curve, designated as B in Figure 102 (right), appears to fit second-order kinetics. (Right) The latter part of the curve designated as C in Figure 102 (right) appears to fit first-order kinetics. The conditions were the same as those used in Figure 101.

Figure 104. Residual plot from the nonlinear fitting of RSSR* decay after the reduction of asparaginase by e-aq at pH 6.9, concurrent first- and second-order decay. The residual plot shows a trend and not random scattering showing that the data do not fit. The conditions were the same as those used in Figure 101.
concurrent second-order decays with and without a linear background drift resulted in less improvement in the fit. Since this complexity could be due, in part, to an overlapping absorbance from the imidazole band, the decay kinetics were again tried at pH 8.43. This pH is above the apparent pKₘ of the active site imidazole that has been demonstrated in methylene blue inactivation profiles¹⁰⁶ and in pH profiles using Asn as the substrate.¹⁰⁵

From these RSSR⁻ decay kinetics it is still difficult to ascertain whether the disulfide decay in asparaginase is really first or second order (Figure 105) because, like the papain kinetics, the limitation of the equipment would not allow more of the decay curve to be measured. There are other problems. First, although the primary structure for the four monomers of this molecule is identical, perhaps their tertiary and quaternary structures are not identical, thus yielding complex kinetics. Second, gentle bubbling with argon resulted in the loss of virtually all asparaginase activity. Unfortunately, this observation was discovered only after the two experiments were made, so little more can be said concerning asparaginase. However, caution should be exercised if asparaginase is attempted again since asparaginase really has four disulfides per molecule,¹⁰⁷ one per monomer, which makes asparaginase a poor candidate for studying RSSR⁻ decay in proteins. Another protein must be found that contains either a single disulfide or many
Figure 105. First- and second-order kinetic analysis for RS5R\textsuperscript{+} decay after the reduction of asparaginase by e\textsuperscript{-}aq at pH 0.43, whole curve analysis. (Left) The data fit first-order decay better than second-order decay; however, there is still some divergence. (Right) Trace used for kinetic analysis.
disulfides in which some can be selectively reduced and blocked to provide a protein with one intact disulfide. There are two possibilities, ribonuclease A (RNase) or carboxypeptidase A. There is a procedure that is reported to cleave and block two of the four disulfides of ribonuclease A, leaving fully active RNase. This would still leave two disulfides, but perhaps one of these disulfides will not react with e⁻aq. Even if both disulfides contribute to the RSSR⁻ decay kinetics, their decay may be significantly different to provide better success with nonlinear fitting.

Carboxypeptidase A has only one disulfide, is relatively cheap, and hence is a good candidate for the study of RSSR⁻ decay in proteins. The single zinc present in the molecule is reported not to compete successfully with the single cystine in carboxypeptidase for e⁻aq since a large fraction of the e⁻aq produced in one pulse resides on the disulfide. This disulfide is also reported to reside on the surface of the molecule and therefore may be very susceptible to changes in ionic strength and solvent polarity. These two parameters could be varied and the characteristics of this single disulfide studied.

RNase. Bovine pancreatic ribonuclease (RNase) is a well-studied protein that catalyzes the cleavage of the phosphodiester linkages of RNA.
RNase is a single-chained molecule with a molecular weight of 13,700 whose amino acid sequence is known. It contains four disulfides and four histidines; two histidines are located at the active site. More pulse radiolysis has been done on RNase than on any other protein. Faraggi et al. have shown that both the imidazole yield and the disulfide yield display pH characteristics with a pKₐ of about 6. Bisby et al. have shown that none of the four disulfides in RNase at neutral pH can be reduced by the CO₂⁻ radical except after repeated pulsing or after heating. It was postulated that the CO₂⁻ radical is physically hindered from reacting with the disulfides until the enzyme has undergone its well-documented thermal denaturation, which may be mimicked by repeated pulsing. The reduction of disulfides by the CO₂⁻ radical occurs relatively slowly when compared to disulfide reduction with e⁻aq with a rate constant of 5 x 10⁸ M⁻¹ s⁻¹ and reacts about 1000 times more slowly with histidine and other amino acids.
The spectrum resulting from the reduction of RNase by e-aq at pH 5.2 showed no disulfide band, only a 355-nm imidazole band (Figure 106). This was expected since the e-aq is reported to react with protonated imidazole and not disulfide at pH 5.2. From thermal denaturation curves for ribonuclease, at 75 °C and pH 5.20, RNase will be fully denatured at this temperature and pH. The spectrum resulting from the one-electron reduction of RNase at pH 5.2 and T = 75 °C (Figure 107) looks very similar to the spectrum taken at room temperature (Figure 106). The major difference is the slight increase in absorbance around 400 nm. It is interesting that this denatured form of the enzyme has only a small absorbance due to disulfide and that this denatured form of RNase has just about the same absorbance at 355 nm as the native form of the enzyme at room temperature. Apparently, denaturing the enzyme did little to reduce or increase the imidazole band at 355 nm or increase the disulfide band at 410 nm.

Since no RSSR⁻ spectrum could be obtained with e-aq at pH 5.2, no RSSR⁻ spectrum was expected when CO₂⁻ was the reducing agent. And, indeed, no RSSR⁻ spectrum was apparent at room temperature or 74 °C (Figures 108 and 109). Yet a rise in the imidazole region is present, and this rise persists when the temperature is raised to 75 °C (Figure 109). A spectrum of the buffer without RNase (Figures 108 and 109, dashed line, and Figure 110) gives a shoulder in the 360-nm
Figure 106. Time-dependent spectrum resulting from $e^-_{aq}$ reacting with RNase at pH 5.2. Notice the 355-360-nm imidazole peak. The conditions used were as follows: $[\text{enz}] = 0.10$ mM, $[e^-_{aq}] = 14$ µM, 10 mM phosphate, 0.1 M tert-butyl alcohol, and argon saturated. $X = 10$ µs, $* = 50$ µs, $\circ = 0.1$ ms, $\bullet = 0.3$ ms, $\ast = 0.5$ ms, and $\times = 0.8$ ms after the end of a 500-ns pulse.

Figure 107. Time-dependent spectrum resulting from $e^-_{aq}$ reacting with RNase at pH 5.2 at 75 °C. Notice the 355-360-nm imidazole peak and the bump around 400 nm. The conditions were the same as those used in Figure 106.
Figure 108. Time-dependent spectrum resulting from CO$_2^-$ reacting with RNase at pH 5.2. Notice that the 360-nm band rapidly decays. The dashed line represents the (yield normalized) spectrum of buffer containing formate taken at 20 µs after the pulse. The conditions for the RNase spectrum used were as follows: [enz] = 0.21 mM, [CO$_2$-aq] = 14 µM, 10 mM phosphate, 0.1 M formate, and H$_2$O saturated. The conditions for the buffer spectrum used were as follows: 0.1 M formate, 10 mM phosphate, pH 8. X = 20 µs, * = 50 µs, o = 0.1 ms, * = 0.3 ms, e = 0.5 ms, and x = 0.8 ms after the end of a 200-ns pulse.

Figure 109. Time-dependent spectrum resulting from CO$_2^-$ reacting with RNase at pH 5.2 and 75 °C. Notice how closely this figure compares to Figure 108 taken at room temperature. The conditions were the same as those used in Figure 108.
Figure 110. Time-dependent spectrum of buffer containing formate. Notice the shoulder at 350 nm. The conditions were the same as those used in Figure 108 for the dashed line except that $x = 2 \mu s$, $+ = 10 \mu s$, $o = 20 \mu s$, $* = 30 \mu s$, $x = 50 \mu s$, and $x = 0.1$ ms after the end of a 300-ns pulse.
region but not a rise as seen in the two RNase plus e^-aq spectra (Figures 106 and 107). A shoulder at 360 nm has never been seen before in the CO_2^- radical spectrum; however, since the magnitude is very small when compared to that of the peak centered near 250 nm, this shoulder could easily have been overlooked. Both the absorbances at 360 and 300 nm decay by second-order kinetics (Figure 111) and are thought to be a result of dimerization to form oxalic acid.\(^5\)

\[
2 \text{CO}_2^- \longrightarrow \text{CO}_2^- + \text{CO}_2^- \quad (54)
\]

Since the extinction coefficient is not known for this region and since extrapolation from published spectra up to this region is difficult and extremely inaccurate, a plot of \(\Delta\text{Abs}/\Delta t\) at 300 nm versus \(\Delta\text{Abs}/\Delta t\) at 360 nm was made (see Figure 112 and Appendix D for derivation). The good linearity is good evidence that the two absorbances decay by the same rate and could belong to the same species.

Only after the CO_2^- spectrum was normalized with respect to dose and path length is it known that the UV end of the spectrum of CO_2^- with RNase cannot be due entirely to the CO_2^- radical; the amplitude of the whole time-dependent spectrum is too high compared to that of the CO_2^- spectrum (Figures 108 and 109). The \(t_\frac{1}{2}\) for the first-order decay of CO_2^- plus RNase spectrum is much shorter than the \(t_\frac{1}{2}\) for
Figure 111. First- and second-order kinetic analysis of the species formed in protein buffer containing formate at pH 8. Both absorbances at 296 nm (left) and 340 nm (right) decay by second-order kinetics. The conditions were the same as those used in Figure 110.

Figure 112. Absorbance/Δt plot for the 350-nm buffer absorbance (x axis) and the 298-nm absorbance (y axis). The linearity suggests that the two absorbances are decaying at the same rate. The conditions were the same as those used in Figure 110.
the second-order decay of CO$_2^-$ in buffer, 280 and 220 µs versus 52 and 77 µs at 296 and 348 nm, respectively. This also suggests that CO$_2^-$ does react with RNase at a site other than the disulfide. The fact that prepulsing is required to see any RSSR$^-$ absorbance at low to neutral pH 89,122 in solutions of RNase is indirect evidence that CO$_2^-$ must react with RNase to cause the conformational change to expose disulfides.

The 360-nm band in the spectrum of RNase taken at pH 5.2 with CO$_2^-$ as the reducing species (Figures 108 and 109) appears to decay more rapidly than the 360-nm band in the RNase spectrum where e$^-_{aq}$ was the reducing species (Figures 106 and 108). This is a strong indication that the band at 360 nm in the pH 5.20 spectrum of RNase is not due to an imidazole. In addition, other amino acid functional groups are purported to be reduced by the CO$_2^-$ radical but at a 1000 times slower rate than with disulfides.89,94 Finally, when the pH is raised to 7.0, the slight rise in the 360-nm region is still present at fast time (Figure 113). If this rise does represent an imidazole, it cannot represent the same imidazole that was reported to have a pK$_{a}$ of 6.0 when e$^-_{aq}$ was the reducing agent$^{10}$ because the absorbance at 1 pH unit above the reported pK$_{a}$ would be very small. There are two interesting features in Figure 113. One is the slight absorbance in the disulfide region and the other is the beginning of an isosbestic point at 390 nm. The decay
Figure 113. Time-dependent spectrum of CO$_2^-$ reacting with RNase at pH 7.0. Notice the shoulder at 350 nm, the beginning of an isosbestic point at 390 nm, and the slight growth of a 410-nm peak. The conditions used were as follows: [enz] = 0.26 mM, [CO$_2^-$] = 14 mM, 10 mM phosphate, 0.5 M formate, and N$_2$O saturated. X = 10 μs, * = 50 μs, o = 0.1 ms, + = 0.3 ms, e = 0.5 ms, and x = 0.8 ms after the end of a 300-ns pulse.

Figure 114. Time-dependent spectrum of CO$_2^-$ reacting with RNase at pH 8.3. Notice the shoulder at 350 nm, the isosbestic point at 370 nm, and the growth of the 410-nm band with time. The insert is the trace representing the isosbestic point at 370 nm. The conditions used were as follows: [enz] = 0.33 mM, [CO$_2^-$] = 14 mM, 10 mM phosphate, 0.1 M formate, and N$_2$O saturated. X = 20 μs, * = 30 μs, o = 50 μs, e = 0.1 ms, * = 0.2 ms, and x = 0.4 ms after the end of a 300-ns pulse.
rates at 296 and 348 nm are first order and are $16 \times 10^3 \text{ s}^{-1}$ and $51 \times 10^3 \text{ s}^{-1}$, respectively, and the formation constant for the 410-nm band is also first order and has a rate of $28 \times 10^3 \text{ s}^{-1}$. There appears to be the beginning of an isosbestic point at 390 nm. The rise in the RSSR$^-$ region and the isosbestic point is even more apparent when the pH was changed to 8.43 (Figure 114). In this case the isosbestic point seems to be located at 370 nm. This looks like a classic example where the species absorbing around 350 nm is decaying to form the species (probably RSSR$^-$) absorbing around 410 nm. However, the decay rates at 327 nm ($41 \times 10^3 \text{ s}^{-1}$, Figure 115) and 348 nm ($51 \times 10^3 \text{ s}^{-1}$, Figure 116) do not compare very well to the formation rate at 409 nm ($9 \times 10^3$, Figure 117), and I am at a loss to explain this.

A plot of observed rate for the formation of the 410-nm band versus [RNase] at pH 8.4 gave a straight line whose slope $= 1 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ (Figure 118). This number is 10 times lower than the reported rate of $5 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$ for the reduction of lipoate by CO$_2$- $^{94,125}$ and 17 times lower than the reported rate of $7 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$ for the reduction of the single disulfide on carboxypeptidase A by CO$_2$-. $^{123}$

The CO$_2^-$ radical must react with other sites on the RNase molecule because Bisby et al. $^{89}$ reported that the RSSR$^-$ spectrum could only be obtained at neutral pH after prepulsing the solution or working above the melting point for RNase. In fact, when the temperature was increased from
Figure 115. Kinetic analysis of the decay of the absorbance at 327 nm, pH 8.34. The conditions were the same as those used in Figure 114.

Figure 116. Kinetic analysis of the decay of the absorbance at 348 nm, pH 8.34. The conditions were the same as those used in Figure 114.
Figure 117. Kinetic analysis of the formation of the absorbance at 405 nm, pH 0.34. The conditions were the same as those used in Figure 114.

Figure 118. Plot of pseudo-first-order formation rate at 410 nm versus RNase concentration gives a straight line. The slope of the line is $1 \times 10^7$ M$^{-1}$ s$^{-1}$ and represents the second-order rate constant for the formation of the 410-nm band in RNase. The buffer contained RNase at the indicated concentration, 10 mM phosphate, 0.1 M formate, pH 8.4, and N$_2$O saturated.
room temperature to 75 °C, the small rise in the 400-nm region (Figure 117) grows into a large disulfide peak centered at 410 nm (Figure 119). This increase in the 410-nm absorbance with increasing pH quite nicely parallels the melting curve for RNase as observed as a decrease in absorbance at 287 nm when a similar experiment using the exact same conditions (except for a lower RNase concentration) was carried out in a scanning spectrophotometer (Figure 120).

Let's explore the possible identity of the two species. The species absorbing at 350 nm could be the CO$_2^-$ radical for three reasons. First, the CO$_2^-$ radical does appear to have a shoulder in the 350-nm region (Figure 110). Second, as the 350-nm band decays the 410-nm band appears (although not at the same rate) (Figures 113 and 114). A charge transfer between a reduced imidazole and a disulfide will not occur$^{94}$ and therefore cannot be used to explain this observation. Third, the CO$_2^-$ radical reacts very slowly with disulfides ($5 \times 10^8$ M$^{-1}$ s$^{-1}$)$^{94,125}$ and is purported to react about 1000 times slower with other amino acid functional groups,$^{89,94}$ and therefore CO$_2^-$ may decay relatively slowly in solutions that contain no accessible disulfides. The product of the decay is most likely the product of reaction 54, oxalic acid. A significant problem with this assignment is that the CO$_2^-$-buffer spectrum is much lower than the CO$_2^-$-RNase spectrum (Figures 108 and 109). The CO$_2^-$ spectrum$^{126}$ and the carbonyl spectrum overlap in this
Figure 119. Time-dependent spectrum of CO$_2^-$ reacting with RNase at pH 7.0 and 75 °C. Notice the massive 410-nm disulfide band. The conditions used were as follows: [enz] = 0.24 mM, [CO$_2^-$] = 14 μM, 10 mM phosphate, 0.1 M formate, and N$_2$O saturated. • = 50 μs, ◦ = 0.1 ms, * = 0.3 ms, • = 0.5 ms, and x = 0.6 ms after the end of a 300-ns pulse.

Figure 120. Melting curves of RNase in the buffer used for pulse radiolysis, monitoring the yield at 410 nm using pulse radiolysis and the absorbance at 287 nm using a spectrophotometer with increasing temperature. The increase in absorption observed at 410 nm (○) compares nicely to the decrease in absorbance observed at 287 nm (•). [enz] = 0.27 mM for pulse radiolysis and [enz] = 34 μM for the steady-state spectroscopy. The buffers containing the enzyme were the same, 10 mM phosphate and 0.1 M tert-butyl alcohol, pH 7.0.
area, and consequently the RNase-CO$_2^-$ spectrum (Figures 108 and 109) could be the sum of the CO$_2^-$ spectrum and carbonyl. This would mean that some CO$_2^-$ must react with protein carbonyls. Unfortunately, no work in this area has been reported, and the reasons may be 2-fold. First, the loss of CO$_2^-$ absorbance would be difficult to measure because the CO$_2^-$ spectrum overlaps the carbonyl radical spectrum. Second, since direct measurement of CO$_2^-$ is difficult (a similar problem when measuring ·OH reactivity), a means of indirectly measuring CO$_2^-$ reactivity must be found. However, the high selectivity of the CO$_2^-$ radical (compared to e$^{-}$aq or ·OH) limits the compounds that can be used as competitors against the protein for CO$_2^-$. For example, the dye methylene blue, one of the few compounds that is known to be reduced by CO$_2^-$, also appears to bind nonspecifically to proteins, thereby complicating the measurement for the rate of CO$_2^-$ reacting with proteins.$^{129}$

The identity of the species absorbing at 410 nm is also difficult. The reductive deaminated product of the N-terminal end of RNase can be ruled out because the yield is displaying the opposite pH dependence that has been reported for simple peptides reduced by the more powerful reducing agent, e$^{-}$aq.$^{8,127}$ More deamination would be expected to occur when the pH is reduced below the normal pKa of 8-10 for the N-terminal end of a protein as it becomes unprotonated.
The absorbing species cannot be due to denatured RNase since $CO_2^-$ will rapidly react with denatured material and thereby instantly give an RSSR$^-$ band as seen in spectra when the temperature is changed to 75 °C (Figure 119).

The presence of the RSSR$^-$ band at pH 8.4 due to "leakage" of e$^{-}$/aq, i.e., e$^{-}$/aq that is not scavenged by $N_2$O, can be ruled out since, like $CO_2^-$ plus denatured RNase, the disulfide would be formed almost instantly, not slowly as seen in Figures 113 and 114. The species absorbing at 410 nm could be due to a disulfide because the absorbance is in the proper region$^{2-4}$ for a disulfide radical anion and $CO_2^-$ radical can reduce disulfides.$^{2-4,94,125}$

The major problem with assigning the 410-nm band to RSSR$^-$ is the strange pH dependence; i.e., at pH 5.2 no RSSR$^-$ band is evident (Figure 108) but at pH 8.4 an RSSR$^-$ band is evident (Figure 114). On the basis of the report$^94$ on the reaction

$$CO_2^- + \text{liophe} \rightarrow \text{liophe}^- + CO_2 \quad (55)$$

the formation rate will not display any pH dependence above pH 6.94. One theory to explain the observed pH dependence is quite similar to the theory proposed by Faraggi et al.$^{10}$ for the observed effect that pH had on both the disulfide band and the imidazole band in spectra obtained for e$^{-}$/aq reduction of RNase. This increase in absorbance at 410 nm with an increase pH above pH 7 may reflect the pH dependence
for another group on RNase that also reacts with the CO$_2^-$ radical and whose product is invisible in the near-UV and visible region of the spectrum. Upon heating or prepulsing, the structural integrity of the molecule is lost as well as some of these "mystery" groups, thereby allowing more CO$_2^-$ to react with disulfides.

Another more plausible explanation is the effect of pH on the structure of RNase. RNase is reported to undergo reversible denaturation below pH 5.\textsuperscript{111,113-115} However, there are reports that this denaturing process is not fully reversible at pH 7 or higher.\textsuperscript{111,125} It is plausible this irreversible denaturation that has been reported at high pH and high temperature may also occur, but to a lesser extent, at room temperature. In a crude attempt to follow the change in the optical properties of RNase under the same conditions used for pulse radiolysis, some steady-state work with a spectrophotometer was tried. Nothing could be seen at room temperature; however, a slight change in the optical properties starting at pH 7 and changing more drastically with increasing pH was observed when the temperature was changed to 75 °C. However, the increase in absorbance is concomitant with clouding of the solution and most likely reflects precipitation of the enzyme since the pI point for the enzyme is 9.45.\textsuperscript{111} If the increase in absorbance at 410 nm with increasing pH has anything to do with the pI being 9.45, it is difficult to understand.
To say the least, the pH dependence of the 410-nm band is confusing. I suggest using the acetone/2-propanol pair with argon or 2-propanol/N₂O to see if the developing 410-nm band is due to disulfide. The resulting CH₃COHCH₃ radical will reduce disulfides. And more work must be done on the reactivity of CO₂⁻ with amino acids. It is still not known how the CO₂⁻ radical is forcing RNase to unfold and expose its disulfide after repeated pulsing.
V. CONCLUSIONS

The conclusion based on the results under PROTEASES is inescapable: in all the enzymes tried, whenever the enzyme was made inactive, the electron-imidazole adduct band at 360 nm was lost except with papain, where the apparent pKₐ for this 360-nm band shifted 4 or more pH units. α-Chymotrypsin, subtilisin, and thiosubtilisin exhibit a 360-nm band but no band with the inactive derivatives PMS-, N-methyl-, and anhydrochymotrypsin and PMS-subtilisin and with subtilisin in the presence of the competitive inhibitor boric acid. These results support an earlier hypothesis that active site histidines can be reduced by e-aq while histidines at other locations appear to be unreactive.⁹,¹⁰ This hypothesis was based on the observation that a 360-nm band was present in trypsin and α-chymotrypsin spectra but not in the spectra of their inactive zymogens, trypsinogen and chymotrypsinogen.⁹ (A summary of the existence of a 360-nm band in proteases can be found in Table 7).

The strength of this hypothesis will only develop as more proteins are studied. But such a study will end with this dissertation unless instrumentation that is capable of taking a spectrum in a single shot is acquired. Such a
Table 7. Summary of the Existence of a 360-nm Band in Proteases

<table>
<thead>
<tr>
<th>protease</th>
<th>360-nm band ?</th>
</tr>
</thead>
<tbody>
<tr>
<td>trypsin$^{9,10}$</td>
<td>yes</td>
</tr>
<tr>
<td>trypsinogen$^{9}$</td>
<td>no</td>
</tr>
<tr>
<td>$\delta$-chymotrypsin</td>
<td>yes</td>
</tr>
<tr>
<td>$\alpha$-chymotrypsin$^{9,10}$</td>
<td>yes</td>
</tr>
<tr>
<td>chymotrypsinogen$^{9}$</td>
<td>no</td>
</tr>
<tr>
<td>PMS-chymotrypsin</td>
<td>no</td>
</tr>
<tr>
<td>CH$_3$-chymotrypsin</td>
<td>no</td>
</tr>
<tr>
<td>anhydrochymotrypsin</td>
<td>no</td>
</tr>
<tr>
<td>subtilisin BPN'</td>
<td>yes</td>
</tr>
<tr>
<td>subtilisin Carlsberg</td>
<td>yes</td>
</tr>
<tr>
<td>thiosubtilisin BPN'</td>
<td>yes</td>
</tr>
<tr>
<td>PMS-subtilisin BPN'</td>
<td>no</td>
</tr>
<tr>
<td>subtilisin BPN' with boric acid</td>
<td>no</td>
</tr>
<tr>
<td>papain</td>
<td>yes</td>
</tr>
<tr>
<td>CH$_3$S-papain</td>
<td>yes(b)</td>
</tr>
</tbody>
</table>

(a) Confirmed in this dissertation. (b) pK$_a$ shift observed.

...system would require smaller quantities of enzyme for study, a severe limitation with the current method, thereby opening up possibilities for studying more expensive proteins. Irrespective of future results, it is surprising that e-aq with a redox potential of ca. -2.7 V$^2$ should appear to have such a high specificity; not only are just active site histidines detected but also covalent or noncovalent modifications at the active site containing these histidines modulate this apparent specificity. Imidazole accessibility to e-aq cannot explain this apparent specificity because the single histidine of lysozyme, which is reported to be solvent accessible on the basis of X-ray diffraction results and not
at the enzyme active site, does not yield the electron-imidazole adduct.\textsuperscript{10}

The primary site of e\textsuperscript{-aq} attachment to proteins may not be the histidines or disulfides. But rather the initial site attachment may occur on the protein surface, perhaps on the carbonyls since they will react with e\textsuperscript{-aq} (equation 15), and is followed by a rapid migration of the electron to its final resting place. Therefore, the specificity of e\textsuperscript{-aq} toward active site histidines could have two explanations. First, an imidazole would be reduced only if there were one or more paths leading from the site(s) of initial e\textsuperscript{-aq} attachment to that imidazole. To explain why covalent or noncovalent changes to the active site of proteases close off these paths to the electron, one would have to conclude that all paths in proteases lead to the active site imidazole. Second, the attached electron is free to migrate throughout the protein molecule until it settles in the deepest potential energy well, either an imidazole or a disulfide. To explain why covalent or noncovalent changes in the active site of proteases result in loss of the imidazole band, one would have to pose that a unique potential surface exists around the active site and this unique potential surface is particularly sensitive to protein structure alterations. Actually, both explanations can be applied to the same molecule with a single electron. For example, after initial attachment the e\textsuperscript{-aq} rapidly migrates through
the molecule until it finds a potential well in which it settles or a unique potential surface that guides the electron to the active site imidazole.

There is circumstantial evidence for electron migration within proteins after attachment of hydrated electrons.\(^2\) Perhaps more evidence could be obtained by using flash photolysis and forcing tryptophan to give up one electron. If the enzyme is carefully chosen such that all tryptophans are not at the surface and the energy of this liberated electron is sufficiently high enough, it might migrate to a disulfide or protonated histidine and reduce it.

From my studies on the effect of solvent on \(\lambda_{\text{max}}\), the spectral features observed in spectra of papain (Figures 31 and 32) at 385 nm (and hopefully in other proteins studied in the future) cannot be due to a disulfide radical anion in a more hydrophobic environment than the disulfide radical anion that absorbs at 410 nm (Figures 31 and 32). Most likely this band represents a disulfide radical anion that has been protonated (like lipoate or lipoamide at low pH, \(\lambda_{\text{max}} = 385\) nm) or a disulfide radical anion that is structurally similar to six-membered ring disulfides (like ox-DTT, \(\lambda_{\text{max}} = 390\) nm).

The decay of disulfide radical anions in proteins needs further study. The disulfide radical anion observed in the e-aq reduction of asparaginase Cd\(_2\)-reduction of papain may be first order, but accurate determination will require
equipment that can be used with very long sweeps. To date simple linear disulfides decay by first-order kinetics and simple cyclic disulfides decay by second-order kinetics (see DISULFIDES). Disulfides in proteins fit the cyclic disulfide category because cleavage of a disulfide in single-chained molecules will not result in two molecules: the two free thiols are still attached to each other. However, if these disulfide radical anions are not on the surface of the protein molecule, they cannot decay by the disproportionate mechanism postulated to be responsible for cyclic decay (Figure 62). Therefore, a slow first-order decay is quite possible.

It is suprising that increasing the temperature above the melting point for RNase did not significantly reduce or increase the 360-nm imidazole band in spectra taken at pH 5.2 (Figures 106 and 107). This observation gives more support to the potential sink argument than the pathway argument as an explanation for electron migration in proteins since one would expect the pathways to change on going from native to denatured enzyme. On the other hand, the fact that there was some indication of disulfide only after the temperature was increased (Figure 107) gives support to the pathway argument since one would not expect temperature to affect potential sinks. Perhaps both arguments are valid. In any event, more experiments are needed.
An interesting sidelight of the RNase work is the presence of the slowly forming 410-nm band in RNase-CO$_2^-$ spectra whose magnitude increases with increasing pH. The pH behavior is confusing and more work must be done. The identity of the 410-nm band as a disulfide could be strengthened by using the CH$_3$C(OH)CH$_3$ radical instead of the CO$_2^-$ radical. This radical is uncharged and reduces disulfides.$^{125}$

For the future I suggest the following experiments:
(1) monitoring of the kinetics for the decay of the single disulfide in carboxypeptidase; (2) exploration of the IR; (3) test the equilibrium idea for cyclic disulfides by varying the temperature and monitoring the 330/400 nm yield ratio; (4) titration of the 410-nm band resulting from the CO$_2^-$ or the CH$_3$CHOH reduction of RNase; (5) determination of the rate constant for the CO$_2^-$ reduction of individual amino acids; (6) if this can be done, then the CO$_2^-$ attachment to proteins can also be done. If the capability of obtaining a highly resolved spectrum in a single shot is possible, the following may be interesting to do: (7) repeat the papain spectrum at pH 7.1; (8) do lipoate or ox-DTT on H$_2$O or ethanol and look for two peaks; (9) repeat the experiment that was originally done by Bisby et al.$^{89}$ on the CO$_2^-$ reduction of RNase by taking the spectrum after the first pulse and the fifth pulse etc.

To complete this conclusion, it is relevant to say much has been done but there still is much to do.
Appendix A: Derivation of the pH Equation To Fit All OD versus pH Data

Given the situation where

\[ HA \rightleftharpoons H + A \] (53)

the equilibrium constant can be defined as

\[ K = \frac{C_A \cdot C_H}{C_{HA}} \] (54)

where \( C_A = [A] \), \( C_{HA} = [HA] \), and \( C_H = [H] \). Assuming that the absorbance (Abs) at a single wavelength is due entirely to HA and A, the following is true based on Beer's law:

\[ \text{Abs} = \varepsilon_{HA} l C_{HA} + \varepsilon_A l C_A \] (55)

\( l \) = path length, \( \varepsilon_{HA} \) = extinction coefficient of HA, and \( \varepsilon_A \) = extinction coefficient of A. In addition, the following expression for the total amount of A (\( C_T \)) must be true.

\[ C_T = C_A + C_{HA} \] (56)
It is possible to combine equations 54 and 56 to yield an equation where \( C_A \) is in terms of \( C_H \) and \( C_T \) and a second equation where \( C_{HA} \) is in terms of \( C_H \) and \( C_T \).

First equation:
\[
C_T = C_A + C_{HA} \quad \text{(rearrangement of equation 56)} \quad (57)
\]
\[
C_T = C_A + \frac{C_H C_A}{K} \quad \text{(substitution into equation 54)} \quad (58)
\]
\[
C_T/(1 + C_H/K) = C_A \quad \text{(rearrangement)} \quad (59)
\]
\[
C_A = \frac{K C_T}{(K + C_H)} \quad \text{(rearrangement)} \quad (60)
\]

Second equation:
\[
C_T = C_A + C_{HA} \quad (56)
\]
\[
C_T = \frac{K C_H}{C_H} + C_{HA} \quad \text{(substitution into equation 54)} \quad (61)
\]
\[
C_{HA} = \frac{C_H C_T}{(K + C_H)} \quad \text{(rearrangement)} \quad (62)
\]

Now substitute equations 60 and 62 into 55:
\[
Abs = \epsilon_{HA} \frac{C_H C_T}{K + C_H} + \epsilon_{A} \frac{K C_T}{K + C_H} \quad (63)
\]

Collecting constants and substituting
\[
Abs = \epsilon_{HA} \frac{C_H}{K + C_H} + \epsilon_{A} \frac{K}{K + C_H} \quad (64)
\]
\[
Abs = a' C_H/(K + C_H) + a'' K/(K + C_H) \quad (65)
\]
Abs is the dependent and \( C_H \) the independent variable. \( a' \), \( a'' \), and \( K \) are the parameters to be fit.
Appendix B: First-Order Decay Derivation

For

\[ C \rightarrow P \quad (66) \]

the rate law is

\[ \frac{-1}{c} \frac{d[C]}{dt} = k[C] \quad (67) \]

where \( c \) is the stoichiometry of \( C \).

The rate law can be integrated to yield

\[ \ln [C] = -ckt + \ln [C]_0 \quad (68) \]

where \([C]\) = concentration at any time and \([C]_0\) = concentration of \( C \) at \( t = 0 \). To convert from concentration units to absorbance units, the following is true:

\[ A = A_\infty \quad (69) \]

so

\[ [C] = (A - A_\infty)/(\varepsilon \ell) \quad \text{and} \quad [C]_0 = (A_0 - A_\infty)/(\varepsilon \ell) \quad (70) \]

where \( A \) = absorbance, \( \varepsilon \) = extinction coefficient of \( C \), and \( \ell \) = path length. Substituting in for \([C]\) and \([C]_0\)
\[ \ln \left( \frac{(A - A_\infty)}{(\varepsilon l)} \right) = -ckt + \ln \left( \frac{(A_0 - A_\infty)}{(\varepsilon l)} \right) \]  

\[ \ln (A - A_\infty) - \ln (\varepsilon l) = -ckt + \ln (A_0 - A_\infty) - \ln (\varepsilon l) \]  

\[ \ln (A - A_\infty) = -ckt + \ln (A_0 - A_\infty) \] (73)

A plot of \( \ln (A - A_\infty) \) versus \( t \) will yield a linear plot and a negative slope where slope = \(-bk\) and intercept = \( \ln (A_0 - A_\infty) \). For nonlinear curve fitting, the antilog of equation 68 is taken:

\[ [C] = [C]_0 e^{-ckt} \] (74)

Substituting in \( A/(\varepsilon l) \) and \( A_0/(\varepsilon l) \) for \([C]\) and \([C]_0\), respectively

\[ \frac{A}{(\varepsilon l)} = \frac{A_0 e^{-ckt}}{\varepsilon l} \] (75)

\[ A = A_0 e^{-ckt} \] (76)

The above equation can be simplified to the form

\[ A = ae^{-bt} \] (77)

where \( a \) and \( c \) are considered to be parameters to be fit.

This means that \( a = A_0 \) and \( b = ck \).
Appendix C: Second-Order Decay Derivation

For

\[ 2C \longrightarrow P \quad (78) \]

the rate law is

\[ \frac{-1}{C} \frac{d[C]}{dt} = k[C] \quad (79) \]

The integrated form is

\[ \frac{1}{[C]} = ckt + \frac{1}{[C]_0} \quad (80) \]

Substituting in \((A - A_\infty)/(\epsilon \ell) = [C]\) and \((A_0 - A_\infty)/(\epsilon \ell) = [C]_0\)

\[ \frac{1}{(A - A_\infty)} = \frac{ck}{\epsilon \ell} t + \frac{1}{(A_0 - A_\infty)} \quad (81) \]

A plot of \(\frac{1}{(A - A_\infty)}\) versus \(t\) will yield a linear plot and a positive slope where the slope = \(ck/(\epsilon \ell)\) and the intercept = \(1/(A_0 - A_\infty)\). The extinction coefficient must be determined by another method to get the rate constant, \(k\). For non-linear curve fitting, equation 81 is first rearranged before the extinction coefficient is substituted into it.
\[ [C] = \frac{A}{(\varepsilon \lambda)} \quad [C]_0 = \frac{A_0}{(\varepsilon \lambda)} \quad (82) \]

\[ [C] = \frac{1}{\frac{\varepsilon \lambda}{t} + \frac{1}{[C]_0}} \quad (83) \]

\[ A = \frac{\varepsilon \lambda}{\frac{\varepsilon \lambda}{A_0} \cdot \frac{A_0}{cA_0k} \cdot \frac{A_0}{cA_0k} + \varepsilon \lambda} = \frac{A_0 \varepsilon \lambda}{cA_0k t + \varepsilon \lambda} \quad (84) \]

\[ A = \frac{A_0}{\frac{cA_0k}{\varepsilon \lambda} t + 1} \quad (85) \]

The above equation can be simplified to the form

\[ A = \frac{a}{b t + 1} \quad (86) \]

where \(a\) and \(b\) are considered to be parameters to be fit.

This means that \(a = A_0\) and \(b = cA_0k/(\varepsilon \lambda)\). Like the linear equation, the extinction coefficient, \(\varepsilon\), must be known to obtain the rate constant, \(k\).

For concurrent first- and second-order decays, equations 77 and 86 were used.

\[ A = a e^{-b t} + \frac{a'}{b' t + 1} + D \quad (87) \]

For concurrent first-order decay, equation 77 was used.

\[ A = a e^{-b t} + a' e^{-b' t} + D \quad (88) \]
And for concurrent second-order decay, equation 86 was used.

\[
A = \frac{a}{bt + 1} + \frac{a'}{b't + 1} + D \quad (89)
\]

All of the nonlinear fitting was tried with or without a background absorbance, \(D (D = 0 \text{ or } D = A_0)\). When a linear drift was applied for background, \(D\) was assigned to be \(Dt\).
Appendix D: Derivation for Comparing Two Second-Order Decays

For

\[ A + A \rightarrow P \]  \hspace{1cm} (90)

the rate law is

\[ \frac{-d[A]}{dt} = k_A[A]^2 \]  \hspace{1cm} (91)

To the first approximation the following is true for very small intervals of \( t \):

\[ \frac{\Delta[A]}{\Delta t} = k_A[A]^2 \]  \hspace{1cm} (92)

For

\[ B + B \rightarrow P' \]  \hspace{1cm} (93)

the following is true:

\[ \frac{\Delta[B]}{\Delta t} = k_B[B]^2 \]  \hspace{1cm} (94)

Solving for \( k_B \)

\[ k_B = \frac{1}{[B]^2} \frac{\Delta[B]}{\Delta t} \]  \hspace{1cm} (95)
If A and B are the same species but absorbing at different wavelengths, then they must decay by the same rate, i.e., $k_A = k_B$. Substituting equation 95 into equation 92

$$\frac{\Delta[A]}{\Delta t} = \frac{[A]^2 \Delta[B]}{[B]^2 \Delta t} \quad (96)$$

Since $k_A = k_B$, then the following must be true also: $[A] = [B]$ and

$$\frac{\Delta[A]}{\Delta t} = \frac{\Delta[B]}{\Delta t} \quad (97)$$

Using Beer's law where $A = \varepsilon_A [A] l$ and $B = \varepsilon_B [B] l$

$$\frac{1}{l \varepsilon_A} \frac{\Delta A}{\Delta t} = \frac{1}{l \varepsilon_A} \frac{\Delta B}{\Delta t} \quad (98)$$

$$\frac{\Delta A}{\Delta t} = \frac{\varepsilon_A \Delta B}{\varepsilon_B \Delta t} \quad (99)$$

A plot of $\Delta A/\Delta t$ versus $\Delta B/\Delta t$ will then yield a straight line whose intercept will be zero and whose slope will be $\varepsilon_A/\varepsilon_B$. If $k_A = k_B$, then the plot will not be linear; the larger the difference between the two $k$'s, the less linear the plot will be.

This type of plot will also display a linear plot if A and B are different species but are connected by a very fast equilibrium and thus A and B appear to be the same.
Monitoring the concentration of B with time will display the same decay kinetics as the concentration of A because of the extremely fast equilibrium constant, $K$, where

$$K = \frac{[A]}{[B]}$$  \hspace{1cm} (101)

Substituting equation 101 into equation 96

$$\frac{\Delta[A]}{\Delta t} = k^2 \frac{\Delta[B]}{\Delta t}$$  \hspace{1cm} (102)

Again substitution into Beer's law:

$$\frac{1}{\lambda \varepsilon_A} \frac{\Delta A}{\Delta t} = k^2 \frac{1}{\lambda \varepsilon_B} \frac{\Delta B}{\Delta t}$$  \hspace{1cm} (103)

$$\frac{\Delta A}{\Delta t} = k^2 \frac{\varepsilon_A}{\varepsilon_B} \frac{\Delta B}{\Delta t}$$  \hspace{1cm} (104)

A plot of $\Delta A/\Delta t$ versus $\Delta B/\Delta t$ will yield a linear plot where the slope is equal to $k^2 \varepsilon_A/\varepsilon_B$ and with a zero intercept.
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