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PROTEIN COIMMOBILIZATION /
REACTIONS OF VICINAL THIOL GROUPS OF PROTEINS

DISSERTATION

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

By

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1997

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ABSTRACT

Most biologically active proteins bind irreversibly to Amberlite XAD-7 polymer beads. Adsorption rates vary due to differences in electrostatic charges. Small amounts of protein are adsorbed exclusively at the periphery of the beads, whereas additional amounts are adsorbed to deeper layers of the same beads. The sequential binding to deeper layers can be used to arrange proteins in a series of concentric layers in the beads. LDH and GDH were thus coimmobilized to generate a NAD⁺/NADH recycling system wherein the conversion of lactate to pyruvate was used to drive the formation of glutamate from α-ketoglutarate and ammonia. The overall efficiency of the system varied with the LDH/GDH ratio and was limited by the amount of LDH. It also varied with the arrangement of the two enzymes and showed the highest efficiency when LDH was on the outermost layer and GDH was on the next inner layer. The system had an optimum pH at ~8.5 and retained >75% of its initial activity after one week.

Dithiothreitol, which is widely used to reduce disulfide bonds in proteins, interferes with most procedures to detect
the resulting monothiols. A rapid and sensitive assay for monothiols in the presence of dithiothreitol was developed using 4,4'-dipyridyl disulfide and arsenite at pH 6. The procedure is much more effective and produces less background than a previous method. Reductions of GSSG and BSA by DTT were characterized in order to demonstrate the use of this procedure.

Model dithiol compounds were S-nitrosated to give mono or dinitrosothiols. Rate constants for their decompositions were determined. Half-lives were ranged from 1 to 7 min for the intramolecular homolysis, and from 3 to 12 min for the intramolecular heterolysis. These reaction rates are correlated to those of some enzyme activity changes after their exposure to NO-donor compounds. S-nitrosation promoted disulfide formation of vicinal thiols on the enzymes may, therefore, account for the effects of NO and nitrovasodilators on those enzymes.
To my parents
and
my husband
ACKNOWLEDGMENTS

I wish to extend my sincerest thanks to my advisor, Dr. Gary E. Means, for his invaluable guidance, constant encouragement, and genuine care during my years in his lab. Without his constant help and support, this work could never have been accomplished. I am profoundly grateful to Dr. Edward J. Behrman, Dr. George A. Barber, and Dr. Wayne J. Becktel for the stimulating discussion and constructive suggestions. I would like to thank Dr. Amanda Simcox for the use of her inverted microscope. I owe special thanks to my friends, Minhong Yang, Peili Zhang, Hangjing Yang, and Joyce Chen, whose understanding and friendship kept me going through my difficult times in these years.

I wish to thank my entire family. To my parents, your never ending love, firm support, and faith in me have and will continue to be the source of the inspiration. I am indebted to my sisters and brother for their assistance and patience which have been provided throughout my many years of schooling. And ultimately, to my husband, Hao Zhang, I deeply appreciate all your confidence, assurance, and sincere care, without which I would never have gone so far.
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CHAPTER 1

KINETICS AND MECHANISM OF ADSORPTION OF PROTEINS TO AMBERLITE XAD-7 POLYMER BEADS

1.1 Introduction

Amberlite XAD-7 is a nonionic macroreticular adsorbent, widely used to adsorb, concentrate, and remove small, hydrophobic and moderately polar, organic molecules from air, water, and biological samples (Anderssen, et al., 1982; Douse, et al., 1984; Vian, et al., 1985). It is a crosslinked polyacrylate bead with both hydrophobic and slightly polar character, a large open-cell pore structure, and a very large, complex, internal surface area (Albright, 1986). It also interacts strongly with many large molecules, including some proteins (Ton, et al., 1979; Carleysmith, et al., 1980a; 1980b; Nagaki, et al., 1991), and has been used as a support for several immobilized enzymes (Carleysmith, et al., 1980a; Hamaguchi, et al., 1985; Ampon, 1987; Ampon and Means, 1988; Cantacuzene and Guerrero, 1989).
Ampon and Means (1987) have described the synthesis of several hydrophobic imidoesters and the adsorption of proteins modified by such to several types of porous polymer beads. Amberlite XAD-7 and XAD-8 were shown to be particularly effective adsorbents of those derivatized proteins, all of which appeared to retain high levels of catalytic activity for long periods of time (Ampon and Means, 1987; Song, et al., 1992).

Subsequent to those reports, it was observed that many native proteins bind to Amberlite XAD-7 with high retention of catalytic activity. In order to understand the kinetics of protein binding to Amberlite XAD-7 beads, we studied the adsorption of eleven native and two chemically modified proteins to the beads and some factors affecting the kinetics of their adsorption. We also examined the distribution of immobilized proteins in the beads to understand the mechanism of their adsorption.

1.2 Materials and methods

1.2.1 Materials

Chicken egg white lysozyme, human serum albumin (HSA), BSA, trypsin, equine myoglobin, cytochrome c, horseradish
peroxidase, yeast alcohol dehydrogenase, *Crotalus adamanteus* amino acid oxidase, porcine pepsinogen, *Aspergillus niger* glucose oxidase, succinic anhydride, picrylsulfonic acid (TNBS), ethylenediamine, 1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide and Amberlite XAD-7 (wet mesh size 20-60) were obtained from Sigma Chemical Company. Naphthol blue black was from Eastman Kodak Company.

1.2.2 Preparation of Amberlite XAD-7

Amberlite XAD-7 polymer beads (specific surface area 450 m²/g, average pore diameter ~90 Å) were washed two or three times with methanol and then dried. They were fractionated by passage through 180 μm and 250 μm sieves, washed for at least 8 hrs with refluxing methanol in a Soxhlet extractor according to Junk et al. (1974) and dried.

1.2.3 Adsorption

The dried beads, 25 to 200 mg in 8 ml screw cap vials, were soaked in 20 mM phosphate buffer, pH 7, for at least 30 min and drained just prior to protein adsorption. Solutions of each protein, 5 ml of 0.2 mg/ml in 20 mM phosphate buffer, pH 7, in some cases containing 0.1 or 0.5 M NaCl, were added to the vials, sealed and gently mixed by slow, continuous rotation end over end (~20 rpm) at 23°C. At various times, 40
μl aliquots of the solution were removed for determination of the protein concentration using a slightly modified BCA procedure (Smith et al., 1985), i.e., employing 0.5 ml of BCA working reagent and 60 min incubation at 37°C. Protein concentrations, expressed as a percentage of the initial concentration, were then plotted versus incubation time and adsorption rates were determined from the initial slopes.

1.2.4 Desorption

Samples of Amberlite XAD-7 (100 mg) with 1% (w/w) of each absorbed protein were washed three times with 20 mM phosphate buffer, pH 7, and drained. Another 2 ml of buffer was added, the vials were sealed and rotated at ~20 rpm and ~23°C. At various time intervals, samples of the supernatant were removed and their protein concentrations were determined as described above.

1.2.5 Protein modification

The succinylation of lysozyme was done by adding 20 mg of succinic anhydride, in ~20 increments with constant stirring, to 40 mg of lysozyme dissolved in 20 ml of pH 9.2, sodium borate buffer. The pH was occasionally readjusted to 9.2 with 0.1 M NaOH. After all of the reagent was added, the solution was stirred for another 20 min and subjected to dialysis
against 1 mM HCl. The extent of succinylation was determined with TNBS (Fields, 1972) and indicated that slightly more than 80% of amino groups were modified.

The reaction of BSA with an amine and a water soluble carbodiimide was done according to Budzynski and Means (1971). BSA, 87 mg, was dissolved in 15 ml of 0.2 M pyridine-HCl buffer at pH 4.5 and 0.53 ml of 0.5 M ethylenediamine was added followed by 174 mg of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide. The reaction was kept on ice overnight and stopped by dialysis against double distilled water. The extent of modification was determined using TNBS and indicated the presence of about 8 additional amino groups per BSA after the modification.

1.2.6 Staining

The procedure for staining protein on the polymer beads was modified from that of Ampon (1987). Samples of Amberlite XAD-7, with various amounts of adsorbed protein were washed with double distilled water followed by 2% acetic acid, and then stained with 0.1% naphthol blue black in 50% ethanol/1% acetic acid for 15 min at room temperature. Excess dye solution was then removed by decantation and the beads were washed alternately with 50% ethanol and 2% acetic acid until the washes were colorless. The stained beads obtained at that
point were stored in 2% acetic acid and were very stable under those conditions.

1.2.7 Coimmobilization of lysozyme and cytochrome c

Lysozyme and cytochrome c were immobilized on the same Amberlite XAD-7 beads as follows: in one vial, 10 mg of lysozyme in 5 ml of phosphate buffer, pH 7, was added to 100 mg of presoaked beads and incubated with mild agitation, as described above, for 24 hrs. The resulting lysozyme-loaded beads were then washed with buffer and drained. A solution containing 1 mg of cytochrome c in 5 ml of the same buffer was added and the beads were again incubated for another 24 hr with mild agitation. In a second vial, the same procedure was followed except that 1 mg of cytochrome c was adsorbed first followed by 10 mg of lysozyme.

1.2.8 Photography

Damp stained or unstained beads were sliced with a razor blade on a glass slide and air dried at room temperature. Photographs of the stained, unstained, sliced and intact beads were taken using an Olympus C-35AD-4 camera attached to an Olympus SZH-ILLD inverted microscope.
1.3 Results

1.3.1 Adsorption of proteins

Figure 1 shows typical curves obtained for the adsorption of six proteins, ranging from ~14,000 to 150,000 Daltons and with isoelectric points from 3.7 to 10.5, to Amberlite XAD-7 in 20 mM phosphate, pH 7.0 and 23°C. Lysozyme, with the highest isoelectric point, was adsorbed the fastest, being more than 90% adsorbed in 1 hr, whereas pepsinogen, with the lowest isoelectric point, was the slowest, being only about 10% adsorbed in the same time. Proteins with isoelectric points in between, such as BSA, had intermediate adsorption rates. Adsorption rates for eleven native proteins, obtained from similar curves, along with their molecular weights and isoelectric points, are shown in Table 1.

Most of the proteins in Table 1 were almost completely adsorbed in 24 hr, including some relatively slowly adsorbed ones such as serum albumin and alcohol dehydrogenase. In the case of trypsin (i.e. 85.4% adsorbed), some of the unadsorbed "protein" probably reflected its partial autolysis during or prior to adsorption. Incomplete adsorption of horseradish peroxidase and glucose oxidase (i.e. ~59% and ~66% adsorbed, respectively) were probably due to their being glycoproteins. Two other glycoproteins were tested for the adsorption, and
Amberlite XAD-7, 100 mg (dry wt.), in 5 ml of 20 mM phosphate buffer, pH 7.0, was gently mixed at room temperature with 1 mg each of lysozyme (○), yeast alcohol dehydrogenase (□), BSA (△), horseradish peroxidase (●), and pepsinogen (■), and protein concentrations were determined at various times, as described in the text.

Figure 1. Adsorption of proteins to Amberlite XAD-7.
Amberlite XAD-7, 100 mg (dry wt.), in 5 ml of 20 mM phosphate buffer, pH 7.0, was gently mixed at room temperature with 1 mg each of ovomucoid (O), concanavalin A (□), and horseradish peroxidase (Δ). Protein concentrations were determined at various times, as described in the text.

Figure 2. Adsorption of glycoproteins to Amberlite XAD-7.
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<th>MW(^a) (Kd)</th>
<th>pI(^a)</th>
<th>Adsorption(^c) Rate x 10(^2) (mg/min)</th>
<th>Percent Adsorption (24 hrs)</th>
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<tr>
<td>Lysozyme</td>
<td>14</td>
<td>10.5</td>
<td>6.9 ± 1.3</td>
<td>99.5</td>
</tr>
<tr>
<td>Trypsin</td>
<td>24</td>
<td>10.1</td>
<td>9.5 ± 0.2</td>
<td>85.4</td>
</tr>
<tr>
<td>Cytochrome c</td>
<td>13</td>
<td>10.0</td>
<td>7.0 ± 0.3</td>
<td>89.2</td>
</tr>
<tr>
<td>Horseradish peroxidase</td>
<td>40</td>
<td>7.2</td>
<td>3.8 ± 0.2</td>
<td>59.2</td>
</tr>
<tr>
<td>Myoglobin</td>
<td>16</td>
<td>6.9</td>
<td>1.9 ± 0.2</td>
<td>95.4</td>
</tr>
<tr>
<td>Amino acid oxidase</td>
<td>140</td>
<td>5.2 - 8.4(^b)</td>
<td>3.5 ± 0.2</td>
<td>82.6</td>
</tr>
<tr>
<td>Alcohol dehydrogenase</td>
<td>150</td>
<td>5.4</td>
<td>2.4 ± 0.1</td>
<td>94.2</td>
</tr>
<tr>
<td>Human serum albumin</td>
<td>68</td>
<td>4.9</td>
<td>1.9 ± 0.2</td>
<td>98.6</td>
</tr>
<tr>
<td>Bovine serum albumin</td>
<td>68</td>
<td>4.8</td>
<td>1.1 ± 0.2</td>
<td>98.4</td>
</tr>
<tr>
<td>Glucose oxidase</td>
<td>150</td>
<td>4.2</td>
<td>1.5 ± 0.1</td>
<td>65.6</td>
</tr>
<tr>
<td>Pepsinogen</td>
<td>41</td>
<td>3.7</td>
<td>0.1 ± 0.1</td>
<td>44.1</td>
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(a) Sober, (1968).
(b) A mixture of at least eight isozymes (Hayes and Wellner, 1969).
(c) Initial adsorption rates were obtained from three independent determinations.

Table 1. Adsorption of proteins to Amberlite XAD-7.
the result is shown in Figure 2. Pepsinogen, the most anionic protein studied, with only about 44% adsorption after 24 hrs, appeared to reflect weak and/or slow adsorption.

1.3.2 Protein desorption

When samples of Amberlite XAD-7 (100 mg), to which 1% (w/w) of the proteins listed in Table 1 were adsorbed, were suspended in 2 ml of phosphate buffer and rotated at 23°C, small amounts of protein were slowly released into the buffer. After six days, protein concentrations of the supernatant solutions ranged from undetectable (i.e. <1 µg/ml) to ~10 µg/ml. When identical Amberlite XAD-7 samples were stored in phosphate buffer at 4°C for the same time, however, no release or desorption of protein was detectable.

1.3.3 Effects of protein/Amberlite XAD-7 ratio on adsorption rates

Adsorption rates increased with increasing amounts of Amberlite XAD-7. Figure 3, for example, shows the adsorption curves obtained with 5 ml of 0.2 mg/ml BSA and 25, 50, 100, 150, and 200 mg of Amberlite XAD-7 and replots of those data according to the relationship, ln[P] = -k_{obs}t, indicating a simple linear or first-order dependence of adsorption rate on protein concentration. Increases in k_{obs}, determined from the
BSA, 1 mg in 5 ml of 20 mM phosphate buffer, pH 7, and 25 (O), 50 (□), 100 (Δ), 150 (●), and 200 (■) mg of Amberlite XAD-7 were gently mixed at room temperature and protein concentrations were determined at various times using the BCA procedure as described in the text. Values of \( \ln A_0/A_t \) were obtained directly from the decreases in \( A_{562} \) readings versus time, as shown in the inset.

**Figure 3.** Effects of BSA/Amberlite XAD-7 ratios on adsorption rates.
slopes of the latter, increased from ~1.5 x 10^{-3} to 1.5 x 10^{-2} s^{-1}, approximately as expected for a first-order dependence on the amount of Amberlite XAD-7.

1.3.4 Effects of chemical modification on adsorption rates

To evaluate the relationship between adsorption rates and isoelectric points, lysozyme, which adsorbed rapidly, and bovine serum albumin, which adsorbed relatively slowly, were chemically modified to alter their net charges and the resulting derivatives' adsorption rates were determined.

Succinylation of 80 to 95% of the seven amino groups of lysozyme, as shown in Figure 4, decreased its adsorption rate to ~40% of native lysozyme, whereas amidation of approximately eight carboxyl groups in BSA with ethylenediamine increased its adsorption rate about 3 fold of loading levels below ~1% and other standard adsorption conditions. Figure 5 shows changes in the adsorption rates after altering the proteins' charge property at different protein/beads ratios. Although the rate change was consistent at all ratios, the extend of the difference was more significant at low protein/beads ratio (i.e., 0.5% (w/w) of adsorption ratio) as compared to a higher ratio (i.e., 4% (w/w) of adsorption ratio).

Adsorption rates of BSA and other slowly adsorbed, anionic, proteins can be increased by adding sodium chloride
BSA, ethylenediamine-amidated BSA (NH-BSA), lysozyme (LYSOZ.), and succinylated lysozyme (SC-LYSOZ.) 1 mg in 5 ml of 20 mM phosphate buffer, pH 7, and 100 mg of Amberlite XAD-7 were gently mixed at room temperature and protein concentrations were determined at various times as described in the text. Values of \( k_{\text{obs}} \) were calculated from the slopes of plots similar to those shown in Figure 3.

**Figure 4.** Effects of chemical modifications on adsorption rates.
BSA (○), ethylenediamine-amidated BSA (●), lysozyme (□), and succinylated lysozyme (■), 1 mg, and various amount of Amberlite XAD-7 were gently mixed at room temperature and protein concentrations were determined at various times as described in the text. Values of $k_{obs}$ were calculated from the slopes of the plot as shown in Figure 3.

Figure 5. Effects of chemical modifications on the adsorption rates at different protein/beads ratio.
to the standard adsorption buffer, whereas lysozyme and other rapidly bound, cationic, proteins are not significantly affected. Figures 6 to 8, for example, show adsorption curves for BSA, amidated BSA which is slightly cationic at pH 7, and succinylated lysozyme in phosphate buffer with and without added salt. Addition of salt in the buffer had little effect on the adsorption of lysozyme to Amberlite XAD-7 beads since it already was adsorbed rapidly.

1.3.5 Distribution of protein in the polymer beads

Amberlite XAD-7 beads with different amounts of adsorbed protein were stained with naphthol blue black, sliced, and examined under a low power microscope. Figure 9 shows a series of photographs of such beads loaded with 0.5%, 1%, 10%, and 35% (w/w) BSA. Exterior surfaces of the beads at all four loading levels were a similar dark blue. Beads not exposed to protein, on the other hand, retained no stain and were white after similar treatment.

Cross sections of the beads, after staining and slicing, showed the blue dye confined to a thin shell including the outer surface of the beads at low protein loading levels (i.e. 0.5 and 1% w/w) (Figures 9A and 9B) and more or less uniformly distributed throughout the beads at high levels (i.e. ~35% w/w) (Figure 9D). Shells of various thicknesses were observed
BSA, 1 mg, in 5 ml of phosphate buffer, pH 7 (O), in buffer plus 0.1 M (□), and in 0.5 M (△) NaCl, was gently mixed with 100 mg (dry wt.) of Amberlite XAD-7, and protein concentrations were determined at various times.

**Figure 6.** Effects of salt on adsorption rates of BSA.
Ethylenediamine-amidated BSA, 1 mg, in 5 ml of phosphate buffer, pH 7 (O), in buffer plus 0.1 M (□), and in 0.5 M (△) NaCl, was gently mixed with 100 mg (dry wt.) of Amberlite XAD-7, and protein concentrations were determined at various times.

**Figure 7.** Effects of salt on adsorption rates of modified BSA.
Succinylated lysozyme, 1 mg, in 5 ml of phosphate buffer, pH 7 (O), in buffer plus 0.1 M (□), and in 0.5 M (△) NaCl, was gently mixed with 100 mg (dry wt.) of Amberlite XAD-7, and protein concentrations were determined at various times.

**Figure 8.** Effects of salt on adsorption rates of modified lysozyme.
Samples of Amberlite XAD-7 with 0.5% (A), 1% (B), 10% (C), and 35% (D) (w/w) of adsorbed BSA were stained with naphthol blue black, sliced and dried. Areas of the beads that look black in the photographs were dark blue when viewed through the microscope.

Figure 9. The distribution of protein in Amberlite XAD-7.
at intermediate loading levels (Figure 9C). The same stain patterns were observed with other studied proteins.

1.3.6 Coimmobilization of lysozyme and cytochrome c

To further understand how proteins adsorb to Amberlite XAD-7, we characterized the adsorption of two similar but readily distinguishable proteins, lysozyme (MW = 14,000 daltons, pI = 10.5), which has no visible color, and cytochrome c (MW = 13,000, pI = 10.0), which is red (Sober, 1968). Figure 10 shows some unstained Amberlite XAD-7 beads with adsorbed cytochrome c, with lysozyme and cytochrome c, adsorbed in that order, and the two adsorbed in the reverse order. When only cytochrome c was present (Figure 10A), the red color was confined to the outer surface of the polymer beads. The intact beads were red but, when sliced, all of the red color was confined to the outer surface. As with other proteins (Figures 10A and 10B), small amounts of cytochrome c appear to adsorb primarily to the outer surface of the beads.

When cytochrome c was adsorbed to Amberlite XAD-7 before adsorbed with lysozyme, the beads were again red and all of that red color was again confined to a thin shell at or very near to the bead surfaces, as shown in Figure 10C. The location of the adsorbed cytochrome c was not affected by the subsequent adsorption of lysozyme.

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(A) Two sliced and one intact bead of Amberlite XAD-7 with 1% (w/w) of cytochrome c are shown. A pale red color, confined to the outermost surface of the beads, is seen as a slight darkening of the surface; (B) a similar preparation with 1% cytochrome c adsorbed after 10% (w/w) of lysozyme is shown against a dark background to reveal the white surface and an interior red ring after slicing; (C) with 1% (w/w) of cytochrome c followed by 10% (w/w) of lysozyme, the red color was again confined to the surface.

Figure 10. The distribution of cytochrome c coimmobilized with lysozyme on Amberlite XAD-7.
When lysozyme was adsorbed to Amberlite XAD-7 followed by cytochrome c, however, the beads had a less intense red color and all of that color, as can be seen in the sliced beads, was located inside the beads at a distance determined by the amount of previously adsorbed lysozyme (Figure 10B). Prior adsorption of lysozyme to Amberlite XAD-7 thus prevented the adsorption of cytochrome c to its outer surface.

1.4 Discussion

Enzymes and other proteins are sometimes immobilized to increase their stability, to physically confine them to a particular region of a surface or support, so as to facilitate their physical separation from reactants, products, and other soluble components of a reaction or process, to allow for their repeated or continuous use in packed beds or other kinds of flow-through reactors, etc. (Klibanov, 1979; Weetall and Royer, 1980; Laskin, 1985; Hartmeier, 1986; Mosbach, 1987a; 1987b; 1988).

Among the techniques used to immobilize proteins, those involving physical adsorption via ionic and/or hydrophobic interactions, are probably the simplest and mildest. Leakage of proteins from supports during long-term storage or under operational conditions is always a concern for immobilized
enzymes (and other proteins) but is usually a special concern when proteins are immobilized by noncovalent interactions (Hartmeier, 1986; Ulbrich, et al., 1991). Noncovalent interactions can be very extensive, however, and are not necessarily weaker or more labile than covalent interactions.

As shown in Figure 1 and Table 1, a wide variety of proteins adsorb readily to Amberlite XAD-7. Only highly anionic proteins (e.g. pepsinogen in Figure 1) and some glycoproteins (as shown in figure 2) were not readily adsorbed. Similarly lipopolysaccharides showed little adsorption to Amberlite XAD-7 (Nagaki, et. al., 1991). Amberlite XAD-7 is a copolymer of trifunctional crosslinker trimethylolpropane trimethacrylate, trimethylolpropane dimethacrylate, and methyl acrylate or other acrylates such as hydroxyethyl methacrylate (Albright, 1972). Therefore, the polarity of the carboxylate and oligosaccharide groups of those negatively charged proteins or glycoproteins presumably interfere with their adsorption to the Amberlite XAD-7 polymer beads which carries carbonyl and probably other polar groups.

At relatively low protein levels (~4% (w/w) and lower), adsorption rates varied with protein concentration and with the amount of Amberlite XAD-7 (Figure 3). Adsorption rates for different proteins, on the other hand, varied as shown in
Table 1, and appeared to correlate with their isoelectric points. Proteins with high isoelectric points, for example, adsorbed more rapidly than those with low isoelectric points, and those with intermediate isoelectric points adsorbed at intermediate rates.

The slow adsorption of proteins with low isoelectric points appeared to be due to their negative electrostatic charge under the conditions of adsorption. Succinylation of lysozyme, which increased its negative charge, for example, sharply reduced its adsorption rate at neutral pH (Figure 4). At pH 3.5, however, where the negative charge was eliminated, it adsorbed nearly as fast as native lysozyme (Miller, 1992). Amidation of BSA with ethylenediamine, which decreased its negative charge at neutral pH, on the other hand, significantly increased its adsorption rate (Figure 4). Similar effects of charge have also been noted for the adsorption of coenzymes to Amberlite XAD-7 (Brunner, et al., 1985). S-Adenosyl-methionine, a cation, adsorbs rapidly whereas NAD⁺ and other related anionic coenzymes do not.

Amberlite XAD-7 is predominantly a hydrophobic adsorbent and strong hydrophobic interactions, not ionic interactions, appear to account for its adsorption of proteins. Sodium chloride thus had little or no effect on the adsorption of lysozyme and actually increased the rates of slowly adsorbed,
anionic, proteins like BSA (Figure 6). Salt effects on the two chemically modified proteins (figures 7 and 8) were less than those on BSA, which reflected, probably, the less negatively charged groups on the proteins. The opposite would, of course, be expected in the case of favorable electrostatic interactions. Accelerated binding in the case of anionic proteins might be due to slightly increased hydrophobic interactions or an increased availability of cations needed to transfer hydrated carboxylate groups into a hydrophobic environment (Tanford, 1980; Warshel, 1981).

Molecular weights of the proteins included in this study varied from ~14,000 to 150,000 Daltons but with little apparent effect on adsorption rates. Myoglobin (~16,500 Daltons, an oblate spheroid of ~44 x 44 x 25 Å (Kendrew, et al., 1960)) and yeast alcohol dehydrogenase (~150,000 Daltons, a prolate spheroid ~110 x 60 x 45 Å (Branden, et al., 1975)), for example, had similar adsorption rates (Table 1). Because the latter’s dimensions are comparable to the 90 Å nominal pore diameter of Amberlite XAD-7, its rapid adsorption was initially surprising. It should be noted, however, that pore diameter and surface area values reported for Amberlite XAD-7 were determined on the dry beads (Albright, 1986) and do not take into account a roughly two-fold increase in volume that takes place upon its suspension in aqueous solution. The
adsorption of much larger proteins may, of course, be limited by pore size.

Due to the large internal surface area of Amberlite XAD-7, it can adsorb relatively large amounts of most proteins. The maximum adsorption capacity for lysozyme, for example, was determined to be about 37% (w/w) (Miller, 1992). As shown in Figure 9D also, approximately 35% (w/w) of BSA was also readily adsorbed. At much lower protein levels (e.g. ~1%) the adsorbed protein appears to be confined to a thin shell around the perimeter of the polymer beads (Figure 9B), a distribution ideally suited for high catalytic activity in immobilized enzyme systems (Park et al., 1981; Borchart and Buchholz, 1984; Dennis, et al., 1984; Do and Hossain, 1986). The well-defined boundaries between those protein layers and the protein-free interiors suggests that adsorption is faster than penetration into the beads (Carleysmith, et al., 1980b). As shown in Figure 5, the difference of adsorption rates among proteins were more substantial at low protein levels than at high protein levels. This may be because at high loading levels, proteins need to penetrate further into the beads in order to find available binding sites. The diffusion limitation, therefore, makes a more significant contribution to the adsorption rate at high protein levels.
The stability of those layers, determined by the same staining procedure after a year or more incubation in some cases, suggests dissociation from the polymer surface is negligible, a result which is in accord with attempts to detect desorption of protein from the beads. Very low amounts of protein detected in the latter cases (data not presented), for example, were highly dependent on the incubation conditions and appeared to reflect the amount of abrasion occurring over various periods of continuous agitation, rather than simple dissociation.

Adsorption of proteins to Amberlite XAD-7 appears to be rapid, strong and, in at least most cases, irreversible. Because it is irreversible and because one protein binding to a site or region appears to preclude the subsequent binding of others, the order of adsorption can be used to achieve different distributions of two or more proteins in the beads. As shown in Figure 10, for example, cytochrome c was adsorbed at the outer surface of the beads or internally depending on the order of its adsorption, either before or after the adsorption of lysozyme.

Therefore, Amberlite XAD-7 beads can be used as a suitable support for the preparation of a multienzyme system where the different enzymes adsorb to the beads in concentric layers and catalyze successive chemical transformations.
CHAPTER 2

COIMMOBILIZATION OF LDH AND GDH ON AMBERLITE XAD-7 BEADS

2.1 Introduction

Many metabolic reactions in cells are catalyzed by multienzyme complexes. The product of one enzyme is the substrate of the next enzyme in such complexes, and is directly delivered from one enzyme to the other without diffusion from the complex. For reactions requiring coenzymes such as NAD'/NADH, NADP'/NADPH, etc., the coenzymes are usually regenerated by another enzyme so as to maintain the metabolic cycles. Systems with multiple enzymes coimmobilized on the same support have, therefore, been designed based on the assumption that the advantages usually associated with natural existing multienzyme complexes, i.e., high enzyme proximity, short substrate transient times, and efficient coenzyme recycling, will also apply to those immobilized systems. Some coimmobilization systems were used in biosensors in which the enzyme specific for the target substance was coimmobilized
with a second enzyme, usually an oxidase, producing hydrogen peroxide that could be detected chemiluminometrically (Tabata and Totani, 1995; Kiba, et al., 1996). In other cases, two enzymatic reactions were coupled so that the coenzymes could be regenerated, for example, NAD'/NADH recycling catalyzed by malate dehydrogenase and lactate dehydrogenase (Srere, et al., 1973) or by glutamate dehydrogenase and glucose dehydrogenase (Chang, 1987).

While immobilized enzymes are used for many purposes, sometimes on a large scale, multienzyme systems are still largely in the laboratory development stage. Beside the problems usually encountered with a single enzyme, there are several additional problems to be considered in the case of multiple enzymes. The amounts and ratios of the individual enzymes in a multienzyme system, for example, can be varied and should, ideally, be adjusted so as to achieve an optimal overall rates. To minimize transient times, enzymes catalyzing successive steps in such a process should also be arranged so as to be in close proximity to each other. These goals are difficult to achieve due to the relatively low and unpredictable efficiencies for the immobilization of most enzymes.

As shown in Chapter 1, Amberlite XAD-7 polymer beads can adsorb large amount of various proteins (i.e., up to ~35 w/w
more or less indiscriminately, as a result of strong hydrophobic interaction. With small amounts of protein (less than ~4 w/w %), the adsorbed proteins appear to be confined to a thin shell at or near the periphery of the porous beads. With multiple proteins, as demonstrated by the coimmobilization of lysozyme and cytochrome c, the different proteins were immobilized in concentric layers arranged according to their order of adsorption. Based on these findings, we coimmobilized lactate dehydrogenase (LDH) and glutamate dehydrogenase (GDH) in various amounts and varied their arrangements in Amberlite XAD-7 beads. We determined their ability to recycle NAD⁺/NADH by following the formation of glutamate from α-ketoglutarate, ammonia, and lactate.

2.2 Materials and methods

2.2.1 Materials

Amberlite XAD-7 (wet mesh size 20-60, specific surface area 450 m²/g, average pore diameter ~90 Å) was obtained from Sigma Chemical Company and pretreated as described in Chapter 1. L-Lactic dehydrogenase (LDH) (EC 1.1.1.27, type XI from rabbit muscle), L-Glutamic dehydrogenase (GDH) (EC 1.4.1.3, type III form bovine liver), bovine serum albumin (BSA)
(fraction V), NAD⁺, NADH, α-ketoglutarate, pyruvate, phenylisothiocyanate, and picrylsulfonic acid (TNBS) were obtained from Sigma Chemical Company. AG 50W-X12 (mesh size 200-400) cation exchange resin was from BIO-RAD Laboratories. D,L-Lactic acid was from Fisher Scientific.

2.2.2 Coimmobilization of LDH and GDH

Solutions of either LDH or GDH, 1 to 3 mg in 5 ml of 20 mM phosphate buffer containing 0.5 mM NAD⁺, pH 7, were added to 100 mg of presoaked Amberlite XAD-7 beads and gently mixed by mild end over end rotation at 23°C. At various times, 40 µl aliquots of the solution were removed for determination of the protein concentration using a slightly modified BCA procedure (Smith, et al., 1985). After complete immobilization of the first enzyme, usually after about 6 hrs, the beads were rinsed several times with buffer and the second enzyme or BSA was added under the same conditions and allowed to adsorb for an additional 6 - 12 hrs. In some cases, LDH was adsorbed first, followed by GDH. In other cases the order was reversed or BSA was adsorbed after the first enzyme and before the second one, therefore, forming a BSA layer between LDH and GDH.
2.2.3 Enzyme assays

Activities of immobilized LDH preparations were determined by incubating them in 5 ml of 2.5 mM pyruvate, 1 mM NADH in 20 mM phosphate at pH 7.4, or in 100 mM of D,L-lactate and 1 mM NAD' in 50 mM phosphate at pH 7.7 in an 8 ml screw capped vial, under continuous end over end rotation at 23°C. At various times, 100 μl aliquots were removed, diluted 10-times, and their absorbencies at 340 nm were determined with a Hewlett Packard 8452A diode-array spectrophotometer. Activities of immobilized GDH were determined under similar conditions by following the conversion of NADH to NAD' in the presence of 10 mM α-ketoglutarate, 1 mM NADH and 50 mM ammonium phosphate at pH 7.7.

2.2.4 Activities of the coimmobilized LDH/GDH

Samples of Amberlite XAD-7 beads (100 mg) with various amounts of immobilized LDH and GDH were incubated in 5 ml of 100 mM D,L-lactate, 10 mM α-ketoglutarate, 1 mM NAD', and 50 mM ammonium phosphate, pH 7.7 in 8 ml screw capped vials. The vials were gently rotated end over end at 23°C, and at various times, 100 μl aliquots were removed and applied to a Bio-Rad AG 50W-X12 cation exchange column (0.5 x 2.5 cm) equilibrated with 20 mM phosphate buffer at pH 7.4. The column was washed with 1.4 ml of the same buffer, eluant were collected and
their glutamate contents were determined by assay with TNBS (Fields, 1972) and, after derivatization with phenylisothiocyanate (Bergman, et al., 1986), by RP-HPLC (4.6 x 250 mm column of Lichrosorb C-18 with a 0 to 36% B (50% acetonitrile) gradient in 25 mM phosphate, pH 6.6, at 1 ml/min).

NAD⁺ and NADH levels of the same reaction solutions were followed by applying the reaction solution directly, without derivatization, to the same HPLC column and eluting under the same conditions. Elutions of NAD⁺ and NADH were monitored at 215 nm and those of Glu derivatives (PTH-Glu) at 254 nm.

2.2.5 pH dependence of immobilized LDH and GDH

Activities of the immobilized LDH, GDH and LDH/GDH preparations at various pH values were determined by following absorbance changes at 340 nm versus time and by following glutamate formation in 50 mM phosphate at pH 6.6, 7.0, 7.4, 7.7, 8.1, and 50 mM pyrophosphate at pH 8.6, 8.8 and 9.1.

2.2.6 Stability of immobilized LDH/GDH

Stabilities of immobilized LDH/GDH preparations were determined as follows. Amberlite XAD-7 adsorbed GDH and LDH were incubated, at 23°C, in 20 mM phosphate buffer, pH 7.4, containing 0.5 mM NAD⁺, with or without various additives and
their activities were determined at various times from rates of glutamate formation. The following additives, 100 mM NaCl, 4 mM β-mercaptoethanol, and both NaCl and β-ME, respectively, were present in some cases so as to determine their effects on stabilities. At various times, the buffers were removed, the beads were washed with fresh buffer, drained, the assay solution was added and the ability to convert α-ketoglutarate into glutamate was determined. After assay, the beads were again washed with buffer, drained, and returned to the original storage conditions.

2.3 Results

2.3.1 Coimmobilization of LDH and GDH

Based on the results obtained from the adsorption of lysozyme and cytochrome c on Amberlite XAD-7, we immobilized two enzymes, LDH and GDH on the same support. Figure 11 shows a time course for the adsorption of 1 mg of LDH to Amberlite XAD-7 beads and for the subsequent adsorption of 1 mg of GDH to the same beads. LDH adsorbed at a moderate rate and approached ~90% completion after 3 hours of incubation. GDH adsorbed to those LDH containing beads at a slower rate, but also approached ~90% adsorption after overnight incubation.
LDH (O), 1 mg in 5 ml 20 mM phosphate buffer containing 0.5 mM NAD⁺ at pH 7.4, was adsorbed to 100 mg of beads for 24 hrs. The beads were washed with fresh buffer, drained. GDH (□), 1 mg in 5 ml same buffer, was incubated with the beads for another 24 hrs.

Figure 11. Time course for the adsorption of LDH and GDH to Amberlite XAD-7 beads.
Activities of immobilized LDH were determined using changes in absorbance at 340 nm to follow both the conversion of NAD⁺ into NADH and, in reverse direction, from NADH to NAD⁺. Figure 12A shows a plot of NADH concentration versus reaction time for the reduction of pyruvate by 100 mg of LDH/GDH beads. The initial activity calculated from the plot was 0.29 μmoles of NADH converted to NAD⁺ per min per mg of LDH. When D,L-lactate was used as the substrate, under the conditions of Figure 12B, the initial conversion rate of NAD⁺ to NADH was 0.17 μmoles per min per mg of GDH, but did not go to completion and gave an equilibrium constant of 3.4 x 10⁻¹² M, which was in good agreement with an earlier value obtained with soluble LDH (i.e., 3.3x10⁻¹² M) (Schwert and Winer, 1963).

The reductive amination of α-ketoglutarate to glutamate catalyzed by 100 mg of LDH/GDH beads is shown in Figure 12C. The enzyme activity calculated from that plot was 0.13 μmoles of NADH converted to NAD⁺ per min per mg GDH.

2.3.2 Glutamate formation by coimmobilized LDH/GDH

The two reactions catalyzed by the immobilized LDH/GDH system are illustrated in Figure 13. The overall reaction which required repetitive cycling of NAD⁺/NADH was followed by measuring the amount of glutamate formed. Initial reaction
Amberlite XAD-7 beads with immobilized enzymes were incubated with substrate solution at room temperature by mild agitation. At various times, an aliquot of the solution was taken to measure the absorbance at 340 nm. (A) LDH activity using NADH and pyruvate as substrates. (B) LDH activity using NAD⁺ and lactate as substrates. (C) GDH activity using NADH, ammonium phosphate, and α-ketoglutarate as substrates.

Figure 12. Activity of immobilized LDH and GDH.
Figure 13. Reactions catalyzed by coimmobilized lactate dehydrogenase (LDH) and glutamate dehydrogenase (GDH).
solutions contained 10 mM α-ketoglutarate, 50 mM ammonium phosphate, and 1 mM NAD⁺, and were initiated by the addition of 100 mM D,L-lactate. Glutamate concentrations at various times were determined by the TNBS assay after removal of ammonium ion on a cation exchange column.

Figure 14 shows the increase in glutamate concentration with reaction time. After 2 hours, 0.52, 2.5, and 3.7 μmoles of glutamate were produced by 0.25, 1, and 2 mg of LDH, respectively, coimmobilized with 1 mg of GDH on Amberlite XAD-7 beads. Figure 15 shows the amount of glutamate produced by varied amounts of LDH at a fixed level of GDH and varied amounts of GDH at a fixed level of LDH after 2 hrs of incubation. When GDH was fixed at 1% (w/w), glutamate production increased with increased amount of LDH. When LDH was fixed at 1% (w/w) and the amount of GDH was increased, glutamate formation was also increased initially but leveled off at a GDH/LDH ratio of about two and, at higher levels, appeared to be governed entirely by the amount of immobilized LDH.

2.3.3 pH optima of immobilized LDH, GDH, and the LDH/GDH system

Figure 16 shows the relative activities of LDH and GDH, separately, and of the LDH/GDH system immobilized on Amberlite
XAD-7 beads, 100 mg immobilized with 1 mg GDH and 0.25 (O), 1 (□), and 2 (△) mg of LDH, were incubated with 5 ml of substrate solution containing 100 mM lactate, 10 mM α-ketoglutarate, 50 mM ammonium phosphate, pH 7.7, and 1 mM NAD⁺ which was added last. At various times, aliquots of the solution were applied to a Bio-Rad AG 50W-X12 cation exchange column to remove ammonium ions. Glutamate content was then determined by the TNBS assay.

Figure 14. Glutamate formation using LDH/GDH Amberlite XAD-7 system.
XAD-7 beads, 100 mg immobilized with 1 mg LDH (O) and different amounts of GDH, or with 1 mg GDH (□) and different amounts of LDH, were incubated for 2 hrs with 5 ml of substrate solution containing 100 mM lactate, 10 mM α-ketoglutarate, 50 mM ammonium phosphate, pH 7.7, and 1 mM NAD⁺ which was added last. The solution was applied to a Bio-Rad AG 50W-X12 cation exchange column to remove ammonium ions. Glutamate content was then determined by the TNBS assay.

**Figure 15.** Glutamate formation using different ratio of coimmobilized LDH/GDH on Amberlite XAD-7 beads.
XAD-7 versus pH. Activities at pH 8 were arbitrarily set at 100%. The optimum for immobilized GDH was between pH 7 to pH 8, whereas activities of LDH increased gradually from pH 6.5 to 9.2. Coimmobilized LDH/GDH on Amberlite XAD-7 had an optimum around pH 8.6. The pH dependencies of the individually immobilized enzymes were not significantly different from the soluble enzymes (Schwert and Winer, 1963), whereas the optimum for the coimmobilized LDH/GDH reflected a combination of the two pH optima.

2.3.4 Stability studies of the LDH/GDH system

The stabilities of the immobilized LDH/GDH were monitored for one week in the presence and absence of both β-mercaptoethanol and NaCl. In phosphate buffer at pH 7 containing 0.5 mM NAD⁺, the activity decreased to about 30% in one week. Including β-mercaptoethanol and NaCl in the buffer, however, increased their stability, as shown in Figure 17. The effects were additive, giving about 78% of the initial activity after 7 days storage when both β-ME and NaCl were present.

2.3.5 NAD⁺/NADH recycling by the LDH/GDH system

Glutamate formation by the LDH/GDH system required repeated NAD⁺/NADH recycling and was accelerated by the
Immobilized enzyme activities were determined as described in the legends of Fig. 12 and Fig. 14. Ammonium chloride was used in place of ammonium phosphate. Buffers used were 50 mM phosphate at pH 6.6, 7.0, 7.4, 7.7 and 8.1, and 50 mM pyrophosphate at pH 8.6, 8.8 and 9.1. (□) LDH activity using pyruvate and NADH as substrates. (Δ) GDH activity using α-ketoglutarate, ammonium ions and NADH. (●) Glutamate formation by coimmobilized LDH/GDH.

Figure 16. pH dependence of immobilized enzyme activities.
Rates of glutamate formation by coimmobilized LDH/GDH on Amberlite XAD-7 beads were determined at various times. The beads were washed with buffer after each assay and stored in 20 mM phosphate buffer (O), with 100 mM NaCl (□), 4 mM β-ME (Δ), and both 100 mM NaCl and 4 mM β-ME (V).

Figure 17. Stability of immobilized LDH/GDH.
addition of either form of that coenzyme. To determine the steady-state levels of the two forms under operating conditions, aliquots of the reaction solutions at various times were taken and subjected to a RP-HPLC. As shown in Figure 18, when NADH was added initially to start the reactions, most of that NADH was converted to NAD⁺ within 30 min to give an NAD⁺/NADH ratio of more than 100/1 and then did not change for at least 24 hrs.

Glutamate concentrations of the solution, determined by RP-HPLC after derivatization with PITC, were also monitored over the same time period. As shown in Figure 19, glutamate contents increased rapidly during the first 30 mins and steadily thereafter for at least 5 hrs, long after the NAD⁺/NADH ratio ceased to change. The fact that glutamate formation far exceeded the amount of NADH initially added showed that the reduced and oxidized forms of the coenzyme were continuously regenerated and reused. Table 2 shows the number of NAD⁺/NADH cycles required to obtain the observed glutamate levels at the different initial NAD⁺/NADH concentrations. The high steady-state ratio of NAD⁺/NADH suggested that the activity of LDH controlled the overall rate of reactions.
Amberlite XAD-7, 100 mg, with 0.5 mg of LDH and 1 mg of GDH immobilized were incubated in 5 ml of substrate solution as described for Figure 13 except 1 mM NADH replaced NAD'. Aliquots of the reaction solution were taken at various times and applied to a C-18 RP-HPLC column. The column was eluted by a gradient of 0 to 36% of 50% acetonitrile in 25 mM sodium phosphate, pH 6.6.

**Figure 18.** HPLC analysis for NAD' and NADH during operation of the LDH/GDH system.
Reaction conditions for immobilized LDH/GDH were the same as in Figure 18. Aliquots of the reaction solution were taken at various times to remove ammonium ions and derivatize Glu with PITC. RP-HPLC conditions were the same as in Figure 18.

Figure 19. HPLC analysis for glutamate formation during operation of the LDH/GDH system.
Initial [NAD\(^+\)] (mM) | Cycles/hr.\(^a\)
---|---
0.1 | 103
0.5 | 156
1.0 | 166

(a) cycles/hr = Glu produced (moles/hr) divided by LDH active sites (moles).

Table 2. Number of NAD\(^+\)/NADH cycles at different initial coenzyme concentrations.

2.3.6 Effect of enzyme arrangement on the efficiency of glutamate formation

To determine the importance of enzyme proximity and orientation on the efficiency of NAD\(^+\)/NADH recycling, we immobilized the two enzymes and, in some cases, BSA in different arrangements on the beads and determined their rates of glutamate formation. Five different arrangements of the two enzymes and BSA, as schematically shown in Figure 20 left panel were examined. In each case, 0.5 mg of LDH and 1 mg of GDH were immobilized on 100 mg of beads. As shown in Figure 20, the lowest overall catalytic efficiency was obtained when
Different enzyme arrangements were achieved by manipulating the sequence of adsorption as described in Materials and Methods. The diagrams on the left show schematically the relative position of the enzyme and BSA layers on the beads. The thickness of the layers in the diagrams do not reflect the actual thickness in the beads. Glutamate concentrations after two hrs of reaction were determined and normalized by setting the highest value to 100%. The results presented are the average of three separate sets of experiments.

Figure 20. Effect of enzyme arrangement on the efficiency of glutamate formation.
the two enzymes were immobilized on separate beads (L+G). The highest efficiency was obtained when they were immobilized in adjacent layers on the same beads with GDH in the outermost layer (i.e., GL). When immobilized in the opposite order, with LDH in the outer layer (i.e., LG), the activity was slightly lower. The presence of an inert, protein layer between the two enzymes (GBL and LBG) also decreased rates of glutamate formation in both cases.

2.4 Discussion

Adsorption of proteins to Amberlite XAD-7 appears to be irreversible, begins at or near the outermost surface of the polymer beads and, with more protein and longer times, gradually proceeds to the center of the bead. As described in Chapter 1, multiple proteins can be arranged in a series of concentric layers in the beads according to the order in which they are adsorbed. Those adsorbed initially are located at or near the periphery of the beads, whereas those adsorbed later are progressively further from the surface.

Lactate dehydrogenase (LDH) and glutamate dehydrogenase (GDH), like other proteins examined previously, adsorbed readily to Amberlite XAD-7 (Figure 11), and retained the catalytic activities (Figure 12) and pH dependencies similar
to those of the soluble enzymes (Figure 16). Together, the two enzymes were able to use lactate to effect the reductive amination of α-ketoglutarate to L-glutamate as shown below.

\[
\begin{align*}
\text{L-lactate} & \quad \text{LDH/GDH} & \quad \text{pyruvate} \\
\alpha\text{-ketoglutarate} & \quad \text{NH}_4^+ & \quad \text{L-glutamate}
\end{align*}
\]

The pH dependence for the latter coupled reactions (Figure 16) appeared to reflect both of the constituent reactions and had a maximum at approximately pH 8.6. As observed with other enzymes immobilized on Amberlite XAD-7, the coimmobilized LDH/GDH system was quite stable, retaining about 78% of its initial overall activity after one week of use in the presence of 100 mM NaCl and 4 mM β-mercaptoethanol (Figure 17).

One of the problems associated with coimmobilizing multiple enzymes is the random distribution of the individual enzymes in the supporting matrix. The manner by which proteins are attached to most supports allows for little or no control of their arrangements in or on the support, the proximity of one relative to the other or, even, in their relative amounts. With Amberlite XAD-7, unlike most other
supports, the amount of an immobilized enzyme can be easily varied, a second, third or forth enzyme can be introduced, the total amounts of the individual enzymes, their relative amounts and their locations in the support beads can be varied by the amount and the order in which they are immobilized. In the case of LDH and GDH, variations in these parameters affected overall rates of lactate-dependent glutamate production.

At a relatively low, fixed, level of GDH, for example, rates of glutamate formation increased more or less linearly with the amount of coimmobilized LDH (Figure 15), suggesting that the formation of NADH was rate-limiting under those conditions. At a relatively low, fixed, level of LDH, and otherwise the same conditions, rates of glutamate formation also increased, initially, with the amount of GDH but leveled off at a GDH/LDH (w/w) ratio of about two. Formation of NADH, again, appeared to be rate-limiting at higher GDH.

NADH levels were in fact very low under most conditions. Even when added in large excess to initiate the reactions, NADH concentrations were rapidly reduced to low levels and then maintained, more or less, while large amount of glutamate were produced (Figures 18 and 19). Repetitive NAD\(^{+}\)/NADH recycling between the immobilized LDH and GDH was clearly necessary to account for the large amounts of glutamate
production after the first 30 min of reaction. Two and ten-fold lower levels of added NADH produced only slight reductions in the observed rates of glutamate production.

The efficiency of NAD'/NADH recycling by different coimmobilized LDH/GDH preparations appeared to vary due to different arrangements of LDH and GDH in the beads. The least efficient arrangement, for example, was that wherein the two enzymes were immobilized on different beads, which were then mixed. Glutamate production in that system presumably required the synthesis of NADH in one polymer bead and its transport to another bead. An additional transport of NAD' back to another LDH bead would then be required for one complete reaction cycle. Rates of glutamate production in that system were relatively low (Figure 20) due, in part, to those relatively, slow transport steps.

When both LDH and GDH were immobilized on the same polymer beads, transport of NAD' and NADH between the two enzymes may occur within a single bead and should be much faster. As shown in Figure 20, rates of glutamate formation were significantly faster in such LDH/GDH beads. Different arrangements of these two enzymes in the beads, however, also affected their activities. When GDH was at the surface with an underlying layer of LDH, for example, the activity was greater than the reverse order. The difference between the
two arrangements may be rationalized as due to greater leakage of NADH from the latter beads. NADH produced near the periphery of a bead can diffuse either more deeply into the bead or out of the bead whereas that produced internally cannot escape without first passing through the GDH layer. Introducing a layer of BSA between the two enzymes to further separate them also increased the NAD+/NADH transport distance, and decreased their rates of glutamate formation. By doing the opposite, commingling the two enzymes in one region in an appropriate ratio, even faster rates of glutamate production should be possible.

Amberlite XAD-7 has several important advantages as a support for enzyme immobilization. It requires no activation and undergoes no spontaneous deactivation process. The procedure is very simple and involves no additional chemicals nor any chemical alterations of the immobilized enzymes. Immobilization results from a spontaneous, irreversible, adsorption of enzymes to the polymer surface, is very efficient, usually quantitative for reasonable amounts (i.e., less than 5 w/w%) of most enzymes, and usually has little or no obvious effect on their catalytic activities. It appears to be particularly well-suited as a support for multienzyme systems as the amounts, location, and arrangement of the enzymes can be easily controlled.
CHAPTER 3

A PROCEDURE FOR THE DETERMINATION OF MONOTHIOLS
IN THE PRESENCE OF DITHIOHREITOL
- AN IMPROVED ASSAY FOR THE REDUCTION OF DISULFIDES

3.1 Introduction

In 1968, Zahler and Cleland (1968) described an assay for disulfides that involved their reduction by DTT, complexing the unreacted DTT with arsenite, and detecting the resulting monothiols with DTNB. While applying that procedure to proteins treated with DTT, we noted some difficulty in distinguishing between thiol groups that react slowly with DTNB due their protein environment and several other slow but significant reactions of DTNB under the same conditions.

In one attempt to solve this problem, we replaced arsenite with phenylarsine oxide, which is known to form even more stable dithiol complexes (Webb, 1966). Reactions of monothiols with DTNB, however, were slower in the presence of phenylarsine oxide and the expected gain due to the greater
stability of dithiol complexes appeared to be offset by a greater tendency to form monothiol complexes.

To pursue the problem further, we examined the pH dependence for the complexation of DTT by arsenite and determined that, although the complex is less stable at lower pH, rates of dissociation decreased even more. To take advantage of the slower dissociation rates, we replaced DTNB with 4-PDS, which can also be used to determine thiol groups and is more effective at low pH (Grassetti and Murray, 1967; Grimshaw, et al., 1979). The procedure that resulted can be used to determine monothiols in the presence of DTT or, as described in the earlier report (Zahler and Cleland, 1968), as a rapid and sensitive assay for disulfide bonds subject to reduction by DTT.

3.2 Materials and Methods

3.2.1 Materials

2,2'-Dipyridyl disulfide (2-PDS), 4,4'-dipyridyl disulfide (4-PDS), 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), dithiothreitol (DTT), glutathione (GSH), oxidized glutathione (GSSG), guanidine-hydrochloride (Gnd-HCl), bovine serum albumin (BSA) (Fraction V), bovine pancreatice ribonuclease A
(RNase A), and bovine insulin were obtained from Sigma Chemical Co. Sodium arsenite was obtained from Allied Chemical & Dye Corp. Bio-Gel P-2 was obtained from Bio-Rad Laboratories, Inc. Solutions of GSH and DTT in phosphate or Tris buffers at various pH values, as indicated later, were prepared immediately prior to use.

Due to the low aqueous solubilities of 2-PDS and 4-PDS (Brocklehurst, 1982), they were dissolved first in a small amount of dimethylformamide (DMF) and then diluted with deionized/double distilled water to make stock solutions of ~10 mM in 5% DMF/H₂O. These solutions were stored in brown glass bottles at room temperature and were stable for at least one month. Accurate concentrations were determined by absorbance at 282 nm for 2-PDS and 248 nm for 4-PDS, using the extinction coefficients of 9.73 x 10⁻³ M⁻¹cm⁻¹ and 1.63 x 10⁻⁴ M⁻¹cm⁻¹, respectively.

3.2.2 Reactions of 2-PDS and 4-PDS with thiols

The procedures for thiol determination was modified from Grassetti & Murray (1967). Thiol contents were calculated by using ε⁺₃₄₂ of 7.06 x 10⁻³ M⁻¹cm⁻¹ and ε⁺₂₂₄ of 1.98 x 10⁻⁴ M⁻¹cm⁻¹ for 2-thiopyridone (2-TP) and 4-thiopyridone (4-TP), respectively. Rate constants for reactions with at least a 50-fold excess of 2-PDS or 4-PDS were determined from increases in A⁺₃₄₂ or A⁺₂₂₄.
respectively, monitored for at least eight half-lives with a Hewlett Packard 8452A diode-array spectrophotometer.

3.2.3 Reaction of arsenite with DTT

Formation of the DTT-AsOH complex was followed at various pH values by monitoring the disappearance of DTT with time as determined by its reaction with 4-PDS. In a typical case, 0.02 mM DTT was incubated with a 10-fold excess of NaAsO₂ at pH 5, 6 or 7 and room temperature. The DTT remaining at time intervals was then determined by transferring aliquots into a solution of 1 mM 4-PDS in 0.1 M phosphate buffer at pH 6. Dissociation of the DTT-AsOH complex was followed in a similar manner by monitoring the release of DTT with either 4-PDS or DTNB. In a typical case, 0.2 mM DTT was preincubated with 1 mM NaAsO₂ for at least 20 min in 0.1 M acetate at pH 5 and 0.1 M phosphate at pH 6 or 7; 0.1 ml of the solution was then added to 1 ml of 1 mM 4-PDS in the same buffer and the absorbance at 324 nm was monitored versus time. Concentrations of uncomplexed DTT were determined by extrapolating plots of A₃2₄ versus time to zero time. Dissociation of the complex at pH 8, in 0.1 M Tris buffer, was followed similarly with 1 mM DTNB and monitored at 412 nm.
3.2.4 Assay for Disulfide Reduction

Reactions of ~0.01 to 0.2 mM GSSG and other disulfides with excess DTT, from ~0.1 to 5 mM, at various pH values can be studied by using sodium arsenite to stop the reaction and then measuring the resulting monothiols with 4-PDS. In a typical case, 0.05 mM GSSG was incubated with 0.5 mM DTT in 20 mM buffer at the indicated pH values. At various times, 0.1 ml of this mixture was diluted ten-fold into 0.9 ml of 1 mM NaAsO₂ in 0.1 M phosphate buffer, pH 6, and incubated for 5-10 min. Monothiol concentrations were then determined by adding 0.05 ml of 10 mM 4-PDS and the absorbance at 324 nm was read after 30-60 sec. Concentrations and volumes of the DTT and arsenite solutions can be easily varied to accommodate concentrations or amounts of disulfide above or below the indicated limits. For disulfide concentrations below 0.01 mM, for example, a more concentrated arsenite solution is highly desirable so as to minimize dilution of the sample. To determine appropriate reaction times and conditions for the reduction of an unknown disulfide, a time-course like those shown in Figure 24 to 26 for the reduction of GSSG is recommended.

To reduce the single mixed disulfide bond in BSA, a small excess of DTT was used; 0.2 mM BSA was reduced by 0.5 mM DTT in 10 mM Tris buffer, pH 8. The reaction was stopped by 10-
fold dilution into 1 mM arsenite, pH 6, and the number of reduced thiol groups was determined as described above.

To determine the effect of Gnd-HCl on reductions of protein disulfide bonds, 0.05 mM BSA, 0.25 mM RNase A and 0.3 mM insulin were incubated with 5 mM DTT (i.e., ~ 5-fold excess as compared to the concentration of disulfide bonds) in 6 M Gnd-HCl/0.1 M Tris buffer at pH 8. At various times, 0.02 ml of the solution was diluted 50-fold into 1 mM NaAsO₂ in 6 M Gnd-HCl/0.1 M phosphate at pH 6 and the number of thiol groups was determined by adding 0.5 mM 4-PDS. The fully reduced BSA (~35 moles -SH/mole BSA) was separated from DTT by passage through a Bio-Gel P-2 column eluted with 6 M Gnd-HCl/0.1 M phosphate, pH 2.7. A 1 ml aliquot of this reduced BSA (~0.4 μM) was incubated with 0, 0.01 and 1 mM NaAsO₂ in 6 M Gnd-HCl/0.1 M phosphate at pH 6 for 5 min. The thiol concentrations were then determined by adding 0.5 mM 4-PDS and monitoring the absorbance at 324 nm versus time.

3.3 Results

3.3.1 The Reactions of 2-PDS and 4-PDS with thiols

2-PDS and 4-PDS are not nearly as popular as DTNB but can be used similarly to determine thiols as shown in equations 1
and 2. They are much more effective at low pH (Grassetti and Murray, 1967; Grimshaw, et al., 1979; Brocklehurst, 1982). Their effectiveness under those conditions stems from their being weak bases and the fact that protonation greatly increases their reactivities. Below pH 7, where protonation of 4-PDS ($pK_a \approx 5.0-5.1$ (Grimshaw, et al., 1979; Brocklehurst, 1982)) becomes significant, reaction rates are significantly faster than those with DTNB and the decline at lower values is also much slower (Figure 21). Between pH 4 and 7, reactions with 4-PDS are also appreciably faster than those with 2-PDS (Figure 21).

Reactions with 4-PDS are also more sensitive than those with DTNB. The 4-TP formed has an absorption maximum at ~324
The reactions of 0.5 mM 2-PDS (□) and 0.5 mM 4-PDS (○) with 0.01 mM GSH were conducted in 0.1 M acetate, pH 4 and 5, and 0.1 M phosphate buffers, pH 6 and 7, at 23°C.

Figure 21. pH dependencies for the reactions of 2-PDS and 4-PDS with GSH.
nm and an extinction coefficient of 19,800 M⁻¹cm⁻¹, which is about 50% greater than that of the TNB dianion obtained similarly from reactions with DTNB. A significant decline in the absorbance of TNB below pH 7 is also avoided by using 4-PDS. The extinction coefficient of 4-TP, with pKₐ values of ~1.4 and 8.6-8.8 (Grimshaw, et al., 1979; Brocklehurst, 1982) does not change from pH ~4 to 7. Therefore, 4-PDS was chosen as the thiol reagent for reactions below pH 7.

3.3.2 The Reaction of DTT with Arsenite

Arsenite can form complexes with dithiols such as DTT (Equation 3) and remove excess DTT after the reduction of a disulfide. Monothiols thus can be directly determined by using thiol reagents like 4-PDS. However, two other reactions,

\[
\text{HO-CH}_2\text{-SH} + \text{AsO}_2^- \xrightleftharpoons[k_1]{k_{-1}} \text{HO-CH}_2\text{S} - \text{As-OH} + \text{OH}^- \quad (3)
\]

DTT          DTT-AsOH

i.e., 4-PDS with DTT dissociated from DTT-AsOH complex and 4-PDS with the complex, interfere with the detection of monothiols using reagents like 4-PDS. To minimize these interferencies, we have studied formation and dissociation of the complex at various pH values. In the presence of at least
a 10-fold excess of arsenite, the disappearance of DTT obeyed good first-order kinetics and the observed pseudo-first-order rate constants varied with both the arsenite concentration and pH. Second-order rate constants, $k_1$ as in equation 3, determined at pH 5, 6 and 7 are presented in Table 3. The reaction at pH 8 was too fast to follow under the same conditions.

To study the dissociation of the complex, the release of DTT from 0.02 mM DTT-AsOH was monitored with 4-PDS (or in some cases DTNB). Figure 22 shows a typical time course at pH 6. Although the reaction is first-order as indicated by the inset of Figure 21, two important features were noticed. First, the reaction appeared to involve a very rapid but small initial increase in absorption due the presence of free DTT. Concentrations of free DTT at equilibrium with various concentrations of arsenite were determined from the plots of $A_{324}$ versus time by extrapolating the first-order process to zero time, as shown in Figure 23. At relatively low arsenite concentrations (< 0.2 mM) those intercepts and the concentrations of free DTT calculated from them were significant and easily determined. They varied slightly with pH, reflecting a slightly less stable complex at low pH (see Figure 23), and with time. The magnitude of this initial fast reaction, as shown in Figure 23, was used to calculate the equilibrium constants shown in Table 3, assuming $K_{eq} =$
Table 3. Equilibrium, Association and Dissociation Rate Constants for the DTT-Arsenite Complex.

<table>
<thead>
<tr>
<th>pH</th>
<th>$k_1 (M^{-1}s^{-1})$</th>
<th>$k_{-1} (s^{-1})$</th>
<th>$K_{eq} (\mu M^{-1})$&lt;sup&gt;c&lt;/sup&gt;</th>
<th>$K_{eq} (\mu M^{-1})$&lt;sup&gt;d&lt;/sup&gt;</th>
<th>$k_2 (M^{-1}s^{-1})$&lt;sup&gt;e&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>7.05</td>
<td>2.3 x 10^{-5}</td>
<td>0.31</td>
<td>0.74</td>
<td>0.072</td>
</tr>
<tr>
<td>6</td>
<td>149</td>
<td>2.1 x 10^{-4}</td>
<td>0.71</td>
<td>3.2</td>
<td>0.21</td>
</tr>
<tr>
<td>7</td>
<td>2,640</td>
<td>5.5 x 10^{-4}</td>
<td>4.8</td>
<td>8.7</td>
<td>0.55</td>
</tr>
<tr>
<td>8</td>
<td>----</td>
<td>7.3 x 10^{-1}</td>
<td>----</td>
<td>12.0</td>
<td>2.9</td>
</tr>
<tr>
<td>8.1&lt;sup&gt;f&lt;/sup&gt;</td>
<td>----</td>
<td>1.5 x 10^{-4}</td>
<td>----</td>
<td>3.0†</td>
<td>2.9&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

(a) in 0.1 M sodium acetate (pH 5), 0.1 M sodium phosphate (pH 6 and 7) and 0.1 M Tris (pH 8) buffers at 23°C; (b) average values from at least three independent experiments with deviations of less than ± 5%; (c) from $k_i/k_{-1}$; (d) from [DTT-AsOH]/[DTT][AsO$_2$H]; e) see Equation 1; (e) from Zahler and Cleland, 1968.
A complete time-course for the reaction of 1 mM 4-PDS with 20 µM DTT after 20 min preincubation with 100 µM NaAsO₂ in 0.10 M phosphate buffer, pH 6, at 23°. The inset shows a first-order plot of the same data.

**Figure 22.** The complete reaction of 4-PDS with the DTT-AsOH complex.
Initial absorbance changes for the reaction of 1 mM 4-PDS with 20 μM DTT after 20 min preincubation with 100 μM NaAsO₂ in 0.10 M acetate, pH 5 (Δ), and 0.10 M phosphate buffers, pH 6 (□) and 7 (○), at 23°C. Concentrations of uncomplexed DTT were determined from the zero time intercepts.

**Figure 23.** The initial reaction of 4-PDS with the DTT-AsOH complex.
Equilibrium constants calculated from $K_{eq} = k_1/k_{-1}$ are also listed in Table 3.

The second observation was that first-order rate constants varied with the pH as well as concentration of 4-PDS or DTNB as shown in Figure 24. In accord with earlier work (Zahler and Cleland, 1968), the variations in $k_{obs}$ appear to reflect reactions of DTNB (at pH 8) and 4-PDS (at pH 5, 6 and 7) with free DTT, formed upon dissociation of the complex (i.e., $k_{-1}$), and a direct reaction of each with the DTT-AsOH complex, in accord with equation 4.

$$\text{Rate} = k_{-1}\text{[complex]} + k_2\text{[4-PDS][complex]}$$

(4)

Values of $k_{-1}$ and $k_2$ determined from such data are presented in Table 3. Although the exact nature of the latter reaction is not known, a 1:1 relationship between the amount of 4-PDS consumed and the added DTT is consistent with the following:

$$4\text{-PDS} + \text{DTT-AsOH} \longrightarrow \text{DTT}_{ox} + \text{AsO}_2\text{H} + 2 \{4\text{-TP}\}$$

(5)

As shown in Table 3, both formation and dissociation of the DTT-AsOH complex increase sharply with pH. When using arsenite to complex dithiols such as DTT in the presence of monothiols, dissociation of the complex and its direct reaction with 4-PDS or DTNB should be as low as possible.
First-order rate constants for the reactions of 4-PDS, at pH 5 (▼), 6 (Δ) and 7 (□), and DTNB, at pH 8 (○), with the DTT-arsenite complex, as described in Figure 21, are plotted versus 4-PDS or DTNB concentration.

**Figure 24.** Rates of reaction of 4-PDS and DTNB with the DTT-arsenite complex.
Lower pH values ensure slow release of DTT. Formation of the DTT-arsenite complex at pH 5, however, is so slow that it proved inconvenient. At pH 6 and the other conditions employed (i.e., 0.02 mM DTT, 1 mM NaAsO₂, 1 mM 4-PDS, room temperature), the reaction of DTT with arsenite is complete in ~5 min (i.e., t₁/₂ ~47 sec), dissociation of the DTT-AsOH complex is still very slow (i.e., t₁/₂ >> 1,000 sec), at least 1,000 times slower than the reaction of monothiols with 4-PDS. These conditions were therefore chosen to follow the reduction of several different disulfides by DTT.

3.3.3 The Reaction of GSSG with DTT

To demonstrate the reduction of a simple disulfide and the use of 4-PDS to detect monothiols in the presence of DTT and arsenite, we examined the reaction of 0.05 mM GSSG at pH 7, 8 and 9 with various concentrations of DTT, as shown in Figure 25 to 27. Aliquots of reactions were stopped at various times by adding 1 mM NaAsO₂ in 0.1 M phosphate buffer at pH 6. In accord with expectations, the reduction proceeded more rapidly at higher pH and higher DTT concentrations. At pH 7 and 5mM DTT, complete reaction was obtained after ~40 min (Figure 25), at pH 8 it was complete in about the same time with 1 mM DTT (Figure 26) and, at pH 9 it was complete in less than 10 min with 0.5 mM DTT (Figure 27).
The reaction solutions contained 0.05 mM GSSG and 5 mM (○), 2.5 mM (□), and 1 mM (△) DTT in 0.05 M phosphate, pH 7, at 23°. At the indicated times, 100 µl samples were diluted ten-fold into 1 mM NaAsO₂ in 0.1 M phosphate buffer, pH 6, and, after 5 min, 1 mM 4-PDS was added and the increase in absorbance at 324 nm was determined.

Figure 25. The reduction of GSSG by DTT at pH 7.
The reaction solutions contained 0.05 mM GSSG and 1 mM (Δ), 0.5 mM (▽), and 0.25 mM (○) DTT in 0.05 M Tris, pH 8, at 23°. At the indicated times, 100 μl samples were diluted tenfold into 1 mM NaAsO₂ in 0.1 M phosphate buffer, pH 6, and, after 5 min, 1 mM 4-PDS was added and the increase in absorbance at 324 nm was determined.

Figure 26. The reduction of GSSG by DTT at pH 8.
The reaction solutions contained 0.05 mM GSSG and 1 mM (△), 0.5 mM (▽), 0.25 mM (○), and 0.1 mM (●) DTT in 0.05 M Tris, pH 9, at 23°. At the indicated times, 100 μl samples were diluted ten-fold into 1 mM NaAsO₃ in 0.1 M phosphate buffer, pH 6, and, after 5 min, 1 mM 4-PDS was added and the increase in absorbance at 324 nm was determined.

**Figure 27.** The reduction of GSSG by DTT at pH 9.
The determined second-order rate constant, at pH 8 and 23°C (i.e. 140 "M min⁻¹), is slightly below that obtained recently under similar conditions (i.e. 186 M⁻¹min⁻¹ at pH 8 and 25°C (Rothwarf and Scheraga, 1992)) by more laborious means. The difference might easily be due to the presence of oxygen in our case. The expected amount of GSH was obtained at completion of reduction. However, at lower DTT concentrations (i.e. < 0.25 mM) and high pH, complete reduction was not attained due, presumably, to the presence of oxygen and some competing oxidation. Excluding oxygen, adding EDTA and/or another chelating agent would probably reduce or eliminate such reoxidation and permit more complete reduction. As will be discussed later, the presence of 6 M Gnd-HCl had little or no effect on either the reduction of GSSG or the detection of GSH under these conditions.

3.3.4 The Reduction of BSA by DTT

In addition to the 34 cysteine residues involved in 17 intramolecular disulfide bonds, BSA has a cysteine at sequence position 34 which is usually found partly in the reduced/thiol form and partly in a nonthiol form, thought to be a mixed disulfide with cystine and/or glutathione (Janatova and Hunter, 1968; Wilson, et al., 1980). To demonstrate the reduction of this complex disulfide and the use of 4-PDS to
detect such in the presence of DTT and arsenite, we examined the reaction of 0.2 mM BSA with 0.5 mM DTT at pH 8. Figure 28 shows absorbance increases obtained after adding 4-PDS to 10-fold diluted aliquots of the BSA/DTT solution after incubation with 1 mM arsenite/0.1 M phosphate at pH 6 for 5 min. The size of the initial rapid rise in $A_{324}$, and the number of thiol groups, calculated from it, increased rapidly, as shown, from ~0.5 initially to ~1.5 (i.e., ~1 -SH group/BSA + ~0.5 cysteine and/or GSH) at 20 min. A slower increase at longer times appeared to reflect further reduction of other disulfide bonds.

To determine whether the same procedure could be used to follow the complete reduction of BSA, 0.05 mM BSA was treated with 5 mM DTT in 6 M Gnd-HCl/0.1 M Tris buffer at pH 8. The number of thiol groups produced was then determined at various times after a 50-fold dilution into 1 mM NaAsO$_2$/6 M Gnd-HCl/0.1 M phosphate, pH 6, with 0.5 mM 4-PDS. As shown in Figure 29, the number of thiol groups increased rapidly to ~10 to 14 per mole of BSA (i.e., ~30 to 40% of the expected thiol content) in ~5 min but no further with longer times (or with a larger excess of DTT). Table 4 shows the similar results obtained when RNase A and insulin were reduced with DTT under the same conditions. Reduction of GSSG under those conditions, however, gave the expected amount of GSH, as shown in Figure 29. To seek possible reasons for the low number of
The reaction solution contained 0.2 mM BSA and 0.5 mM DTT in 0.01 M Tris buffer, pH 8, at 23°. At 0 (○), 5 (□) and 20 min (△), 100 μl samples of the solution were diluted ten-fold into 1 mM NaAsO₂ in 0.1 M phosphate buffer, pH 6, and, after 10 min, 0.3 mM of 4-PDS was added and the absorbance at 324 nm was monitored.

Figure 28. The reduction of BSA by DTT.
The reaction solutions contained 0.94 mM GSSG (○) or 0.05 mM BSA (□) (i.e., 0.9 mM disulfide bonds) and 5 mM DTT in 6 M Gnd-HCl/0.1 M Tris, pH 8, at 23°. At various times, 20 μl samples of the solution were diluted 50-fold into 1 mM NaAsO₂ in 6 M Gnd-HCl/0.1 M phosphate buffer, pH 6. After 10 min, 0.5 mM of 4-PDS was added and A_{324} was determined.

Figure 29. The reduction of GSSG and BSA by DTT in the presence of 6 M Gnd-HCl.
detectable thiol groups in reduced proteins, we separated fully reduced BSA from excess DTT by gel-filtration on a Bio-Gel P-2 column. Thiol contents were then determined with 4-PDS in 6 M Gnd-HCl/0.1 M phosphate, pH 6, with and without the presence of arsenite. In the absence of arsenite, the reaction with 4-PDS was rapid and the absorption increase at 324 nm after ~1 min gave approximately the expected number of thiol groups (i.e., ~30 to 37 of the theoretical 35), as shown in Table 4. After 5 min incubation with 0.01 and 1 mM arsenite, however, only 75% and 30% of the thiol groups, respectively, were free to react rapidly with 4-PDS, as shown in Figure 30. As in the case of DTT, the initial rapid increase in absorbance presumably reflects the reactions of uncomplexed thiol groups and is followed by a further slow reaction that reflects, largely, the dissociation of arsenite-dithiol complexes. Similar results were also obtained with fully reduced RNase A in the presence of arsenite (data not shown). These results suggest that some thiol groups in denatured proteins also form complexes with arsenite under the described conditions.
Table 4. Thiol groups detected in reduced proteins with 4-PDS in the presence of 1 mM NaAsO$_2$/6 M Gnd-HCl, pH 6.

<table>
<thead>
<tr>
<th>proteins$^a$</th>
<th>Number of thiols per mole of protein</th>
<th>absence of NaAsO$_2$$^b$</th>
<th>presence of NaAsO$_2$$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>bovine serum albumin</td>
<td>30-37 (35$^a$)</td>
<td>10-14</td>
<td></td>
</tr>
<tr>
<td>ribonuclease A</td>
<td>7-8 (8$^a$)</td>
<td>3-4</td>
<td></td>
</tr>
<tr>
<td>insulin</td>
<td>--- (6$^a$)</td>
<td>3-3.6</td>
<td></td>
</tr>
</tbody>
</table>

(a) Proteins were reduced by 5 mM DTT for at least 20 min in 6 M Gnd-HCl/0.1 M Tris, pH 8;
(b) The excess DTT was removed by gel-filtration on a Bio Gel P-2 column at pH 2.7 and the proteins were the diluted into 6 M Gnd-HCl/0.1 M phosphate, pH 6, prior to the addition of 0.5 mM 4-PDS;
(c) The excess DTT was "removed" by dilution into 1 mM NaAsO$_2$/6 M Gnd-HCl/0.1 M phosphate, pH 6, prior to the addition of 0.5 mM 4-PDS;
(d) The number of cysteine residues in the proteins.
0.4 μM reduced BSA, collected from a Bio-Gel P-2 column, was incubated with 0 (○), 0.01 (□), and 1 (Δ) mM NaAsO₂ in 6 M Gnd-HCl/0.1 M phosphate buffer, pH 6, for 5 min, then 0.5 mM 4-PDS was added to the solution and $A_{324}$ was monitored.

**Figure 30.** The reaction of thiols on fully reduced BSA with arsenite in the presence of 6 M Gnd-HCl.
3.4 Discussion

Thiol groups are required for the biological activities of many proteins and are one of the most frequent targets for protein modification. DTNB and, to a lesser extent, 2-PDS and 4-PDS are widely used to determine thiol groups in proteins, and other aspects of their presence, such as the number of fast, slow, and unreactive thiol groups, as part of an overall strategy to determine three-dimensional structure, effects of modification on biological activity, relationships between individual thiol groups and biological activity, to determine the effects of substrates, allosteric effectors and other ligands on such modifications and so on.

DTT and other thiols are frequently required to activate or stabilize enzymes and other proteins. As one of the strongest thiol reducing agents, DTT is also commonly used to effect selective or, sometimes, complete reduction of disulfide bonds in proteins (Bewley and Li, 1969; Shapira and Arnon, 1969; Shechter, et al., 1973). As pointed out by Zahler and Cleland (1968), it should be possible to determine the number of monothiol groups with DTNB, or another suitable thiol reagent, in such cases after complexation of the DTT with excess arsenite. It should also be possible, in principle, to determine the number of fast, slow and unreactive thiol groups.
Slow reactions of DTNB with thiols can be difficult to characterize, however, due to its slow hydrolysis (Equation 6) and disproportionation (Equation 7) and, usually, to oxygen and transition metal ion-dependent reoxidation of TNB back to DTNB (Equation 9) (Janatova and Hunter, 1968; Riddles et al., 1979). The former reaction (Equation 6) is first-order in hydroxide ion and is usually rate-determining. Its rate increases rapidly with pH and is easily observed at pH ~8 or above as a slow increase in A$_{412}$ in the absence of a thiol or long after the reaction with a thiol is complete. Reoxidation of TNB (Equation 9) is usually observed as a slow decline in A$_{412}$ after the reaction of DTNB with a thiol is complete. It can be eliminated or greatly reduced by carefully excluding oxygen, by adding EDTA and/or by otherwise complexing or eliminating transition metal ions.

\[
\begin{align*}
2 \text{ArSSAr} + 2 \text{OH}^- & \rightarrow 2 \left[\text{ArSOH}\right] + 2 \text{ArS}^- \quad (6) \\
2 \left[\text{ArSOH}\right] & \rightarrow \text{ArSO}_2^- + \text{ArS}^- + 2 \text{H}^+ \quad (7) \\
2 \text{ArSSAr} + 2 \text{OH}^- & \rightarrow \text{ArSO}_2^- + 3 \text{ArS}^- + 2 \text{H}^+ \quad (8) \\
\text{M}^{n-} + 2 \text{ArS}^- + 1/2 \text{O}_2 + 2 \text{H}^+ & \rightarrow \text{ArSSAr} + \text{H}_2\text{O} \quad (9)
\end{align*}
\]

where ArSSAr = DTNB, ArS$^-$ = TNB
Two other reactions give rise to even faster increases in $A_{412}$ in the presence of DTT and arsenite. One appears to involve the reaction of DTNB with DTT as it is released from the arsenite complex (Equation 10) and the other appears to involve a direct reaction of DTNB with the DTT-arsenite complex (Equation 5). Both of these reactions increase at higher pH (see Figures 23 and 24) and, together, account for significant increases in $A_{412}$ under the conditions usually employed. All four of the described background reactions (i.e. Equations 6, 7, 9 and 10) can be suppressed at low pH, but the reactivity of DTNB with thiols is severely compromised below pH ~7.

At pH 6, 4-PDS reacts rapidly with thiols to give a chromophore (i.e. 4-TP) with a strong absorbance at ~324 nm and only low levels of background reactions (Grassetti and Murray, 1967; Grimshaw, et al., 1979; Brocklehurst, 1982). Reaction rates are typically much faster than those with either 2-PDS or DTNB and the extinction coefficient of the resulting chromophore is considerably more intense. Dissociation of the DTT-arsenite complex and its oxidation by
4-PDS are both very slow at pH 6 (see Table 3). Hydrolysis and disproportionation of 4-PDS appears to be similar to DTNB (see Equations 7, 8 and 9) but much slower ($k_{obs} \approx 7 \times 10^{-10} \text{M}^{-1}\text{s}^{-1}$ at pH 6) and reoxidation of 4-TP to 4-PDS has not been observed.

4-PDS is an effective but rather seldom used reagent for the spectrophotometric determination of thiols (Grassetti and Murray, 1967). It is most effective at pH values between ~3 and 7 and, as just described, has a number of advantages as compared to DTNB. At pH 6, where it reacts very rapidly with thiols, it reacts only very slowly with the DTT-arsenite complex and the dissociation of that complex is also very slow. Under such conditions, it can be used as a part of a convenient and sensitive assay for thiols and, by introducing excess arsenite, for the selective determination of monothiols in the presence of DTT. Under the latter conditions, it should be possible to monitor the formation or disappearance of monothiol groups during reductions and reoxidations of proteins and other disulfide compounds by DTT/DTT$_{os}$, without the need to first separate DTT. A very similar procedure can also, presumably, be used to determine monothiols in the presence of other strong dithiol reducing agents (e.g. see (Singh and Whitesides, 1994)) that form stable arsenite complexes (Zahler and Cleland, 1968; Delnomdedieu, et al., 1993).
To demonstrate this procedure, we used it to follow the reduction of two quite different disulfides by DTT. Figures 25 to 27, for example, show the reduction of GSSG by several different concentrations of DTT at three different pH values. Figure 28 shows the absorbance increases obtained upon adding 4-PDS, and excess arsenite, to bovine serum albumin at different times during its treatment with 0.05 mM DTT. In both cases, there was a rapid and very obvious reaction of the monothiol(s) with 4-PDS but, due to the presence of 1 mM arsenite, almost no reaction with the large excess of DTT. The formation of monothiol groups was therefore clearly and easily observed above a negligible background.

It is worth mentioning that cysteine-34 of BSA is located in a hydrophobic crevice in the amino-terminal domain adjacent to and slightly protected by histidine 39 and glutamate 82 (Carter and Ho, 1994). It reacts very slowly with DTNB and other anionic disulfides (Janatova and Hunter, 1968; Wilson, et al., 1980) but more rapidly with 2-PDS and other, relatively small, cationic disulfides (Svenson and Carlsson, 1975; Wilson, et al., 1980; Pedersen and Jacobsen, 1980). We are aware of only one report, that did not present any rate data, of its reaction with 4-PDS (Fantl, 1970). Selective reduction of the cysteine 34-mixed disulfide of BSA is complicated by the presence of 17 other potentially
susceptible disulfide bonds and the detection of its reduction is made difficult by the low reactivity of cysteine 34. As shown in Figure 28, the reduction of this mixed disulfide bond can be easily monitored using the present procedure. Therefore, excessive reduction of other disulfide bonds of the protein can be avoided.

Using the same assay procedure to follow the complete reduction of several proteins (i.e., BSA, RNase A and insulin with 5 mM DTT, 6 M Gnd-HCl; see Figure 29 and Table 4), however, gave significantly lower than expected numbers of thiol groups. The exact numbers varied, particularly, with the concentration of arsenite employed and appeared to reflect significant complexation of the reduced and denatured proteins by arsenite. Although reduced and thoroughly denatured proteins are usually thought to have little or no fixed three-dimensional structure, they are clearly not monothiols and should be expected to react to some extent with arsenite. For the three selected model proteins, BSA, RNase A and insulin, the extent of such reaction is, perhaps, surprisingly large and could not be ignored. Similar problems should probably be anticipated in the case of other reduced and denatured proteins. It is worth recalling that arsenite is a potent inhibitor of many so-called dithiol enzymes due, presumably, to its reactions with thiol groups that are closely spaced in
the three-dimensional structure but not necessarily in the amino acid sequence (Webb, 1966; Klempere and Pickart, 1989).

Table 3 summarizes kinetic and equilibrium data for the DTT-arsenite complex obtained at pH 5, 6, 7 and 8 and that of Zahler and Cleland (1968) at pH 8.1. Our value and their value for $k_2$ at pH ~8 are in very good agreement but the values for $k_{-1}$ are inexplicably quite different. We checked our value several times, using both DTNB and 4-PDS (data not presented), and have no explanation for the large difference. The true value is of some importance in that dissociation of the complex is one of the main background reactions in these assays at pH ~8.

Two sets of values for $K_{eq}$ are given in Table 3. Those determined from $k_2/k_{-1}$ were quite reproducible but consistently half or less of those determined from equilibrium concentrations of free DTT under the same conditions. Estimates of free DTT, on the other hand, varied significantly with the length of the incubation time. At relatively short incubation times (< 5 min, depending on the concentration and pH), concentrations of uncomplexed DTT decreased steadily with time and appeared to be approaching a lower equilibrium value. After ~5 min and for up to 30 min, concentrations of free DTT continued to decline but much more slowly.

We note that the DTT-arsenite complex has been shown to be a rather complicated bicyclic complex, with arsenic
coordinated to both thiol groups and the oxygen of one hydroxyl moiety (Cruse and James, 1972). We speculate that the initial complexation may involve the formation of both this tridentate complex and a bidentate complex, similar to that proposed by Zahler and Cleland (1968), that then rearranges, slowly, to the more stable tridentate complex. Rates of complex formation would then reflect a composite of two processes and give rise to incorrect values of \( K_{eq} \); the slow decrease in free DTT after \( \sim 5 \) min would presumably reflect rearrangement to the tridentate complex.

The \( K_{eq} \) value reported by Zahler and Cleland (1968) was obtained from concentrations of free DTT after correction for a small amount of a monothiol impurity or something that resembled a monothiol in respect to its interactions with arsenite. We observed no evidence for a monothiol in DTT we used and our values, based on free DTT concentrations after 20 min incubation, are not corrected for such. Because of the variations in free DTT with time, we considered whether or not a monothiol or some other type of heterogeneity of the DTT might be affecting our results but prefer the explanation presented above. It is of some note that their \( K_{eq} \) value, obtained after 5 min incubation, is similar to that we might have obtained after a similar incubation period but without a monothiol correction.
CHAPTER 4

THE DECOMPOSITION OF S-NITROSATED DITHIOLS:
A MODEL FOR VICINAL NITROSOThIOLS OF ENZYMES

4.1 Introduction

Nitric oxide (NO), nitrovasodilators like nitroglycerin, and many other NO-donor compounds are known to affect many physiological and pathophysiological processes (Knowles and Moncada, 1992). Many enzymes are also affected by these compounds (Park, 1988; Lepoivre, et al., 1991; Lei, et al., 1992; Molina, et al., 1992). Relaxation of smooth muscles by NO and NO-donors is thought to be due to their activation of a large heterodimeric soluble guanylate cyclase (sGC) with approximately 30 thiol groups and one heme moiety (Arnold, et al., 1977; Katsuki, et al., 1977; Waldman, et al., 1988). Creatine kinase (Gross, et al., 1996) and protein kinase C (Gopalakrishna, et al., 1993) are inactivated by S-nitrosothiols. Type I adenylyl cyclase (Duhe, et al., 1994) is no longer activated by calmodulin after treatment with NO-donors.
Activation of sGC by NO or NO-donors is usually attributed to the binding of NO to its heme moiety (Ignarro, et al., 1982; Ignarro, 1991). Oxidations of thiol groups, in some cases (Kamisaki, et al., 1986; Niroomand, et al., 1989; Wu, et al., 1992; Stamler, et al., 1992; Liu, et al., 1995), also stimulated sGC activity, however, without the involvement of NO or any NO-donor compounds. None of the other mentioned enzymes has a heme moiety but all have multiple thiol groups and may have one or more pairs of closely spaced or vicinal thiol groups. In all cases, the effects of NO-donors are reversed by treatment with DTT.

Under physiological conditions, S-nitrosothiols undergo rapid transnitrosation in the presence of thiols (Park, 1987; Meyer, et al., 1994). Both low molecular weight thiols and the thiol groups of proteins appear to undergo such reactions (Zhang and Means, 1996). In cases of vicinal thiol groups in the enzymes mentioned above, an NO moiety might be transferred to either one or both of the thiol groups. It is important, therefore, to understand the properties of such derivatives, in particular, the nature and kinetics of the reactions which they undergo. Here, we present results pertaining to the stability of the mono and dinitroso derivatives of DTT, DTT(NO), and DTT(NO)$_2$, and three other dithiols as models for what may transpire upon the S-nitrosation of vicinal thiol
groups in proteins. These results suggest a mechanism whereby S-nitrosation might affect temporary changes in the activity of an enzyme by facilitating disulfide formation. The results of this study also provide valuable information as to how DTT, and other dithiols, may promote NO formation from simple S-nitrosothiols and sodium nitroprusside (Richter, et al., 1994; Cleeter, et al., 1994).

4.2 Materials and Methods

4.2.1 Materials

Dithiothreitol (DTT), dithioerythritol (DTE), DL-6,8-thiocetic acid (TA), and 2,3-dimercaptopropanol (DMP), GSH, and NaNO₂ were obtained from Sigma Chemical Co. Solutions of dithiols were prepared daily and the concentrations of the reduced form were determined by the reaction with DTNB using the extinction coefficient of 13,600 M⁻¹cm⁻¹ (Ellman, 1959).

4.2.2 S-nitrosation of dithiols

S-nitrosations were carried out by mixing thiols with sodium nitrite at pH 1.5 to 2 and room temperature (Zhang and Means, 1996). S-nitrosoglutathione (GSNO) was prepared by mixing equimolar concentration of GSH and NaNO₂, usually 20 mM of each in 40 μM HCl, for 10 min. The pH of the solution was
adjusted to neutral and EDTA was added to give a final concentration of 10 mM GSNO/1 mM EDTA, ~pH 7. For dinitrosothiols (e.g., DTT(NO)$_2$), a slight excess (~2 to 5%) of nitrite to dithiol was used. Mononitrosated dithiols (e.g., DTT(NO)) were prepared similarly but with at least a 20-fold excess of thiol (DTT:NaNO$_2$ > 10:1). The pH of those solutions were maintained at ~2 until used. S-nitrosations of dithiols were followed, in each case, by monitoring their UV-visible absorptions with a Hewlett-Packard 8452 spectrophotometer. Products of S-nitrosated dithiols were analyzed by RP-HPLC using a Lichrosorb C-18 column eluting with a 0 to 60% acetonitrile gradient in 10 mM phosphoric acid. S-nitrosothiols were also analyzed by mass spectroscopy on a PE Sciex API 300 mass spectrometer.

4.2.3 Kinetic studies

Decompositions of dinitrosothiols were followed by monitoring absorbance changes at 334 nm, the maximum wavelength of an S-nitroso moiety. Aliquots of dinitrosothiols, prepared just prior to the reaction, were diluted to a final concentration of 0.25 mM in 100 mM Tris-HCl buffer, 1 mM EDTA, pH 7.4. Absorbencies at 334 nm were monitored with the Hewlett-Packard 8452 spectrophotometer. For very unstable dinitrosothiols, S-nitrosation was carried out directly in the
cuvette. Absorbance changes, in those cases, were followed immediately after the addition of a small aliquot of sodium nitrite to 0.25 mM of dithiol in 40 µM HCl. First-order rate constants \( (k) \) were calculated from plots of \( \ln(A/A_0) \) versus time.

Decompositions of mononitroso derivatives were followed under two conditions. First, 0.1 mM of each freshly made mononitrosated dithiol was mixed with 1 to 3 mM of the respective free dithiol in 100 mM Tris-HCl buffer, 1 mM EDTA, pH 7.4 and absorbencies at 334 nm were followed with time. Second, 0.1 mM of GSNO were used as an NO donor and mixed with 1 to 3 mM of each dithiol in the same, pH 7.4, buffer. Absorbance changes at 334 nm were again followed with time. Values of \( k_{cs} \) were obtained by linear regression analysis from plots of \( \ln(A/A_0) \) versus time. First-order rate constants \( (k) \) and second-order rate constants \( (k_2) \) were obtained from the intercepts and slopes, respectively, of plots of \( k_{cs} \) versus dithiol concentration. A pH dependence for the decomposition of DTT(NO) in the presence of 1 mM DTT was obtained similarly by diluting freshly prepared DTT(NO) into a series of buffers, i.e., 100 mM acetate at pH 5.2, phosphate at pH 6 and 6.8, Tris-HCl at pH 7.4, 8.0, and 8.6, pyrophosphate at pH 9.15, and carbonate at pH 9.4 and 10.2.
4.2.4 Analyses of decomposition products

Products from the homolysis and heterolysis of nitrosated dithiols were analyzed by RP-HPLC on a C-18 column eluted with a linear gradient from 0 to 60% acetonitrile in 10 mM phosphoric acid at 1 ml/min for 30 min. Gaseous products of the reactions were analyzed with a Finnigan MAT-900 GC-MS spectrometer.

4.3 Results and Discussion

Spectra of DTT(NO) and DTT(NO)$_2$, as shown in Figure 31, show characteristic S-nitrosothiol absorption peaks at ~334 nm and 544 nm (inset). Peak intensities of DTT(NO), ~950 M$^{-1}$cm$^{-1}$ at 334 nm and ~18 M$^{-1}$cm$^{-1}$ at 544 nm, were similar to those of GSNO and other mononitrosothiols and approximately half of those of DTT(NO)$_2$. The dinitrosothiols were not isolated in pure forms, however, S-nitrosations were complete as evidenced by HPLC analysis and by electrospray mass spectra of DTT(NO)$_2$ and DTE(NO)$_2$, m/z 230 (M + NH$_4^+$) and m/z 235 (M + Na$^+$).

Decomposition of S-nitrosothiols by homolysis involves the generation of NO, thyl radicals and finally disulfides. Rates are influenced by factors such as metal ions, light, pH, temperature, and the structure of the S-nitrosothiols (Oae and Shinhama, 1983; Sexton, et al., 1994; McaAninly, et al., 1993;
DTT(NO) (---) was made by mixing DTT and NaNO₂ at final concentrations of 2.5 mM and 0.25 mM, respectively, in 40 μM HCl. DTT(NO)₂ (---) was made similarly at final concentrations of 0.25 mM DTT and 0.52 mM NaNO₂ in 40 μM HCl. Inset: 10 mM DTT(NO) (---) and DTT(NO)₂ (---).

Figure 31. Spectra of DTT(NO) and DTT(NO)₂.
Mathews and Kerr, 1993; Zhang, 1996). Among these factors, metal ions have the most profound effects, particularly on S-nitroso compounds with an adjacent amino group (Zhang, 1996). After the elimination of free metal ions by including EDTA in the solutions, simple S-nitrosothiols are reasonably stable with half-lives ranging from 13.2 h to 46.5 h for S-nitrosocysteine (Komiyama and Fujimori, 1997) and GSNO (Zhang, 1996), respectively, and are pH independent. It was suggested that the first step in the homolysis of S-nitrosothiols (Equation 11a) involves the formation of a thiyl radical followed by rate-determining dimerization of thiyl radicals to give the disulfide (Equation 11b) (Bainbrigge, et al., 1997). Reactions between thiols and S-nitrosothiols are thought to proceed by nucleophilic attack of the thiol anion on the S-nitrosothiol to produce a disulfide and NO\(^{+}\) (Equation 12a). The latter combine to give N\(_2\)O as final product (Equation 12b). Rates of this heterolytic reaction are dependent on thiol concentration and pH.

\[
\begin{align*}
RSNO & \rightleftharpoons NO + Rs^* \quad (11a) \\
2Rs^* & \rightarrow RSSR \quad (11b) \\
RSNO + RS^- & \rightarrow RSSR + NO^+ \quad (12a) \\
2NO^- + 2H^+ & \rightarrow N_2O + H_2O \quad (12b)
\end{align*}
\]
When the thiol in question is a dithiol, homolysis and heterolysis, as shown in equations 13 and 14, respectively, become intramolecular reactions and are expected to be faster.

\[ \text{SNO}_2 \xrightarrow{\text{SNO}} \text{S} \text{S} + 2\text{NO} \]  
(13)

\[ \text{SNO}_2 \xrightarrow{\text{SNO}} \text{S} \text{S} + \text{NO}^- \]  
(14)

We have examined the mono and dinitrosated derivatives of five dithiol compounds (shown in Figure 32): dithiothreitol (DTT), dithioerythritol (DTE), DL-6,8-thioci acid (TA), 2,3-dimercaptopropanol (DMP), and glycol dimercaptoacetate (GDMA).

Figure 33 shows typical time courses for the homolyses of \( \text{DTT(NO)}_2 \) and \( \text{DTE(NO)}_2 \). First-order rate constants, calculated from plots of \( \ln(A/A_0) \) versus time as shown in the inset of Figure 33, are listed in Table 5. The decomposition of \( \text{DTT(NO)}_2 \), \( \text{DTE(NO)}_2 \), and \( \text{TA(NO)}_2 \) were 2 to 3 orders of magnitude faster than those of most monothiols. Half-lives thus were 6.4, 7.4, and 1.1 min, respectively, as compared to \( \sim 6 \) h for \( \text{DMP(NO)}_2 \) and 12.8 h for the S-nitroso derivative of \( \beta\)-mercaptopoethanol (\( \beta\)-ME) under similar conditions.

Since a large excess of dithiol relative to the NO-donor was required to obtain mononitrosothiol derivatives,
Figure 32. Compounds used in this study as models for vicinal thiols in proteins.
Freshly prepared DTT(NO)_2 (—) and DTE(NO) (---) were diluted into 100 mM Tris-HCL buffer, 1 mM EDTA, pH 7.4, to a final concentration of 0.25 mM. Absorbencies at 334 nm were monitored versus time. Inset: First-order plots for the homolysis of DTT(NO)_2 (○) and DTE(NO) (□).

**Figure 33.** Homolysis of DTT(NO)_2 and DTE(NO)_2.
<table>
<thead>
<tr>
<th>Thiols</th>
<th>Homolysis $k \times 10^3$ (s$^{-1}$)</th>
<th>Heterolysis $k_1 \times 10^3$ (s$^{-1}$)</th>
<th>$k_2$ (M$^{-1}$s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DTT</td>
<td>1.8</td>
<td>0.9 $^b$</td>
<td>0.78 $^b$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.0 $^c$</td>
<td>0.78 $^c$</td>
</tr>
<tr>
<td>DTE</td>
<td>1.6</td>
<td>0.4 $^b$</td>
<td>0.42 $^b$</td>
</tr>
<tr>
<td>TA</td>
<td>10.0</td>
<td>3.2 $^c$</td>
<td>20.2 $^c$</td>
</tr>
<tr>
<td>DMP</td>
<td>0.032</td>
<td>n/d$^d$</td>
<td>103.6 $^d$</td>
</tr>
<tr>
<td>GDMA</td>
<td>15.0</td>
<td>7.21 $^c$</td>
<td>3.05 $^c$</td>
</tr>
<tr>
<td>β-ME</td>
<td>0.015</td>
<td>n/d$^d$</td>
<td>0.05</td>
</tr>
<tr>
<td>Cys$^e$</td>
<td>0.015</td>
<td>n/d$^c$</td>
<td>0.03</td>
</tr>
</tbody>
</table>

(a) Abbreviations for the dithiols: DTT, dithiothreitol; DTE, dithioerythritol; TA, DL-6,8-thiolic acid; DMP, 2,3-dimercaptopropanol; GDMA, glycol dimercaptoacetate
(b) For synthesized mononitrosated dithiols.
(c) For reactions of GSNO and dithiols.
(d) Not detectable.
(e) Data from Komiyama and Fujimori, 1997.

Table 5. Decomposition rate constants for S-nitrosated mono and dithiols at pH 7.4.
intramolecular heterolyses of DTT(NO) and other mononitrosated dithiols were conducted in the presence of a large excess of DTT or the other dithiols and also were followed by monitoring absorbencies at 334 nm (Figure 35). Under such conditions the intramolecular heterolysis is accompanied, however, by an intermolecular heterolysis reaction. Rate constants for the two competing processes, the intramolecular and intermolecular reactions, $k_1$ and $k_2$, respectively, can be obtained from the intercepts and slopes of plots of $k_{obs}$ versus dithiol concentrations (Figure 35).

Two completely different procedures were used to produce and follow the decomposition of mononitrosated dithiols. First, DTT(NO) and other mononitrosated dithiols, which had been prepared from NaNO$_2$ and a ten-fold molar excess of dithiol at ~pH 2 (see Materials and Methods) were adjusted to neutral pH and allowed to decay in the presence of various concentrations of free dithiol. Figure 34 shows time courses for two such reactions. In accord with the rate equation for these heterolyses shown as Equation 15, values of $k_{obs}$, calculated from the slopes of pseudo first-order plots (inset), were then plotted against the DTT or DTE concentration as shown in Figure 35.

\[
\text{rate} = k_1[D\text{TT(NO)}] + k_2[D\text{TT(NO)}][\text{DTT}]
\]  

(15)
Freshly prepared DTT(NO) (---) and DTE(NO) (---), 0.1 mM, were mixed with 1 mM DTT and DTE, respectively, in 100 mM Tris-HCl buffer, 1 mM EDTA, pH 7.4. Absorbencies at 334 nm were monitored versus time. Inset: Pseudo first-order plots of heterolysis of DTT(NO) (O) and DTE(NO) (□).

**Figure 34.** Heterolysis of DTT(NO) and DTE(NO).
Values of $k_{\text{obs}}$ for the reactions of DTT(NO) (○) and DTE(NO) (□), 0.1 mM, with 0.9 to 3 mM of DTT and DTE, respectively, in 100 mM Tris-HCl, 1 mM EDTA, pH 7.4, obtained from pseudo first-order plots as shown in Figure 3 (inset) are plotted versus dithiol concentrations.

**Figure 35.** The observed decomposition rate constants ($k_{\text{obs}}$) as a function of dithiol concentrations.
The intercepts of the lines in Figure 35 were the first-order rate constants ($k_1$) for the intramolecular reaction of mononitrosated dithiols and the slopes were the second-order rate constants ($k_2$) for the intermolecular reactions of DTT(NO) and DTE(NO) with the corresponding dithiols. Values of $k_1$ and $k_2$ for all four mononitrosated dithiols under these conditions and for the decomposition of S-nitroso-β-mercaptoethanol and S-nitrosocysteine in the presence of excess β-mercaptopethanol and cysteine, respectively, are presented in Table 5.

Heterolyses of mononitrosated dithiols were also studied under similar conditions using GSNO as a donor of NO. Because rates of transnitrosation, under most conditions, are 2 to 3 orders of magnitude faster than those obtained for heterolysis, addition of GSNO to a large excess of DTT resulted in a rapid formation of DTT(NO). Decomposition of that DTT(NO) was again followed by monitoring absorbencies at 334 nm and values of $k_1$ and $k_2$ were obtained from plots of $k_{obs}$ versus DTT concentration according to equation 15. In the case of DTT(NO), values of $k_1$ and $k_2$ under such conditions were in fact very similar to those obtained previously as shown in Table 5. Half-lives for the intramolecular heterolysis of DTT(NO), DTE(NO), and TA(NO), calculated from $k_1$, were 12.1, 28.9, and 3.6 minutes, respectively.
Unlike the homolysis of DTT(NO)₂ and other dinitrosothiols, rates of the heterolytic decomposition of mononitrosated dithiols increased rapidly with pH in accord with the nucleophilic mechanisms of Equations 9a and 11. Figure 36 shows the pH dependence of the heterolysis of DTT(NO) in the presence of ten-fold excess of DTT.

Exceptionally slow intramolecular decomposition of both DMP(NO) and DMP(NO)₂, as indicated by an undetectable $k_i$ for the heterolysis of DMP(NO) and a very low decomposition rate for the homolysis of DMP(NO)₂, suggests formation of the cyclic, oxidized, form of DMP is very difficult. Intermolecular heterolysis of DMP(NO), however, was rapid, consistent with the observation of polymeric reaction products and nothing resembling a monomeric disulfide form of DMP by RP-HPLC.

In accord with their faster intramolecular rates, only cyclic disulfide forms were produced by homolysis or heterolysis of the other nitrosated dithiols as shown (e.g., Figure 37). Under anaerobic conditions, NO (m/z 30) was the principle (>95%) gaseous product from the homolysis of DTT(NO)₂, whereas N₂O (m/z 44) was the main product (>95%) from the heterolysis of DTT(NO). Mixtures of DTT(NO) and DTT(NO)₂ gave both gaseous products and a 10-fold excess of GSNO over DTT was required for the efficient formation of NO.
Freshly prepared DTT(NO), 0.1 mM, was reacted with 1 mM DTT in 100 mM buffer at various pH values containing 1 mM EDTA. Pseudo first-order rate constants, $k_{obs}$, obtained as shown in Figure 3 (inset) are plotted versus pH.

**Figure 36.** The observed decomposition rate constant of DTT(NO) as a function of pH.
DTT(NO) (A) and DTT(NO)$_2$ (B) were made as described in the text. Samples of 20 μl of each solution before (top) and after (bottom) decomposition were subjected to RP-HPLC using a C-18 column and eluted with a 0 to 60% acetonitrile gradient in 10 mM phosphoric acid at flow rate of 1 ml/min.

**Figure 37.** RP-HPLC profiles for the heterolysis of DTT(NO) and the homolysis of DTT(NO)$_2$. 

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Since our purpose was to study dithiols as models of vicinal thiol groups in proteins, we were particularly interested in the rates of intramolecular homo- and heterolysis. Those rates for the mono and dinitrosated derivatives of DTT, DTE, TA, and DMP, which all appear to be reasonable models for vicinal thiol groups in proteins, vary by more than three orders of magnitude due to differences in the distance between their respective thiol groups, their geometric and stereochemical relationships, their pKa values and their inherent reactivities. In all but the last case, proximity resulted in intramolecular rates two to three orders of magnitude greater than those observed in the case of simple mononitrosothiols. Half-lives for the intramolecular heterolysis of DTE(NO), DTT(NO) and TA(NO) ranged from 3 to 30 min and those for homolysis of the corresponding dinitroso derivatives ranged from 1 to 10 min. Changes in enzyme activities over similar periods of time following exposure to nitrovasodilators may be due to similarly rapid formation of disulfide bonds between vicinal thiol groups (shown in Figure 38). The environments of two thiol groups and their spatial relationship to each other would determine the rate of such a process in the case of a specific enzyme and might serve as a timing device allowing for transient changes in activity over a predetermined period of time.
Figure 38. A possible pathway for enzyme regulation through S-nitrosation of vicinal thiol groups.

(1) transnitrosation
(2) disulfide bond formation
(3) disulfide bond cleavage
(4) protein degradation
S-Nitrosation promoted disulfide formation may activate an enzyme or inactivate an enzyme. S-nitrosothiols are known to activate guanylate cyclase but to inactivate many other enzymes such as protein kinase C (Gopalakrishna, et al., 1993) and creatine kinase (Gross, et al., 1996). The active form of an individual enzyme in regard to the chemical form of their vicinal thiols, i.e., reduced, S-nitrosated, or oxidized, may vary. The vicinal thiols need not necessarily be located close to the active site of the enzyme. Structural changes triggered by their nitrosation/oxidation/reduction can be propagated to the active site by way of a conformational change. This type of enzyme regulation may be reversible, as shown in Figure 38, through regeneration of the active form, or irreversible due to rapid degradation of a specific form of the enzyme. Although the precise mechanism for a particular enzyme can not be proposed here, this study suggests a possible new pathway for enzyme regulation through S-nitrosation of vicinal thiols. The physiological effect of endogenous NO-donors or the pharmacological effects of exogenous NO-donor compounds might be mediated via this type of enzyme regulation.
REFERENCES


