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Biophysical studies of plastocyanin

Durell, Stewart Richard, Ph.D.
The Ohio State University, 1989

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Biophysical Studies of Plastocyanin

DISSERTATION

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

By
Stewart Richard Durell, B.S.

* * * * *

The Ohio State University
1989

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Elizabeth L. Gross, Ph.D., Advisor
Biophysics Program
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1989
DEDICATION

To my parents and teachers,
whose love and attention
will always be felt
ACKNOWLEDGEMENTS

I especially wish to thank Dr. Elizabeth L. Gross for the generous giving of herself and her knowledge. I would also like to thank Dr. Robert T Ross for also giving of his time and inspiration to me. I am also indebted to Steven A. Molnar, April S. Curtiss, George P. Anderson, and James E. Draheim for being such good friends and teachers.
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Fields of Study

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2) The use of computers to solve scientific problems.

3) Elucidating the structure and function of biomacromolecules.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>DEDICATION</td>
<td>11</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>III</td>
</tr>
<tr>
<td>VITA</td>
<td>iv</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>ix</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>xiii</td>
</tr>
<tr>
<td>ABBREVIATIONS</td>
<td>xiv</td>
</tr>
<tr>
<td>CHAPTER</td>
<td></td>
</tr>
<tr>
<td>I. INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>The Structure of PC</td>
<td>1</td>
</tr>
<tr>
<td>The Effect of Oxidation State and pH on the</td>
<td>8</td>
</tr>
<tr>
<td>Conformation and Activity of PC</td>
<td>18</td>
</tr>
<tr>
<td>Absorption and CD Spectroscopy of PC</td>
<td>20</td>
</tr>
<tr>
<td>Factor Analysis of the Near-UV Absorption of PC</td>
<td>22</td>
</tr>
<tr>
<td>Modeling of the Electrostatic Potential Field of PC</td>
<td>22</td>
</tr>
<tr>
<td>II. PROCEDURES AND METHODS</td>
<td>26</td>
</tr>
<tr>
<td>Absorption and CD Spectroscopy</td>
<td>26</td>
</tr>
<tr>
<td>Isolation of PC and Concentration Determination</td>
<td>26</td>
</tr>
<tr>
<td>Spectroscopic Measurements</td>
<td>27</td>
</tr>
<tr>
<td>Near and Far-UV Absorption</td>
<td>27</td>
</tr>
<tr>
<td>Near and Far-UV CD</td>
<td>30</td>
</tr>
</tbody>
</table>

- vi -
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>FIGURE</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. The 3-dimensional structure of spinach PC</td>
<td>3</td>
</tr>
<tr>
<td>2. The position of PC in the electron transport chain</td>
<td>4</td>
</tr>
<tr>
<td>3. The secondary structure of PC</td>
<td>6</td>
</tr>
<tr>
<td>4. The copper center in spinach PC</td>
<td>7</td>
</tr>
<tr>
<td>5. The oxidized and reduced copper centers at neutral pH</td>
<td>10</td>
</tr>
<tr>
<td>6. The reduced copper center at pH 7.8 and pH 3.8</td>
<td>11</td>
</tr>
<tr>
<td>7. Cut-away representation of PC</td>
<td>13</td>
</tr>
<tr>
<td>8. Direct view of the east face site of PC</td>
<td>14</td>
</tr>
<tr>
<td>9. The effect of pH on the electron transport activity of PC</td>
<td>16</td>
</tr>
<tr>
<td>10. The effect of pH on the oxidation of PC via the east face site</td>
<td>17</td>
</tr>
<tr>
<td>11. Typical file for nonlinear model fitting with SAS and WYLBUR</td>
<td>37</td>
</tr>
<tr>
<td>12. Schematics of the 2-way, 3-way, and 4-way data arrays</td>
<td>40</td>
</tr>
<tr>
<td>13. Near-UV absorption spectra of parsley PC</td>
<td>61</td>
</tr>
<tr>
<td>14. Near-UV absorption spectra of spinach PC</td>
<td>62</td>
</tr>
<tr>
<td>15. Near-UV absorption of phenylalanine and tyrosine amino acids</td>
<td>64</td>
</tr>
<tr>
<td>16. Reduced - oxidized difference spectra of parsley and spinach PC</td>
<td>68</td>
</tr>
<tr>
<td>17. Near-UV absorption spectra of Cu(I) metallothionein</td>
<td>70</td>
</tr>
<tr>
<td>18. pH 6.8 - pH 5.2 difference spectra of reduced PC</td>
<td>72</td>
</tr>
<tr>
<td></td>
<td>Description</td>
</tr>
<tr>
<td>---</td>
<td>-----------------------------------------------------------------------------</td>
</tr>
<tr>
<td>19</td>
<td>pH dependence of the absorption at 278 nm of reduced PC</td>
</tr>
<tr>
<td>20</td>
<td>The Hydrogen bonding of Tyr 83</td>
</tr>
<tr>
<td>21</td>
<td>Aromatic residue conformation in oxidized and reduced spinach PC</td>
</tr>
<tr>
<td>22</td>
<td>Aromatic residue conformation in reduced PC at pH 7.8 and pH 3.8</td>
</tr>
<tr>
<td>23</td>
<td>Far-UV absorption of model secondary structures of poly-L-lysine</td>
</tr>
<tr>
<td>24</td>
<td>Predicted far-UV absorption of the different chromophores in PC</td>
</tr>
<tr>
<td>25</td>
<td>Far-UV absorption of oxidized parsley PC</td>
</tr>
<tr>
<td>26</td>
<td>Far-UV absorption of oxidized parsley and spinach PC</td>
</tr>
<tr>
<td>27</td>
<td>Far-UV CD spectrum of oxidized spinach PC at pH 7.0</td>
</tr>
<tr>
<td>28</td>
<td>Far-UV CD spectra of PC and model protein secondary structure</td>
</tr>
<tr>
<td>29</td>
<td>Near-UV CD of oxidized and reduced parsley PC at pH 7.0 and 5.0</td>
</tr>
<tr>
<td>30</td>
<td>Near-UV CD of oxidized and reduced spinach PC at pH 7.0 and 5.0</td>
</tr>
<tr>
<td>31</td>
<td>pH dependence of the near-UV CD spectrum of reduced parsley PC</td>
</tr>
<tr>
<td>32</td>
<td>pH dependence of the CD of reduced parsley PC at 255 nm</td>
</tr>
<tr>
<td>33</td>
<td>Near-UV CD of Cu(I) metallothionein and reduced PC at neutral pH</td>
</tr>
<tr>
<td>34</td>
<td>Fluorescence of reduced spinach PC at three pH values</td>
</tr>
<tr>
<td>35</td>
<td>Effect of pH on the tyrosine fluorescence of reduced spinach PC</td>
</tr>
<tr>
<td>36</td>
<td>Effect of pH on the emission band shape of reduced spinach PC</td>
</tr>
<tr>
<td>37</td>
<td>RMS residual vs. number of components for the different models</td>
</tr>
<tr>
<td>38</td>
<td>Residual matrices for the bilinear models</td>
</tr>
</tbody>
</table>
39. Residual matrices for the trilinear models ........................... 138
40. Residual matrices for the quadrilinear models ....................... 139
41. First three SVD basis vectors of the bilinear data array . 143
42. 2 and 3 wavelength basis vector fits to the absorption targets .................................................. 144
43. Example of the indeterminacy of bilinear models .............. 146
44. Fixed bilinear model of spinach PC absorption ............. 150
45. 2-component trilinear model without non-negativity constraint .................................................. 152
46. 2-component trilinear model with non-negativity constraint .................................................. 153
47. 2-comp. quadrilinear model without non-negativity constraint .................................................. 154
48. 2-comp. quadrilinear model with non-negativity constraint .................................................. 155
49. 3-component trilinear model without non-negativity constraint .................................................. 157
50. 3-component trilinear model with non-negativity constraint .................................................. 158
51. 3-comp. quadrilinear model without non-negativity constraint .................................................. 159
52. 3-component quadrilinear model of spinach PC absorption 163
53. Effect of copper atom charge on the potential field of spinach PC ........................................ 175
54. East face sites of spinach and poplar PC ...................... 178
55. Three main sites of EDA chemical modification on PC 180
56. $\Delta E^0$ vs. inverse distance from the copper atom for EDA mods. ........................................ 184
57. Change of the potential field of spinach PC due to EDA mod. ........................................ 185
58. Conformation of Glu 68 in spinach PC .............................. 187

- xi -
59. Contour map of the potential field of oxidized spinach PC ........................................... 191
60. Parallel slices thru the potential field of oxidized spinach PC .................................... 192
61. Contour map of the potential field of reduced spinach PC ........................................... 193
62. Effect of ionic strength on the potential field of oxidized spinach PC ........................ 196
63. Effect of protein dielectric constant on the field of oxidized spinach PC ..................... 197
64. Effect of pH on the potential field of oxidized spinach PC .......................................... 199
65. Dipole approximation of the potential field of oxidized spinach PC ............................. 201
66. Bound waters in the crystal structure of oxidized poplar PC ...................................... 206
# LIST OF TABLES

<table>
<thead>
<tr>
<th>TABLE</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. pKa Values for the Absorption and Activity of PC</td>
<td>76</td>
</tr>
<tr>
<td>2. Effect of Oxidation State on the Aromatic Residue Orientations</td>
<td>84</td>
</tr>
<tr>
<td>3. Effect of pH on the Aromatic Residue Orientations in Reduced PC</td>
<td>86</td>
</tr>
<tr>
<td>4. Distances Between the Aromatic Residues</td>
<td>88</td>
</tr>
<tr>
<td>5. Predictions of the Fixed Bilinear Model</td>
<td>148</td>
</tr>
<tr>
<td>6. Predictions of the 3-Component Quadrilinear Model</td>
<td>162</td>
</tr>
<tr>
<td>7. Electrostatic Effects on the pKa of Nitro-Tyrosine</td>
<td>176</td>
</tr>
<tr>
<td>8. Electrostatic Effects on the Redox Potential of PC</td>
<td>182</td>
</tr>
<tr>
<td>9. Comparison of the Linear and Nonlinear Poisson-Boltzmann Eqs.</td>
<td>204</td>
</tr>
</tbody>
</table>
ABBREVIATIONS

CD ....................... Circular Dichroism
EDA ...................... Ethylenediamine
IMSL ........................ International Math and Statistic Libraries
IRCC ...................... Instruction and Research Computing Center
NMR ....................... Nuclear Magnetic Resonance
NTyr ....................... Nitro-tyrosine
PC ......................... Plastocyanin
UV  ......................... Ultra Violet
CHAPTER I
INTRODUCTION

This document describes physical-chemical studies of the protein Plastocyanin (PC). PC is an important component in the photosynthetic electron transport chain of plant chloroplasts, and in some species of cyanobacteria. It is important to understand the mechanisms of photosynthesis, because they are the means by which sunlight is converted to chemical energy, and by which animal life is sustained on this planet. In this regard, the impetus of these studies was to better understand the mechanisms by which PC functions in the photosynthetic electron transport chain. As an added benefit, the information gained about the function of PC also increases the general understanding of the relationship between structure and function in the reaction among proteins.

THE STRUCTURE OF PC

Figure 1 on page 3 displays the 3-dimensional structure of spinach PC. The role of PC in the transport chain is to shuttle an electron from Cytochrome f of the Cytochrome b_{6}/f complex to the P700 reaction center of the Photosystem I complex (Boulter et al., 1977; Haehnel et al., 1980). Figure 2 on page 4 illustrates the position of PC in the
photosynthetic electron transport chain. PC is a relatively small protein of 10.5 kiloDaltons, and is soluble in aqueous solution. PC is located in the lumen of the thylakoid membrane, where it can make contact with its reaction partners. Although PC interacts with intrinsic membrane complexes, it is not believed to be directly bound to the lipid phase of the membrane.

The electron transporting function of PC is carried out by a single bound copper atom (see Figure 1). The copper atom can exist in either the oxidized (Cu(II)) or reduced (Cu(I)) states. The copper-ligand coordination complex of PC is a typical Type I copper site (Freeman, 1981). These sites absorb intensely at ca. 600 nm in the oxidized state, and samples of them have a deep blue color. This is presumably the origin for the suffix "cyanin" in the name of PC. PC belongs to a family of "blue" copper proteins, which includes: Azurin, Stellacyanin, and possibly Cytochrome c oxidase in mitochondria (Freeman, 1981).

X-ray crystallography has been used to determine the 3-dimensional crystal structure of PC (Guss & Freeman, 1983; Guss et al., 1986). At the present time, poplar PC is the only species for which the crystal structure has been determined. The PC protein is mostly composed of strands of parallel and anti-parallel β-sheet, but there is also a single turn of α-helix. The overall conformation of PC can best be described as two β-sheets in a compressed sandwich configuration. Figure 3 on page 6 illustrates the sandwich structure. Recently, 2-dimensional nuclear magnetic resonance (NMR) techniques have been
Figure 1: The 3-dimensional structure of spinach PC
Figure 2: The position of PC in the electron transport chain
used to determine the structure of PC in aqueous solution (Wright, 1989). Although these studies are still evolving, the solution structure of PC is believed to be very similar to its crystal structure.

In the view of PC shown in Figure 1 on page 3, the copper atom is located at the top of the molecule. This end of the protein has been designated the "north pole" (Freeman, 1981). The amino acid ligands to the copper are His 37, Cys 84, His 37, and Met 92. Figure 4 on page 7 displays four space-filling representations of PC, which are the front, back, right, and top sides of the molecule as oriented in Figure 1 on page 3. These figures demonstrate that the copper-ligand center is buried slightly below the surface of the protein. The copper center has the appearance of being held by the finger-like β-loops of the protein. These figures also demonstrate where the copper center is exposed to the solvent. The His 87 ligand at the top of the protein has the greatest solvent exposure of the four ligands.
Figure 3: The secondary structure of PC
Figure 4: The copper center in spinach PC. The residues of the copper center are highlighted. a) Front; b) back; c) right; d) top side.
The Effect of Oxidation State and pH on the Conformation and Activity of PC

When light strikes the chloroplast, a proton gradient is established across the thylakoid membranes. This results in an acidic environment in the lumen of the thylakoids, with pH values ranging from pH 4.5 to pH 5.5 (Haehnel et al., 1981). Thus, it would be interesting to understand the effects that pH changes have on the conformations of oxidized and reduced PC, and how this may affect the electron transporting activity of PC with its reaction partners (Guss et al., 1986; Sykes, 1985).

X-ray crystallography has been used to determine the effects that the oxidation state of the copper center and the environmental pH have on the structure of PC (Guss et al., 1986). It was found that the protein portion of the molecule is not significantly affected by the changes in oxidation state or pH, (from pH 7.8 to pH 3.8). In contrast, the coordination geometry of the copper center was found to be significantly affected by both of these parameters. The oxidized copper center is in a pseudo-tetrahedral coordination. Reduction of the copper atom results in a further distortion of the tetrahedral geometry, in which the Cu(I)-His 87 and Cu(I)-His 37 ligand bond lengths are increased by 0.15 Å and 0.08 Å, respectively. Figure 5 on page 10 displays the superimposed structures of the oxidized and reduced copper centers at neutral pH. The lowering of the environmental pH does not affect the conformation of the oxidized copper center, but does affect the conformation of the reduced copper
center. Figure 6 on page 11 displays the superimposed structures of the reduced copper centers at pH 7.8 and pH 3.8. At low environmental pH, the copper atom has moved away from the His 87 ligand, and assumes a trigonal-planar coordination with the other three ligands. The distance between the copper atom and His 87 becomes 3.15 Å, which is large enough to allow for the protonation of the imidazole ring, and indicates that the Cu(I)-His 87 ligand bond has been broken (Guss et al., 1986). The cleavage of this ligand bond has also been observed by NMR spectroscopy (Markley et al., 1975; Kojiro & Markley, 1983). A second conformer exists at low pH where the His 87 imidazole ring rotates 180° about its stem and hydrogen bonds to a water molecule.

Based on the crystallographic refinements at intermediate pH values, Guss et al. (1986) concluded that the reduced copper center is in equilibrium between high and low pH conformations, and does not assume intermediate conformations.

There are two locations on the PC molecule which have been identified as active sites for the association and reaction with other proteins and inorganic reagents. These are at the north pole (described above), and at a second site on the right side of the protein called the east face (Sykes, 1985). In general, negatively charged reagents associate with PC at the north pole site, and positively charged reagents associate at the east face site. Figure 7 on page 13 and Figure 8 on page 14 display the north pole and east face sites. For reagents which bind at the north pole site, electron transfer with the copper atom is believed to occur through the exposed
Figure 5: The oxidized and reduced copper centers at neutral pH. The two structures are superimposed.
Figure 6: The reduced copper center at pH 7.8 and pH 3.8.
edge of the His 87 ligand (Handford et al., 1980). The amino acid residues surrounding the north pole are mostly non-polar, and form a hydrophobic patch which is conserved in the sequences of other plastocyanin species (Freeman, 1981). There is evidence that PC associates with photosystem I at the north pole site (Anderson et al., 1987). For reagents which bind at the east face site, electron transfer with the copper atom is believed to occur by a tunneling mechanism through the partially exposed Tyr 83 residue (Coleman et al., 1978). There are a series of acidic amino acid residues (42-45 & 59-61) which form a negatively charged patch at the east face site. This feature is conserved in the sequences of other plastocyanin species (Sykes, 1985). There is evidence that PC associates with cytochrome f at the east face site (Anderson et al., 1987).

Initial experiments indicated that the redox potential of the copper center increases as the environmental pH is lowered below pH 6.0 (Katoh, 1962). This effect was subsequently shown, by kinetic experiments using inorganic redox reagents, to be due to a decrease in the activity of reduced PC at low pH with no corresponding effect on oxidized PC (Segal & Sykes, 1978). Figure 9 on page 16 displays the effect of environmental pH on the rate of reduction of oxidized PC, and on the rate of oxidation of reduced PC (Sykes, 1985).

The rate of oxidation of reduced PC at the north pole site is governed by a single pKa value; whereas, the rate of oxidation at the east face site is governed by both the north pole pKa value and an
Figure 7: Cut-away representation of PC. The acidic residues of the east face, Tyr 83, and the copper center are indicated.
Figure 8: Direct view of the east face site of PC. This is the same orientation as the "right side" view in Figure 4 on page 7.
independent east face pKa value. Figure 10 on page 17 displays the
effect of pH on the rate of oxidation of PC via the east face site, and
the two titration curves fitted to the data. From NMR titration
studies of the protonation of the His 87 ligand, it was determined that
the pKa value for the change in conformation of the reduced copper
center is the same as the pKa value for the switch-off in activity of
the north pole site (Markley et al., 1975; Kojiri & Markley 1983;
Sykes, 1985). This lead to the interpretation that the breaking of the
Cu(II)-His 87 ligand bond at low pH, and the resultant change in copper
coordination, is responsible for the inactivation of the reduced copper
center for electron transport at either the north pole or east face
sites (Sinclair-Day et al., 1985). This also affirmed that electron
transport at the north pole site occurs through the partially solvent
exposed His 87 ligand.
Figure 9: The effect of pH on the electron transport activity of PC. The reduction of oxidized PC by ferrocyanide (▲), the rate of oxidation of reduced PC by ferricyanide (○). The data were obtained from Sykes (1985).
Figure 10: The effect of pH on the oxidation of PC via the east face site. The fitted titration curves of the north pole and east face sites are shown as dashed lines. The data were obtained from Sykes (1985).
ABSORPTION AND CD SPECTROSCOPY OF PC

As discussed above, the main purpose of the studies described in this document is to understand the mechanisms by which PC functions in the electron transport chain of chloroplasts. It is an accepted axiom in biology that structure dictates function. For this reason, it was desirable to determine how the change in oxidation state of the copper center and the different environmental pH values affect the structure of PC in solution. The X-ray crystallography studies described above have already provided a rough picture of the structure of the protein, and its responses to the changes in these parameters. However, the function of PC in vivo may be regulated by subtle conformational changes in the tertiary structure of the protein which are missed by the crystallographic analysis.

Absorption and circular dichroism (CD) spectroscopy in the ultraviolet (UV) region (190-320 nm) was chosen to study the structure of PC. These optical methods are particularly suited for monitoring the responses of the structure of PC in solution to changes in the oxidation state of the copper center and the environmental pH. They also have the advantage that the acquisition and analysis of the spectra can be achieved in a considerably shorter time and with less material than is required for other methods, such as NMR spectroscopy. The chromophores in PC which absorb in the UV region are the peptide bonds, the tyrosine and phenylalanine amino acid residues, and the copper center. The absorption and CD of the peptide bonds is a good monitor of the secondary structure of the protein; whereas, the
absorption and CD of the aromatic residues and the copper center is a good monitor of the tertiary structure of the protein. Since the copper center and Tyrosine 83 are located at the north pole and east face (see Figure 7 on page 13), the absorption and CD of these two chromophores provides a good means to monitor the localized conformations of these two important reaction sites.

The UV absorption and CD studies presented here are an extension of work started in Dr. Gross's Laboratory at the Ohio State University (Drahelm et al., 1985; Drahelm et al., 1986). These earlier studies examined the spectra of spinach, poplar and lettuce PC. These species were found to have qualitatively similar spectra, with similar variations due to the change in oxidation state of the copper center and environmental pH. However, due to the overlapping of the spectra of the chromophores, there was some difficulty in assigning the bands to specific chromophores in the protein. This limited the ability to determine structural information about PC. The studies presented here are expanded to include the absorption and CD of parsley PC, which has significantly different spectral features than the other three PC species previously studied. These differences in the spectra are shown to be valuable in assigning the bands in the spectra to specific chromophores in the protein. These studies also show how the oxidation state and pH dependencies of the absorption and CD spectra can be used to elucidate the band assignments. The comparison of the PC spectra with that of copper metallothioneins, from yeast and Neurospora (Weser et al., 1977; Beltramini & Lerch, 1983), is also used to assign the spectral bands.
FACTOR ANALYSIS OF THE NEAR-UV ABSORPTION OF PC

As described above, the overlapping of the spectra of the chromophores in PC limits the ability to derive structural information. In particular, it is desirable to isolate the absorption of the copper center and Tyrosine 83 from the superimposed absorption spectrum. To this end, this document describes the use of mathematical factor analysis to separate the independent components in the absorption spectrum of PC. The factor analysis procedures were conducted using the standard bilinear model, as well as using the recently developed trilinear and quadrilinear models. These studies also demonstrate the relative advantages of using the different models. The methods used here were primarily developed by Dr. Lee and Dr. Ross, in Dr. Ross' laboratory at the Ohio State University (Marchiarullo & Ross, 1985; Lee, 1988).

Bilinear factor analysis is a traditional method for separating independent components in a data set. Many examples of the bilinear factor analysis of spectroscopic measurements can be found in the chemistry literature (Malinowski & Howery, 1980; Ramos et al., 1986), but only a few pertaining to the absorption of proteins (Shrager & Hendler, 1982; Hofricter et al. 1983, 1985; Halaka et al. 1985). However, the bilinear model has the disadvantage that it does not uniquely define the responses of the components to the independent variables. This well-known problem is referred to as the "fundamental indeterminacy" of bilinear models, and as the "rotation problem" of bilinear factor analysis (Kruskal, 1983). In the applications to
protein absorption cited above, the components are determined by assuming a particular form for the response of the components to one of the independent variables. For example: If it is known that the protein contains a titratable group, then it is assumed that the response of this group to the change in pH is described by the standard Henderson-Hasselbalch titration model. Thus, the ability to identify the components in the data set by bilinear factor analysis is dependent on the particular application, and by how much is already known about the components. The bilinear factor analysis of the absorption spectra of PC is presented here partially to demonstrate the uncertainty that arises in determining the components.

The use of trilinear and quadrilinear models in factor analysis is still new (Kroonenberg, 1983a,b), especially for the analysis of spectroscopic data. There are a few examples of trilinear factor analysis of fluorescence measurements (Appellof & Davidson, 1981; Callis, 1984; Lee, 1988). To our knowledge, this is the first published application of trilinear and quadrilinear factor analysis of absorption spectra. The advantage of using the trilinear and quadrilinear models is that they uniquely define the responses of the components to the independent variables. These models are said to have the "intrinsic axis property" (Kruskal, 1983). Therefore, trilinear and quadrilinear factor analysis can be used to determine the independent components in the data without the use of outside information about them. Trilinear and quadrilinear factor analysis are applicable to any system where the response variable can be measured as
a function of three or four non-interacting independent variables.

Since this approach is new, the derivation of the different models as they apply to absorption measurements is included, as well as a brief description of the mathematical algorithm used to calculate the components.

**MODELING OF THE ELECTROSTATIC POTENTIAL FIELD OF PC**

It is widely recognized that electrostatic forces influence the rate of reaction between electrically charged, biologically important molecules in solution (Warshel & Russell, 1984; Matthew, 1985; Honig et al., 1986). The electrostatic effects can be broadly categorized into (a) long-range interactions influencing the rate of association, (b) short-range interactions influencing the relative orientations of the reactants, and (c) direct influences upon the activities of the molecules (e.g., the change in redox potential of a metalloprotein). Studies previously carried out in Dr. Gross' laboratory, at the Ohio State University, have specifically measured the effects of electrostatic interactions on the reaction rate of PC, on the the redox potential of the copper center, and on the pKa value of nitro-Tyrosine 83 (Anderson et al., 1987; Anderson et al, 1985).

To better understand the electrostatic interactions that affect activity, it is important to be able to determine the 3-dimensional electrostatic potential field inside and surrounding the protein. However, the calculation of the electrostatic potential field is
complicated by the fact that proteins are heterogeneous, anisotropic material, and are generally less electrically polarizable than the surrounding solvent. Historically, a simplified view of the system was pursued, in which the protein and solvent are treated as homogeneous, isotropic media, possessing different macroscopic dielectric constants (Matthew, 1985; Gilson et al., 1985). The protein was modeled as a low dielectric spherical cavity, suspended in the high dielectric solvent. In this case, the electrostatic potential field due to the charge distribution of the protein could be calculated by analytically solving Poisson's equation (Tandford & Kirkwood, 1957).

Recently, this approach has been expanded to account for the irregular, 3-dimensional shape of the protein (Warwicker & Watson, 1982; Rogers & Sternberg, 1984; Zauher & Morgan, 1985; Klapper et al., 1986). This provides for a more accurate description of the protein/solvent dielectric boundary of the system. One important consequence of this is that the charged atoms in the protein are treated with the proper degree of solvent exposure, pursuant to their locations relative to the protein surface. Due to the irregularly shaped dielectric boundary, the electrostatic potential field is calculated by numerically solving the Poisson-Boltzmann equation. It has not been until recently, with the accessibility of high-speed computers, that a computationally intensive problem such as this could be accomplished in a reasonable amount of time. The boltzmann term in the equation was added to model the distribution of electrolyte around the solvent exposed charged atoms.
A number of test cases have shown that this approach can provide accurate predictions of electrostatic effects which have been observed in a series of proteins (Klapper et al., 1986; Rogers et al., 1985; Sharp et al., 1987a; Sharp et al., 1987b; Sternberg et al., 1987; Gilson & Honig, 1988; Bashford et al., 1988). Studies reported in this document extend the test of this approach to the prediction of electrostatic effects which have been observed in PC. In particular, this approach is used to calculate the effect of the change in oxidation state of the copper center on the pKa value of nitro-Tyrosine 83, and the effects of ethylenediamine chemical modification of acidic amino acid residues on the redox potential of the copper center. The electrostatic potential field of PC is calculated with the DelPhi-v.2 computer program, which was obtained from the laboratory of Dr. B. Honig at Columbia University (Klapper et al., 1986).

Studies are also presented in this document which examine the shape of the electrostatic potential field surrounding PC, and the effects on the field of the values chosen for the protein dielectric constant and the solvent ionic strength parameters. It is well known that the shape of the electrostatic potential field can influence where other charged reagents contact the protein surface (Koppenol & Margoliash, 1982; Allison et al., 1985; Sharp et al., 1987a). It is thus very important to know the shape of the electrostatic potential field of PC to determine the locations of the active site domains. The results of this analysis can be correlated with other studies performed in Dr. Gross' laboratory, in which reaction rate kinetic measurements of
modified charge forms of PC were used to determine the active site locations (Anderson et al., 1987).

Recently, Rush et al. (1988) reported on studies to determine the locations of the active sites on PC for the reactions with hexacyanoferrate (III), tris(1,10-phenanthroline)cobalt (III), and ferrocytochrome c. To this end, they used a simplified dipole moment approximation to determine the shape of the electrostatic potential field surrounding PC. This work was an application of the approach developed by Koppenol & Margoliash (1982) to determine the locations of the active domains on cytochrome c. The results obtained by Rush et al. were somewhat in variance to those obtained in Dr. Gross' laboratory (Anderson et al., 1987), and by other researchers (Cookson et al., 1980; Handford et al., 1980; King et al., 1985). However, the method used by Rush et al. to calculate the electrostatic potential field of PC did not take into account the lower dielectric polarizability of the protein, or the ionic strength of the solvent. The final study in this section examines the dipole electrostatic potential field used by Rush et al., and compares it with the field calculated by the method presented in this document. This is done to try to explain the discrepancies in the determination of the active domain locations on PC.
CHAPTER II
PROCEDURES AND METHODS

ABSORPTION AND CD SPECTROSCOPY

Isolation of PC and Concentration Determination

Plastocyanin was isolated from commercially obtained parsley leaves by the method of Graziani et al. (1974), except for the following changes. Instead of a Tris concentration gradient, a solution of 50 mM Tris and 0.2 M NaCl was used to elute PC from the DEAE-cellulose column. The final purification of PC was accomplished by means of a Pharmacia FPLC, using a mono Q HR 5/5 anion exchange column. After this step, the 278/597 nm absorption purity ratio was ca. 2 for oxidized parsley PC at pH 7.0. This ratio was higher for parsley PC than for spinach and poplar PC (ca. 1.2), due to the extra tyrosine residue at position 62. PC concentrations were determined by measuring the magnitude of the visible absorption band at 597 nm, using an extinction coefficient of 4.9 mM⁻¹cm⁻¹, after complete oxidation with excess ferricyanide.
Spectroscopic Measurements

Plastocyanin samples were either oxidized or reduced by the addition of excess ferricyanide or sodium ascorbate, respectively. These redox reagents were removed prior to spectroscopic measurements by gel filtration, using a Biogel P-10 column. This step also served to perform the buffer exchanges. The percent oxidation of the PC samples was determined by measuring the absorption at 597 nm before and after the addition of excess ferricyanide. During the course of the experiments, the percent oxidation of the PC samples did not vary significantly, and was typically within 10% of complete oxidation or reduction. The spectra were mathematically corrected to represent 100% oxidized and reduced samples of PC.

Near and Far-UV Absorption

An Aminco DW-2a was used to measure near-UV absorption spectra (240-350 nm), and a Cary 118-C was used to measure far-UV absorption spectra (190-240 nm). Both instruments are dual-beam spectrophotometers. Standard 1 cm quartz cuvettes were used for the near-UV measurements, and 1 mm quartz cuvettes were used for the far-UV measurements. The shorter pathlength was used for the far-UV measurements to allow for greater light penetration through the sample, and thus a larger signal-to-noise ratio.

Samples of PC were prepared in two different ways for the absorption measurements. The first method was to suspend the PC, using the gel filtration method described above, into 10 mM concentrations of the pH
buffer. HEPES buffer was used for measurements at pH 7.0 and Citrate buffer was used for measurements at pH 5.0. The PC concentration was typically 20 µM. The absolute absorption spectrum was then measured by placing the PC solution and the plain buffer solution in the sample and reference cuvettes, respectively. A baseline for the spectrum was then measured by substituting more of the plain buffer solution for the PC solution in the sample cuvette. The second method was to first prepare a concentrated solution of PC (typically 80 µM) in 10 mM Tris-succinate buffer at pH 7.0. A concentrated solution of the Tris-succinate buffer, adjusted to the desired pH and 100 mM ionic strength, was placed in both the sample and reference cuvettes. The Tris-succinate buffer was good for pH ranges between ca. pH 4.0 and pH 8.5. Aliquots of the PC solution were then added to the sample cuvette. This step affected the change in environmental pH of PC. The final concentration of PC and the pH were measured after the experiment. The second method is less time consuming than the first, because it does not require a separate gel filtration step to prepare each unique sample of PC, for the different oxidation states and environmental pH values.

Reduced minus oxidized difference absorption spectra of PC were measured directly by first placing portions of oxidized PC, from the same stock solution, into both the sample and reference cuvettes. This assured that both cuvettes had identical concentrations of PC. A baseline of the spectrum was then obtained. A few crystals of sodium-borohydrate were then added to the sample cuvette to reduce the
PC in the solution. However, the sodium-borohydrate also causes hydrogen gas bubbles to form in the solution, and care must be taken to assure that the bubbles have dissipated before making an absorption measurement. Another problem is that the sodium-borohydrate acts like a base, and can significantly alter the pH of the solution. For this reason, a concentrated buffer (typically 200 mM) was used to inhibit the shift in pH. However, the resultant high ionic strength causes the PC molecules to aggregate together at low pH values (ca. pH 5.0), which is detectable by the cloudy appearance of the solution. Once these problems were overcome, a reduced minus oxidized difference absorption spectrum was obtained. A concentrated PC solution (ca. 80 μM) was used to assure that the differences in the spectra of reduced and oxidized PC were measurable.

The pH difference absorption spectra of PC were directly measured in a similar manner to that described above. Identical solutions of either oxidized or reduced PC, in Tris-succinate buffer at pH 7.0, were placed in both the sample and reference cuvettes, and a baseline of the spectrum was obtained. The pH of the sample solution was then reduced by the addition of microliter amounts of 1 N hydrochloric acid, and then was directly measured with a pH electrode. The pH difference spectrum was then measured. This method could also be used to obtain a titration of the absorption spectrum. Since the differences in the spectrum of PC due to changes in pH are significantly smaller than those due to the change in oxidation state, it is especially important to use a concentrated PC solution.
Near and Far-UV CD

Both near-UV and far-UV circular dichroism (CD) spectra were measured with a Jasco 500-A spectropolarimeter. A poor signal-to-noise ratio was typically a problem with the CD measurements. The main factor which influences the signal-to-noise ratio is the intensity of light that is transmitted by the sample and onto the photomultiplier tube. Since the system is designed to maintain a constant current through the tube, reduced light intensities result in higher photomultiplier voltages and smaller signal-to-noise ratios. As a rule of thumb, accurate data can be obtained using a photomultiplier voltage up to 600 Volts.

The concentration of PC must be adjusted to allow for sufficient light penetration, but still maintain a measurable signal. For example: a dilute concentration of PC allows for more light to pass through the sample, but results in a decreased signal strength. The noise in the spectrum can also be reduced by increasing the time constant of the response. However, this requires that the wavelength scan-rate also be reduced, and makes for very long experiments (ca. 40 minutes per spectrum). By experience, certain parameters have been found to be suitable for making CD measurements of PC. In the near-UV region, a concentration of 50 μM PC should be used in a 1 cm cuvette. In the far-UV region, a concentration of 5 μM PC should be used in a 1 mm cuvette. One should not be tempted to use the 0.1 mm cuvette for far-UV measurements, because the problems associated with filling and emptying it out-weigh the benefit of increased light penetration.
These concentrations of PC allow for the use of either the 0.5 or 1.0 mdeg/cm sensitivity scale. The use of a more sensitive scale tends not to be profitable.

The CD measurements were made using PC samples prepared in a similar manner to those for the absorption measurements. The spectral range was set to include at least one region devoid of CD signal. This was done to allow for the alignment of the CD spectrum with its baseline. The baseline of the spectrum was measured using plain buffer solution. The baseline was subtracted from the sample spectrum to negate contributions from the buffer, and to center a rotated spectrum. Since the Jasco is a single-beam instrument, the recorded spectrum may become disoriented, or rotated, from the centerline of the chart paper. The titration experiments were conducted by adding microliter amounts of 1 N hydrochloric acid or sodium hydroxide to adjust the pH of the sample.

Near-UV Fluorescence

Fluorescence measurements were made with a SLM SPF 500c scanning spectrofluorometer. Data were collected in the ratio mode. PC concentrations of up to 15 μM were used. The fluorescence signal was determined to be linear up to a PC concentration of ca. 20 μM. The PC samples were prepared the same way as for the absorption measurements described above.
Materials
DEAE-cellulose and Biogel P-10 were obtained from Biorad laboratories. HEPES and Tris were obtained from Sigma Chemical Co. All other chemicals were of reagent grade.

DATA FITTING

Titration Models
The standard titration model (i.e., the Henderson-Hasselbalch equation) was used as a basis to derive models for the effect of environmental pH on the absorption, CD and fluorescence of PC. The full derivation of the models are shown here for clarity. Equation (1) describes the dissociation reaction of a single proton from PC,

\[ \text{PCH} \rightarrow \text{H}^+ + \text{PC} \]  

(1)
The equilibrium constant of this reaction is defined in Equation (2).

\[ K_a = \frac{[\text{H}^+][\text{PC}]}{[\text{PCH}]} \]  

(2)
The pKa and pH parameters are formed by taking the base 10 logarithm of both sides of (2).

\[ -\log(K_a) = \log([\text{H}^+]) - \log(\frac{[\text{PC}]}{[\text{PCH}]})) \]  

(3)

\[ pK_a = \text{pH} - \log(\frac{[\text{PC}]}{[\text{PCH}]})) \]  

(3)

Using simple algebraic manipulations and the conversion to the natural logarithm, (3) becomes.
where \( \ln(10) \) is the natural logarithm of 10. Equation (5), which is the conservation of mass law, is used to relate the concentrations of \( PC^- \) and \( PCH \).

\[
[PCH]_{\text{total}} = [PCH] + [PC]
\]

\[
[PCH] = [PC]_r - [PC]
\]

The expression for \([PCH]\) in (5) is substituted into the left-hand side of (4).

\[
\frac{[PC]_r - [PC]}{[PC]} = e^{(pK_a - pK_b)\ln(10)}
\]

\[
\frac{[PC]_r}{[PC]} - 1 = e^{(pK_a - pK_b)\ln(10)}
\]

\[
\frac{[PC]}{[PC]_r} = \frac{1}{1 + e^{(pK_a - pK_b)\ln(10)}}
\]

The next steps are to relate the fraction of dissociated \( PC \) (i.e., the left-hand side of (6)) to the spectroscopic signal. The equations are derived here for the CD of \( PC \), measured in the terms of the ellipticity. The derivations for the absorption and fluorescence intensities are analogous. As expressed in Equation (7) on page 34, the observed ellipticity is equal to the sum of ellipticity due to the protonated and unprotonated forms of \( PC \) in the sample.

\[
\theta = [\theta_p][PCH] + [\theta_u][PC]
\]
In (7), \([\theta_i]\) and \([\theta_h]\) are the molar ellipticity constants of the low and high pH forms of PC (i.e., the protonated and unprotonated forms). The expression for \([P_{CH}]\) in (5) on page 33 is substituted into (7) on page 33, and an expression for \([PC]\) is derived.

\[
\theta = [\theta_i]([PC]_r - [PC]) + [\theta_h][PC]
\]

\[
\theta = [\theta_i][PC]_r + ([\theta_h] - [\theta_i])[PC]
\]

\[\text{(8)}\]

\[
[PC] = \frac{\theta - [\theta_i][PC]_r}{[\theta_h] - [\theta_i]}
\]

Finally, (8) is combined with (6) on page 33 to form the model for the effect of pH on the CD of PC.

\[
\frac{\theta - [\theta_i][PC]_r}{([\theta_h] - [\theta_i])[PC]_r} = \frac{1}{1 + e^{(pKa-pH)\ln(10)}}
\]

\[\text{(9)}\]

\[
\theta = [PC]_r\left(\frac{[\theta_h] - [\theta_i]}{1 + e^{(pKa-pH)\ln(10)} + [\theta_i]}\right)
\]

**Multiple Proton Model**

In some instances, however, the transition between the high and low pH endpoints of the spectroscopic titration was sharper than that of the single proton dissociation model. In these cases, a modified model was used, which accounts for a greater number of protons. The overall dissociation reaction is described in Equation (10) on page 35,

\[
P_{CH_n} \rightarrow PC + nH^+
\]

\[\text{(10)}\]
where \( n \) is the number of protons involved. In the extreme case that the binding sites are infinitely cooperative, all of the protons would bind or dissociate at the same time. Infinite cooperativity is also defined as when the Hill constant equals \( n \). The apparent equilibrium constant for this "all-or-none" reaction is defined in Equation (11) (Cantor and Schimmel, 1980).

\[
K_a^n = \frac{[PC][H^+]^n}{[PCH_n]} \quad (11)
\]

Using the same derivation as above, (12) is the model for the effect of pH on the CD of PC due to infinitely cooperative proton dissociation. The effect on the titration curve of having \( n \) values greater than 1 is to sharpen the transition without shifting the pKa value.

\[
\theta = [PC]_T \left( \frac{[\theta_1] - [\theta_0]}{1 + e^{n(pKa-pH)ln(10)} + [\theta_0]} \right) \quad (12)
\]

Data Fitting Procedure

The data of the titration experiments are a spectroscopic signal as a function of environmental pH. The desired parameters, which characterize the titration, are the pKa value, the \( n \) value, and the molar signal constants for the protein in the low and high pH endpoint forms. In the models of equations (9) on page 34 and (12) the pKa and \( n \) values are nonlinear parameters, and the molar signal constants are linear parameters.
The titration models were fit to the data with a commercial nonlinear regression computer program. This program was the NLIN procedure in the Statistical Analysis System (SAS) software package. Use of the SAS software was through the Instruction and Research Computing Center (IRCC), at Ohio State University. The software was installed on the IBM 3081-D mainframe computer, and was accessed through the WYLBUR batch processing environment.

Figure 11 on page 37 shows a printout of a typical command stream file for doing the nonlinear fitting procedure. The first three lines, that start with "//", are job control language (JCL) statements which tell the operating system to initiate the use of the SAS software. The next three lines define the data set and the variables, which are the spectroscopic signal and pH. Note that each SAS statement must end in a semicolon. The following lines contain the data values in the X vs. Y format. The PROC statement calls the NLIN procedure, and sets some options. The remaining lines define the desired regression. The PARAMETER statement provides initial values for some of the parameters. The MODEL statement defines the nonlinear model. It is necessary to provide the program with the first partial derivatives of the model with respect to each of the parameters to be fitted. These are in the Der. statements. For further information on using this procedure the SAS User's guides should be consulted. These manuals can be found at IRCC.
```
// JOB,
// REGION=2048K
// EXEC SAS
//SYSIN DD *
DATA=ONE;
INPUT CD PH;
CARDS;
-5  5.0
-4  5.2
...

PROC NLIN METHOD=MARQUARDT;
PARAMETERS EL=-5 EH=8 PKA=6.0;
F=LOG(10);
ED=EXP(F*(PH-PKA));
D=1+ED;
MODEL CD=50*((EL-EH)/D)+EH);
DER.EL=50*(1/D);
DER.EH=50*(1/(1/D));
DER.PKA=50*(((EL-EH)*((1/D)*2)*ED*F);
OUTPUT PREDICTED=PY RESIDUAL=RY;
PROC PLOT;
   PLOT CD*PH='D' PY*PH='P' /OVERLAY;
//
```

Figure 11: Typical file for nonlinear model fitting with SAS and WYLBUR.
Typical output of the regression procedure includes an iteration history, the fitted values, some basic statistics, and a plot of the original and fitted curves.

**FACTOR ANALYSIS**

The data used for the factor analysis experiments were compiled from near-UV absorption spectra (250-320 nm) of four different species of PC: spinach, poplar, lettuce, and parsley PC. For each species of PC, the spectra of four different sample preparations were used. These were the spectra of 100% oxidized PC at pH 5.0 and at pH 7.0, and 100% reduced PC at pH 5.0 and at pH 7.0. Thus, the data were the absorption of PC as a function of four independent variables: the absorption wavelength, the plant species, the oxidation state of the copper center, and the pH of the solution. The data values were normalized to be expressed as the millimolar extinction coefficients (mM⁻¹cm⁻¹) of PC.

The absorption spectra of parsley PC used for the data set were the same as is presented in Chapter III. Whereas, the absorption spectra of poplar, spinach, and lettuce PC were obtained from Draheim et al. (1986). Although the spectra were obtained from two different sources, they are believed to be of equal accuracy. This is because both sets of spectra were obtained in this laboratory, using similar methods. Of special significance, is that all of the PC samples were purified by FPLC chromatography (see Section I) prior to the absorption measurements. Typically, the sample solution consisted of a 20-50 μM
concentration of a single species of PC, and a 10 mM concentration of the pH buffer. The spectra are believed to be free of artifacts caused by protein aggregation, because Beers law was found to be obeyed up to a PC concentration of 120 μM. The maximum error in the data was determined to be 0.3 mM⁻¹cm⁻¹, by analyzing the variation in absorption of independently prepared PC samples (see Draheim et al., 1986). The spectrum of Neurospora metallothionein was taken from Beltramini & Lerch (1986).

For bilinear, trilinear, and quadrilinear factor analysis the variables were combined to express the absorption data as 2-way, 3-way, and 4-way arrays. Figure 12 on page 40 provides a graphical representation of these arrays. A 2-way array corresponds to a rectangle, with two orthogonal coordinates. A 3-way array corresponds to a rectangular box, with three orthogonal coordinates. A 4-way array corresponds to a 4-dimensional analog of a rectangular box, with four orthogonal coordinates. A 4-dimensional cube is known as a tesseract.

For bilinear analysis, the wavelength variable was assigned to one coordinate of the 2-way array, and the combination of the other three variables assigned to the other coordinate. This second coordinate has 16 values, which represent all the possible combinations of one of the four plant species of PC in one of the two oxidation states and at one of the two environmental pH values. Since this combined variable only refers to chemical aspects of PC it is referred to as the "chemical-condition" variable. The values of the chemical-condition...
Figure 12: Schematics of the 2-way, 3-way, and 4-way data arrays.
variable are also the identities of the 16 spectra compiled to form the data, as indicated above.

For trilinear analysis, the wavelength variable, the plant species variable, and the combination of the oxidation state and pH variables were assigned to the three coordinates of the 3-way data array. The combined oxidation state and pH coordinate has four values, which represent all the possible combinations of PC in one of the two oxidation states and at one of the two environmental pH values. For quadrilinear analysis, the wavelength, plant species, oxidation state, and pH variables were each assigned to a separate coordinate of the 4-way data array.

All procedures were coded in FORTRAN 77. The bilinear analysis was executed by the programs FA1, FA2 and FA3. These were modified versions of programs originally obtained from Dr. R. T. Ross (presently at the Ohio State University). IMSL (International Mathematical and Statistical Libraries; Houston, TX; USA) subroutines were used for the singular value decomposition (SVD) and other matrix algebra operations. The programs were run on an IBM 3081-D mainframe computer, belonging to IRCC at the Ohio State University. The trilinear and quadrilinear analyses were executed by the programs PARAFAC3 and PARAFAC4, which were developed by Dr. C. -H. Lee (1988) while at the Ohio State University. These programs were run on a CRAY X-MP/24 supercomputer at the Ohio Supercomputer Center.
Bilinear Analysis

The procedure for bilinear analysis has two main parts: derivation of a basis vector set of the absorption data, and transformation of the basis set to find the linear combinations which represent the components. The first part is accomplished by the closed-form SVD operation (Marchiarullo & Ross, 1985; Golub & Reinsch, 1970). This operation factors the 2-way data array, \( A_{2\text{-way}} \) (I by J), into the product of three matrices, such that

\[
A_{2\text{-way}} = U S V
\]  

(13)

where \( U \) is an I by I matrix of orthonormal column vectors, \( S \) is an I by J diagonal matrix of positive singular values, and \( V \) is a J by J matrix of orthonormal row vectors. The row and column labels of \( A \) are assigned as the absorption wavelengths and chemical conditions, respectively. The column vectors of \( U \) form a basis for the column vectors of \( A \) (which are the PC absorption spectra), and the row vectors of \( V \) form a basis for the row vectors of \( A \) (which are the dependence of the PC absorption on the chemical condition). The SVD decomposition is unique, except for sign convention and permutation of the basis vectors. The column and row vectors of \( U \) and \( V \) are the same as the eigenvectors of the symmetric matrices \( A A^t \) and \( A^t A \) (Kruskal, 1983), which are described in Principal Component Analysis (Cochran & Horne, 1977).

The number of non-zero singular values obtained by the SVD operation equals the rank of the data array. If the data were completely free of
error, then this would also be the number of independent components in the data. However, measurements generally contain a degree of error and all of the singular values are non-zero. This fact complicates the determination of the number of real components. Several methods have been proposed to determine the number of components (Warner et al., 1977; Malinowski & Howery, 1980; Shrager & Hendler, 1982; Lin & Liu, 1978). These methods exploit the property of the SVD operation to partially separate the non-random variation in the data (which generally represents the real spectral information) from the random variation in the data (which generally represents the noise). When the basis vector pairs (i.e., column of U and associated row of V) are sorted into descending order of the value of their associated singular value, each successive vector pair has a smaller signal-to-noise ratio. The information required to reproduce the real absorption components is generally contained in a subset of the leading basis vector pairs, and the remaining vectors pairs represent only noise.

The method used to determine the number of spectral components is based on the analysis of the quality of data reproduction using bilinear models with an increasing number of components. The minimum number of components needed to satisfactorily model the original data is taken to be the number of real absorption components. The set of fitted value arrays that are formed with the bilinear model using an increasing number of components is defined by (14) on page 44.

\[
(A(F)) = U(F) S(F) V(F); \quad F = 1 \to J
\]  

(14)
where the $U(F)$, $S(F)$, and $V(F)$ matrices contain subsets of the initial $F$ basis vectors and singular values of $U$, $S$, and $V$. The quality of data reproduction is judged by examining the residuals between the original and fitted values. The criteria for a satisfactory model are that the residuals appear random and that the residuals are smaller than the experimental error.

The set of residuals for each fitted values array are compared by calculating the root-mean-square (RMS) residual of each set. The RMS residual is the same as the standard deviation between the original data and fitted values, and is calculated using (15).

$$\text{RMS}(F) = \frac{\sum_{i=1}^{I} \sum_{j=1}^{J} (a_{ij} - a_{ij})^2}{d_f(F)_{2\text{-way}}}$$

(15)

and

$$d_f(F)_{2\text{-way}} = (I - F)(J - F)$$

where $d_f(F)_{2\text{-way}}$ is the number of degrees of freedom for the bilinear model. Use of the RMS statistic is appropriate when the error in the data is normally distributed, of uniform variance, and uncorrelated. While this may not be true, these methods are commonly used in non-linear least squares fitting procedures (Bates & Watts, 1988).

Since both the real absorption component vectors and the SVD basis vectors are basis sets of the absorption data, the real absorption component vectors can be formed by linear combinations of the SVD basis vectors. This procedure is referred to as a basis set transformation.
\[
A_{2-\text{way}} = (U(F) S(F) T) (T^{-1} V(F)) = \mathbf{E} \mathbf{A}^t
\]

In (16) \( F \) is the number of components, \( \mathbf{E} \) and \( \mathbf{A} \) contain the predicted wavelength and chemical-condition-dependent parameter vectors of the components. However, due to the "fundamental indeterminacy" of bilinear models (see Chapter VI), the correct transformation matrix, \( T \), can not be determined without including additional information about the real components. The criteria and constraints used to select the correct transformation matrix are dependent on the type of data being studied, and how much is already known about the components.

One constraint, which was used in all cases, was that the predicted extinction coefficients and concentrations must be non-negative. This constraint limits the choice of possible transformation matrices, since some transformations would produce negative components. A second constraint, which was also used in all cases, was that the column vectors of the transformation matrix have a constant euclidean length. This normalization constraint fixes a parameter in each column vector of \( T \).

In some instances, one or more entire column vectors of \( T \) were fixed by requiring the spectrum of a component to be as close as possible to the "target" spectrum of a known, or suspected chromophore. For more about "target testing" in spectroscopic applications of factor analysis, see Malinowski & Howery (1980). The near-UV absorption spectra of aqueous tyrosine monomer and Neurospora metallothionein were used as the absorption targets. The fixed component spectrum was then
the least squares fit of the SVD wavelength basis vectors to the target spectrum. The least squares fitting was done with either IMSL subroutines or with Equation (17), which is a familiar formulation in linear algebra.

\[ T_f = (U(F)^t U(F))^{-1} U(F)^t Y \]

and

\[ E_f = U(F) T_f \]

In (17), \( T_f \) is the fixed column of the transformation matrix, \( Y \) is a column vector of length \( I \) which contains the target spectrum, and \( E_f \) is the fixed spectrum of the \( f^{th} \) component.

The normalization and target constraints fix \((f-1)(f-n)\) elements of the \( n^2 \) elements of \( T \), where \( n \) is the number of targets used. A non-linear function minimizing routine was then used to determine values for the remaining elements of \( T \), which comply with the non-negativity constraint. This routine was developed by Dr. R. T. Ross, based on the work of Nelder & Mead (1964) and Box (1965). The minimizing routine minimized a penalty assigned to trial transformation matrices based on the negative magnitude of the predicted components.

When there were only one or two free elements of \( T \), a grid search routine was substituted for the minimization routine. The penalty was evaluated for each node of a finely spaced grid of possible element values. In this way, information about the range of solutions for the
components was also determined. When there were more than two free elements in \( T \), the range of solutions were determined by the grid routine in a localized area around the solution determined by the minimizing routine.

**Trilinear and Quadrilinear Analyses**

The trilinear and quadrilinear models are shown in Equation (18). See Chapter VI for the derivation of these models.

\[
a_{yk} = \sum_{F=1}^{P} e_{y} x_{y} y_{F}
\]

\[
a_{yul} = \sum_{F=1}^{P} e_{y} x_{y} y_{F} z_{u}
\]

(18)

In these equations, \( a \) represents the absorption of PC as a function of the independent variables; and \( b, x, y, \) and \( z \), represent the responses of the independent spectral components to a single independent variable. Unlike the bilinear model, the component vectors in the trilinear and quadrilinear models are uniquely defined (see Chapter VI). Therefore, the component vectors in these models could be determined simply by finding the least-squares fits to the data. Thus, the absorption components are treated as parameters to be fitted. This procedure is expressed mathematically by Equation (19) on page 48, which is easily derived from (18).
The hat character indicates the fitted parameters. (19) was solved by first taking the partial derivative with respect to each of the spectral component parameters (i.e., all of the $z_{1f}, x_{jf}, y_{kf}$ and $z_{1f}$). This resulted in a set of non-linear equations, which were solved by an iterative minimization procedure described by Lee (1988). For the cases where negative parameters resulted, the equations were solved using the non-negativity constraint. Finally, the predicted components were obtained by multiplying the fitted parameters according to the models in (18) on page 47. Each term in the summation of these models corresponds to a different independent component. Due to the normalization of the data described above, the absorption of the components were in the units of the millimolar extinction coefficient ($\text{mM}^{-1}\text{cm}^{-1}$).

The quality of data reproduction of the trilinear and quadrilinear models was examined in the same manner as for the bilinear model. The trilinear and quadrilinear models with increasing number of components were determined by solving (19) with increasing values of $F$. The fitted value arrays were determined by multiplying the sets of fitted...
component parameter values according to the models of (18) on page 47, and summing the independent component vectors. The RMS residuals for each fitted values array was determined analogously to (15) on page 44, with the number of degrees of freedom for the different models determined by (20)

$$\text{df}(F)_{3{\text{way}}} = IJK - (I + J + K - 2)$$

and

$$\text{df}(F)_{4{\text{way}}} = IJKL - (I + J + K + L - 3)$$

ELECTROSTATIC MODELING

Atomic Coordinates of PC

The atomic coordinates of oxidized poplar PC (Populus nigra var. italica) (Guss & Freeman, 1983) were obtained from the Brookhaven Protein Data Bank (Bernstein et al., 1977). At the present time, this is the only species for which the crystal structure of PC has been determined. The water molecules identified in the crystal structure were not included in our calculations. The atomic coordinates of spinach PC were derived from poplar PC by making the appropriate amino acid residue substitutions with the computer program MOLEDT (Polygen Corp.; Waltham, MA 02254; USA). This entailed the substitution of 22 out of 99 amino acid residues. The orientations of the original side chains were used to orientate the new residues. The program MOLEDT was also used to add hydrogen atoms to the structure when the partial
atomic charges were used. The amino acid sequences of spinach and poplar PC were obtained from Sykes (1985). This approach was used to form the structure of spinach PC because the two PC species have the same number of amino acids, and their absorption and CD spectra indicate that they have nearly identical secondary and tertiary structures (see Chapters III and IV).

Electrostatic Potential of PC

The electrostatic potential field of PC was calculated using the computer program DelPhi-v.2 (Klapper et al., 1986; Gilson et al., 1987). This program uses a macroscopic, continuum approach to the dielectric properties of the protein and solvent (Gilson et al., 1985). The atomic coordinates of the protein are mapped onto a 3-dimensional grid. United atom van der Waals radii are assigned to the heavy atoms to define the surface of the protein. The program allows for the assignment of different dielectric constants to the protein and the solvent. The protein surface defines the dielectric boundary. The program also allows for the assignment of solvent ionic strength and Stern layer thickness (the closest approach of solvated ions to the protein surface) (Gilson & Honig, 1988). Fractional electrostatic charges are mapped onto the grid in such a way as to provide spherically symmetric distributions around the charged atoms of the protein. The electrostatic potential field of the protein is then calculated by numerically solving the Poisson-Boltzmann equation by the finite difference method. The program was executed on a CONVEX C1.
computer in the vector mode. The average calculation required 10 minutes of CPU time.

A cubic grid with 65 units along each side was used. The PC molecule was mapped onto the grid such that the geometric center of the molecule was at the center of the grid. The scale was adjusted so that the longest dimension of the molecule was 50% of the side length of the grid. This resulted in a scale factor of 1.6 grid units/A. The position of PC in the grid and the scale factor were varied to examine their influence on the calculated electrostatic potential. The dielectric constant value of the solvent was set to 80. The dielectric constant value of the protein was varied from 1 to 80 to examine its influence on the calculated electrostatic potential. The ionic strength of the solvent and the Stern layer thickness were also varied to examine their effects on the calculated potential. The atoms of PC were given either formal or partial electrostatic charge assignments. The formal charge assignments were as follows: the NZ nitrogen atoms of the lysine residues and the backbone nitrogen atom of the N-terminal residue were +1.0e; the copper atom was +2.0e or +1.0e for oxidized or reduced PC; the OD and OE oxygen atoms of the aspartate and glutamate residues and the backbone oxygen atoms of the C-terminal residue were -0.5e; and the SG sulfur atom of the Cys 84 ligand to the copper was -1.0e. Partial atomic charges were taken from the AMBER package (Weiner et al., 1984). The electrostatic potential was calculated using an approximation of the nonlinear Poisson-Boltzmann equation described by Jayaram et al. (Jayaram et al., 1989). Boundary values
for the potential at the edges of the grid were assigned using a
coulombic/Debye-Huckel approximation (Klapper et al., 1986).
Iterations of the finite difference procedure were continued until the
maximum change in potential at any of the grid points was less than 1.0
X 10^-4 kT/e.

Electrostatic Effects on the pKa of NTyr 83.
The effect of the change in electrostatic potential on the pKa of a
titratable group can be derived from thermodynamics. The equations
will be derived for a general case first, and then applied to the pKa
of NTyr 83. The deprotonation reaction of a titratable group is
described in Equation (21),

\[ \text{RH}^n \rightarrow \text{R}^{n-1} + \text{H}^+ \]

where \( n \) is the electric charge of the protonated species. The change
in free energy of this reaction is described in Equation (22)

\[ \Delta G = \Delta G^0 + RT \ln \left( \frac{[\text{R}^{n-1}] [\text{H}^+]}{[\text{RH}^n]} \right) \]

At equilibrium, the argument of the logarithm equals the equilibrium
constant, and \( \Delta G \) equals zero.
\[ \Delta G^0 = -RT \ln(K_a) \]
\[ \Delta G^0 = -RT \ln(10) \log(K_a) \]
\[ \Delta G^0 = RT \ln(10) \text{pKa} \]
\[ \text{pKa} = \frac{1}{RT \ln(10)} \Delta G^0 \]

From (23) it can be seen that a change in the standard free energy change of the reaction would result in a change in the pKa value.

\[ \Delta \text{pKa} = \frac{1}{RT \ln(10)} \Delta \Delta G^0 \]

The assumption is made that the change in the \( \Delta G^0 \) of the deprotonation reaction is due solely to a change in electrostatic energy.

\[ \Delta \Delta G^0 = \Delta \Delta W' \]

The change in electrostatic energy for the deprotonation reaction is expressed in (26),

\[ \Delta W' = (q_{R^n-1} V - q_{RH^n} V) Na \]
\[ \Delta W' = \Delta q V Na \]
\[ \Delta \Delta W' = -le \Delta V Na \]

where \( q \) is the charge at the titratable group, \( V \) is the potential at the group, \( Na \) is Avogadro's number, \( \Delta q \) is taken to be exactly \(-le\), and \( e \) is the positive elementary charge. (26) shows that the change in electrostatic energy of the deprotonation reaction can be due to a change in potential at the reaction site. Equations (24), (25), and
(26) can be used to express the change in the pKa value as a function of the change in potential,

$$
\Delta \text{pKa} = \frac{-1eNA}{RT \ln(10)} \Delta V
$$

(27)

$$
\Delta \text{pKa} = \frac{-1}{(kT/e) \ln(10)} \Delta V
$$

where $k$ is the Boltzmann constant.

With the change in potential measured in the units of $kT/e$.

Equation (28) was used to calculate the change in the pKa value of NTyr 83 due to the reduction of the copper center:

$$
\Delta \text{pKa}_{\text{ox-red}} = \frac{-1}{\ln(10)} \Delta V_{\text{ox-red}}
$$

(28)

where $\Delta V_{\text{ox-red}}$ is the change in potential at the hydroxyl group between oxidized and reduced PC, and the absolute temperature is set at 298°K.

One consequence of using the nonlinear approximation of the Poisson-Boltzmann equation instead of the linear approximation is that the superposition principle and the single source charge method can not be used to calculate the electrostatic effects (Gilson & Honig, 1988). Therefore, the value of $\Delta V_{\text{ox-red}}$ was obtained by subtracting the potentials calculated for oxidized and reduced PC, with formal charges assigned to all of the atoms. To represent the oxidized and reduced states of the copper center, the copper atom was assigned either 1.0e or 2.0e, respectively. The atomic coordinates of oxidized PC were used
to calculate the electrostatic potential of both oxidized and reduced PC. This was done to avoid different orientations of the molecule in the grid, which could lead to numerical error. The crystal structures of oxidized and reduced PC are nearly identical at pH 7.8 (Guss et al., 1986), and the effect of not using the reduced PC coordinates is expected to be minimal.

Electrostatic Effects on the Redox Potential of the Copper Center

A similar thermodynamic treatment can be used to derive the effect of changes of electrostatic potential on the redox potential of the copper center. Equation (29) describes the reduction reaction.

\[
\text{PC}_{(ox)} + e^- \rightarrow \text{PC}_{(red)}
\]

(29)

The standard free energy change of the reaction is described in Equation (30),

\[
\Delta G^0 = -FE^0
\]

\[
E^0 = \frac{-1}{F} \Delta G^0
\]

(30)

\[
\Delta E^0 = \frac{-1}{e\text{Na}} \Delta \Delta G^0
\]

where \( F \) is the Faraday constant and \( E^0 \) is the redox potential. The change in electrostatic energy of the reduction reaction is described in Equation (31) on page 56,
\[ \Delta W' = (q_{\text{red}} V - q_{\text{ox}} V) \text{Na} \]

\[ \Delta W' = \Delta q_{\text{red-ox}} V \text{Na} \] \hspace{1cm} (31)

\[ \Delta \Delta W' = \Delta q_{\text{ox-red}} \Delta V \text{Na} \]

where \( \Delta q_{\text{red-ox}} \) is the change in electric charge of the copper atom between the reduced and oxidized states, and \( V \) is the potential at the copper atom due to the other charges in the protein. If it is again taken that the change in standard free energy change of the reduction reaction is due solely to a change in electrostatic energy, then (30) on page 55 and (31) can be combined using (25) on page 53.

\[ \Delta E^0 = \frac{-\Delta q_{\text{red-ox}}}{e \text{Na}} \Delta V \text{Na} \] \hspace{1cm} (32)

For \( \Delta V \) measured in the units of \( kT/e \), Equation (33) was used to calculate the change in redox potential of the copper center due to the EDA chemical modification of acidic residues,

\[ \Delta E^0_{\text{(mod-cont)}} = \Delta V_{\text{(mod-cont)}} 25.7 \text{ (mVolts/kT/e)} \] \hspace{1cm} (33)

where \( \Delta q_{\text{ox-red}} \) is taken to be \(-1e\), and \( \Delta V_{\text{(mod-cont)}} \) is the change in potential at the copper atom between the modified and control forms of PC. The factor 25.7 converts from the change in potential calculated by the program in the units of \( kT/e \) into the units of mVolts.

For the same reason as for the \( \Delta pK_a \) described above, the value of \( \Delta V_{\text{(mod-cont)}} \) was obtained by subtracting the potentials calculated for
EDA modified and control PC, with formal charges assigned to all of the atoms. The chemical modification of a carboxyl group was represented by assigning 0.5e charges to both of the oxygen atoms. This is a net change in charge of 2.0e for the modified group. The coordinates of oxidized PC were used to calculate the electrostatic potential of both oxidized and reduced PC.

**GRAPHICAL REPRESENTATIONS OF PC**

Pictures of the 3-dimensional structure of PC were ultimately derived from the atomic coordinates of the crystal structure of poplar PC. These coordinates were obtained from the Brookhaven Protein Data Bank (Berstein et al., 1977). Stick figure and space-filling representations of PC were produced with the QUANTA computer program (Polygen Corp.; Waltham MA 02254; USA). The QUANTA manual should be consulted for the procedures for producing the pictures. This program was run on a CDC 910-Silicon graphics 4D/70GT graphic workstation at the Ohio Supercomputer Center.

Three different devices were used to produce hard copy of the pictures. The QUANTA program provides for the formation of graphic output files in either Hewlett-Packard Graphics Language (HPGL) or Postscript formats. The HPGL files were plotted on a Hewlett-Packard 7550A pen plotter attached to the graphics workstation. The UNIX command for sending the HPGL file to the plotter was: cat (filename) >/dev/plotter. The Postscript files were output on a Texas Instrument
Omnilaser 2115 laser printer at the Ohio Supercomputer Center. Color pictures of PC could be made be directly dumping the image on the screen to a Tektronix 4693D color laser printer, which was also connected to the graphics workstation. It should be noted that the Silicon Graphics system is known for its high resolution and dazzling colors. The image was dumped by remotely logging onto the workstation, and issuing the command: printfscreen cmap.

Contour maps of the electrostatic potential field of PC were produced with the CONTOUR2D and CONTOUR3D programs, which were included with the DelPhi-v2 software package (described previously). The programs were run on the CONVEX C1 computer. In order to compile the programs on this system, the AED display subroutines in the gcs.f subprogram had to be removed. The programs produced HPGL format graphic files, which were output on the Hewlett-Packard plotter described above. The programs could also produce images of the electrostatic potential field directly on the computer screen of the SUN workstations, if the Tektronix 1410 emulator in the SUNVIEW window system was used.
CHAPTER III
THE NEAR AND FAR-UV ABSORPTION OF PC

Near-UV absorption spectroscopy has been used here to study the tertiary conformation of PC. Particular emphasis has been placed on monitoring the conformational effects of changing the oxidation state of the copper center and the environmental pH. The near-UV energy region of the absorption spectrum is a good indicator of tertiary conformational changes, because it is responsive to environmental changes of the aromatic amino acid residues and the copper center of the protein. However, the task of deducing structural information from changes in the absorption spectrum of PC is complicated by the fact that the spectra of the chromophores are overlapped, and it is not always clear which chromophores experience the environmental changes. For this reason, substantial emphasis is placed on trying to identifying the specific chromophores in PC which are responsible for the different absorption bands in the spectrum.
THE NEAR-UV ABSORPTION SPECTRUM

Figure 13 on page 61 displays the near-UV absorption spectra of oxidized and reduced parsley PC at pH 6.8 and pH 5.2. Figure 14 on page 62 displays the same set of spectra for spinach PC. The spectra of these two PC species are qualitatively similar in shape, and in the response to changes in the oxidation state of the copper center and the environmental pH. In particular: the spectra all have a peak at ca. 278 nm with a shoulder at ca. 284 nm, which is characteristic of the absorption of tyrosine residues; the absorption of reduced PC is greater than that of oxidized PC in the entire near-UV region; the absorption of reduced PC is greater at pH 6.8 than at pH 5.2. The spectra of poplar and lettuce PC have been found to be qualitatively similar, with the same responses to changes in the oxidation state and the environmental pH (data not shown) (Draheim et al., 1986).

To analyze the absorption bands in the spectra of PC it is convenient to divide the wavelength range into three regions: I) 250-265 nm, II) 265-290 nm, and III) 290-320 nm. The definition of these regions is based on the absorption bands of the phenylalanine and tyrosine residues in the protein. Figure 15 on page 64 shows the near-UV absorption spectra of these two amino acid residues in aqueous solution, and the three regions of the spectrum (Donovan 1969). From this it can be seen that in the absorption spectrum of PC phenylalanine residues contribute primarily to region I, and that the tyrosine residues contribute primarily to region II. The fine structure of absorption peaks in region I is due to the phenylalanine residues;
Figure 13: Near-UV absorption spectra of parsley PC. Oxidized PC, pH 6.8 (——); oxidized PC, pH 5.2 (- - -); reduced PC, pH 6.8 (…….); reduced PC, pH 5.2 (-.-.-)
Figure 14: Near-UV absorption spectra of spinach PC. Oxidized PC, pH 6.8 (-----); oxidized PC, pH 5.2 (-- -- --); reduced PC, pH 6.8 (-----); reduced PC, pH 5.2 (-.-.-)
however, region I may also contain residual absorption from the $n\rightarrow\pi^*$
electronic transitions of the peptide bonds, which have a peak at ca. 220 nm. The absorption in region III of the spectrum must be due to
electronic transitions in the copper center, since there are no other
cromophores in PC which could conceivably absorb in this region.
Figure 75: Near-UV absorption of phenylalanine and tyrosine amino acids. Phenylalanine (-----); tyrosine (---). The left-hand scale is for the phenylalanine absorption, and the right-hand scale is for the tyrosine absorption. The spectra were taken from Brown (1980).
The Absorption of Oxidized PC

The near-UV absorption spectrum of oxidized PC at pH 7.0 has been found to be nearly identical to that of APO-PC, in which the copper atom has been removed (Tamilarasan and McMllin, 1986; Anderson et al., 1989). This indicates that electronic transitions in the copper center do not contribute to the absorption spectrum of oxidized PC at this pH value. Therefore, the absorption in region II of the spectrum of oxidized PC must be due primarily to the tyrosine residues. The average extinction coefficient of the tyrosine residues can be calculated by dividing the extinction coefficient of PC by the number of tyrosines present in the protein. At 278 nm, the wavelength of the tyrosine absorption peak, the extinction coefficients of oxidized parsley and spinach PC at pH 6.8 are 7.3 and 5.0 mM⁻¹cm⁻¹, respectively. Since there are 4 tyrosines in parsley PC and 3 tyrosines in spinach PC, the average extinction coefficients of these residues in parsley and spinach PC are 1.8 and 1.7 mM⁻¹cm⁻¹, respectively. These values correspond to the 1.8 mM⁻¹cm⁻¹ extinction coefficient observed for tyrosine in ethanol solution, but are significantly greater than the 1.4 mM⁻¹cm⁻¹ extinction coefficient observed for tyrosine in water (Donovan, 1969; Gratzer, 1968). This indicates that at least some of the tyrosine residues in the oxidized PC protein experience a hydrophobic environment.

The 3 tyrosine residues in the sequence of spinach PC are conserved in the sequence of parsley PC. If it is assumed that the three common tyrosine residues are in correspondingly similar environments, then the
extinction coefficient of the extra tyrosine residue in parsley PC, Tyr 62, can be estimated by subtracting the extinction coefficients of parsley and spinach PC. In this manner, the extinction coefficient of Tyr 62 is found to be 2.3 mM$^{-1}$cm$^{-1}$. This value is significantly larger than the average extinction coefficients of the other tyrosine residues, and of that observed for tyrosine in ethanol. Due to the lack of information in this area, it is not known whether this is a reasonable extinction coefficient for a tyrosine residue or not.

The Absorption of Reduced PC
The absorption of PC in the near-UV region increases upon reduction of the copper center (Figure 13 on page 61 and Figure 14 on page 62). Tamilarasan and McMllin (1986) have suggested that this increase in absorption is due to electronic transitions that occur selectively in the reduced copper center. Citing the relatively large magnitude of the absorption changes, they rejected the idea, proposed by Draheim et al. (1986), that changes in the absorption of the aromatic amino acid residues contribute to the enhanced absorption of reduced PC. Tamilarasan and McMllin (1986) suggested three types of electronic transitions which could cause the absorption of the reduced copper center: 1) Cu(I) 3d$\rightarrow$4s atomic orbital transitions; 2) Cu(I)$\rightarrow$histidine ligand charge transfer transitions; 3) Cu(I)$\rightarrow$solvent charge transfer transitions. Examples of these types of transitions have been observed in model reduced copper atom organometallic compounds (Payne et al., 1984; Sorrell & Borovik, 1986; David et al., 1978). However, it is
unknown what the wavelength maxima or extinction coefficients of the absorption of analogous electronic transitions in the copper center of PC would be.

The enhanced absorption of reduced PC can be better examined from the reduced minus oxidized difference spectra. Figure 16 on page 68 displays this difference spectra for both parsley and spinach PC at the two pH values. It is seen that the difference spectra of both species are qualitatively similar in shape, and in the response to the environmental pH. In region II the difference spectra are larger at pH 6.8 than at pH 5.2; whereas, in regions I and III the difference spectra are relatively independent of the pH. Thus, the increase in absorption of PC that occurs upon reduction of the copper center has both pH-dependent and pH-independent components. The difference spectrum at pH 5.2 is the pH-independent component, and the increase in absorption of the difference spectrum in region II at pH 6.8 is the pH-dependent component. This indicates that the enhanced absorption of reduced PC is due to at least two different electronic transitions, one which is effected by the environmental pH and one which is not. It is also likely that these transitions are located in at least two different chromophores in PC. It should be noted that the pH-dependent component of the difference spectrum is in region II, which is also the region of maximum tyrosine absorption. One possibility is that the reduced copper center and the tyrosine residues comprise these two different chromophores.
Figure 16: Reduced - oxidized difference spectra of parsley and spinach PC. Parsley PC, pH 6.8 (----); spinach PC, pH 6.8 (.....); parsley PC, pH 5.2 (- - -); spinach PC, pH 5.2 (---).
It is observed that the reduced minus oxidized difference spectrum of PC at pH 5.2 is similar to the absorption spectrum of the copper metallothionein protein. Figure 17 on page 70 displays the near-UV absorption spectra of copper metallothionein from yeast and Neurospora (Weser et al., 1977; Beltramini & Lerch 1983). Each protein contains multiple copper atoms, which are ligated by cystein residues in pseudo-tetrahedral coordination geometries. These copper centers normally assume the reduced Cu(I) oxidation state. The differences in the wavelength maxima for the peaks in the absorption spectra of the two different species of metallothionein (254 vs. 280 nm) may be due to small differences in the coordination geometries of the copper centers. Since yeast and Neurospora metallothionein are both devoid of aromatic amino acid residues, their near-UV absorption has been attributed by default to Cu(I)→Cys charge transfer transitions in the metal centers (Beltramini & Lerch, 1983). This interpretation has been questioned by Tamilarasan and McMillin (1986); however, these authors do not suggest an alternative source for the absorption. The similarity of the pH-independent reduced minus oxidized difference spectra of PC with the absorption spectrum of the metallothioneins indicates that Cu(I)→Cys 84 ligand charge transfer transitions may also be responsible for the absorption of the reduced copper center in PC.
Figure 17: Near-UV absorption spectra of Cu(I) metallothionein. Neurospora (—); yeast (-- - -). The spectra were obtained from Weser et al. (1977) and Beltramini and Lerch (1983).
The Enhanced Absorption of Tyrosine Residues in Reduced PC

As seen in Figure 13 on page 61 and Figure 14 on page 62, the environmental pH has a larger effect on the absorption of reduced PC than on that of oxidized PC. This can also be seen from the pH 6.8 minus pH 5.2 difference spectra of oxidized and reduced PC displayed in Figure 18 on page 72. Qualitatively similar difference spectra are observed for the parsley and spinach species. The absorption of oxidized PC can be considered to be pH-independent, since the variation due to the pH change is within the limit of error of measuring the spectra (± 4%). Thus, the pH-dependent component to the reduced minus oxidized difference spectrum (Figure 16 on page 68) can be attributed completely to a pH effect on the absorption of reduced PC, and not to that on the absorption of oxidized PC. It is also observed that the pH difference spectrum of reduced PC resembles the absorption of a tyrosine residue, with a peak at 278 nm and a shoulder at 285 nm. This indicates that the absorption of the tyrosine residues in reduced PC is pH-dependent. It also confirms the suggestion made in the previous section that tyrosine residues contribute to the increase in absorption of PC that occurs upon reduction of the copper center.

Figure 19 on page 74 displays the effect of pH on the absorption of reduced parsley and spinach PC at 278 nm. This wavelength was selected because it is at the peak of the tyrosine absorption band. The solid lines represent the fits to the data of the modified Henderson-Hasselbalch model (12) on page 35. The fitted pKa values for the pH titrations of reduced parsley and spinach PC are 6.1 and 5.4,
Figure 18: pH 6.8 - pH 5.2 difference spectra of reduced PC. Reduced parsley PC (-- --); reduced spinach PC (--.--); oxidized parsley PC (--.--); oxidized spinach PC (------).
respectively. It should be noted that the fit to the spinach data appears inaccurate due to a bad point, and that a more accurate pKa value would be closer to 5.5. The fitted n value, the number of protons transferred in an infinitely cooperative system, is 2 for both curves. These pKa values are similar to those observed for the pH dependence of the rate of oxidation of reduced PC via the east face reaction site (6.1 for parsley PC and 5.7 for spinach PC) (see Chapter I) (Sykes, 1985). Since Tyr 83 is located at the east face site, this result specifically implicates Tyr 83 as a contributor to the pH-dependent change in absorption of reduced PC. This also indicates that the same mechanism which alters the electron transport activity through the east face site also affects the absorption of Tyr 83.

As described by Sykes (1985), the pKa value for the pH dependence of the rate of oxidation of reduced PC via the east face site is a composite of two separate, pH-dependent mechanisms, which affect the activities of the north pole and east face sites (see Chapter I). The pKa values for the north pole and east face sites are 5.7 and 5.8 in parsley PC, and 4.9 and 5.6 in spinach PC. Table 1 on page 76 correlates the different pKa values discussed so far. For the parsley species the argument can be made that the absorption of Tyr 83 is affected by the pH-dependent states of both the east face and the north pole sites. This is because the pKa of the absorption is the same as the composite pKa value, and not the independent east face pKa value. This is also indicated by the fact that the absorption titrates with the value of n equal to 2. However, this argument can not be made for
Figure 19: pH dependence of the absorption at 278 nm of reduced PC. Parsley (△); spinach (□). The solid lines are the fits to the data.
spinach PC, because the pKa values for the change in absorption and the
electron transport activity through the east face site are different.
This discrepancy may be due to error in the absorption data and a poor
fit of the titration model. The signal to noise ratio is smaller for
the titration of the spinach PC absorption than for the parsley PC
absorption.

There are three possible mechanisms by which the absorption of the
tyrosine residues in PC could be enhanced: 1) a decrease in the
environmental polarity; 2) the formation of a hydrogen bond; 3)
hyperchromicity due to electronic transition moment coupling (Donovan,
1969; Strickland et al., 1972). All of these mechanisms could be
induced by relatively small, tertiary conformational changes of the
protein, which are localized at the sites of the tyrosine residues. A
decrease in environmental polarity could be caused by a decrease in
solvent exposure of the tyrosine residues. A hydrogen bond could be
formed by the repositioning of an acceptor group in the appropriate
vicinity of the tyrosine hydroxyl group. While, hyperchromicity could
be affected by repositioning of the tyrosine rings relative to the
other chromophores.

The formation of a hydrogen bond is a likely cause for the increase
in absorption of Tyr 83 at high pH. Previously, Farver and Pecht
(1981) have pointed out that in the crystal structure of PC, Tyr 83 is
in an appropriate position to form a hydrogen bond with the carboxylate
group of Glu 59, which is one of the conserved acidic residues at the
Table 1

$pK_a$ Values for the Absorption and Activity of PC

<table>
<thead>
<tr>
<th>PC species</th>
<th>Parsley</th>
<th>Spinach</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absorption at 278 nm</td>
<td>6.1</td>
<td>5.4</td>
</tr>
<tr>
<td>Activity via east face</td>
<td>6.1</td>
<td>5.7</td>
</tr>
<tr>
<td>north pole mechanism</td>
<td>5.7</td>
<td>4.9</td>
</tr>
<tr>
<td>east face mechanism</td>
<td>5.8</td>
<td>5.6</td>
</tr>
</tbody>
</table>
east face. Figure 20 on page 78 shows the structure of the east face site of spinach PC, and the possibilities of hydrogen bond formation. In the modified view, the side chain of Glu 59 has been rotated to position the carboxylate group 2.7 Å from the hydroxyl proton of Tyr 83, which is an average hydrogen bond distance. The side-chain of Asp 42 has been rotated to position its carboxyl group 3.4 Å from Tyr 83. An additional small rotation of the Tyr 83 side-chain would be required to hydrogen bond with Asp 42.

A hydrogen bonding mechanism could also explain why the enhanced absorption of Tyr 83 is pH dependent. Lowering of the pH would eventually protonate the hydrogen bond acceptor and sever the hydrogen bond. This would result in a decrease in the absorption of the tyrosine residue. The pKₐ values for the protonation of the hydrogen bond acceptor would thus be the same pKₐ values as for the change in absorption of Tyr 83 in the two PC species (6.1 in parsley and 5.4 in spinach). Since for parsley PC the pKₐ value for the activity of the east face site is different from that for the change in absorption (5.8 versus 6.1), the involvement of Tyr 83 in a hydrogen bond must not affect the activity of the east face site. If the supposition of Coleman et al. (1978) is correct, that electron transport at the east face site occurs through Tyr 83, then the formation of a hydrogen bond must not affect the efficiency of this mechanism. It should be noted that Tyr 83 is replaced by a phenylalanine in the Scenedesmus obliquus species of PC, for which there is no hydroxyl group to form a hydrogen bond (Sykes, 1985). It would be interesting to compare the absorption
Figure 20: The Hydrogen bonding of Tyr 83. The orientations of the Glu 59 and Asp 42 side-chains have been modified to show the possibility of hydrogen bonding with Tyr 83.
of S. obliquus with the other species of PC in order to determine the variable absorption of the other tyrosine residues besides Tyr 83. Incidentally, it should also be noted that position 82 is a phenylalanine residue in the spinach, poplar, and parsley species, and a tyrosine residue in the S. obliquus species. It should be investigated whether coupling of the electron densities of these two proximal aromatic residues plays a role in creating an electron transfer path to the copper center.

As seen in Figure 18 on page 72, the pH-dependent change in the extinction coefficient of reduced PC at 278 nm is 2.8 mM\(^{-1}\)cm\(^{-1}\) for parsley PC and 1.5 mM\(^{-1}\)cm\(^{-1}\) for spinach PC. A larger difference extinction coefficient for parsley PC is consistent with the existence of an extra tyrosine residue (Tyr 62) in this species. However, the magnitudes of the pH difference extinction coefficients are considerably larger than what would be expected from the change in tyrosine absorption alone. The maximum changes in the extinction coefficient of a tyrosine residue due to changes in environmental polarity and hydrogen bonding are predicted to be only 0.7 mM\(^{-1}\)cm\(^{-1}\) and 0.35 mM\(^{-1}\)cm\(^{-1}\), respectively (Donovan, 1968; Strickland et al., 1972). Considering that the crystal structure of PC is substantially the same in the two oxidation states and at different pH values (Guss & Freeman, 1983; Guss et al., 1986), it is unlikely that any of the tyrosine residues undergo the maximal change in environmental polarity. Therefore, there must be a contribution of the reduced copper center, in addition to that of tyrosine, to the pH-dependent component of the enhanced absorption of reduced PC.
The fact that the reduced copper center contributes to both the pH-dependent and pH-independent components of the enhanced absorption of reduced PC can be explained in two ways. The first possibility is that the same electronic transition contributes to both components, but that the intensity of this transition is greater in the high pH conformation of the reduced copper center than in the low pH conformation (see Chapter I). As shown in Figure 5 on page 10, lowering the environmental pH results in the change in conformation of the reduced copper center from a pseudo-tetrahedral to a trigonal coplanar coordination. As a result, at low pH the Cu-His 87 bond is broken, and the Cu-Met 92 bond is strengthened. Therefore, a Cu→His 87 charge transfer transition could not be the source of this absorption because it would not contribute to the absorption at the lower pH values measured. The second possibility is that there are two electronic transitions in the reduced copper center which contribute to the absorption, one which is affected by the change in conformation of the reduced copper center and one which is not. In this case, one possibility would be that a Cu(I)→His 87 charge transfer transition provides the pH-dependent absorption, and a Cu(I)→His 37 or Cu(I)→Cys 84 charge transfer transition provides the pH-independent absorption.

The Possibility of Hyperchromic Effects

It has been pointed out by Draheim et al. (1986) that the phenylalanine residues in PC are ideally oriented to cause hyperchromism of their near-UV electronic transitions. They proposed that slight changes in
the orientations of these residues between oxidized and reduced PC, and between different environmental pH values, could significantly affect the absorption of these residues, and thus contribute to the enhanced absorption of reduced PC. Hyperchromism and hypochromism is caused by the coupling of electronic transitions of different energies in proximal chromophores (Donovan, 1986; Tinoco, 1962; Cantor, C. R. & Timasheff, 1982). For example: the intensity of a near-UV electronic transition in a phenylalanine residue could be either enhanced or diminished by the coupling with transitions of different energy in the other aromatic residues, the copper center, or in the peptide bonds. The magnitude of the hyperchromic effect is inversely proportional to the cube of the distance between the chromophores, and is also dependent on the relative orientations of the transition moments.

Due to the large number of chromophores in PC, and the complex ensemble of coupled transitions, it is not possible to accurately predict hyperchromic effects in the aromatic amino acids of PC. However, by examining the changes in the orientations of the aromatic residues between oxidized and reduced PC, and for reduced PC at different environmental pH values, it might be possible to predict which residues are likely to be involved in hyperchromicity. For this analysis, the crystal structures of poplar PC are used to determine the orientations of the phenylalanine and tyrosine residues. In regard to the aromatic residues, the only differences between the poplar, parsley and spinach PC species are that Phe 70 is a tyrosine residue in parsley and spinach PC, and that parsley PC has an extra tyrosine at location 62.
Figure 21 on page 83 displays the change in orientations of the aromatic residues between oxidized and reduced PC. The change in orientation of each residue in relation to the other aromatic residues is given in Table 2 on page 84. The maximum change in the relative orientation is 8 degrees, and the average of the changes is calculated to be 4 degrees. The larger changes in orientation occur between Phe 14, Phe 19, Phe 29, Phe 35, Phe 82, and Tyr 83. Figure 22 on page 85 displays the changes in the orientations of the aromatic residues between the crystal structures of reduced PC at pH 7.8 and pH 3.8. Table 3 on page 86 gives the relative changes in orientations of the residues. Again, the largest change in relative orientation is 8 degrees, and the average change is approximately 3 degrees. Therefore, the conformations of the aromatic residues are altered approximately the same amount by the change in redox state and in environmental pH. The largest changes in orientation occur between Phe 19, Phe 29, Phe 35, Phe 41, Phe 70, Phe 82, and Tyr 83.

As noted above, the degree to which the electronic transition moments couple is inversely proportional to the cube of the distance between the chromophores. Predictions of which aromatic residues are more likely to be coupled to other aromatic residues can thus be made by examining the distances between them. Table 4 on page 88 displays the distances between the centers of the aromatic rings of the phenylalanine and tyrosine residues in the crystal structure of oxidized poplar PC. The five closest pairs of aromatic residues are Phe 82-Phe 14, Phe 82-Phe 41, Phe 29-Phe 14, Tyr 80-Phe 41, and Phe
Figure 21: Aromatic residue conformation in oxidized and reduced spinach PC.
Table 2

Effect of Oxidation State on the Aromatic Residue Orientations

The values are the differences in the angles (degrees) between the unit normals of the ring planes between oxidized and reduced poplar PC.

<table>
<thead>
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<th>14</th>
<th>19</th>
<th>29</th>
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<td></td>
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<tr>
<td>Phe 29</td>
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<tr>
<td>Phe 35</td>
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<tr>
<td>Tyr 80</td>
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<tr>
<td>Tyr 83</td>
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<td>-2</td>
<td>-5</td>
<td>-3</td>
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Figure 22: Aromatic residue conformation in reduced PC at pH 7.8 and pH 3.8.
Table 3

Effect of pH on the Aromatic Residue Orientations in Reduced PC

The values are the differences in the angles (degrees) between the unit normals of the ring planes between reduced poplar PC at pH 7.8 and pH 3.8.

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<tr>
<td>Phe 70</td>
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<td>-7</td>
<td>-1</td>
<td>4</td>
<td>-3</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tyr 80</td>
<td>0</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>-1</td>
<td>0</td>
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<tr>
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<td>-4</td>
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<tr>
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<td>-1</td>
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</table>
82-Phe 19. Each of these residues, except Tyr 80, has also been observed as having the largest changes in relative orientation. If hyperchromicity is a factor in the oxidation state and pH-dependent absorption of PC, then these residues would be the most likely contributors.
Table 4

Distances Between the Aromatic Residues

The values are the distances (angstroms) between the aromatic ring centers in oxidized poplar PC at pH 6.0.

<table>
<thead>
<tr>
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<tr>
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<td>7.02</td>
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<td>19.88</td>
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<td>Phe 41</td>
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<td>9.58</td>
<td>6.99</td>
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<td>Phe 70</td>
<td>10.98</td>
<td>13.13</td>
<td>6.65</td>
<td>15.96</td>
<td>8.06</td>
<td>0</td>
<td></td>
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<tr>
<td>Tyr 80</td>
<td>13.97</td>
<td>10.49</td>
<td>11.46</td>
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<td>6.09</td>
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<td>5.56</td>
<td>10.37</td>
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<td>Tyr 83</td>
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<td>15.04</td>
<td>11.92</td>
<td>18.82</td>
<td>9.05</td>
<td>12.87</td>
<td>13.64</td>
<td>8.70</td>
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</table>
THE FAR-UV ABSORPTION SPECTRUM

Far-UV (180-240 nm) absorption spectroscopy is used here to study the secondary structure of PC. The absorption in the far-UV region is due primarily to the peptide bond chromophores. The absorption of these chromophores is known to be significantly affected by excitonic transition moment coupling. The different types of secondary structures of the protein put the peptide bonds into different relative orientations, which affect the electronic coupling, and thus the far-UV absorption spectrum. Figure 23 on page 90 demonstrates these changes for the absorption of poly-lysine in the α-helix, β-sheet, and random coil secondary structures. In going from the α-helix to the β-sheet structures, the magnitude of the absorption peak of the peptide bonds increases approximately 60%, and red-shifts approximately 5 nm.

The far-UV (180-240 nm) region of the absorption spectrum of PC is due to the peptide bonds, phenylalanine, and tyrosine chromophores. Due to a lack of information, it is not known what contribution the copper center chromophore might make to the absorption in this region. There are approximately ten times more peptide bond chromophores in PC than there are aromatic amino acid residues. Figure 24 on page 91 shows the predicted relative contributions of the peptide bonds and the aromatic residues to the spectrum, based on the absorption of model compounds. At 190 nm, the sum of the absorption of the phenylalanine and tyrosine residues is approximately 60% of the absorption of the peptide bonds.
Figure 23: Far-UV absorption of model secondary structures of poly-L-lysine. α-helix (——); β-sheet (.....); random coil (----). The data were taken from Cantor and Schimmel (1980).
Figure 24: Predicted far-UV absorption of the different chromophores in PC. Absorption spectrum of parsley PC. 96 peptide bonds (- - -); 4 tyrosines (----); 6 phenylalanines (-----).
Figure 25 on page 93 shows the far-UV absorption spectrum of oxidized parsley PC at pH 7.0 and pH 5.0. It should be known that this data is the result of a single set of measurements, and that repeated experiments need to be conducted to confirm the accuracy. Nearly identical spectra were obtained for reduced PC at these two pH values. Thus, unlike in the near-UV region, the far-UV absorption spectrum of PC is more affected by the change in environmental pH than by the change in oxidation state. In going from pH 7.0 to pH 5.0 there is a small increase in the magnitude of the absorption peak (ca. $0.1 \times 10^6$ M$^{-1}$cm$^{-1}$), and a blue-shift of approximately 3 nm. This change in the far-UV absorption spectrum could be due to a slight change in the $\beta$-sandwich secondary structure of the PC molecule at the two different environmental pH values.

Figure 26 on page 95 compares the far-UV absorption spectra of oxidized parsley and spinach PC at pH 7.0. Again it should be noted that these spectra are the results of just a single set of measurements. Similar to the parsley species, the spectrum of spinach PC at pH 7.0 was not significantly affected by the reduction of the copper center. The absorption peak in the spectrum of spinach PC is smaller than that of parsley PC by ca. $0.5 \times 10^6$ M$^{-1}$cm$^{-1}$, and is blue-shifted by ca. 2 nm. This is true despite the fact that these two species contain nearly the same number of peptide bond chromophores (98 vs. 96). These differences in the far-UV absorption spectrum may reflect differences in the secondary structures of the two proteins. A difference in secondary structure would not be surprising, considering
Figure 25: Far-UV absorption of oxidized parsley PC. pH 7.0 (-----); pH 5.0 (--.--).
that there are two less amino acids in parsley PC, and that there are 28 substitutions in the sequences. However, the differences in the far-UV spectrum may also be due to changes in the orientations and/or environments of the aromatic residues, or to the conformation of the copper center.
Figure 26: Far-UV absorption of oxidized parsley and spinach PC. Parsley PC (---); spinach PC (- - -);
CD spectroscopy was used to investigate the structure of PC in solution, and to determine the effects that the changes in the oxidation state of the copper center and the environmental pH have on the structure of the protein. CD is defined as the difference in absorption of left-handed and right-handed circularly polarized light (Cantor & Schimmel, 1980). The CD spectrum of a chromophore overlaps its absorption spectrum, although the CD bands may be of positive or negative magnitude. Thus, for the PC protein, the far-UV CD spectrum is due to the electronic transitions of the peptide bonds, aromatic residues, and possibly the copper center; and the near-UV CD spectrum is due to electronic transitions of just the aromatic residues and the copper center. The phenomenon of CD occurs for chromophores which are optically active. Optical activity is an intrinsic property of chromophores which have asymmetric molecular structure. However, optical activity may also be induced in symmetrical chromophores by the presence of an asymmetrically charged environment, or by transition moment coupling with other chromophores (Tinoco, 1962; Sears & Beychok, 1973). Thus, the CD spectrum of a chromophore is dependent on both its environment and its position with respect to the other chromophores.
The far-UV CD spectrum of a protein is primarily influenced by the relative orientations of the peptide bond chromophores, and is therefore used to monitor the secondary structure. Whereas, the near-UV CD spectrum is primarily influenced by the environments and relative orientations of the aromatic amino acid residues, and is therefore used to monitor the tertiary structure.

THE FAR-UV CD SPECTRUM OF PC

Figure 27 on page 98 displays the far-UV CD spectrum of oxidized spinach PC. The spectrum has a positive band with a peak at 195 nm, and a broad negative band with a minimum at 223 nm. There is a cross-over point from positive to negative ellipticity at 212 nm. Nearly identical spectra have been observed for parsley and poplar PC (Drahelm et al., 1986). This indicates that these three PC species have the same net secondary structure. It should be noted that this is in contrast to the far-UV absorption spectra presented in Chapter III, which does indicate some difference in the secondary structures of spinach and parsley PC. However, as also noted in that Chapter, more experiments need to be done to confirm the far-UV absorption results. The far-UV CD spectra of parsley, spinach, and polar PC are found to be unaffected by the change in oxidation state of the copper center and the change in environmental pH from 7.0 to pH 5.0. This indicates that the net secondary structure of PC in solution is unaffected by these changes. This was also found for the secondary structure of poplar PC in the crystal state (Guss & Freeman, 1983; Guss et al., 1986).
Figure 27: Far-UV CD spectrum of oxidized spinach PC at pH 7.0. The PC concentration is 50 μM, the cell pathlength is 0.1 mm. The data were obtained from Draheim et al., 1986.
It is known from X-ray crystallography (Guss & Freeman, 1983) that the secondary structure of PC is primarily composed of \( \beta \)-pleated sheet. However, the far-UV CD spectrum of PC is found to be significantly different from the CD spectrum of model \( \beta \)-sheet proteins. Figure 28 on page 100 compares the far-UV CD spectrum of oxidized spinach PC with that of model \( \alpha \), \( \beta \), and random coil proteins (Saxena & Wetlaufer, 1971). The spectrum of PC is red-shifted ca. 5 nm from that of the model \( \beta \)-sheet, and has a significantly larger peak-to-trough magnitude ratio (3.8 vs. 1.4). The unique far-UV CD spectrum of PC has been attributed to the fact the \( \beta \)-structure is in a compressed barrel configuration (Manning & Woody, 1986). This sandwich-like structure is shown in Figure 3 on page 6. The close association of the two \( \beta \)-sheets causes an asymmetrically charged environment for the peptide bond chromophores, and allows for the additional coupling of transition moments in opposite sides of the sandwich.

The unique far-UV CD spectrum of PC indicates that it is a good monitor of the relative orientations of the strands in the \( \beta \)-sandwich. Small changes of the distance and orientation between the sheets would likely be reflected in the CD spectrum. This adds emphasis to the conclusions from the analysis of the far-UV CD spectra presented above, that the net secondary structures of parsley, spinach, and poplar PC are the same, and that they are unaffected by the changes in oxidation state and pH. This invariance in the net secondary structures occurs
Figure 28: Far-UV CD spectra of PC and model protein secondary structure. a) α-helix (---), β-sheet (-- --), and random coil (.....). The data were obtained from Saxena & Wetlaufer, 1971. b) Spinach PC (---) and model β-sheet (-----). The spectra are scaled to the same positive peak height.
despite differences in the amino acid sequences of these three PC species. There are 22 amino acid substitutions between spinach and poplar PC, and 34 substitutions and two deletions between parsley and poplar PC (Sykes, 1985). The conservation of the net secondary structure suggests that it is important to the function of PC.

In many types of physical investigations of proteins it is important to know the 3-dimensional structures. To date, poplar PC is the only species for which the crystal structure has been determined (Guss & Freeman, 1983; Coleman et al., 1978; Guss et al., 1986). Although differences may exist, the crystal structure is generally an acceptable first approximation for the solution structure of the protein. For the case of PC, the similarity of the far-UV CD spectra of parsley, spinach, and poplar PC, and the invariance to oxidation state and pH changes, supports the use of the crystal structure of poplar PC to approximate the solution structures of spinach and parsley PC (Johnson, 1988).

**THE NEAR-UV CD SPECTRUM OF PC**

Figure 29 on page 103 displays the near-UV CD spectra of oxidized and reduced parsley PC at pH 5.0 and pH 7.0. As with the absorption spectra described in Chapter III, the CD spectra can be conveniently divided into three wavelength regions, which correspond to the absorption of the aromatic amino acid residues in the protein. Region I is 250-265 nm, region II is 265-290 nm, and region III 290-320 nm.
Phenylalanine residues absorb primarily in region I and tyrosine residues absorb primarily in region II. The CD in region III must be due to the copper center, since this is the only chromophore in PC which could absorb in this region.
Figure 29: Near-UV CD of oxidized and reduced parsley PC at pH 7.0 and 5.0. The PC concentration is 50 μM and the cell pathlength is 1 cm. Oxidized PC, pH 7.0 (——); oxidized PC, pH 5.0 (-- --); reduced PC, pH 7.0 (.....); reduced PC, pH 5.0 (---).
The Near-UV CD of Oxidized PC

The near-UV CD spectrum of oxidized PC is much less affected by the change in environmental pH than is the CD spectrum of reduced PC. This result was also observed for the near-UV absorption of PC, and indicates that the tertiary structure of oxidized PC is less affected by pH changes than is that of reduced PC. The CD spectrum of oxidized parsley PC can be described as follows: There is a positive band in region I with a maximum at 252 nm, a negative band in region II with a minimum at 275 nm, and a broad, weak positive band in region III with a maximum at 305 nm. All three of these bands have multiple peaks, which indicates the contributions of multiple electronic transitions.

As described in Chapter III, the absorption spectrum of oxidized PC can be interpreted as due solely to the aromatic amino acid and peptide chromophores, and not to electronic transitions in the copper center. If the oxidized copper center is indeed devoid of electronic transitions which absorb in the near-UV region, then the CD spectrum of oxidized PC can be interpreted analogously. In this case, the positive band in region I would be due primarily to the phenylalanine residues, and the negative band in region II would be due primarily to the tyrosine residues. The assignment of the tyrosine band is confirmed by the near-UV CD spectrum of APO-spinach PC (data not shown), in which the copper atom has been removed (Anderson et al., 1989). The spectrum of APO-PC has a negative band in region II similar to that of oxidized PC. The spectrum of APO-PC also has a small positive band in region I,
which is smaller than that in the spectrum of oxidized PC. It is uncertain whether this smaller band is real or not, due to the error in the measurement. The reduction, or elimination, of this CD band in the spectrum of APO-PC would indicate that there are electronic transitions in the copper center which do contribute to region I of the CD spectrum of oxidized PC. However, caution must be taken in making this conclusion, because it is not certain whether the APO-PC sample maintained the native protein conformation. The reduction, or elimination, of the CD band in the spectrum of APO-PC could also be due to changes in the orientations of the phenylalanine residues. In any event, the weak, broad CD band in region III indicates that there are some electronic transitions in the oxidized copper center which contribute to the spectrum of oxidized PC. This is because there are no other chromophores in PC which could absorb in this region.

The Near-UV CD of Reduced PC

The near-UV CD spectrum of parsley PC is significantly altered by the reduction of the copper center. The effect that this reduction has on the CD spectrum is also dependent on the environmental pH. At pH 7.0, reduction of the copper center results in a 220% increase in the positive CD band in region I, and a red-shift of the wavelength maximum by ca. 5 nm. This red-shift is also seen for the spectrum in regions II and III, but the changes in the magnitudes of these other bands are much smaller. At pH 5.0, reduction of the copper center results in the inversion of the polarity of the CD bands in regions I and III. The
near-UV CD spectrum of reduced parsley PC at pH 5.0 can be described as follows: In region I there is a large negative band with a minimum at 258 nm, and in regions II and III these is a small positive band with a maximum at 270 nm and a large negative band with a minimum at 288 nm.

For comparison, Figure 30 on page 107 displays the near-UV CD spectra of oxidized and reduced spinach PC at pH 7.0 and pH 5.0. Qualitatively similar CD spectra are observed for poplar and lettuce PC (data not shown) (Drahelm et al., 1986). Except for the reduced oxidation state at pH 5.0 and differences in the absolute magnitudes of the peaks, the near-UV CD spectra of spinach PC is very similar to that of parsley PC. The pattern of alternating positive and negative bands in the three wavelength regions, and the relative magnitudes of the bands, are similar for the different PC species. In addition, the CD spectrum of each species displays the large increase in magnitude of the positive band in region I that occurs upon reduction of the copper center at pH 7.0. These similarities in the CD spectra indicate that the net tertiary conformations of the four PC species are similar in the oxidized state, and in the reduced state at pH 7.0.

In contrast, the negative CD band in region I of the spectrum of reduced parsley PC at pH 5.0 is not observed in the CD spectra of the other species under these conditions. Instead, the CD spectra of reduced spinach, poplar, and lettuce PC at pH 5.0 have a positive band in region I which has a maximum at 250 nm. The magnitude of this band is in-between that of oxidized PC and reduced PC at pH 7.0. However,
Figure 30: Near-UV CD of oxidized and reduced spinach PC at pH 7.0 and 5.0. The PC concentration is 50 μM and the cell pathlength is 1 cm. Oxidized PC, pH 7.0 (-----); oxidized PC, pH 5.0 (-- --); reduced PC, pH 7.0 (-----); reduced PC, pH 5.0 (--- --). The data were obtained from Draheim et al. (1986).
It appears that the CD spectrum of at least reduced spinach PC does have a negative band in region I when the pH is lowered below pH 4.5. Unfortunately, an accurate spectrum for reduced spinach PC could not be obtained under these acidic conditions due to the rapid and irreversible denaturation of the protein. This result suggests that lowering the environmental pH has the same effect on the CD spectrum of reduced spinach PC as it does on the CD spectrum of reduced parsley PC, but that the effect occurs at a lower range of pH values.

The Effect of pH on the CD of Reduced PC

The effect of pH on the CD of reduced PC can be better understood from a titration experiment. Figure 31 on page 109 displays the near-UV CD spectrum of reduced parsley PC as a function of environmental pH, between pH 7.0 and pH 5.0. The entire near-UV CD spectrum decreases monotonically with the reduction of the environmental pH, except at 278 nm, where there is an isodichroic point. It should be observed that 278 nm is also the wavelength maximum of the tyrosine absorption band. This indicates that either the CD of the tyrosine residues in reduced PC is unaffected by the reduction of pH or that there is the phenomenon of exciton coupling, where an inflection point occurs at the absorption maximum (Cantor and Schimmel, 1980).

More can be learned about the effect of pH by fitting the change in the CD spectrum of reduced parsley PC with the standard titration model. Figure 32 on page 111 displays the fit of the Henderson-Hasselbalch model (12) on page 35 to the combination of three
Figure 31: pH dependence of the near-UV CD spectrum of reduced parsley PC. The PC concentration is 50uM and the cell pathlength is 1 cm. a) The pH values for the spectra are, from top to bottom, 7.0, 6.7, 6.4, 6.1, 5.8, 5.7, 5.5, 5.4, 5.1, 5.0, and 4.9. b) An interpolated, 3-dimensional representation of the data.
independent data sets of the CD vs. pH at 255 nm. This wavelength was selected because it is where the largest change in the spectrum occurs. The value of \( n \) in the Henderson-Hasselbalch model was set to 1, which assumes that only a single proton transfer is involved in the reaction. The fitted pKa value is 5.7. Similar curve fitting was done for the CD vs. pH data at wavelengths spaced every 2.5 nm between 250 nm and 320 nm. In region I the average fitted pKa was 5.7 ± 0.2, and in both regions II and III the average fitted pKa's were 5.9 ± 0.4.

The change in the near-UV CD spectrum of reduced PC must be due to some pH dependent change in the tertiary conformation of PC. The fact that the change in the CD spectrum can be adequately modeled by the Henderson- Hasselbalch equation using a single pKa value and \( n \) equal to 1 indicates that the change in the protein conformation is a transition between two states: i.e., high and low pH conformations. This conclusion is also supported by the existence of an isodichroic point at 278 nm (Figure 31 on page 109). If stable intermediate conformations existed they probably would possess a unique CD spectrum and be detected as a deviation from the titration model.
Figure 32: pH dependence of the CD of reduced parsley PC at 255 nm. Three independent data sets are shown. The concentration is 50 μM and the cell pathlength is 1 cm. The solid line is the linear least squares fit to the three data sets.
The Reduced Copper Center Chromophore

The cause of the change in the CD spectrum of reduced PC can be determined by considering information gained about PC from a variety of different sources. From the analysis of the near-UV absorption of PC described in Chapter III, it is known that copper center of PC only contributes to the absorption spectrum when it is in the reduced oxidation state. Thus, it must be suspected that the change in the CD spectrum of reduced PC compared to that of oxidized PC is due to the addition of the reduced copper center chromophore. It was determined that the reduced copper center absorbs primarily in region I, but also absorbs in regions II and III. This corresponds with the finding that the largest change in the CD spectrum between oxidized and reduced PC, at both pH 7.0 and pH 5.0, occurs in region I, and that smaller changes occur in the other two regions. Likewise, the largest change in the CD spectrum of reduced PC due to the change in environmental pH also occurs in region I, with smaller changes in the other regions. These similarities between the near-UV CD and absorption spectra of PC indicate that the reduced copper center is a large contributor to the change in the CD spectrum between oxidized and reduced PC, and the pH dependent change of the spectrum of reduced PC. However, contributions of the aromatic amino acid residue chromophores to these changes in the CD spectrum can not be ruled out.

Once it is determined that the reduced copper center chromophore contributes to the CD spectrum of reduced PC, the question becomes: why
Is the CD spectrum of reduced PC pH dependent? The answer to this question can be found by examining information gained about PC from X-ray crystallography and NMR studies. As described in Chapter I, the conformation of the copper center in reduced poplar PC is dependent on the environmental pH (Guss et al., 1986). Figure 5 on page 10 shows that as the pH is lowered from pH 7.8 to pH 3.8 the coordination of the copper atom changes from pseudo-tetrahedral to trigonal coplanar. This change in coordination involves the severing of the Cu(I)-His 87 ligand bond, and the protonation of the histidine. Titration experiments of the NMR spectrum of reduced PC in solution have been done to determine the pKa values for the protonation of the histidine ligand, and thus the change in conformation of the reduced copper center (Markley et al., 1975; Kojiro & Markley, 1983). These pKa values are found to vary for different species of PC. In reduced parsley PC the pKa value is 5.7, and in reduced spinach PC the pKa value is 4.9 (Sykes, 1985).

The pKa value of 5.7 for the change in conformation of the reduced copper center is the same as the fitted pKa value for the change in the CD spectrum of reduced parsley PC in region I (Figure 32 on page 111). This important result indicates that the change in the CD spectrum of reduced PC in region I is due to the pH dependent change in conformation of the reduced copper center. The change in conformation is also likely responsible for the changes in the CD spectrum of reduced PC in regions II and III. This is because the reduced copper center absorbs in these regions, and the small difference between the average fitted pKa values for the three wavelength regions (0.2 pH units) could easily be due to experimental error.
That the pH-dependent change in conformation of the reduced copper center is responsible for the change in the CD spectrum of reduced PC is supported by two other observations described above. The first observation is that the change in the CD spectrum of reduced spinach PC occurs at a lower range of pH values than it does for reduced parsley PC. This can be explained by the fact that the pKa value for the change in the conformation of the reduced copper center is also lower in spinach PC (4.9 vs. 5.7). At pH 5.0, less than 45% of the reduced copper centers of reduced spinach PC would be in the low pH conformation. If the CD spectrum of reduced spinach PC has the same magnitude ratio of the high and low pH bands in region I as does that of reduced parsley PC (Figure 29 on page 103), then at pH 5.0 the CD spectrum of reduced spinach PC would be expected to have a positive CD band in region I, that is of smaller magnitude than the band at pH 7.0. As can be seen in Figure 30 on page 107, this is exactly what occurs in the CD spectrum of reduced spinach PC at pH 5.0. Since the CD spectra of reduced poplar and lettuce PC at pH 5.0 are similar to that of reduced spinach PC, these other two PC species can also be expected to have pKa values for the change in conformation of their reduced copper centers below pH 5.0. The second observation is that the pH dependent change in the CD spectrum follows a simple two state model. This corroborates the conclusion of Guss et al. (1986) that the change in the conformation of the reduced copper center of PC is a transition between two states, and is not a smooth transition with stable intermediates.
Transition Moment Assignment

The next question becomes: what are the identities of the electronic and magnetic transitions that cause the absorption and CD of the reduced copper center chromophore. As described in Chapters I and III, there are a variety of possible electronic transitions in the reduced copper center. The possibilities are a) electronic transitions between the atomic orbitals of the copper center (e.g., 3d→4s, Rydberg transitions), b) charge transfer transitions between the copper atom and any of the four ligands (His 37, Cys 84, His 87, and Met 92), and c) charge transfer transitions between the copper atom and the solvent.

To aid in the analysis, these transitions can be classified according to the effect that the pH-dependent change in conformation of the copper center has upon them. For example: the intensity of a Cu(I)→ligand charge transfer transition would likely be affected by a change in the distance between the copper and the ligand. Thus, the electronic transitions can be separated into two groups: those which are and are not affected by the change in conformation of the copper center.

The transitions which would definitely be affected by the change in conformation of the reduced copper center are Cu(I)→His 87 and Cu(I)→Met 92 charge transfer transitions. A Cu(I)→His 87 charge transfer transition could only exist in the high pH conformation, because this ligand bond is severed in the low pH conformation. Whereas, a Cu(I)→Met 92 charge transfer transition would likely only exist in the low pH conformation, because this ligand bond is of an
unusually large length in the high pH conformation (Guss et al., 1986). Electronic transitions localized at the copper atom and charge transfer transitions between the copper atom and the solvent would also likely be affected by the change in conformation of the copper center. The relative energies of the hybridized molecular orbitals of the copper atom would most likely be affected by the change in coordination geometry of the copper center from pseudo-tetrahedral to trigonal coplanar. Therefore, the energy and/or intensity of an electronic transition localized at the copper atom (e.g., Rydberg transition) would likely be affected by the change in conformation of the reduced copper center. The intensity of a Cu(I)→solvent charge transfer transition would also likely be affected by the change in location of the copper atom, and thus a change in the distance to the nearest solvent molecules. Conversely, Cu(I)→Cys 84 and Cu(I)→His 37 charge transfer transitions would not likely be affected by the change in conformation of the copper center, since the lengths of these ligand bonds are nearly unchanged (Guss et al., 1986).

The most obvious possibility is that the pH-dependent CD spectrum of reduced parsley PC is due to one or more of the pH-dependent electronic transitions (i.e. Cu(I)→His 87 & Met 92, Rydberg, and Cu(I)→solvent). One possible scenario is that the Cu(I)→His 87 charge transfer transition causes the positive band in region I of the CD spectrum of reduced PC at high pH. The lowering of pH would result in the breaking of the Cu(I)-His 87 bond, and thus eliminate the contribution of this transition from the spectrum. In addition, the Cu(I)→Met 92 charge
transfer transition may cause the negative band in region I of the CD spectrum of reduced PC at low pH. Raising the pH would eliminate the contribution of this transition from the spectrum. Alternatively, the Cu(I)→Met 92 charge transfer transition may cause the negative CD band in region III of the spectrum of reduced parsley PC at low pH. Optical activity, and thus CD, may be induced in these charge transfer transitions by the existence of an asymmetric electrically charged environment or by the coupling with other proximal transitions (Tinoco, 1962; Sears & Beychok, 1973).

One problem of assigning the pH-dependent bands in region I of the CD spectrum of reduced PC to Cu(I)→His 87 and Cu(I)→Met 92 charge transfer transitions is that corresponding changes are not observed in this region of the absorption spectrum. As described in Chapter III, region I of the near-UV absorption spectrum of reduced PC is nearly independent of the environmental pH. It is unlikely the the loss of absorption due to the elimination of the Cu(I)→His 87 charge transfer transition at low pH is exactly compensated for by the increase in absorption due to the Cu(I)→Met 92 charge transfer transition. A possible explanation would be if the optical activity of these transitions is magnetically allowed. The optical activity of an electronic transition is proportional to the dot product of its electric and magnetic transition moments (Cantor & Schimmel, 1980). A magnetically allowed transition is one in which the magnitude of the magnetic moment is much larger than the magnitude of the electric moment. In this case, the existence of these charge transfer transition would not be detected in the absorption spectrum of PC.
It is also possible that the pH-dependent CD spectrum of reduced PC is due to the pH-independent charge transfer transition (i.e. Cu(I)→Cys 84 and Cu(I)→His 37). Since the distances between the copper atom and these ligands are not significantly affected by the change in conformation of the reduced copper center, the electric transition moments, and thus absorption, of these transitions would not be changed. However, the optical activity of these charge transfer transition could still be affected by the change in conformation of the copper center. Although the bond lengths are unaffected, the movement of the copper atom between the high and low pH conformations relocates the Cu(I)-His 37 and Cu(I)-Cys 84 bonds in space. This could result in changes of the charged environments and the coupling with other transitions, which would affect the optical activity.

Another possibility is that the movement of the copper atom alters the relative orientations of the electric and magnetic moments of the charge transfer transitions. As noted above, the optical activity of an electronic transition is proportional to the relative orientations of its electric and magnetic transition moments. This change in relative orientation could be caused by torsional forces applied to the ligand bonds, or by the change in molecular orbital structure between the two coordination states of the copper center. Therefore, since the optical activity of the Cu(I)→His 37 and Cu(I)→Cys 84 charge transfer transitions could be altered without affecting the magnitudes of their electrical moments, these transition could be responsible for both the pH-dependent CD spectrum and the pH-independent absorption spectrum of reduced PC.
The possibility that a Cu(I)$\rightarrow$Cys 84 charge transfer transition contributes to the CD spectrum of reduced PC is supported by the comparison with the CD spectra of the Cu(I) metallothionein proteins (Waser et al., 1977; Beltramini & Lerch, 1983). These metallothionein proteins contain multiple copper atoms, which are ligated by cystein residues in a pseudo-tetrahedral geometry. Figure 33 on page 120 displays the near-UV CD spectra of yeast and Neurospora Metallothionein, and the comparison with reduced parsley PC at pH 7.0. Although there is a shift in the spectra of the two metallothionein species, their CD spectra can be loosely described as having positive bands in regions I and III, and negative bands in region II. By default, the near-UV CD spectra of the Cu(I) metallothioneins is assigned to Cu(I)-Cys ligand charge transfer transitions, since there are no other chromophores in these proteins which absorb in this region. It is observed that the CD spectrum of reduced PC at pH 7.0 is most similar to that of yeast Cu(I) metallothionein. The differences in the peak wavelength maxima of these two spectra may be due to small differences in the coordination geometries of the copper centers. It should be noted, however, that this comparison does not rule out the possibilities that copper atomic orbital transitions or electronic coupling between the copper center and Phe 35 and Tyr 62 (in parsley) contribute to the altered CD spectra of reduced PC.
Figure 33: Near-UV CD of Cu(I) metallothionein and reduced PC at neutral pH. a) Yeast (-----) and Neurospora (-----) metallothioneins. b) yeast (-----) and reduced parsley PC (-----) at pH 7.0.
CHAPTER V
THE EFFECT OF PH ON THE FLUORESCENCE OF REDUCED PC

Fluorescence spectroscopy was undertaken to further elucidate the effects of changes in environmental pH on the tyrosine residues in PC. The studies were done on spinach PC, which has three tyrosine residues. The only chromophores in PC which could conceivably contribute to the fluorescence spectrum are the tyrosine residues and the copper center. It is fortunate that there are no tryptophane residues in PC, since their fluorescence would overshadow that of the tyrosine residues. The fluorescence yield of tyrosine is sensitive to the electrical polarity of the environment, and to the hydrogen bonding of its hydroxyl proton. Thus, the fluorescence spectrum is a good potential indicator of conformational changes in PC.

Figure 34 on page 122 shows the excitation and emission spectra of reduced spinach PC at pH 7.0, pH 5.0, and pH 4.3. The fluorescence yield of reduced PC increases with the reduction of pH. The excitation spectra have peaks at ca. 282 nm, and the emission spectra have peaks at ca. 305 nm. These bands are typical of what is observed for tyrosine residues in proteins. Therefore, there is no indication of fluorescence from the reduced copper center chromophore in this wavelength region.
Fluorescence of reduced spinach PC at three pH values. pH 7.0 (-----), pH 5.0 (- - -), pH 4.3 (......). a) emission spectra (278 nm excitation); b) excitation spectra (305 nm emission).
Figure 35 on page 124 shows the pH titration behavior of the fluorescence of reduced spinach PC at 305 nm. The data were fit with the Henderson-Hasselbalch model (12) on page 35 with the n parameter set to 1. The pKa of the fitted curve is 4.6. There is some uncertainty in the fit to the data due to the inability to obtain points at lower pH values, where rapid PC denaturation occurs. In the figure, the fitted curve extends above the data point at pH 4.3. If this data point were in reality the maximum extent of the increase in magnitude of the fluorescence, then a steeper curve, with a greater n value, would be required to fit the data.

It should be observed that the fitted pKa value for the change in fluorescence is similar to the intrinsic pKa value for the protonation of a glutamate residue (ca. 4.5). Therefore, the change in tyrosine fluorescence yield observed for PC is likely due to the protonation of carboxyl groups in glutamate residues at low environmental pH. It is known that the fluorescence yield of a tyrosine residue in a protein is increased by the reduction of environmental polarity, and is decreased by the involvement of the hydroxyl proton in a hydrogen bond. One possibility is that one or more of the tyrosine residues is hydrogen bonded to a glutamate residue. At low pH the glutamate residue would be protonated, thus breaking the hydrogen bond and causing an increase in fluorescence yield. Contrary to this hypothesis, however, is the observation that the band-width of the emission spectra are unaffected by the change in pH. Figure 36 on page 126 compares the shape of the emission spectra displayed in Figure 34 on page 122, by scaling the
Figure 35: Effect of pH on the tyrosine fluorescence of reduced spinach PC. The solid line is the fit to the data.
peaks to the same arbitrary height. There is some evidence that hydrogen bonding causes a widening of the tyrosine emission band by ca. 3 nm (Lux et al., 1985).

Another possibility is that the protonation of the glutamate residues at low pH results in a decrease in the charged environment felt by the tyrosine residues. This elimination of charges would reduce the effective polarity of the environment, and thus cause an increase in the tyrosine fluorescence yield. It has been observed for a variety of cases that the fluorescence of a tyrosine residue can be quenched by the presence of charged functional groups nearby in the protein (Edelhoch et al., 1968).

Still another possibility is that the fluorescence of the tyrosine residues is quenched by energy transfer to the reduced copper center. As seen in Figure 13 on page 61 and Figure 14 on page 62, the reduced copper center provides a broad, but weak, absorption band in region III of the spectrum, which overlaps the tyrosine fluorescence band. In this case, it would be expected that the pH-dependent change in conformation of the reduced copper center would affect the degree of energy transfer, and thus the quantum yield of the tyrosine fluorescence. However, in spinach PC the pKa value for the change in fluorescence is different from the pKa value for the change in conformation of the reduced copper center (4.6 versus 4.9). Yet, this does not rule out the possibility of this type of quenching, because energy transfer is dependent on the relative orientation of the
Figure 36: Effect of pH on the emission band shape of reduced spinach PC. pH 7.0 (—), pH 5.0 (— — —), and pH 4.3 (…….). The peaks were scaled to the same height.
electronic transitions and the change in fluorescence could be due to a pH dependent change in the conformations of the tyrosine residues.

Since the fluorescence yield of tyrosine is sensitive to the formation of a hydrogen bond, it makes a good test of the hypothesis developed in Chapter III that Tyr 83 is hydrogen bonded to an acidic residue at the east face site. If Tyr 83 is hydrogen bonded at high pH, there should be a corresponding reduction of the fluorescence yield with the same pH dependence. The change in absorption of reduced spinach PC was found to titrate with a pKa value of 5.4 (Figure 19 on page 74). From Figure 35 on page 124 it can be seen that there is indeed a change in the fluorescence yield of PC in the pH 5.0 to pH 6.0 region, which contains the absorption pKa value. The magnitude of the change in fluorescence is significantly smaller in the pH 6.0 to pH 5.0 region than in the pH 5.0 to pH 4.0 region. This could be due to the situation where the fluorescence of only Tyr 83 is affected in the former region, and the fluorescence of all of the tyrosine residues is affected in the latter region. This would lead to the interpretation that the change in fluorescence of PC is due to two pH dependent events: the hydrogen bonding of Tyr 83 with a pKa value of 5.4, and the change in environmental polarity of all of the tyrosine residues with a pKa value somewhere between 4.5 and 5.0. The invariance of the emission band-width of PC to pH (Figure 36 on page 126) could be explained by the small expected change in the fluorescence band-width of Tyr 83 being overshadowed by the fluorescence of the other two tyrosine residues in the protein.
CHAPTER VI
FACTOR ANALYSIS OF THE NEAR-UV ABSORPTION OF PC

This Chapter describes the use of mathematical factor analysis to separate the components in the near-UV absorption spectrum of PC. In Chapter III it was shown that the near-UV absorption spectrum of PC is heavily superimposed, with contributions from the phenylalanine, tyrosine, and copper center chromophores. The absorption of the copper center and Tyr 83 were also shown to be dependent on the oxidation state of the protein and on the environmental pH. It would be desirable to quantitatively separate the contributions of these two chromophores from the absorption spectrum of PC, because they are located at the north pole and east face reaction sites (see Figure 7 on page 13). From the responses of the absorption of the copper center and Tyr 83 to changes in oxidation state and environmental pH, information could be gained about the states of the reaction sites.

The factor analysis is conducted with bilinear, trilinear, and quadrilinear models of the absorption of PC. Traditionally, factor analysis has only utilized bilinear models. However, these models often do not provide for a unique separation of the components. Recently, research has been conducted on the use of the higher order,
trilinear and quadrilinear models for factor analysis. These models have the advantage that they do provide for a unique separation of the components, as long as the independent variables are non-interacting. This report is the first to use the trilinear and quadrilinear models for the factor analysis of the absorption of a protein. For this reason, considerable space is provided to show how the models are constructed.

The bilinear, trilinear, and quadrilinear models utilize two, three, and four independent variables, respectively. The factor analysis procedures determine the components in the absorption data which respond uniquely to at least two of the independent variables. For this reason, variables were chosen which distinguish the the absorption of the copper center and the tyrosine residues. The data set used for the factor analysis was the absorption of PC as a function of the light wavelength variable and three chemical-condition variables. The chemical-condition variables were the plant species of PC, the oxidation state of the copper center, and the environmental pH. The wavelength variable was selected because the spectral components likely have different absorption spectra, and it is desirable to know what these spectra are. The plant species variable was selected because the different plastocyanins have different numbers of tyrosine residues (2 in poplar PC, 3 in spinach and lettuce PC, and 4 in parsley PC), and this would help to distinguish the absorption of the tyrosine residues. The oxidation state variable was selected to distinguish the absorption of the copper center. The environmental pH variable was selected to
distinguish the variable absorption of Tyr 83 and the reduced copper center.

**CONSTRUCTION OF THE BILINEAR, TRILINEAR, AND QUADRILINEAR MODELS**

The absorption of light is commonly modeled as a bilinear product of two independent parameters: the extinction coefficient and the concentration of a chromophore. For a sample with \( F \) different chromophores, Beer's law can be expressed as

\[
a_i = D \sum_{j=1}^{F} \varepsilon_y c_j
\]

where \( a_i \) is the absorption of the sample at wavelength \( i \), \( \varepsilon_y \) is the extinction coefficient of the \( f \) chromophore at wavelength \( i \), \( c_j \) is the concentration of the \( f \) chromophore, and \( D \) is the pathlength of the light beam through the sample.

In a protein, the extinction coefficients and spectral forms of the chromophores may be affected by interactions with their local environments. In turn, these interactions may be affected by conformational changes of the protein, or by the direct influence of changes in the chemical condition of the protein and/or the surrounding solvent. In the models discussed here, a chromophore with multiple spectral forms is treated as if it consisted of multiple chromophores with concentrations dependent on the chemical conditions. If the effects of different chemical-condition variables do not interact, then
the chromophore concentrations can be separated into the product of terms representing the effects of each chemical-condition variable (i.e. a separation of variables). Taking into account the variation of one, two, and three non-interacting chemical-condition variables, the concentration term in (34) on page 130 can be expressed as

\[ c_{ij} = x_{ij} \]

or

\[ c_{ijk} = x_{ij}y_{jk} \]

(35)

or

\[ c_{ijk} = x_{ij}y_{jk}z_{ik} \]

Substituting (35) into (34) on page 130 forms bilinear, trilinear, and quadrilinear models for the absorption measured as a function of two, three, and four independent variables, as indicated by i, j, k, and l.

\[ a_{ij} = D \sum_{r=1}^{F} e_{ij}x_{ij} \]

\[ a_{ijk} = D \sum_{r=1}^{F} e_{ij}x_{ij}y_{jk} \]

(36)

\[ a_{ijk} = D \sum_{r=1}^{F} e_{ij}x_{ij}y_{jk}z_{ik} \]

The third model in (36) was proposed by Harshman (1970) and by Carroll & Chang (1970), and is referred to as a Parafac (Parallel Factors) or CANDECOMP (Canonical Decomposition) model in the psychometric
literature (Kroonenberg, 1983a,b; Law, et al., 1984; Kruskal, 1983).

The first and second models of (36) on page 131 can be thought of as two and four variable equivalents, respectively, of the PARAFAC-CANDECOMP model.

Up to this point, the absorption has been described as the sum of contributions of all of the chromophores. However, some of the chromophores may be indistinguishable in terms of their spectra and/or the effects of the chemical-condition variables on their concentrations. To be distinguishable, the absorption of the chromophores must respond uniquely to at least two of the independent variables (Kruskal, 1977). Therefore, the components which are definable in the models of (36) on page 131 must differ in respect to at least two of the independent variables, and may be the superpositions of indistinguishable chromophores. Thus, the number of independent components in the data may be the same as the number of chromophores in the protein, but may also be less.

The first model in (36) on page 131 written in matrix notation becomes

\[ A_{2\text{-way}} = DX^t \]  \hspace{1cm} (37)

where \( E \) is an \( I \) by \( F \) matrix, \( X \) is a \( J \) by \( F \) matrix, \( I \) and \( J \) are the total number of different absorption wavelengths and chemical conditions, \( F \) is redefined as the number of independent components, and \( t \) represents the matrix transpose operation. The column vectors of \( E \)
are the wavelength-dependent extinction coefficients of the components (i.e., the spectra of the components) and the column vectors of X are the chemical-condition-dependent concentrations of the components.

The "fundamental indeterminacy" of bilinear models (Kruskal, 1977, 1983) can be seen by the fact that $A_{2\text{-way}}$ is unaffected by the transformation of E and X by any nonsingular F by F matrix $T$ and its inverse $T^{-1}$, respectively.

$$A_{2\text{-way}} = D(E'T)(T^{-1}X't) = D'E'X't$$

where $E'$ and $X'$ are the transformed matrices of E and X. The transformation of E and X affects both the shape of the spectra and the responses to the chemical condition of the components in the model. Thus, the bilinear model does not uniquely define the spectra or the responses to the chemical condition of the real absorption components in the data.

The second and third models in (36) on page 131 can be expressed as the sum of the Kronecker products of the component vectors (Callis, 1984),

$$A_{3\text{-way}} = D \sum_{f=1}^{F} E_f \times X_f \times Y_f$$

and

$$A_{4\text{-way}} = D \sum_{f=1}^{F} E_f \times X_f \times Y_f \times Z_f$$
where the subscripted $f$ indicates the column vectors of the $I$, $J$, $K$, and $L$ by $F$ matrices $E$, $X$, $Y$, and $Z$, respectively, and $\times$ denotes the Kronecker product. The "intrinsic axis property" of these models is seen by the fact that the only possible transformations which do not affect $A_{3 \times m}$ and $A_{4 \times n}$ are permutation and scalar multiplication of the component vectors (Kruskal, 1977, 1983). These transformations would affect the order and lengths of the component vectors, but not their orientations in euclidean space (Kruskal, 1977, 1983). Thus, the spectra and chemical-condition-dependent responses of the real components in the data are defined by the trilinear and quadrilinear models.

**ANALYSIS OF THE RESIDUALS**

Figure 37 on page 135 displays the RMS residuals for the bilinear, trilinear, and quadrilinear models with 1 to 5 components. All of the 1 and 2-component models have an RMS residual larger than the maximum error in the PC absorption spectra (0.3 mM$^{-1}$cm$^{-1}$). The RMS residuals for the trilinear and quadrilinear models are not significantly affected by the application of the non-negativity constraint. To a first approximation, the curves are biphasic, with cusps occurring for the models with three components. For models with the same number of components, the quality of the fit improves in the order of the quadrilinear, trilinear, and bilinear models.
Figure 37: RMS residual vs. number of components for the different models. Bilinear model (—); trilinear model (—); quadrilinear (—–) model. The trilinear and quadrilinear models were derived with the non-negativity constraint.
Figure 38 on page 137, Figure 39 on page 138, and Figure 40 on page 139 displays the original minus fitted values, residual matrices of the 2 and 3-component bilinear, trilinear, and quadrilinear models. The chemical condition variables of the trilinear and quadrilinear models have been combined to form 2-way matrices to enable the graphic representations of the residuals of these models. The change from two to three components in the bilinear, trilinear, and quadrilinear models results in randomization of the residuals in the 250-296 nm wavelength region. However, a systematic deviation is still seen in the residuals in the 296-320 nm region. It should be noted, though, that the magnitude of the absorption of PC in this latter region of the spectrum is relatively small (see Figure 13 on page 61 and Figure 14 on page 62), and the magnitudes of the residuals are generally less than the maximum error in the spectra.

Residual analysis is important for determining whether a model accurately represents the data and for determining the number of independent components. Two important criteria are that the distribution of the residuals are random and that the RMS residual is less than the maximum error in the spectra. Two main results indicate that there are three independent components in the data: (a) Three components are required to reduce the RMS residuals below the maximum magnitude of error in the absorption spectra; (b) the rate of RMS residual reduction with the incorporation of greater numbers of components is biphasic, and decreases for more than 3 components in the model (Figure 37 on page 135). The fact that the same results occur in
Figure 38: Residual matrices for the bilinear models. 2-component (top); 3-component (bottom).
Figure 39: Residual matrices for the trilinear models. 2-component (top); 3-component (bottom).
Figure 40: Residual matrices for the quadrilinear models.  
2-component (top); 3-component (bottom).
the bilinear, trilinear, and quadrilinear models is further
confirmation. The first, rapid phase of the RMS residual reduction is
interpreted as fitting of both the real components and the noise in the
data: the second, slow phase is interpreted as fitting of only the
noise.

For two components to be resolvable, they must be distinguished by
unique responses to at least two of the independent variables (Kruskal,
1977), with the magnitude of this distinction being greater than the
error (0.3 mM⁻¹cm⁻¹). In a bilinear model the components must have
different spectra and be affected differently by a change of the
chemical-condition variable (either in PC species, oxidation state, or
pH). In the trilinear and quadrilinear models, two distinguishable
components may have the same spectra if they are affected differently
by two of the independent chemical-condition variables.

Factor analysis using trilinear and quadrilinear models was
undertaken to determine whether the PC species, oxidation state, and
environmental pH variables are non-interacting, and if so, to exploit
the intrinsic axis property of these models to uniquely define the
components (see previous section). For the models with the same number
of components, the progressive increase of the RMS residuals for the
bilinear, trilinear, and quadrilinear models indicates that the PC
species, oxidation state, and pH variables do interact. It is clear
that the absorption wavelength variable (i.e., the setting of the
monochrometer) does not interact with the chemical-condition variables.
However, the fact that the increase of the RMS residual is relatively small, and that similar components occur in the three types of models (see following sections), indicate that these variables are sufficiently non-interactive to validate the use of the trilinear and quadrilinear models to separate the components in the absorption of PC.

**BILINEAR FACTOR ANALYSIS**

Figure 41 on page 143 displays the first three wavelength and chemical-condition SVD basis vectors of the 2-way absorption data array. The vectors are normalized to a euclidean length of 1. The singular values corresponding to these basis vectors are 124.90, 11.39, and 4.47, and the sum of all 16 singular values is 148.53. The first wavelength basis vector is the average of the 16 PC absorption spectra, and depicts recognizable features of the data (see Figure 13 on page 61 and Figure 14 on page 62). Evident in this vector is the tyrosine absorption band with the characteristic peak at 278 nm and shoulder at 284 nm (Donovan, 1969). The small absorbance above 290 nm is not due to tyrosine, but is attributed to absorption of the copper center (Drahaim et al., 1986). The magnitude in the 250-265 nm region is due to the combination of absorption from the copper center, the tyrosine residues, and the phenylalanine residues. Absorption from the n-π* transitions of the peptide bonds may also contribute to this region. The fine structure in this region is due to the absorption of the phenylalanine residues (Drahaim et al., 1986). The second basis vector has a negative peak at 280 nm and a shoulder at 286 nm, both of which
are slightly red-shifted from the corresponding structures in the first basis vector. The third basis vector is less regular than the others, with oscillations occurring over relatively short wavelength intervals.

Figure 42 on page 144 displays the least squares fit of linear combinations of the first two and three wavelength basis vectors to the tyrosine and Neurospora metallothionein absorption targets. For both targets, including the third basis vector only slightly improves the quality of fit. The tyrosine target spectrum has been red-shifted 2.0 nm from the spectrum of tyrosine in water to have a wavelength maximum at 280 nm. This shift results in a 60% reduction of the RMS residuals for the two basis vector fit. The fitted tyrosine spectra show the characteristic tyrosine absorption peak and shoulder at 278 nm and 284 nm, respectively. The basis vectors reproduce the general form of the Neurospora metallothionein target spectrum; however, there are significant deviations in the fine structure of these spectra. The fitted spectra do not have the peak maximum at 252 nm of the target spectrum, but instead have small peaks at 276 nm and 284 nm.

The attempt to determine the wavelength and chemical-condition-dependent component vectors by linear combinations of the first two and three SVD basis vector pairs using only the normalization and non-negativity constraints results in a broad range of possible solutions (not shown). The variation is too large to derive any conclusions about the components. The additional use of one of the two
Figure 41: First three SVD basis vectors of the bilinear data array. First (-----); second (.....); third (.....). The vectors are normalized to have an euclidean norm of 1. a) Wavelength-dependent vectors. b) Chemical-condition-dependent vectors.
Figure 42: 2 and 3 wavelength basis vector fits to the absorption targets. First 2 (---); first 3 (.....). The spectra are normalized to have an euclidean length of 1. a) Tyrosine target; b) yeast metallothionein target.
absorption target criteria still does not provide for an interpretable range of solutions. For example: Figure 43 on page 146 displays the range of possible values for the components of a two basis vector pair transformation using the tyrosine target, normalization, and the non-negativity constraints. In this case, only a single element of the transformation matrix is not fixed. The variation in the spectrum of the second component is characterized by the change of magnitude and the existence of peaks and fine structure in the 250-300 nm region (most notably the peak at 278 nm). The associated variation in the chemical-condition-dependent vector of the tyrosine component (not shown) is characterized by the change from greater magnitude in oxidized PC than reduced PC to greater magnitude in reduced PC than oxidized PC. In contrast to the 2-component case, totally non-negative components could not be determined for a 3-component bilinear model using both the tyrosine and Neurospora metallothionein targets.

To have a bilinear model for analysis, a 2-component bilinear model was formed by using both the tyrosine and Neurospora metallothionein absorption targets. Since all of the elements of the transformation matrix are defined by the targets this model is referred to as the "fixed bilinear model." The wavelength-dependent component vectors of the fixed bilinear model are the same as the two SVD basis vector fits to the targets shown in Figure 42 on page 144. Table 5 on page 148 shows the predicted extinction coefficients at selected wavelengths for the two components of the fixed bilinear model in the 16 PC absorption spectra. The extinction of the tyrosine component varies among the
Figure 43: Example of the indeterminacy of bilinear models. The wavelength dependency of the first component is fixed by the tyrosine absorption target. The solid lines represent the range of the second component. The vectors have arbitrary euclidean length.
four PC species according to the number of tyrosine residues in each (2 in poplar PC, 3 in spinach and lettuce PC, and 4 in parsley PC). This can especially be seen in the <TYR> column of Table I, in which the average tyrosine extinction coefficient for a particular oxidation state and pH shows little variation for the different PC species. For each PC species, the extinction coefficient of the tyrosine component is significantly greater in the reduced state at pH 7.0 than for the other three combinations of oxidation state and pH, for which there are smaller variations. The metallothionein component is characterized by a greater magnitude in reduced PC than in oxidized PC, with smaller variations in each oxidation state due to changes of pH and PC species. Even in the oxidized state, however, the metallothionein component contributes significantly to the absorption of PC in the main tyrosine absorption region (265-290 nm). Using the prediction for oxidized spinach PC at pH 7.0 as an example, the metallothionein component contributes 24% to the net absorption at 278 nm.

Using the spectra of spinach PC as an example, Figure 44 on page 150 displays the effects of oxidation state and pH on the components of the fixed bilinear model. At pH 5.0, the increase in extinction of the PC spectrum upon reduction is due solely to the increase of the metallothionein component: the reduced minus oxidized difference extinction coefficients at 278 nm of the tyrosine and metallothionein components are -0.1 and 2.3 mM\(^{-1}\)cm\(^{-1}\), respectively. At pH 7.0 the tyrosine component contributes 15% to the increase in the PC spectrum upon reduction: the reduced minus oxidized difference extinction
Table 5

Predictions of the Fixed Bilinear Model

The values are the extinction coefficients (mM$^{-1}$cm$^{-1}$) of the Tyrosine (TYR) and Metallothionein (MT) Components.

<table>
<thead>
<tr>
<th>PC species</th>
<th>TYR 278 nm</th>
<th>&lt;TYR&gt;</th>
<th>MT 278 nm</th>
<th>TYR 260 nm</th>
<th>MT 260 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poplar</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ox5</td>
<td>2.7</td>
<td>1.3</td>
<td>2.1</td>
<td>0.9</td>
<td>3.2</td>
</tr>
<tr>
<td>ox7</td>
<td>2.4</td>
<td>1.2</td>
<td>1.7</td>
<td>0.8</td>
<td>2.6</td>
</tr>
<tr>
<td>red5</td>
<td>2.6</td>
<td>1.3</td>
<td>4.6</td>
<td>0.9</td>
<td>7.1</td>
</tr>
<tr>
<td>red7</td>
<td>3.4</td>
<td>1.7</td>
<td>4.5</td>
<td>1.2</td>
<td>6.9</td>
</tr>
<tr>
<td>Spinach</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ox5</td>
<td>4.3</td>
<td>1.4</td>
<td>1.8</td>
<td>1.5</td>
<td>2.8</td>
</tr>
<tr>
<td>ox7</td>
<td>4.6</td>
<td>1.5</td>
<td>1.4</td>
<td>1.6</td>
<td>2.2</td>
</tr>
<tr>
<td>red5</td>
<td>4.2</td>
<td>1.4</td>
<td>4.1</td>
<td>1.5</td>
<td>6.3</td>
</tr>
<tr>
<td>red7</td>
<td>5.1</td>
<td>1.7</td>
<td>4.3</td>
<td>1.8</td>
<td>6.6</td>
</tr>
<tr>
<td>lettuce</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ox5</td>
<td>4.0</td>
<td>1.4</td>
<td>1.9</td>
<td>1.4</td>
<td>2.9</td>
</tr>
<tr>
<td>ox7</td>
<td>3.9</td>
<td>1.3</td>
<td>1.7</td>
<td>1.4</td>
<td>2.6</td>
</tr>
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<td>4.1</td>
<td>1.4</td>
<td>4.5</td>
<td>1.4</td>
<td>6.9</td>
</tr>
<tr>
<td>red7</td>
<td>4.5</td>
<td>1.5</td>
<td>4.1</td>
<td>1.6</td>
<td>6.3</td>
</tr>
<tr>
<td>parsley</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ox5</td>
<td>6.1</td>
<td>1.5</td>
<td>1.6</td>
<td>2.2</td>
<td>2.5</td>
</tr>
<tr>
<td>ox7</td>
<td>5.7</td>
<td>1.4</td>
<td>1.8</td>
<td>2.0</td>
<td>2.7</td>
</tr>
<tr>
<td>red5</td>
<td>5.7</td>
<td>1.4</td>
<td>4.6</td>
<td>2.0</td>
<td>7.1</td>
</tr>
<tr>
<td>red7</td>
<td>7.7</td>
<td>1.9</td>
<td>5.2</td>
<td>2.7</td>
<td>8.1</td>
</tr>
</tbody>
</table>

*The <TYR> label denotes the average extinction coefficients of the tyrosine residues determined by dividing by the value for TYR by the number of tyrosine residues in that PC species (2 in poplar PC, 3 in spinach and lettuce PC, and 4 in parsley PC).*
coefficients at 278 nm of the tyrosine and metallothionein components are 0.5 and 2.9 mM$^{-1}$cm$^{-1}$, respectively. A similar pattern occurs for the other species. In the spectra of poplar, lettuce, and parsley PC at pH 7.0 the tyrosine component contributes 27, 20, and 37%, respectively, to the total increase of extinction at 278 nm upon reduction. In the 250-265 nm region of the PC spectrum the increase of extinction is due primarily to the increase of the metallothionein component at both pH 5.0 and pH 7.0.
Figure 44: Fixed bilinear model of spinach PC absorption. Original spectrum (---), predicted spectrum (-----), tyrosine component (---), and metallothionein component (-----). a) Oxidized PC, pH 5.0; b) oxidized PC, pH 7.0; c) reduced PC, pH 5.0; and d) reduced PC, pH 7.0.
TRILINEAR AND QUADRILINEAR FACTOR ANALYSIS

2-Component Models.

Figure 45 on page 152, Figure 46 on page 153, Figure 47 on page 154, and Figure 48 on page 155 show the wavelength and chemical-condition dependencies of the 2-component trilinear and quadrilinear models with and without the non-negativity constraint. These are the sets of vectors which are multiplied together to form the predicted components of the models (This procedure is described in Chapter II). Without the non-negativity constraint, the spectra of the tyrosine components in the 2-component trilinear and quadrilinear models are negative in the 250-255 nm wavelength region. The fact that the non-negativity constraint changes the spectra and extinction coefficients of the components of the trilinear and quadrilinear models without significantly altering the RMS residuals indicates that the components are not completely defined by the models. This topic was already discussed in the residual analysis section of this Chapter. The indeterminacy of the components is due to the error in the PC spectra and the interaction among the effects of the variables.

With the non-negativity constraint, the spectral forms of the components of the 2-component trilinear and quadrilinear models are nearly identical to the components of the fixed bilinear model. Since the components of the fixed bilinear model are derived from the tyrosine and metallothionein absorption targets, the components of these models are designated as the "tyrosine" and "MT" components. The
Figure 45: 2-component trilinear model without non-negativity constraint. Tyrosine (——) and MT (-----) parameter vectors a) wavelength dependency, b) species dependency, c) combined oxidation state and pH dependency.
Figure 46: 2-component trilinear model with non-negativity constraint. Tyrosine (— - -) and MT (— — —) parameter vectors. a) wavelength dependency, b) species dependency, c) combined oxidation state and pH dependency.
Figure 47: 2-comp. quadrilinear model without non-negativity constraint. Tyrosine (——) and MT (---------) parameter vectors. a) wavelength dependency, b) species dependency, c) oxidation state dependency, and d) pH dependency.
Figure 48: 2-comp. quadrilinear model with non-negativity constraint. Tyrosine (---) and MT (-----) parameter vectors. a) wavelength dependency, b) species dependency, c) oxidation state dependency, and d) pH dependency.
extinction coefficients of the components in the constrained trilinear and quadrilinear models are also similar to those of the fixed bilinear model, and respond similarly to the changes of the PC species, oxidation state, and pH variables (data not shown). In particular, (a) the magnitude of the tyrosine component corresponds to the number of tyrosine residues in each PC species; (b) the magnitude of the tyrosine component is largest in the spectra of reduced PC at pH 7.0; (c) the magnitude of the MT component is much larger in the spectra of reduced PC than of oxidized PC. Unlike the fixed bilinear model, in the quadrilinear model the magnitude of the tyrosine component in the spectra of reduced PC at pH 5.0 is closer to that observed in the spectra of reduced PC at pH 7.0 than to that in the spectra of oxidized PC.

3-Component Models.

Figure 49 on page 157, Figure 50 on page 158, and Figure 51 on page 159 show the wavelength and chemical-condition dependencies of the 3-component trilinear and quadrilinear models with and without the non-negativity constraint. The vectors of the quadrilinear model come out completely non-negative, and therefore, do not require the constraint. The wavelength dependent, spectral forms of the components in the two 3-component models are very similar to each other. However, in the trilinear model, the non-negativity constraint shifts the predicted contribution of the MT component in the spectra of parsley PC to zero in the trilinear model. This is considered an invalid result, and the 3-component trilinear model is not considered further.
Figure 49: 3-component trilinear model without non-negativity constraint. Tyrosine (-----), MT (-----), and 3rd (-----) parameter vectors. a) wavelength dependency, b) species dependency, c) combined oxidation state and pH dependency.
Figure 50: 3-component trilinear model with non-negativity constraint. Tyrosine (——), MT (— —), and 3rd (……) parameter vectors. a) wavelength dependency b) species dependency, c) combined oxidation state and pH dependency.
Figure 51: 3-comp. quadrilinear model without non-negativity constraint. Tyrosine (-----), MT (-----), and 3rd (-----) parameter vectors. a) wavelength dependency b) species dependency, c) oxidation state dependency, and d) pH dependency.
The components of the 3-component quadrilinear model (Figure 51 on page 159) are designated as the tyrosine, MT, and 3rd components, based on the correspondence to the 2-component models. In particular, the spectrum of the tyrosine component in this model is nearly identical to that of the tyrosine components in the 2-component models. The spectrum of the MT component has the same shoulders at 278 and 284 nm as in the 2-component models, but drops sharply at 292 nm, to form a second broad band in the 294-320 nm region. The spectrum of the 3rd component appears to be a mixture of the tyrosine and MT components, showing a sharp decrease in the 250-260 nm region (similar to the MT component) and a peak and shoulder at 280 and 286 nm (which are red-shifted 2.0 nm from the spectrum of the tyrosine component). The wavelength maxima of these peaks correspond to the wavelength minima of the peaks in the second SVD basis vector (Figure 41 on page 143).

Table 6 on page 162 shows the predicted extinction coefficients at selected wavelengths of the three components of the 3-component quadrilinear model. The extinction coefficients of both the MT and 3rd components are considerably greater for reduced PC than for oxidized PC. In each oxidation state the magnitude of the MT component is greater at pH 5.0 than at pH 7.0, and the magnitude of the 3rd component is greater at pH 7.0 than at pH 5.0. The effects of oxidation state and pH changes on the magnitude of the tyrosine component are significantly different from that observed for the fixed bilinear model. In particular, the magnitude of the tyrosine component is larger in the spectra of oxidized PC than of reduced PC, and in each
oxidation state, larger at pH 5.0 than at pH 7.0. However, the
response of the sum of the tyrosine and 3rd components is similar to
that of the tyrosine component in the fixed bilinear model.

Using the spectra of spinach PC as an example, Figure 52 on page 163
displays the effects of the oxidation state and pH on the components of
the 3-component quadrilinear model. At both pH 5.0 and pH 7.0, the
increase in extinction of the PC spectrum upon reduction is due to the
combined increase of the MT and 3rd components. The reduced minus
oxidized difference extinction coefficients at 278 nm of the MT and 3rd
components are 1.6 and 2.4 mM⁻¹cm⁻¹ at pH 5.0, and 1.2 and 3.7 mM⁻¹cm⁻¹
at pH 7.0. Reduction of PC results in a decrease of extinction of the
tyrosine component. The magnitude of this decrease is the same at pH
5.0 as it is at pH 7.0. In the spectra of poplar, spinach, lettuce,
and parsley PC at pH 7.0 the reduced minus oxidized difference
extinction coefficients at 278 nm of the tyrosine component are -0.8,
-1.7, -1.5, and -2.2 mM⁻¹cm⁻¹.
Table 6

Predictions of the 3-Component Quadrilinear Model

The values are the extinction coefficients (mM$^{-1}$cm$^{-1}$) of the Tyrosine (TYR), Metallothionein (MT), and 3rd Components

<table>
<thead>
<tr>
<th>Wavelength</th>
<th>278 nm</th>
<th>260 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC species</td>
<td>TYR</td>
<td>&lt;TYR&gt;</td>
</tr>
<tr>
<td>Poplar</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ox5</td>
<td>2.1</td>
<td>1.0</td>
</tr>
<tr>
<td>ox7</td>
<td>1.9</td>
<td>0.9</td>
</tr>
<tr>
<td>red5</td>
<td>1.1</td>
<td>0.6</td>
</tr>
<tr>
<td>red7</td>
<td>1.0</td>
<td>0.5</td>
</tr>
<tr>
<td>Spinach</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ox5</td>
<td>4.1</td>
<td>1.4</td>
</tr>
<tr>
<td>ox7</td>
<td>3.7</td>
<td>1.2</td>
</tr>
<tr>
<td>red5</td>
<td>2.2</td>
<td>0.7</td>
</tr>
<tr>
<td>red7</td>
<td>2.0</td>
<td>0.7</td>
</tr>
<tr>
<td>lettuce</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ox5</td>
<td>3.6</td>
<td>1.2</td>
</tr>
<tr>
<td>ox7</td>
<td>3.3</td>
<td>1.1</td>
</tr>
<tr>
<td>red5</td>
<td>2.0</td>
<td>0.7</td>
</tr>
<tr>
<td>red7</td>
<td>1.8</td>
<td>0.6</td>
</tr>
<tr>
<td>parsley</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ox5</td>
<td>5.3</td>
<td>1.3</td>
</tr>
<tr>
<td>ox7</td>
<td>4.8</td>
<td>1.2</td>
</tr>
<tr>
<td>red5</td>
<td>2.9</td>
<td>0.7</td>
</tr>
<tr>
<td>red7</td>
<td>2.6</td>
<td>0.7</td>
</tr>
</tbody>
</table>

*The <TYR> label is the same as in Table 1. +3rd denotes the sum of TYR and 3rd components.
Figure 52: 3-component quadrilinear model of spinach PC absorption. Original spectrum (---), predicted spectrum (-----), tyrosine component (--), MT component (....), and 3rd component (-.-.-). a) Oxidized PC, pH 5.0; b) oxidized PC, pH 7.0; c) reduced PC, pH 5.0; d) reduced PC, pH 7.0.
DISCUSSION OF THE MODELS AND THE ABSORPTION COMPONENTS

2-Component Models.

The fundamental indeterminacy of the bilinear model results in a broad range of possibilities for the components corresponding to the same fit to the data. Unlike the trilinear and quadrilinear models, a unique solution cannot be determined solely by fitting the bilinear model to the data. Criteria must be imposed to determine the correct linear combination of the SVD basis vectors that form the real absorption components. In this case, the accuracy of the predicted components depends on the accuracy of the criteria used. The requirement that the absorption components be non-negative is a general property of absorption spectroscopy, and therefore, should not distort the results. If there is an absorption region where only a single component absorbs then the non-negativity criterion alone will fully define all of the components (Malinowski & Howery, 1980). However, the components in the near-UV spectrum of PC are heavily superimposed and absorption targets are required.

The tyrosine target was selected because it is known that tyrosine absorption is a major component in 265-290 nm wavelength region of the PC spectrum (Drahelm et al., 1985). The Neurospora metallothionein target (Beltramini & Lerch, 1981, 1986) was selected to approximate the absorption component due to the copper center in the spectrum of reduced PC (see Chapter III). The absorption of the metallothionein protein is due to electronic transitions occurring in reduced
copper-sulfur centers, which may be analogous to transitions in the reduced copper center of PC. The appropriateness of these targets for a 2-component model, and thus the accuracy of the predicted components of the fixed bilinear model, is indicated by the fact that nearly identical components occur in the 2-component trilinear and quadrilinear models, for which outside criteria are not used.

The small deviations between the targets and the fitted spectra (Figure 42 on page 144) provide some insight into the chromophores in PC. The fact that the absorption band of the fitted tyrosine component is broader than the absorption band of the target suggests that the spectra of the different tyrosine residues are slightly shifted due to environmental perturbations. The deviation of the spectrum of the MT component from the 250 nm peak in the MT target may be because this band is blue-shifted in the spectrum of PC. The possible variability of the wavelength maximum of this transition is indicated by the fact that it is shifted to 280 nm in the spectrum of yeast MT (Rupp et al., 1979) (see Figure 17 on page 70). The shoulder peaks at 278 and 284 nm in the metallothionein component spectrum are likely due to a small amount of tyrosine absorption, and not a predicted feature of the absorption of the reduced copper center. The fact that the absorption of Neurospora and yeast MT is due to electronic transitions in their reduced copper-sulfur centers suggests that electronic transitions associated with the Cu(I)-Cys 84 and/or Cu(I)-Met 92 ligand bonds are responsible for the absorption of the reduced copper center of PC (see Chapters III and IV).
The fixed bilinear model is used to analyze the tyrosine and MT components of the 2-component models because it is free of the error caused by the interaction of the chemical-condition variables (see residual analysis section). The tyrosine component is interpreted as a superposition of the absorption of the tyrosine residues in PC. This is supported by three results: (a) The spectrum of the tyrosine component is very close to that of a tyrosine residue; (b) the magnitude of the tyrosine component corresponds to the number of tyrosine residues in each PC species; (c) the average extinction coefficients at 278 nm of the tyrosine residues (Table 5 on page 148) correspond to the extinction coefficients of tyrosine in water and in ethanol (1.4 and 1.8 mM⁻¹cm⁻¹, respectively) (Gratzer, 1968). The MT component is interpreted as the superposition of the absorption of the copper center, phenylalanine, and possibly, peptide bond chromophores. In the 250-265 nm region of the spectrum of oxidized PC, the MT component represents the absorption of only the phenylalanine and peptide bond chromophores. The increase of the MT component in the spectra of reduced PC represents the absorption of the reduced copper center. The fact that the magnitude of the increase upon reduction of the MT component is nearly identical in the four PC species, indicates that the structure of the copper center is also nearly identical in these four different species.
3-Component Model.

The 3-component quadrilinear model further resolves the absorption of the tyrosine residues and the copper center. The red-shifted tyrosine absorption peaks in the spectrum of the 3rd component (at 280 and 286 nm) indicate that the 3rd component represents the absorption of perturbed tyrosine residues. It is known that environmental perturbation and hydrogen bonding of the hydroxyl moiety can cause a red-shift of up to 4.0 nm in the absorption spectrum of tyrosine (Bailey et al., 1968; Strickland et al., 1972; Donovan, 1969) (see Chapter III). The unshifted tyrosine absorption peaks in the spectrum of the tyrosine component (at 278 and 284 nm) indicate that the tyrosine component represents the absorption of unperturbed tyrosine residues. This interpretation is also supported by the observation that the sum of the tyrosine and 3rd components responds to the chemical-condition variables in a manner analogous to that of the tyrosine component in the 2-component models. The response of the MT component to the oxidation state variable, and the similarity to the spectra in the 250-285 nm region of the MT components of the 2-component models, indicate that the MT component in this model also represents the superimposed absorption of the copper center, phenylalanine, and peptide bond chromophores.

The decrease of the tyrosine component and the increase of the 3rd component upon reduction of PC indicates that a greater number of tyrosine residues are perturbed in reduced PC than in oxidized PC. The change in magnitude of the tyrosine component between that in oxidized
and reduced PC indicates that one additional tyrosine is perturbed in reduced poplar, spinach, and lettuce PC, and that two additional tyrosines are perturbed in reduced parsley PC (see Table II). The pH independence of the decrease in magnitude of the tyrosine component upon reduction of PC indicates that the same number of tyrosine residues are affected at pH 5.0 as at pH 7.0. Therefore, the pH dependence of the increase in magnitude of the 3rd component upon reduction of PC indicates that the extinction of the perturbed tyrosine residues is greater at pH 7.0 than at pH 5.0.

The shape of the spectrum of the 3rd component deviates from that of tyrosine in the 250-265 nm wavelength region ((No Figure with ID af7f) found) This indicates that the 3rd component represents more than just the absorption of the perturbed tyrosine residues. One possibility is that the 3rd component represents the superimposed absorption of coupled electronic transitions belonging to a tyrosine residue and another type of chromophore. The coupling of electronic transitions provides an alternate explanation for the red-shift of the tyrosine absorption peaks in the spectrum of the 3rd component. The increase of the 3rd component upon reduction of PC indicates that the dipole strength of the second electronic transition is affected by the oxidation state of the copper center. The most likely situation is that this electronic transition is located in the copper center, and is directly affected by the pH and oxidation-state-dependent changes of the conformation of the copper center (Guss & Freeman, 1983; Guss et al., 1986) (see Chapter IV). The tyrosine residues most likely to be
involved in the coupling are the ones closest to the copper center. This is tyrosine 83 in all four PC species and tyrosine 62 in parsley PC.

Since the MT and 3rd components are distinguished by their responses to the independent variables, the copper center contributions to these components must be due to at least two different electronic transitions. The presence of the 3rd component in the spectrum of oxidized PC indicates that the electronic transition which couples to the tyrosine is present in both the oxidized and reduced copper centers, but has less dipole strength in the oxidized copper center. This interpretation is supported by the observation that the predicted extinction of the tyrosine component in the spectra of oxidized PC is less in the 3-component model than in the 2-component models. However, the magnitude of the 3rd component in the spectra of oxidized PC is relatively small, and its presence may instead be an artifact due to the interactions of the chemical-condition variables. If this is the case, then the electronic transition which couples to the tyrosine is likely absent in the oxidized copper center.

The superposition of the spectra of coupled electronic transitions explains why the increase in extinction at 278 nm of the 3rd component upon reduction of PC is larger than that of the tyrosine component in the fixed bilinear model. In the fixed bilinear model, the increase of the tyrosine component is due to the increase in extinction of the perturbed tyrosine residues in reduced PC. In the quadrilinear model,
the increase of the 3rd component is due to the combination of the 
extinction of the coupled copper center transition, the increase in the 
number of perturbed tyrosine residues, and the enhanced absorption of 
the perturbed tyrosine residues (see Chapter III).

The results of the 2 and 3-component models support the conclusions 
drawn in Chapter III, that the reduction of PC results in a 
pH-dependent increase of the net absorption of the tyrosine residues. 
At pH 7.0, the increase in the absorption of PC upon reduction is due 
to both the reduced copper center and the tyrosine residues. As 
discussed in Chapter III, the enhanced absorption of the tyrosine 
residues can be explained by changes in the hydrophobicity of the local 
environments and/or by changes in the hydrogen bonding of the hydroxyl 
moieties. In turn, these interactions may be caused by local 
conformational changes and/or by deprotonation of proximal carboxylate 
groups at high pH. Model studies have indicated that the extinction 
coefficient of a tyrosine at 278 nm may increase a maximum of 0.7 
mM$^{-1}$cm$^{-1}$ by the decrease of environmental polarity, and may increase a 
maximum of 0.3 mM$^{-1}$cm$^{-1}$ by the formation of a hydrogen bonding 
(Donovan, 1969; Strickland et al., 1972). These mechanisms would be 
applicable whether or not the affected tyrosines are electronically 
coupled to the copper center. If the tyrosines are coupled, then the 
increase of extinction may also be due to dipole-strength exchange 
among the coupled transitions.
In the fixed bilinear model, the magnitudes of the reduced-minus-oxidized difference extinction coefficients at 278 nm of the tyrosine component are exceptionally large for poplar and parsley PC (see Table I). This suggests that more than one of the tyrosine residues in these proteins are perturbed by the change in oxidation state. However, this is unlikely for poplar PC, considering that one of its two tyrosine residues (Tyr 80) is hydrogen bonded to the backbone, on the opposite side of the protein from the copper center (Guss & Freeman, 1983). The most likely tyrosine residue to be affected by the reduction of the copper center is Tyr 83, which is located in the negatively charged, east face reaction site adjacent to the copper center in all four species of PC (see Figure 7 on page 13). The involvement of Tyr 83 was also indicated in Chapter III, by the finding that the absorption increases of reduced spinach and parsley PC at 278 nm titrate with pKa values similar to the those for the rate of oxidation of reduced PC via the east face site (Durell et al., 1988). The pH dependence of this oxidation reaction has been shown to be a composite of pH-dependent changes at the copper center and east face reaction sites (Sykes, 1985). Thus, the similarity of the pKa values further supports the interpretation that the 3rd component is due to the absorption of coupled electronic transition in the reduced copper center and Tyr 83. In parsley PC, Tyr 62 is also located near the copper center, and may also contribute to the observed effects in that species.
CHAPTER VII
THE ELECTROSTATIC POTENTIAL FIELD OF PC

This Chapter deals with the calculation of the electrostatic potential field of PC. Knowledge of the potential field is important for understanding the electrostatic forces which act in the association and interaction of PC with other charged reagents in solution. Knowledge of the potential field of PC is also necessary to determine the contributions of electrostatic interactions to the thermodynamic parameters of the redox potential and the pKa values of the active sites, which also affect the electron transporting activity of PC.

A macroscopic approach is used to model the protein and solvent system. In this approach, the protein and solvent are treated as continuous, isotropic material, that possess bulk dielectric constants. The solvent is taken to have a greater dielectric constant than the protein. The surface of the protein, determined by X-ray crystallography, is used to define the dielectric boundary. The crystal structure is also used to define the locations of the atomic charges. The electrostatic potential field is calculated by numerically solving the Poisson-Boltzmann equation. This procedure was carried out with the DelPhi computer program, which was obtained from Dr. B. Honig's laboratory at Columbia University (Klapper et al., 1986).
The first section of this Chapter is concerned with evaluating the accuracy of calculating the electrostatic potential field by this approach. This is done by comparing electrostatic effects already observed in PC with the predicted effects. These are the effect of oxidation state of the copper center on the pKa of nitro-tyrosine 83, and the effect of chemical modification of acidic residues on the redox potential. The second section of this Chapter is concerned with examining the shape of the electrostatic potential field surrounding PC, and the effects that the selected protein dielectric constant and solvent ionic strength have on it. The third section of this Chapter describes a series of experiments meant to further investigate the limits of this approach for calculating electrostatic interactions.

**CALCULATION OF ELECTROSTATIC EFFECTS IN PC**

**Electrostatic Effects on the pKa of NTyr 83.**

The first test of the method was to predict the electrostatic effect of the change in oxidation state of the copper center on the pKa of nitro-tyrosine 83 (NTyr 83). Previously, Gross et al. have reported on the formation of a NTyr 83 modified form of PC, which is produced by the addition of tetrannitromethane to PC in solution (Gross et al., 1985). Tyr 83 is chemically modified by the binding of a nitro group to the ortho carbon of the phenol ring. The pKa value of a nitro-tyrosine molecule in aqueous solution is ca. 7.0 (Means & Feeney, 1971). The pKa value of Ntyr 83 in oxidized spinach PC is raised to
8.3, due to hydrophobic and/or electrostatic effects at the east face site. Reduction of PC, which changes the formal charge of the copper center by 1.0e, results in the raising of the pKa value to 8.6 (Anderson et al., 1985).

Figure 53 on page 175 displays contour maps of the electrostatic potential fields of oxidized and reduced spinach PC, and the difference between these two fields. The 2-dimensional cross sections through the protein are the same in each map, and contain the copper atom, the hydroxyl oxygen atom of Tyr 83, and the center of the molecule. The change in charge of the copper atom affects the potential throughout the molecule, and provides a significant change in potential at Tyr 83 (ca. 0.80 kT/e). The effect of the dielectric boundary between the protein and solvent is clearly seen. In the higher dielectric constant region of the solvent, the magnitudes of the potential and the field gradient are both reduced.

Table 7 on page 176 compares the experimentally measured and calculated effects, for spinach and poplar PC, of the change in oxidation state of the copper center on the pKa of NTyr 83. The influences of the ionic strength, protein dielectric constant, and Stern layer thickness parameters are also shown. The variation in the calculated ΔpKa value due to different orientations of the PC molecule in the grid was less than 0.04 pH units, which is below the error of the measurements (0.05 pH units). Identical results were obtained using the formal and partial atomic charge assignments.
Figure 53: Effect of copper atom charge on the potential field of spinach PC. a) oxidized PC, b) reduced PC, and c) oxidized minus reduced field. The parameters are: protein/solvent dielectric constants = 2/80, ionic strength = 30 mM, The thick line represents the surface of the protein. Iso-potential lines are shown for 4.0, 2.0, 1.0, 0.5, 0.25, -0.25, -0.5, -1.0, -2.0, & -4.0 kT/e.
Table 7

Electrostatic Effects on the pKa of Nitro-Tyrosine 83

The values indicate the calculated change in the pKa value of NTyr 83 between oxidized and reduced PC.

Experimental $\Delta$pKa$_{(\text{ox-red})}$, (ionic strength = 0.03 M)

<table>
<thead>
<tr>
<th></th>
<th>spinach</th>
<th>poplar</th>
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<tr>
<td></td>
<td>-0.30 + 0.05</td>
<td>-0.30 + 0.05</td>
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Calculated $\Delta$pKa$^a$

Effect of protein dielectric constant

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<tbody>
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<td>spinach</td>
<td>-0.35</td>
<td>-0.24</td>
<td>-0.17</td>
<td>-0.13</td>
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<tr>
<td>poplar</td>
<td>-0.33</td>
<td>-0.24</td>
<td>-0.17</td>
<td>-0.13</td>
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Effect of ionic strength

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<th>0.15 M</th>
<th>0.30 M</th>
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<tbody>
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<td>spinach</td>
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<td>-0.32</td>
<td>-0.31</td>
</tr>
<tr>
<td>poplar</td>
<td>-0.33</td>
<td>-0.35</td>
<td>-0.34</td>
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Effect of Stern layer

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<tbody>
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<td>-0.35</td>
<td>-0.37</td>
</tr>
<tr>
<td>poplar</td>
<td>-0.35</td>
<td>-0.33</td>
<td>-0.34</td>
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$^a$Unless otherwise noted, the parameters are: ionic strength = 0.03 M, protein dielectric constant = 2, solvent dielectric constant = 80, and Stern layer thickness = 2 A.
The calculated values agree with the measured values when a
dielectric constant of 2 to 4 for the protein interior is used. This
range of values is in accord with estimates of the dielectric constant
of proteins made by other researchers (Gilson & Honig, 1986). When a
dielectric constant of 10 for the protein is used, then the calculated
ΔpKa is only 50% of the experimental value. These results demonstrate
the importance of treating the protein as a lower dielectric medium
than the solvent for calculating electrostatic effects.

In this application, the change in the protein dielectric constant
has a larger effect on the calculated ΔpKa value than does the changes
in the ionic strength and Stern layer parameters. This is due to the
fact that the copper atom is buried below the surface of the protein,
and its electrostatic effect on NTyr 83 is propagated primarily through
the protein interior (Figure 53 on page 175). This is also due to the
fact that, in the crystal structure of PC, Tyr 83 is partially shielded
from contact with the solvent.

Both the experimental and calculated ΔpKa values are nearly
identical for the structures of spinach and poplar PC. This is true
despite the fact that there are amino acid substitution at the east
face between these two species, which cause a difference in the shape
of their dielectric boundaries near Tyr 83. In particular, Ser 45 and
Ser 53 in poplar PC are replaced by Glu 45 and Ala 53 in spinach PC.
Figure 54 on page 178 shows the east face surfaces of these two PC
species. Thus, this computational approach is found to respond
correctly to the change in the shape of the dielectric boundary.
Figure 54: East face sites of spinach and poplar PC. a) poplar PC, b) spinach PC. The region of greatest change is indicated on the spinach structure.
Electrostatic Effects on the Redox Potential of PC

The second test of the method was to predict the electrostatic effects that ethylenediamine (EDA) chemical modification of acidic residues in PC has on the redox potential of the copper center. Previously, Anderson et al. (1987) have reported on the formation of a series of singly and doubly EDA chemically modified forms of PC, which are produced by the reaction of EDA with the carboxyl groups of aspartate and glutamate residues in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide. The modification changes the formal charge of these residues from $-1.0e$ to $+1.0e$. They have observed that this modification causes a positive shift in the redox potential of the copper center in PC, and that the magnitude of this effect is dependent on the location of the EDA chemical modification relative to the copper center (Anderson et al., 1987).

There are three main sites of acidic residues on the PC molecule where the EDA modification occurs. These sites are: 1) Asp 42, Glu 43, Asp 44, & Glu 45 (east face); 2) Glu 59, Glu 60, & Asp 61 (east face); and 3) Glu 68 (north Pole). These sites are shown in Figure 55 on page 180. In this application, only forms of PC which have a single modification at one of the three sites is studied. However, for sites 1 and 2, it is not known which particular carboxylate group of the set is modified.
Figure 55: Three main sites of EDA chemical modification on PC. The view is of the east face and back side of the protein.
Table 8 on page 182 compares the experimentally measured and calculated changes in the redox potential of spinach PC due to EDA chemical modification. The effects of the protein dielectric constant, ionic strength, and Stern layer parameters are also presented. The variation in the calculated $\Delta E^0$ value due to different orientations of the PC molecule in the grid was less than 2 mVolts, which is significantly below the error of the measurement (5 mVolts). Identical results were obtained using the formal and partial atomic charge assignments.

The calculated $\Delta E^0$ values agree with the experimental values for the EDA modification of residues 42, 45, and 61 when a protein dielectric constant of 2 to 4 is used. Conversely, the calculated $\Delta E^0$ values for the modification of the other residues at the first two sites are at least 50% below the experimental values. Figure 56 on page 184 plots the calculated $\Delta E^0$ due to modification versus the inverse of the distance from the copper atom for each of the residues tested. Except for Glu 68, the residues fall into two groups: Those for which the calculated effects agree with the experimental values (42, 45, & 61), and those for which they do not (43, 44, 59, & 60). The slope of the lines through these two groups of points is proportional to the effective dielectric constant of the media between the residues and the copper atom. The line through the first group has a greater slope than that through the second group. Since electric potential is inversely proportional to the dielectric strength of the media, as described by Coulombs law, the steeper regression line
Table 8

Electrostatic Effects on the Redox Potential of PC

The values are the calculated change in the $\Delta E^0$ (millivolts) of PC due to EDA chemical modified of specific acidic residues.

<table>
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<th>ASP</th>
<th>GLU</th>
<th>ASP</th>
<th>GLU</th>
<th>GLU</th>
<th>OLU</th>
<th>ASP</th>
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<td>43</td>
<td>44</td>
<td>45</td>
<td>59</td>
<td>60</td>
<td>61</td>
<td>68</td>
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Experimental $\Delta E^0$ (ionic strength = 0.13 M, pH 7.0)*

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Calculated $\Delta E^0$ b

Effect of protein dielectric constant

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Effect of ionic strength

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Effect of Stern layer

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<tr>
<td>3A</td>
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<td>12</td>
<td>11</td>
<td>12</td>
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aFor the first two sites it is not know which of the residues and in what relative proportions they are modified.
bUnless otherwise noted, the parameters are: ionic strength = 0.13 M, protein dielectric constant = 2, solvent dielectric constant = 80, and Stern layer thickness = 2 Å.
indicates a smaller effective dielectric constant.

As can be seen in Figure 7 on page 13 and Figure 8 on page 14, the separation of the residues into two groups is due to a differential degree of solvent exposure. The residues for which the calculated effects agree are at least partially shielded from the solvent, and thus their electrostatic influences are propagated primarily through the interior of the protein. Since the dielectric constant of the protein is significantly less than that of the solvent (2 vs. 80), these residues have a greater electrostatic effect on the copper center. This can also be seen by the fact that the calculated effects of these residues are substantially more affected by the change in the protein dielectric constant than by the changes in the ionic strength and Stern layer parameters. This point is further illustrated by Figure 57 on page 185, which shows the differences in the electrostatic potential field of spinach PC due to EDA modification at Asp 42 and Glu 43. Since Glu 43 has a greater degree of solvent exposure, the change in electrostatic potential at the copper center due to EDA chemical modification of this residue is significantly less than that for Asp 42.

The calculated $\Delta E^0$ value due to EDA modification of Glu 68 is significantly larger than the experimental value when a low protein dielectric constant is used, but agrees well when the dielectric constant is raised to 80 (which is the value for the solvent). Figure 58 on page 187 shows that in the crystal structure of PC Glu 68 lies
Figure 56: $\Delta E^0$ vs. inverse distance from the copper atom for EDA mods. The data is for each acidic residue at the three modification sites.
Figure 57: Change of the potential field of spinach PC due to EDA mod. a) EDA modification of Asp 42, b) EDA modification of Glu 43.
along the surface of the protein, and is thus partially shielded from the solvent. One possibility is that, the conformation of the Glu 68 side chain assumes a different conformation in solution which leads to greater solvent exposure and screening of its charged carboxylate group. Figure 58 on page 187 also displays an altered conformation of the Glu 68 side chain which has been rotated $140^\circ$ around the CB-CG bond away from the protein surface. The calculated $\Delta E^0$ value due to EDA modification of Glu 68 in this altered conformation is 12 mVolts when a protein dielectric constant of 2 is used. This agrees with the experimental value of 8 mVolts to within the error of the measurement (5mVolts). This indicates that the Glu 68 side chain may indeed assume a different conformation in the solution structure of PC than in the crystal structure.

If it is assumed that the orientations of residues 42-45 and 59-61 for PC in solution are the same as those in the crystal structure, than the calculated results for the first two modification sites would indicate that residues 42, 45 and 61 are preferentially modified by the EDA reagent. However, in light of the findings for Glu 68 caution must be taken in accepting this assumption.

These results corroborate the findings of Anderson et al. (1987) that charges on the residues at the north pole and east face sites influence the redox potential of the copper center. This is especially important considering that the difference in redox potential between PC and its reactants affects the rate of electron transport (Tollin et
Figure 58: Conformation of Glu 68 in spinach PC. a) crystal structure, b) altered conformation.
al., 1986). It must be considered that the activity of PC \textit{in vivo} may be regulated by changes in the electrostatic potential at the copper center due to associations with cytochrome f and photosystem I at the north pole and/or east face sites.

There is currently a debate in the scientific community as to what are the best methods to calculate electrostatic effects in biomacromolecules. It is clear that a microscopic approach provides the most realistic description of the system (Warshel & Russell, 1984). The microscopic method does not use bulk dielectric constants, but rather accounts explicitly for the electric polarization of each atom and chemical bond in the system. However, the microscopic approach is very complex, and at the current state of technology, requires too much computing power to model the PC-solvent system in a reasonable amount of time. For this reason, the macroscopic approach was used, which is conceptually and computationally much simpler and quicker. However, the macroscopic approach is known to have significant limitations in calculating electrostatic effects. In general, the macroscopic approach is most accurate in calculating charge-charge interactions among groups which are located away from the dielectric boundary. The abrupt polarization discontinuity at the boundary leads to inaccurate calculations of the potential. It would be expected that the calculated $\Delta E^0$ values are less accurate than the $\Delta pK_a$ values (Table 7 on page 176 and Table 8 on page 182), because the carboxylate groups of the acidic residues are right at the dielectric boundary and Tyr 83 is partially shield from the solvent. However, the calculated results for
both parameters are within the experimental error (0.05 pH units and 5 mVolts), and it is not possible to determine which is more accurate. There is an additional uncertainty in the calculated $\Delta$ values due to the uncertainty of the ratio of modification of each residue in the first two sites. Nevertheless, support is growing for the acceptance of the macroscopic approach used here for determining the electrostatic potential field of proteins in the surrounding solvent. Algorithms similar to the DelPhi program are routinely being included in electrostatic application programs developed by other researchers.

**ELECTROSTATIC POTENTIAL FIELD OF PC.**

The results presented above indicate that the current approach can adequately predict the magnitude of electrostatic effects in PC when the protein is treated as a low dielectric constant medium. This provides justification for extending this approach to study the 3-dimensional potential field surrounding PC in solution. Figure 59 on page 190 displays a contour map of a cross section through the potential field of oxidized spinach PC, which was calculated using dielectric constants of 2 and 80 for the protein and solvent, respectively. The other parameters used are an ionic strength of 150 mM and a Stern layer thickness of 2 Å. This slice through the protein contains the copper atom, hydroxyl oxygen atom of Tyr 83, and the center of the molecule (the same as in Figure 53 on page 175 and Figure 57 on page 185), and as such, displays the potential at the north pole and east face sites. This is also a similar orientation of the PC.
molecule to that in Figs. 1a and 1b.

Figure 60 on page 192 indicates the 3-dimensional shape of the potential field surrounding the protein by displaying six parallel cross sections at +15, +10, +5, -5, -10, and -15 A from the slice in Figure 59. For the association of PC with other charged reagents, it is important to observe that the electrostatic potential over the north pole and east face sites are of different sign: i.e., the potential at the north pole is positive and the potential at the east face is negative. There are actually two localized regions of positive potential at the top of the molecule, which are due to the 1.0e charge of Lys 30 (left side) and to the 2.0e charge of the copper atom (right side). The negative potential region at the east face is due primarily to the negative charges of residues 42-45 and 59-61.

Figure 61 on page 193 displays the potential field of reduced spinach PC, which was calculated the same way as for oxidized PC except that the copper atom was assigned a charge of only 1.0e. It is seen that even with a reduced charge on the copper atom, a small region of positive potential is preserved over the north pole site.

It has been postulated that the acidic residues at the east face cause a negative potential at that site, and that this is why positively charged reagents (e.g., cobalt phenanthroline) bind at the east face site and negatively charged reagents (e.g., ferricyanide) bind at the north pole site (Sykes, 1985). The electrostatic potential
Figure 59: Contour map of the potential field of oxidized spinach PC. The parameters are: protein/solvent dielectric constants = 2/80, ionic strength = 150 mM, Iso-potential contour lines are shown for 4.0, 2.0, 1.0, 0.5, 0.25, -0.25, -0.5, -1.0, -2.0, & -4.0 kT/e.
Figure 60: Parallel slices thru the potential field of oxidized spinach PC. The field is the same as that in Figure 59. The planes are displaced at a) +15Å, b) +10Å, c) +5Å, d) -5Å, e) -10Å, & f) -15Å.
Figure 61: Contour map of the potential field of reduced spinach PC. The parameters are the same as for oxidized PC in Figure 59, except that the charge of the copper atom was assigned the value of 1.0e instead of 2.0e.
field of PC calculated by the current method fully supports these previous observations, and demonstrates that negatively charged reagents would experience an electrostatic attraction to the north pole site.

The effect of the solvent ionic strength on the calculated electrostatic potential field of PC is demonstrated in Figure 62 on page 195. These fields were calculated the same way as for the field in Figure 59 on page 191 except that the ionic strength was set to zero, 15, and 300 mM. At zero ionic strength the potential field is dominated by the net negative, formal charge of the PC molecule (−8.0e for oxidized spinach PC). At this ionic strength the potential at the surface of the molecule is not less negative than −1.0kT/e. Under these conditions, negatively charged reagents would be repelled from contact with all surfaces of PC. Increasing the ionic strength to only 15 mM substantially alters the shape of the potential field surrounding PC. The negative potential begins to recede from the top of the molecule, and reveals two small regions of positive potential above the surface of the protein. This trend in the change of the field is continued as the ionic strength is increased. At 300 mM ionic strength the negative potential due to the acidic residues at the east face (42-45, 59-61) is more localized at that site, and the two regions of positive potential extend further from the top of the molecule. Thus, it is observed that increasing the ionic strength has the effect of diminishing the contributions of the solvent exposed charged residues to the net potential field of the protein. It is clear that the shape
of the potential field of PC is dependent on the ionic strength, and that this effect should be included in the modeling of the interactions of PC with other charged reagents.

Figure 63 on page 197 displays the potential field that results from using the same dielectric constant for the protein as for the solvent. The field was calculated with the same parameters as for that in Figure 59 on page 191 except that the protein dielectric constant was set to 80 instead of 2. The major difference between the two fields is at the north pole site, where the magnitude of the positive potential is smaller when using a protein dielectric constant of 80. This is due to the diminished influence of the buried copper atom charge on the net potential field. In contrast, the dielectric constant of the protein does not significantly affect the potential surrounding the east face, since the charged residues at that site extend into the solvent.

In the plant chloroplast, PC is located in the lumen of the thylakoid membrane. Under conditions of strong illumination the environment of PC becomes acidic (ca. pH 4.5) (Ort & Melandri, 1982). This low pH range is comparable to the intrinsic pKa values for the protonation of the side chain carboxylate groups of the aspartate and glutamate residues, 4.0 and 4.5 respectively (Shire et al., 1974). For the acidic residues at the east face site (42-45 and 59-61) it has been postulated that their effective pKa values are higher (ca. 5.6) due to "proton sharing" as a result of their close proximity (Sykes, 1985). This can also be understood as a cooperative, electrostatic effect of
Figure 62: Effect of ionic strength on the potential field of ox spinach PC. a) zero M, b) 0.015 M, and c) 0.300 M. The other parameters are the same as in Figure 59 on page 191.
Figure 63: Effect of protein dielectric constant on the field of ox spn PC. The parameters are the same as in Figure 59 on page 191, except that the protein dielectric constant was set to 80.
the charge of each residue on the pKa values of the other proximal residues. In any event, at low pH some of the acidic residues will become protonated and their contributions to the potential field will be eliminated.

Figure 64 on page 199 displays a very simple approximation of the potential field of PC in a low pH environment. This field was calculated with the same parameters as for the field in Figure 59 on page 191 except that the magnitude of the negative charge assigned to the acidic residues was reduced by 50%. As would be expected, the negative potential at the east face site is reduced and the positive potential at the north pole site is increased. Under these conditions, negatively charged reactants would experience a greater electrostatic attraction to the north pole of PC than at higher pH conditions. This is in agreement with the findings of Takabe et al. (1983), who found that the rate of reaction between PC and P700 increases with the reduction of environmental pH. As described in the Introduction, the reaction domain of P700 is believed to be negatively charged and associate with PC at the north pole site (Anderson et al., 1987).

In a recent paper by Rush et al. (1988) a dipole moment approximation was used to model the electrostatic potential field of PC. This was an application of the approach developed by Koppenol and Margollash (1982) to determine the location of the active binding domain of cytochrome c. The dipole moment was calculated from the centers of positive and negative charge, which were determined
Figure 64: Effect of pH on the potential field of oxidized spinach PC. The parameters are the same as in Figure 59 on page 191, except that the charges of the acidic residues are reduced by 50%.
analogously to the calculation of the center of mass. However, this method does not account for the different degrees of solvent exposure of the charged residues, and thus charge screening, due to their different positions relative to the surface of the protein. Neither does this method account for the lower polarizability of the protein compared to the solvent, or the solvent ionic strength.

Figure 65 on page 201 displays the electrostatic potential field of oxidized spinach PC derived from the dipole moment approximation described by Rush et al. This field was calculated by assigning the total positive and negative formal charges of the PC molecule (9.0e and -17.0e) to the locations of the centers of positive and negative charge in the protein, and by setting both the protein and solvent dielectric constants to 80 and the ionic strength to zero. The shape of the potential field most closely resembles that of PC calculated at zero ionic strength (Figure 62 on page 196), in that the protein is surrounded by negative potential. However, at higher ionic strength values the potential field of PC calculated by the current method significantly deviates from that of the symmetrical dipole case. In particular, the model used by Rush et al. does not account for the regions of positive potential which develop over the top of the molecule. It is suspected that these differences in the potential fields calculated by the two different methods would affect the analysis of experiments designed to determine the locations of the active binding sites on the PC molecule for other charged reagents.
Figure 65: Dipole approximation of the potential field of ox spinach PC. The parameters used to calculate the field were: protein/solvent dielectric constants = 80/80 and zero ionic strength.
FURTHER EVALUATION OF THE METHOD

The Linear versus Non-linear Poisson-Boltzmann Equation

The difference between the linear and non-linear Poisson-Boltzmann equations is in the approximation of the exponential Boltzmann term, which describes the distribution of electrolyte in the solvent. In the linear equation only the first term of the series expansion of the exponential function is used; whereas, in the non-linear equation the first three terms of the expansion are used. The superposition principal, which states that the potential at a point is equal to the sum of the contributions of each charge separately, is only valid for the potentials calculated with the linear equation (Gilson & Honig, 1988). The potential calculated with the non-linear equation takes into account the effects of all of the charges on the polarization of the electrolyte concentration. This is why the single source charge method is valid when using the linear equation, but not when using the non-linear equation (see Chapter II).

The differences in the calculated electrostatic effects between using the linear and non-linear equations were investigated for the pKa of NTyr 83 and the redox potential. Table 9 on page 204 shows comparisons of the calculated ΔpKa and ΔE° values for a few of the conditions listed in Table 7 on page 176 and Table 8 on page 182. The single source charge method was used with the linear equation to calculate the ΔpKa and ΔE° values. The copper atom was assigned a charge of 1e to represent the difference between the oxidized and
reduced states. Equation (40) on page 203 was used to calculate the ΔpKa of NTyr 83,

\[ ΔpKa = \frac{1}{\ln 10} V_{OH} \]  

where \( V_{OH} \) is the potential at the hydroxyl group in the units of kT/e.

Equation (41) was used to calculate the ΔE° due to EDA modification of acidic residues,

\[ ΔE° = (V_A + V_B) \cdot 25.7 \text{mVolts/kT/e} \]  

where \( V_A \) and \( V_B \) are the potentials at the two oxygen atoms of the carboxylate group in the units of kT/e. To calculate the effect of the modification of a lysine group Equation (42) would be used,

\[ ΔE° = Δq(\text{mod-cont}) \cdot V_{NZ} \cdot 25.7 \text{mVolts/kT/e} \]  

where \( Δq(\text{mod-cont}) \) is the change in charge of the lysine residue and \( V_{NZ} \) is the potential at the site of the NZ atom.

The variations in the calculated effects between the linear and non-linear equations are 0.01 pH units and 1 mVolts for the ΔpKa and ΔE° values. These variations are well within the experimental error (±0.05 pH units and ±5 mVolts). Thus, for these specific cases the single source charge method and the linear Poisson-Boltzmann equation can be used to calculate the electrostatic effects. This leads to a substantial savings in time for calculating the ΔE° values, because the potentials at all of the sites of chemical modification can be
Table 9
Comparison of the Linear and Nonlinear Poisson-Boltzmann Eqs.

Comparisons are made for the effect of the copper center oxidation state on the pKa of NTyr 83 and EDA chemical modification on the redox potential of PC. The values listed under the non-linear equation are the same as those in Table 7 on page 176 and Table 8 on page 182.

### Calculated ΔpKa of NTyr 83 in Spinach PC

<table>
<thead>
<tr>
<th>Protein dielectric constant</th>
<th>2</th>
<th>4</th>
<th>10</th>
<th>80</th>
</tr>
</thead>
<tbody>
<tr>
<td>non-linear</td>
<td>-0.35</td>
<td>-0.24</td>
<td>-0.17</td>
<td>-0.13</td>
</tr>
<tr>
<td>linear</td>
<td>-0.36</td>
<td>-0.25</td>
<td>-0.18</td>
<td>-0.12</td>
</tr>
</tbody>
</table>

### Calculated ΔE° of Spinach PC due to EDA Chemical Modification

<table>
<thead>
<tr>
<th>Sites of modification</th>
<th>ASP</th>
<th>GLU</th>
<th>ASP</th>
<th>GLU</th>
<th>GLU</th>
<th>O LU</th>
<th>ASP</th>
<th>O LU</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>protein dielectric constant = 2</td>
<td>42</td>
<td>43</td>
<td>44</td>
<td>45</td>
<td>59</td>
<td>60</td>
<td>61</td>
<td>68</td>
</tr>
<tr>
<td>non-linear</td>
<td>13</td>
<td>4</td>
<td>3</td>
<td>11</td>
<td>10</td>
<td>11</td>
<td>22</td>
<td>52</td>
</tr>
<tr>
<td>linear</td>
<td>12</td>
<td>4</td>
<td>3</td>
<td>10</td>
<td>9</td>
<td>10</td>
<td>21</td>
<td>52</td>
</tr>
</tbody>
</table>
determined simultaneously from a single run of the program. A note of caution should be taken in assuming that the results of the linear and non-linear equations would be the same in all situations. A substantial difference could occur in situations of large charge magnitude at the surface of the molecule, where large electrolyte gradients would occur. The non-linear equation is expected to provide a more accurate representation of the electrolyte concentration.

The Effect of the Water Molecules Bound in the Crystal Structure

Water molecules which are immobilized due to contact with the surface can often be identified in the crystal structure of a protein. In the crystal structure of oxidized poplar PC 45 water molecules are identified. Figure 66 on page 206 displays the locations of the water molecules in the crystal structure of poplar PC. Since these water molecules are at least partially immobilized, it is not clear whether they should be treated as part of the bulk solvent, or as part of the protein surface. This is important for the spatial definition of the dielectric boundary.

To measure the effect of including the bound water molecules the ΔpKa of Ntyr 83 was calculated with and without them included in the atomic coordinates of poplar PC. The single source charge method was used, with a 1.0 e charge on the copper atom. The calculated ΔpKa value was -0.42 and -0.36 pH units with and without and the water molecules. This variation is slightly above the experimental error (±0.05). Thus the crystal waters do have an effect on the
Figure 66: Bound waters in the crystal structure of oxidized poplar PC. The waters which appear directly over Tyr 83 are really on the other side of the protein.
calculations. It is worth noting that the values calculated without the waters is closer to the experimentally measured ΔpKa (0.30). It is surprising that the water molecules have such a large effect considering that they are not very near to Tyr 83.

Consideration of Grid Error

Due to the finite representation of the atoms and charges on the grid, the calculation of the potential near to a charged atom is inaccurate. However, the change in potential near to a charged atom due to a change in the charge of a distance atom is accurate. To illustrate this point: the potential at the copper atom was measured for oxidized spinach PC in two different positions in the grid. The potential in the default position was 554.953 kT/e, and the potential at the copper atom for the protein shifted 0.5 grid units in the x, y, and z directions was 691.023 kT/e. However, the calculation of the charge in potential at the copper atom due to EDA modification of an acidic residue was independent of any type of offset of the protein in the grid.

The consequence of this grid error is that it may not be possible to compare potentials between two, or more, different sets of atomic coordinates. For example: to determine the electrostatic contribution to the change in the pKa value of the copper ligand His 87 between spinach and parsley PC, it would be desirable to compare the potentials at this ligand between the two PC species. However, this can not be done because the two different sets of atomic coordinates assume
different orientations in the grid, and it would not be known how much of the difference in potential is due to the grid error. This problem also arises in the calculation of the mutual interaction of proximal acidic residues on their pKa values. Since potential values must be calculated close to the charges of the other residues, it would not be known how much of the measured values are due to grid error.
CHAPTER VIII
CONCLUSIONS

SPECTROSCOPIC MEASUREMENTS

Absorption, CD, and fluorescence spectroscopies can be important tools for monitoring the secondary and tertiary structures of PC. To understand how PC functions as an electron carrier in the thylakoid lumen it is important to know how the conformation is affected by the change in oxidation state and environmental pH. The spectroscopic properties of the aromatic residues and the copper center can provide important information about localized areas of the protein. In particular, the copper center and Tyr 83 are ideally located to be probes of the north pole and east face reaction sites. However, obtaining this information is hampered by the fact that the spectral bands are superimposed, and it is not always clear which of the chromophores are responding to the perturbations. To help separate and assign the spectral bands of PC the absorption and CD spectra of different PC species were compared. It was also useful to correlate the effects of the change in oxidation state and environmental pH on the spectra of the different species.

For the comparison of the spectra of different PC species to be meaningful, it was first necessary to determine how the structures of
the different PC proteins are related. From the far-UV CD spectra it was found that spinach, poplar, and parsley PC have the same net secondary structures. This is true despite the fact that there are amino acid substitutions and deletions in the sequences of these different PC species. The unique far-UV CD spectrum of PC has been attributed to the close 3-dimensional arrangement of the $\beta$-sheets in the protein. To the extent that this is a measure of tertiary structure, the three PC species have the same net tertiary structure. This last conclusion is supported by the fact that the different species have qualitatively similar near-UV absorption and CD spectra. Thus, it is appropriate to use the 3-dimensional structure of poplar PC, as determined by X-ray crystallography, as an approximation of the structures of spinach and parsley PC. This enables the determination of the locations of the chromophores in spinach and parsley PC by analogy to the sequence and structure of poplar PC.

From far-UV CD measurements it was determined that the net secondary structure of PC in solution is invariant to the changes in the oxidation state of the copper center and the environmental pH. This agrees with the findings of the crystallographic studies. In contrast, the near-UV absorption and CD measurements indicated that the tertiary conformation of PC is affected by changes in both the oxidation state and pH. The conformation of reduced PC is substantially more affected by the change in pH than is the conformation of oxidized PC. From consideration of both the near and far-UV absorption and CD measurements it is concluded that the conformational changes in PC are
localized at specific locations, and are not drastic rearrangements of the secondary structural elements.

The pH-dependent change in the near-UV CD spectrum of reduced PC is primarily due to the change in conformation of the reduced copper center. This was first observed for the parsley PC species because the pKa value (5.7) for the change in conformation is unusually high, and falls well within the range of pH values for which the protein can be studied without denaturation (pH 9.0–5.0). Similar pH-dependent changes are believed to occur in the reduced copper centers of spinach and poplar PC at lower pH values (ca. 4.9). These results demonstrate that the conformational change of the reduced copper center which was observed in the crystal structure of poplar PC also occurs for spinach and parsley PC in solution.

The increase in the near-UV absorption of PC that occurs upon reduction of the protein has contributions from both the reduced copper center and the tyrosine residues. It was not possible to determine if the phenylalanine residues contribute to the absorption change. The majority of the absorption is due to the reduced copper center; however, the tyrosine residues make a significant contribution in region II of the spectrum. The enhanced absorption of the tyrosine residues is found to be pH dependent, being greater at high pH (ca. pH 7.0) than at low pH (ca. pH 5.0). By the comparison of the magnitudes of the variable tyrosine absorption, it is concluded that at least two tyrosine residues are involved in spinach PC, and three tyrosines are
involved in parsley PC. It is also concluded that the reduced copper center makes a contribution to the pH-dependent change in absorption, as well as being the source of the pH-independent change. This indicates that the near-UV absorption of the reduced copper center is due to at least two electron transitions: One which is pH-dependent and one which is not.

From comparison of the pKa values it is concluded that the absorption of the tyrosine residues is affected by the pH-dependent states of both the reduced copper center and the east face sites. The involvement of the east face site specifically implicates Tyr 83 as being one of the affected residues. It is shown that hydrogen bonding of the hydroxyl proton to the carboxylate groups of Glu 59 or Asp 42 are likely causes for the enhanced absorption of Tyr 83.

From the comparison of the spectra of PC with that of the reduced copper metallothioneins (from yeast and neurospora) it is concluded that the enhanced absorption and altered CD spectra of reduced PC is at least partially due to a Cu(I)->Cys 84 charge transfer transition. By analysis of the pH-dependent change in conformation of the reduced copper center it is shown that this transition could be a source of both the pH-dependent CD spectrum and the pH-independent enhanced absorption of reduced PC. Charge transfer transitions to the His 87 and Met 92 ligands are also shown to be potential sources of the pH-dependent spectral changes if their optically activity is magnetically allowed.
FACTOR ANALYSIS

The mathematical factor analysis procedures were able to separate the near-UV absorption spectrum of PC into three components. These components were interpretable as unperturbed tyrosine residues, a combination of the reduced copper center and the phenylalanine residues, and the coupled electronic transitions of the reduced copper center and Tyr 83. These results are in accord with the conclusions of the spectroscopic analysis discussed above. In particular, they indicate that the absorption of Tyr 83 is enhanced in reduced PC, and that this effect is pH dependent. The coupling of electronic transitions also explains why the absorption of Tyr 83 is dependent on the pH-dependent states of both the copper center and the east face site. In addition, the involvement of the copper center in two of the components supports the theory that there are at least two separate electronic transitions which cause the absorption of the reduced copper center.

It is determined that the use of the trilinear and quadrilinear models provide a significant advantage over the bilinear model for the factor analysis of the absorption of PC. It is believed that this recent innovation will be of great benefit to spectroscopic analysis efforts. In the absence of independent knowledge about the components, the use of the bilinear model is limited to just determining the number of components. The trilinear and quadrilinear models are applicable to any multiplicative system where the response variable can be measured as a function of three or four non-interacting independent variables.
For the case of the absorption of PC a small degree of interaction was found between the species, oxidation state, and pH variables. Fortunately, the magnitude of interaction was not large enough to preclude the successful prediction of the components. This result speaks to the substantial degree of conformational homology between the different PC species.

**ELECTROSTATIC MODELING**

The macroscopic approach used by the DelPhi program provides accurate predictions of electrostatic effects on pKa values and the redox potential of PC. It is important to assign a lower dielectric constant to the protein material than to the solvent. A protein dielectric constant value in the range of 2 to 4 is found to be appropriate. It is also found important to consider that charged residues which are at the protein surface may assume different conformations from that in the crystal structure when the protein is in solution.

The magnitude and shape of the electrostatic potential field of PC is dependent on the ionic strength of the solvent. As the ionic strength is increased the potential due to the charged residues exposed to the solvent becomes more localized around those residues, and the whole potential field becomes more heterogeneous. However, even at low ionic strength values the shape of the potential field is considerably different from that derived from a dipole moment approximation of the charges in the protein. A significant feature of the potential field,
which has not been previously observed, is that at ionic strength values above ca. 20 mM there is a region of positive potential over the north pole active site of PC. This positive potential would enhance the association of negatively charged reagents to that site. It is concluded that for the modeling of the electrostatic potential field of PC, and for the simulation of electrostatic interactions between PC and other charged reagents, it is important to account for the different polarizabilities of the protein and the solvent, and for the solvent ionic strength.


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