PLASTOCYANIN CONFORMATION

An Analysis Of Its Near Ultraviolet Absorption And Circular Dichroic Spectra

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ABSTRACT The near-ultraviolet absorption and circular dichroic spectra of plastocyanin are dependent upon the redox state, solution pH, and ammonium sulfate concentration. This dependency was observed in plastocyanin isolated from spinach, poplar, and lettuce. Removal of the copper atom also perturbed the near-ultraviolet spectra. Upon reduction there are increases in both extinction and ellipticity at 252 nm. Further increases at 252 nm were observed upon formation of apo plastocyanin eliminating charge transfer transitions as the cause. The spectral changes in the near-ultraviolet imply a flexible tertiary conformation for plastocyanin. There are at least two charge transfer transitions at ~295–340 nm. One of these transitions is sensitive to low pH's and is attributed to the His 87 copper ligand. The redox state dependent changes observed in the near-ultraviolet spectra of plastocyanin are attenuated either by decreasing the pH to 5 or by increasing the ammonium sulfate concentration to 2.7 M. This attenuation cannot be easily explained by simple charge screening. Hydrophobic interactions probably play an important role in this phenomenon. The pH and redox state dependent conformational changes may play an important role in regulating electron transport.

INTRODUCTION

Plastocyanin (PC)[1] is a 10.5 kD "blue copper" type I protein that acts as a mobile electron carrier between the cytochrome b_6/f complex and Photosystem I (Boulter et al., 1977; Freeman, 1981). The crystal structures of the oxidized, reduced, and apo forms of PC have been determined (Freeman, 1981; Guss and Freeman, 1983; Garrett et al., 1984). The copper atom appears to be coordinated to two histidines, a cysteine and a methionine. EXAFS measurements show that for the oxidized form the methionine residue may not be a true ligand (Scott et al., 1982). Most investigators believe that PC contains two binding sites for its reaction partners (Cookson et al., 1980; Farver et al., 1982). One binding site is near His 87, which is a ligand to the copper, while the other binding site is near Tyr 83. Since the reduction of PC by $[Ru(NH_3)_5(py)]^{2+}$ is inhibited by $[Pt(NH_3)_6]^{4+}$, Tyr 83 is probably the dominant reaction site (Chapman et al., 1983*a*). In addition, two patches of negatively-charged residues (No. 42– 45 and No. 59–61) in PC are conserved in higher plant species but are replaced with neutral or positively charged residues in some cyanobacterial species (Boulter et al., 1977).

Previously, this laboratory has shown that in solution PC undergoes conformational changes both upon changing redox state and upon EDA chemical modification (Draheim et al., 1985) as determined by near-UV absorption, circular dichroism (CD) and fluorescence measurements. These conformational changes are probably quite small in magnitude (subtle) but Gross et al. (1985) have provided evidence that they are global in scale. The crystal structures of oxidized and reduced PC appear to be nearly identical at neutral pH's (Freeman, 1981; Guss and Freeman, 1983). However, it should be noted that suspending PC in high concentrations of ammonium sulfate attenuates the spectroscopic changes observed in solution upon reduction. Currently, Freeman et al. are attempting to form the PC crystals without using ammonium sulfate (Freeman, personal communication). There is no a priori reason for the solution structures and crystal structures of a protein to be identical. Furthermore, conformational changes of only 0.1 Å to 0.2 Å could significantly affect the CD, absorption and fluorescence spectra of a protein.

A primary goal of this work is to present an evaluation of the contributions of the aromatic residues (phenylalanine and tyrosine in the case of PC) and charge-transfer (CT) transitions (due to the copper site) to the near-UV absorption and CD spectra of PC. The near-UV CD spectrum of a protein is very sensitive to subtle conformational changes.

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[1] *Abbreviations used in this paper*: plastocyanin, (PC); ultraviolet, (UV); circular dichroism, (CD); fast protein liquid chromatography, (FPLC); extended x-ray absorption fine spectra, (EXAFS); the reaction center chlorophyll of Photosystem I, (P700); charge transfer, (CT); Hepes, (4-(2-hydroxymethyl)-1-piperazineethanesulfonic acid); ethlylenediamine, (EDA); penta-amine pyridineruthenium(II), $[Ru(NH_3)_5(py)]2^+$.

If the pH of the thylakoid lumen can regulate the activity of PC with its reaction partners, then changing the pH should affect the tertiary conformation of PC. In this manuscript the term subtle refers to conformational changes that are small in magnitude but are still likely to be important with respect to biological function. The far-UV CD spectra of oxidized and reduced PC were measured both to monitor possible redox-state dependent secondary structure changes, and to explore the possibility of coupling to transitions in the near-UV.

Since PC is thought to be located on the inside of the thylakoid membrane it should experience a three-unit decrease in pH upon illumination (Ort and Melandri, 1982; Boulter et al., 1977). Such a change in pH could affect both the conformation and activity of PC and thus regulate photosynthetic electron transport. Upon decreasing the pH there is lengthening of the bond between His 87 and the copper atom for reduced PC (Guss and Freeman, 1983). However, the structure of oxidized PC is relatively pH independent. This increase in bond length has been correlated with the creation of a redox-inactive form of reduced PC (Freeman, 1981). In addition, Chapman et al. (1983*a–c*) have suggested that for reduced PC there is a cooperative effect between the negative patch and the active site. This region is also one of the most probable sites of EDA chemical modification which affects the K_M and V_{Max} of the PC/P700 interaction (Burkey and Gross, 1981; Takenaka and Takabe, 1984). These results suggest that perturbation of charges 18 Å distant from the copper atom can influence the copper site.

The fact that reduced PC can assume a redox-inactive form at low pH is paradoxical since there is evidence that PC should be active under these conditions. Takabe et al. (1983) have established that the rate of the reaction of reduced PC with $P700^+$ is maximal at pH 4.7. Furthermore, since PC is located in the lumen of the thylakoid it must routinely experience a pH of 5 during illumination. It was decided to examine the relationship between pH and redox state on the conformation of PC in solution because of its importance in photosynthetic electron transport.

METHODS

Isolation of Plastocyanin

PC was isolated from spinach according to the method of Davis and San Pietro (1979). The PC was further purified using a Pharmacia FPLC with a mono Q HR 5/5 anion exchange column. The final A278/A597 ratio for oxidized spinach PC was 1.12. Apo spinach PC was formed using the method of Scawen et al. (1975). An identical procedure was used to isolate PC from lettuce.

The above procedure was used to isolate PC from young poplar leaves of (*Populus deltoides*) with the following exceptions: (*a*) 0.5% polyvinylpyrilodine was added to the chloroplast isolation medium and the PC was treated with 60% $(NH_4)_2SO_4$ to precipitate the contaminating proteins prior to the DEAE chromotoghraphy; (*b*) *P. deltoides* (cottonwood) was used instead of the *P. nigra*. *P. nigra* was the species for which the crystal structure was determined. However, amino acid analysis showed no significant difference in the amino acid composition of the two species, particularly for the aromatic amino acids. In the case of poplar PC, the final A278/A597 ratio was 0.9 due to its lower tyrosine content.

PC concentrations were determined after each experiment using an extinction coefficient at 597 nm of 4.9 mM^{-1} cm^{-1} after the addition of excess ferricyanide. PC was oxidized using $K_3Fe(CN)_6$, which was subsequently removed by gel filtration on a Biogel P-10 column. PC was reduced using sodium ascorbate, which was removed by gel filtration on Biogel P-10. (Biorad Laboratories, Richmond, CA).

Spectral Measurements

The absorption and circular dichroic (CD) spectral measurements were performed using a Cary 118 C spectrophotometer and a Jasco 500A spectropolarimeter, respectively. PC (15–35 μM) was suspended in 10 mM concentrations of the buffers indicated. Both the absorption and the CD spectra of PC varied linearly with concentration between 15 and 100 μM indicating that aggregation effects do not contribute to our results.

In this study each spectral measurement was performed at least twice for any single PC preparation. For any given PC preparation the absorbance in the near-UV was reproducible to within three significant figures. This would be ~1% of the signal of the absorption peak at the 278 nm which is also comparable to the machine noise. For the far-UV the absorbance was reproducible to within two significant figures. When comparing one PC preparation to another there was no more than a 3% variation in the absorbance at 278 nm provided the FPLC purification step was performed. If only the G75 purification step was performed there was a variation among the PC preparations of as much as 19%. The spectral measurements shown in this manuscript were performed on at least two different preparations of FPLC purified PC. The baseline of the Cary-118C spectrophotometer was carefully flattened using matched cuvettes loaded with the appropriate buffer solutions.

In the wavelength range 210–400 nm the machine noise for the Jasco 500 Å spectropolarimeter was typically 5–8% of the signal for the PC concentrations used in this study. Between 190 and 210 nm the machine noise was typically 10% of the signal. Each CD measurement was performed at least twice on any single PC preparation and a center line drawn through the noise did not vary by more than 2%. When comparing one PC preparation to another the signal variation was ~5% for FPLC purified PC. The solvent CD baselines were recorded and carefully subtracted from the sample CD recordings. The spectral curves shown in this manuscript are typical spectral measurements, not averages of several measurements.

Computer Graphics Display of Poplar PC

A graphical display of the three dimensional structure of oxidized poplar PC was made using the x-ray coordinates obtained from the Broohaven Protein Data Bank, (Chemistry Dept., Brookhaven National Laboratory, Upton NY) together with a commercial engineering wireframe modeling graphics package IDEAS. Further details of the graphics methodology will be published elsewhere (Duane et al., 1985).

Materials

DEAE-cellulose and Biogel P-10 were obtained from Biorad Laboratories. HEPES and Tris were obtained from Sigma Chemical Co. (St. Louis, MO). All other chemicals were of reagent grade.

RESULTS

Fig. 1 *a* displays a color coded view of the oxidized poplar PC molecule. Some of the residues have been numbered in order to provide a frame of reference. Poplar PC contains seven phenylalanine and two tyrosine residues. Phe 70 is replaced with a third tyrosine in spinach and lettuce PC. Residues Phe 29 and Phe 70 appear to be in a stacked configuration. This view also shows the proximity of Phe

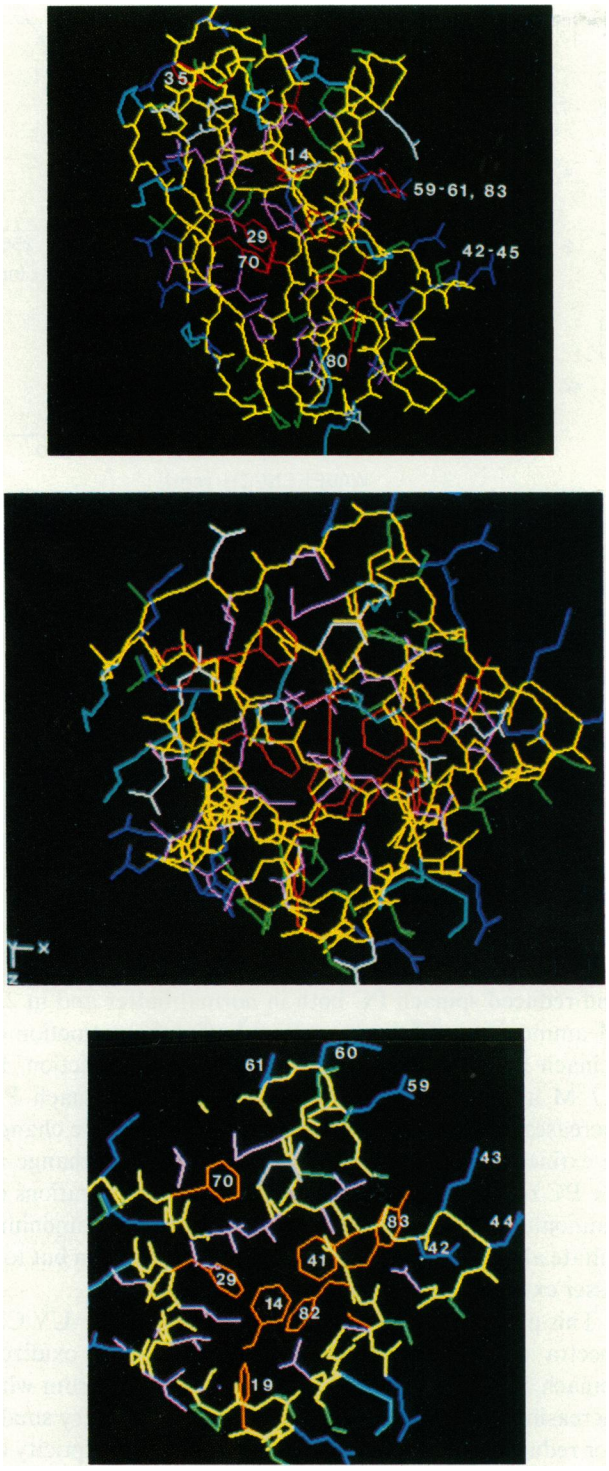

FIGURE 1 (*a*) A color coded computer drawn view of oxidized poplar PC. Some of the individual residues have been numbered. This is the normal view of the molecule often refered to as its "front face." The color code is: yellow, the protein backbone and carbonyl; magenta, nonpolar residues; red, aromatic residues; green, uncharged polar residues and proline; dark blue, negatively charged residues; light blue, positively charged residues; white, asparagine and glutamine. (*b*) A color coded computer drawn view of poplar PC identical to Fig. 1 *a* but rotated 90°. This shows the "top" view of the molecule. (*c*) An 11 Å "slice" of the view of poplar PC displayed in Fig. 1*b*.

14 and Phe 35 to the copper atom. The distances between the centers of the aromatic rings of Phe 14 and Phe 35 to the copper atom are 6.9 Å and 9.4 Å, respectively. The structural alignment of Phe 14 to the copper center in PC is homologous to the alignment of Phe 15 in azurin to its copper center (Chothia and Lesk, 1982). This view also shows the highly conserved negatively charged residues No. 42–45 and No. 59–61.

Fig. 1 *b* displays another view of the oxidized poplar PC molecule. The negatively charged residues No. 42–45 and No. 59–61 can be easily seen as a possible binding or recognition site for PC. Fig. 1 *c* displays PC in the same orientation as that shown in Fig. 1 *b* but now as an 11 Å "slice." Obviously, many of the aromatic residues of PC are located at the same physical level on the crystallographic *Y*-axis and some exhibit a relatively high amount of end-to-end orientation. This could have important implications in relation to the mutual interactions of these aromatic residues.

Fig. 2 shows the near-UV absorption spectra of spinach PC (oxidized, reduced, and apo). The extinction of PC increases significantly upon reduction throughout the near-UV below 310 nm. This phenomenon was the subject of a previous study (Draheim et al., 1985). Some minor absorption bands (~320–330 nm) were either lost or diminished upon forming apo PC. Removal of the copper atom also caused an 11% decrease in extinction at 278 nm relative to reduced PC. However, this decrease was much smaller than the 56% increase in extinction at 278 nm caused by the reduction of oxidized PC. It should be noted that the shape of the difference spectrum of reduced minus apo spinach PC (Fig. 2, insert) was very similar to a tyrosine absorption spectrum in this region. The extinction of apo PC at 252 nm is significantly higher than that of either oxidized or reduced PC. At 252 nm the extinction of apo PC is 39% greater than that of reduced PC. The PC

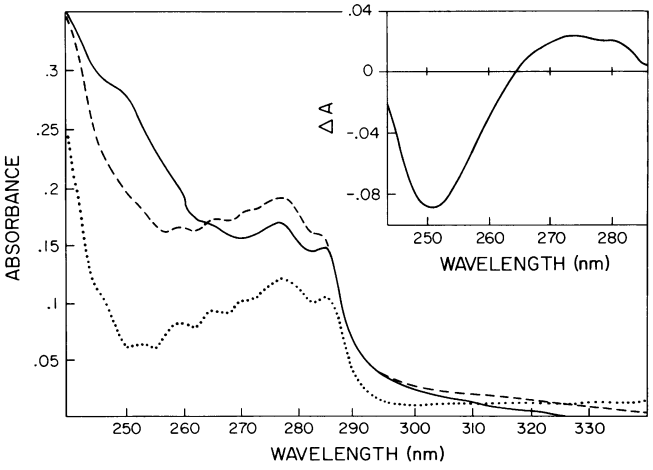

FIGURE 2 The near-UV absorption spectra of spinach PC. Oxidized (· · · ·). Reduced (– – –). Apo (——). The concentration was 20 μM. 10 mM Hepes pH 7.0. *Insert.* A difference absorption spectrum of spinach PC (reduced minus apo).

extinction at 252 nm is sensitive to the state of the copper atom since it decreases as the charge at the copper site increases going from apo to reduced to oxidized PC.

Fig. 3 shows the near-UV CD spectra of spinach PC (oxidized, reduced, and apo). There are at least two redox state dependent CD bands (~295 nm and 320 nm). The CD band at ~320 nm vanishes upon forming apo PC while the positive band at ~295 nm becomes negative. Both these bands may be attributed to CT transitions. The ellipticity of spinach PC at 278 nm and 284 nm exhibits only minor changes upon reduction. Since the extinction of these transitions increase upon reduction, the net rotatory strength must decrease. Upon forming apo PC the negative ellipticity of the 278 nm and 284 nm transitions increase 55%. Upon reduction PC exhibits a large increase in ellipticity at 252 nm. This is a true change in rotatory strength since the percent increase in extinction is less than the percent increase in ellipticity. The formation of apo PC causes a further increase in ellipticity of 30% at 252 nm together with a small blue shift of that CD band to 250 nm. Thus, the positive ellipticity at 252 nm increases in going from oxidized to reduced to apo PC.

Fig. 4 shows the far-UV CD spectra of oxidized and reduced spinach PC. When the far-UV CD spectrum of PC is measured in a 0.1 mm optical cell there were no observable changes upon reduction. The samples used had absolutely identical concentrations. The far-UV CD spectrum of reduced PC was initially measured and then the sample was oxidized with ferricyanide in the optical cell. To within 0.5 nm the crossover wavelength (214 nm) did not change upon changing the redox state. Normally, the crossover wavelength is very sensitive to changes in the secondary structure of a protein. When the far-UV CD spectrum of apo PC was measured (not shown) it exhibited an identical crossover wavelength. In addition, the ratio of the positive ellipticity at 198 nm to the negative ellipticity at 225 nm was identical for holo and apo PC. When the

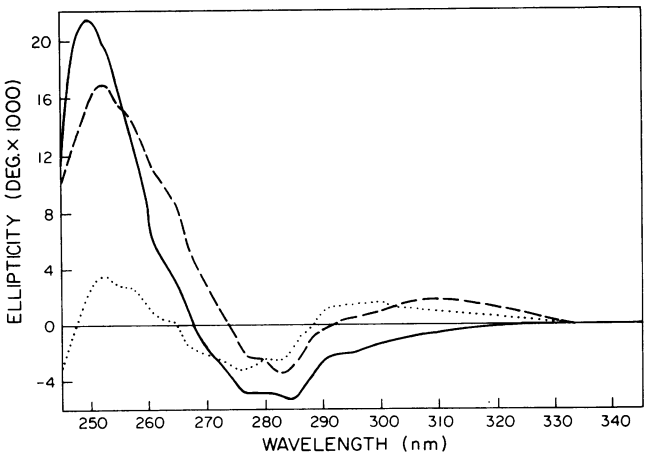

FIGURE 3 The near-UV CD spectra of spinach PC. Oxidized (· · · ·). Reduced (– – –). Apo (——). The concentration was 50 μM. 10 mM Hepes pH 7.0.

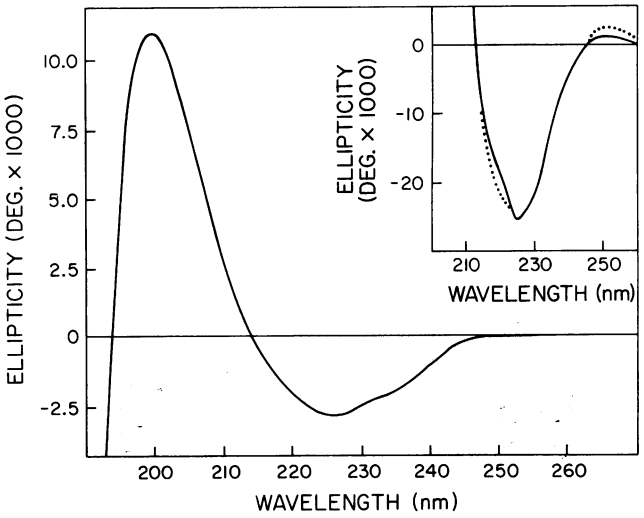

FIGURE 4 The far-UV CD spectrum of spinach PC. The concentration was 50 μM. 10 mM Hepes pH 7.0. The cell pathlength was 0.1 mm. *Insert.* The same as in Fig. 4 but in a 1.0 mm pathlength cell. Oxidized (——). Reduced (· · · ·).

far-UV CD spectrum of holo PC was measured in a 1.0 mm optical cell some minor differences could now be observed (Fig. 4, insert). Upon reduction a small negative CD band appeared at ~220 nm. Because of the scaling, the magnitude of this CD band is of the same order as the magnitude of the positive CD band at 252 nm due to the reduction of oxidized PC. In the 1.0 mm optical cell the crossover wavelength for the PC far-UV CD spectrum was at 214 nm and also did not vary with redox state.

Fig. 5 shows the near-UV absorption spectra of oxidized and reduced spinach PC both in normal buffer and in 2.7 M ammonium sulfate. In normal buffer the extinction of spinach PC increases 56% at 278 nm upon reduction. In 2.7 M ammonium sulfate the extinction of spinach PC increases only 38% at 278 nm upon reduction. The change in extinction in the near-UV associated with a change in the PC redox state is attenuated at high concentrations of ammonium sulfate. Lower concentrations of ammonium sulfate also attenuated the change in PC extinction but to a lesser extent.

This phenomenon was also observed in the near-UV CD spectra of spinach PC shown in Fig. 6. For oxidized spinach PC the change in its near-UV CD spectrum with increasing ammonium sulfate concentration is very small. For reduced spinach PC there is a decrease in ellipticity at 252 nm of 19% upon increasing the ammonium sulfate concentration to 2.7 M. 100 mM $CaCl_2$ did not measurably affect the near-UV absorption and CD spectra of spinach PC.

Fig.7 shows the near-UV absorption spectra of oxidized and reduced spinach PC at pH 5 and pH 7. At pH 7 the extinction of spinach PC increases 56% at 278 nm and 200% at 252 nm upon reduction. At pH 5 the extinction of spinach PC increases upon reduction only 28% at 278 nm and 104% at 252 nm. Upon lowering the pH the conforma-

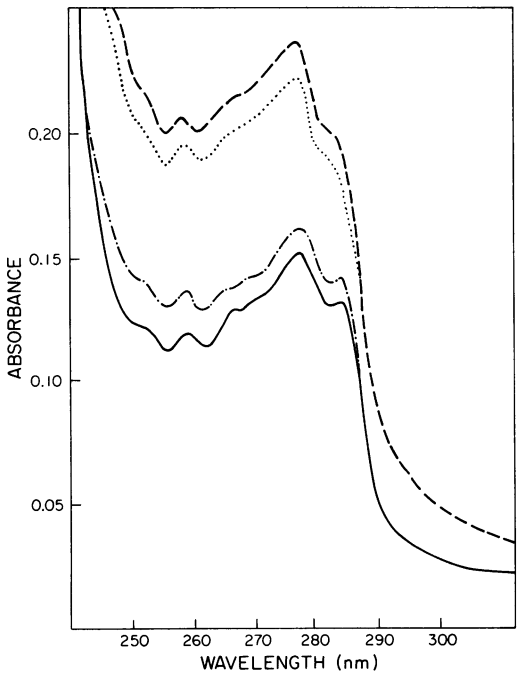

FIGURE 5 The near-UV absorption spectra of spinach PC. Oxidized (—). Oxidized, 2.7 M ammonium sulfate (–·–). Reduced (– – –). Reduced, 2.7 M ammonium sulfate (· · · ·). The concentration was 25 μM. 10 mM Hepes pH 7.0.

tional change associated with changing the redox state of PC is attenuated.

Fig. 8 shows the near-UV CD spectra of oxidized and reduced spinach PC at pH 5 and pH 7. The redox state dependent changes in ellipticity at 252 nm are very sensitive to pH. At pH 7 there is a fourfold increase in ellipticity at 252 nm upon reduction while at pH 5 there is only a onefold increase. This is a severe attenuation of the spectroscopic change associated with changing the redox state

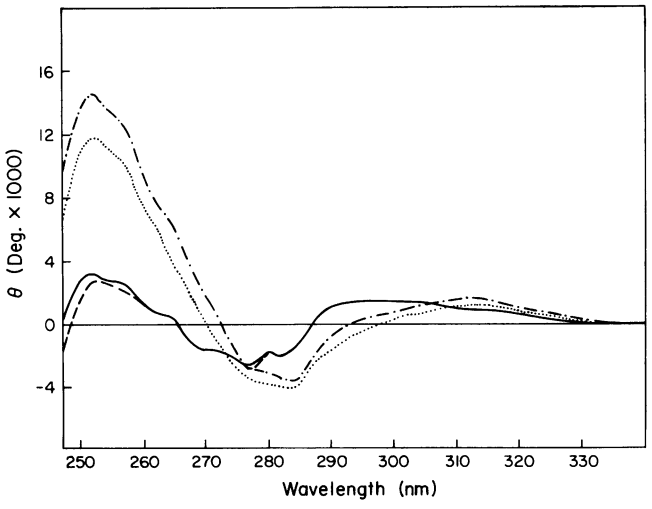

FIGURE 6 The near-UV CD spectra of spinach PC. Oxidized (—). Oxidized, 2.7 M ammonium sulfate (– – –). Reduced (–·–). Reduced, 2.7 M ammonium sulfate (· · · ·). The concentration was 50 μM. 10 mM Hepes pH 7.0.

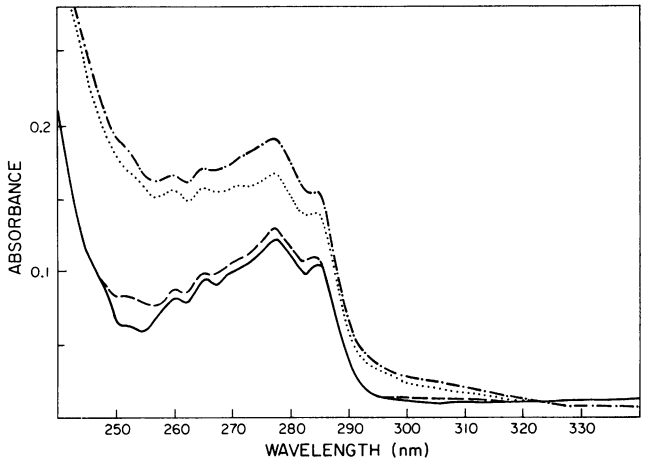

FIGURE 7 The near-UV absorption spectra of spinach PC. Oxidized, pH 7.0 (—). Oxidized, pH 5.0 (– – –). Reduced, pH 7.0 (–·–). Reduced, pH 5.0 (· · · ·). The concentration was 20 μM. 10 mM Hepes and Citrate.

of PC. The effect of pH was greater on the reduced form of PC than on the oxidized form. Lowering the pH also caused a minor blue shift of the wavelength of this large positive CD band to ~250 nm. Lowering the pH had less effect on the ellipticity of spinach PC at 278 nm and 284 nm. pH also had little effect on the CD band ~320 nm. In contrast, lowering the pH had a large effect on the CD band ~295 nm. For reduced PC the ellipticity at 295 nm is more negative at pH 5 than at pH 7. For oxidized PC the ellipticity at 295 nm is more positive at pH 5 than at pH 7. Thus the redox state dependent changes in ellipticity at 295 nm are larger at pH 5 than at pH 7.

Similar spectral changes were observed in PC from other species. Figs. 9 and 10 show the near-UV spectra of poplar PC and Figs. 11 and 12 show the near-UV spectra of lettuce PC. With few exceptions, lowering the pH also induced an attenuation of the spectroscpic change in intensity in the near-UV spectra of these species observed upon changing the redox state. It should be noted that the

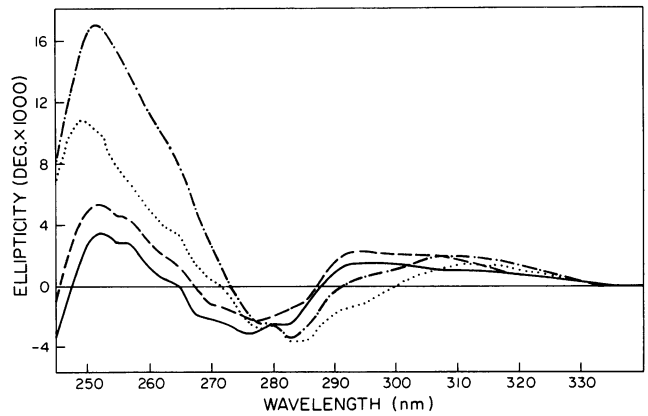

FIGURE 8 The near-UV CD spectra of spinach PC. Oxidized, pH 7.0 (—). Oxidized, pH 5.0 (– – –). Reduced, pH 7.0 (–·–). Reduced, pH 5.0 (· · · ·). The concentration was 50 μM. 10 mM Hepes and Citrate.

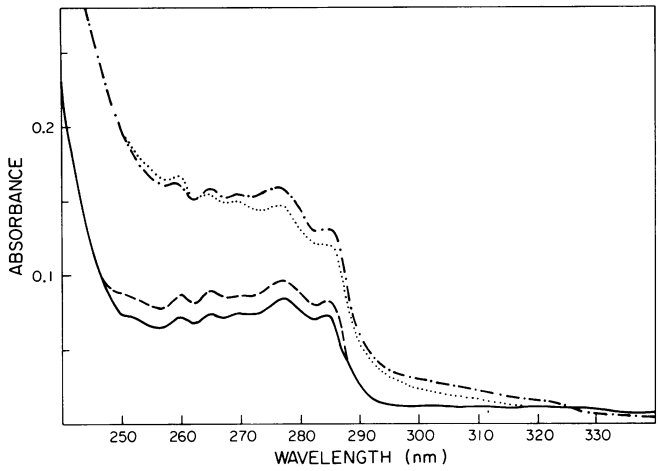

FIGURE 9 The near-UV absorption spectra of poplar PC. Oxidized, pH 7.0 (—). Oxidized, pH 5.0 (– – –). Reduced, pH 7.0 (–·–). Reduced, pH 5.0 (· · · ·). The concentration was 20 μM. 10 mM Hepes and Citrate.

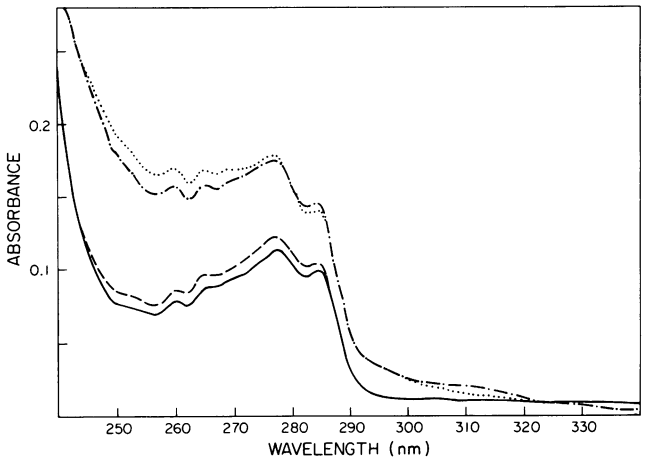

FIGURE 11 The near-UV absorption spectra of lettuce PC. Oxidized, pH 7.0 (—). Oxidized, pH 5.0 (– – –). Reduced, pH 7.0 (–·–). Reduced, pH 5.0 (· · · ·). The concentration was 20 μM. 10 mM Hepes and Citrate.

far-UV CD spectra of PC from these species were also identical to that of spinach PC. These results suggest that the spectroscopic changes due to changing the pH are part of a general phenomenon in PC and not just related to spinach. Although the near-UV spectra of these other species are very similar to spinach PC, they are not identical. Some of the differences observed in the near-UV spectra between poplar PC and the other two species are probably due to poplar PC having one more phenylalanine residue and one less tyrosine residue. This is especially noticeable in the near-UV CD spectrum of oxidized poplar PC at 278 nm. Another difference is that each species of PC exhibits a different A278/A597 ratio for its oxidized form. This ratio is 0.90 for poplar PC and 1.23 for lettuce PC as opposed to 1.12 for spinach PC. All species of PC used in this study were equally and extensively purified according the stated methods. To date all species of PC studied exhibit small but definite CD bands at ~295 nm and 320 nm.

DISCUSSION

Figs. 2, 5, 7, 9, and 11 show that for PC from several species, the near-UV absorption spectrum is dependent upon the redox state of the copper atom. Upon reduction there are significant increases in extinction throughout the near-UV below 310 nm. These increases can be explained as light scattering artifacts, charge transfer (CT) bands, or conformational changes in the protein that alter the environments of the aromatic residues and/or interactions between these aromatic residues.

Light scattering artifacts can be ruled out for the following reasons: (*a*) Reduced and apo spinach PC each exhibit a very flat absorption spectrum in the visible region from 330 to 800 nm; (*b*) No changes in the far-UV absorption spectrum of spinach PC were detected upon

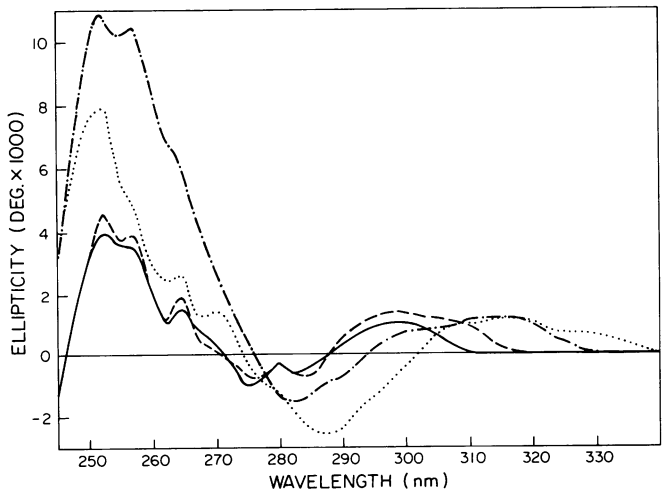

FIGURE 10 The near-UV CD spectra of poplar PC. Oxidized, pH 7.0 (—). Oxidized, pH 5.0 (– – –). Reduced, pH 7.0 (–·–). Reduced, pH 5.0 (· · · ·). The concentration was 50 μM. 10 mM Hepes and Citrate.

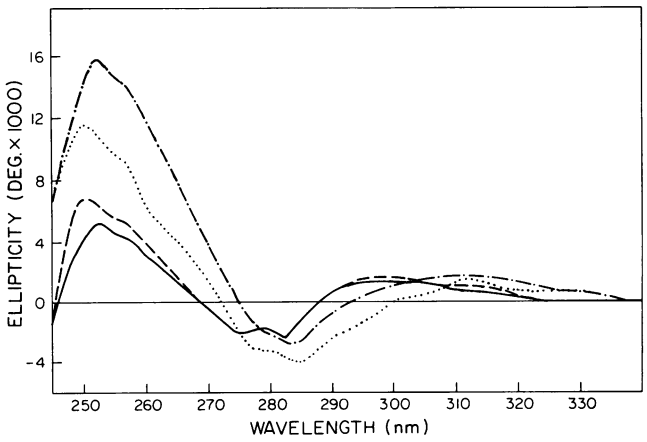

FIGURE 12 The near-UV CD spectra of lettuce PC. Oxidized, pH 7.0 (—). Oxidized, pH 5.0 (– – –). Reduced, pH 7.0 (–·–). Reduced, pH 5.0 (· · · ·).

reduction; (*c*) At neutral pH spinach PC has a net charge of −7, −8, and −9 for its oxidized, reduced, and apo forms respectively. It is doubtful that PC aggregates under the conditions used in this study.

The change in the near-UV absorption spectrum of PC could be due to CT bands, which would obviously be dependent upon the redox state of the copper atom. Assuming that the ligands to the copper form a distorted tetrahedral structure (Solomon et al., 1980), a metal-to-ligand type of CT band is possible that occurs in the Cu(I) state vs. the Cu(II) state. However, removal of the copper atom caused only an 11% decrease in extinction at 278 nm relative to reduced spinach PC. This is much less than the 56% increase in extinction due to the reduction of oxidized PC. Thus only ~20% of the redox state dependent change in extinction at 278 nm can be attributed to CT bands. The shape of the difference spectrum (reduced minus apo spinach PC) was very similar to a tyrosine absorption spectrum. This suggests that the CT band at 278 nm (assuming it exists) must have an absorption envelope similar to that of a tyrosine residue. This is unlikely for a simple CT band. Upon forming apo PC the extinction at 252 nm increases significantly over that of either oxidized or reduced PC. The observation that the extinction increases (not decreases) upon removal of the copper atom indicates that the change at 252 nm upon reduction cannot be due to a CT transition.

All three species of PC exhibit absorption maxima or shoulders at 284, 278, 273, 269, 266, 259, 252, and 248 nm. The maxima at 284 nm and 278 nm can be attributed to transitions due to the tyrosine residues while the remaining maxima can be attributed to transitions due to the phenylalanine residues (Donovan, 1969). Assuming that there is a CT contribution to the near-UV absorption spectrum of spinach PC, at least 80% of the redox state dependent change in extinction at 278 nm must be attributed to its three tyrosine residues. Increasing the environmental hydrophobicity of a tyrosine residue can increase its extinction coefficient by 0.700 mM^{-1} units (Donovan, 1969). For spinach PC the average tyrosine extinction coefficient would have to increase by 1.10 mM^{-1} units upon reduction. While a hydrophobic mechanism is mathematically possible for the large increase in extinction at 278nm, it could entail significant translocations of the PC tyrosine residues. By this mechanism the tyrosine residues would be exposed to the aqueous phase in oxidized PC and shielded from the aqueous phase in reduced PC. Large simultaneous translocations of all three of the tyrosine residues are unlikely since the secondary structure of PC does not change upon reduction (Draheim et al., 1985). In addition, reduction of PC caused similar changes in extinction in the spectral region usually associated with transitions due to phenylalanine residues. Phenylalanine residues are less sensitive to environmental hydrophobicity than are tyrosine residues. Hydrophobic interactions typically increase the extinction of a phenylalanine residue by only 0.04 mM^{-1} units (Donovan, 1969). It is highly unlikely that a change in the hydrophobic environments of the PC phenylalanine residues could cause the large changes in extinction observed ~252 nm upon reduction of the copper atom.

A more likely explanation for the large redox state dependent changes in extinction in the near-UV involves the mechanism of hyperchromicity. There are many aromatic-aromatic interactions possible as observed in Figs. 1 *a* and 1 *c*. These include both card-stacking and end-to-end orientations. Many of the aromatic residues are located within an 11 Å "slice." Any increase in extinction in one spectral region must be matched by a decrease in extinction in another region. For example, phenylalanine has transitions in the far-UV (188 nm and 205 nm). A small amount of borrowing could greatly affect the near-UV without measurably altering the far-UV absorption spectrum. In order to produce the increase in extinction observed in the near-UV absorption spectrum of PC at 252 nm, the extinction in the far-UV would have to decrease only 1% due to the presence of the 99 peptide transitions. A 1% change in extinction is smaller than the experimental error in the far-UV region.

Figs. 3, 6, 8, 10, and 12 show that for PC from several species, the near-UV CD spectrum is also dependent upon the PC redox state. The largest change in ellipticity occurs at ~252 nm. Transitions in this spectral region are usually associated with phenylalanine residues. There are several possible explanations for this phenonmenon. Mechanisms involving light scattering artifacts, CT bands, and drastic changes in the hydrophobic environments of the phenylalanine residues can be ruled out using arguments similar to those discussed above for the changes in the near-UV extinction of PC. Fig. 3 indicates that the ellipticity at 252 nm increases as the charge at the copper site decreases. This is analogous to the change observed in Fig. 2.

One possible explanation for these changes in ellipticity involves the one-electron state mixing mechanism (Sears and Beychok, 1973). An asymmetric charged environment can induce optical activity in an otherwise optically inactive chromophore (e.g. the benzene ring of phenylalanine). Optical activity is induced through a reduction in the symmetry of the chromophore. This allows the mixing of one of the chromophore's "magnetic" transitions with one of its "electric" transitions. By this mechanism a positive CD band at one wavelength must be matched with a negative CD band at another wavelength. Because of their proximity to the copper atom, the residues Phe 14 and Phe 35 are good candidates for such a dependency. Fig. 4 shows the far-UV CD spectra of oxidized and reduced spinach PC. Upon reduction there is no measurable change in the secondary structure of PC. However, some minor differences between the two redox forms could be observed at ~220 nm (Fig. 4, insert). Upon reduction a small negative CD band appeared ~220 nm relative to the oxidized spectrum. Since there is no detectable change in the

crossover wavelength, the change in ellipticity near 220 nm cannot be interpreted as a change in the secondary structure. The magnitude of this negative CD band is of the same order as the positive CD band induced at 252 nm due to the reduction of oxidized PC. One possible interpretation is that upon reduction the negative CD band at 220 nm is coupled to the positive CD band at 252 nm through a one-electron mechanism. Because of the scaling it is normally very difficult to observe the change in ellipticity at 220 nm due to changing the PC redox state.

Another possible mechanism for the large increases in ellipticity at ~252 nm involves the dipole-dipole coupled oscillator mechanism. By this mechanism the interaction of two or more separate chromophores creates a positive CD band at one wavelength and a matching negative CD band at another wavelength. The difference absorption spectrum of reduced minus oxidized spinach PC (not shown) exhibits a peak at 216 nm. An individual histidine residue exhibits a transition at 217 nm (Donovan, 1969). It is possible that Phe 14 and/or Phe 35 are coupled to one of the histidine ligands. Phe 14 is 7.6 Å and 9.7 Å distant from His 37 and His 87 respectively (ring-center to ring-center). The large ellipticity at 252 nm is also observed in apo spinach PC. This implies that CT transitions cannot be an important factor. However, a purely ligand histidine transition could still exist and be coupled to a phenylalanine residue transition. Such a histidine transition would probably be sensitive to the charge at the copper site and thus sensitive to the redox state of the molecule. Although both mechanisms are possible, at this time we favor the one-electron mechanism since the CD band is centered at ~220 nm rather than 216 nm.

Since there are no measurable changes in the secondary structure of PC upon changing its redox state, the spectroscopic changes observed in the near-UV must involve subtle conformational changes. However, small/subtle conformational changes can occur at many places throughout the molecule. There is some evidence that this is the case for spinach PC (Gross et al., 1985). It should be noted that using the x-ray crystallographic structures, the calculated far-UV CD spectrum of PC does not reproduce the measured spectrum. The reference set of protein structures was that of Chen et al. (1974). This suggests that the secondary structure of PC is somewhat unusual and therefore does not transform according to most "normal? proteins. Perhaps the large amount of proline (six residues, colored green in Figs. 1 *a–c*) in a relatively small protein is responsible.

The ellipticity of spinach PC exhibits only minor changes at 278 nm and 284 nm upon reduction. This spectral region is usually associated with transitions due to tyrosine residues. Since the extinction increases, the net rotatory strength of the tyrosine residues must decrease upon reduction. Upon forming apo PC there were further changes at 278 nm. These results support the interpretation that the PC tyrosine residues are also sensitive to the charge at the copper site.

There are some minor absorption bands at ~300–330 nm in all species of PC measured that appear to be redox state dependent. Removing the copper atom perturbed the absorbance in this region slightly. Because of their location it is difficult to assign these bands to aromatic residue transitions. All species of PC also exhibit at least two positive CD bands at ~295 nm and 320 nm that are also redox state dependent. At least one of these CD bands vanishes upon forming apo PC. This suggests that at least one CT band exists between 295 and 330 nm for spinach PC. Model histidine-Cu compounds exhibit transitions in this region (Schugar, 1983). Figs. 8, 10, and 12 also show the effect of pH on the CD bands at 295–330 nm. At least one of these CD bands is sensitive to a decrease in pH. This is consistent with the interpretation that these bands are due to either histidine transitions and/or histidine CT bands. It is possible to speculate that CT bands due to His 87 are responsible for the pH effects on the CD spectra at~ 295 nm. His 87 is accessible to the media while His 37 is not (Kojiro and Markley, 1983). X-ray crystallography indicates that there is a change in the orientation and distance of the His 87 ligand to the copper atom with lowering the pH for the reduced form (Freeman, 1981).

Suspension of spinach PC in 2.7 M ammonium sulfate altered its near-UV absorption and CD spectra as shown in Figs. 5 and 6. The redox state dependent changes in the near-UV absorption and CD spectra of PC were attenuated by high concentrations of ammonium sulfate. By implication, the conformational changes associated with these spectroscopic changes were also attenuated. 100 mM $CaCl_2$ did not measurably affect the near-UV spectra of spinach PC. This implies that the spectroscopic changes due to the ammonium sulfate cannot be explained by simple charge screening. Hydrophobic interactions may play an important role in this solvent system. Moreover, the cation requirement for the interaction of PC with P700 cannot be explained by divalent cation induced conformational changes in PC.

The redox state dependent changes in the near-UV absorption and CD spectra of PC are also attenuated upon lowering the pH as observed in Figs. 7–12. By implication, the associated conformational changes are attenuated. Simple charge screening effects probably do not play an important role since these spectroscopic changes appear to mimic those associated with high concentrations of ammonium sulfate. Lowering the pH actually neutralizes charges, which is a slightly different mechanism. As the pH is decreased, a larger percentage of the carboxyl groups become protonated. These protonated carboxyl groups will behave on the average more like typical uncharged polar residues and will exhibit properties similar to such residues. Hydrophobic interactions will be relatively enhanced under these circumstances. The near-UV spectrum of the

reduced form of PC is more sensitive to pH and ammonium sulfate than that of the oxidized form. This suggests that the conformation of the reduced form is more sensitive (flexible) to these perturbations. The oxidized form of PC is also slightly perturbed by these environmental factors.

The implications of a redox state dependent conformation for PC were previously discussed (Draheim et al., 1985). The effect of pH on the PC conformation has some implications with respect to the PC/P700 interaction. The rate of the PC/P700 interaction is maximal at pH 4.7 (Takabe et al., 1983). At this pH the reduced form of PC has been described as redox inactive (Freeman, 1981). The change in the PC near-UV spectra observed upon lowering the pH could be attributed to a redox inactive form. Since the redox inactive form of reduced PC is presumably more prevalent at pH 5, it should start to dominate the near-UV spectra at that lower pH. The near-UV spectrum of reduced PC is more similar to oxidized PC at pH 5 than at pH 7. This suggests that the tertiary conformation of the redox inactive form of reduced PC is more similar to that of oxidized PC than is the redox active form of reduced PC which is more prevalent at pH 7. The tertiary conformation of reduced PC at pH 5, the redox inactive form (and thus its associated spectrum), would presumably be "somewhere in between" that of oxidized and reduced PC at pH 7. The spectroscopic changes noted above could also be due to a redox active form of PC whose tertiary structure is pH dependent. Of course a third possibility is that both are occuring. At all pH's there should be an equilibrium between the redox active form and the redox inactive form of PC. Although the effect of pH on the oxidized form of PC is smaller than that on the reduced form, lowering the pH definitely affects the conformation of the oxidized form of PC.

CONCLUSIONS

PC exhibits significant changes in its near-UV absorption and CD spectra upon oxidation and reduction. These changes have been observed in several species and cannot be due to light scattering. Metal-to-ligand CT transitions are probably responsible for the spectroscopic changes at 295–340 nm. At least one transition in this region is sensitive to pH and is thus attributed to His 87. The increase in both extinction and ellipticity upon reduction between 250–270 nm (phenylalanine region) cannot be due to CT bands since there are further increases upon removal of the copper atom. CT bands can explain only 20% of the increase in extinction at 278 nm (tyrosine region). The source of the change in ellipticity is most likely the one-electron mechanism. An asymmetric electric field can induce optical activity in a chromophore. Altering the charge at the copper site will affect this asymmetric electric field and thus alter the CD.

The near-UV absorption and CD spectra of all species of PC studied were also sensitive to pH. Although pH has a greater effect on the spectra of its reduced form, the tertiary structures of both oxidized and reduced PC are sensitive to pH. Upon lowering the pH there is an attenuation of the spectroscopic changes associated with changing the redox state. Presumably, there is also an attenuation of the corresponding conformational changes. It is unlikely, that the effect of lowering the pH is due to simple charge screening. An enhancement of hydrophobic interactions following a pH decrease is a more likely possibility.

Note added in proof: The Far-UV spectrum shown in Fig. 4 agrees fairly well with that calculated for PC assuming a twisted β-pleated sheet conformation. 1986. (Manning, M.C., and R.W. Woody. *Biophys. J.* 49:(2, pt.2) 296*a*(Abstr.)

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