Biochimica et Biophysica Acta 808 (1985) 437–447
Elsevier

BBA 41800

Plastocyanin conformation. The effect of nitrotyrosine modification and pH

E.L. Gross, G.P. Anderson, S.L. Ketchner and J.E. Draheim

Department of Biochemistry, The Ohio State University, Columbus, OH 43210 (U.S.A.)

(Received January 21st, 1985)

Key words: Plastocyanin; Electron transport; Chemical modification; Nitrotyrosine; (Spinach, Poplar, Lettuce)

Plastocyanin isolated from several species including spinach, poplar, and lettuce showed conformational changes both upon reduction and upon lowering the pH as determined by near-ultraviolet absorption and fluorescence measurements. The fluorescence excitation maximum was at 278 nm for all species of plastocyanin measured. In the case of spinach, the emission maximum was at 310–312 nm, similar to a tyrosine residue in solution. The fluorescence intensity increased 22% upon reduction of plastocyanin at pH 7.0. In poplar plastocyanin, the emission maximum was shifted to 335 nm and increased only 10% upon reduction. The 335 nm emission peak observed in poplar plastocyanin is attributed to Tyr 80 which is hydrogen bonded to a carbonyl group on the protein backbone. Tyr 83 was also shown to undergo fluorescence changes upon reduction since the redox state-dependent fluorescence changes decreased for a nitrotyrosine (nitrotyrosine-plastocyanin) derivative of this residue. These results show that the east face of the molecule, which contains both Tyr 80 and 83 as well as a possible binding site [1,2], undergoes conformational changes upon reduction. These conformational changes may be involved in promoting smooth electron transport between plastocyanin and its reaction partners. Both the absorption and fluorescence were found to be pH dependent. The quantum yield for fluorescence increased sharply below pH 6 for both oxidized and reduced spinach plastocyanin. This may be related to the appearance of a redox-inactive form of reduced plastocyanin [3]. The conformational changes observed at low pH may provide a mechanism for control of electron transport by the proton gradient. Low concentrations of $CaCl_2$ (10 mM) had no effect on plastocyanin fluorescence. However, addition of 2.7 M $(NH_4)_2SO_4$ eliminated the redox-dependent fluorescence changes.

Introduction

Plastocyanin is a 10.5 kDa 'blue copper' protein functioning in the chloroplast electron-transport chain between cytochrome *f* and P-700, the photoactive chlorophyll in Photosystem I [3–5]. The crystal structures of both oxidized and reduced plastocyanin have been determined [3,6,7]. The copper is coordinated to four ligands (two histidines, a cysteine and a methionine) in a distorted tetrahedral geometry (Fig. 1). It has been proposed [1,2] that plastocyanin contains two binding sites for its reaction partners: (1) His 87 at the top of the molecule and (2) the region near Tyr 83 at the side. Plastocyanin also contains two patches of negative charges (residues Nos. 42–45 and Nos. 59–61) which are conserved in higher plant plastocyanins but are replaced by neutral or positively charged residues in cyanobacterial plastocyanins [1].

We have previously shown that plastocyanin in solution undergoes conformational changes upon

Abbreviations: Cyt, cytochrome; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid.

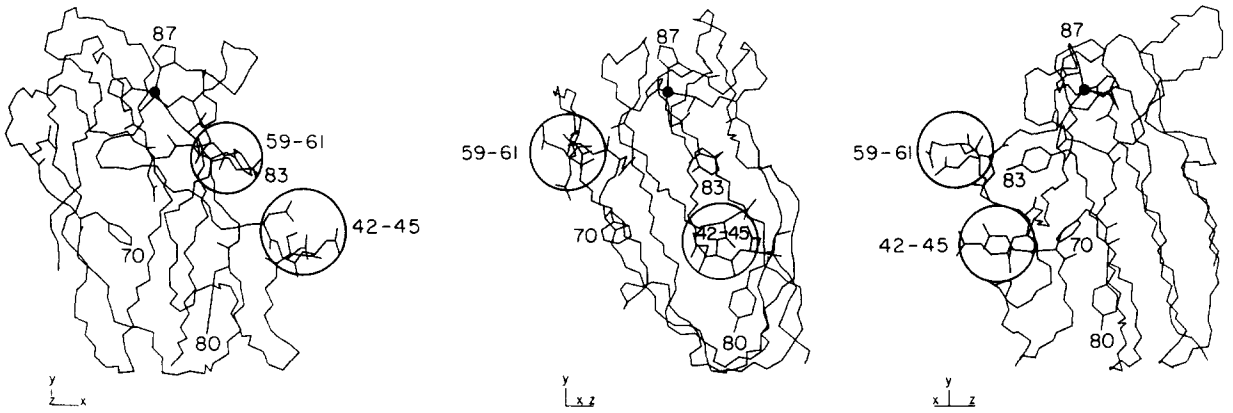

Fig. 1. Computer graphics representation of poplar plastocyanin. The X-ray coordinates for oxidized poplar (*Populus nigra*) plastocyanin [3] were obtained from the Brookhaven Protein Data Bank and displayed using a commercial graphics program called SUPERTAB. The peptide backbone, the histidine ligands to the copper, the tyrosine residues, phenylalanine 70 (which is a tyrosine residue in spinach) and the highly conserved negatively charged residues at positions 42–45 and 59–61 are shown. See Materials and Methods for further details.

oxidation and reduction [8,9] as determined by near-ultraviolet absorption and circular dichroism measurements. In contrast, the crystal structures of oxidized and reduced plastocyanin appear to be nearly identical at neutral pH values [3]. A similar situation exists for mammalian cytochrome *c* which has been shown to undergo larger redox state-induced conformational changes in solution than in the crystal [10,11]. The observation that high concentrations of ammonium sulfate attenuate redox state-induced conformational changes in plastocyanin explains part of this paradox [9].

It has been postulated that plastocyanin acts as a mobile electron carrier [12,13] shuttling electrons between the cytochrome b_6/f complex and Photosystem I which contains P-700. The redox state-linked conformational changes could be important in promoting smooth electron transport under these circumstances via differential binding of the oxidized and reduced species of plastocyanin to its reaction partners. For example, it would be advantageous for reduced plastocyanin to bind more tightly to P-700 than oxidized plastocyanin.

Plastocyanin is thought to be located on the inside of the thylakoid membrane [14,15]. Because of this, it should experience a 3 unit decrease in pH upon illumination [16]. The decrease in pH could affect both the conformation and activity of plastocyanin which could, in turn, regulate photosynthetic electron transport. Decreasing the pH had an effect on the crystal structure of reduced but not oxidized plastocyanin [3]. In particular, decreasing the pH increased the length of the bond between His 87 and the copper (Fig. 1). This change was originally correlated with the appearance of a redox-inactive form of reduced plastocyanin [17]. More recently [18], the region of highly conserved negative charges located at residues Nos. 42–45 has also been implicated in causing the redox-inactive form of reduced plastocyanin. If this is true, then changes in residues Nos. 42–45, which are 1.8 nm distant from the copper, can influence the copper site. These results suggest that plastocyanin is a flexible molecule in which 'conformational changes' at one site can be felt at another.

The redox-inactive form of reduced plastocyanin is interesting because there is evidence that plastocyanin should be active at low pH. First, Takabe et al. [19] have found that the rate of the reaction of reduced plastocyanin with $P\text{-}700^+$ is maximal at pH 4.7, under conditions in which plastocyanin should be inactive. Second, since plastocyanin is located in the lumen of the thylakoid, it must routinely experience a pH of 5 during illumination. It would be unreasonable for one redox state to be inactive under these condi-

tions. Because of the importance of plastocyanin in electron transport, we decided to examine the relationship between pH and redox state on the conformation of plastocyanin in solution. It was decided to use fluorescence for these studies because plastocyanin contains only a few tyrosine residues (three for spinach and two for poplar [3,4]) and no tryptophan residues. Each of the tyrosine residues is located in a different part of the plastocyanin molecule and in a different type of environment (Fig. 1 of Ref. 3). Tyr 83 sticks out into the medium whereas Tyr 80 is on the inside and hydrogen-bonded to the peptide carbonyl of residue 76. Tyr 70 in spinach (replaced by a phenylalanine in poplar) is located near other aromatic residues (not shown) and has one side exposed to the medium [7]. The fact that the tyrosines are in different environments may allow us to differentiate between the fluorescence of each tyrosine and thus, determine which parts of the molecule undergo conformational changes. In addition, a chemically modified plastocyanin will be prepared containing a nitrotyrosine derivative of Tyr 83. Since the nitrotyrosine moiety is non-fluorescent [20], it will allow us to evaluate the contribution of Tyr 83 to the fluorescence spectra.

Materials and Methods

Isolation of plastocyanin

Plastocyanin was isolated from spinach according to the method of Davis and San Pietro [21]. The plastocyanin was further purified using a Pharamcia FPLC with a Mono Q HR 5/5 anion exchange column. The final A_{275}/A_{597} ratio for oxidized spinach plastocyanin was 1.12. The FPLC purification was necessary for the fluorescence studies in order to remove the last trace of contaminating tryptophan proteins. An identical procedure was used to isolate plastocyanin from lettuce.

The same procedure was used to isolate plastocyanin from poplar (*Populus deltoides*) except that 0.5% polyvinylpyrilodone was added to the chloroplast isolation medium and the plastocyanin was treated with 60% $(NH_4)_2SO_4$ to precipitate the contaminating proteins prior to the DEAE chromatography. *P. deltoides* (cottenwood) was used instead of the *P. nigra* which 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 the aromatic amino acids. In the case of poplar, the final A_{275}/A_{597} was 0.9 due to its lower tyrosine content.

Plastocyanin concentrations were determined using an extinction coefficient at 597 nm of 4.9 $mM^{-1} \cdot cm^{-1}$ after addition of excess ferricyanide. Plastocyanin was oxidized using $K_3Fe(CN)_6$ which was subsequently removed by gel filtration on a Biogel P-10 column. Plastocyanin was reduced using sodium ascorbate which was removed by gel filtration on Biogel P-10.

Absorption measurements

Absorption measurements were carried out using a Cary 118 C spectrophotometer. Plastocyanin (15–25 μM) was suspended in 10 mM concentrations of the buffers indicated. Plastocyanin concentrations were determined at the end of each experiment by measuring the absorbance at 597 nm after addition of excess ferricyanide. Absorption spectra were normalized to 20 μM. The absorbance of plastocyanin was found to vary linearly with concentration between 20 and 100 μM, indicating that aggregation effects do not contribute to our results.

Fluorescence measurements

Fluorescence spectra were measured on a Spex Fluorolog fluorometer using a Bascomb-Turner electronic recorder and an Apple II computer for data processing. All fluorescence spectra were corrected for water Raman, incomplete oxidation or reduction and differences in concentration. The correction for water Raman was necessary, since it contributed up to 40% of the total emission at 310 nm.

The fluorescence excitation spectra were monitored at 310 nm for spinach and lettuce plastocyanin and 340 nm for poplar plastocyanin. The slit width for the excitation monochromator was 2 nm. Excitation spectra were corrected for the wavelength dependence in lamp intensity. The excitation wavelength for the emission spectra was 285 nm and the slit width for the emission monochromator was 10 nm. The shape of the emission spectra were independent of the excitation wavelength.

Plastocyanin (10–25 μM) was suspended in 10 mM concentrations of the buffers indicated. The plastocyanin concentrations were determined at the end of the experiment as described above and the spectra were normalized to 20 μM.

Chemical Modification of plastocyanin

A nitrotyrosine derivative of spinach plastocyanin was prepared by a modification of the procedure of Davis and San Pietro [21]. 50 μl of tetranitromethane were added in 10 μl aliquots every 10 min to a stirred 2.5 ml solution of 0.3 mM plastocyanin in 50 mM Tris-Cl (pH 8.5) + 100 mM NaCl. The total reaction time was approx. 1 h. The nitrotyrosine-plastocyanin was then separated from unmodified and denatured plastocyanin on the FPLC as described above. The number of nitrotyrosine residues per plastocyanin molecule was determined by measuring the absorption of the nitrotyrosine-plastocyanin at 360 nm and 597 nm. This number was found to be near unity. The location of the label was determined by monitoring the nitrotyrosine absorption following trypsin digestion and HPLC separation of the tryptic peptides. Heat denatured plastocyanin and nitrotyrosine-plastocyanin were digested for 1 h with 1% DPCC-treated trypsin. The peptides were then separated using reverse phase chromatography on a LDC HPLC with an Anspec RP-8 column. The peptides were eluted using a 0–45% gradient of a 9 : 1 acetonitrile/0.1% trifluoroacetic acid in water solution. The identity of the peptides were determined using a Beckman 119 CL Amino Acid Analyzer. The nitrotyrosine-containing peptide was identified as peptide 6 which contains Tyr 83. No other peptides were labelled.

Computer graphics display of poplar plastocyanin

A graphical display of the three-dimensional structure of oxidized poplar plastocyanin was made using the X-ray coordinates obtained from the Brookhaven Protein Data Bank, Chemistry Department, Brookhaven National Laboratory, Upton, N.Y. 11973 together with a commercial engineering wireframe modeling graphics package called SUPERTAB which is part of the IDEAS package.

SUPERTAB is available to universities through grants from its developer (Structural Dynamics Research Col, Milford, OH). A processor was developed to adapt SUPERTAB for the display of biological molecules. Further details of the graphics will be published elsewhere [23] and are also available from the Brookhaven Protein Data Bank.

Materials

DEAE-cellulose and Biogel P-10 were obtained from Biorad Laboratories. Hepes, Tris and DPCC-treated trypsin were obtained from the Sigma Chemical Co. HPLC grade acetonitrile and water were obtained from Burdick & Jackson Laboratories Inc. All other chemicals were of reagent grade.

Results

Near-ultraviolet absorption spectra for oxidized and reduced spinach and poplar plastocyanin

The near-ultraviolet absorption spectrum of spinach plastocyanin (Fig. 2) shows peaks or shoulders at the following wavelengths: 284, 278,

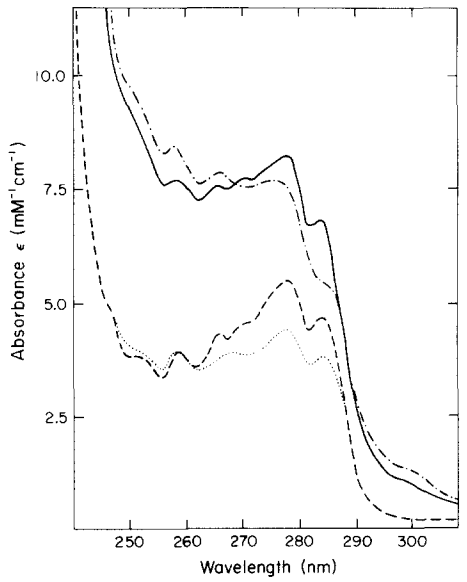

Fig. 2. Near-ultraviolet absorption spectra were determined for oxidized and reduced spinach and poplar plastocyanin (at 20 μM) suspended in 10 mM Hepes buffer (pH 7.0). Other conditions were as described in the Materials and Methods section. ———, Reduced spinach plastocyanin; - - - - - -, oxidized spinach plastocyanin; ·–·–·, reduced poplar plastocyanin; ·····, oxidized poplar plastocyanin.

273, 269, 266, 259, 252 and 248 nm. The peaks at 284 and 278 nm can be attributed to the tyrosine residues and the rest to the phenylalanine residues [24]. The small absorption bands observed for reduced plastocyanin between 290 and 310 nm may be due to histidine charge transfer bands because the associated circular dichroic bands disappear upon removal of the copper [9]. Reduction of spinach plastocyanin caused a 50% increase in the absorbance at 278 nm. The absorbance at 278 nm is less for poplar than for spinach plastocyanin as predicted from the difference in amino acid composition. The absorbance of poplar plastocyanin increased 70% upon reduction.

A comparison of reduced plastocyanin with non-denatured apo-plastocyanin showed that, at wavelengths below 270 nm, the absorbance of apo-plastocyanin was greater than that for reduced plastocyanin indicating that charge transfer bands can not be responsible for the absorbance increases observed upon reduction of the protein (not shown). At 278 nm, the absorbance of apo-plastocyanin was slightly less than that observed for reduced plastocyanin. However, if the total decrease in absorbance observed at 278 nm upon forming apo-plastocyanin were due to charge transfer bands, they would account for only one-fifth of the absorbance increase observed upon reduction of plastocyanin.

Fluorescence excitation and emission spectra of spinach and poplar plastocyanin

Fig. 3a shows the fluorescence excitation spectra of the oxidized and reduced forms of spinach plastocyanin at pH 7.0. Upon reduction, there was a 40% increase in the fluorescence at the excitation maximum of 278 nm. The shape of the excitation spectra resembles the absorption spectrum of tyrosine in solution indicating the tyrosine residues in plastocyanin are responsible for the fluorescence. It is unlikely that there is any significant fluorescence from the copper center charge transitions in this region, since such fluorescence (if it occurred at all) would be emitted from the lowest excited state (approx. 775 nm for copper in its oxidized state). The fluorescence excitation spectrum of poplar plastocyanin has the same shape as that of spinach (not shown). However, the fluorescence intensity is only 35% that of spinach, and

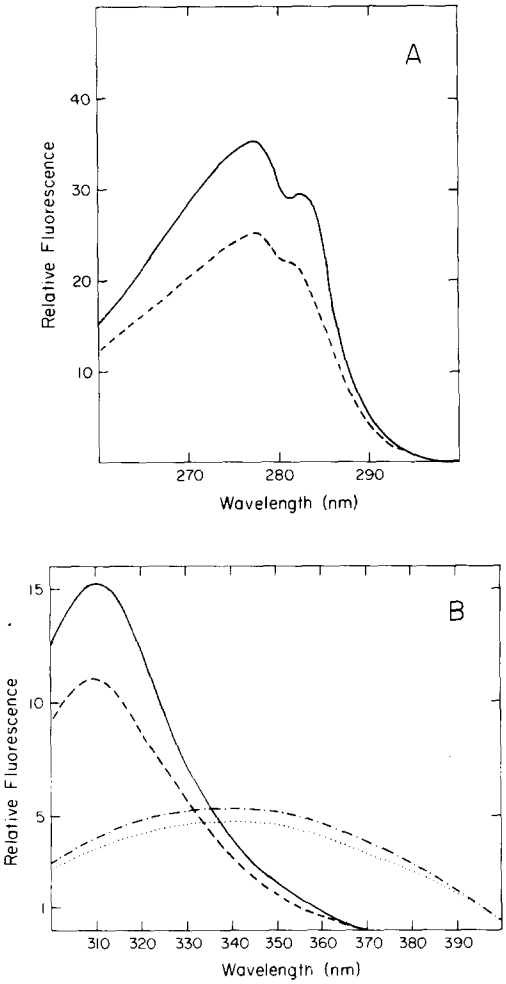

Fig. 3. Fluorescence excitation and emission spectra for plastocyanin. Spinach or poplar plastocyanin (at 20 μM) was suspended in 10 mM Hepes buffer (pH 7.0). Other conditions were as described in the Materials and Methods section. (A) Excitation spectra: ———, Reduced spinach plastocyanin; - - - - - -, oxidized spinach plastocyanin. (B) Emission spectra: ———, reduced spinach plastocyanin; - - - - - -, oxidized spinach plastocyanin; ·—·—·, reduced poplar plastocyanin; · · · · · ·, oxidized poplar plastocyanin.

there is only an 18% increase upon reduction.

The fluorescence emission spectra of oxidized and reduced spinach and poplar plastocyanin are shown in Fig. 3b. Both oxidized and reduced spinach plastocyanin showed an emission maximum at 310 nm which is identical to that observed for tyrosine in solution. Identical results were obtained for lettuce plastocyanin (not shown). The fluorescence emission intensity for oxidized poplar

plastocyanin was only 48% of that of oxidized spinach plastocyanin. Also, there was only a 10% increase in fluorescence upon reduction. The shift in emission spectrum to longer wavelengths has been interpreted as being due to the formation of a deprotonated tyrosine (tyrosinate) in the excited state (exciplex). This type of emission spectrum was observed for adrenodoxin [25] and was attributed to the presence of hydrogen-bonded tyrosine which should promote exciplex formation. For this reason, we have attributed the 340 nm emission band in poplar plastocyanin to Tyr 80. Thus, Tyr 80 undergoes conformational changes upon reduction of plastocyanin.

The absorption and fluorescence properties of spinach plastocyanin containing a nitrotyrosine derivative of Tyr 83

We evaluated the contribution of Tyr 83 by preparing a nitrotyrosine (nitrotyrosine-plastocyanin) derivative [21] of that residue. Peptide mapping (Anderson, G.P., Draheim, J.E., Ketchner, S. and Gross, E.L., unpublished data) showed that Tyr 83 was the only residue modified. The nitrotyrosine residue produced is non-fluorescent [20] and has two new absorption bands at 360 and 420 nm, respectively. Therefore, a comparison of the absorption and fluorescence properties of control and nitrotyrosine (nitrotyrosine-plastocyanin) should allow us to determine the absorption and fluorescence properties of Tyr 83 (Table I). The absorption and fluorescence intensities of Tyr 83 were calculated from the difference between control plastocyanin and nitrotyrosine-plastocyanin and from comparison to model nitrotyrosine-Tyr amides in solution. It can be seen that the increase in absorption for nitrotyrosine-plastocyanin is larger than the control indicating that most of the absorption increase is due to either Tyr 70 or 80. At least some of the absorption change must be due to Tyr 80, since it is also observed in poplar plastocyanin (Fig. 2) which lacks Tyr 70. The contribution of Tyr 70 can be estimated from the difference in absorbance at 284 nm between spinach and poplar plastocyanin. This difference increases by 44% when plastocyanin is reduced indicating that Tyr 70 also undergoes redox state-dependent absorbance changes. In contrast, most of the change in fluorescence intensity is due to Tyr 83. The relative quantum yield of fluorescence of Tyr 83 increases upon reduction of plastocyanin whereas that for the remaining portion of the protein decreases. The increase of fluorescence may be due to Tyr 83 moving into a more hydrophobic environment when plastocyanin is reduced [26]. In any case, these results show that Tyr 83 is involved in the redox state-dependent

TABLE I

THE EFFECT OF CHEMICAL MODIFICATION ON THE ABSORPTION AND FLUORESCENCE PROPERTIES OF SPINACH PLASTOCYANIN

Absorbance at 278 nm and the fluorescence intensities were determined as described for Figs. 2 and 3b and in the Materials and Methods section, except that plastocyanin was suspended in 10 mM citrate buffer (pH 6.0). The absorbance measurements were corrected for the nitrotyrosine absorbance at 278 nm by using the ratio of 278 to that at 340 nm obtained for model nitrotyrosine compounds. The quantum yields of fluorescence were calculated using the following formula.

$$\phi_{\text{plastocyanin}} = \frac{\phi_{\text{tyr}} * (1 - 10^{-\text{tyr}_{\text{abs}}}) * f_{\text{plastocyanin}}}{f_{\text{tyr}} * (1 - 10^{-\text{plastocyanin}_{\text{abs}}})}$$

where ϕ_{tyr} is the quantum yield of fluorescence for tyrosine in water, namely 0.25; tyr_{abs} is the absorbance of tyrosine; $\text{plastocyanin}_{\text{abs}}$ is the absorbance of plastocyanin; $f_{\text{plastocyanin}}$ and f_{tyr} are the fluorescence intensities of plastocyanin and tyrosine, respectively. Spinach plastocyanin was chemically modified with tetranitromethane as described in the Materials and Methods section. The absorbance and fluorescence intensity of Tyr 83 were calculated from the difference between these quantities for control and nitrotyrosine-plastocyanin. + refers to an increase; − refers to a decrease.

Oxidation state	Control	Type of plastocyanin	
		Nitrotyrosine-plastocyanin	Tyr-83_{calc}
Absorbance			
Oxidized	0.111	0.057	0.054
Reduced	0.180	0.117	0.063
% Change on reduction	+62	+105	+17
Fluorescence intensities			
Oxidized	154	77	77
Reduced	211	95	116
% Change in reduction	+37	+23	+51
Fluorescence quantum yields			
Oxidized	0.0150	0.0137	0.0145
Reduced	0.0137	0.0088	0.0185
% Change in reduction	−9	−36	+28

conformational changes. The fluorescence results are confirmed by circular dichroism measurements [22]. The involvement of Tyr 83 in conformational changes is of interest, since this residue may be involved in one of the binding sites for plastocyanin's reaction partners [1,2].

The absence of the 312 nm peak in poplar plastocyanin also suggests that Tyr 70 is involved in the conformational changes. However, small species-dependent changes in the amino acid sequence of plastocyanin could change the geometry of the hydrogen bond between Tyr 80 and the peptide carbonyl group of residue No. 76 which would prevent exciplex formation. Thus, Tyr 80 may also fluoresce at 312 nm in spinach (Fig. 3), lettuce (not shown) and parsley [27].

The effect of pH upon the absorbance and fluorescence of spinach plastocyanin

The absorbance at 278 nm was almost pH independent for oxidized spinach plastocyanin (Fig. 4). In contrast, the absorbance for reduced plastocyanin increased from pH 5 to 7 and decreased again at higher pH's. The absorbance increased upon reduction for all pH's tested.

The effect of pH on fluorescence intensity is more complicated (Fig. 5). In the case of reduced plastocyanin, the fluorescence intensity was highest at pH 5, decreasing sharply between pH 5 and 6 and more slowly between pH 6 and 9. The decrease in fluorescence intensity between pH 5 and 6 was also observed for oxidized plastocyanin. However, the fluorescence intensity increased again between pH 6 and 9. Because of this, the fluorescence change observed upon reduction changes in both magnitude and sign as a function of pH. For example, the 22% increase observed at pH 7 became a 14% decrease at pH 9. There is almost no change in fluorescence upon reduction at pH 8.

Similar results were obtained for poplar plastocyanin (Table II). In this case, a small decrease in fluorescence was observed at pH 7.0 both at 312 and 335 nm. These results contrast with those presented in Fig. 3 probably due to the fact that pH 7 is very close to the crossover point in poplar. The fluorescence of poplar plastocyanin at

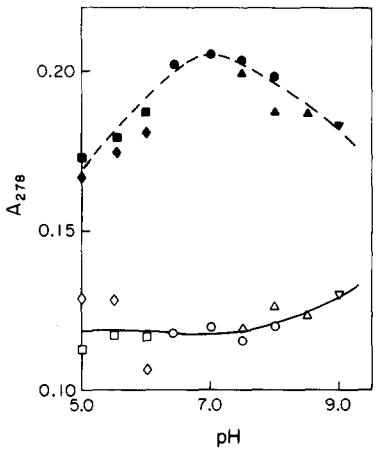

Fig. 4. The effect of pH on the absorbance of tyrosine residues in spinach plastocyanin. Oxidized and reduced spinach plastocyanin was suspended in 10 mM concentrations of the buffers indicated below after which the absorbance at 278 nm was determined. Other conditions were as described for Fig. 2 and in the Materials and Methods section. Buffers used: ▽, glycine-HCl; △, Tris-HCl; ○, Hepes; □, succinate; ◇, citrate. Open symbols refer to oxidized plastocyanin; closed symbols refer to reduced plastocyanin.

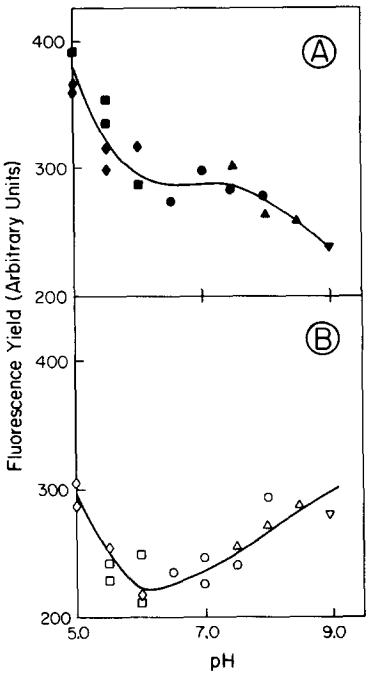

Fig. 5. Fluorescence intensity as a function of pH for spinach plastocyanin. The fluorescence intensity was determined as a function of pH. Conditions were as described for Figs. 3 and 4 and in the Materials and Methods section. The excitation wavelength was 278 nm and the emission wavelength was 310 nm. (A) Reduced spinach plastocyanin; (B) oxidized spinach plastocyanin.

TABLE II

THE EFFECT OF SPECIES, pH AND ADDITION OF SALTS ON THE FLUORESCENCE YIELD OF PLASTOCYANIN

Fluorescence intensities were determined as described in the Materials and Methods Section. The excitation wavelength was 278 nm. The error in determining individual fluorescence intensities was ±4%. The following buffers were used: 10 mM Hepes (pH 7.0); 10 mM citrate (pH 5.0). The values for the fluorescence intensity are given in arbitrary units. +denotes an increase in fluorescence; −denotes a decrease in fluorescence.

pH	Salt	Concn. (M)	Wavelength (nm)	Fluorescence intensity		% Change upon reduction
				oxidized	reduced	
Poplar						
7.0	–	–	312	97	81	−17
7.0			335	105	94	−12
5.0			312	146	183	+25
5.0			335	151	170	+13
Spinach						
7.0	–	–	312	199	221	+11
7.0	$CaCl_2$	0.01	312	197	219	+11
7.0	$CaCl_2$	0.1	312	192	176	−8
7.0	$(NH_4)_2SO_4$	2.7	312	108	108	0

312 and 335 nm was greater at pH 5 than that at pH 7.0. In addition it increased upon reduction. The ratio of the emission at 312 nm to that at 335 nm also increased slightly suggesting that exciplex formation is pH dependent.

These results become more comprehensible if we consider relative quantum yields for fluorescence (Fig. 6). The quantum yields for fluorescence incorporate both changes in absorbance and fluorescence intensity. The data can be divided into two pH regions. Increasing the pH from 5 to 6 caused a decrease in the quantum yield of fluorescence for both oxidized and reduced plastocyanin. Above pH 6, the quantum yield for oxidized plastocyanin remained constant, whereas that for reduced plastocyanin continued to decrease with increasing pH. Most important, however, is the observation that the total quantum yield for fluorescence decreased upon reduction for all pH values tested. However, the contribution of the various tyrosine residues is pH dependent. The quantum yield for oxidized and reduced nitrotyrosine-plastocyanin increased 33% and 106%, respectively when the pH was lowered from 6 to 5. In contrast, the quantum yield for Tyr 83 in oxidized plastocyanin decreased by 12% upon lowering the pH, while for reduced plastocyanin it increased 17%. These results show that at least two of the tyrosine residues are involved in the pH-dependent conformational changes.

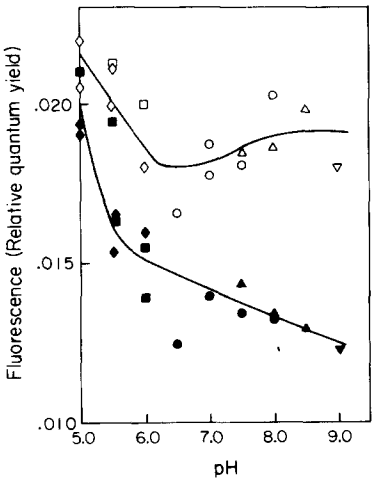

Fig. 6. The effect of pH on the quantum yield for fluorescence for spinach plastocyanin. Conditions were as described for Figs. 2, 3, 4 and 5 and in the Materials and Methods section. Open symbols refer to oxidized plastocyanin and closed symbols refer to reduced plastocyanin. Fluorescence quantum yields were determined as described for Table I.

Cu^{2+} cannot be a quencher under these circumstances, since the quantum yield for fluorescence actually increases upon oxidation of the protein. Furthermore, with respect to fluorescence intensity, at high pH values the oxidized plastocyanin fluoresces more than the reduced form, whereas the converse is true at low pH. This would

imply that Cu^{1+} is the quencher at high pH, while Cu^{+} is the quencher at low pH.

The effect of salts on the fluorescence of spinach plastocyanin

Salts have been shown to promote the interaction of plastocyanin with P-700 [28–31], while inhibiting that between plastocyanin and cytochrome *f* [32]. These results have been interpreted in terms of charge screening [33]. However, this does not rule out effects on the conformation of the electron carriers themselves. For this reason, the effect of salts on plastocyanin fluorescence was studied. It was found that concentrations of $CaCl_2$ (10 mM) sufficient to saturate the interaction of plastocyanin with P-700 had no effect on plastocyanin fluorescence (Table II). Thus, salt-induced conformational changes in plastocyanin cannot be required for its binding to P-700. However, the addition of 100 mM $CaCl_2$ decreased the fluorescence of reduced plastocyanin suggesting that reduced plastocyanin is more sensitive to environmental influences than is oxidized plastocyanin. Addition of 2.7 M $(NH_4)_2SO_4$ decreased the fluorescence intensity for both oxidized and reduced plastocyanin and completely eliminated the increase in fluorescence observed upon reduction. This is interesting, since poplar plastocyanin is usually crystallized from 2.7 M $(NH_4)_2SO_4$. Thus, one would expect the redox state-dependent conformational changes to be damped in the crystal. These results add further support to our hypothesis that the conformation of plastocyanin in solution is not identical with that observed in the crystal. The changes in fluorescence observed at high salt concentrations are probably due to changes in hydrophobic interactions rather than simple charge screening.

Discussion

Fluorescence as a monitor of conformational changes in plastocyanin

The near-ultraviolet absorbance and fluorescence spectra depicted above show that plastocyanin undergoes conformational changes upon changes in redox state, pH and salt concentrations. These results present a picture of plastocyanin as a flexible molecule capable of responding to changes in its environment.

Spatial location of the conformational changes in plastocyanin

A comparison of the absorbance changes for spinach and poplar plastocyanin implicates both Tyr 70 and 80 in the redox state-dependent conformational changes. An analysis of the fluorescence spectra for spinach, poplar and spinach plastocyanin containing a nitrotyrosine derivative of Tyr 83, shows that Tyr 80 and 83 are involved in the conformational changes. These results taken together confirm our previous findings [9] that the conformational changes are global in nature. In addition, the fluorescence studies suggest that the conformation of the protein in the neighborhood of Tyr 80 may be species dependent. The involvement of Tyr 83 in the conformational changes is important for two reasons. First, this part of the molecule has been proposed as a potential binding site for one of the reaction partners of plastocyanin [1,2]. Second, it has also been implicated in the formation of the redox-inactive form of reduced plastocyanin observed at low pH values [18]. The extent of the conformational changes are probably small (< 0.1 nm) since there were no corresponding changes in secondary structure (Draheim, J.E. and Gross, E.L., unpublished results). Nonetheless, they may be sufficient to alter the binding of plastocyanin with its reaction partners. For example, it would be useful if oxidized plastocyanin bound more tightly to reduced Cyt *f* than reduced plastocyanin. The converse would be true for P-700. Thus the conformational changes could be involved in promoting smooth electron transport.

The effect of pH on the conformation of plastocyanin

The absorbance measurements indicate that the reduced form of plastocyanin is more sensitive to pH than is the oxidized form. These results agree with the crystallographic data. However, the fluorescence measurements indicate that both the oxidized and reduced forms of plastocyanin are sensitive to pH, especially below pH 6. Thus, the conformation of plastocyanin will be different when chloroplasts are illuminated and the internal thylakoid pH drops to approx. 5 than during the dark. In fact, the high-fluorescent form observed at low pH may represent a 'redox-inactive form'. If this is true, how does one account for the high

rates of P-700$^+$ reduction observed at low pH values? There are two possible explanations. First, there may be two forms of plastocyanin present at low pH, one of which is redox-inactive and one of which can reduce P-700$^+$ with very high rates (Eqn. 1):

$$\begin{array}{l} \text{plastocyanin}(Cu^{1+})_{pH5,I} \leftrightarrow \text{plastocyanin}(Cu^{1+})_{pH5,A} \\ \quad\quad\quad\quad \updownarrow \\ \text{plastocyanin}(Cu^{2+})_{pH5,A} + \text{P-700}^+ \\ \quad\quad\quad\quad \updownarrow \\ \text{plastocyanin}(Ca^{2+}) + \text{P-700} \end{array} \quad (1)$$

where A and I refer to active and inactive conformations respectively. If this is true, the fluorescence experiments are monitoring the inactive form, since it would predominate at low pH values.

The second possibility is that the redox-inactive form is converted to a redox-active form upon binding to P-700 (Eqn. 2):

$$\begin{array}{l} \text{plastocyanin}(Cu^{1+})_{pH7} \\ \quad\quad\quad\quad \updownarrow \\ \text{plastocyanin}(Cu^{1+})_{pH5,I} + \text{P-700}^+ \\ \quad\quad\quad\quad \updownarrow \\ \text{plastocyanin}(Cu^{2+})_{pH5,A} - \text{P-700}^+ \\ \quad\quad\quad\quad \updownarrow \\ \text{plastocyanin}(Cu^{2+}) + \text{P-700} \end{array} \quad (2)$$

This would prevent reduced plastocyanin from giving its electron to any but its physiological reaction partner and, thus, control electron transport. Further experiments are required to distinguish between these two models. In either case, the structure and function of plastocyanin is influenced by the protonmotive force. This, in turn, could provide a means for controlling the rate of electron transport through this part of the electron-transport chain by affecting the binding constants of oxidized and reduced plastocyanin with its reaction partners.

Conclusions

In conclusion, it has been shown that plastocyanin undergoes conformational changes in response to reduction, lowering the pH and addition of high concentrations of salts. This indicates that the plastocyanin molecule is flexible and capable of responding to changes in its environment. Tyr 80 and 83 were found to be definitely involved in these conformational changes. There is also some evidence that Tyr 70 in involved. These conformational changes may be involved in promoting smooth electron transport between Cyt *f* and plastocyanin as well as allowing electron transport to be regulated by the protonmotive force.

Acknowledgments

We thank Stephanie Bail for her expert technical assistance. This research was supported in part by Grant 1 R01 GM30560 from the N.I.H.

References

1 Cookson, D.J., Hayes, M.T. and Wright, P.E. (1980) Biochim. Biophys. Acta 591, 162–176
2 Hanford, P.M., Hill, H.A.O., Lee, W., Henderson, R.A. and Sykes, A.G. (1980) J. Inorg. Biochem. 13, 83–88
3 Freeman, H.C. (1981) Coordination Chem. 21, 29–51
4 Boulter, D., Haslett, B.G., Peacock, D., Ramshaw, J.A.M. and Scawan, M.D. (1977) Int. Rev. Biochem. 13, 1–40
5 Solomon, E.I. (1983) in Copper Coordination Chemistry, Biochemical and Inorganic Perspectives (Karlin, K.D. and Zubieta, J., eds.), pp. 1–22, Adenine Press, Guilderland, NY
6 Coleman, P.M., Freeman, H.C., Guss, J.M., Murata, M., Norris, V.A., Ramshaw, J.A.M. and Venkatappa, M.P. (1978) Nature 272, 319–324
7 Guss, J.M. and Freeman, H.C. (1983) J. Mol. Biol. 169, 521–563
8 Gross, E., Draheim, J., Pan, R., Tripathy, B., Anderson, G., Rellick, L. and Duane, J. (1984) in Advances in Photosynthesis Research (Sybesma, C., ed.), Vol. I, pp. 537–540, Martinus Nijhoff/Dr. W. Junk Publishers, Dordrecht, The Netherlands
9 Draheim, J.E., Anderson, G.P., Pan, R.L., Rellick, L.M., Duane, J.W. and Gross, E.L. (1985) Arch. Biochem. Biophys. 237, 110–117
10 Salemme, F.R. (1977) Annu. Rev. Biochem. 46, 299–329
11 Sudha, B.P., Dixit, N. and Vanderkooi, J.M. (1984) in Current Topics in Bioenergetics, Vol. 13, pp. 159–204, Academic Press, New York
12 Olsen, L.F. (1982) Biochim. Biophys. Acta 582, 482–490
13 Takano, M., Takahashi, M. and Asada, K. (1982) Arch. Biochem. Biophys. 218, 369–375
14 Hauska, G., Berzborn, R.J., McCarty, R.E. and Racker, E. (1971) J. Biol. Chem. 246, 3524–3531
15 Haehnel, W., Berzborn, R.J. and Andersson, B. (1981) Biochim. Biophys. Acta 637, 389–399
16 Ort, D.R. and Melandri, A. (1982) in Photosynthesis. Energy Conversion by Plants and Bacteria (Govindjee, ed.), Vol. I, pp. 539–487, Academic Press, New York

17 Segal, M.G. and Sykes, A.G. (1978) J. Am. Chem. Soc. 100, 4585–4592
18 Chapman, S.K., Watson, A.D. and Sykes, A.G. (1983) J. Chem. Soc. Dalton Trans. 1983, 2543–2548
19 Takabe, T., Ishikawa, H., Niwa, S. and Itoh, S. (1983) J. Biochem. 94, 1901–1911
20 Williams, R.T. (1959) J.R. Inst. Chem. 83, 611–626
21 Davis, D.J. and San Pietro, A. (1979) Anal. Biochem. 95, 254–259
22 Reference deleted
23 Duane, J., Rellick, L. and Gross, E.L. (1985) Engineering Design Graphics J., in the press
24 Donovan, J.W. (1979) in Physical Principles and Techniques in Protein Chemistry (Leach, S.J., ed.), Part A, pp. 102–170, Academic Press, New York
25 Lim, B.T. and Kimura, T. (1980) J. Biol. Chem. 255, 2440–2444
26 Giancotti, V., Quadrifoglio, F., Cowgill, R.W. and Crane-Robinson, C. (1980) Biochim. Biophys. Acta 624, 60–65
27 Graziani, M.T., Agro, A.F., Rotillo, G., Barra, D. and Mondovi, B. (1976) Biochem. 13, 804–808
28 Lien, S. and San Pietro, A. (1979) Arch. Biochem. Biophys. 194, 128–137
29 Lockau, W. (1979) Eur. J. Biochem. 94, 365–373
30 Tamura, N., Yamamoto, Y. and Nishimura, M. (1980) Biochim. Biophys. Acta 592, 536–545
31 Haehnel, W., Propper, A. and Krause, H. (1980) Biochim. Biophys. Acta 593, 384–399
32 Niwa, S., Ishikawa, H., Nikai, S. and Takabe, T. (1980) J. Biochem. (Tokyo) 88, 1177–1183
33 Barber, J., Mills, J. and Love, A. (1977) FEBS Lett. 74, 174–181