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The interaction of nitrotyrosine-83 plastocyanin with cytochromes *f* and *c*: pH dependence and the effect of an additional negative charge on plastocyanin

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Spinach plastocyanin was selectively modified using tetranitromethane which incorporates a nitro group *ortho* to the hydroxyl group of tyrosine 83 (Anderson, G.P., Draheim, J.E. and Gross, E.L. (1985) Biochim. Biophys. Acta 810, 123–131). This tyrosine residue has been postulated to be part of the cytochrome *f* binding site on plastocyanin. Since the hydroxyl moiety of nitrotyrosine 83 is deprotonated above its p*K* of 8.3, it provides a useful modification for studying the effect of an extra negative charge on the interaction of plastocyanin with cytochrome *f*. No effect on cytochrome *f* oxidation was observed at pH 7 under conditions in which the hydroxyl moiety is protonated. However, the rate of cytochrome *f* oxidation increased at pH values greater than 8, reaching a maximum at pH 8.6 and decreasing at still higher pH values. The increase was half-maximal at pH 8.3 which is the p*K* for the hydroxyl moiety on nitrotyrosine 83. In contrast, the rate of cytochrome *f* oxidation for control plastocyanin was independent of pH from pH 7 to 8.6. These results show that increasing the negative charge on plastocyanin at Tyr-83 increases the ability to react with cytochrome *f*, supporting the hypothesis that cytochrome *f* interacts with plastocyanin at this location. In contrast, the reaction of Ntyr-83 plastocyanin with mammalian cytochrome *c* was independent of pH, suggesting that its mode of interaction with plastocyanin is different from that of cytochrome *f*. A comparison of the effects of Ntyr-83 modification of plastocyanin with the carboxyl- and amino-group modifications reported previously suggests that plastocyanin binds to cytochrome *f* in such a way that electrons could be donated to plastocyanin at either of its two binding sites.

Introduction

Plastocyanin is a 10.5 kDa copper protein which functions in the chloroplast electron transport chain by transferring electrons from cytochrome *f* to P700 in Photosystem I [1–3]. The crystal structures of both oxidized [4,5] and reduced [6] plastocyanin have been determined. The copper atom is coordinated to two histidine residues (37 and 87), a cysteine residue (84) and a methionine (92) in a distorted tetrahedral geometry.

NMR studies [7,8] revealed the existence of two potential binding sites on plastocyanin for reaction partners. One site (Site 1) is located at the top of the plastocyanin molecule as shown in Fig. 1. Electron transfer at this site occurs through His 87 by an outer sphere mechanism. Site 2 is located at Tyr-83 on the right-hand side of the molecule as shown in Fig. 1. Electron transfer at this site occurs via a tunneling mechanism. Tyr 83 is surrounded by two patches of negatively charged residues (42–45 and 59–61) which are highly conserved in higher plant plastocyanins [1]. These negatively charged residues produce a negatively charged electrostatic potential field in the vicinity of Binding Site 2 [9] which, in turn, attracts positively charged electron donors and acceptors such as $Co(phen)_3^{2+,3+}$ to this location [3].

The existence of two potential binding sites raises the question as to which of the site(s) are used by reaction partners such as cytochrome *f*, P700 or mammalian cytochrome *c*. We have used a chemical modification

Abbreviations: CDNB, 4-chloro-3,5-dinitrobenzoic acid; DEAE, diethylaminoethyl; EDA, ethylenediamine; FPLC, fast protein liquid chromatography; Ntyr-83 plastocyanin, plastocyanin modified with a nitro group at tyrosine 83; P700, reaction center chlorophyll of Photosystem I.

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approach to answer this question. Our initial studies [10–12] involved the modification of carboxyl groups on plastocyanin using ethylenediamine (EDA) plus a water-soluble carbodiimide. This reaction replaces negatively charged carboxyl groups with positively charged amino groups. Three singly modified forms were obtained containing 1 mol of EDA in either the 42–45 cluster, the 59–61 cluster or at residue 68. The locations of these modifications are shown in Fig. 1. All three forms of modified plastocyanin showed inhibition of cytochrome *f* oxidation. These results were expected, since the binding site on cytochrome *f* for plastocyanin is positively charged [13,14], although the cytochrome *f* molecule as a whole [15] has a net negative charge. Plastocyanin molecules modified at residues 42–45 and 59–61 showed greater inhibition of cytochrome *f* oxidation than did those modified at residue 68 [12]. We concluded from these results that cytochrome *f* interacts with plastocyanin at Site 2. A similar conclusion was reached by Morand et al. [16] by sequencing the cross-linked portions of a covalent adduct formed between plastocyanin and cytochrome *f* [17] and by Beoku-Betts and Sykes [18] also using chemical modification.

NMR experiments have also shown that cytochrome *c* interacts at Site 2 [19]. However, this conclusion has recently been challenged by Rush et al. [20] using a dipole model for the interaction between the two proteins.

Our initial chemical modification studies were incomplete in that only three chemically modified forms were obtained, all of which involved replacing negative charges by positive charges. For this reason, a complementary experiment was designed in which positively charged amino groups on plastocyanin were replaced by negatively charged carboxyl groups using 4-chloro-3,5-dinitrobenzoic acid (CDNB) [21]. Four singly modified forms were obtained with the label located at residues 1, 54, 71 and 77, respectively. None of these modifications is located near Binding Site 2. If replacing a negative charge by a positive charge caused inhibition of cytochrome *f* oxidation, it was expected that replacing a positive charge with a negative charge would increase the rate of cytochrome *f* oxidation. Instead, inhibition of cytochrome *f* oxidation was observed varying from 0% to 71% depending on location of the label. Simple explanations such as steric hindrance and shifts in redox potentials were ruled out. A hypothesis was developed implicating both the net charge on the plastocyanin molecule and the charge at the binding site for cytochrome *f* in determining the rate of interaction of the two molecules. In particular, charges at the binding site 'recognize' the positively charged binding site on cytochrome *f*, whereas those at other locations 'recognize' the net negative charge on the cytochrome *f* molecule.

If this model is correct, then stimulation of cytochrome *f* oxidation should be observed if an extra negative charge is incorporated in plastocyanin at Binding Site 2. However, incorporation of a negative charge at that location proved to be difficult. It was not possible to prepare a CDNB derivative of plastocyanin labelled at 81 which would be close to Binding Site 2. We also attempted to add a negative charge to Binding

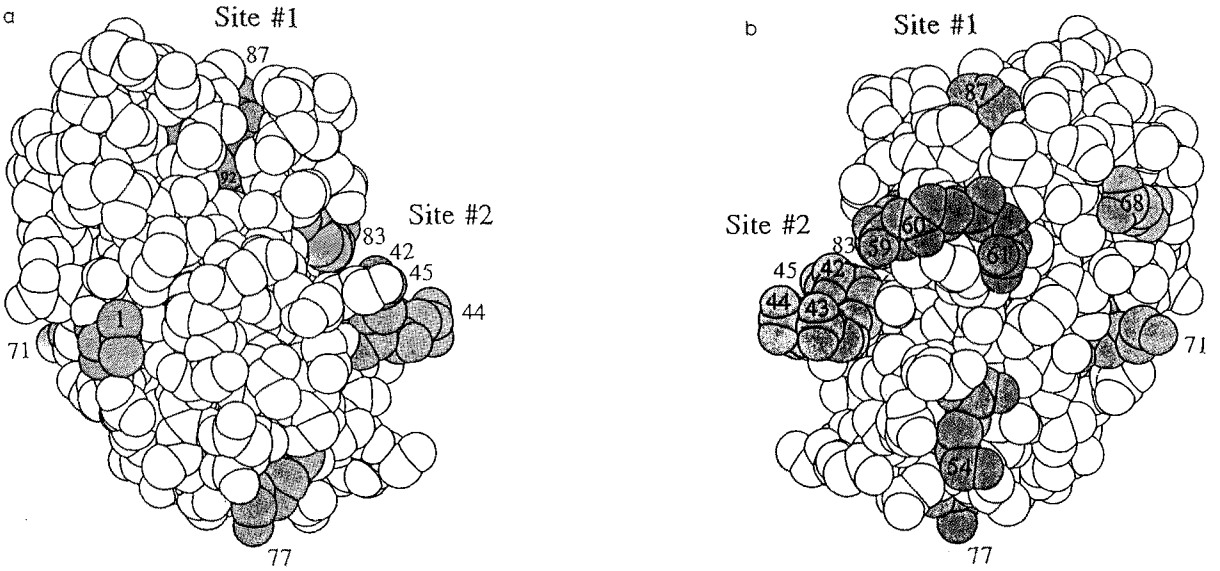
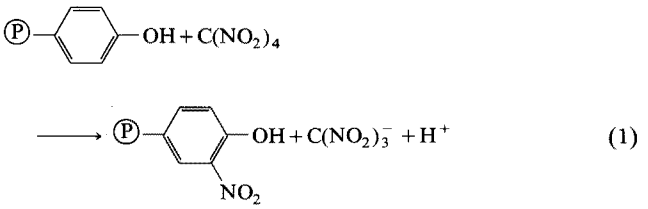


Fig. 1. Molecular graphics representation of spinach plastocyanin. X-ray coordinates of poplar plastocyanin [5] were obtained from the Brookhaven Protein Data Bank [30]. 22 substitutions were made to convert poplar to spinach plastocyanin. The plastocyanin molecule is displayed using the Quanta program from Polygen, which is available to us courtesy of the Ohio Supercomputer Center. The program was run on a Control Data-Silicon Graphics 4D.70 GT graphics workstation. The EDA modification sites at residues 42–45, 59–61 and 68 are shown as are the CDNB modification sites at residues 1, 54, 71 and 77. The nitrotyrosine modification site at Tyr-83 is also shown as are the two potential binding sites.

Site 2 by making a nitrotyrosine derivative of Tyr-83 (Ntyr-83 plastocyanin) [22], reducing the nitro group to an amino group [23], and reacting with CDNB. This also failed, possibly for steric reasons. As a final attempt, we decided to make use of the dissociation properties of the nitrotyrosine moiety of Ntyr-83 plastocyanin itself. Previously [2], we have shown that reaction of plastocyanin with tetranitromethane [24] results in the selective incorporation of a nitro moiety *ortho* to the hydroxyl group of Tyr-83 according to Eqn. 1.



(1)

Incorporation of the nitro group lowers the pK of the tyrosine hydroxyl from around pH 10 to pH 7 [24]. However, the pK of the nitrotyrosine residue is sensitive to the surrounding electrostatic field. The pK of nitrotyrosine-83 was found to be pH 8.3 for oxidized plastocyanin [22] as a result of the negative electrostatic field generated by the carboxyl-containing residues at Binding Site 2 [9]. The pK was increased to 8.6 upon reduction of the copper center, again due to electrostatic effects. The importance of these results for this study is that, in the case of Ntyr-83 plastocyanin, there will be an additional negative charge at Binding Site 2 at pH values greater than 8.3, allowing us to use this system to test the effect of an additional negative charge at Binding Site 2 on the interaction of plastocyanin with both cytochrome *f* and cytochrome *c*. Our results are presented below.

Methods

Isolation and chemical modification of plastocyanin

Plastocyanin was isolated as described by Davis and San Pietro [25] without further purification. NTyr-83 plastocyanin was prepared as previously described [22]. 0.3 mM plastocyanin was incubated in 50 mM Tris-HCl (pH 8.5) in the presence of 0.1 M NaCl and 0.5 mM potassium ferricyanide in a total volume of 3.0 ml. Five aliquots of 20 μl tetranitromethane were added at 10 min intervals for a total incubation time of 50 min. The reaction was carried out at room temperature in the hood. The reaction was stopped by chromatography on a Sephadex G-25 column in 10 mM succinate buffer (pH 6.0). Ntyr-83 plastocyanin was purified by anion-exchange FPLC using a mono-Q anion-exchange column in 25 mM Tris-HCl (pH 8.2) using a linear NaCl gradient from 0 to 500 mM. As described previously, three peaks were obtained consisting of unmodified plastocyanin, plastocyanin containing a single nitro group at Tyr-83, and denatured plastocyanin containing multiple modifications. Both the unmodified plastocyanin peak and the Ntyr-83 plastocyanin peak were further purified as follows. The peaks were divided into two parts: an A portion containing material from the beginning of the peak to the point of maximal absorption and a B portion containing material from the point of maximal absorption to the end of the peak. Each portion was rechromatographed separately. The rechromatographed B portions of both the Ntyr-83 plastocyanin peak and the unmodified plastocyanin peak were used for the studies described below.

The extent of incorporation was determined by measuring the absorption of the nitrotyrosine moiety at 360 nm using an extinction coefficient of 2.8 $mM^{-1} \cdot cm^{-1}$ for the Ntyr-83 plastocyanin in 10 mM succinate buffer (pH 6.0). This value was compared to the concentration of plastocyanin which was determined by measuring the extinction at 597 nm in the presence of excess ferricyanide using an extinction coefficient of 4.9 $mM^{-1} \cdot cm^{-1}$. The ratio of these two values represents the extent of incorporation. The unmodified plastocyanin fraction recovered from the FPLC fractionation contained less than 0.08 mol nitrotyrosine per mol plastocyanin. The average incorporation for the Ntyr-83 plastocyanin was 1.1 mol nitrotyrosine per mol plastocyanin. The method for determining the location of the nitrotyrosine moiety is described in Ref. 22.

Determination of the redox potential of Ntyr-83 plastocyanin.

The redox potentials of control and Ntyr-83 plastocyanin was determined using the method of Gross et al. [21]. The redox potentials were determined at pH 7.0,8.6 and 9.0. For pH 7.0 and 8.6, the buffer used was 25 mM Tris-succinate and for pH 9.0 the buffer was 25 mM Tris-HCl. Sufficient NaCl was added to give an ionic strength of 0.30 M in each case.

Isolation of cytochrome f and cytochrome c

Monomeric cytochrome *f* was isolated from turnip leaves according to the method of Molnar et al. [26], through the DEAE purification step. The cytochrome *f* prepared using this method has a purity index of 0.7. Cytochrome *c* from horse heart (Type VI) was used without further purification and was dissolved in 25 mM Tris-succinate buffer (pH 7.0).

Kinetics of cytochrome f and cytochrome c oxidation

The interaction of either cytochrome *f* or cytochrome *c* with plastocyanin was determined by measuring the oxidation kinetics of the cytochrome component using the stopped flow attachment for the Aminco DW 2a spectrophotometer as previously described [22]. The Aminco DW-2a was operated as a single beam instru-

ment. The signal from the photomultiplier was fed to a Keithley Model 427 Current Amplifier and monitored using an oscilloscope. The signal from the current amplifier was fed to a Bascomb-Turner Model 2110 electronic recorder with a data acquisition rate of one data point per millisecond and one data point per 5 ms for cytochrome *f* and cytochrome *c* respectively. Data were analyzed on a Kaypro computer using the program Hyperplot. For both cytochrome *c* and *f*, cytochrome oxidation was monitored at 422 nm using a slit width of 6 nm.

Second-order rate constants were determined using pseudo-first-order conditions. The cytochrome and plastocyanin concentrations were 0.5 μM and 2.5 μM, respectively. These conditions were sufficient to drive the cytochrome *f* reaction nearly to completion. Unless otherwise specified, the reactions were carried out in 25 mM Tris-succinate at the indicated pH with sufficient NaCl added to give the desired ionic strength. The reaction temperature was 22° C. Each value reported represents the average of at least five separate samples.

Cytochrome *f* was reduced by reaction with excess sodium ascorbate which was removed by concentration in an Amicon cell. The reduced state was maintained provided the cytochrome *f* sample was stored at 4° C prior to use. Cytochrome *c* was reduced by reaction with excess sodium ascorbate which was removed by dialysis against 25 mM Tris-succinate buffer (pH 7.0) for 1–2 h under nitrogen. The cytochrome *c* was stored under nitrogen to maintain the reduced state. Plastocyanin was oxidized using an excess of potassium ferricyanide which was removed using the Amicon cell.

Materials

Bio-Gel P-10 was obtained from Bio-Rad Laboratories. Tris and cytochrome *c* were obtained from the Sigma. All other reagents were of reagent grade.

Results

The pH dependence of the interaction of cytochrome f with Ntyr-83 plastocyanin

The rate of cytochrome *f* oxidation was determined as a function of pH for control and Ntyr-83 plastocyanin (Fig. 2). In the case of control PC, the rate of cytochrome *f* oxidation was independent of pH from pH 7 to 8.6 but decreased slightly at higher pH values. Similar results were observed for the unreacted plastocyanin recovered from the reaction mixture after FPLC chromatography, except that the rates of cytochrome *f* oxidation were slightly higher for the unreacted plastocyanin than for control plastocyanin at all pH values tested. These results show that increasing the pH to values as high as 8.6 did not affect the interaction of plastocyanin with cytochrome *f*.

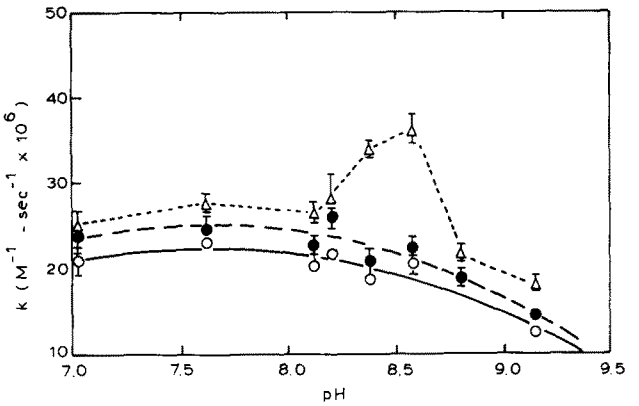


Fig. 2. The effect of pH on the interaction of Ntyr-83 plastocyanin with cytochrome *f*. The reaction was carried out as described in the Methods section. 25 mM Tris-succinate buffer was used to maintain the pH at the values indicated. Sufficient NaCl was added in each case to bring the ionic strength to 0.3 M. Each value presented represents the average of at least five separate determinations. ○, Control plastocyanin; ●, unreacted plastocyanin recovered from the reaction mixture after FPLC; △, Ntyr-83 plastocyanin.

In contrast the rate of interaction of cytochrome *f* with Ntyr-83 plastocyanin showed the predicted pH dependence. Almost no effect was observed for the nitrotyrosine modification at pH values less than 8, in agreement with previous results [22]. These results reflect the fact that the tyrosine hydroxyl is protonated under these conditions causing no change in the charge at Binding Site 2. The absence of any effect also shows that the addition of the nitro group to Tyr-83 causes neither steric hindrance of cytochrome *f* binding nor interference with electron transfer.

However, in contrast to control plastocyanin, the rate of cytochrome *f* oxidation for the Ntyr-83 derivative increased above pH 8, reaching a maximum at pH 8.6, and decreased again at still higher pH values. Half-maximal increases were observed about pH 8.3, which is the p*K* for the nitrotyrosine-83 moiety for oxidized plastocyanin [22]. Thus, the increases in the rate of cytochrome *f* oxidation parallel the appearance of the negative charge on the hydroxyl group of Ntyr-83.

The effect of nitrotyrosine modification on the midpoint redox potential of plastocyanin

The question arises as to whether the increase in the rate of cytochrome *f* oxidation could be due to a modification-induced change in the redox potential of plastocyanin [27]. To answer this question, the redox potentials were determine for control plastocyanin, the unmodified plastocyanin recovered from the reaction mixture and Ntyr-83 plastocyanin at pH 7.0, 8.6 and 9.0. The midpoint redox potential for control plastocyanin was found to be $+372 \pm 5$ mV, which is in agreement with previous results [28]. The midpoint redox potential was found to be independent of pH between 7.0 and 9.0 (not shown). In addition, the mid-

point redox potentials of the Ntyr-83 plastocyanin and the unreacted plastocyanin recovered from the reaction mixture were identical to that of control plastocyanin at all pH levels. Therefore, the increase in the rate of cytochrome *f* oxidation observed at pH 8.3 and 8.6 is not due to a change in the midpoint redox potential. The observation that the addition of an extra negative charge had no effect on the midpoint redox potential is consistent with the results previously obtained for the CDNB-plastocyanin derivatives [21] and with the electrostatic calculations of Durell et al. [9].

The pH dependence of the interaction of mammalian cytochrome c with Ntyr-83 plastocyanin

Since it has been postulated that both cytochrome *f* [12,16] and cytochrome *c* [19] bind at Binding Site 2 (but see Ref. 20), similar results should be observed for cytochrome *c* oxidation. The rate of cytochrome *c* oxidation was independent of pH between 7.0 and 8.6 (Table I) indicating, as in the case of cytochrome *f*, that increasing the pH does not interfere with either binding or electron transfer. However, in contrast to the results observed for cytochrome *f*, there was no increase in the rate of cytochrome *f* oxidation above pH 8 for Ntyr-83 plastocyanin. Thus, the interaction of cytochrome *f* and cytochrome *c* with plastocyanin must differ in either the binding or electron transfer step.

TABLE I

The effect of pH on the interaction of Ntyr-83 plastocyanin with cytochrome c

Plastocyanin	k ($M^{-1}s^{-1}$)($\times 10^6$)			
pH:	7.0	8.0	8.3	8.6
Control [a]	1.8±0.1	1.9±0.2	2.0±0.3	1.8±0.1
Ntyr-83	2.2±0.3	2.2±0.2	1.9±0.2	1.9±0.2

[a] Control plastocyanin consisted of the unreacted fraction of the reaction mixture separated by FPLC. Reaction conditions consisted of 25 mM Tris-succinate buffer at the pH values indicated plus sufficient NaCl to bring the reaction mixture to 0.3 M ionic strength. The exact values of the pH were measured after the reaction. Other conditions were as described in the Methods section. Each value represents the average of at least five separate determinations.

The effect of ionic strength on the interaction of Ntyr-83 plastocyanin with cytochrome f and cytochrome c

If the increase in reaction rate observed for Ntyr-83 plastocyanin is due to an increase in binding resulting from the additional negative charge, then the effect should be ionic-strength-dependent. The ionic strength dependence of the increase in cytochrome *f* oxidation is shown in Table II. It can be seen that the degree of stimulation observed for Ntyr-83 increases with decreasing ionic strength. Thus, these results are consistent with the hypothesis that an additional negative charge at Tyr-83 increases the binding of plastocyanin to cytochrome *f*. In contrast, there was no significant increase in the rate of cytochrome *c* oxidation except a very low ionic strength (0.075 M). These results confirm that the mode of interaction of cytochrome *c* with plastocyanin differs from that of cytochrome *f*.

Discussion

The pH dependence of the rate of cytochrome *f* oxidation using Ntyr-83 plastocyanin shows that the addition of an additional negative charge to Binding Site 2 affects the interaction of plastocyanin with cytochrome *f*. One question which arises is whether the soluble cytochrome *f* isolated from turnip leaves corresponds to the cytochrome *f* which is part of the cytochrome *b/f* complex. To answer this question, we have prepared a monoclonal antibody to isolated turnip cytochrome *f*, which cross-reacts with isolated spinach cytochrome *f* and also with cytochrome *f* in the cytochrome *b/f* complex (Gross, Molnar, Curtiss, Berg and Reuter, unpublished data). In addition, we have sequenced small regions of the turnip cytochrome *f* molecule and have found the amino acid sequences of these

TABLE II

The effect of ionic strength of the interaction of Ntyr-83 plastocyanin with cytochrome c and cytochrome f

pH	Ionic strength (M)	k ($M^{-1}s^{-1}$)($\times 10^6$)				
		cytochrome *c*		cytochrome *f*		
		control [a]	Ntyr-83	control	Ntyr-83	% of control
7.0	0.3	2.6±0.2	2.9±0.2	29.6±1.9	27.6± 1.6	93
8.6	0.075	9.6±0.5	11.6±0.7	–	–	–
8.6	0.225	–	–	47.2±3.0	93.4±11.3	198
8.6	0.3	3.2±0.3	2.9±0.4	24.2±1.8	37.0± 3.0	153
8.6	0.5	2.2±0.1	2.4±0.3	11.1±0.4	13.5±0.6	122

[a] Control plastocyanin for the kinetic studies consisted of the unreacted plastocyanin separated from the reaction mixture by FPLC. Reaction conditions consisted of 25 mM Tris-succinate buffer at the indicated pH plus sufficient NaCl to obtain the desired ionic strength. pH values were measured after the conclusion of the reaction. Other conditions were as described in the Methods section. Each value reported represents the average of at least five separate determinations.

regions to be identical to the corresponding regions of spinach.

A second question is whether the results obtained are an artifact of the high pH values used in this experiment. The following considerations suggest that the results are valid. First, the rates of cytochrome *f* oxidation for control plastocyanin are independent of pH up to pH values above 8.6. Second, in the CDNB modification studies, plastocyanin was incubated at pH 9 for 17 h without a decrease in activity [21]. Third, the second order rate constants obtained for cytochrome *f* oxidation compare favorably with those obtained previously [12,22,29], particularly in view of the high ionic strengths used in our study. Finally, the fact that stimulation, not inhibition, was observed shows that there is no steric hindrance involved in adding the nitro group to the tyrosine, nor is there a general deleterious effect of adding an additional negative charge to the plastocyanin molecule.

A third question concerns whether the observed stimulation of cytochrome *f* oxidation is due to an increase in the binding to cytochrome *f*, an increase in the rate of electron transfer or both. Unfortunately, the rapid rates of the reaction coupled with the low binding constant for the two reactants preclude determining the K_m for cytochrome *f*-plastocyanin binding. However, the ionic strength dependence of the observed stimulation of cytochrome *f* oxidation is consistent with a binding phenomenon, although an effect on electron transfer can not be ruled out. In any case, Site 2 must be involved either in the initial recognition of cytochrome *f* by plastocyanin or in the electron transfer. Thus, these results confirm the earlier results of Anderson et al. [12] and Morand et al. [16].

A fourth question concerns the reason for the decrease in reaction rates observed above pH 8.6. The decreases can be attributed to the Ntyr-83 plastocyanin molecule since they are not observed for control plastocyanin. An effect of pH on cytochrome *f* would be observed in both cases. We have previously observed that Ntyr-83 plastocyanin is less stable than control plastocyanin at pH levels above 8.3. A decrease in stability resulting from the additional negative charge at Site 2, would cause a decrease in reaction rates compared to the control under the same conditions.

A fifth question concerns the reason for the difference in the behavior of Ntyr-83 plastocyanin with cytochrome *f* and cytochrome *c*. The results obtained support the conclusion that cytochrome *f* and cytochrome *c* either bind to different sites on the plastocyanin molecule or donate electrons at different sites. Further studies are necessary to differentiate between these alternatives. However, in either case, it may be possible to reconcile the results of Morand et al. [16] with those of Rush et al. [20]. In any case, these results show that cytochrome *c* can not be used as a simple model for cytochrome *f* either in kinetic experiments or computer modeling of protein-protein interactions.

Model of the binding of plastocyanin to cytochrome f

To date, we have prepared three types of chemically modified plastocyanin: (A) those modified at acidic residues (42–45, 59–61 and 68) [12]; (B) those modified at lysine residues (54, 71 and 77) [21] and (C) Ntyr-83 plastocyanin [22]. These modifications can be divided into three classes with respect to their effect on cytochrome *f* oxidation. The first class consists of those modifications located in the vicinity of binding Site II. These include the EDA modifications at residues 42–45 and 59–61 and the nitrotyrosine modification of residue 83. The observation of inhibition of cytochrome *f* oxidation for the EDA derivatives and stimulation for Ntyr-83 plastocyanin at high pH indicates that the initial binding of plastocyanin with cytochrome *f* occurs at Site II. These conclusions are consistent with those of Beoku-Betts and Sykes [18] and Morand et al. [16]. These results also show that Site II on plastocyanin reacts with positive charges on cytochrome *f*, which is consistent with the hypothesis that the heme cleft in cytochrome *f* is surrounded by positively charged residues [13].

The second class of modifications consists of the CDNB derivatives modified at residues 1, 54 and 77. In each case, inhibition of cytochrome *f* oxidation was observed, indicating that the amino groups in question interact with negatively charged residues on cytochrome *f*. Originally [21], we proposed that these amino groups 'recognize' the net negative charge on cytochrome *f*. However, since not all modifications show inhibition (i.e., CDNB Lys-71 plastocyanin), we now suggest that there may be specific interactions between certain positively charged residues on plastocyanin and negatively charged residues on cytochrome *f*.This may be particularly true in the case of CDNB Lys-77 plastocyanin. We do not think that the inhibition observed is due to a wrong orientation, since stimulation was observed for cytochrome *c* oxidation for all the CDNB-modified forms.

EDA Glu-68 plastocyanin belongs to a third class of modifications. It is not located at Site 2 but shows inhibition of cytochrome *f* oxidation. We propose that Glu-68 also reacts with positive charges on cytochrome *f*, possibly near the heme cleft. If this is so, then the initial binding site for cytochrome *f* would not be directly at Tyr-83 but more towards the backside of the molecule (Fig. 1b). In this case, cytochrome *f* could donate electrons at either Site I or Site II.

Conclusions

The rate of cytochrome *f* oxidation using Ntyr-83 as an electron acceptor showed an increase above pH 8

with a pK of 8.3. These results are consistent with an increase in the binding of Ntyr-83 to cytochrome f at high pH as a result of the additional negative charge at Binding Site 2. However, an effect on electron transfer can not be ruled out. The lack of an effect using cytochrome c as the electron donor suggests that cytochrome c interacts with plastocyanin in a different manner than does cytochrome f.

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References

1 Boulter, D., Haslett, B.G., Peacock, D., Ramshaw, J.A.M. and Scawen, M.D. (1977) Int. Rev. Biochem. 13, 1–40.
2 Freeman, H.C. (1981) Coord. Chem. 21, 29–51.
3 Sykes, A.G. (1985) Chem. Soc. Rev. 14, 283–315.
4 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.
5 Guss, J.M. and Freeman, H.C. (1983) J. Mol. Biol. 169, 521–563.
6 Guss, J.M., Harrowell, P.R., Murata, M., Norris, V.A. and Freeman, H.C. (1986) J. Mol. Biol. 192, 361–387.
7 Cookson, D.J., Hayes, M.T. and Wright, F.E. (1980) Biochim. Biophys. Acta 591, 162–176.
8 Hanford, P.M., Hill, H.A.O., Lee, R.W.-K., Henderson, R.A. and Sykes, A.G. (1980) J. Inorg. Biochem. 13, 83–88.
9 Durell, S.R., Labanowski, J.K. and Gross, E.L. (1990) Arch. Biochem. Biophys. 277, 241–254.
10 Burkey, K.O. and Gross, E.L. (1981) Biochemistry 20, 5495–5499.
11 Burkey, K.O. and Gross, E.L. (1982) Biochemistry 21, 5886–5890.
12 Anderson, G.P., Sanderson, D.G., Lee, C.H., Durell, S., Anderson, L.B. and Gross, E.L. (1987) Biochim. Biophys. Acta 894, 386–398.
13 Takenaka, K. and Takabe, T. (1984) J. Biochem. 96, 1813–1821.
14 Takabe, T., Ishikawa, H., Niwa, S. and Tanaka, Y. (1984) J. Biochem. 96, 385–393.
15 Gray, J.C. (1978) Eur. J. Biochem. 92, 133–141.
16 Morand, L.Z., Frame, M.K., Kim, K., Colvert, D.A., Johnson, D., Krogmann, D.W. and Davis, D.J. (1989) Biochemistry 28, 8039–8047.
17 Davis, D.J. and Hough, K. (1983) Biochem. Biophys. Res. Commun. 116, 1000–1006.
18 Beoku-Betts, D. and Sykes, A.G. (1985) Inorg. Chem. 24, 1142–1147.
19 King, G.C., Binstead, R.A. and Wright, P.E. (1985) Biochim. Biophys. Acta 806, 262–271.
20 Rush, J.D., Levine, F. and Koppenol, W.H. (1988) Biochemistry 27, 5876–5884.
21 Gross, E.L., Curtiss, A., Durell, S.R. and White, D. (1990) Biochim. Biophys. Acta 1016, 107–114.
22 Anderson, G.P., Draheim, J.E. and Gross, E.L. (1985) Biochim. Biophys. Acta 810, 123–131.
23 Kenner, R.A. and Neurath, H. (1971) Biochemistry 10, 551–557.
24 Means, G.E.and Feeney, R.E. (1971) in Chemical Modification of Proteins, pp. 183–186, Holden-Day, San Francisco.
25 Davis, D.J. and San Pietro, A. (1979) Anal. Biochem. 95, 254–259.
26 Molnar, S.A., Anderson, G.P. and Gross, E.L. (1987) Biochim. Biophys. Acta 894, 327–331.
27 Meyer, T.E., Przysieki, C.T., Watkins, J.A., Bhattacharyya, A., Simondson, R.P., Cusanovitch, M.A. and Tollin, G. (1983) Proc. Nat. Acad. Sci. USA 80, 6740–6744.
28 Sanderson, D.G., Anderson, L.G: and Gross E.L. (1986) Biochim. Biophys. Acta 852, 269–278.
29 Wood, P.M. (1974) Biochim. Biophys. Acta 357, 370–379.
30 Bernstein, F.C., Koetzle, T.F., Williams, G.J.B., Meyer, Jr. E.F., Brice, M.D., Rogers, J.R., Kennard, O., Shimanouchi, T. and Tasumi, M. (1977) J. Mol. Biol. 112, 535–542.