Interaction of Cytochrome c with the Blue Copper Proteins, Plastocyanin and Azurin*

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Mary Ann Augustin, Stephen K. Chapman, D. Martin Davies, and A. Geoffrey Sykes
From the Department of Inorganic Chemistry, The University, Newcastle upon Tyne, NE1 7RU, England

Samuel H. Speck and E. Margoliash
From the Department of Biochemistry, Molecular Biology and Cell Biology, Northwestern University, Evanston, Illinois 60201

Bimolecular rate constants have been determined for the reaction of native horse cytochrome c, eight 4-carboxy-2,6-dinitrophenyl (CDNP-) cytochromes c singly modified at lysines 7, 13, 25, 27, 60, 72, 86, or 87 and one 2,3,6-trinitrophenyl cytochrome c singly modified at lysine 13, with the blue copper proteins, plastocyanin (from parsley leaves) and azurin (from Pseudomonas aeruginosa). Plastocyanin, a protein having a negative charge of about -7, yields a bimolecular rate constant with native ferrocytochrome c of $1.5 \times 10^4$ M$^{-1}$ s$^{-1}$, which decreases with the modified cytochromes c to a minimum of $7.5 \times 10^2$ M$^{-1}$ s$^{-1}$ for the CDNP-lysine 13 derivative. Conversely azurin, a protein with an overall negative charge of about $-1$ to $-2$, exhibits a bimolecular rate constant with native ferrocytochrome c of $6.8 \times 10^3$ M$^{-1}$ s$^{-1}$ at pH 6.1 and $4.0 \times 10^5$ M$^{-1}$ s$^{-1}$ at pH 8.6, which increase upon modification of the cytochrome c to a maximum of $4.1 \times 10^4$ M$^{-1}$ s$^{-1}$ at pH 6.1 and $2.7 \times 10^6$ M$^{-1}$ s$^{-1}$ at pH 8.6, for the CDNP-cytochrome c modified at lysine 72. This behavior indicates that: 1) the reaction of cytochrome c occurs at a negatively charged site on plastocyanin, whereas azurin behaves as a positively charged reactant, the electrostatics governing to a large extent the relative reactivities of the modified cytochromes c; 2) in both cases the interaction domain on cytochrome c is located on the "front" surface of the protein and encompasses the solvent accessible edge of the heme prosthetic group, as is the case for all the reactions of cytochrome c with its mitochondrial protein redox partners, as well as for small inorganic redox complexes; and 3) the bimolecular rate constants for plastocyanin and azurin are orders of magnitude slower and the effects of lysozyme modifications far smaller than for the reactions with physiological systems, indicating that: (a) the electric fields generated by the reactants do not align them, prior to electron transfer, as effectively as for the physiological reaction partners of cytochrome c; and (b) there is an absence of a precise molecular fit between cytochrome c and the nonphysiological redox partners.

As discussed in the accompanying paper, a variety of lysine-modified cytochromes c have served to map in some detail the area of the protein which interacts with its mitochondrial redox partners, as well as with several small inorganic redox complexes and the superoxide radical anion (1-19). In every case, including the negatively as well as the positively charged inorganic complexes, the interaction area was located on the positively charged "front" surface of the molecule and included a large proportion if not all of the solvent accessible edge of the heme prosthetic group, the most likely site of electron transfer. It would thus appear that, notwithstanding the large influence of electrostatic interactions on these electron exchanges, there is only one general location on cytochrome c at which electrons can be transferred irrespective of the charge on the reactants. Nevertheless, the question remained whether this would be the case with nonphysiological redox proteins, as it is with the small molecules.

The present paper presents the kinetics of reaction of native horse cytochrome c, of eight mono-CDNP- and of one mono-TNP modified horse cytochromes c with the blue copper proteins, plastocyanin and azurin. It is shown that even though the interaction domain on the surface of azurin is positively charged and that on plastocyanin is negatively charged, the corresponding site of interaction on cytochrome c is the same positively charged front surface of the molecule that services all other redox reactions.

**EXPERIMENTAL PROCEDURES**

**Materials**—Plastocyanin was isolated from parsley leaves by the method of Pleminar and Benda (20). The protein was purified and stored at a concentration of $5 \times 10^{-4}$ M, as previously described. Purified *Pseudomonas aeruginosa* azurin, stored in ammonium sulfate buffer at a concentration of $5 \times 10^{-5}$ M, was obtained from Microbiological Products (Porton, United Kingdom). The oxidized copper(II) forms of plastocyanin and azurin both gave ratios of absorbance maxima, $A_{625}/A_{625}$ and $A_{625}/A_{625}$, respectively, of 1.7 ± 0.1. The reduced copper(I) forms of the proteins do not absorb in the visible region. The concentrations of plastocyanin and azurin were determined using millimolar extinction coefficients of 4.5 mm M$^{-1}$ cm$^{-1}$ at 597 nm (21, 22) and 4.8 mm M$^{-1}$ cm$^{-1}$ at 625 nm (23), respectively. A few crystals of potassium ferricyanide were added to the plastocyanin and azurin solutions to ensure complete oxidation, and the solutions were dialyzed against the appropriate buffers at 4 °C with three changes.

The preparation and purification of the mono-CDNP horse cytochromes c modified at lysines 7, 13, 25, 27, 60, 72, 86, or 87 and of the mono-CDNP horse cytochrome c modified at lysines 13, 25, 27, 60, 72, 86, or 87 and of the...
lysine 13 TNP-modified horse cytochrome c was carried out as previously described (11, 24, 29). Prior to kinetic analysis, the native and modified cytochrome c preparations were reduced with minimal sodium ascorbate and separated from excess reducing agent and the small proportion of polymeric forms of the proteins, which may accumulate upon prolonged storage and/or lyophilization, by gel filtration on Sephadex G-50 Superfine (Pharmacia), as previously described (26). Reduced cytochrome c in 0.10 M sodium chloride, pH 7, was diluted, as required, with buffer to concentrations in the range of 0.5 × 10⁻⁶ M to 2.0 × 10⁻⁶ M.

Kinetics—All experiments were run at 25 °C in either Tris/maleate (pH 5.0 to 9.0) or MES (pH 6.0 to 7.2) buffers, with the copper proteins in 10-fold excess over cytochrome c. The buffer, 10 mM, was present in both reactant solutions and the ionic strength was adjusted to 0.10 M with sodium chloride. Earlier work demonstrated that these two buffers give identical rate constants at the same pH. The oxidation of ferrocytochrome c was monitored at 417 nm (Δ reduced-oxidized = 40 mm⁻¹ cm⁻¹) (28) using a DIONEX D-110 stopped flow spectrophotometer, and the data were stored and analyzed as described in the accompanying paper (19). For each cytochrome c preparation at least three concentrations of plastocyanin or azurin were employed, and at each concentration eight determinations of kₖₒₜₜ were obtained, yielding standard deviations of <5% of the mean.

RESULTS

Plots of the change in absorbance, as ln(Aₖ - Aₐ) versus time, were linear for greater than four reaction half-lives, over a range of concentrations of plastocyanin of 20 to 120 μM, and azurin of 10 to 60 μM, ranges previously observed to exhibit first order kinetics in the reactions with native cytochrome c. The bimolecular rate constants determined from the dependence of kₖₒₜₜ on the concentration of oxidant, plastocyanin (Fig. 1) or azurin (Fig. 2), were found to be independent of the concentration of ferrocytochrome c over a 2-fold range (1 to 2 μM).

The bimolecular rate constants for the reaction of native and modified horse cytochromes c with plastocyanin and azurin are listed in Table I. Since the reaction of native cytochrome c with plastocyanin is known to be independent of pH in the range of 6.0 to 8.2, the present experiments were carried out only at pH 7.6 in Tris/maleate buffer. However, the bimolecular rate constant for the reaction of azurin with native cytochrome c exhibits a mild pH dependence with an acid dissociation constant, pKₐ, of about 6.9. Therefore, the reactions of the lysine-modified cytochromes c with azurin were examined at pH 6.1 (MES buffer) (Fig. 2) and pH 8.6 (Tris/maleate buffer) (Fig. 3), namely at either extremity of the pH profile. Notwithstanding the limited analysis carried out at pH 8.6, owing to the small amounts of derivatized cytochromes c available, it appears that the effects of lysine modification are similar to those observed at pH 6.1, even though the rate constants are all smaller at the higher pH. This is expected, since a change in pH over this range would not change the location of the interaction domain on cytochrome c.

In the case of plastocyanin, except for the lysine 60 derivative, the effect of changing the charge on a lysyl residue from +1 to −1 by introducing the CDNP moiety, leads to a decrease in the bimolecular rate constant (Table I). This indicates that the molecular surface of plastocyanin that interacts with cytochrome c is negatively charged. For the reaction of azurin with cytochrome c, the opposite is observed, the CDNP modification leading to an increase in the bimolecular rate constant. Thus, even though azurin has a slight net negative charge (29), the surface interacting with cytochrome c must be positively charged.

The decreasing order of reactivities of the CDNP-cytochrome c is negatively charged. For the reaction of azurin with cytochrome c, the opposite is observed, the CDNP modification leading to an increase in the bimolecular rate constant. Thus, even though azurin has a slight net negative charge (29), the surface interacting with cytochrome c must be positively charged.

The decreasing order of reactivities of the CDNP-cytochrome c after modification is plastocyanin> native azurin> CDNP-native azurin> CDNP-plastocyanin> azurin. The numbers in the plot refer to the sequence positions of the CDNP-modified lysyl residues, except where noted. Assay conditions: 10 mM MES, pH 6.1 buffer adjusted to an ionic strength of 0.10 M with sodium chloride; 25 °C.

Fig. 1. Relationship between the observed pseudo-first order rate constants (kₖₒₜₜ) and the concentration of copper(II) plastocyanin. Assay conditions as given under "Experimental Procedures." The numbers refer to the sequence positions of the CDNP-modified lysyl residues, except where noted.

Fig. 2. Relationship between the observed pseudo-first order rate constants (kₖₒₜₜ) and the concentration of copper(II) azurin. The numbers in the plot refer to the sequence positions of the CDNP-modified lysyl residues, except where noted. Assay conditions: 10 mM MES, pH 6.1 buffer adjusted to an ionic strength of 0.10 M with sodium chloride; 25 °C.
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Second order rate constants for the oxidation of native and modified ferrocytochrome c by copper(II) plastocyanin ($k_2$) and copper(II) azurin ($k_3$).

| Derivative | $10^{-3} k_2$ | $10^{-3} k_3$ | $\ln(k_3$(native$) / k_3$(derivative))$ | $\ln(k_2$(native$) / k_2$(derivative))$ |
|------------|---------------|---------------|------------------------------------|------------------------------------|
| Native     | 15            | 6.6           | 0.000                              | 0.022                              |
| CDNP-60    | 15            | 6.8           | 0.000                              | 0.022                              |
| CDNP-72    | 13            | 41            | 0.143                              | 1.838                              |
| CDNP-7     | 12            | 8.1           | 0.223                              | 0.201                              |
| TNP-13     | 9.8           | 12.6          | 0.426                              | 0.635                              |
| CDNP-87    | 9.0           | 7.4           | 0.510                              | 0.105                              |
| CDNP-25    | 8.5           | 13.3          | 0.556                              | 0.083                              |
| CDNP-86    | 8.6           | 8.1           | 0.556                              | 0.201                              |
| CDNP-27    | 8.1           | 32            | 0.616                              | 1.564                              |
| CDNP-13    | 7.5           | 27            | 0.693                              | 1.386                              |

* $10$ mM Tris/maleate, pH 6.1, at $25$ °C, $I = 0.10$ m (NaCl).

* $10$ mM Tris/maleate, pH 8.6, at $25$ °C, $I = 0.10$ m (NaCl).

With both blue copper proteins, as with inorganic complexes, the effect of the TNP lysine-13 modification of cytochrome c is about half that of the CDNP lysine-13, consistent with the replacement of a $1^+$ by $1^-$ charge in the latter case (Table I). This suggests that electrostatic interactions play a part in the formation of an adduct prior to electron transfer. The influence of lysine modifications on the oxidation of ferrocytochrome c by plastocyanin is that expected for the reaction of the strongly positively charged cytochrome c with a negatively charged redox partner. From the amino acid composition cytochrome c is estimated to have a charge of -2.0. The contours were drawn with approximately equal spacing between the modified residues.
mated that plastocyanin has a net charge of about -7 at this pH. However, the region of largest effect is located around lysine-13 and lysine-27 (Fig. 4) rather than lysine-72 and lysine-13, as observed for negatively charged complexes of the type Fe(2CN)xXn-1, where X is cyanide, 4-aminopyridine, or imidazole (19). As with the small negatively charged inorganic complexes, the magnitude of the effects observed, namely the ratios of rate constants (Table 3), is smaller for plastocyanin than for azurin, which like the positively charged Co(phen)32+ exhibits increased reactivity with the lysine-modified derivatives of cytochrome c.

The region of greatest effect of lysine modification in the case of azurin is around lysine-72 (Fig. 5). This is on the opposite side of the exposed heme edge from lysine-27, which is in the region of greatest effect for the positively charged Co(phen)32+ (19). It is also quite different from the pattern observed for the strongly negatively charged plastocyanin (Fig. 4). The differences in reactivity patterns between plastocyanin and azurin with the cytochrome c derivatives do not necessarily indicate that different sites on cytochrome c are involved in electron transfer, such as was implied for the reactions of the positively and negatively charged small inorganic complexes (19). Rather, they may simply reflect differences in the distribution of charged and uncharged residues on the surfaces of the two blue copper proteins which result in different charges as well as other secondary interactions. Indeed, the reactions of both plastocyanin and azurin with cytochrome c are nonphysiological; namely, the proteins have not been subjected to the selective evolutionary pressures that would have resulted in co-adaptation of the tertiary structures of their respective electron transfer domains. This is obvious from the comparatively small bimolecular rate constants for these reactions as compared to the close to diffusion limited electron exchange reactions between cytochrome c and its mitochondrial redox partners (31-36). Similarly, various lysine modifications have small effects on the reactions with the blue copper proteins as compared to the massive effects observed with the physiological systems (1-14, 17). Notwithstanding these differences, it is clear that the reaction of inorganic complexes and the two blue copper proteins with cytochromes c modified at lysines 72, 13, or 27, which are closest to the heme edge, have the largest effect, indicating that these reactants utilize the same site for electron transfer as do the protein systems of mitochondria.

The bimolecular rate constants for the oxidation of ferrocyanocobalt(II) c by azurin are dependent on pH, the pK, for the transition being at pH 6.9. The low pH form is more reactive than the high pH form. This behavior is opposite to that observed for the reaction of azurin with Co(phen)33+, but is the same as observed for the reaction with Fe(CN)x3- (37). Azurin has an isoelectric point of 5.4 (30) and so is negatively charged at both pH values used in this study, though from a consideration of amino acid composition the magnitude of the charge is small and is at most -1 or -2 (38). This discrepancy between the effects of pH on the reactivity of azurin with the inorganic complexes, as compared to that with cytochrome c, may result from the deprotonation of azurin enhancing non-productive binding of cytochrome c to a negatively charged domain on the surface of azurin, thereby decreasing the frequency of productive encounters at the positively charged electron transfer site on azurin. It has previously been observed that redox reactions between azurin and negatively charged bacterial C-type cytochromes have higher rate constants than reactions between azurin and positively charged cytochromes (22, 39).

It should be emphasized that while the overall net charge of the redox proteins is of importance in determining the collision frequency, the rate of productive encounters appears to be largely determined by the spatial orientation of all the charged groups on the molecule with respect to the electron transfer site. Indeed, bovine cytochrome c1 is very similar to azurin in net charge, its pI being about 5.5 (40). From the amino acid sequence the net charge of the protein is estimated to be close to neutral in the neutral pH range (41). However, the second order rate constant for the reaction of bovine cytochrome c1 with horse cytochrome c is several orders of magnitude faster than the reaction of horse cytochrome c with azurin (13, 36). From the decreased reactivities of the CDNP-modified cytochromes c with cytochrome c compared to the reaction of native horse cytochrome c, it is clear that a negatively charged domain on cytochrome c is involved in the productive interaction with the positively charged front face of cytochrome c (13), in contrast to the results obtained with azurin.

Information is also available on the site used for electron transfer on plastocyanin. In other work (42) it has been shown that the complexes, Co(NH3)63+ and Pt(NH3)2+, function as redox inactive blocking agents on copper (I) plastocyanin in the oxidation by Co(phen)33+. From proton NMR line-broadening effects, using Cr(III) complexes with identical ligand sets, it has been demonstrated that these complexes associate preferentially at or close to tyrosine 83 on the copper(I) plastocyanin (43, 44). Similar behavior is expected for copper(II) plastocyanin where consecutive negatively charged residues in positions 42 to 45 on the protein are believed to function as a binding site. Therefore, since Co(NH3)63+ and particularly Pt(NH3)2+ block the oxidation of ferrocyanocobalt(II) c by plastocyanin, it would appear that cytochrome c exchanges electrons at the same site on plastocyanin. Indeed, preliminary proton NMR line-broadening experiments (44) indicate that this site is involved in cytochrome c binding. Tyrosine 83 is located some 16 to 15 Å away from the copper active site. From these considerations one would estimate that the copper and iron centers in the productive plastocyanin-cytochrome c complex are 17 to 22 Å apart at the time of
electron transfer. However, electrons are delocalized from the iron(II) of cytochrome c over the porphyrin ring and are presumed to be available for outer sphere reactions at the exposed heme edge (45), making the distance over which transfer has to occur much smaller.

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