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Preferred Sites on Cytochrome *c* for Electron Transfer with Two Positively Charged Blue Copper Proteins, *Anabaena variabilis* Plastocyanin and Stellacyanin†

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ABSTRACT: Rate constants for the reactions of horse cytochrome *c* (E'_0 of +260 mV) with the copper proteins *Anabaena variabilis* plastocyanin (E'_0 of +360 mV) used as oxidant and stellacyanin (E'_0 of +187 mV) used as reductant have been determined at 25 °C, pH 7.5 and 7.0, respectively, and an ionic strength of 0.10 M (NaCl). These rate constants were also measured with eight different singly substituted 4-carboxy-2,6-dinitrophenyl (CDNP) horse cytochrome *c* derivatives, modified at lysine-7, -13, -25, -27, -60, -72, -86, or -87 and with the trinitrophenyl (TNP) derivative modified at lysine-13. The influence of the modifications on the bimolecular rate constants for these reactions defines the region on the protein that is involved in the electron-exchange reactions and demonstrates that the preferred site is at or near the solvent-accessible edge of the heme prosthetic group on the "front" surface of the molecule. Both reactions are strongly influenced by the lysine-72 modification to the left of the exposed heme edge and, to this extent, behave similar to the earlier studied reaction with azurin. These effects span only an order of magnitude in rate constants and are thus many times smaller than those for the physiological protein redox partners of cytochrome *c*. While the preferred sites of reaction on the surface of cytochrome *c* for small inorganic complexes appear to be dependent only on the net charge of the reactants, with the copper proteins additional factors intervene. These influences are discussed in terms of hydrophobic patches and the distribution of charges on the surface of the four copper proteins so far examined.

A variety of singly modified lysine derivatives of horse cytochrome *c* have provided a valuable means of mapping out regions on the protein that react with different redox partners (Staudenmayer et al., 1976, 1977; Smith, et al., 1977; Ng et al., 1977; Ferguson-Miller et al., 1978; Kang et al., 1978; Ahmed et al., 1978; Speck et al., 1979, 1981; Stonehuerner et al., 1979; Webb et al., 1980; Osheroff et al., 1980; Smith, et al., 1980; Konig, et al., 1980; Butler et al., 1981, 1982, 1983; Ahmed & Millett, 1981; Koppenol & Margoliash, 1982; Augustin et al., 1983). In addition to studies with natural mitochondrial electron-exchange partners, other reactions examined (Butler et al., 1983) include those with nonphysiological protein partners, such as parsley plastocyanin, estimated to have a charge of −7 for the Cu(II) state at pH 7, and *Pseudomonas aeruginosa* azurin, with a charge of −1 for the Cu(II) state and a p*I* of 5.4, assuming only one of the two free histidines carries a +1 charge. In the case of plastocyanin, it was observed that replacement of the +1 lysine charge at neutral pH, by the −1 charge of the 4-carboxy-2,6-dinitrophenyl-(CDNP-) substituted derivatives resulted in a decrease in rate constant, as expected on the basis of simple electrostatic considerations. Remarkably, with azurin, the rate constants of the modified cytochromes *c* increased, suggesting that azurin behaves as a positively charged oxidant and/or that factors besides charge are contributing. Therefore, it was decided to investigate further these phenomena by employing two other blue (type 1) single copper proteins (Lappin, 1981), namely plastocyanin from the blue-green algae *Anabaena variabilis*,

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with a charge of +3 at pH 7 for the Cu(II) state, (Aitken, 1975) and stellacyanin from the lacquer tree, with a charge of +7 for the Cu(II) state (Bergman et al., 1977; Reinhammer, 1970). Because of the magnitude of their reduction potentials (+370 and +187 mV, respectively), compared to that of cytochrome *c* (+260 mV), the plastocyanin Cu(II) oxidation of cytochrome *c*(II), and the stellacyanin Cu(I) reduction of cytochrome *c*(III) were studied.

Materials and Methods

Proteins. The preparation and purification of single CDNP (4-carboxy-2,6-dinitrophenyl) and TNP (2,4,6-trinitrophenyl) lysine-modified derivatives of horse heart cytochrome *c* were performed by those methods previously described (Osheroff et al., 1980; Brautigan et al., 1978a,b). These were all purified to the point that they were 99% homogeneous or better (Osheroff et al., 1980; Brautigan et al., 1978a). For studies with cytochrome *c*(II), the protein was reduced by addition of a minimal amount of sodium ascorbate (BDH, AnalaR). Separation from excess reducing agent and any polymeric protein material was by gel filtration on a 1 × 10 cm column of Sephadex-G50 (Sigma Chemicals) in Tris/HCl at pH 7.5. The reduced protein was stored under N_2. To convert to cytochrome *c*(III), a minimal amount of $K_3[Fe(CN)_6]$ (BDH AnalaR) was added and excess oxidant and polymeric material were removed by ion-exchange chromatography on a Whatman carboxymethylcellulose column (CM52) in Tris/HCl at pH 7.0 at an appropriate ionic strength. Procedures for growing *A. variabilis* (Aitken, 1975; Kratz & Myers, 1955) and the isolation of photosynthetic particles from the alga were carried out as described by Lightbody and Krogman (1967). Final purification of the *A. variabilis* plastocyanin Cu(II) was achieved by ion-exchange chromatography on Sephadex CM-52 (Sigma Chemicals) using phosphate buffer at pH 7.5 and a gradient of 1–50 mM. Protein with absorption peak ratios at 278–597 nm, $A_{278}/A_{597} \leq 1.2$, was used for the kinetic studies. Concentrations of plastocyanin Cu(II) were determined spectrophotometrically by using an absorption coefficient of 4500 M^{-1} cm^{-1} at 597 nm.

Stellacyanin from the Japanese lacquer tree, *Rhus vernicifera*, was obtained from the acetone powder supplied by Saito and Co., Tokyo, by the method of Reinhammer (1970, 1972). The protein was handled as described previously (Sisley et al., 1983). Protein with absorbance peak ratios $A_{280}/A_{604} \leq 5.9$ was used for the kinetic experiments. Reduction was carried out with a minimal amount of sodium ascorbate followed by dialysis against 1 L of the required buffer for at least 24 h, with three changes. Anaerobic (N_2) conditions were employed to avoid oxidation of the stellacyanin Cu(I). Concentrations were determined as stellacyanin Cu(II) by using an absorption coefficient of 4080 M^{-1} cm^{-1} at 604 nm.

Stoichiometry. The three proteins studied are known to undergo one-electron redox changes. Absorbance changes were consistent with 1:1 reactions as in eq 1 and 2:

cytochrome *c*(II) + plastocyanin Cu(II) → cytochrome *c*(III) + plastocyanin Cu(I) (1)

stellacyanin Cu(I) + cytochrome *c*(III) → stellacyanin Cu(II) + cytochrome *c*(III) (2)

The favorable thermodynamic driving force and the greater than 10-fold excesses of the copper proteins ensured that the reactions proceeded to ≥97% completion.

Kinetics. All experiments were carried out at 25.0 ± 0.1 °C with the ionic strength adjusted to $I = 0.100 \pm 0.001$ M with NaCl. The copper proteins were in greater than 10-fold excess over cytochrome *c*. Oxidations and reductions of native and chemically modified cytochrome *c* by plastocyanin Cu(II) and stellacyanin Cu(I) were carried out at pH 7.5 and 7.0, respectively, by using 10–20 mM Tris-HCl (Sigma Chemicals) buffer. With plastocyanin, a pH of 7.5 rather than 7.0 was chosen to ensure that the measurements were done in a region of pH independence of the reaction rates. The reactions of stellacyanin do not exhibit any pH dependence in the pH 7–8 range (Kratz & Myers, 1955). The kinetics were monitored at 416 nm, the peak of the Soret band of reduced cytochrome *c*, at which wavelength the protein exhibits the highest difference in the absorbancies of the ferro- and ferric forms ($\Delta\epsilon = 4 \times 10^4$ M^{-1} cm^{-1}). A Dionex D-110 stopped-flow spectrophotometer was used, and the data were analyzed by a standard kinetic first-order treatment (linearity of plots for ≥4 half-lives), yielding values for k_{obsd}. At each relevant concentration five repeat traces were obtained, and the first-order rate constants k_{obsd}, were averaged. Limited supplies of the modified proteins did not enable the effects of TNP lysine-13 and CDNP lysine-25 cytochromes *c* to be investigated in the case of the reaction of stellacyanin Cu(I) and cytochrome *c*(III). This deficiency is not crucial to the interpretation of the results.

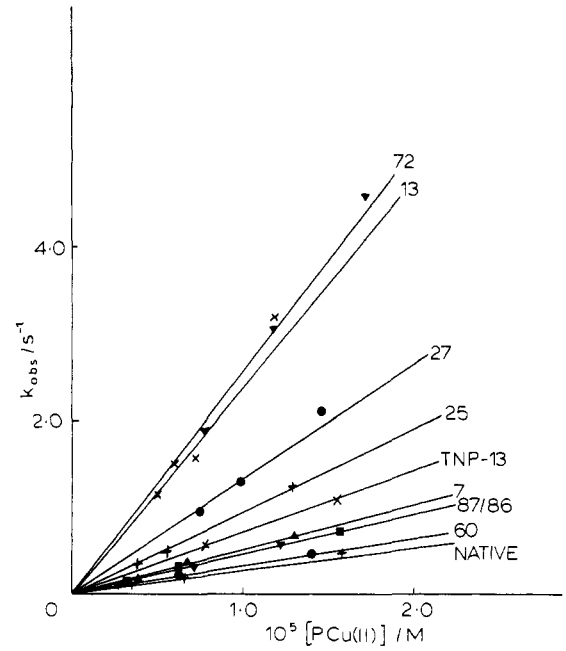

FIGURE 1: Relationship between observed first-order rate constants, k_{obsd}, and the concentration of *A. variabilis* plastocyanin (reactant in excess) for the plastocyanin Cu(II) oxidation of cytochrome *c*(II) modified derivatives at 25 °C, pH 7.5 (20 mM Tris-HCl), and *I* adjusted to 0.10 M with NaCl.

Results

The linear dependencies of the first-order rate constants, k_{obsd}, on the concentration of the copper proteins (reactant in excess) are shown in Figure 1 for the oxidation of cytochrome *c*(II) by plastocyanin Cu(II) and in Figure 2 for the reduction of cytochrome *c*(III) by stellacyanin Cu(I). For each modified cytochrome *c* derivative, at least two different copper protein concentrations were used, which were in the range 0.3×10^{-5} M to 1.7×10^{-5} M, for plastocyanin Cu(II), and 0.55×10^{-5} M to 1.25×10^{-5} M for stellacyanin Cu(I). Second-order rate constants, *k*, obtained from the slopes of the lines in Figures 1 and 2, are listed in Tables I and II, respectively. All the modified cytochromes *c* reacted more rapidly than the native horse protein with the copper proteins, as expected from the

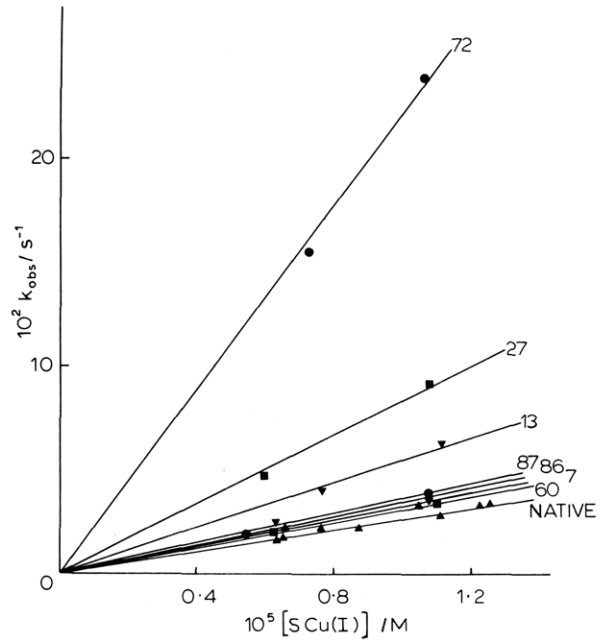

FIGURE 2: Relationship between observed first-order rate constants, k_{obsd}, and the concentration of stellacyanin (reactant in excess) for the stellacyanin Cu(I) reduction of cytochrome *c*(III) modified derivatives at 25 °C, pH 7.0 (10 mM Tris-HCl), and *I* adjusted to 0.10 M with NaCl.

Table I: Second-Order Rate Constants, *k*, for Oxidation of Native and Modified Horse Cytochrome *c*(II) by *A. variabilis* Plastocyanin Cu(II) at 25 °C, pH 7.5 (Tris-HCl), and *I* = 0.10 M (NaCl)

cytochrome *c* derivative	$10^{-4}k$ ($M^{-1}\ s^{-1}$)	R^a
native	3.3	
CDNP-60	3.8	0.14
CDNP-72	25.9	2.06
CDNP-7	5.2	0.45
TNP-13	7.1	0.77
CDNP-87	4.6	0.33
CDNP-25	9.6	1.06
CDNP-86	4.6	0.33
CDNP-27	13.3	1.39
CDNP-13	24.0	1.98

[a] *R* is defined in eq 3 in the text.

Table II: Second-Order Rate Constants, *k*, for Reduction of Native and Modified Cytochrome *c*(III) by *Rhus vernicifera* Stellacyanin $Cu(I)_2$ at 25 °C, pH 7.0 (Tris-HCl), and *I* = 0.1 M (NaCl)

cytochrome *c* derivative	$10^{-3}k$ ($M^{-1}\ s^{-1}$)	R^a
native	2.4	
CDNP-60	3.2	0.29
CDNP-72	22.7	2.25
CDNP-7	3.3	0.32
CDNP-87	3.6	0.41
CDNP-86	3.5	0.38
CDNP-27	8.6	1.28
CDNP-13	5.5	0.83

[a] *R* is defined in eq 3 in the text.

net positive charge of each of the reactants in both cases. The increasing order of the reactivities of the CDNP- and TNP-modified cytochromes *c*(II) with plastocyanin Cu(II) was native < CDNP-60 < CDNP-86 = CDNP-87 < CDNP-7 < TNP-13 < CDNP-25 < CDNP-27 < CDNP-13 < CDNP-72, and the rate constants span a range of 7.8-fold. The order of increasing reactivities for the reaction of stellacyanin Cu(I) with cytochrome *c*(III) is native < CDNP-60 < CDNP-7 < CDNP-86 < CDNP-87 < CDNP-13 < CDNP-27 < CDNP-72. Again, the rate constants span a similar range of 9.5-fold.

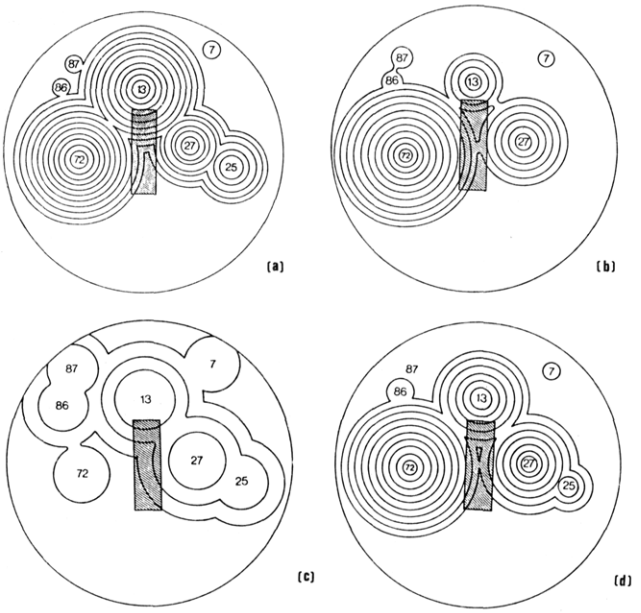

FIGURE 3: Diagrammatic reactivity contour maps on a planar projection of the front hemisphere of cytochrome *c* for the reactions of cytochrome *c*(II) with (a) *A. variabilis* plastocyanin Cu(II), (b) stellacyanin Cu(I) with cytochrome *c*(III), (c) cytochrome *c*(II) with parsley plastocyanin Cu(II), and (d) cytochrome *c*(II) with azurin Cu(II). The latter two are taken from Augustin et al. (1983). The shaded rectangle represents the exposed edge of the heme prosthetic group, and the numbers refer to the positions of the α-carbon atoms of the modified lysine residues of cytochrome *c*.

Reactivity contour maps for the two sets of reactions, depicted in Figure 3a,b, illustrate the influence of the different modifications, and are based on the ratio *R*, defined in eq 3.

$$R = \ln [k(\text{derivative})/k(\text{native})] \quad (3)$$

The values of *R* are listed in Tables I and II. Each contour on a line joining two centers of modification represents a difference of 0.2 unit in *R*. Thus, the lysine residue surrounded by the largest number of concentric circles is the position of greatest influence. Contour maps drawn in the same way for the previously studied (Augustin et al., 1983) reactions of plastocyanin Cu(II) prepared from a higher plant parsley, and azurin Cu(II) with cytochrome *c*(II) are also shown in parts c and d of Figure 3. The lysine-60 modification, located on the "back" surface of the protein, namely the surface diametrically opposed to that containing the exposed heme edge, is the only modification not shown in these figures. As found in many other studies (Ferguson-Miller et al., 1978; Osheroff et al., 1980; Konig et al., 1980; Speck et al., 1981; Butler et al., 1981, 1982, 1983; Koppenol & Margoliash, 1982; Augustin et al., 1983) this modification has no significant effect on rate constants determined for a variety of physiological and non-physiological systems.

It should be emphasized that the modifications employed in all these studies have little or no effect on the reduction potential of cytochrome *c*, the values remaining within 5 mV of that for the native proteins (Osheroff et al., 1980; Brautigan et al., 1978b); microscopic reversibility requires that the same pattern of reactivity holds for the reverse reactions.

Discussion

The reactivities of *A. variabilis* plastocyanin and of stellacyanin are enhanced by the CDNP modifications on cytochrome *c*, which cause a decrease of the net positive charge by 1 unit, as expected for reactions between two proteins that are both positively charged. Also, for *A. variabilis* plastocyanin Cu(II), the rate constant for the TNP lysine-13 modification,

which replaces a +1 with a zero charge, is intermediate between that for the native and the CDNP lysine-13 modified protein, with a charge change to −1, again consistent with electrostatics playing an important overall role in these reactions.

However, in addition to these major directional trends, the pattern of rate constants observed is useful in defining with some precision the regions on the molecular surface of cytochrome *c* that have an influence on the reactions studied. Four copper proteins, all nonphysiological redox partners for cytochrome *c*, have now been studied. These carry charges of −7 for plastocyanin Cu(II)(parsley), −1 for azurin Cu(II), +3 for plastocyanin Cu(II) (*A. variabilis*), and +7 for stellacyanin Cu(II). Inspection of the reactivity contour diagrams in Figure 3, suggests that whereas for the latter three lysine-72 has the largest influence on the reaction, parsley plastocyanin Cu(II) exhibits somewhat different behavior.

Interestingly, in similar studies of the modified cytochrome *c* derivatives with inorganic complexes, it was found that the negatively charged $[Fe(CN)_6]^{3-}$ is most influenced by lysine-72 to the left of the exposed heme edge, whereas positively charged $[Co(phen)_3]^{3+}$ is most influenced by lysine-27 to the right of the exposed heme edge, on the front surface of the molecule (Butler et al., 1983). Studies with $[Fe(EDTA)]^{2-}$ and the sepulchrate cage complex $[Co(sep)]^{2-}$, as reductants for cytochrome *c*(III), are in agreement with this picture and reinforce it as well (Armstrong et al., 1986). Thus, it is possible that the region on the surface of cytochrome *c* capable of accepting or donating an electron is not very limited and extends to both sides of the exposed heme edge. The negatively charged small inorganic reactants would tend to impact at the most positively charged part of that area, while the positively charged small reactants would tend to approach the least positively charged area still capable of electron exchange. Such a situation would explain the earlier observations (Butler et al., 1983; Armstrong et al., 1986).

However, in considering the reactivity of cytochrome *c* with the copper proteins, such a simple explanation does not appear to suffice, and the net overall charge on the copper protein is apparently not the only factor determining site selectivity. Indeed, for proteins, the electrostatic field at any point will be influenced by the distribution of the often widely dispersed charges on the surface of the molecule, and other factors may intervene, such as the hydrophobicity of surface patches, which may favor certain interaction geometries. In this connection, it should be noted that in the present experiments the rate constants maintained their second-order character under all conditions tested, so that any secondary rearrangements of the reactants following their initial collision could not have been rate-limiting.

Higher plant plastocyanin, of which parsley, spinach, and French bean are the most investigated, have been shown to have two sites for electron transfer (Lappin et al., 1979; Chapman et al., 1983a,b; Sinclair-Day et al., 1985). These are described as the adjacent (north) site, close to histidine-87, which is 6 Å from the copper, and the remote (east) site, close to tyrosine-83, which is 10–12 Å from the copper. The latter is part of a negative patch incorporating carboxylate residues 42–45 (Guss & Freeman, 1983), which serves as a recognition site for positively charged reagents. Negatively charged complexes, such as $[Fe(CN)_6]^{3-}$, and very likely the high-potential iron sulfur protein, with a charge of −3, react at the adjacent histidine-87 site (Chapman et al., 1984), which is hydrophobic with no charged residues available. From a variety of evidence, including the effect of pH, inhibition by $[Pt(NH_3)_6]^{4+}$, and the effect of Cr(III) modifications, as well as the evidence provided by important NMR studies (Cookson et al., 1980a,b; Hanford et al., 1980), it has been concluded that positively charged complexes, such as $[Co(phen)_3]^{3+}$ and cytochrome *c*(II)(8+), react at the remote tyrosine-83 site (Chapman et al., 1983; King et al., 1985). Thus, the negative charges on parsley plastocyanin Cu(II) at the remote tyrosine-83 site may well be influential in determining the preferred location for reaction on the cytochrome *c* surface, shown in Figure 3c.

On the other hand, *A. variabilis* plastocyanin is a basic protein of charge +3 having fewer carboxylate residues. Reactivity patterns of this protein with a series of positively and negatively charged complexes suggest that some reactivity is retained at the remote tyrosine-83 site (Sinclair-Day et al., 1985; Jackman et al., personal communication). As compared to parsley plastocyanin, only residue 42 of the residue 42–45 patch remains negatively charged (Aitken, 1975), but residue 85, which is close to tyrosine-83 is now a glutamic acid, and the proximity of aspartic acid-42 and glutamic acid-85 to tyrosine-83 may be important (Jackman et al., personal communication).

Our work has established that *A. variabilis* plastocyanin Cu(II), azurin Cu(II), and stellacyanin Cu(I) have preferential reactivity in the region of lysine-72, apparently a site similar to that used by $[Fe(CN)_6]^{3-}$. On the other hand, parsley plastocyanin Cu(II) exhibits a different reactivity pattern, probably because of the influence of the negatively charged remote site discussed above, and the lysine-87/86, -13, and -27 regions on cytochrome *c* become more important than that of residue 72. With *A. variabilis* (Figure 3a), there is a greater involvement of lysine-13, -27, and -25 than for stellacyanin and azurin, but this is in the opposite direction to the similar effects observed with parsley plastocyanin, and the implications of this different behavior are not fully understood. More detailed assignments will have to wait on studies with cytochromes *c* fitted at the various lysines with affinity-labeled moieties.

To summarize, with small inorganic reagents, the preferred site for reaction on cytochrome *c* is apparently according to the overall charge of the reagent. In reactions with the copper proteins, the involvement of two protein surfaces, with their varying degrees of hydrophobicity and the wide ranging distribution of charges, brings in other factors. Some pattern of selectivity has emerged but it is not in terms of overall charge, since the positively charged copper proteins use the same region as the negatively charged $[Fe(CN)_6]^{3-}$ and not that reacting with the positively charged $[Co(phen)_3]^{3-}$. Whether the hydrophobic patches on azurin, stellacyanin, and *A. variabilis* plastocyanin are responsible for the favorable interaction at the lysine-72 area on cytochrome *c* remains to be ascertained.

Registry No. Cytochrome *c*, 9007-43-6; L-lysine, 56-87-1.

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