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# The Electron-Transfer Site of Spinach Plastocyanin†

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ABSTRACT: Two sites for electron transfer have been proposed for plastocyanin: one near the copper ion and the other close to the acid patch formed by residues 42–45. Calculations of electrostatic properties of spinach plastocyanin and ionic strength dependences of electron-transfer reactions of this protein have been used to distinguish between these two sites. Calculations show that the electric potential field of spinach plastocyanin is highly asymmetric and that the protein has a dipole moment of 360 D. The negative end of the dipole axis emerges between the negative patches formed by residues 42–45, which is thought to be the cation binding site, and residues 59–61. The angles between the dipole vector and vectors from the center of mass to the copper ion and to the acid patch are 90° and 30°, respectively. The angle between the dipole vector and a line from the center of mass to the site of electron transfer is evaluated from the ionic strength dependence of electron-transfer rates at pH 7.8 with the help of equations developed by Van Leeuwen et al. [van Leeuwen, J. W., Mofers, F. J. M., & Veerman, E. C. I. (1981) *Biochim. Biophys. Acta 635*, 434] and Van Leeuwen [van Leeuwen, J. W. (1983) *Biochim. Biophys. Acta 743*, 408]. The angles found are 85°, 110°, and 75 ± 15° for reactions with tris(1,10-phenanthroline)cobalt(III), hexacyanoferrate(III), and ferrocytochrome *c*, respectively. The electric potential field calculations suggest that the hexacyanoferrate(III) interaction angle corresponds to a unique site of minimum repulsion at the hydrophobic region of the protein surface, close to the copper ion. An interaction angle of 65 ± 15° was determined for (4-carboxy-2,6-dinitrophenyl)lysine 13 ferrocytochrome *c*. The angles found indicate that electron transfer could take place within the hydrophobic region of plastocyanin near the copper ion, possibly via His 87, but not at Tyr 83 or the negative patches. The ionic strength dependence of the association constant between hexaamminecobalt(III) and cuprous plastocyanin was consistent with cation binding near the negative patch formed by residues 42–45.

Plastocyanins are copper-containing proteins that were first discovered in green algae by Katoh (1960). The function of the protein is to carry electrons from cytochrome *f* in photosystem II to pigment $P700^+$ in photosystem I (Anderson, 1982). Recently, the spatial structure of poplar plastocyanin has been determined at 1.6-Å resolution (Guss & Freeman, 1983). It shows that the protein, which consists of a single peptide chain of 99 residues, has a cylindrical shape. Due to an excess of acidic residues the protein has a net charge of $-8e$[1] or $-9e$, depending on the oxidation state of the metal. The copper atom is embedded 6 Å within the protein and is coordinated by methionine 92, cysteine 84, and histidines 87 and 37. The distorted tetrahedral arrangement of nitrogen and sulfur donors is responsible for the intense blue color of the oxidized protein as well as the high reduction potential, $E°'$[Pc(II)/Pc(I)] = 360 mV at neutral pH (Katoh, 1960). These characteristics are general for higher plant plastocyanins, and the structures of poplar plastocyanin and spinach plastocyanin, which has been used in this study, are almost certainly closely related (Ulrich & Markley, 1978).

The electron-transfer reactions of plastocyanins with cytochrome *f* (Farver & Pecht, 1981; Wood, 1974), flavodoxin (Tollin et al., 1986), azurin (Wood, 1974), and cytochrome *c* (Wood, 1974; Augustin et al., 1983) as well as small molecules such as tris(1,10-phenanthroline)cobalt(III) (Segal & Sykes, 1978; Lappin et al., 1979; Holwerda et al., 1980), ferricyanide (Segal & Sykes, 1978; Lappin et al., 1979), ferrous EDTA (Wherland et al., 1975), flavin (Tollin et al., 1983), flavin semiquinone (Meyer et al., 1987), and ascorbate

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[1] Abbreviations: $e$, elementary charge in coulombs; dtpa, diethylenetriaminepentaacetate; $k_B$, Boltzmann's constant; $k_0$, bimolecular rate constant at zero ionic strength; $k_{inf}$, bimolecular rate constant at infinite ionic strength; $n$, number of negative charges; $p$, number of positive charges; phen, 1,10-phenanthroline; $\bar{r}_P$ and $\bar{r}_N$, radius vectors from the center of mass to the centers of positive and negative charge, respectively; $2A = 1.02$ (eq 5); CDNP, 4-carboxy-2,6-dinitrophenyl; CM, center of mass; Fe(II)*c* and Fe(III)*c*, ferro- and ferricytochrome *c*, respectively; $\mathbf{P}_1$ and $\mathbf{P}_2$, dipole moments of spinach plastocyanin and horse cytochrome *c* in C·m, respectively; Pc(I) and Pc(II), cuprous and cupric plastocyanin, respectively; $T$, temperature in K; $R_1$, radius of plastocyanin; $R_2$, radius of inorganic reactant in eq 3 or cytochrome *c* in eq 4; $R = R_1 + R_2$; $Z_i$, number of elementary charges; $\epsilon_0$, permittivity constant; $\epsilon$, static dielectric constant; $\theta$, angle between dipole vector and vector from center of mass to site of electron transfer; $\kappa = 0.33\mu^{1/2}A^{-1}$; $\mu$, ionic strength.

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(Takabe et al., 1980) have been studied. The subject has been reviewed recently (Sykes, 1985). Kinetic studies by Sykes and co-workers (Segal & Sykes, 1978; Lappin et al., 1979) have revealed that the oxidation of cuprous plastocyanin is strongly pH dependent in the range of pH 4–7. This is apparently caused by the protonation of a ligand which destabilizes the cupric state. Some of these studies have shown that positively charged oxidants exhibit limiting kinetics (Segal & Sykes, 1978; Lappin et al., 1979) as well as inhibition by redox-inert cationic metal complexes (Beoku-Betts et al., 1983; Sykes, 1985) which might indicate that electron transfer follows binding of the oxidant or reductant. The principal cation binding site for small metal complexes has been identified by NMR studies (Cookson et al., 1980) as a site very close to the consecutively acidic residues 42–45, which we will refer to as the acid patch. This negatively charged area is a conserved feature of plastocyanins and is about 19 Å from the copper ion. Ferricytochrome *c* also binds at this site (Cookson et al., 1980). Though all of these reagents are rather weak redox agents, generally having reduction potentials that differ less than 100 mV from the copper ion, the observed binding of redox agents at the acid patch suggests that plastocyanin may use this site for some of its electron-transfer activity (Beoku-Betts et al., 1983; King et al., 1985). Similar suggestions have been made on the basis of experiments with plastocyanins chemically modified at residues 42–45 (Farver et al., 1982; Burkey & Gross, 1982). It has been suggested that the acid patch is the recognition site for the photosystem I $P700^+$ reaction center (Farver et al., 1982). However, recent protein modification studies (Anderson et al., 1987) show that this area and residues 59–61 are involved in binding to cytochrome *f* and that the reaction domain of photosystem I binds near His 87. The hydrophobic area is the site where electron transfer would appear to be most facile because of its proximity to the copper atom. Paramagnetic shifts of the proton resonances in Phe 35 and His 87 imidazole indicate that the hexacyanochromate(III) anion binds at the hydrophobic region (Cookson et al., 1980), and its redox-active analogue, the hexacyanoferrate(III) anion, might reasonably be expected to also use this site to transfer electrons. According to a kinetic model for the quenching of long-lived states of chromium and ruthenium complexes by plastocyanin, electron transfer from the hydrophobic pathway is about 10 times faster than from the acidic patch at low protein concentrations (Brunschwig et al., 1985).

There exist a number of ionic strength studies of reactions involving plastocyanin in the literature. Interpretations in terms of net charges are often (Rosenberg et al., 1976), but not always (Segal & Sykes, 1978), in reasonable agreement with the amino acid composition. Interaction site charges (Rosenberg et al., 1976; Cummins & Gray 1977; Tollin et al., 1986; Anderson et al., 1987) have been derived from simplified forms of eq 5 (see below) or the Marcus–Debye equation (Wherland & Gray, 1976). Binding between inorganic complexes and plastocyanin has also been described in terms of local charges (Chapman et al., 1983). Aside from theoretical objections (Koppenol, 1980), one might question the usefulness of the local or interaction site charge approaches since they do not shed light on location of these charges on the surface of the protein.

In the present study we investigated the ionic strength dependences of the reactions of hexacyanoferrate(III) and tris(1,10-phenanthroline)cobalt(III) with cuprous plastocyanin, and of horse heart ferrocytochrome *c* with cupric plastocyanin, for the purpose of determining the actual electron-transfer site of spinach plastocyanin. This approach applies to proteins that possess a sizable dipole moment oriented at a known angle relative to the interaction site of the, preferably highly charged, redox probe (Koppenol, 1980; Van Leeuwen et al., 1981; Van Leeuwen, 1983; Rush et al., 1987). As such, it is a refinement of those theories that assume spherically symmetrical charge distributions and take into account net charges only. The ionic strength dependence of a rate constant is now considered a function not only of net charge and radius but also of dipole moment and, most importantly, the site of reaction relative to the dipole vector. Spinach plastocyanin, which we have taken to be a structural analogue of the poplar protein, has a dipole moment of 360 D oriented at a 30° angle to the acid patch and almost orthogonally to the hydrophobic site (see below). The choice of ferrocytochrome *c*, tris(1,10-phenanthroline)cobalt(III), and hexacyanoferrate(III) as redox probes is advantageous because of their high charges and known binding domains, and because the kinetics of their reactions with plastocyanins have been previously investigated (Sykes, 1985). Also, detailed electrostatic calculations of ferrocytochrome *c* and its 12 CDNP-lysyl derivatives are available (Koppenol & Margoliash, 1982). These singly modified proteins have been used to characterize reaction domains on the surface of cytochrome *c* (Koppenol & Margoliash, 1982). In all cases investigated, these interaction domains included the solvent-accessible heme edge. In the present study we have used cytochromes *c* modified at position 13 and at position 72 to investigate the possibility of specific electrostatic interactions between the lysine residues surrounding the electron-transfer domain of cytochrome *c* and carboxylate groups on the plastocyanin surface.

## Materials and Methods

*Equations.* Electric potential fields were calculated as described for bovine Cu/Zn superoxide dismutase (Koppenol, 1981) and tuna and horse cytochrome *c* (Koppenol et al. 1978; Koppenol and Margoliash, 1982). The potential $V(\vec{r})$ is given by

$$V(\vec{r}) = (1/4\pi\epsilon\epsilon_0)\sum_i (q_i/|\vec{r} - \vec{r}_i|) \quad (1)$$

At pH 7 the residues assumed to be positively charged are lysines and the N-terminal amino group. Spinach plastocyanin does not contain arginines. The metal is assigned either a $+1e$ or a $+2e$ charge. Aspartic acids, glutamic acids, the sulfur of cysteine 84, and the C-terminal carboxylic acid group are considered to be negatively charged. Coordinates of poplar plastocyanin (Guss & Freeman, 1983) were obtained from the Brookhaven Protein Data Bank (Bernstein et al., 1977). If the amino acid sequence of the spinach protein (Scawen et al., 1975) indicated a glutamic acid where poplar plastocyanin has an aspartic acid, the coordinates of aspartic acid were used, and vice versa. The positive charge of lysine 71 of the spinach protein was placed at the coordinates of atom OE2 of glutamic acid 71 of poplar plastocyanin. The negative charge of glutamic acid 45, which is a serine in the poplar protein, was arbitrarily placed 5 Å away from the $\beta$-carbon of serine along a line from the center of mass to this atom.

Dipole moments are obtained with

$$\mathbf{P} = n(\vec{r}_P - \vec{r}_N)e + (p - n)\vec{r}_N e \quad (2)$$

as previously described (Koppenol & Margoliash, 1982). Since the protein backbone contains only two turns of $\alpha$-helix (Guss & Freeman, 1983), the contribution from peptide bond dipoles could be ignored.

The use of the kinetic ionic strength effect in determining reaction sites of large molecules with asymmetric charge

distributions depends on the fact that the electrostatic energy of an ion in the field of a dipole is a function of its location. The screening by ionic strength of the electric potential field due to a monopole is different from that due to a dipole such that at higher ionic strength significant deviations from Brønsted–Debye–Hückel behavior can be attributed to this effect. Equations 3–5 are used for analyses of ionic strength data. Equation 3, derived by Van Leeuwen et al. (1981), is

$$\ln k = \ln k_0 + \frac{Z_1 Z_2 e^2}{4\pi\epsilon_0\epsilon k_B T R}\left(1 - \frac{e^{-\kappa R_2}}{1 + \kappa R_1}\right) + \frac{Z_2 e \mathbf{P}_1 \cos\theta}{4\pi\epsilon_0\epsilon k_B T R^2}\left(1 - \frac{1 + \kappa R}{1 + \kappa R_1} e^{-\kappa R_2}\right) \quad (3)$$

applicable to the reactions of a small ion with a larger dipolar protein and has been used previously for reactions of cytochrome *c* with transition metal complexes (Rush et al., 1987). The monopole screening function in eq 3 was derived by Debye (1943). The interaction angle $\theta$ is defined as the angle between the dipole vector and a line from the center of mass to the site of electron transfer. Other symbols used in eq 1–5 are described in footnote 1.

An equation, also due to Van Leeuwen (1983), is derived for the interaction between large dipolar molecules in which a third term is necessary to describe the dipole–dipole potential energy, which is a function of the location of the reaction sites relative to the dipole vectors:

$$\ln k = \ln k_{inf} - [Z_1 Z_2 + (ZP)(1 + \kappa R) + (PP)(1 + \kappa R)^2]\frac{e_2}{4\pi\epsilon_0\epsilon k_B T R} f(\kappa) \quad (4)$$

in which the monopole–dipole interaction is given by

$$(ZP) = (Z_1 \mathbf{P}_2 \cos\theta_2 + Z_2 \mathbf{P}_1 \cos\theta_1)/eR$$

the dipole–dipole interaction by

$$(PP) = \mathbf{P}_1 \mathbf{P}_2 \cos\theta_1 \cos\theta_2/(eR)^2$$

and the ionic strength function $f(\kappa)$ by

$$f(\kappa) = \frac{1 - e^{-2\kappa R_2}}{2\kappa R_2(1 + \kappa R_1)}$$

Equation 4 has been applied to reactions of cytochrome *c* with proteins (Van Leeuwen, 1983), although in the absence of detailed electrostatic description of both redox partners. At low ionic strengths, eq 3 reduces to the Brønsted–Debye–Hückel equation:

$$\log k = \log k_0 + \frac{2 Z_1 Z_2 A\sqrt{\mu}}{1 + 6.1\sqrt{\mu}} \quad (5)$$

The application of eq 3 and 4 for evaluating the interaction angles require that the amino acid sequence and structure of the reactant(s) be known. The dipole moments of cytochrome *c* and the CDNP-lysine 13 and 72 derivatives were recalculated (Koppenol & Margoliash, 1982) with the assumption that the heme propionic acid side chains are ionized. $\theta$ is 30° for cytochrome *c*, as previously verified in reactions with small molecules (Rush et al., 1987). A radius of 18.5 Å was used for cytochrome *c* and includes the radius of a water molecule.

Since the protein radius appears to the second power in eq 3 and 4, the effective radius of the cylindrical plastocyanin molecule was estimated by averaging the distances of the charged atoms, which extend into the solvent, to the center of mass, which yielded a radius of 15.1 Å. This dimension agreed well with the solvated radius of 15.9 Å obtained from a molecular weight based formula by Tanford (1961), and 15.5 ± 0.5 Å was taken as the radius for calculations.

*Materials.* Plastocyanin was isolated from fresh spinach leaves by the method of Katoh (1971). The preparation was eluted twice from DEAE-Sephacel anion-exchange columns with phosphate buffer and stored frozen as a concentrated solution. The peak ratio $A_{278}/A_{596} = 1.7$ of cupric plastocyanin indicated satisfactory purity. Concentrations were calculated from $\epsilon_{596} = 4.9 \times 10^3$ $M^{-1}$ $cm^{-1}$ (Malkin & Malmström, 1970). Horse heart ferricytochrome *c*, type VI, was obtained from Sigma. The reduced proteins were obtained by ascorbate or dithionite treatment followed by gel filtration using a nitrogen-saturated Tris-HCl buffer of pH 7.8 for most preparations. The (4-carboxy-2,5-dinitrophenyl)lysyl 13 and 72 modified ferricytochromes *c* were prepared as described previously (Brautigan et al., 1978a,b; Osheroff et al., 1980b) and supplied by Professor E. Margoliash, Northwestern University. Tris(1,10-phenanthroline)cobalt(III) perchlorate was prepared by literature methods (Schilt & Taylor, 1959) and its purity determined by measurements of its absorption spectrum. Potassium ferricyanide and hexaamminecobalt(III) were commercial reagents as were the other common chemicals used. Ferrous diethylenetriaminepentaacetate was prepared by dissolving $Fe^{II}(NH_4)_2(SO_4)_2$ in a deaerated solution with a 1.3:1 excess of the ligand. Water was purified by reverse osmosis and ion exchange (Mar Cor).

*Kinetic Experiments.* Kinetic data were obtained by stopped-flow spectrophotometry as previously described (Rush et al., 1987). Temperatures were maintained by a water bath at 25 °C in most experiments. Rates of reactions between inorganic oxidants and cuprous plastocyanin were determined with an excess of oxidant (>10:1) at two or three concentrations of the oxidant. Rates determined at a particular concentration of oxidant were the average of three to five experiments. In most experiments with plastocyanin the pH was maintained at 7.8 ± 0.1 with Tris-HCl to prevent interference from the inactive form of the protein which is predominant below pH 5.7 (Segal & Sykes, 1978). NaCl was used to maintain ionic strength, with corrections made for added buffer. Rate constants determined at high (>0.5 M) ionic strength were not expected to fit any equation involving the Debye–Hückel theory. Empirically, however, the agreement between theory and experiment at these high ionic strengths was remarkable. Reactions were monitored at 596 nm, the absorbance maximum of cupric plastocyanin. Rate data were also obtained at constant tris(1,10-phenanthroline)cobalt(III) concentration in the presence of four to five different concentrations of the binding cation hexaamminecobalt(III). From these experiments a binding constant for the interaction of hexaamminecobalt(III) with plastocyanin was derived. The reaction between $Fe^{II}dtpa^{3-}$ and CDNP-lysine 13 ferricytochrome *c* was performed at pH 6.5 in a phosphate-buffered medium to prevent complications from the heme crevice $pK_a$ of 8.1 (Osheroff et al., 1980a).

The reduction of cupric plastocyanin by ferrocytochrome *c* was measured with an excess of the latter and monitored at 550 or 417 nm, maxima in the spectrum of ferrocytochrome *c*. The dependence was first-order in both reactants as was observed in all the redox systems studied. With the exception of a few experiments at pH 7.4 in phosphate buffer, all plastocyanin rate data were obtained in a Tris-HCl/NaCl medium. The reactions of the CDNP-lysine-modified cytochromes were performed with an excess of the plastocyanin (three different concentrations) and monitored at 417 nm.

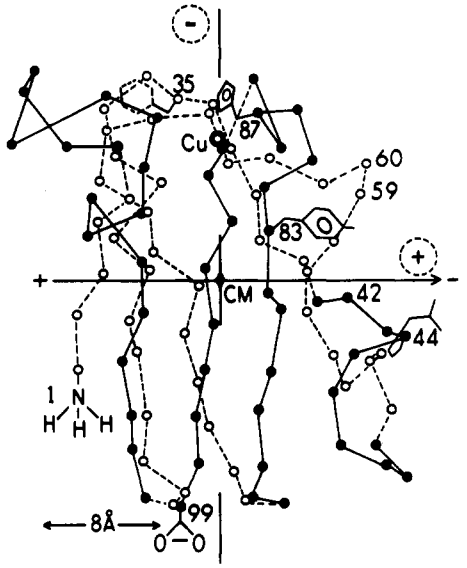


FIGURE 1: Projection of the spatial structure (Guss & Freeman, 1983) of plastocyanin in a center-of-mass coordinate system. The $x$ axis is coincident with the dipole moment axis, and the $y$ axis passes through the copper atom. The positive pole of the dipole axis crosses the protein surface close to the $\beta$-carbon of valine 3 and the negative pole near the nitrogen of phenylalanine 41. A-carbon positions projecting foward from the plane of the page are indicated with filled circles. The regions where cation and anion binding sites are located (Cookson et al., 1980) are indicated as are the side chains of solvent-exposed residues, Phe 35, His 87 (imidazole), Tyr 83, and residues 42–45.

Table I: Orientations with Respect to the Dipole Moment Vector and Distance from the Copper Site of Some Plastocyanin Surface Residues

| surface residue(s) | interaction angle[a] (deg) | distance from Cu (Å) |
|---|---|---|
| His 87 (center imidazole) | 86 | 3.2 |
| Phe 35 (center phenyl) | 112 | 9.4 |
| Pro 36 ($\alpha$-carbon) | 91 | 6.1 |
| Tyr 83 (center phenol) | 38 | 9.9 |
| residues 42–45[b] | 30 | 19.0 |
| residues 59–61[b] | 34 | 13.9 |

[a] The angles given are with respect to the *negative* end of the dipole axis. [b] From the average of the coordinates of the negative charges.

## RESULTS

*Dipole Moment.* The asymmetric charge distribution leads to dipole moments of 358 and 361 D for cupric and cuprous plastocyanin, respectively. The dipole vector is at a right angle (89.5°) to a line from the center of mass to the copper ion, as shown in Figure 1. The angle between the dipole moment of cuprous plastocyanin and that of the cupric form is small, 7.5°. Table I shows the interaction angles relative to the dipole vector for significant residues on the protein surface and the cation/anion binding sites (Cookson et al., 1980).

*Electric Potential Field.* Because of a high negative net charge of $-8e$ on cupric plastocyanin, the electric potential field at any appreciable distance from the protein surface is negative. The $-1k_BT/e$ surface is nearly spherically symmetrical, reflecting only the net charge. However, close to the surface the field is not homogeneous due to the asymmetric distribution of surface charges. Figure 2 shows the electric potential field at two cross sections through the protein. The field increases from $-1k_BT/e$ to $-4k_BT/e$ close to the protein surface. The orientation of the protein in Figure 2 is the same as that in Figure 1. The domain of cation binding in the vicinity of residues 42–45 corresponds to a maximum in the attractive potential of the electrostatic field. Moreover, the field is relatively homogeneous in this region of the protein surface

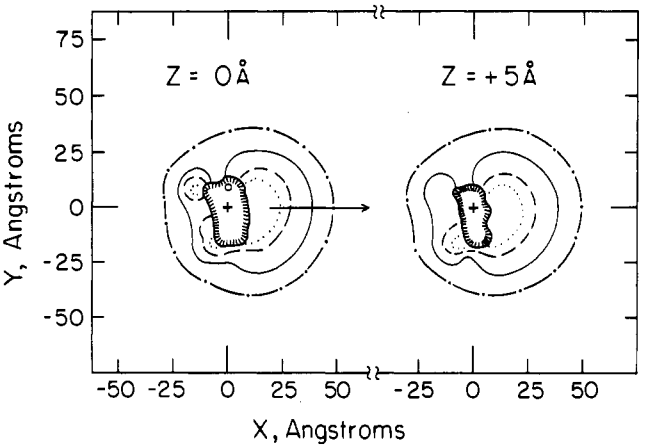


FIGURE 2: Two parallel cross sections 5 Å apart of the electrostatic field of oxidized spinach plastocyanin at zero ionic strength. The protein is oriented as in Figure 1 with respect to the center of mass and the dipole moment vector. The outer equipotential line (dot dashed) indicates a $-1.5k_BT/e$ potential. More inward potentials of $-2k_BT/e$ (continuous), $-3k_BT/e$ (dashed, and $-4k_BT/e$ (dotted) are found. Cross section $Z = 0$ Å passes through the center of mass. The copper atom is indicated by a circle.

through which the negative end of the dipole axis passes. The surface site of minimum repulsion for anions does not correspond to the positive end of the dipole but is located within the hydrophobic region proximal to the copper atom. The $-2k_BT/e$ contour intersects the surface in the vicinity of residues Phe 35 and His 87 and corresponds to the locus of anion binding determined by previous NMR studies (Cookson et al., 1980). Other cross sections of the electric potential field of spinach plastocyanin did not reveal sites more favorable to anion interactions. As discussed before (Koppenol & Margoliash, 1982), the numerical values of the potential calculated for a homogeneous dielectric constant and an ionic strength of zero might be incorrect, but the shape of the electric potential field around a spherical protein is thought to be a good approximation of the actual situation.

*Kinetic Ionic Strength Dependences.* Reactions 6 and 7 showed second-order kinetics over the range of oxidant concentrations used, $2 \times 10^{-5}$–$4 \times 10^{-4}$ M. The second-order

$$Fe(CN)_6^{3-} + Pc(I) \rightarrow Fe(CN)_6^{3-} + Pc(II) \quad (6)$$

$$Co(phen)_3^{3+} + Pc(I) \rightarrow Co(phen)_3^{2+} + Pc(II) \quad (7)$$

rate constants at ionic strengths from 0.005 to 1 M are shown in Table II. At higher tris(1,10-phenanthroline)cobalt(III) concentrations, reaction 7 exhibits rate saturation (Segal & Sykes, 1978; Lappin et al., 1979), which has been interpreted as electron transfer within a protein–oxidant complex. We prefer to regard this as inhibition due to formation of an unreactive complex of protein with oxidant, and therefore the data are reported as a second-order rate constant. Low concentrations of oxidant were used so as to avoid complications from this effect, especially at the lower ionic strengths where ion pairing of the reactants becomes stronger.

The data from Table II are plotted according to the simple Brønsted–Debye–Hückel theory, eq 5, in Figure 3. For $\mu <$ 0.05 M the slopes of data for reactions 6 and 7 give net charges on cuprous plastocyanin of $-8.6e$ and $-9.2e$, respectively, which agree well with the theoretical charge of $-9e$. This basic agreement is important since it indicates that various potentially complicating effects of ionic strength such as protein conformational changes, ion binding, or catalysis are not entering the rate laws for the reactions. The data are then fitted according to eq 3 with the parameters listed in Table III. The

Table II: Ionic Strength Dependences of Second-Order Rate Constants[a]

| reaction | $\mu$ (M) | $k$ ($M^{-1}$ $s^{-1}$) |
|---|---|---|
| $Fe(CN)_6^{3-}$ + Pc(I) | 0.0055 | $2.8 \times 10^3$ |
| | 0.011 | $5.5 \times 10^3$ |
| | 0.026 | $1.55 \times 10^4$ |
| | 0.052 | $3.3 \times 10^4$ |
| | 0.10 | $7.0 \times 10^4$ |
| | 0.25 | $1.51 \times 10^5$ |
| | 0.50 | $2.2 \times 10^5$ |
| | 1.0 | $3.1 \times 10^5$ |
| $Co(phen)_3^{3+}$ + Pc(I) | 0.006 | $6.8 \times 10^4$ |
| | 0.011 | $3.3 \times 10^4$ |
| | 0.021 | $1.33 \times 10^4$ |
| | 0.052 | $4.4 \times 10^3$ |
| | 0.105 | $1.77 \times 10^3$ |
| | 0.25 | $7.0 \times 10^2$ |
| | 0.50 | $4.7 \times 10^2$ |
| | 1.00 | $2.8 \times 10^2$ |
| Fe(II)*c* + Pc(II) | 0.021 | $4.5 (\pm 0.5) \times 10^7$ |
| | 0.031 | $2.4 \times 10^7$ |
| | 0.045 | $1.1 \times 10^7$ |
| | 0.062 | $6.3 \times 10^6$ |
| | 0.085 | $4.0 \times 10^6$ |
| | 0.105 | $3.2 \times 10^6$ |
| | 0.20 | $1.55 \times 10^6$ |
| | 0.40 | $8.3 \times 10^5$ |
| | 0.80 | $6.0 \times 10^5$ |
| | 2.0 | $5.5 \times 10^5$ |
| CDNP-Lys 13 Fe(II)*c* + Pc(II) | 0.05 | $6.3 \times 10^6$ |
| | 0.10 | $2.7 \times 10^6$ |
| | 0.20 | $1.5 \times 10^6$ |
| | 0.40 | $8.9 \times 10^5$ |
| | 0.80 | $7.2 \times 10^5$ |
| CDNP-Lys 72 Fe(II)*c* + Pc(II) | 0.10 | $2.0 \times 10^6$ |

[a] Conditions: [metal complex] $\geq 10$[Pc(I)]; [Pc(I)] $\simeq 5$ $\mu$M. During the native cytochrome *c* experiments [cytochrome *c*] $\geq 3$[Pc]. Pc(II) (4–15 $\mu$M) was in approximately 8-fold excess during the modified cytochrome *c* experiments. The error in these measurements is estimated to be approximately 5% or smaller, except as indicated.

interaction angles for hexacyanoferrate(III) and tris(1,10-phenanthroline)cobalt(III) are 110° and 85°, respectively. In Figure 3 are also plotted the expected ionic strength dependences for a 90°, 180°, and 30° interaction. The latter dependence is expected for an electron transfer via the acid patch cation binding site and yields a significantly greater slope than is observed.

*Binding Constants of $Co(NH_3)_6^{3+}$ with Cuprous Plastocyanin.* It has been proposed that the inhibition of electron-transfer reactions in plastocyanins by binding cations results from competition for the reaction site (Sykes, 1985). We therefore evaluated the inhibition constants, $K_i$, for reaction 7 in the presence of hexaamminecobalt(III) according to a mechanism involving reaction 7 and the reactions:

$$Co(NH_3)_6^{3+} + Pc(I)^{9-} \overset{K_i}{\rightleftharpoons} [Pc(I)Co(NH_3)_6]^{6-} \quad (8)$$

$$Co(phen)_3^{3+} + [Pc(I)Co(NH_3)_6]^{6-} \rightarrow [Pc(II)Co(NH_3)_6]^{5-} + Co(phen)_3^{2+} \quad (9)$$

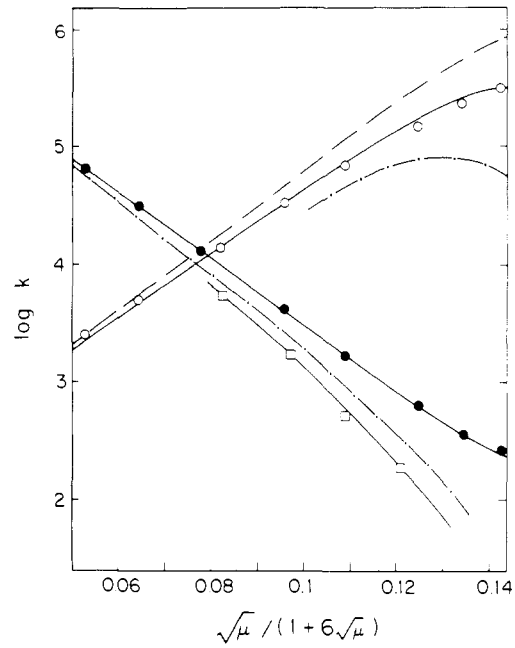


FIGURE 3: Second-order rate constants as a function of ionic strength for the reactions of $Fe(CN)_6^{3-}$ (○) and $Co(phen)_3^{3+}$ (●) with cuprous plastocyanin. Solid curves through the data are obtained from eq 3 with the parameters listed in Table III. The dashed curve is indicated for an interaction angle of 90°. The dot-dashed curves are calculated for an interaction angle of 180° in the hexacyanoferrate(II) reaction (upper) and for an interaction angle of 30° (lower) in the tris(1,10-phenanthroline)cobalt(III) reaction. Also shown on the same scale are the binding constants $K_i$ (□) for hexaamminecobalt(III) with cuprous plastocyanin. The calculated dependence is obtained from a binding site interaction angle of 30°.

In the presence of the inhibitor, the appparent second-order rate constant $k'$ decreases since $k_9$ is expected to be less than $k_7$ if only because the negative charge on the protein–metal complex is 3 units less than that of the protein. The dependence of $k'$ on $k_7$, $k_9$, and $K_i$ is $k' = (k_7 + k_9K_i[Co(NH_3)_6^{3+}])/(1 + K_i[Co(NH_3)_6^{3+}])$. Figure 4 shows the experimental data obtained at ionic strengths 0.025, 0.05, 0.1, 0.2, and 0.5 M as well as recalculated curves obtained by using the values of $K_i$ and $k_9$ listed in Table IV. The experimental values of $K_i$ are also plotted in Figure 3 for comparison with the ionic strength dependence of $k_7$. The binding constants for hexaamminecobalt(III) to cuprous plastocyanin depend on ionic strength as expected for a site at a 30° orientation with respect to the dipole moment vector. This is consistent with the supposition that the acid patch is the preferred binding location. The values of $K_i$ are ~55% of that calculated from the Eigen equation (Moore & Pearson, 1981), indicating that the association of hexaamminecobalt(III) and cuprous plastocyanin is mainly electrostatic.

The values of $k_7$ and $K_i$ depend differently on the ionic strength, which indicates that the binding and the electron-transfer site are not identical. The ionic strength dependence

Table III: Ionic Strength Fitting Parameters

| | reactant | | | plastocyanin[a] | | |
|---|---|---|---|---|---|---|
| | radius (Å) | dipole (D) | $\theta$ (deg) | radius (Å) | $\theta$ (deg) | $k_0$ ($k_{inf}$) ($M^{-1}$ $s^{-1}$) |
| $Fe(CN)_6^{3-}$ | 3 | | | 16 | 110 | 80 |
| $Co(phen)_3^{3+}$ | 5 | | | 16 | 85 | $1.8 \times 10^6$ |
| Fe(II)$c^{6+}$ (native) | 18.5 | 300 | 30 | 16 | 70 | $(1.65 \times 10^5)$ |
| Fe(II)$c^{6+}$ (native) | 18.5 | 300 | 30 | 15 | 83 | $(2.0 \times 10^5)$ |
| Fe(II)$c^{4+}$ (CDNP-lysine 13) | 18.5 | 240 | 30 | 15.5 | 60 | $(2.0 \times 10^5)$ |
| Fe(II)$c^{4+}$ (CDNP-lysine 13) | 17.0 | 240 | 30 | 15 | 75 | $(2.3 \times 10^5)$ |

[a] On the basis of the amino acid composition, the net charges of cupric and cuprous plastocyanin are $-8e$ and $-9e$, respectively. The dipole moment of plastocyanin is 360 D.

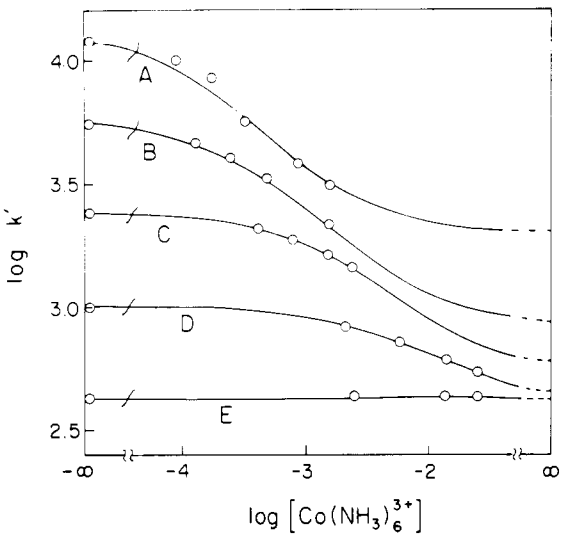


FIGURE 4: Hexaamminecobalt(III)-dependent inhibition of the reaction between tris(1,10-phenanthroline)cobalt(III) and cuprous plastocyanin at ionic strengths of 0.025 M (A), 0.05 M (B), 0.1 M (C), 0.2 M (D), and 0.5 M (E). The calculated curves were obtained from the values of $k_9$ and $K_i$ in Table IV.

Table IV: Ionic Strength Dependence of Binding ($K_i$) between $Co(NH_3)_6^{3+}$ and Cuprous Plastocyanin and Oxidation of $[Pc(I)Co(NH_3)_6^{3+}]$ by $Co(phen)_3^{3+}$ [a]

| ionic strength (M) | $K_i$ ($M^{-1}$) | $k_9$ ($M^{-1}$ $s^{-1}$) |
|---|---|---|
| 0.025 | $5.3 \times 10^3$ | $2.2 \times 10^3$ |
| 0.05 | $1.7 \times 10^3$ | $9.0 \times 10^2$ |
| 0.10 | $5.2 \times 10^2$ | $6.0 \times 10^2$ |
| 0.20 | $1.9 \times 10^2$ | $4.5 \times 10^2$ |
| 0.50 | | $\sim 4.2 \times 10^2$ |

[a] Conditions: $[Co(phen)_3^{3+}] = 0.1$–$0.2$ mM; $[Pc(I)] = 5$ μM; $[Co(NH_3)_6^{3+}] \geq 10[Pc(I)]$.

of reaction 9 is not as simple. Though obtained by extrapolation, it is clear that values of $k_9$ decrease in an approximately normal manner in the ionic strength range of 0.025–0.05 M, but $k_9$ appears to become constant at about 400 $M^{-1}$ $s^{-1}$ as the ionic strength approaches 0.5 M. A possible explanation for this is given in the Discussion.

*Reaction of Ferrocytochrome c with Cupric Plastocyanin.* Though the ionic strength data for reactions 6 and 7 are consistent with an electron-transfer site within the hydrophobic region of the protein, reaction 10 may be a more realistic model

$$Fe(II)c + Pc(II) \rightarrow Fe(III)c + Pc(I) \qquad (10)$$

of the biological reduction of cupric plastocyanin with cytochrome *f*, although the latter is, in contrast to cytochrome *c*, negatively charged. Reaction 10 was measured at pH 7.4–7.8 over the ionic strength range from 0.021 to 2.0 M and was found to be first-order in cytochrome *c* (not shown). Second-order rate constants are listed in Table II. The error is estimated not to exceed ±5% except for the rate constant obtained at 0.021 M, which has an error of ±10% owing to instrumental limitations. The rapidly increasing rate did not permit measurements at lower ionic strength which would have been valuable in determining accurately the interaction angle according to eq 4. In spite of the complexity of eq 4, the parameters needed to evaluate the ionic strength dependence of reaction 10 are known with some accuracy. Values of $k_{10}$, plotted against $f(\kappa)$ and ionic strength in Figure 5A, were fitted to eq 4 for various values of the plastocyanin radius between 15 and 16 Å with $k_{10}$(inf) as an adjustable parameter in the fit. The mean relative error in calculated/experimental values

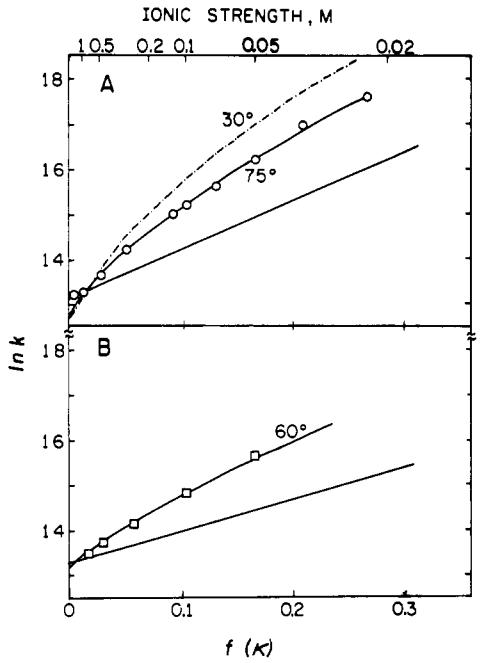


FIGURE 5: Ionic strength dependences for (A) reaction 10, ferrocytochrome *c* + cupric plastocyanin, and (B) CDNP-lysine 13 ferrocytochrome *c* + cupric plastocyanin. In (A) and (B) the calculated fit to the data with the parameters in Table III is plotted against ionic strength (upper ordinate) and $f(\kappa)$ (lower ordinate) with the help of eq 4. In (A) two other curves are plotted to show the sensitivity of the dependences to the protein dipole. The dot-dashed curve is obtained for an interaction angle with the plastocyanin dipole of 30° as for an "acid patch" interaction site ($k_{inf} = 2 \times 10^4$ $M^{-1}$ $s^{-1}$). The linear solid line is obtained when the protein dipoles of ferrocytochrome *c* and plastocyanin are neglected entirely in eq 4.

was optimized at about 4% for the different radii, and the resulting interaction angles are given in Table III. The uncertainty limit in each of these angles was taken to be the value of $\theta$ at which the mean relative error exceeded 10% with the best possible fit. The result is an interaction angle of 75 ± 15°. The difference between the upper and lower range of this angle is caused by the inclusion (lower) or exclusion (upper) of a water molecule between proteins. For purposes of comparison, we also show in Figure 6A the ionic strength dependence expected for a reaction occurring via the acid patch on plastocyanin at an interaction angle of 30°.

*Reactions of CDNP-lysine 13 and 72 Ferrocytochromes c with Cupric Plastocyanin.* The CDNP-lysine 13 and 72 modified ferrocytochromes have net charges 2*e* less than the native protein and both also have smaller dipole moments (Koppenol & Margoliash, 1982). In a number of kinetic studies, these modifications near the heme crevice have been found to reduce the activity of cytochrome *c* toward negatively charged protein redox partners, often by 1 or 2 orders of magnitude (Koppenol & Margoliash, 1982) when strong electrostatic binding precedes electron transfer. In order to compensate for the change in charge and dipole moment of the CDNP-lysine 13 derivative, the rate constant at high ionic strength must be evaluated. In a 0.1 M NaCl medium the rate constants of the modified proteins with cupric plastocyanin were determined to be $2.7 \times 10^6$ $M^{-1}$ $s^{-1}$ for CDNP-lysine 13 Fe(II)*c* and $2.0 \times 10^6$ $M^{-1}$ $s^{-1}$ for CDNP-lysine 72 Fe(II)*c*, compared to $3.2 \times 10^6$ $M^{-1}$ $s^{-1}$ for the native protein. These rate decreases are marginal and may be a result of decreased bulk electrostatic attractions between the cationic cytochrome derivatives and plastocyanin. To determine the dipole magnitude and its angle with respect to the electron-transfer site, the ionic strength dependence of its reaction with the small

Table V: Ionic Strength Dependence of the Reduction of CDNP-lysine 13 Ferricytochrome *c* by $Fe^{II}dtpa^{3-}$

| ionic strength (M) | $k_{11}$ ($M^{-1}$ $s^{-1}$) (exptl) | $k_{11}$ ($M^{-1}$ $s^{-1}$) (calcd)[a] |
|---|---|---|
| 0.0055 | $2.8 \times 10^5$ | $2.5 \times 10^5$ |
| 0.011 | $1.46 \times 10^5$ | $1.6 \times 10^5$ |
| 0.0225 | $8.2 \times 10^4$ | $9.2 \times 10^4$ |
| 0.055 | $4.8 \times 10^4$ | $5.3 \times 10^4$ |
| 0.11 | $2.4 \times 10^4$ | $2.6 \times 10^4$ |
| 0.51 | $7.6 \times 10^3$ | $7.9 \times 10^3$ |
| 1.0 | $5.5 \times 10^3$ | $5.3 \times 10^3$ |

[a] Calculated values of $k_{11}$ were obtained from eq 3 with the following parameters for CDNP-lysine 13 ferricytochrome *c*: $R_1 = 18.5$ Å, $Z_1 = +5e$, $P_1 = 240$ D, and $\theta_1 = 30°$; and for $Fe^{II}dtpa^{3-}$, $R_2 = 4$ Å and $Z_2 = -3e$, with $k_{inf} = 2.48 \times 10^3$ $M^{-1}$ $s^{-1}$.

anionic redox probe $Fe^{II}dtpa^{3-}$ was measured from 5.5 mM to 1.0 M. Second-order rate constants for reaction 11 are listed

$$Fe^{II}dtpa^{3-} + \text{CDNP-lysine 13 Fe(III)}c \rightarrow Fe^{III}dtpa^{2-} + \text{CDNP-lysine 13 Fe(II)}c \quad (11)$$

in Table V together with values of **P** and $\theta$ calculated from eq 3. The dipole moment and interaction angle cannot be determined independently from ionic strength data alone, but a best fit was obtained from a dipole magnitude of 240 D, which is higher than the 180 D calculated from structural data, and an interaction angle of 30° with respect to the solvent-exposed heme edge, which is somewhat less than the computed angle (~54°). The difference between the fitted and computed parameters may reflect the uncertainty in the location of the CDNP group. The fitted parameters were used to evaluate the ionic strength dependence of the reaction between CDNP-lysine 13 ferrocytochrome *c* and plastocyanin according to eq 4. Second-order rate constants are listed in Table III and plotted as a function of $f(\kappa)$ in Figure 5B along with a calculated curve. The angle between the electron-transfer site and the plastocyanin dipole moment vector, approximately 65 ± 15°, was evaluated by the same procedure used for reaction 10. The range of values for the rate constants at infinite ionic strength for the reactions of the native and modified ferrocytochromes *c* with plastocyanin are similar within experimental error, $(2.0 \pm 0.4) \times 10^5$ $M^{-1}$ $s^{-1}$. The calculated dipole moment of the CDNP-lysine 72 derivative is 208 D (vs 180 D for CDNP-lysine 13 ferrocytochrome *c*). This is sufficient to account for its reduced activity at $\mu = 0.1$ M, and although the ionic strength dependence of $k_{10}$ for CDNP-lysine 72 ferrocytochrome c was not measured, the rate constant at infinite ionic strength is expected to fall within the same range.

The charged residues of plastocyanin extend, on average, 15.1 Å from the center of mass, but some distances are as little as 13 Å and others as much as 17 Å. The distance of closest approach could therefore vary, depending on reactant and site of electron transfer. This leads to an uncertainty in $\theta$ which is particularly important in the case of cytochrome *c*, where there is a substantial interaction of the cytochrome *c* dipole with the net charge of plastocyanin. Inclusion of the extremes in radius introduces a further uncertainty of 10° in the values of $\theta$.

## Discussion

The interaction angles for the reactions of spinach plastocyanin with $Fe(CN)_6^{3-}$, $Co(phen)_3^{3+}$ and native and CDNP-lysine 13 ferrocytochrome *c* are 110°, 85°, 75°, and approximately 65°, respectively. These angles are with respect to the negative pole of the dipole axis. They do not specify unique sites but rather a band of sites equatorial to the dipole vector of the molecule in Figure 1. On the basis of the ionic strength dependences it is possible to exclude sites such as the acid patch and tyrosine 83 as viable electron-transfer areas as well as all other sites that are close to the sites where the dipole axis emerges from the protein surface. The relative reactivities of the modified ferrocytochromes indicate a site (or sites) with a minimum of specific charge interaction. It would be tempting to conclude on the basis of the similar values of $k_{inf}$ obtained for the reactions of the native and CDNP-lysine-modified cytochromes that a region relatively free of carboxylate residues on the plastocyanin surface, such as the hydrophobic region, is the site of electron transfer. However, it is more likely that only a mechanism in which strong and specific electrostatic binding between surface residues of the redox partners is involved can be reliably excluded. This is consistent with the conclusions from other kinetic studies of the redox reaction that preassociation between ferrocytochrome *c* and plastocyanin is weak (Sykes, 1985). The hydrophobic region in the upper part of the molecule centered around the histidine 87 imidazole seems to fit the interaction angle and the electric potential field criteria best. This area also includes the region where the distance from the surface to copper atom is shortest (6 Å). The short distance is probably critical when the difference in reduction potential between the reactants is small (Marcus & Sutin, 1985; Isied, 1984).

The interaction angles for hexacyanoferrate(III) and tris(1,10-phenanthroline)cobalt(III) fit the location of Phe 35 and His 87 imidazole residues, respectively, on the hydrophobic surface of the protein. The data for these small complexes seem sufficiently sensitive to distinguish two electron-transfer sites for oppositely charged ion. The use by the protein of two electron-transfer sites close together may simply be due to its asymmetric electrostatic field (Figure 2), which causes ions to approach from a direction of minimum repulsion. The angle of 110° indicates significant "tilting" away from the strong repulsive field on the right side of the protein and coincides with the anion binding region. If Phe 35 is the reaction site for anions, the electron could be transferred to His 37 via Pro 36 along the peptide chain. For cations that approach the hydrophobic area along the negative pole of the dipole axis, the solvent-exposed imidazole (Guss & Freeman, 1983) of histidine 87 may be the first reactive site encountered, the anionic electron-transfer site being less favored electrostatically. The results for native and CDNP-lysine 13 ferrocytochromes *c* are also consistent with electron transfer at a common site but not sufficiently sensitive to exclude others.

Our results do not imply that plastocyanin cannot undergo redox via remote sites, if those sites are determined by strong binding between the protein and the redox partner, or if the Gibbs energy change for electron transfer is sufficient to overcome a distance barrier. For instance, it has been shown by product analysis that chromium(II) reacts, at least in part, near the acid patch (Farver & Pecht, 1981; Farver et al., 1982). This reagent is an example of a powerful reducing agent. A NMR study (King et al., 1985) has shown that the rate of electron exchange within a cuprous plastocyanin–ferricytochrome *c* complex, which presumably is formed by electrostatic attraction between lysines around the heme edge of ferricytochrome *c* and the acid patch of plastocyanin, saturates when the complex is fully formed. It is reasonable that complexation by ferricytochrome *c* would inhibit electron transfer by excess protein in solution much more thoroughly than a small cation could, but this is not conclusive evidence that electron transfer occurs in the dominant binding orientation. Random motions within the electrostatically bound complex would permit exposure to other potential sites. It is possible that for the reactions in the present study redox within the hydrophobic region is preceded by ligation at the cation

binding site (Beoku-Betts et al., 1983) followed by reorientation of the protein–cation complex. This use of the acid patch as an intermediate recognition site by cationic reactants would not influence, or be detected by, the kinetic ionic strength dependences, although it could partly account for the inhibition by inert cations (Cookson et al., 1980) bound at this site.

The application of the kinetic ionic strength effect for evaluation of reaction sites obviously depends on the absence of specific ion influences or protein conformational changes which could hopelessly complicate interpretation of the data. At high ionic strength, some association of electrolyte ions with the inorganic complexes is expected. However, this should not be a significant source of error unless ion association were to change the reactivity of the inorganic complex by other than simply electrostatic factors, which is not expected for substitutionally inert systems. The validity of eq 3 and 4 is dependent on the assumptions made in the Debye–Hückel theory (Debye & Hückel, 1923), notably the linearization of the Poisson–Boltzmann equation. Extensive calculations indicate that for weakly charged polyelectrolytes with surface potentials smaller that approximately $|4|k_BT/e$ the linearization is valid (Lampert & Crandell, 1979) and that the counterion distributions around a polyelectrolyte are not very dependent on the shape of the polyelectrolyte (Guêron & Weisbuch, 1980). Indeed, a simplified form of eq 3 and 4, the Brønsted–Debye–Hückel equation (eq 5), yields to a first approximation the net charge of a polyelectrolyte (Koppenol et al., 1978; Feinberg & Ryan, 1981). This is general evidence that the net charge determines the bulk electrostatic properties of a polyelectrolyte, rather than local charges. Essential also is an equilibrium atmosphere of counterions. Since the reactions reported here have rates that are much less than diffusion-controlled, we believe that this condition is met. A possible source of error due to the nonuniform radius of plastocyanin has been discussed. Although this introduces an uncertainty in the interaction angles evaluated for cytochrome *c*, it does not affect our main conclusion that the interaction of the redox partners with the dipole moment of plastocyanin is small and that therefore electron transfer is not likely to take place at the acid patch.

The reaction between tris(1,10-phenanthroline)cobalt(III) and cuprous plastocyanin liganded with hexaamminecobalt(III) shows a complex ionic strength dependence which suggests that the reactivity of the protein complex is increasing at high ionic strength even as the bulk electrostatic attraction between the reactants is decreasing. This may be due to a change in the strength of the electrostatic association between inorganic complex and protein. The cation–protein complex differs from a simple ion pair in that it is an oriented ion pair, and as seems likely from Figure 2, this is a consequence mainly of the protein's dipole moment. As the orienting power of the asymmetric charge distribution decreases with increasing ionic strength, the bound cation is free to change its position more readily in response to an approaching reactant. In an idealized case, where the potential function at the surface of the macromolecule is a simple function of its angle, $\theta$, with respect to the dipole, the probability of finding the cation between angles $\theta$ and $\theta + d\theta$ is given by eq 12 (Böttcher, 1973). $W_P$ is the

$$\int_{\theta_2}^{\theta_1} \mathbf{P}(\theta)\, d\theta = \frac{2R^2 \int_{\theta_2}^{\theta_1} \exp(-W_P/k_BT) \sin\theta/d\theta}{2R^2 \int_{180°}^{0°} \exp(-W_P/k_BT) \sin\theta/d\theta} \qquad (12)$$

dipolar potential energy given by the third term on the right side of eq 3. The solutions for values of $W_P$ calculated for the cuprous plastocyanin–$Co(NH_3)_6^{3+}$ complex at several ionic strengths are shown in Figure 6. For ionic strengths below 10 mM screening is weak and the orienting force does not change very much so that the ionic strength dependence of reaction 9 should be as predicted by eq 3 or 5. However, $W_P$ decreases rapidly as the ionic strength increases and the ion "delocalization" on the protein's surface approaches the isotropic case shown in curve D of Figure 6. In this model the increasing ability of the entering charged reactant to polarize the protein complex into a more favorable orientation of the bound cation may more than offset the diminishing Coulombic attraction at ionic strengths greater than 0.5 M.

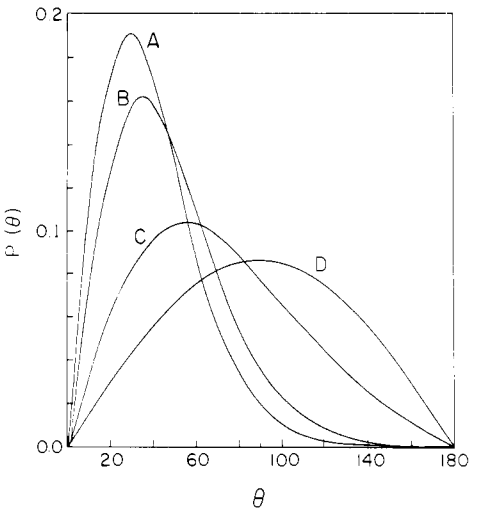


FIGURE 6: Angular probability distribution as a function of the ionic strength of a tripositive cation bound electrostatically to the surface of a protein with dimensions and dipole moment of plastocyanin. The values of $\theta$ in the ordinate are relative to the negative direction of the dipole moment vector. Values of $\mathbf{P}(\theta)$ are computed from eq 12 by using the dipolar energies (A) $W_P = 3.5kT$, approximately constant for values of $\mu < 0.01$ M; (B) $W_P = 2.5kT$, $\mu = 0.1$ M; (C) $W_P = 0.85kT$, $\mu = 1$ M; and (D) $W_P = 0$ at infinite ionic strength where the distribution is isotropic.

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