Catalysis of plastocyanin electron self-exchange by redox-inert multivalent cations

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Electron self-exchange in solutions of the 'blue' copper protein plastocyanin is catalysed by the redox-inert multivalent cations Mg^{2+} or $Co(NH_3)_6^{3+}$. Measurements of specific ^{1}H-NMR line broadening with 50% reduced solutions in the presence of these cations show that electron exchange proceeds through encounters of cation-protein complexes which dissociate at high ionic strength. In the presence of 8 mM (5 equivalents/total protein) $Co(NH_3)_6^{3+}$, with 10 mM cacodylate (pH*6.0) as background electrolyte, the bimolecular rate constant at 25°C is 7×10^4 $M^{-1} \cdot s^{-1}$. For comparison, the 'electrostatically screened' rate constant measured in 0.1 M KCl in the absence of added multivalent cations is $\sim 4 \times 10^3$ $M^{1} \cdot s^{-1}$.

Plastocyanin Electron self-exchange NMR Protein-protein interaction Multivalent cation Blue copper protein

1. INTRODUCTION

Rates of protein-protein intermolecular electron-transfer processes, for which large donor/acceptor separation in the transition state may yield small electronic transmission coefficients, are greatly increased through precursory complex formation. Specific binding requirements, involving spatially optimised electrostatic, hydrophobic and hydrogen-bonding interactions, can provide much of the selectivity that is evident in biological electron-transfer systems. Some of the best studied examples involve electron transfer between cytochrome *c* and cytochrome *c* oxidase [1,2], cytochrome *c* peroxidase [3,4], or cytochrome b_5 [5–7]. In each case, rate enhancement occurs through the intermolecular interaction of complementary binding domains. Thus the ring of conserved lysine residues surrounding the heme crevice of cytochrome *c* may be specifically engaged by spatially compatible domains of carboxylate residues on the reaction partner. For non-physiological partners, the absence of spatial complementarity can lead to decreased rate constants [8,9].

An interesting case to consider is protein-protein electron-transfer systems, both physiological and non-physiological, for which binding domains may be intrinsically non-complementary. This may, to a first approximation, give rise to localised or even extensive repulsive interactions which will retard electron transport. On the other hand such non-complementarity may be readily modulated; for example, by the presence of small ions which may bind and alter electric field patterns around and within the interaction domains. There are some interesting observations in the behaviour of the 'blue' copper protein, plastocyanin, which are relevant to this theme. Plastocyanin from higher plants carries a significant net negative charge, some of which is conservatively located on the 'east' side of the molecule in the region of residues 42–45 [10]. From NMR [11,12] and kinetic [13] studies it is postulated that this constitutes a binding site for cations of the type $M(NH_3)_6^{3+}$ and $M(phen)_3^{3+}$ (M = Co,Cr). It is, furthermore, widely recognised [14–19] that electron transfer from eukaryotic plastocyanin to the $P700^+$ centre is stimulated by Mg^{2+}. On the other hand, the reac-

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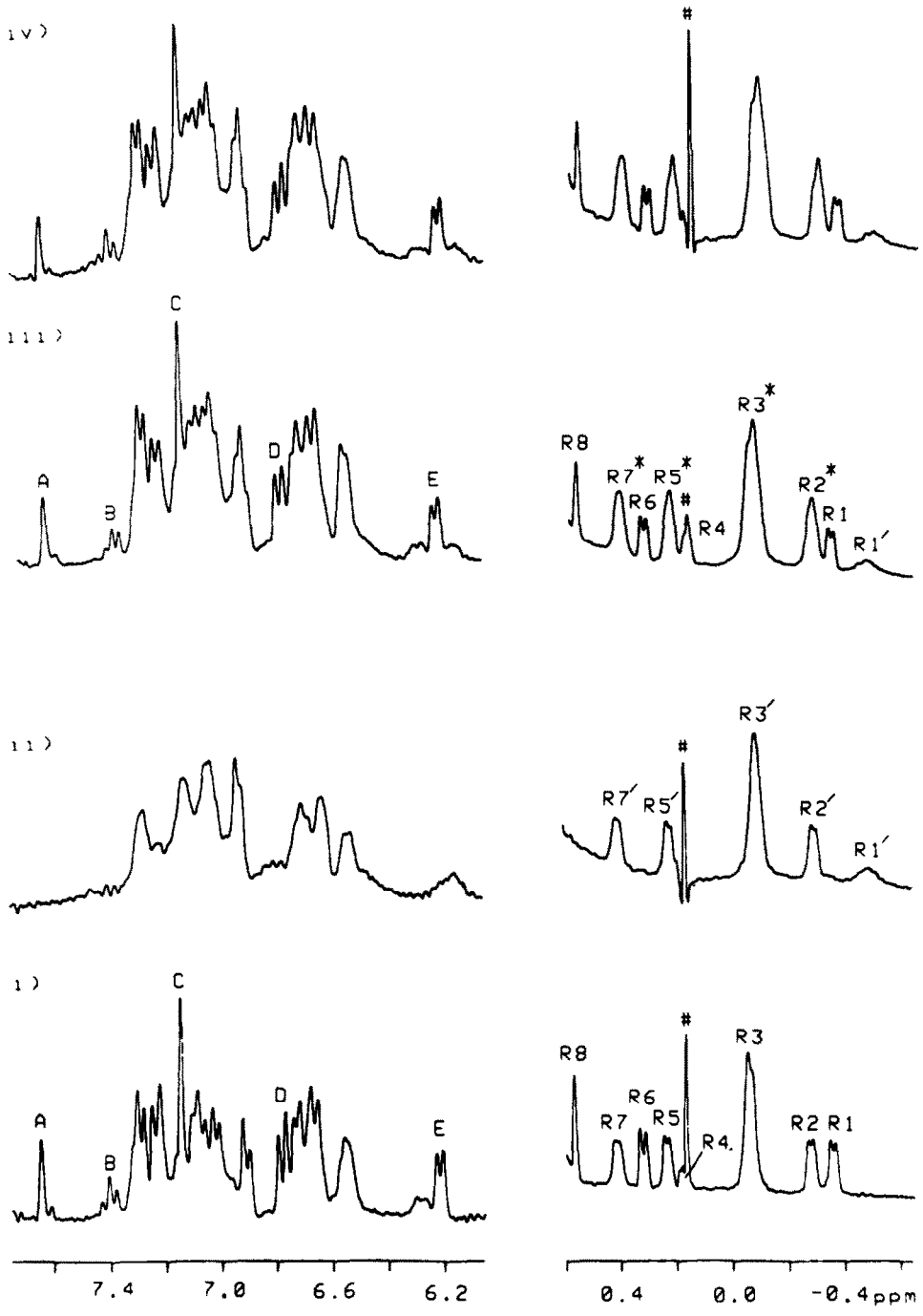


Fig.1. The aromatic and ring current shifted methyl group (vertical scale × 1/2) regions of the resolution enhanced 300 MHz ^{1}H-NMR spectra of (i) reduced plastocyanin; (ii) oxidised plastocyanin; (iii) 50% oxidised plastocyanin and (iv) a spectrum obtained by addition of (i) and (ii). The resonance labels referred to in the text are indicated. The sharp singlet resonance indicated by # which obscures a doublet resonance R4 in (i) is due to an impurity. This was present in relatively large concentration when spectra (i) and (ii) were recorded but was largely removed for measurement of spectrum (iii). All spectra were recorded in 10 mM cacodylate buffer, pH* 6.0, at 25°C.

tions of *Anabaena variabilis* plastocyanin [15] or specifically-modified spinach plastocyanin [17], which do not have this negative domain, have no Mg^{2+} requirement. In an apparently analogous manner, heterogeneous electron-transport between spinach plastocyanin and the negatively charged 'edge' surface of pyrolytic graphite electrodes (which is rich in acidic C-O functionalities) is promoted by free multivalent cations, including Mg^{2+} [20].

For protein-protein electron self-exchange processes the problem of non-complementary domain repulsion may become serious whenever high surface-charge densities are encountered. This indeed appears to be the case with plastocyanin, for which the electron self-exchange process in 10 mM phosphate buffer, as measured by NMR, is slow [21]. In view of our electrochemical findings and our interest in the possible modulatory properties of multivalent cations in physiological systems, we sought to examine this fundamental process in greater detail. In this paper we present evidence that electron self-exchange in spinach plastocyanin is indeed catalysed by redox-inert multivalent cations, including Mg^{2+}.

2. MATERIALS AND METHODS

Plastocyanin was isolated from spinach (*Spinacia oleracea*) using a modification of the procedure of Borchert and Wessels [22]. The final gel filtration was carried out using a column (90 × 2.6 cm) of LKB Ultragel AcA 202. The pure protein ($A_{278}/A_{597} = 1.28$) was concentrated, desalted and exchanged against D_2O (99.8% 2 H_2O, Merck, Sharpe and Dohme) by extensive diafiltration using an Amicon 8MC unit fitted with a YM5 membrane. The final protein, concentration 6 mM, was stored as frozen pellets in liquid nitrogen. All reagents were of Analar or Aristar grade and pH values are quoted as direct meter readings (pH*) uncorrected for the deuterium isotope effect.

Samples of plastocyanin for NMR were prepared by exchanging protein stock solution into 10 mM sodium cacodylate/DCl buffer, pH* 6.0, by diafiltration. Fully oxidised and reduced samples were obtained by treatment of the solutions with a small excess of potassium ferricyanide or sodium ascorbate prior to diafiltration. Partially oxidised samples, free of inorganic redox contaminants, were then made up by mixing the pure solutions in the required amounts. The concentration and degree of oxidation of the protein samples were determined from the absorbance at 597 nm for the fully oxidised form (slight excess of ferricyanide) for which $\epsilon_{597} = 4.9\ mM^{-1} \cdot cm^{-1}$ [23], and the difference as compared to the untreated solution, respectively. To avoid slow atmospheric oxidation of protein NMR samples, tubes were flushed with Ar gas and sealed with rubber septa.

Hexaamminecobalt(III) chloride ($Co(NH_3)_6Cl_3$) [24] and hexaammineplatinum(IV) chloride ($Pt(NH_3)_6Cl_4$) [25] were prepared by literature methods. A sample of $Pt(NH_3)_6Cl_4$ was converted into the more soluble fluoride salt by equilibrating dilute solutions with anion exchange resin (IRA-402) in the fluoride form. Solid $Pt(NH_3)_6F_4$ was recovered by precipitation with absolute ethanol. A sample of $MgCl_2 \cdot 6H_2O$ was freeze-dried from 99.8% D_2O several times to exchange coordinating H_2O. Final concentrated stock solutions were freshly made up using D_2O.

Proton NMR spectra were recorded on the Bruker WH-300 spectrometer of the Oxford Enzyme Group. The Fourier transform mode and quadrative phase detection were utilised. Free induction decays (FIDS) were accumulated in 8K memory and Fourier transformed in 16K memory after zero-filling; 800–1600 transients were collected per spectrum. Suppression of the solvent HDO peak was achieved by presaturation with gated irradiation. Line widths were measured from FIDS unmanipulated for resolution or sensitivity enhancement. Chemical shifts are quoted in parts per million (ppm) downfield from DSS (sodium 2,2-dimethyl-2-silapentane-5-sulphonate). All spectra were recorded at 25°C.

3. RESULTS AND DISCUSSION

Aromatic and ring current shifted methyl group (RCSM) regions of the ^1H spectra of reduced and oxidised plastocyanin are shown in fig.1(i) and (ii). All spectra shown here were obtained with 10 mM cacodylate, pH* 6.0, as the sole buffer-electrolyte. Corresponding spectra for a 1 : 1 mixture (fig.1[iii]) and a simulated addition of reduced and oxidised forms (fig.1[iv]) are also shown. The close similarity between experimental and simulated 1 : 1 spectra

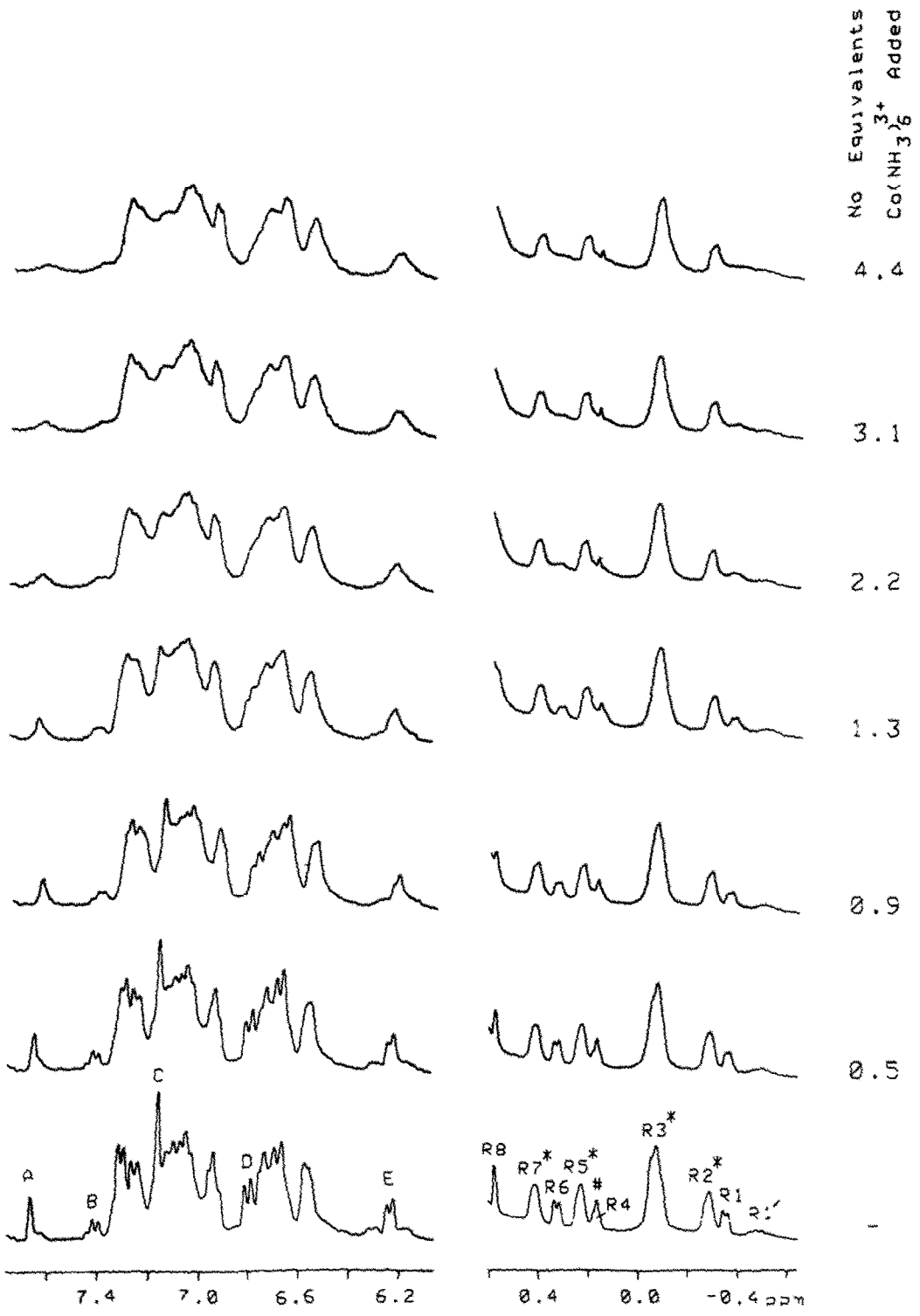


Fig.2. The effect of $Co(NH_3)_6^{3+}$ on partially oxidised plastocyanin. Figure shows the aromatic and ring current shifted methyl group (vertical scale × 1/2) regions of the resolution enhanced 300 MHz ^{1} H-NMR spectrum of 50% oxidised spinach plastocyanin in 10 mM cacodylate buffer, pH* 6.0, at 25°C. The number of equivalents $Co(NH_3)_6^{3+}$ to total plastocyanin for each spectrum is indicated. An impurity peak is denoted by #.

shows that electron exchange between oxidised and reduced forms of the protein under conditions of low ionic strength is slow on the NMR timescale, as previously reported [21]. With reference to fig.1(iii), peaks A, B, C, D and E in the aromatic region and peaks R1, R6 and R8 in the RCSM region are well-resolved resonances attributable to the diamagnetic reduced form of the protein (cf. spectrum [i]). Peak R4 is also specific to the Cu(I) form but here it is obscured by an impurity peak (denoted by #) at the same chemical shift. Peak R1′ is the only well resolved resonance attributable only to the oxidised form (cf. spectrum [ii]). Peaks R2*, R3*, R5* and R7* are superpositions of the resonances for both oxidised and reduced forms which have almost identical chemical shifts in either case, e.g. R2* = (R2 + R2′)/2.

Upon addition of $Co(NH_3)_6^{3+}$ to the 1:1 reduced/oxidised plastocyanin solution in 10 mM cacodylate, differential broadening of peaks occurs in these regions of the spectrum, as shown in fig.2. Some peaks are broadened more effectively than others. It is important at this stage to note that no broadening was apparent upon addition of $Co(NH_3)_6^{3+}$ to solutions of the fully reduced protein. Thus broadening is neither attributable to induced dimerisation, or to any relaxation process directly related to binding of the diamagnetic species $Co(NH_3)_6^{3+}$. Ring current shifted resonances R4, R6 and R8, and peaks A, B, C and D in the aromatic region are broadened almost beyond detection upon addition of 4 equivalents of $Co(NH_3)_6^{3+}$. Peak R1 is also broadened significantly but, more importantly, also shifts to merge with peak R1′ in a broad coalescence. It is also possible to detect the emergence of some doublet character in signals R2* and R5*, indicating some exchange narrowing of signals R2 and R2′, R5 and R5′.

Of these signals of interest, certain resonances have been assigned to specific amino acid residues. That of R8 is due to the CH_3 group of Met 92 [26] while A and C in the aromatic region arise from protons C-2 and C-4 of His 37 [27]. Both of these amino acids are involved in coordination of Cu, thus they are observed only in the reduced form.

We attribute these differential broadening effects to a profound increase in the rate of electron self-exchange (eqn 1) between oxidised and reduced forms of the protein,

$$Pc(I) + {}^*Pc(II) \longrightarrow Pc(II) + {}^*Pc(I) \quad k_{ex} \qquad (1)$$

where k_{ex} is the electron self-exchange rate constant as defined in eqn 2.

$$\text{Rate} = k_{ex}\,[Pc(I)]\,[Pc(II)] \qquad (2)$$

For resonances that are broad in the oxidised spectrum, line broadening of the reduced-form signals due to electron exchange is related to the exchange relaxation time by eqn 3.

$$\tau^{-1} = \pi \Delta\nu_{1/2} \qquad (3)$$

where $\Delta\nu_{1/2}$ is the exchange-induced line broadening [28]. Since $\tau^{-1} = k_{obs}$ = Rate/[Pc(I)], we can readily determine k_{ex}. In our case [Pc(I)] = [Pc(II)].

Line broadening, as measured using resonance A in the aromatic region, is also induced by the addition of Mg^{2+} ($MgCl_2$) or, to a much less extent, by raising the formal ionic strength (>0.1 M) using KCl. Experiments carried out using $Pt(NH_3)_6^{4+}$ showed this to be a particularly effective catalyst for electron exchange. Investigations in this case, however, were severely limited by the slow appearance of some turbidity and precipitation of a white insoluble material. The dependence of line broadening upon the nature and concentration of added cations is shown in fig.3. Typical rate constants (k_{ex}) obtainable are: 4×10^3 $M^{-1} \cdot s^{-1}$ (0.1 M KCl); 2.7×10^4 $M^{-1} \cdot s^{-1}$ (20 mM $MgCl_2$); 7×10^4 $M^{-1} \cdot s^{-1}$ (8 mM $Co(NH_3)_6Cl_3$). In the latter case, determination of higher rate constants using the line broadening of resonance A was not feasi-

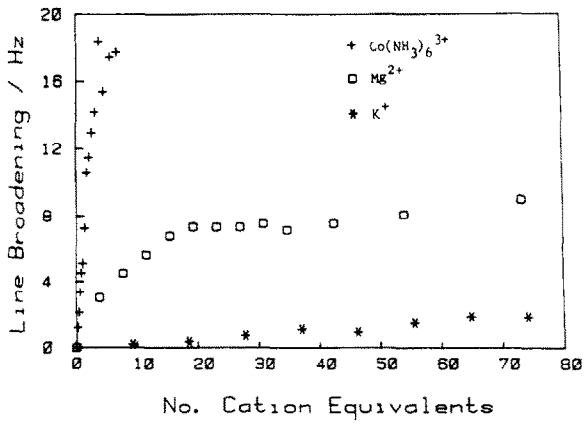
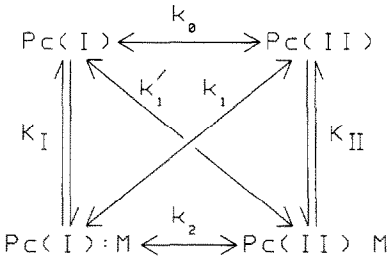


Fig.3. Line broadening of resonance A (His 37) induced by the addition of cations of differing charges to a 1:1 mixture of Pc(I) and Pc(II). Total [Pc] = 1.6 mM, 10 mM cacodylate buffer, pH* 6.0, 25°C.

ble. The value quoted should therefore at this stage be regarded as providing a conservative estimate of the degree of rate enhancement that may be obtained through catalysis by multivalent cations. In the case of Mg^{2+}-catalysed exchange, the rate constant appears to be a limiting value.

In these experiments no attempt was made to standardise formal ionic strength. Consequently each addition represents an increase in this variable. The greatly amplified effect of multivalent cations does, however, indicate that catalysis is not proceeding generally through electrostatic screening in accordance with Debye-Hückel theory (although this may certainly account for the rate increases observed upon large additions of KCl). A more likely rationale is the formation of cation-protein 'ion pairs' which can engage very much more effectively in protein-protein association prior to electron transfer. In order to make a simple test for this we conducted an experiment in which a 1:1 mixture of Pc(I) and Pc(II) (each 0.8 mM) in 10 mM cacodylate was treated with $Co(NH_3)_6^{3+}$ (2 mM) to produce an intermediate (8 Hz) line broadening. When the ionic strength was increased to 0.1 M by addition of a saturated solution of KCl, the exchange broadening decreased to 4 Hz. This observation is fully consistent with participation, in the electron-exchange process, of electrostatically bound cation-protein complexes which dissociate at high ionic strength. In accordance with the implications of NMR [11,12] and kinetic [13] studies we expect that $Co(NH_3)_6^{3+}$ will associate at or near the conserved negatively-charged domain incorporating residues 42–45. At pH 7.5, I = 0.10 M (NaCl), 25°C, the reported [13] association constant for $Co(NH_3)_6^{3+} \cdot Pc(I)$ complexation is 470 M^{-1}. Within our lower ionic strength range, we expect this to represent a minimum value. Although Mg^{2+} binding has not previously been reported, some weak association, with the possibility for inner-sphere complexation, does seem likely.

For the electron exchange processes which may be operative we propose the general scheme as outlined below (scheme 1). Here k_o represents electron exchange between free protein molecules. This is very small at low ionic strength [21], but becomes appreciable at 0.1 M KCl. The rate constants k_1 and k_1' represent electron exchange between free and cation(M)-bound protein molecules, for which a single cation could in effect 'bridge' two plastocyanin cation-binding domains within an electron-transfer encounter complex. Finally, k_2 represents electron exchange between two cation-protein complexes for which local charge compensation has occurred and where specific 'double bridging' involving additional cation-protein interactions becomes a possibility. In a more general sense, protein-protein association through hydrophobic interactions is expected to become more effective once electrostatic repulsion has been quenched and some relaxation of contact domain solvation has occurred. In preliminary quantitative studies [29] to determine the mechanism of these exchange processes we have so far been unable to detect the decrease in exchange at high cation concentrations that would be expected for the situation where k_1, $k_1' > k_2$.



Scheme 1.

In conclusion, our results demonstrate for a fundamental process, that of electron self-exchange between protein molecules, the extent to which the binding of small multivalent ions may promote protein-protein encounter complex formation and thus modulate electron-transfer. For physiologically abundant divalent cations such as Mg^{2+}, this modulatory activity is clearly of some relevance.

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