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NMR and kinetic characterization of the interaction between French bean plastocyanin and horse cytochrome *c*

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French bean plastocyanin is shown by stopped-flow kinetics to oxidize horse cytochrome *c* with *k* (298 K, $I = 0.10$ M) = $5.1 \cdot 10^6$ $M^{-1} \cdot s^{-1}$. The activation parameters demonstrate a satisfactory isokinetic correlation with those previously reported for plastocyanin-cytochrome *f* reactions. NMR line broadening and shifts of the hyperfine shifted resonances of cytochrome *c*(III) reveal that strong 1:1 complexes are formed with plastocyanin. The negative patch of plastocyanin and the heme edge region of cytochrome *c* are shown to be the interacting sites by the hyperfine shift perturbations and competitive binding experiments with Gd^{3+}, which associates selectively with the negative patch of plastocyanin. Complexation of plastocyanin and cytochrome *c* causes a small change in the heme electronic structure, but there is no NMR or optical evidence for significant conformational changes at either metal center. The rate of the reverse electron-transfer reaction within the plastocyanin-cytochrome *c* complex has been directly measured by NMR line broadening (k_{rev} (298 K) = 87 s^{-1}). A rate for the forward intracomplex electron-transfer reaction (k_f (298 K) = $4.8 \cdot 10^3$ s^{-1}) has been calculated from k_{rev} and the optically measured equilibrium constant.

Introduction

In recent years, there has been considerable effort directed towards understanding the mechanism, specificity and control of biological electron-transfer reactions. Of particular interest is the possibility that proteins functioning in linear electron-transport chains may possess two distinct reaction sites. Unfortunately, the large size and membrane-associated nature of most electron-transfer components makes detailed biophysical studies of their interactions quite difficult. Much work has consequently focused on the small, soluble proteins.

Plastocyanin is a small (M_r 10 600) blue copper protein from the photosynthetic electron-transport chain. Solution of the X-ray structure of poplar plastocyanin originally led to the suggestion of two surface regions as possible electron-transfer sites [1,2]. One site was at the 'northern' [3] end of the molecule, where one edge of the copper ligand His 87 lies exposed to solvent within a region of conserved hydrophobic residues. Electron transfer could proceed by a normal outer sphere mechanism [4] at this site. The second putative site was the 'negative patch', a cluster of conserved acidic residues near the solvent-exposed sidechain of Tyr 83, some 1.2 nm from the copper. It was assumed that a quantum-mechanical tunnelling process would be operative in reactions involving this site

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Abbreviations: Mops, 4-morpholinepropanesulphonic acid; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

[1]. Subsequent kinetic measurements [5–7], NMR binding studies [8,9], competitive inhibition experiments (Ref. 10 and King, G. and Wright, P.E., unpublished data) and chromium labelling experiments [11] have provided a consistent body of evidence that the negative patch is a functional binding and electron-transfer site for positively charged inorganic complexes.

Several groups have reported kinetic studies of the reaction between plastocyanin and its physiological partner, cytochrome *f* [12–15]. Electrostatic interactions were reported to be important [13]. Competitive inhibition experiments [15] suggest that the electron-transfer interaction involves the negative patch of plastocyanin and a positively-charged region of the cytochrome. It would be of interest to look for conformational/dynamic changes that may occur on interaction of these proteins using ^{1}H-NMR techniques. Unfortunately, such NMR investigations are difficult due to the relatively high molecular weight of cytochrome *f*. One approach to this problem is to use a smaller protein to model interactions at the negative patch of plastocyanin.

Cytochrome *c* (M_r 12 500) is considered to be a suitable candidate as it has the potential to form complementary charge interactions with plastocyanin. In addition, it shares the *c*-type active site with cytochrome *f* and is very well characterized. The plastocyanin-cytochrome *c* system may also be useful as a general model of protein-protein electron transfer interactions.

The first kinetic studies of the oxidation of cytochrome *c* by parsley plastocyanin [12] demonstrated that the reaction is rapid ($k = 10^6$ $M^{-1} \cdot s^{-1}$). Further kinetic experiments with blocking reagents [10] and lysine modified cytochrome *c* derivatives [16,17] suggested that the negative patch of plastocyanin and the heme edge region of cytochrome *c* are the interacting sites. Cross-linking of the proteins with a water-soluble carbodiimide [18] leads to the same conclusions.

NMR studies of the solution conformation and interactions of French bean plastocyanin are being pursued in this laboratory. In this paper, we report an initial study of its interaction with horse cytochrome *c* using stopped-flow kinetics in conjunction with NMR. Combination of these techniques allows us to characterize several rates within the overall reaction scheme that are not accessible with the stopped-flow technique alone. This study forms the basis for future investigations of the interaction by more extensive NMR studies and computer graphics simulation.

Experimental procedures

Proteins

Plastocyanin was prepared from French bean leaves by a modification of the method of Ramshaw et al. [19]. Protein with an A_{278}/A_{597} ratio of 1.1–1.2 was used at all times. Concentrations were determined using $\varepsilon_{597} = 4500$ $M^{-1} \cdot cm^{-1}$ for the oxidized form [20].

Horse heart cytochrome *c* (Sigma type VI) was purified before use by ion exchange chromatography on CM-cellulose [21]. Concentrations were determined using ε_{416} and ε_{550} equal $129.1 \cdot 10^3$ and $29.5 \cdot 10^3$ $M^{-1} \cdot cm^{-1}$, respectively, for the reduced protein and ε_{410} and ε_{530} equal $106.1 \cdot 10^3$ and $11.0 \cdot 10^3$ $M^{-1} \cdot cm^{-1}$, respectively, for the oxidized form [22].

Reduced forms of each protein were prepared by addition of a minimum quantity of solid sodium dithionite, which was subsequently removed by desalting on a Sephadex G-15 column or by ultrafiltration in an Amicon cell with a Diaflo YM-5 membrane.

NMR spectra

NMR spectra were recorded with a Bruker WM-400 spectrometer over a spectral width of 30 kHz at 3.7 Hz/point resolution. Chemical shifts were referenced to internal dioxan at 3.750 ppm. Temperature calibration was performed with an ethylene glycol sample [23]. Quoted pH values are uncorrected meter readings.

Several NMR titrations were performed by adding aliquots of a solution of plastocyanin (2–4 mM) plus cytochrome *c* (0.75–1 mM) to a solution of cytochrome *c* (0.75–1 mM) in an NMR tube and acquiring spectra after each addition.

Proton relaxation enhancement experiments [24] were performed using a Bruker HX 90 spectrometer. The longitudinal relaxation time (T_1) of the solvent resonance from a 90% H_2O/10% ^2H_2O mixture at pH 6.0, 298 K was monitored on addition of aliquots of cytochrome *c* to a solution

containing plastocyanin (100 μM) and the lanthanide Gd^{3+} (100 μM).

Stopped-flow kinetics

Stopped-flow experiments were performed with a Beckman DU-2 spectrophotometer fitted with an Aminco-Morrow stopped flow attachment. The reaction was monitored at 417 nm, where $\Delta\varepsilon_{red\text{-}ox} = 4.0 \cdot 10^4\ M^{-1} \cdot cm^{-1}$ for cytochrome *c* [17]. Traces were photographed from a Tektronix D-15 storage oscilloscope. Between two and seven traces were analysed per experimental point. Plots of $\log(A_\infty - A_t)$ vs. *t* were linear over at least three half-lives. Observation cell temperature was regulated to ± 0.2 K with a Colora cryo-thermostat. All experiments were conducted with both proteins in 10 mM Tricine buffer (pH 7.5) (90 mM NaCl).

Electronic spectra

Spectra covering the range from the near infrared to the ultraviolet region were recorded with a Cary 17I spectrophotometer interfaced to a Commodore PET computer. Baselines were digitially subtracted from the spectrum of each sample. Spectral simulation for a mixture of proteins was achieved by computer addition of various proportions of spectra from the individual redox forms of each protein.

Results

Oxidation of Fe(II)cytochrome c by Cu(II) plastocyanin

Fig. 1A summarizes data for the temperature dependence of the rate of oxidation of Fe(II)cytochrome *c* by Cu(II)plastocyanin. A fit of the Eyring plot yields k (298 K) $= 5.1 \pm 0.3 \cdot 10^6\ M^{-1} \cdot s^{-1}$, $\Delta H^\dagger = 32 \pm 2\ kJ \cdot mol^{-1}$ and $\Delta S^\dagger = -10 \pm 6\ J \cdot K^{-1} \cdot mol^{-1}$ at $I = 0.10$ M. The observed rate is fast, given that the partners are nonphysiological, and is some 4-times that reported previously for the reaction involving parsley plastocyanin [10,12,17]. This difference in reactivity between species may be caused by the French bean protein forming a stronger precursor complex with the cytochrome. The rate is, however, approximately one order of magnitude slower than that of the plastocyanin-cytochrome *f* reaction.

Comparison of the activation parameters ob-

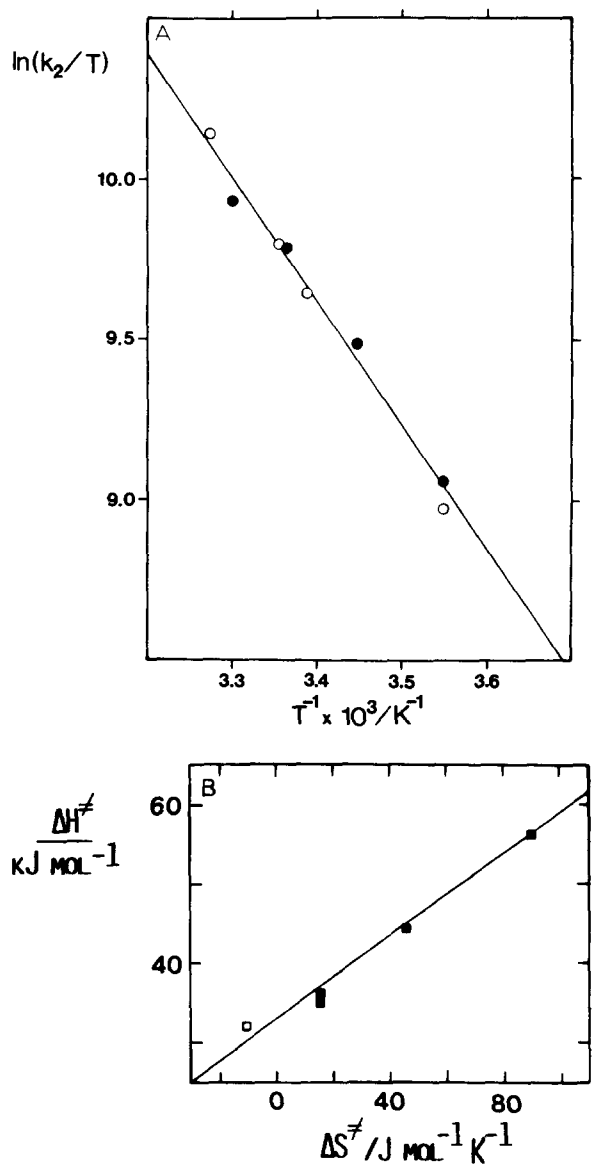

Fig. 1. (A) Eyring plot for the reduction of Cu(II)plastocyanin by Fe(II) cytochrome *c*. Data from two separate experiments are included. (B) Isokinetic correlation of the activation parameters (□) with those previously reported for reactions of plastocyanins with cytochromes *f* (■) [12–14].

tained here with those previously reported [12–14] for plastocyanin-cytochrome *f* reactions reveals a satisfactory isokinetic correlation [25] (Fig. 1B). A linear fit of the present data with those of the plastocyanin-cytochrome *f* reactions gives a value for the compensation temperature (slope) of 251 ±

20 K and a correlation coefficient c of 0.981, while the physiological reactions alone yield a value of 277 ± 8 K, with $c = 0.998$. Isokinetic correlations have been frequently observed for electron-transfer reactions between metalloproteins and inorganic reagents [25–28]. The compensation temperatures found in the present work are in the range (250–320 K) reported for a number of protein reactions [29]. The observation of an isokinetic correlation for plastocyanin-cytochrome reactions is of interest. It has been suggested that such a relationship is indicative of a common reaction mechanism [25]. However, alternative interpretations are possible [29]. The present correlation is based on activation parameters for a single plastocyanin-cytochrome c reaction. Measurement of activation parameters for reactions of plastocyanin from other species is required before it can be assumed to be generally valid.

Effect of Cu(II)plastocyanin on the hyperfine shifted resonances of Fe(III)cytochrome c

The hyperfine shifted resonances of Fe(III)cytochrome c are perturbed on addition of Cu(II)plastocyanin in unbuffered ^2H_2O at pH 7.5, 298 K (Fig. 2). These resonances have been previously assigned to protons of the heme group and the axial ligands [30–33]. Titration of Cu(II) plastocyanin results in a general broadening of all resonances (Fig. 2a–c), as expected for a decrease in rotational correlation time caused by complexation. The behaviour of the heme methyl-8 resonance at 35.33 ppm (M8) is typical. Its linewidth increases linearly from 30 to 45 Hz near [plastocyanin(II)]/cytochrome c(III)] = 1 and then remains constant (Fig. 3a). If diamagnetic dipolar relaxation in the M_r 12 500 protein contributes approx. 10 Hz to the linewidth of the heme methyl-8 resonance (determined from its linewidth in the spectrum of Fe(II)cytochrome c), then paramagnetic relaxation is responsible for approx. 20 Hz. Simplistically, complexation with plastocyanin may produce a species of M_r 23 100 which tumbles 1.8-times more slowly, with an M8 resonance linewidth of $1.8 \times 10 + 20 = 38$ Hz. However, such an analysis is only applicable for spherical proteins so that the observed linewidth of 45 Hz is reasonable for what must be a markedly non-spherical complex whose motions are char-

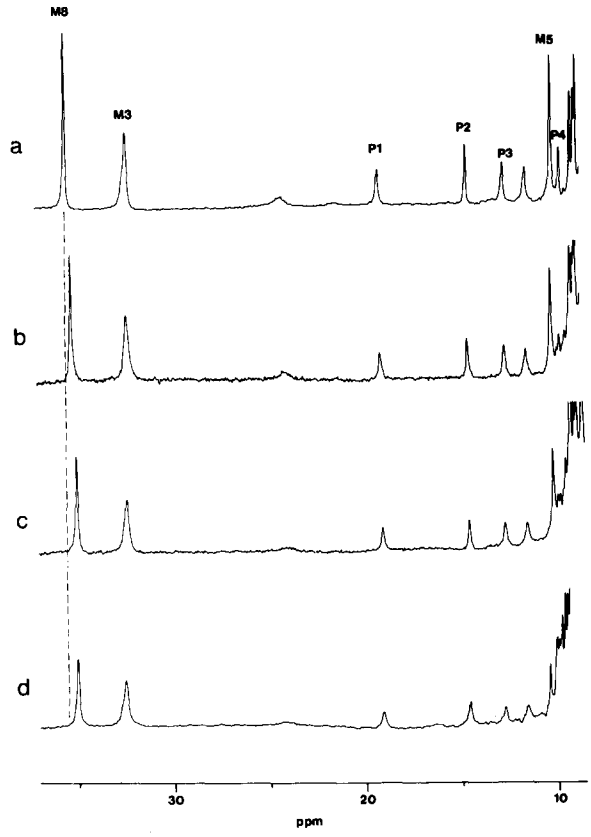

Fig. 2. Low-field region of the NMR spectra of (a) 1.0 mM Fe(III) cytochrome c plus (b) 48% Cu(II)plastocyanin (c) 101% Cu(II) plastocyanin (d) 109% Cu(I)plastocyanin. Spectra were recorded at 298 K in unbuffered ^2H_2O (pH 7.5). Resonance identification scheme from Ref. 29. P_1–P_4, peak designations for propionates, as given in Ref. 29; M_5, heme methyl-5 resonance.

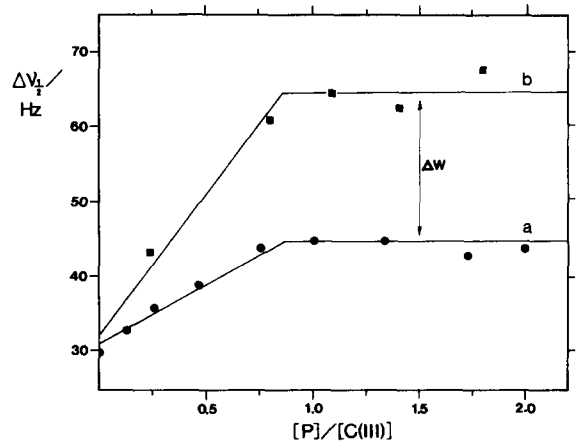

Fig. 3. Linewidth data for the heme methyl-8 resonance of Fe(III) cytochrome c on titration with (a) Cu(II)plastocyanin (b) Cu(I) plastocyanin. The linewidth difference ΔW is due to exchange broadening caused by reverse intracomplex electron transfer. P, plastocyanin, C, cytochrome c.

acterized by several different correlation times. It is notable that the Cu(II) atom of plastocyanin does not cause preferential broadening of any hyperfine shifted resonances. This observation places an approximate lower limit of 1.2 nm on the Cu-heme proton distances.

In addition to general broadening, several cytochrome *c* resonances are observed to shift specifically on addition of plastocyanin (Figs. 2 and 4). The resonances of heme methyls -8, -3, and -5 at 35.33 (M8), 32.13 (M3) and 10.08 (M5) ppm are shifted by −0.44, +0.15 and −0.14 ppm between free and complexed forms. Resonances from the ε-methyl group of Met 80 at −24.16 ppm (S-CH_3)

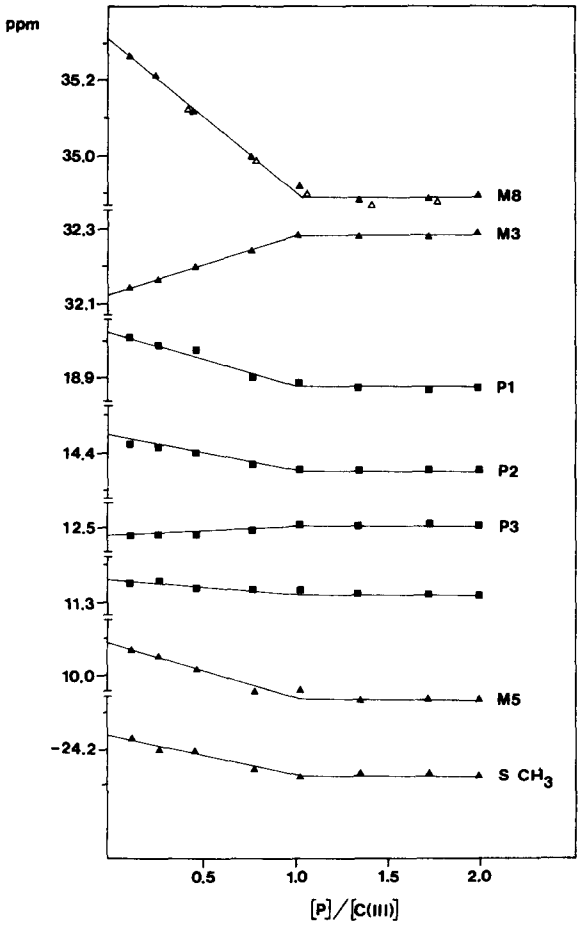

Fig. 4. Chemical shift data for the hyperfine shifted resonances of Fe(III)cytochrome *c* on titration with Cu(II)plastocyanin (▲,■) and Cu(I)plastocyanin (△). Resonance labelling corresponds to Fig. 2. P, plastocyanin; C, cytochrome *c*.

and those assigned to βCH groups at 19.03 (P 1) and 14.45 (P 2) ppm are similarly shifted by −0.11, −0.16 and −0.10 ppm, respectively (P_1–P_4 are peak designations for propionates, as given in Ref. 29). Other resolved signals shift by less than 0.04 ppm. It is notable that only the lower field resonance of the P 1, P 3 and P 2, P 4 pairs, which have been tentatively assigned to the βCH_2 groups of the two heme propionates [30,31,33], shifts significantly.

Similar behaviour is observed when the titration is performed at 313 K (10 mM NaCl). Shift and linewidth changes again reach a plateau near [plastocyanin(II)]/[cytochrome *c*(III)] = 1. The M8 resonance linewidth increases from 30 Hz to a final value of 44 Hz in the plateau region.

The linear changes in chemical shifts and linewidths that saturate near [plastocyanin(II)]/[cytochrome(III)] = 1 are indicative of 1 : 1 complexation with a large (more than 10^4 M^{-1}) association constant. This is particularly significant in view of the fact that both proteins carry high opposite charges, and may have been able to form complexes of higher stoichiometry. The observation of steady shifting of resonances shows that exchange between the chemical shifts of the free (δ_f) and complexed (δ_c) forms is fast, with the complex dissociation rate $k_{off} \gg 2\pi(\delta_f - \delta_c)_{max} \gg 1000\ s^{-1}$.

The shift behaviour of the heme methyl resonances shown in Fig. 4 is identical to that reported for the interaction of Fe(III)cytochrome *c* with cytochrome *c* peroxidase [34] and cytochrome b_5 [35,36]. Both of these proteins are considered to bind at the heme edge region of cytochrome *c*. It is apparent that plastocyanin also interacts at this site.

The binding site on plastocyanin was identified by proton relaxation enhancement competition experiments. High resolution NMR studies show that the lanthanide Gd^{3+} and other positively charged inorganic reagents bind quite selectively to the negative patch of plastocyanin. The association constant for binding of Gd^{3+} is approx. $8.3 \cdot 10^3$ M^{-1} in unbuffered solution at pH 6.0. Details of these experiments will be presented elsewhere. The paramagnetic relaxation enhancement of the solvent water proton resonance is dramatically reduced when Fe(III)cytochrome *c* is

titrated into a solution containing Cu(II)plastocyanin and Gd^{3+} in a 1:1 ratio. The observed enhancement factor ε_1^* [24] is reduced from an initial value of 3.6 to a value of 2.3 when all components are present at equal concentrations. Fe(III)cytochrome *c* thus efficiently displaces Gd^{3+} from the negative patch. When the [cytochrome *c*(III)]/[Gd^{3+}] ratio reaches 4.0, ε_1^* approaches unity, which indicates that Gd^{3+} is almost wholly uncomplexed. Analysis of the data yields a value for the association constant for the plastocyanin-cytochrome *c* complex of approx. $1.5 \cdot 10^4$ M^{-1} in unbuffered 90% H_2O/10% ^2H_2O (pH 6.0).

The binding sites identified for both proteins in the present work are in agreement with those determined using other methods [10,16–18].

Effect of Titration with Cu(I)plastocyanin

Addition of Cu(I)plastocyanin to Fe(III)cytochrome *c* results in a pattern of resonance shifts indistinguishable from that caused by Cu(II) plastocyanin under the same conditions (Fig. 2d). The behaviour of the heme methyl-8 resonance is shown as an example in Fig. 4. General broadening of resonances also occurs, but the effect is considerably greater than that observed on titration with Cu(II)plastocyanin. The broadening ΔW which is in excess over that observed for complexation with Cu(II)plastocyanin (Fig. 3) is ascribed to exchange averaging from the thermodynamically unfavorable (reverse) electron-transfer reaction plastocyanin(I) · cytochrome *c*(III) → plastocyanin(II) · cytochrome *c*(II). The fact that the excess broadening is independent of plastocyanin concentration at [plastocyanin(I)]/[cytochrome *c*(III)] > 1 indicates that reverse electron transfer occurs only within the complex and not by extra collisional events, for which a normal first order concentration dependence would be expected. For a system in slow exchange the relationship between the exchange rate and the broadening caused by the exchange process is given by $\Delta W = k_{rev}/\pi$ [37]. The heme methyl-8 resonance of Fe(II)cytochrome *c* occurs at 2.19 ppm [32], producing a shift difference between the oxidized and reduced forms of 13.2 kHz at a field of 400 MHz. Slow exchange conditions must apply for the M8 signal as the chemical shift difference is 150-times greater than the exchange rate of approx. 90 s^{-1} apparent from the value of ΔW in Fig. 3. The linewidth of the heme methyl-8 signal is thus affected by the complexation reaction (fast exchange) and the reverse electron-transfer reaction (slow exchange).

The excess broadening caused by Cu(I) plastocyanin increases markedly with temperature, and is due to normal thermal activation of the reverse intracomplex electron transfer process. The increased broadening at observed high temperatures cannot be due to the spin state equilibrium described previously by Ångström et al. [33], which does not occur at pH 7.5, the pH at which the present experiments were performed. Nor can it be due to the alkaline transition, which does not cause line broadening with increasing temperature [33].

Fig. 5 shows data for the temperature dependence of the reverse electron-transfer rate calculated from ΔW for a solution with a [plastocyanin(I)]/[cytochrome *c*(III)] ratio of 1.5 in 10 mM Mops (pH 7.5). This [plastocyanin(I)]/[cytochrome *c*(III)] ratio lies well within the zero order region of Fig. 3b where only intracomplex electron transfer contributes to ΔW. No attempt has been made to allow for the line sharpening that occurs due to a decrease in solvent viscosity with increasing temperature, which is only of the order of the digital resolution over the temperature range studied. The uncertainty in linewidth measurement becomes considerably larger as the linewidth increases above approx. 50 Hz. A fit of the Eyring plot yields k_{rev} (298 K) = 87 ± 3 s^{-1}, $\Delta H^\dagger = 30.9 \pm 0.9$ kJ · mol^{-1} and $\Delta S^\dagger = -104 \pm 3$ J · K^{-1} · mol^{-1}.

The temperature dependence of the hyperfine shifted resonances in the Cu(I)plastocyanin · Fe(III)cytochrome *c* complex is very similar to that of the free cytochrome [33]. Fig. 6 shows the data for selected resonances on a scale similar to that of Ref. 33.

Electronic spectrum of the complex

Aliquots of Cu(II)plastocyanin and Fe(II)cytochrome *c* solutions in ^2H_2O (pH 7.5) were mixed in a 1:1 ratio and allowed to equilibrate before recording the electronic spectrum. A trial and error summation of spectra from the four contribut-

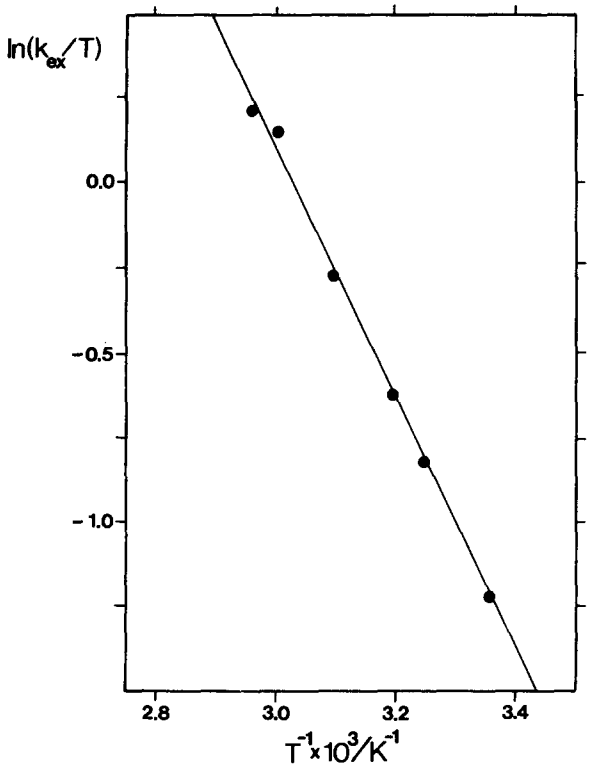

Fig. 5. Eyring plot for the reverse electron-transfer reaction within the Cu(I)plastocyanin-Fe(III)cytochrome *c* complex. Rates were calculated from the excess broadening ΔW observed for the heme methyl-8 resonance in the solution with a [plastocyanin]/[cytochrome *c*(III)] ratio of 1.5 in 10 mM Mops (pH 7.5).

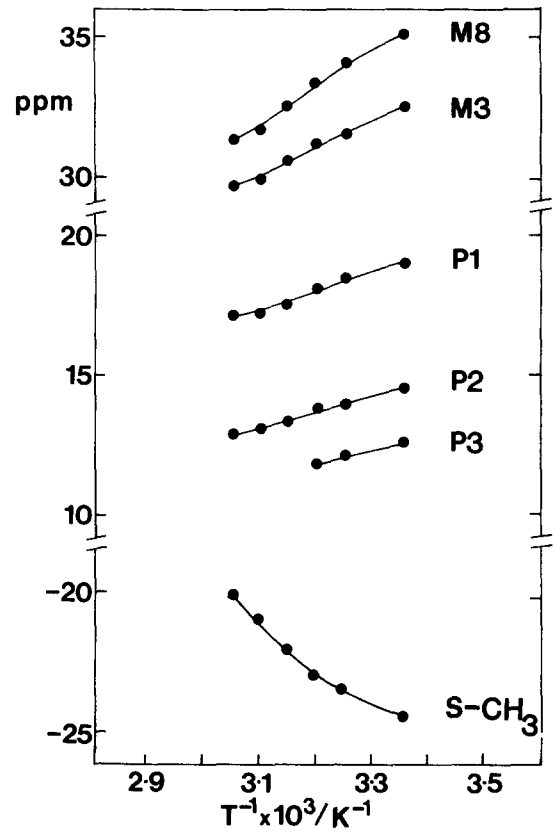

Fig. 6. Temperature dependence of chemical shifts in the Cu(I) plastocyanin-Fe(III)cytochrome *c* complex. Conditions as for Fig. 5. P_1–P_3, peak designations for propionates, as given in Ref. 29; M_3, M_8, heme methyl-3 and methyl-8 resonances.

ing protein components produced a final spectrum in good agreement with the observed spectrum of the equilibrium mixture (Fig. 7). No significant perturbations could be observed for any absorptions, including the Soret bands of the cytochrome. No intervalence charge transfer bands with molar absorptivity ε greater than the present experimental detection limit of approx. 20 $M^{-1} \cdot cm^{-1}$ could be located in the range 500–1600 nm. This finding is not unexpected in the light of the reported ε of 0.35 $M^{-1} \cdot cm^{-1}$ for the intervalence charge-transfer transition in the cytochrome *c*-cytochrome *c* peroxidase complex [38]. Static spectrophotometry is insufficiently sensitive to detect these very weak transitions in the presence of much stronger chromophoric absorptions [38].

The simulated spectrum of the equilibrium mixture which is in closest agreement with the experi-

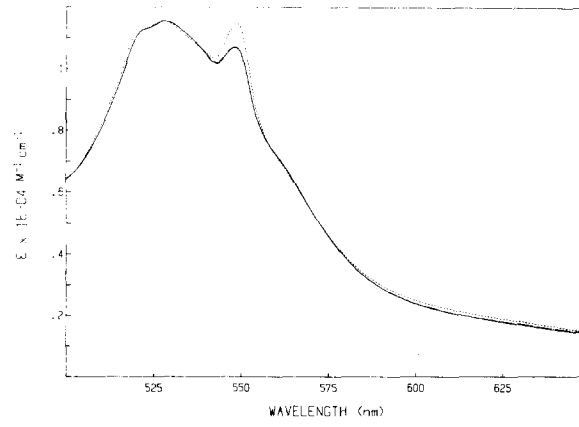

Fig. 7. Experimental absorption spectrum of an equilibrium mixture of plastocyanin and cytochrome *c* (———) is almost identical to a simulated spectrum with $K_{eq} = 55$, $\Delta E_M = 103$ mV (·–·–·) (these lines almost merge in the figure). The fit for $K_{eq} = 29.8$, $\Delta E_M = 87$ mV (- - - - -) is poor.

mental spectrum (Fig. 7) gives an equilibrium constant K_{eq}^{obs} of 55 ± 5 M^{-1}. This corresponds to a redox potential difference ΔE_M of 103 ± 5 mV. Since the complex is strong, K_{eq}^{obs} is a measure of the redox potential difference within the complex, rather than ΔE_M between the free reactants. It is significant that a K_{eq} of 29.8 (corresponding to $\Delta E_M = 87$ mV, a value obtained from the published E_M of plastocyanin [39]) fits the observed spectrum quite poorly (Fig. 7).

Discussion

Kinetic parameters

The overall reaction can be described by the equation:

$$\text{plastocyanin(II)} + \text{cytochrome } c\text{(II)}$$

$$\underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{plastocyanin(II)}\cdot\text{cytochrome } c\text{(II)}$$

$$\underset{k_{rev}}{\overset{k_f}{\rightleftharpoons}} \text{plastocyanin(I)}\cdot\text{cytochrome } c\text{(III)}$$

$$\underset{k_2}{\overset{k_{-2}}{\rightleftharpoons}} \text{plastocyanin(I)} + \text{cytochrome } c\text{(III)}$$

where $K_A = k_1/k_{-1}$, $K_D = k_2/k_{-2}$ and $K_{eq}^{obs} = k_f/k_{rev} = K_{eq} = k_f K_A/k_{rev} K_D$. The bimolecular rate constant $k = k_f K_A$. The rate parameters obtained in the present experiments and those calculated from them are summarized in Table I. The forward intracomplex electron transfer rate k_f (calculated from K_{eq}^{obs} and k_{rev}) is found to be $4.8 \pm 0.6 \cdot 10^3$ s^{-1}, which is comparable to the rate of $2.1 \cdot 10^4$ s^{-1} reported for the $Fe(CN)_6^{3-}$-Fe(II)cytochrome *c* complex [40]. It is some 200-times faster than the forward rate in the $Co(1,10\text{-phen})_3^{3+}$-Cu(I)plastocyanin complex measured for both the parsley [5–7] and French bean (Cotton, B., King, G., and Wright, P.E., unpublished data) proteins, which also involves the negative patch of plastocyanin [7–9]. Preliminary computer graphics docking simulations of the interaction of plastocyanin with cytochrome *c* predict a Cu–Fe distance of approx. 1.8 nm, with a more reasonable thiolate ligand to heme edge distance of approx. 1.2 nm (Wright, P.E., McClarin, J. and Langridge, R., unpublished data; Freeman, H.C., Getzoff, E.D. and Tainer, J.A., unpublished data). The electron-transfer rate over this distance is remarkably fast in a case where the reactants are nonphysiological, and have not had the chance to evolve mutually optimal interaction geometries.

The association constant K_A is calculated (from the bimolecular rate constant, k and k_f) to be $1.1 \pm 0.3 \cdot 10^3$ M^{-1} in 10 mM tricine (pH 7.5, 90 mM NaCl). Proton relaxation enhancement results indicate that K_A increases to approx. 10^4 M^{-1} in unbuffered solution. K_A and K_D are expected to be approximately equal, since the groups that participate in the complexation reaction are quite distant from the redox sites. The negative free-energy change associated with K_A and K_D is indicative of the complementary charge interactions responsible for complexation. Small conformational and dynamic differences between redox states [41] could of course contribute to small differences between K_A and K_D which could not be detected in the present experiments.

TABLE I

RATE PARAMETERS FOR THE REACTION OF FRENCH BEAN PLASTOCYANIN WITH HORSE CYTOCHROME *c*

Rate parameter	Value	ΔG_0 (kJ·mol^{-1})	$\Delta G^{\dagger}$ (kJ·mol^{-1})
$K_{eq}^{obs} = k_f/k_{rev}$	55	−9.9	
k_f	$4.8 \cdot 10^3$ s^{-1} [a]		52.0 [a]
$k = K_A \cdot k_f$	$5.1 \cdot 10^6$ $M^{-1} \cdot s^{-1}$		35.0
$K_A \approx K_D$ [b]	$1.1 \cdot 10^3$ M^{-1} [a]	−17.0 [a]	
k_{rev}	87 s^{-1}		61.9
$K_D \cdot k_{rev}$	$9.6 \cdot 10^4$ $M^{-1} \cdot s^{-1}$ [a]		44.9 [a]

[a] Values calculated from experimental data.
[b] In 10 mM tricine (pH 7.5), $I = 0.10$ M (NaCl).

Effect of complexation on active site structure

The observed perturbation of the hyperfine shifted resonances of Fe(III)cytochrome *c* is clear evidence that complexation with plastocyanin causes a small change in the electronic structure of the heme. The −0.44 ppm shift of the heme methyl-8 resonance is almost identical to shifts reported on complexation with cytochrome b_5 [36] and cytochrome *c* peroxidase [34]. The shifts are consistent with a small change in the angle of the electronic *g*-tensor on complexation. They cannot be explained by simple electronic repulsion of the

heme electron cloud by the negative patch of plastocyanin. The change in heme electronic structure is sufficiently small to be unobservable in the optical spectrum.

There is no evidence that complexation with plastocyanin causes conformational changes at the active site of the cytochrome. The heme methyl-3 resonance of free Fe(III)cytochrome *c* is somewhat exchange-broadened (Ref. 42 and Fig. 2a) by a slow process that might influence the Fe-Met 80 bond [33]. Complexation does not appear to affect the exchange-broadening (Fig. 2). Also, the Met 80 methyl resonance (S-CH_3) shows an apparently identical deviation from Curie law behaviour in the complex (Fig. 6) to that reported for the free protein [33]. The implication is that conformational changes (if any) responsible for the deviation from Curie law behaviour are identical in free Fe(III)cytochrome *c* and within the complex.

More detailed NMR investigations which extend to resonances in the diamagnetic envelopes of all redox forms of the proteins confirm the suggestion that complexation causes only minimal structural changes at the active sites (King, G. and Wright, P.E., unpublished data).

Redox potentials within the complex

It may be expected that electrostatic complexation of plastocyanin with cytochrome *c* could cause changes in the redox potential of each metal centre. There is evidence that intraprotein electrostatics can play a role in the control of E_M in cytochromes *c* [43,44]. Simple electrostatic calculations [45] can be performed to gauge the influence of surface residues on the redox potential of plastocyanin. Allowing for a charge change of 0 to +1 and a physically reasonable dielectric constant of 50 [46], each of the negative patch residues 42, 43, 44, 59 and 61 is calculated to contribute an average of approx. $-1.5\ kJ \cdot mol^{-1}$ or a ΔE_M of -16 mV to the redox potential of the copper centre. Carbodiimide linking of two or more ethylenediamine molecules to plastocyanin carboxyl groups has been reported to cause an increase in E_M of at least 40 mV [47,48], even though optical [47] and NMR (Cotton, B., Walsh, M. and Wright, P.E., unpublished data) spectra suggest that structural changes at the copper site are insignificant.

The present experiments are equivocal on the effect of complexation on E_M for two reasons. Static spectrophotometry of a four-component mixture is not a very satisfactory measure of K_{eq} (and hence ΔE_M) as the concentrations of all species must be determined using relatively imprecise molar absorptivities. Secondly, reported E_M values for proteins can be considerably dependent on the experimental method and conditions. The originally reported E_M for French bean plastocyanin of 347 ± 1 mV was superseded by a value of 360 ± 2 mV [49]. The same authors reported an E_M for horse cytochrome *c*, which is at variance with the generally accepted value of 260 mV [49]. It is thus not possible to be sure of the E_M values that pertain under the present conditions.

A 25 mV decrease in E_M has been previously reported for cytochrome *c* chemically crosslinked to plastocyanin [18]. Whether this change is wholly electrostatic in origin is unclear. Spectroelectrochemical experiments conducted under constant conditions are necessary to determine directly the effect of the demonstrated electrostatic complexation on E_M values.

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References

1 Colman, P.M., Freeman, H.C., Guss, J.M., Murata, M., Norris, V.A., Ramshaw, J.A.M. and Venkatappa, M.P. (1978) Nature 272, 319–324
2 Freeman, H.C. (1981) in Coordination Chemistry, Vol. 21 (Laurent, J.L., ed.), pp. 29–51, Pergamon Press, Oxford
3 Guss, J.M. and Freeman, H.C. (1983) J. Mol. Biol. 169, 521–563
4 Sutin, N. (1973) in Inorganic Biochemistry (Eichhorn, G.L., ed.), pp. 611–653, Elsevier, Amsterdam
5 Segal, M.G. and Sykes, A.G. (1977) J. Chem. Soc. Chem. Commun. 764–765
6 Segal, M.G. and Sykes, A.G. (1978) J. Am. Chem. Soc. 100, 4585–4592
7 Lappin, A.G., Segal, M.G., Weatherburn, D.C. and Sykes, A.G. (1979) J. Am. Chem. Soc. 101, 2297–2301

8 Cookson, D.J., Hayes, M.T. and Wright, P.E. (1980) Biochim. Biophys. Acta 591, 162–176
9 Handford, P.M., Hill, H.A.O., Lee, W.-K., Henderson, R.A. and Sykes, A.G. (1980) J. Inorg. Biochem. 13, 83–88
10 Chapman, S.K., Davies, D.M., Watson, A.D. and Sykes, A.G. (1983) in ACS publication No. 211 (Chisholm, M.H., ed.), pp. 177–197, American Chemical Society, Washington
11 Farver, O. and Pecht, I. (1981) Proc. Natl. Acad. Sci. USA 78, 4190–4193
12 Wood, P.M. (1974) Biochim. Biophys. Acta 357, 370–379
13 Niwa, S., Hiroshi, I., Nikai, S. and Takabe, T. (1980) J. Biochem. (Tokyo) 88, 1177–1183
14 Tanaka, K., Takahashi, M. and Asada, K. (1981) Plant Cell. Physiol. 22, 33–39
15 Beoku-Betts, D., Chapman, S.K., Knox, C.V. and Sykes, A.G. (1983) J. Chem. Soc. Chem. Commun. 1150–1152
16 Chapman, S.K. and Sykes, A.G. (1983) Inorg. Chim. Acta 79, 187–188
17 Augustin, M., Chapman, S.K., Davies, D.M., Sykes, A.G., Speck, S.H. and Margoliash, E. (1983) J. Biol. Chem. 258, 6405–6409
18 Geren, L.M., Stonehuerner, J., Davis, D.J. and Millett, F. (1983) Biochim. Biophys. Acta 724, 62–68
19 Ramshaw, J.A.M., Brown, R.H., Scawen, M.D. and Boulter, D. (1973) Biochim. Biophys. Acta 303, 269–273
20 Milne, P.R. and Wells, J.R.E. (1970) J. Biol. Chem. 245, 1566–1574
21 Brautigan, D.L., Ferguson-Miller, S. and Margoliash, E. (1978) Methods Enzymol. 53, 128–164
22 Lemberg, R. and Barrett, J. (1973) The Cytochromes, Academic Press, London
23 Friebolin, H., Schilling, G. and Pohl, L. (1979) Org. Magn. Reson. 12, 569–573
24 Dwek, R.A. (1973) NMR in Biochemistry, Clarendon Press, Oxford
25 Wherland, S. and Gray, H.B. (1977) in Biological Aspects of Inorganic Chemistry, (Dolphin, D., ed.), p. 289, John Wiley, New York
26 McArdle, J.V., Coyle, C.L., Gray, H.B., Yoneda, G.S. and Holwerda, R.A. (1977) J. Am. Chem. Soc. 99, 2483–2489
27 Yoneda, G.S., Mitchel, G.L., Blackmer, G.L. and Holwerda, R.A. (1978) Bioinorg. Chem. 8, 369–386
28 Reid, L.S. and Mauk, A.G. (1982) J. Am. Chem. Soc. 104, 841–845
29 Lumry, R. and Rajender, S. (1970) Biopolymers 9, 1125–1227
30 Redfield, A.G. and Gupta, R.K. (1971) Cold Spring Harbor Symp. Quant. Biol. 36, 405–411
31 McDonald, C.C. and Phillips, W.D. (1973) Biochemistry 12, 3170–3186
32 Keller, R.M. and Wüthrich, K. (1978) Biochim. Biophys. Acta 533, 195–206
33 Ångström, J., Moore, G.R. and Williams, R.J.P. (1982) Biochim. Biophys. Acta 703, 87–94
34 Gupta, R.K. and Yonetani, T. (1973) Biochim. Biophys. Acta 292, 502–508
35 Miura, R., Sugiyama, T., Akasaka, K. and Yamano, T. (1980) Biochem. Int. 1, 532–538
36 Eley, C.G.S. and Moore, G.R. (1983) Biochem. J. 215, 11–21
37 Wüthrich, K. (1976) NMR in Biological Research: Peptides and Proteins, North-Holland, Amsterdam
38 Potasek, M. (1978) Science 201, 151–153
39 Sailasuta, N., Anson, F.C. and Gray, H.B. (1979) J. Am. Chem. Soc. 101, 455–458
40 Stellwagen, E. and Shulman, R.G. (1973) J. Mol. Biol. 80, 559–573
41 Moore, G.R., Williams, R.J.P., Chien, J.C.W. and Dickinson, L.C. (1980) J. Inorg. Biochem. 12, 1–15
42 Burns, P.D. and La Mar, G.N. (1979) J. Am. Chem. Soc. 101, 5844–5846
43 Smith, H.T., Staudenmeyer, N. and Millett, F. (1977) Biochemistry 16, 4971–4974
44 Moore, G.R. (1983) FEBS Lett. 161, 171–175
45 Schulz, G.E. and Schirmer, R.H. (1979) in Principles of Protein Structure, pp. 30 and 31, Springer-Verlag, Berlin
46 Rees, D.C. (1980) J. Mol. Biol. 141, 323–326
47 Burkey, K.O. and Gross, E.L. (1981) Biochemistry 20, 5495–5499
48 Burkey, K.O. and Gross, E.L. (1982) Biochemistry 21, 5886–5890
49 Taniguchi, V.T., Sailasuta-Scott, N., Anson, F.C. and Gray, H.B. (1980) Pure Appl. Chem. 52, 2275–2281