The modulation of cytochrome *c* electron self-exchange by site-specific chemical modification and anion binding

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Received 14 July 1986

The site-specific chemical modification of horse heart cytochrome *c* at Lys-13 and -72 using 4-chloro-3,5-dinitrobenzoic acid (CDNB) increases the electron self-exchange rate of the protein. In the presence of 0.24 M cacodylate (pH* 7.0) the electron self-exchange rate constants, k_{ex}, measured by a ^{1}H NMR saturation transfer method at 300 K, are 600, 6×10^3 and 6×10^4 $M^{-1} \cdot s^{-1}$ for native, CDNP-K13 and CDNP-K72 cytochromes *c* respectively. Repulsive electrostatic interactions, which inhibit cytochrome *c* electron self-exchange, are differentially affected by modification. Measurements of ^{1}H NMR line broadening observed with partially oxidised samples of native cytochrome *c* show that ATP and the redox inert multivalent anion $Co(CN)_6^{3-}$ catalyse electron self-exchange. At saturation a limiting value of $\sim 1.4 \times 10^5$ $M^{-1} \cdot s^{-1}$ is observed for both anions.

Cytochrome c *Chemical modification* *Electron transfer* *NMR* *Protein-protein interaction* *ATP*

1. INTRODUCTION

The formation of protein-protein complexes plays an important role in determining the kinetics of many metalloprotein electron transfer reactions [1]. Protein association, by facilitating electronic coupling of the donor and acceptor, gives rise to 'intracomplex' electron transfer at a rate determined largely by the thermodynamic driving force and the distance between redox sites [2]. More effective association results when the proteins possess electrostatically or hydrophobically compatible surface regions. For example, a computer-simulated model of the cytochrome *c*-cytochrome *c* peroxidase complex [3] suggests that the ring of conserved lysine residues surrounding the haem of cytochrome *c* interacts with spatially complementary carboxylates of the peroxidase. In this case the matching of protein surfaces leads to the close approach of the haem groups assumed to be necessary for rapid electron transfer. Furthermore, experimental evidence from chemical modification studies supports the role of cytochrome *c* surface lysines in physiological electron transfer [4–6].

In contrast, electron transfer between non-physiological reaction partners often proceeds via an encounter complex formed by proteins which possess non-complementary or even electrostatically repulsive surfaces. The relatively high density of positively charged lysines surrounding the cytochrome *c* haem crevice inhibits protein self-association and the rate of electron self-exchange, as measured by NMR [7], is therefore comparatively slow. The importance of elec-

Abbreviations: ATP, adenosine triphosphate; CDNB, 4-chloro-3,5-dinitrobenzoic acid; CDNP, 4-chloro-3,5-dinitrophenyl; $Co(CN)_6^{3-}$, hexacyanocobaltate(III); DSS, sodium 2,2-dimethyl-2-silapentane-5-sulphonate; K, lysine

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/86/$3.50

trostatic factors in retarding this exchange process is revealed, firstly, in the modulatory effect of Cl^- on the exchange rate [8] and, secondly, in the observation that bacterial cytochromes *c*, which carry a lower net charge, have electron self-exchange rates considerably higher than that of horse heart cytochrome *c* [9,10]. Similar considerations apply to other redox proteins, such as plastocyanin [11,12].

Here, we describe how the cytochrome *c* electron self-exchange rate may be substantially changed by site-specific modification of Lys-13 and -72 with CDNB. In addition the effects of anions (cacodylate, ATP and $Co(CN)_6^{3-}$) on the self-exchange rates of the native protein are considered. The aims of the study are to determine the protein-protein contact region for the reaction and to elucidate the dependence of the cytochrome *c* self exchange rate on non-complementary surface structure.

2. EXPERIMENTAL

Horse heart cytochrome *c* (type VI, Sigma), purified to remove deamidated forms [13], was chemically modified by the method of Osheroff et al. [14]. A 5-fold molar excess of CDNB in 0.2 M boric acid-NaOH (pH 9.0) was reacted with cytochrome *c* (final concentration 1 mM). Resolution of the reaction mixture by ion-exchange chromatography (CM 32, 7.7 × 150 cm, Whatman) into a series of well defined fractions enabled the fastest migrating mono-CDNP substituted band to be subsequently resolved into two homogeneous components, CDNP-K13 and CDNP-K72 cytochrome *c*.

NMR samples, free of chloride, phosphate and other contaminants, were prepared by extensive diafiltration (Amicon 8MC ultrafiltration cell) of pure native, CDNP-K13 and CDNP-K72 cytochromes with cacodylic acid-NaOD pH* 7.0 in D_2O (Merck, Sharp and Dohme; 99.8%). Fully reduced samples were prepared by treating the proteins with excess sodium dithionite prior to diafiltration. Partially oxidised samples were obtained by mixing oxidised and reduced protein solutions. In addition to spectrophotometric measurement of protein concentration, oxidised/reduced cytochrome *c* ratios were determined by integration of the Met-80 methyl resonances in NMR spectra. ATP (disodium dihydrogen salt) and $K_3Co(CN)_6$ were used without further purification.

NMR spectra were recorded on a Bruker AM-500 spectrometer operating in a Fourier transform mode. Chemical shifts are quoted in ppm downfield from DSS. Electron self-exchange rates were determined either by a saturation transfer method following that used by Gupta et al. [8], or by measuring electron exchange induced line broadening. In the former, the lifetime of the protein in the reduced state was determined, in a partially oxidised sample, from the reduction in intensity of the Met-80 methyl resonance (−3.25 ppm) resulting from pre-irradiation of the corresponding resonance of the oxidised protein (+24 ppm). At equilibrium [17]

$$I'/I = \frac{\tau}{\tau + T_1} \quad (1)$$

where I' and I are the intensities of the Met-80 resonance measured with and without presaturation of the resonance of the oxidised protein, τ is the lifetime of the protein in the reduced state and T_1 is the longitudinal relaxation time of the reduced Met-80 methyl resonance measured in the absence of the oxidised form. I' and I were obtained from spectra recorded with the decoupler applied first on- and then off-resonance. A spectral width of 30000 Hz was employed and 540–1200 transients per spectrum were accumulated in 16K memory. T_1 values were determined using an inversion-recovery pulse sequence [18] to accumulate free induction decays (FIDs) of 10000 Hz sweep width in 8K memory. Exponential functions were then fitted to a minimum of 10 data points obtained for each protein.

Exchange rates, comparable in magnitude to the inverse of the natural linewidth of the Met-80 methyl resonance, were determined by measuring the line broadening of the resonance due to electron exchange. In this case the inverse state lifetime is related to $\Delta V_{1/2}$, the induced line broadening, according to [18]

$$\tau^{-1} = \Delta V_{1/2} \quad (2)$$

Linewidths were measured from FIDs unmanipulated for resolution enhancement. If the bimolecular electron self-exchange rate, k_{ex}, is

defined by,

$$k_{ex} = \text{observed rate}/[Ox][Red] \quad (3)$$

where [Ox] and [Red] are the concentrations of oxidised and reduced cytochrome *c*, then

$$k_{ex} = 1/\tau[Ox] \quad (4)$$

3. RESULTS

The T_1 values of the Met-80 methyl protons were found to be 0.65,0.83 and 0.12 for native, CDNP-K13 and CDNP-K72 cytochrome *c*, respectively. These values probably reflect only small differences in the orientations of the Met-80 ligand since 1- and 2-dimensional proton NMR studies [18] reveal no significant modification-induced conformation changes. Saturation transfer effects were observed and interpreted, using Met-80 methyl T_1 values, to determine the self-exchange rates of native, CDNP-K13 and CDNP-K72 cytochromes *c* at different concentrations of sodium cacodylate. Fig.1 shows the results of these experiments in the form of Debye-Hückel plots for which the ionic strengths were calculated solely from the sodium cacodylate concentrations. At zero ionic strength the k_{ex} values of native, CDNP-K13 and CDNP-K72 cytochromes *c* are ~100, ~1000 and 2×10^4 $M^{-1}\cdot s^{-1}$, respectively. The self-exchange rates for all three proteins show a similar dependence on ionic strength and at $I = 0.24$ M the values of k_{ex} are ~600, ~6000 and 6×10^4 $M^{-1}\cdot s^{-1}$.

The effects of ATP and $Co(CN)_6^{3-}$ on the electron self-exchange rate of native cytochrome *c* were determined by measuring the exchange-induced line broadening of the Met-80 methyl resonance of ferrocytochrome *c* in an oxidised/reduced protein mixture. Addition of ATP and $Co(CN)_6^{3-}$ to partially oxidised samples was found to substantially increase resonance linewidths. Fig.2 shows the effect of ATP on the

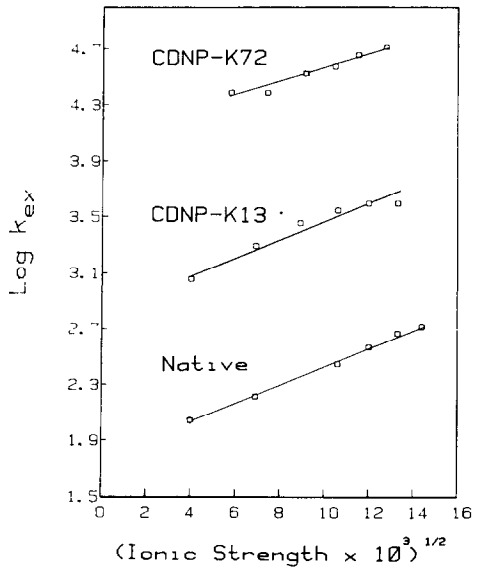

Fig.1. Debye-Hückel plots of log k_{ex} vs (ionic strength)$^{1/2}$ obtained by saturation transfer. All measurements were performed at 300 K and pH* 7.0 at protein concentrations of 4 mM (native), 1 mM (CDNP-K13) and 0.8 mM (CDNP-K72). Ionic strengths were calculated from the concentration of sodium cacodylate.

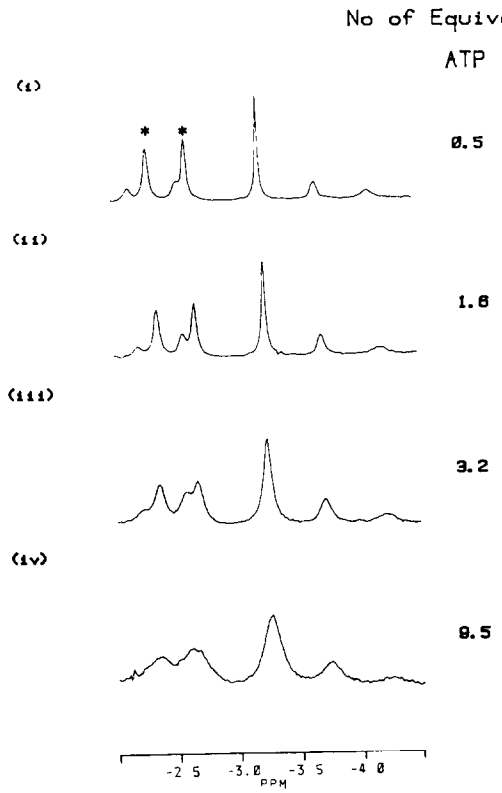

Fig.2. 500 MHz ^{1}H NMR spectra of partially oxidised native cytochrome *c* (total protein concentration 4 mM, 300 K, 20 mM sodium cacodylate, pH* 7.0) in the presence of different ATP concentrations. Resonances of oxidised protein are denoted by (*).

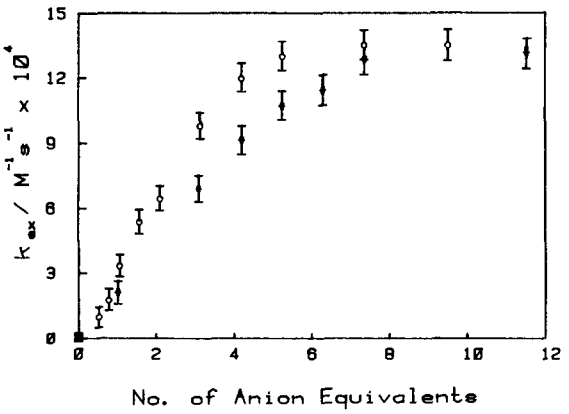

Fig.3. Effects of $Co(CN)_6^{3-}$ (*) and ATP (○) on the electron self-exchange rate, k_{ex}, determined from line broadening measurements made on the Met-80 methyl resonance of ferrocytochrome *c* (total protein concentration 4 mM, 300 K, 20 mM sodium caodylate, pH* 7.0).

ferrocytochrome *c* Met-80 methyl resonance. Addition of the anions to fully reduced samples resulted in only small increases in the resonance linewidth and these were considered when determining the excess broadening due to electron exchange. Fig.3 shows the effect of ATP and $Co(CN)_6^{3-}$ on the bimolecular self-exchange rates determined from line broadening. In both cases saturation occurs and the same limiting value of $\sim 1.4 \times 10^5$ $M^{-1} \cdot s^{-1}$ is observed for k_{ex}.

4. DISCUSSION

The observed increases in the cytochrome *c* electron self-exchange rate induced by the K13 and K72 CDNP modifications are, strictly, related to electron transfer transition state-protein ground state energy differences. However, on the basis of structural studies it is reasonable to assume similar ground state energies for the native and CDNP-modified proteins. The location of CDNP-modified lysines close to the partially exposed haem edge suggests, in conjunction with the observed effects on k_{ex}, that the protein-protein contact region is centred on the haem crevice. This lends experimental support to a proposed model for the encounter complex [2] where the haem edge-haem edge distance is estimated to be 9.4 Å; the presence of a large aromatic group on the surface of the protein should not reduce this distance. It is likely, therefore, that k_{ex} is increased principally through the direct involvement of the CDNP-lysyl groups in promoting protein association rather than through a major enhancement of the first-order ('intracomplex') electron transfer. To a first approximation this may be explained by the reduction in protein-protein electrostatic repulsion but the formation of specific electrostatic bridges between the CDNP carboxylates and the lysine side chains of the protein partner may further stabilise the binary complexes formed.

The difference in the electron self-exchange rates of CDNP-K13 and CDNP-K72 cytochromes *c* may reflect a greater involvement of the Lys-72 side chain in protein-protein interaction. This is consistent with the observation that the reactivities of the protein with negatively charged reagents decrease in the order, native> CDNP-K13>CDNP-K72 cytochromes *c* [19,20]. An intramolecular electrostatic salt bridge between the CDNP-K13 carboxylate and surface Lys-86 or -87 may account for its less effective modulation of reactivity.

Raising the ionic strength of the solution by increasing the concentration of background electrolyte also reduces electrostatic repulsion between protein molecules. The effect of NaCl on the self-exchange rate of cytochrome *c* has been reported [8] and is greater than that of sodium cacodylate. Chloride is known to bind to the surface of the protein [21] and it is possible that neither electrolyte functions solely by screening charge in the Debye-Hückel sense. Instead, effective association may result from the relatively weak binding of the anions at the haem crevice with the association constant for chloride being greater than that for cacodylate. It is reasonable to suppose that protein modification has little effect on this binding.

The catalytic effects of ATP and $Co(CN)_6^{3-}$ on k_{ex} may be attributed to the relatively strong binding of these anions to the surface of cytochrome *c*. ^1H NMR studies using the $Cr(CN)_6^{3-}$ relaxation probe analogue of $Co(CN)_6^{3-}$ have shown that two high-affinity binding regions exist close to the haem edge [22]. Additionally, an association constant of ~ 200 M^{-1} ($I = 0.12$ M) has been independently determined by ^{59}Co NMR [23]. We suggest that catalysis of the self-exchange reaction by $Co(CN)_6^{3-}$ results from anion binding at one or both of these previously characterised regions. The presence of up to two anions at the

protein-protein interface of the encounter complex promotes more effective association. A haem edge binding domain has also been proposed for phosphates and nucleotide triphosphates [24] and the effect of ATP on k_{ex} may be interpreted in a similar way. Association constants of 50–400 M^{-1} have been reported for ATP and phosphate binding to cytochrome *c* [25,26]. It is of interest that the values of k_{ex} measured at saturation for the ATP and $Co(CN)_6^{3-}$ binding are very similar.

Although the physiological significance of anion binding to cytochrome *c* remains unclear, ATP binding has been shown to inhibit the reaction of cytochrome *c* with cytochrome *c* oxidase [27]. It is therefore unlikely that electron transfer at the oxidase involves a rate-limiting, ATP-modulated, self-exchange between bound and free cytochrome [28].

In conclusion it should be noted that although enhanced electron self-exchange rates are discussed predominantly in terms of electrostatic interactions, whether by CDNP-lysyl modifications or specific anion binding, there may also be changes in the hydrophobic contact contribution to the association energy.

ACKNOWLEDGEMENTS

This is a contribution from the Oxford Enzyme Group of which R.J.P.W. and H.A.O.H. are members. Additional support was provided by SERC postdoctoral (D.W.) and postgraduate (D.W.C) grants.

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