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Effects of NO_2-modification of Tyr83 on the reactivity of spinach plastocyanin with cytochrome *f*

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We have investigated the electron transfer (ET) reactions between turnip cytochrome *f*, and the native and NO_2-Tyr83-modified forms of spinach plastocyanin (PCu) at 10.0°C and ionic strength 0.200 M(NaCl), in both directions as a function of pH. The PCu(II)/cytochrome *f*(II) rate constants in the pH-range 4–6.8 reflect active and remote binding site protonation. At higher pH, NO_2-Tyr83 and positively charged residues on cytochrome *f* are deprotonated, and both native and NO_2-modified PCu exhibit a composite rate constant variation in this pH range. When framed by ET theory this pattern is fully understandable in terms of variations in reduction potentials and electrostatic interactions, caused by the protonation equilibria. The rate constant ratio k^{nitro}/k^{native} is, however, only 1.04 for the PCu(II)/cytochrome *f*(II) reactions in spite of a 18 mV higher reduction potential for NO_2-Tyr83-modified PCu. This is much lower than the value of 1.42 expected from ET theory solely on the basis of such a reduction potential effect. A similar effect is seen for PCu(I)/cytochrome *f*(III) for which the low-pH k^{nitro}/k^{native} ratio is 0.51. Notable but smaller effects are also observed for the small reaction partners $[Fe(CN)_6]^{3-/4-}$ and $[Co(phen)_3]^{3+/2+}$. The effect of NO_2-modification in addition to the reduction potential effect can be resolved into a small reorganization energy increase around the copper atom and a smaller electronic transmission coefficient for ET through the Cu/Cys84/Tyr83 sequence. The former effect dominates in the reactions with the small reaction partners, while the electronic effects contribute significantly for PCu/cytochrome *f*, supporting the concept that the PCu/cytochrome *f* ET is at the remote PCu binding site.

Introduction

Plastocyanin (PCu) and cytochrome *f* are natural reaction partners in the photosynthetic electron transport chain. Cytochrome *f* (M_r 29000) is part of the membrane-bound cytochrome b_6/f-complex (plastoquinol : plastocyanin oxidoreductase) [1,2], but can be isolated and purified in a monomeric water-soluble form [3]. PCu (one type-1 copper, M_r 10500) is a water-soluble metalloprotein which transfers electrons from cytochrome *f* to P700 in Photosystem I (PS I) [4].

No three-dimensional structure is available for cytochrome *f*. Crystallographic three-dimensional structures are available for PCu from poplar [5,6] (Fig. 1) and the green alga *Enteromorpha prolifera* [7] while the NMR three-dimensional structure is reported for the green alga *Scenedesmus obliquus* [8]. As expected from the amino-acid sequences and studies of PCu from different sources, the overall three-dimensional structure including the active site structure is conserved. We therefore refer the spinach PCu structure to the structures already obtained.

A number of previous studies on electron transfer (ET) properties of higher plant PCu have suggested that at least two, rather different, binding sites on the surface are involved in ET reactions with small inorganic redox complexes [4]. One site is adjacent to the copper atom, with weak electrostatic interactions (also described as the 'north' site of the molecule). The other one is a remote ('east') site of negatively charged

Abbreviations: PCu, spinach plastocyanin; PS I, Photosystem I; P700, reaction center of Photosystem I; ET, electron transfer.

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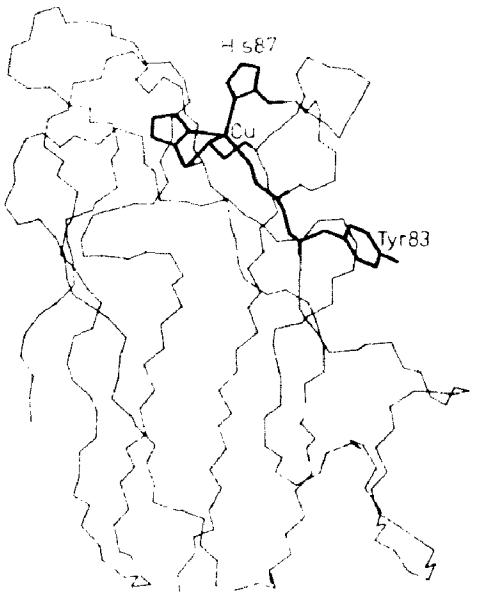

Fig. 1. Poplar PCu structure [5,6]. The copper ligands His37, Cys84, His87 and Met92 as well as the Cu, Cys84, Tyr83 path are indicated by bold lines.

amino acid residues surrounding the solvent-exposed Tyr83. The possibility of PCu using two electron transfer binding sites also in biological electron transfer has been extensively studied by various approaches, such as protein modification and kinetics [9–12], NMR [13,14], protein cross-linking [15–19], and theoretical calculations [20].

The ET properties of cytochrome *f*, as studied by use of small inorganic redox complexes with different charges, suggest that there is a common binding site on cytochrome *f* [21]. Despite an overall negative charge at neutral pH there are indications of a positively charged binding site on cytochrome *f* [21,22], conceivably as a ring of positive charges around an exposed heme edge as in the three-dimensional structure of cytochrome *c* [23]. A positively charged binding site is also involved in ET between PCu and cytochrome *f* as indicated from the ionic strength dependence of the second-order rate constants [24].

The ET reaction between PCu and cytochrome *f* is very fast [25]. At low pH (< 7.5) the rate constants decrease with decreasing pH for both the PCu(II)/cytochrome *f*(II) and the PCu(I)/cytochrome *f*(III) reactions [24,26]. This indicates that the cytochrome *f* binding site is close to the negatively charged 'east' site of PCu, provided that the number of ionizable groups in this pH-range at the PCu binding site is larger than at the binding site on cytochrome *f*. The pH-dependence can thus be ascribed to two physical effects, i.e., an electrostatic and a free enthalpy effect, the latter due to active site protonation on PCu(I). The two effects compensate each other in the reaction between PCu(II) and cytochrome *f*(II) which explains the slower decrease in rate constants as pH is decreased than for the reverse reaction between PCu(I) and cytochrome *f*(III) where the two effects reinforce each other. At high pH (> 7.5) the PCu reduction potential is constant, whereas the cytochrome *f* reduction potential decreases with increasing pH due to a change in the iron ligand environment [22] and probably also because of rapid conformational changes in the protein structure as seen for cytochrome *c* [27]. Furthermore cytochrome *f* becomes less positively charged as pH increases. The overall decreasing rate constants for the reactions in both directions with increasing pH then indicate that the inter-reactant electrostatic effect of changing pH is stronger than the reduction potential effect.

An appealing view for the function of PCu in photosynthesis could be that PCu is first bound to cytochrome *f* by the negatively charged remote binding site and receives electrons through Tyr83 at this site. PCu is subsequently bound to PS I (P700) also by the remote binding site. The PS I binding site for PCu has been shown to be the PS I-F polypeptide [15,28,29]. However, this protein does not contain electron acceptor prosthetic groups, and its function is therefore likely to be docking of PCu into the right position for adjacent site ET (His87) from PCu directly to $P700^+$. This view would be in line with the fact that a ternary complex between PS I, cytochrome *f* and PCu cannot be formed [30].

The reactivity of NO_2-Tyr83 modified PCu with small inorganic redox complexes has previously been investigated [12], where it was shown that: (a) NO_2-modification of Tyr83 increases the reduction potential by about 20 mV at pH < 7; (b) deprotonation of the phenolic group on NO_2-Tyr83 modified PCu leads to a decrease of the reduction potential by 20–25 mV; (c) the $[Fe(CN)_6]^{4-/3-}$ couple appears to be subjected to only a fraction of the negative charge developed on the phenol group deprotonation, indicating that this couple reacts at the adjacent site away from the Tyr83; (d) in contrast, the positively charged reaction partner $[Co(phen)_3]^{3+}$ is exposed to a substantial attractive potential on phenol group deprotonation, indicating that this reaction partner has an ET site close to Tyr83; (e) these observations could be framed by theoretical calculations pointing to a facile ET pathway through the Cu/Cys84/Tyr83 sequence [20].

In the present work we have extended these investigations to PCu and NO_2-Tyr modified PCu ET with its natural reaction partner cytochrome *f* in the pH-range 4.0–9.3. We have disentangled reduction potential, inter-reactant interaction and electronic effects of NO_2-modification and of surface residue protonation by careful use of electron transfer theory, and noted some intriguing differences between the ET behaviour of cytochrome *f* and small reaction partners towards PCu.

Materials and Methods

Purification of proteins

Spinach (*Spinacea oleracea*) PCu was isolated and purified from frozen leaves as described elsewhere [31]. Turnip cytochrome *f* was purchased from Sigma and purified on FPLC using the following conditions: buffer A, 20 mM Tris-HCl (pH 7.5); buffer B, 20 mM Tris-HCl (pH 7.5) + 0.5 M NaCl, using a 12 ml linear gradient from 0–24% buffer B, with a flow rate of 1.0 ml/min on a Mono Q (HR 5/5) anion-exchange column. Purified cytochrome *f* shows an absorbance ratio A_{554}/A_{278} of 0.93–0.95 in the reduced state. All chemicals used were of analytical grade and made up in Milli-Q water at room temperature.

NO_2-modification and purification of modified spinach plastocyanin

NO_2-Tyr83 modified PCu was obtained as previously described [12] with minor modifications. Residual tetranitromethane adsorbed to the protein after gel-column separation, was released by adding sodium ascorbate. When developing of yellow colour due to the trinitromethane-anion had ceased the protein was reoxidized by $[Fe(CN)_6]^{3-}$ and extensively ultrafiltrated (Amicon, YM5 membrane) into 20 mM Bis-Tris-HCl (pH 6.0) before purification. NO_2-Tyr83-modified PCu was purified on a Q-Sepharose HiLoad 16/10 High Performance (Pharmacia) column equilibrated with 20 mM Tris-HCl (pH 7.5) buffer using a 600 ml linear gradient from 80 to 200 mM NaCl in 20 mM Tris-HCl (pH 7.5) with a flow rate of 2.0 ml/min at 4°C.

Determination of protein concentrations

The concentration of cytochrome *f* was determined spectrophotometrically at 554 nm ($\epsilon = 27700\ M^{-1}\ cm^{-1}$) [30] in the reduced state and the concentrations of native and NO_2-Tyr83 modified PCu in the oxidized form at 597 nm. The 'molar' absorption coefficient is only known for native PCu ($\epsilon_{597} = 4500\ M^{-1}\ cm^{-1}$) [4]. It is assumed that the absorption changes in the S → Cu(II) transition on NO_2-modification can be ignored due to the considerable distance between the copper atom and the site of modification. In comparison, NO_2-substitution in $[Fe(phen)_3]^{2+}$ where the coupling between the Fe-atom and the nitro group is much stronger, increases the 'molar' absorption coefficient of the strong visible charge transfer transition by only 4% [32].

Kinetics

A Hi-Tech Scientific stopped-flow spectrophotometer PQ/SF-53 (Salisbury, UK) equipped with On Line-Instrument-Systems (Jefferson, U.S.A.) stopped-flow data acquisition software was used. In order to perform kinetic measurements over a broad range of conditions with limited amount of proteins which are scarce or unstable in certain pH-ranges (both of which apply to NO_2-Tyr83 modified PCu), we have developed jointly with Hi-Tech Scientific, a new device for the stopped-flow spectrophotometer, which allows simultaneous mixing of three solutions. As shown in this work such a three-syringe stopped-flow setup makes it possible to measure the rate constants over a broad range of for example pH with only one solution of each protein under investigation. The three-syringe stopped-flow mode was obtained by placing a stopped-flow pre-mixer (Hi-Tech Scientific, NA3951) in front of the integral-mixer. This device (the stopped-flow pre-mixer) gives a dead vol. of only 35 μl between the pre-mixer and the integral-mixer.

Kinetic measurements were performed using the three-syringe setup in connection with a pH-jump procedure. The proteins under investigation were made up in 1 mM Mes (pH 6.7), $I = 0.200$ M(NaCl) for the kinetics experiments and mixed in the stopped-flow apparatus with a third buffer of 58 mM buffer concentration, the pH of the solution after mixing being determined by pH of the latter. The kinetics were studied under first-order conditions, with the Cu-proteins in at least 10-fold excess. The reactions were followed by monitoring the change in cytochrome *f* concentration at 422 nm ($\Delta\epsilon_{422} = 1.20 \cdot 10^5\ M^{-1}\ cm^{-1}$, determined by conventional spectrophotometry), absorbance changes for NO_2-Tyr83 modified PCu only affecting the amplitude. To slow down rates sufficiently and enable rate constants to be determined by the stopped-flow technique, the measurements were performed at 10.0 ± 0.1°C with the ionic strength adjusted to 0.200 M(NaCl). The final buffer concentration was 20 mM and the cytochrome *f* concentrations $(0.25–0.30) \cdot 10^{-6}$ M.

In all cases, second-order rate constants (k) were obtained from experimental first-order values (k_{obs}) in accordance with the rate law (Eqn. 1).

$$rate = k_{obs}[\text{cytochrome } f] = k[\text{plastocyanin}][\text{cytochrome } f] \qquad (1)$$

In no cases did the pH-jump procedure cause any transient effects due to interfering protonation/deprotonation events. Neither did the data show any deviation from mono-exponential behaviour due to, say slow conformational changes of cytochrome *f* at high pH. Such changes would, certainly, have been detected by our data acquisition procedure. The proteins were brought to the desired oxidation state by use of 10–20-fold molar excess of sodium ascorbate or $[Fe(CN)_6]^{3-}$, followed by ultrafiltration (Amicon, membrane YM5) against at least $1.5 \cdot 10^4$-fold excess of buffer.

The 58 mM buffers used, were at pH 4.0–5.0, sodium acetate trihydrate to which 0.2 M HCl was added to the required pH; pH 6.0–7.0, Mes to which 0.2 M NaOH was added; pH 7.5–8.5, Tris to which 0.2 M HCl was added; pH 9.3, a borate solution obtained from disodium tetraborate decahydrate, to which 0.2 M NaOH was added.

Results

The reactions between PCu and cytochrome *f* are very fast, and must be monitored at lower temperature (10°C) and at higher ionic strength (0.2 M (NaCl)), compared to studies of their individual kinetics with small inorganic redox complexes where 25°C and I = 0.1 M (NaCl) have commonly been used.

The pH-dependence of the second-order rate constants for the natural forward (Cu(II)/Fe(II)) and for the reverse (Cu(I)/Fe(III)) reactions involving native and NO_2-Tyr83 modified PCu are shown in Fig. 2 and Fig. 3, respectively.

Differences in reduction potentials between cytochrome *f* and its reaction partners (native and NO_2-Tyr83-modified PCu) and differences in the reduction potentials between PCu and NO_2-Tyr83-modified PCu as a function of pH, calculated from the second-order rate constants are shown in Table I. The reduction potential differences obtained are in agreement with the pseudo-first-order reactions studied proceeding to more than 90% completion, which is also justified by the observed absorbance changes (data not shown). The reduction potential pattern furthermore agrees with the values obtained in the studies with the small inorganic redox complexes [12]. At pH 7 the reduction potential difference between native and NO_2-Tyr83 modified PCu is found to be 18 and 16 mV for the $[Fe(CN)_6]^{4-/3-}$ and the $[Co(phen)_3]^{3+/2+}$ couples respectively, which agrees very well with the value of 17.9 mV obtained in the present study with cytochrome *f*, although the temperature is lowered from 25°C to 10°C. It therefore appears that native and NO_2-Tyr83-modified PCu follow the same trend on the temperature change.

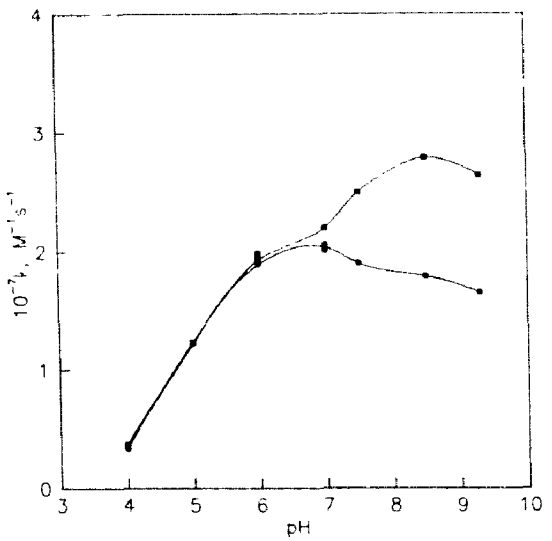

Fig. 2. The variation of rate constants k (10°C) with pH for the cytochrome *f*(II) reduction of native spinach PCu(II) (●) and NO_2-Tyr83-modified PCu(II) (■); I = 0.200 M (NaCl).

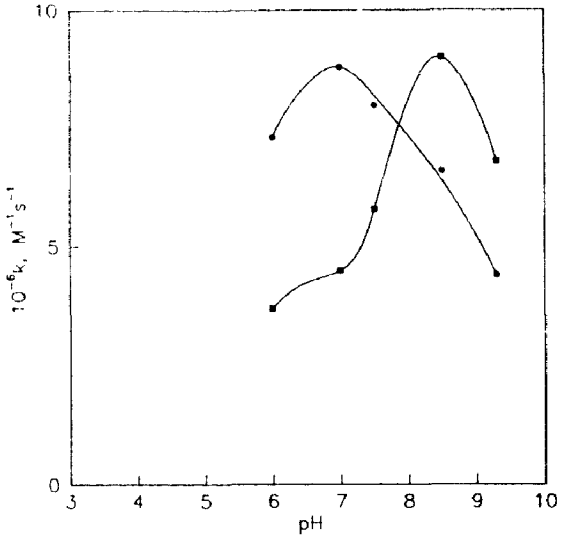

Fig. 3. The variation of rate constants k (10°C) with pH for the cytochrome *f*(III) oxidation of native spinach PCu(I) (●) and NO_2-Tyr83-modified PCu(I) (■); I = 0.200 M (NaCl).

Considering first the Cu(II)/Fe(II) system, the overall pattern in Fig. 2 appears at first somewhat complicated but can be resolved into thermodynamic, electrostatic and electronic effects, when inserted into the framework of ET theory. In the pH-range 4–6 the pH profiles reflect protonation at the negatively charged remote binding site (Fig. 1) on PCu. At high pH both NO_2-Tyr83-modified PCu and cytochrome *f* are further deprotonated. The protonation equilibria are reflected in the reduction potentials and electrostatic interactions of the species involved and induce rate constant changes for ET in both directions which either reinforce or oppose each other. This is the origin

TABLE I

Calculated differences in reduction potentials between cytochrome f and reaction partners and differences in the reduction potential between PCu and NO_2-Tyr83-modified PCu as a function of pH from the reactions between native and NO_2-Tyr83-modified PCu with cytochrome f at 10°C and I = 0.200 M (NaCl)

pH	PCu	NO_2-Tyr83-PCu	
	ΔE (mV)	ΔE (mV)	$\Delta\Delta E$ (mV)
7.0	20.8	38.7	17.9
7.5	21.1	35.6	14.5
8.5	24.3	27.6	3.3
9.3	32.3	33.1	0.8

of the partly non-parallel rate constant variation with pH.

Another interesting feature to be noted relates to the rate constant ratio k^{nitro}/k^{native}. The rate constant for the Cu(II)/Fe(II) reaction is virtually unaffected by NO_2-modification at low pH in spite of the 18 mV reduction potential increase. This behaviour differs from that of the small reaction partners, from which a ratio of 1.2–1.3 (cf. Table II) might have been expected. It is, on the other hand, in accordance with reported observations for the reactions between cytochrome *f*(II) and both PCu(II) and NO_2-Tyr83 modified PCu(II) [33,34]. Moreover, from the reduction potential change of 18 mV alone, a ratio of 1.42 would have been expected (cf. Discussion section). Other than pure reduction potential effects must therefore accompany NO_2-modification and provide a rate constant decrease for the NO_2-Tyr83 modified PCu(II)/cytochrome *f*(II) ET reaction of approximately the same magnitude as the increase from the reduction potential change. Similar, notable but smaller effects, 1.42/(1.2–1.3) = 1.09–1.18 (cf. Table II) in addition to the reduction potential effects are observed for the small reaction partners [12].

These observations are in fact what prompted the investigations of the reverse reactions, PCu(I)/cytochrome *f*(III) and NO_2-Tyr83 modified PCu(I)/cytochrome *f*(III) where data for the latter have not been reported before. The effects which lead to a decrease of the rate constant for PCu(II)/cytochrome *f*(II) on NO_2-modification in addition to the reduction potential effects would be expected to induce, jointly with the reduction potential change approximately twice the effect on the rate constant of the reverse reaction.

This expectation is completely borne out by the data in Fig. 3 and Table III. These data are limited to pH higher than 6, due to the rather endothermic nature of the reaction at lower pH. The observed ratio k^{nitro}/k^{native} at pH = 6.0 is 0.51, and very close to the expected value. An approximately similar rate constant decrease thus appears consistently in the forward and reverse experimental PCu/cytochrome *f* rate constants on NO_2-modification, in addition to the reduction potential effect.

The rate constants for oxidation of PCu(I) and NO_2-Tyr83-modified PCu(I) with cytochrome *f*(III) vary in a non-parallel fashion with pH. As for PCu(II)/cytochrome *f*(II) and NO_2-Tyr83-modified PCu(II)/cytochrome *f*(II), this can be traced to mutually reinforcing and counteracting reduction potential and surface charge variations in the different pH regions.

TABLE II

Second-order rate constant ratios (k^{nitro}/k^{native}) as a function of pH for the reactions between NO_2-Tyr83 modified and native PCu with small inorganic redox complexes

Rate constants from Ref. 12.

pH	Reduction with		Oxidation with	
	$[Fe(CN)_6]^{4-}$	$[Co(phen)_3]^{2+}$	$[Fe(CN)_6]^{3-}$	$[Co(phen)_3]^{3+}$
4.00		1.27	0.53	0.57
4.50	1.34		0.50	0.53
5.00		1.19	0.56	0.60
5.50	1.33		0.57	0.54
5.75			0.60	0.57
6.30			0.64	0.60
6.50			0.62	0.62
Average	1.34	1.23	0.57	0.58

TABLE III

Comparison of rate constant ratios (k^{nitro}/k^{native}) for the reactions of native PCu and NO_2-Tyr83 modified PCu with cytochrome f at low pH

pH	Reduction with cytochrome *f*(II)	Oxidation with cytochrome *f*(III)
4.00	1.09	
5.00	1.01	
6.00	1.02	0.51
Average	1.04	0.51

Discussion

We have provided new experimental data for the ET reaction between PCu and cytochrome *f*, and between NO_2-Tyr83-modified PCu and cytochrome *f* in both the natural forward and the reverse directions, as a function of pH. A key observation is that the k^{nitro}/k^{native} rate constant ratio takes low values for both the forward and reverse reactions, namely about two thirds of what could be expected solely from the 18 mV increase in reduction potential on NO_2-modification.

The data can be referred to reduction potential changes and to changes of the protein surface charges when pH is varied, and to reduction potential effects and electronic structural changes of PCu on NO_2-modification. A suitable frame for these observations is ET theory [35] which we shall use in the following way. The diabatic bimolecular ET rate constant is given by [36]:

$$k = \kappa \frac{\omega_{el}}{2\pi} \Delta V \exp\left\{ -\left[w_r + \frac{(E_r + \Delta G_0 + w_p - w_r)^2}{4E_r} \right] / k_B T \right\} \qquad (2)$$

where the activation free energy incorporates E_r, the overall molecular, protein and solvent reorganization free energy ΔG_0, the reaction free energy, and the inter-reactant work terms in the reactants' and prod-

uets' state, w_r and w_p, respectively. k_B is Boltzmann's constant and T the temperature. The pre-exponential factor contains the effective nuclear vibrational frequency, ω_{eff}, and the volume of the reaction zone, $\Delta V'$, precise prescriptions for which are available [36,37]. In addition, it contains the electronic transmission coefficient, κ, which takes small values in the diabatic limit of weak donor–acceptor center interactions, but approaches unity in the adiabatic limit of strong interactions. Our general conception of the reaction between PCu and cytochrome f is that ET proceeds at the remote binding site of PCu [21,24,26]. Recent theoretical calculations [20] have shown that this path is dominated by electronic coupling along the Tyr83/Cys84/Cu sequence (Fig. 1) and that κ in Eqn. 2 is small and clearly belongs to the diabatic limit (our unpublished data).

We focus here on *changes* in the rate constant on pH-variation and NO_2-modification. The changes are reflected in ΔG_0, w_r and w_p, in E_r, and as it turns out, also in the transmission coefficient, κ. We therefore exploit Eqn. 2 in the approximate form

$$\frac{k^{\text{mutio}}}{k^{\text{native}}} = \frac{\kappa^{\text{mutio}}}{\kappa^{\text{native}}} \exp\left(\frac{1}{4} \frac{E_r^{\text{mutio}} - E_r^{\text{native}}}{k_B T} \right) \times \exp\left(\frac{1}{2} \frac{\Delta G_0^{\text{mutio}} - \Delta G_0^{\text{native}}}{k_B T} \right) \times \exp\left(-\frac{1}{2} \frac{w_r^{\text{mutio}} + w_p^{\text{mutio}} - w_r^{\text{native}} - w_p^{\text{native}}}{k_B T} \right) \quad (3)$$

This form is obtained from Eqn. 2 with the reservations what $\Delta V'$ and ω_{eff} are unchanged on surface group protonation or NO_2-modification, and the quadratic ΔG_0-term in Eqn. 2 is omitted. The latter is a good approximation when $\Delta G_0 + w_p - w_r \ll 4E_r k_B T$. These conditions can be substantiated by the following observations. By their definition [37,39] $\Delta V'$ and ω_{eff} are little affected by the protein modifications. The processes are approximately thermoneutral, ΔG_0 being in the range 0–70 mV (cf. Table 1). E_r, w_r and w_p are not directly available but can be estimated by molecular and dielectric models. For all reasonable parameters [40,41] such models provide E_r-values in the range 48–97 kJ/mol for the PCu reactions with small reaction partners and 24–34 kJ/mol for the reactions with cytochrome f, in either case safely ensuring that the condition $\Delta G_0 \ll 4E_r k_B T$ is valid. Screened Coulomb work terms between local surface charges on two solvated spherical dielectric globules are also small [41] and furthermore compensate each other in the reactants' and products' states. The interactions would be significant in a strongly nonlocal solvent region of low short-range dielectric constant. We shall disregard such effects, which could, however, be incorporated in a modified analysis [42]. With the above reservations the advantage of Eqn. 3 is then that the electronic, thermodynamic, structural and surface charge effects are separated very simply. The reduction potential effects are furthermore available from independent data, and the rate constant variations therefore provide quantitative estimates of the work term, and of structural or electronic changes. Eqns. 2 and 3 apply to the PCu and NO_2-Tyr83 modified PCu ET reactions with both the small reaction partners and cytochrome f.

We first refer briefly to our previous data [12] for the reactions of native and NO_2-Tyr83 modified PCu with $[Fe(CN)_6]^{3-/4-}$ and $[Co(phen)_3]^{3+/2+}$. Table II summarizes $k^{\text{mutio}}/k^{\text{native}}$ at pH < 7. The ratio arising solely from the 20 mV reduction potential increase on NO_2-modification of PCu is 0.68 for oxidation and 1.48 for reduction. The observed values are 0.58 for oxidation while it is 1.34 and 1.23 for reduction with $[Fe(CN)_6]^{3-/4-}$ and $[Co(phen)_3]^{3+/2+}$, respectively. In addition to the reduction potential effect, NO_2-modification of PCu therefore induces further rate constant decreases by a factor of 0.83–0.90 for both oxidation and reduction of PCu. As no charge evolves on NO_2-modification in this pH-range, work term changes are insignificant and the additional rate constant decrease must be due to changes of either the transmission coefficient or the reorganization free energy. We shall show below that NO_2-modification could in fact be accompanied by a small decrease of the transmission coefficient (up to a factor of two or so) for the remote site Cu/Cys84/Tyr83 ET path. Since this path dominates a substantial part of the $[Co(phen)_3]^{2+/3+}$ kinetics, changes of the transmission coefficient could be a possible origin of the observed effect for this complex. It is, however, striking that a notable rate constant decrease is also found for $[Fe(CN)_6]^{3-/4-}$ which most likely reacts exclusively at the adjacent site, far from the site of NO_2-modification. Other effects, originating from small structural changes and formally represented by a reorganization free energy increase of about 1.0 kJ/mol must therefore be invoked for $[Fe(CN)_6]^{3-/4-}$. Such effects would also apply to $[Co(phen)_3]^{2+/3+}$, but are not as clearly distinctive as to electronic transmission coefficient effects or molecular structural effects as for $[Fe(CN)_6]^{3-/4-}$.

In comparison, the ratio between the rate constants of deprotonated NO_2-Tyr83-PCu at pH ≥ 9 and its protonated form at pH < 7, $k^{\text{depro}}/k^{\text{pro}}$, is 1.48 for oxidation with $[Fe(CN)_6]^{3-}$ and 0.53 for reduction with $[Fe(CN)_6]^{4-}$ [12]. Correction for the reduction potential decrease of 25 mV on NO_2-Tyr83 modified PCu deprotonation [12] leaves a 10% decrease for $k^{\text{depro}}/k^{\text{pro}}$ for both oxidation and reduction. In addition to small reorganization free energy changes, these effects can, however, be caused by repulsive work terms from the negative charge evolved as the remote ET site on

NO_2-modification, even though $[Fe(CN)_6]^{3-/4-}$ ET occurs at the adjacent site. Data for ET between *Anabaena variabilis* PCu and $[Co(phen)_3]^{3+}$, which occurs predominantly at the adjacent site (unpublished data), electrostatic models for the solvated globular protein [41], and numerical calculations [44] thus show that the potential from the remote site negative charge evolved, extends to the adjacent site and can be of the observed magnitude.

The PCu and NO_2-Tyr83 modified PCu reactions with cytochrome *f* are much faster ($k \sim 10^7$ M^{-1} s^{-1}) than ET between PCu and the small reaction partners, where the rate constants are approximately 10^3, 10^4 and 10^5 M^{-1} s^{-1} for $[Co(phen)_3]^{2+}$, $[Fe(CN)_6]^{4-}$ and $[Ru(NH_3)_5py]^{2+}$, respectively. Since all the reactions are almost thermoneutral, these differences must arise from different reorganization free energies, work terms or transmission coefficients. $[Co(phen)_3]^{3+/2+}$ are the only reaction partners that undergo major intramolecular reorganization on ET. This effect, rooted in the Co(III)/Co(II) spin change, has been estimated to about 40 kJ/mol [36,45] which explains the rate difference between $[Co(phen)_3]^{2+}$ and $[Ru(NH_3)_5py]^{2+}$ (Eqn. 2). $[Fe(CN)_6]^{3-/4-}$ like $[Ru(NH_3)_5py]^{3+/2+}$ undergoes insignificant intramolecular structural changes, but is accompanied by changes in the ion pair formation pattern [46,47]. This could well amount to the 24 kJ/mol needed to account for the rate constant differences. A similar quantity emerges, however, from repulsive electrostatic image forces when the strongly charged $[Fe(CN)_6]^{4-}$ is close to the protein [41]. They are insignificant for the doubly charged ions. In comparison with both these effects transmission coefficient differences between $[Fe(CN)_6]^{4-}$ and the doubly charged ions is insignificant, since the less favourable transmission coefficients for the latter are compensated by favourable work terms at the negatively charged remote binding site. The rate constants for the three small reaction partners are thus understandable in terms of reorganization free energy and work term differences and are little dependent on the particular form of these quantities.

Secondly, the larger radius and dielectric nature of cytochrome *f* compared with the small reaction partners imply that the solvent/protein reorganization free energy is smaller. For example, if the two proteins are represented as spheres of radius 15 Å and dielectric constant $\epsilon_{sp} = 5$, $E_r = 25$ kJ/mol emerges (larger if ϵ_{sp} increases). In comparison E_r for the small reaction partners are in the range 39–48 kJ/mol if they are represented by conducting spheres with their crystallographic radii and PCu as a dielectric globule of radius 15 Å. This difference causes an order of magnitude larger rate for cytochrome *f* than for $[Ru(NH_3)_5py]^{2+}$. The remaining difference is most likely due to more attractive work terms, caused by favourable orientation of complementary charges at the binding sites of the two proteins.

We proceed next to the new PCu/cytochrome *f* and NO_2-Tyr83-PCu data. The small k^{nitro}/k^{native} ratio of 1.04 (Table III) obtained for cytochrome *f*(II) reduction of the Cu-proteins in the whole pH-range up to 6 is striking both in view of the expected value of 1.48 from the reduction potential effect and the values of 1.2–1.3 for the small reaction partners. k^{nitro}/k^{native} for cytochrome *f*(III) oxidation of the proteins is 0.51, while as noted, the reduction potential effect would correspond to 0.68. As for the small reaction partners, NO_2-modification thus induces similar rate constant decreases for PCu/cytochrome *f* ET in both directions, in addition to reduction potential effects, but now amounting to a factor of 0.7–0.75, notably larger than for the small reaction partners.

If the reorganization effect observed for the small reaction partners is associated with small structural changes near the Cu-atom on NO_2-modification, then a similar effect is expected for the cytochrome *f* reactions. This leaves a rate constant ratio of 1.04 (1.2–1.3) = 0.80–0.87 for the PCu/cytochrome *f* reactions in both directions, most naturally associated with the electronic transmission coefficient κ in Eqn. 2 since ω_{rf}, ΔV and the work terms are all insignificantly affected by NO_2-modification. This supports that PCu/cytochrome *f* ET is at the remote binding site of PCu, as suggested also by ionic strength [24], protein modification [22], and NMR data [13,14]. We have checked this (unpublished data) by quantum chemical calculations of the electron exchange matrix elements for the Cu/Cys84/Tyr83 ET path, analogous to our previous calculations for native PCu. The result substantiates that there could in fact be a small decrease of the electronic factor (up to a factor of two) on NO_2-modification. The effect arises primarily from a smaller electron exchange matrix element between Cys84 and Tyr83, but the magnitude is at the limit of what can be achieved by such calculations. A transmission coefficient change for the $[Co(phen)_3]^{3+/2+}$ reactions could also be expected, but would be smaller than for cytochrome *f*. These reactions occur only partly at the remote binding site which is the one sensitive to electronic effects of NO_2-modification. The smaller radii also enable these reaction partners, without affecting the formal kinetics, to exploit different remote binding sites, such as implied by NMR data for the binding of positively charged reaction partner analogues [48]. Such a pattern would exhibit different and overall less sensitivity of the donor-acceptor overlap to NO_2-modification than for cytochrome *f* which is conceivably locked in a narrower intermolecular configuration range.

The pH-profiles of the PCu/cytochrome *f* and NO_2-Tyr83 modified PCu/cytochrome *f* reactions at

pH ≤ 7 are determined by protonation of the negatively charged residues and (for PCu(I)) the His87 ligand, similar to those of the $[Co(phen)_3]^{3+/2+}$ couple. At higher pH they are, however, different because NO_2-Tyr83 PCu and cytochrome *f* are here both engaged in protonation equilibria, reflected in their reduction potentials and surface charges. The reduction potential variation in the pH-range 7–9.3, obtained from the observed forward and reverse rate constants of cytochrome *f*, are shown in Table I. Subject to certain reservations as to the possible conformational equilibria of cytochrome *f* (cf. below), these data enable us to separate the reduction potential effects from surface charge effects on the rate constant variation with pH. Slow interconversion between different cytochrome *f* conformers is unlikely as this would give rise to biphasic behaviour in the rate pattern. Fast interconversion might imply that more than a single conformer is present in solution. However, the basic feature of the analysis below needs modification only if other rate parameters than the reduction potential and inter-reactant work terms are notably different for the different conformers. With these reservations the overall pH-dependence of the rate constants in the pH-range 7–9.3 is thus composed of the following specific effects on the individual reactants:

(a) The reduction potential of cytochrome *f* decreases with increasing pH by 12 mV in the pH range 7–9.3. This effect facilitates reduction of the PCu's and retards their oxidation.

(b) Positively charged surface amino acids near the cytochrome *f* heme group are deprotonated in the same pH range (7–9.3), weakening electrostatic attraction between cytochrome *f* and the PCu's and thereby lowering the rate constant. Reduction potential and surface charge effects arising from cytochrome *f* deprotonation therefore reinforce each other in PCu oxidation but counteract in their reduction. These are the only protonation effects which determine the pH profile for the reactions of cytochrome *f* with native PCu. From Figs. 2 and 3 the rate constants for both oxidation and reduction of native PCu are noted to decrease with increasing pH in this range, but more rapidly for PCu(I) oxidation than for PCu(II) reduction. This is fully in line with the points above that the electrostatic inter-reactant work term effect is stronger than the effect from the reduction potential decrease of the cytochrome *f* couple. From the cytochrome *f* reduction potential decrease of 12 mV in the pH-range 7.0–9.3 (Table I) the expected rate constant ratio k(pH 9.3)/k(pH 7.0) is 1.26 for reduction of PCu(II) and 0.79 for oxidation of PCu(I). The observed values are 0.80 and 0.50, respectively, leaving for both forward and reverse rate constants a decrease by a factor of 0.63 due to the weakened electrostatic work terms, caused by deprotonation of cytochrome *f* surface groups. The corresponding free energy change is 2.2 kJ/mol which is equivalent to fully screened interaction between two single charges separated by 13 Å.

(c) The reduction potential of NO_2-Tyr83-modified PCu decreases in the pH-range 7–9.3 by 17 mV (Table I) due to deprotonation of NO_2-modified Tyr83 at the remote binding site. This effect raises the rate constant for oxidation by cytochrome *f*(III), but lowers the rate constant for reduction by cytochrome *f*(II).

(d) The electrostatic interaction between deprotonated NO_2-Tyr83-modified PCu and local positive surface charges on cytochrome *f* increases in the pH range 7–9.3, leading to more favourable inter-reactant interaction and higher rate constants for the NO_2-Tyr83-modified PCu/cytochrome *f* reactions in the pH range 7–9.3. The reduction potential decrease of 17 mV for NO_2-Tyr83 modified PCu is partly compensated by the reduction potential decrease of 12 mV for cytochrome *f*, leaving a reaction free energy increase of 0.5 kJ/mol for the oxidation of NO_2-Tyr83 modified PCu(I) and a similar decrease for reduction of NO_2-Tyr83-modified PCu(II). At the same time, since both cytochrome *f* and NO_2-Tyr83-modified PCu are deprotonated, the work term changes arising from each reactant as pH is increased from 7 to 9.3, are also partly compensated, as long as the charge on the cytochrome *f* binding site remains positive.

All these effects are summarised in Table IV. They can be related to Figs. 2 and 3 in two ways. First, the initial rise in the rate constant for both the forward

TABLE IV

Qualitative effects of increasing pH on rate constants at pH ≈ 7 for the reactions with cytochrome f

	Reduction with cytochrome *f*		Oxidation with cytochrome *f*	
	PCu	NO_2-Tyr83 mod. PCu	PCu	NO_2-Tyr83 mod. PCu
a: Decreasing cytochrome *f* E.	increase	increase	decrease	decrease
b: Deprotonation on cytochrome *f*	decrease	decrease	decrease	decrease
c: Decreasing E for NO_2-Tyr83 PCu		decrease		increase
d: Increasing negative charge on NO_2-Tyr83 PCu		increase		increase

and reverse reactions in the pH range 7–9.3 suggests that electrostatic interactions dominate over reduction potential effects which would be reflected in opposite directions for the two reactions. Secondly, the fact that the rate constant increases indicate that NO_2-Tyr83-modified PCu deprotonation is more important than deprotonation of cytochrome *f* in this pH range. At higher pH the rate constants decrease, and faster for oxidation of NO_2-Tyr83-modified PCu(I) than for reduction of NO_2-Tyr83 modified PCu(II). In this higher pH range, cytochrome *f* deprotonation dominates, and the small reduction potential effects are only weakly reflected.

The data finally offer the following information. If the reduction potential and charge effects arising from cytochrome *f* are the same for PCu and NO_2-Tyr83 modified PCu, then the rate constant ratio *k*(pH 9.3)/*k*(pH 7.0) of 0.80 and 0.50 for NO_2-Tyr83 modified PCu(II) reduction and NO_2-Tyr83-modified PCu(I) oxidation, respectively, arise from the cytochrome *f* part of the system. The reduction potential decrease of 17 mV for NO_2-Tyr83-modified PCu when going from pH 7 to 9.3 gives additional rate constant ratios of 1.41 for oxidation of NO_2-Tyr83-modified PCu(I) and 0.71 for reduction of NO_2-Tyr83-modified PCu(II). Together with the experimental rate constant ratios of 1.20 for NO_2-Tyr83-modified PCu(II) reduction and 1.51 for NO_2-Tyr83-modified PCu(I) oxidation this gives a rate constant increase of about a factor of two for both NO_2-Tyr83 modified PCu(II)/cytochrome *f*(II) and NO_2-Tyr83 modified PCu(I)/cytochrome *f*(III) ET reactions. This effect is associated solely with the additional negative charge at the remote binding site of NO_2-Tyr83-modified PCu on deprotonation and again supports the view that PCu/cytochrome *f* and NO_2-Tyr83-modified PCu/cytochrome *f* ET is at the remote PCu site. The corresponding work term is 35 mV, i.e., larger than for the charges involved in cytochrome *f* deprotonation. The two kinds of proton must therefore be exposed differently to the electric field of their reaction partner.

Conclusion

It can be concluded that the comprehensive data for the reactions of PCu and NO_2-Tyr83 modified PCu towards small reaction partners and cytochrome *f* from our present communication and Ref. 12 have provided a detailed molecular ET pattern. Tyr83-NO_2 modification is accompanied by reduction potential, structural, and electronic changes, while PCu, NO_2-Tyr83 PCu, and cytochrome *f* protonation equilibria are accompanied by reduction potential and inter-reactant interaction effects. All these effects have been consistently and quantitatively resolved by judicious use of ET theory, of which Eqns. 2 and 3 are general and model insensitive formal representations, with little use of external parameters. A notable merit of this approach is thus that unambiguous information about many of the crucial microscopic factors which determine the ET rates of metalloproteins can be obtained using the whole set of data on pH- and NO_2-modification effects.

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