Determination of Reaction Site Charge on Plastocyanin from the Cyanobacterium *Anabaena variabilis* Based on Electrostatic Interactions with Small Inorganic Complexes

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In order to investigate whether plastocyanin (PC) from the cyanobacterium *Anabaena variabilis*, like PC from green algae and higher plants, can exhibit dual path electron transfer (ET) we have determined the reaction site charge of *A. variabilis* PC. Kinetic measurements of the reactions between PC and $[Co(phen)_3]^{2+}$, $[Co(phen)_3]^{3+}$ and the neutral, sulfonated analogue $[Co(phen\text{-}SO_3)_3]$ as a function of pH (6.3–8.8) and ionic strength (0.05–0.20 M) are reported. These data are supplemented by the midpoint potentials for *A. variabilis* in the same pH and ionic strength ranges. Three different approaches to reaction site charge determination are used. These rest on the ionic strength dependence of work terms and reaction free energy, on a comparison of the rate constants for the oxidation of PC by $[Co(phen)_3]^{3+}$ and $[Co(phen\text{-}SO_3)_3]$, and on the pH profile of the ET rate constants. Consistent values for the reaction site charge are obtained, with only a weak pH dependence. The state of protonation of His-59 ($pK_a = 7.3$), situated at the conceivable remote reaction site, thus does not affect the reactivity of PC to any significant extent. These results are consistent with the absence of dual path reactivity for *A. variabilis* PC and a reaction pattern dominated by ET at the adjacent site close to the copper atom, for small positively charged reaction partners.

Plastocyanin (PC) is a small (M 10500) metalloprotein containing a single Type-1 copper centre per molecule.[1] The biological role of PC is photosynthetic electron transfer (ET) from the cytochrome b_6/f complex to photosystem I,[2,3] and it seems to be rather universal as PC has been isolated from higher plants as well as algae and cyanobacteria.[4]

The crystal structures of poplar plastocyanin in both oxidation states have been known for some time.[5–7] On the basis of sequence homology and many detailed spectroscopic studies[8,9] it has been assumed that structural data for poplar PC is also representative of PC from other sources. This view has been further substantiated by the crystal structure of PC from the green alga *Enteromorpha prolifera*,[10] the gross structure of which, including the copper centre and the negatively charged remote site (*vide infra*), is analogous to that of poplar PC.

The availability of structural data has made PC an attractive choice for studies on biological ET. (For two recent reviews, see Refs. 11 and 12.) Studies with small inorganic complexes as reaction partners suggest that PC from higher plants and green algae exploits two ET pathways,[13–17] and this conclusion is supported by NMR line-broadning experiments.[18] One pathway involves His-87 at a hydrophobic adjacent site (Fig. 1) 6 Å from the copper centre, while the

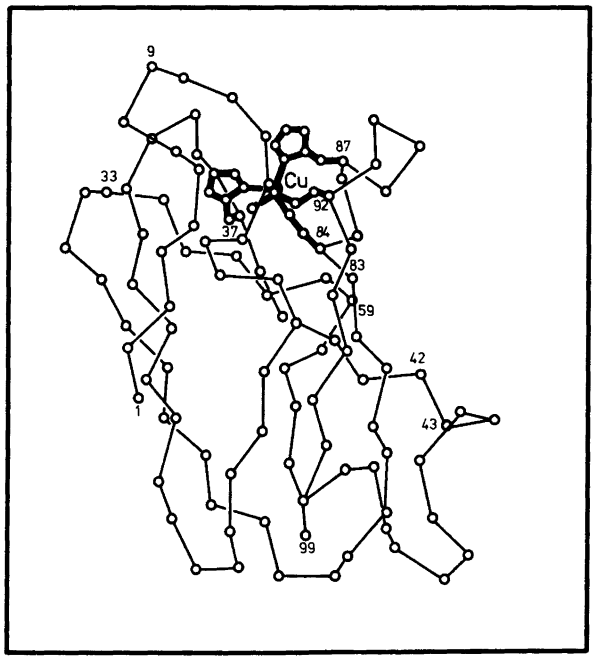

Fig. 1. The α-carbon chain of poplar plastocyanin.

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other one involves Tyr-83 and a negatively charged patch (residues 42–45 and 59–61) at a remote site, 15 Å from the copper centre. Owing to the cylindrical shape of PC, the adjacent and remote sites are often referred to as the north and east sites, respectively. Negatively charged reaction partners, such as hexacyanoferrate, $[Fe(CN)_6]^{3-/4-}$, react at the north site, while reaction partners with local positive charges, such as tris(1,10-phnanthroline)cobalt, $[Co(phen)_3]^{2+/3+}$, and the natural reaction partner cytochrome f,[19,20] react predominantly at the east site.

The negatively charged patch is strongly conserved in PC from all sources but the cyanobacterium *Anabaena variabilis*. While PC from other sources has a nominal charge between −5 and −7 at the east site,[11] this charge is +1 on *A. variabilis* PC, estimated from the amino acid sequence at neutral pH.[21] Likewise, the overall charge of PC from other sources is between −8 and −10,[11] whereas the overall charge of PC from *A. variabilis* is +1/+2 depending on the oxidation state.[21] Consequently, PC from *A. variabilis* and PC from other sources constitute a system with closely related structure and function, but where charge and charge distribution are different. This feature makes comparative studies of PC from *A. variabilis* and other sources particularly well suited for investigating the effects of electrostatic interactions in biological ET. The fact that positively charged reaction partners can react at the east site, despite the much longer distance to the copper centre than from the north site, can be explained by favourable work terms and by a relatively facile electronic transmission coefficient (*vide infra*). As the negatively charged patch at the east site is not conserved in *A. variabilis* PC, it is of considerable interest to determine whether reaction partners such as $[Co(phen)_3]^{2+/3+}$ still react at the east site. We have investigated this question by stopped-flow kinetic studies on reactions (1)–(3), where $[Co(phen\text{-}SO_3)_3]$ is the neutral

$$PC(I) + [Co(phen)_3]^{3+} \xrightarrow{k_1} PC(II) + [Co(phen)_3]^{2+} \quad (1)$$

$$PC(II) + [Co(phen)_3]^{2+} \xrightarrow{k_{-1}} PC(I) + [Co(phen)_3]^{3+} \quad (2)$$

$$PC(I) + [Co(phen\text{-}SO_3)_3] \xrightarrow{k_2} PC(II) + [Co(phen\text{-}SO_3)_3]^- \quad (3)$$

sulfonated analogue of $[Co(phen)_3]^{3+}$, tris(5-sulfonato-1,10-phenanthroline)cobalt(III).

The $[Co(phen)_3]^{3+}/[Co(phen\text{-}SO_3)_3]$ couple has appeared to be a useful probe for work terms of ET reactions involving both inorganic complexes[22] and proteins,[23] and is in crucial respects well represented by the simplest diabatic ET theory. We have obtained second-order rate constants and midpoint potentials in the pH range 6.3–8.8 and in the ionic strength range 0.05–0.20 M. The pH range was chosen because *A. variabilis* PC has a histidine residue, His-59, the pK_a of which is 7.3,[24] located at the conceivable east site. His-59 is the only ionizable group with a pK_a value in this pH range, and ET at the east site is thus here expected to be particularly sensitive to pH changes. The pH range was chosen to cover the whole range from full protonation to full deprotonation of His-59. The reported pK_a value of 7.3 refers to a NMR titration in D_2O. With the recommended corrections,[25] the value in H_2O is then 7.5–7.6, and both values are thus at the centre of the pH range used.

We have determined the reaction site charge on *A. variabilis* PC from the data in three different ways. One of these rests on the ionic strength dependence of both work terms and driving force for reactions (1) and (2), the second one on a comparison of the reaction rates for reactions (1) and (3), while the third one rests on rate constant comparisons in a pH range where His-59 is either fully protonated or fully deprotonated. Two important results emerge. One is that consistent reaction site charges are obtained by the different approaches, and that PC reacts as a positively charged species, also when pH > pI, indicative of the significance of local positive charges. Since these are located around the north site, we suggest that ET between *A. variabilis* PC and the Co complexes occurs predominantly at this site. The other result is that the effects of His-59 protonation on the rate constants for reaction with $[Co(phen)_3]^{2+/3+}$ are smaller than expected if an additional charge evolves at the reaction site. This substantiates the result that the reaction site can not be close to His-59.

Experimental

A culture of *A. variabilis* (strain 27892) was kindly provided by Prof. A. G. Sykes of the University of Newcastle upon Tyne, UK. Procedures for growing *A. variabilis* and isolating PC were as previously described.[26] PC with an absorbance ratio $A_{278}/A_{579} \leq 1.15$ was used for the kinetic experiments. Protein concentrations were determined spectrophotometrically using $\varepsilon_{597} = 4650\ M^{-1}\ cm^{-1}$ in the oxidized state.[27] $[Co(phen)_3](ClO_4)_3$ was prepared by a modified literature method.[28] It was found essential to avoid evaporation, and the solution was instead refluxed for 15 min. The complex was characterized by the following absorbance peak positions: 330, 4680; 350, 3700; and 459, 99 (nm, $M^{-1}\ cm^{-1}$).[29] $[Co(phen)_3]^{2+}$ was prepared by adding phenanthroline (in a five-fold excess over Co) to a solution of $CoCl_2$. $[Co(phen\text{-}SO_3)_3]$ was synthesized as previously described.[22] 20 mM MES (2-[*N*-morpholino]ethanesulfonic acid) buffers were used for measurements at pH 6.3 and 7.0 and 20 mM TRIS [tris(hydroxymethyl)aminoethane] buffers at pH 8.3 and 8.8. The ionic strength was adjusted using NaCl. All solutions were made up in Milli-Q water (Millipore), and all chemicals were of analytical grade.

Midpoint potentials for the $[Co(phen)_3]^{2+/3+}$ couple were measured potentiometrically with a glassy carbon electrode and a K401 calomel electrode (Radiometer) equipped with a saturated sodium chloride bridge (SSCE) at ambient temperature (23–25 °C). The potentials were determined from

a plot of E vs. log [Ox]/[Red] with $[Co]_{total} = 5\times10^{-4}$ M. The reactions were studied using a PQ/SF-53 stopped-flow apparatus (Hi-Tech) combined with 4300S stopped-flow data acquisition software (OLIS On-Line Instrument Systems) by measuring absorbance changes at 597 nm. The temperature was 25 °C. Oxidized and reduced protein samples were obtained by adding an excess (5–10-fold) of $[Fe(CN)_6]^{3-}$ or sodium ascorbate, respectively, followed by extensive ultrafiltration (model 8200 fitted with a YM-5 membrane, Amicon). Protein concentrations were $(0.5–1.0)\times10^{-5}$ M, and the inorganic complexes were in sufficient excess (at least 30-fold) to ensure pseudo-first-order and non-equilibrium kinetics (>95 % conversion). The latter was checked by the absorbance changes. Second-order rate constants were obtained from the experimental pseudo-first-order rate constants, k_{obs}, according to eqn. (4).

$$v = k_{obs}[PC] = k[Co][PC] \quad (4)$$

Results

Second-order rate constants for the oxidation and reduction of PC by $[Co(phen)_3]^{3+}$ and $[Co(phen)_3]^{2+}$, respectively, were determined as a function of pH in the range 6.3–8.8 and of ionic strength in the range 0.05–0.20 M. The results are shown in Tables 1 and 2 and summarized in Fig. 2.

The rate constant for the oxidation of PC by $[Co(phen-SO_3)_3]$ was measured in the ionic strength range 0.05–0.20 M at pH 7.0. The second-order rate constant is 2.39×10^4 M^{-1} s^{-1}, and is independent of the ionic strength.

Table 1. The dependence of second-order rate constants (in M^{-1} s^{-1}) for oxidation of plastocyanin from *Anabaena variabilis* by $[Co(phen)_3]^{3+}$ on pH and ionic strength (NaCl) at 25 °C.

pH	*I*/M			
	0.05	0.10	0.15	0.20
6.3	456	565	609	651
7.0	527	650	705	756
8.3	623	741	773	822
8.8	640	752	785	826

Table 2. The dependence of second-order rate constants (in M^{-1} s^{-1}) for reduction of plastocyanin from *Anabaena variabilis* by $[Co(phen)_3]^{2+}$ on pH and ionic strength (NaCl) at 25 °C.

pH	*I*/M			
	0.05	0.10	0.15	0.20
6.3	167	240	301	349
7.0	184	232	293	342
8.3	173	243	292	338
8.8	181	243	295	344

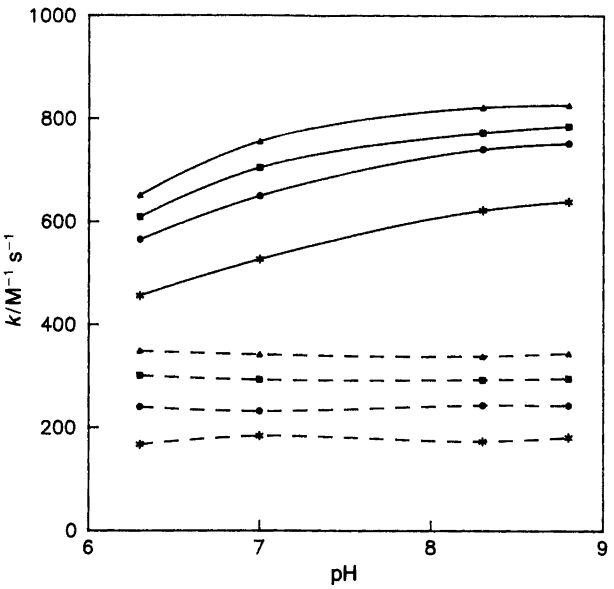

Fig. 2. Second-order rate constants for the oxidation (——) and reduction (----) of *Anabaena variabilis* plastocyanin by $[Co(phen)_3]^{3+}$ and $[Co(phen)_3]^{2+}$, respectively. * I = 0.05 M; ● I = 0.10 M; ■ I = 0.15 M and ▲ I = 0.20 M. Ionic strength adjusted with NaCl. T = 25 °C.

The midpoint potentials vs. SHE for the $[Co(phen)_3]^{2+/3+}$ couple as a function of pH (pH 6.3–8.8) and ionic strength (I = 0.05–0.20 M) in a sodium chloride medium are shown in Table 3. The midpoint potential for the reference electroder (SSCE) was taken to be 236 mV vs. SHE.

The equilibrium constant, K, for reaction (5) is given by

$$PC(I) + [Co(phen)_3]^{3+} \overset{K}{\rightleftharpoons} PC(II) + [Co(phen)_3]^{2+} \quad (5)$$

the ratio k_1/k_{-1}. From the Nernst equation the midpoint potential for PC is given by eqn. (6). Table 4 shows the

$$E^{\circ\prime}_{PC} = E^{\circ\prime}_{Co^{2+/3+}} - 0.059 \log K \quad (6)$$

midpoint potentials of PC as a function of pH and ionic strength.

Table 3. The dependence of the midpoint potential (in mV vs. SHE) for the $[Co(phen)_3]^{2+/3+}$ couple on pH and ionic strength (NaCl).

pH	*I*/M			
	0.05	0.10	0.15	0.20
6.3	366	359	357	355
7.0	364	356	351	352
8.3	364	358	353	351
8.8	363	359	354	351

Table 4. The dependence of the midpoint potential (in mV vs. SHE) of plastocyanin from *Anabaena variabilis* on pH and ionic strength (NaCl).

pH	I/M			
	0.05	0.10	0.15	0.20
6.3	340	337	339	339
7.0	337	330	328	332
8.3	331	329	328	328
8.8	331	330	329	329

Estimation of reaction site charge

The ionic strength dependence of the rate constants, a comparison of the rate constants for the two Co(III) complexes and the pH profile offer a basis for determination of the reaction site charge on PC. From a subsequent comparison with the real charge at the two most conceivable sites, i.e. the north and the east sites, we shall be in a position to assess which of the two is likely to dominate the overall reaction.

Estimation based on ionic strength dependence. We use eqn. (7) as an expression for the second-order rate constant

$$k = \varkappa_{el}\frac{\omega_{eff}}{2\pi}\Delta V \exp\left[-\left(w_r + \frac{(E_r + \Delta G^\circ + w_p - w_r)^2}{4E_r}\right)\frac{1}{RT}\right] \quad (7)$$

known from ET theory,[30-33] where $\varkappa_{el}$ is the electronic transmission coefficient, ω_{eff} is the effective vibrational frequency, ΔV is the reaction zone, w_r is the reactants' and w_p the products' work terms, E_r is the total reorganization free energy, ΔG° is the reaction free energy, R is the gas constant, and T is the temperature.

Further analysis is significantly simplified if approximation (8) is invoked, valid if $E_r >> (\Delta G^\circ + w_p - w_r)$. ΔG°

$$(E_r + \Delta G^\circ + w_p - w_r)^2 \approx E_r^2 + 2E_r(\Delta G^\circ + w_p - w_p - w_r) \quad (8)$$

and the work term combination are relatively small for all the reactions. E_r is the sum of the solvent, protein and cobalt reorganization free energies. Any reasonable model will lead to large values of these quantities,[33,34] and the above condition is thus met.

We use the specific work term in the form of eqn. (9),[35,36]

$$w = \frac{e^{-\varkappa r} Z_1 Z_2 e^2 N}{1 + \varkappa r\, 8\pi\varepsilon_0\varepsilon_r r} \quad (9)$$

where r is half the distance of closest approach of the two reactants, Z_1 and Z_2 are the charges of the two reacting species, e is the charge of the electron, N is Avogadro's number, ε_0 is the vacuum permittivity, ε_r is the relative permittivity of the medium, and $\varkappa$, the inverse Debye screening length, is given by eqn. (10), I being the ionic strength.

$$\varkappa = (2N^2e^2I/\varepsilon_0\varepsilon_r RT)^{1/2} \quad (10)$$

We now make the following assumptions: (A) The charges in eqn. (9) are regarded as local charges, i.e. Z_1 is the charge on the Co complexes, and Z_2 refers to ionized amino acids on the protein surface and the copper atom. (B) The reaction distance is the sum of the radius of the Co complex (7 Å),[33] the diameter of a chloride ion (3.6 Å), and an O–H bond length (1 Å). A suitable average r is thus 6 Å. (C) The bulk solvent permittivity value, $\varepsilon_r = 78$, is used.

More detailed interaction potentials, with correspondingly more parameters, can be introduced by available procedures, but this is not warranted by the data. Such models include, for example, dielectric image effects from the protein,[37] non-local dielectric effects,[38] or specific molecular structural features of the protein surface.

Inserting eqns. (9) and (10) in eqn. (7) converts eqn. (7) into eqn. (11), which is convenient, as the reaction free

$$\ln k + \frac{\Delta G^\circ}{2RT} = A - \frac{e^{-\varkappa r}}{1 + \varkappa r}\frac{1}{2RT}\frac{e^2N}{8\pi\varepsilon_0\varepsilon_r}(Z_1^pZ_2^p + Z_1^rZ_2^r) \quad (11)$$

energy also exhibits an ionic strength dependence (Tables 3 and 4). The A-term incorporates all other I-independent factors from eqn. (7), and the superscripts p and r denote the products' and reactants' states. From a plot of $\ln k + \Delta G^\circ/2RT$ vs. $e^{-\varkappa r}/(1 + \varkappa r)$ the charge combination $Z_1^pZ_2^p + Z_1^rZ_2^r$ for the reacting species can be calculated. These plots always had correlation coefficients larger than 0.995. Interpretation of the charge combination, however, requires that we invoke a relation between the protein charges Z_2^r and Z_2^p. We can do this in two ways. We can either assume that the charge of the copper atom is sufficiently diffuse that the change in copper charge upon oxidation/reduction does not affect the reaction partner, i.e. $Z_2^r = Z_2^p$. Alternatively, the reaction site electrostatic field can include a full charge from the Cu(I) and two full charges from the Cu(II) ion, i.e. $Z_2^r = Z_2^p - 1$ for the oxidation of PC and $Z_2^r = Z_2^p + 1$ for the reduction of PC. The reaction site charges obtained from these calculations are shown in Table 5.

Estimation based on the ratio between the rate constants for the reactions with $[Co(phen)_3]^{3+}$ and $[Co(phen\text{-}SO_3)_3]$. The second approach to reaction site charge estimation is based on the ratio between the rate constants for eqns. (1) and (3). Structures and other properties are very similar for the two Co complexes, which can therefore be expected to have similar reorganization free energies, transmission coefficients, reaction volumes and effective vibrational frequencies.[22,23] We further assume that the two Co complexes react at the same surface region on *A. variabilis* PC. This is

Table 5. Reaction site charges on plastocyanin from *Anabaena variabilis* reacting with $[Co(phen)_3]^{2+/3+}$.

	pH			
	6.3	7.0	8.3	8.8
Reduced PC[a]	+1.2	+1.0	+1.0	+0.9
Oxidized PC[a]	+2.2	+2.0	+2.0	+1.9
Reduced PC[b]	+1.6	+1.4	+1.4	+1.3
Oxidized PC[b]	+1.6	+1.4	+1.4	+1.3

[a]Assuming $Z_2^r = Z_2^p - 1$ and $Z_2^r = Z_2^p + 1$ for oxidation and reduction of PC, respectively. [b] Assuming $Z_2^r = Z_2^p$ for oxidation and reduction of PC.

substantiated by the consistency of the results emerging from the reaction site charge calculations, and by the fact that being an uncharged species, $[Co(phen\text{-}SO_3)_3]$ is likely to react close to the copper centre, i.e. at the north site. The two complexes then constitute a useful probe for the work terms, and we obtain eqn. (12) from eqn. (7), where

$$\frac{k_2}{k_1} = \exp\left(-\frac{\Delta\Delta G^\circ}{2RT}\right)\exp\left(-\frac{w_p^s + w_r^s - w_p^{us} - w_r^{us}}{2RT}\right) \qquad (12)$$

the superscripts s and us denote the substituted and unsubstituted Co complex, respectively, and $\Delta\Delta G^\circ$ is the reaction free energy difference between reactions with $[Co(phen)_3]^{3+}$ and $[Co(phen\text{-}SO_3)_3]$.

In this comparison we refer the rate constants and reaction free energies to zero ionic strength, pH 7.0, extrapolated from eqn. (11) and Tables 3 and 4. The appropriate rate constant and reaction free energy for reaction (1) are then 167 M^{-1} s^{-1} and −2.9 kJ mol^{-1}, respectively. The midpoint potentials of the $[Co(phen)_3]^{2+/3+}$ and $[Co(phen\text{-}SO_3)_3]^{0/-1}$ couples in 0.05 M sodium 4-toluene sulfonate, NaTs, have been determined as 397 and 496 mV vs. SHE, respectively.[22] The value for $[Co(phen)_3]^{2+/3+}$ is lower in a chloride medium owing to ion pair formation.[29] We have therefore ascertained that the midpoint potential for the $[Co(phen)_3]^{2+/3+}$ couple is independent of ionic strength in the 0.05–0.20 M range in NaTs media at pH 7.0. We thus use $\Delta\Delta G^\circ = -9.5$ kJ mol^{-1} in eqn. (12). Using eqns. (9) and (12) we calculate a reaction site charge for reduced PC of 1.2 ($Z_2^r = Z_2^p - 1$) or 1.7 ($Z_2^r = Z_2^p$) at pH 7.0.

Estimation based on the effects of His-59 protonation. The ratio of the rate constants for oxidation of PC at pH 8.8 and 6.3 is 1.40 at $I = 0.05$ M, and from eqn. (7) this corresponds to a decrease in the reaction free energy and/or work terms of 17 mV. As the reaction free energy decrease is equivalent to 6 mV (Tables 3 and 4), only 11 mV are to be explained by work terms in changing the pH from 6.3 to 8.8. This value decreases to 6 mV for $I = 0.20$ M. In comparison, the work term changes corresponding to the evolution of a full charge, calculated from eqn. (8), are 35 and 17 mV for $I = 0.05$ M and 0.20 M, respectively, for an inter-reactant distance of 12 Å.

Discussion

Unlike PC from green algae and higher plants, *A. variabilis* PC does not have a negative charge accumulation at any specific surface region. There is therefore no *a priori* expectation that *A. variabilis* PC should possess the dual path ET pattern of other PCs. We have substantiated this by investigations of the electric field to which an incoming small positively charged reaction partner is exposed, using three different approaches. One rests on the ionic strength dependence of the rate constants in a pH range (6.3–8.8) for which a single positive charge evolves at His-59, located at the conceivable east site. The second approach is based on a comparison of the rate constants of two structurally similar Co(III) complexes with different charges, which are sensitive probes for work terms and reaction site charges. The final approach is based on rate constant comparisons of the His-59 protonated and deprotonated form of *A. variabilis* PC.

The reaction site charges obtained are significant, positive and with only weak pH dependence, and consistent values of the reaction site charge at pH 7.0 are obtained by the different methods. The data are all unambiguously in line with reaction at the north site, where reaction partners are exposed to a positive electric field due to the copper atom, and two positively (Lys-9 and Lys-33) and one negative (Glu-85) charged amino acid. The reaction site charges agree with such a distribution considering the variable charge separations, but much less with the charge distribution at the east site, dominated by one positively (Lys-60) and one negatively (Asp-42) charged amino acid, and the fractionally positive His-59. The reaction site charge would therefore be smaller for east site ET, and the pH dependence would be much more pronounced than observed.

The pH range takes His-59 from almost complete protonation to almost complete deprotonation. The work terms for east site ET are therefore expected to change by an amount corresponding to a full proton charge. The actual work term changes of the rate constants corrected for redox potential are much smaller. From studies of the reactivity of NO_2–Tyr-83 modified spinach PC towards $[Co(phen)_3]^{3+}$, here largely at the east site, it has been shown that protonation/deprotonation of NO_2–Tyr-83 at the east site is in fact reflected in the work terms as evolution of a full electronic charge.[17] The work term changes presently observed are thus small compared with expectations for ET at a site where a full charge is evolved, but are in line with a site remote from His-59, i.e. at the north site, such as indicated by the ionic strength data and the $[Co(phen)_3]^{3+}/[Co(phen\text{-}SO_3)_3]$ comparison. All these observations support the assumption that ET between *A. variabilis* PC and $[Co(phen)_3]^{2+/3+}$ is dominated by north site reaction. The fact that PC also behaves as a positively charged species at pH > pI (pI = 7.8)[27] emphasizes that the estimated charges

must be regarded as reaction site and not as overall protein charges.

While the effect of His-59 protonation is small it is not negligible. From both ionic strength and pH dependences it amounts to 0.2–0.3 electronic charges. Partial reaction at the east site is unlikely, owing to the unfavourable electronic transmission coefficient (*vide infra*), and these effects most likely reflect long-range interactions with His-59. This view is supported by the observation that oxidation of PC from the green alga *Scenedesmus obliquus*, which also contains His-59, by the north site reactant $[Fe(CN)_6]^{3-}$ is affected by His-59 protonation,[16] as the rate-constant increase with increasing pH is smaller than expected solely from the PC midpoint potential decrease. This is in accordance with the negatively charged reaction partner being exposed to less attractive work terms owing to His-59 deprotonation. The effect is also of the same order (a few millivolts) as for *A. variabilis* PC, while the effect on *S. obliquus* oxidation by $[Co(phen)_3]^{3+}$ is larger, reflecting that $[Co(phen)_3]^{3+}$ reacts here both at the east and north sites.

His-59 protonation is also reflected in Fig. 2, which shows that the rate of PC reduction is virtually independent of pH, while the rate of oxidation increases with increasing pH. When the pH is changed from 6.3 to 8.8 the midpoint potential difference between $[Co(phen)_3]^{2+/3+}$ and PC increases. This raises k_1 but lowers k_{-1}. On the other hand, the pH change reduces the positive charge to which a reacting species approaching PC is exposed, and this increases both k_1 and k_{-1}. The two effects are antagonistic for PC reduction, giving approximately pH-independent rate constants, but cooperate for PC oxidation, raising the rate constants with increasing pH.

A. variabilis PC can be compared with PC from green algae and higher plants, for which ET occurs at both reaction sites, despite the considerably longer distance from the east (15 Å) than from the north site (6 Å). This dual path feature can be explained by two factors.[39] (1) Theoretical calculations based on superexchange and extended Hückel theory show that the electronic transmission coefficient, $\varkappa_{el}$, is only about 50 times smaller for the east that for the north site. (2) There are favourable work terms for positively charged species at the highly negatively charged east site. The electronic disfavour of the east site reaction path is thus compensated by attractive work terms for positively charged reaction partners. Owing to amino acid homology, the transmission coefficient for the east site reaction path is expected to be similar for green algae, higher plant and *A. variabilis* PC. However, with the absence of the negative charges at the east site, the crucially important attractive work terms are no longer available to assist ET at this site. In fact, with two positively (His-59 and Lys-60) and only one negatively charged residue (Asp-42) the work term for positively charged species at this site is repulsive and it is understandable that only the north site is exploited.

We notice a final point bearing on the nature of the ET site of *A. variabilis* PC. pK_a of this site has been determined by oxidation with $[Fe(CN)_6]^{3-}$ and $[Co(phen)_3]^{3+}$ at different pH, giving values of 5.0 and 5.4, respectively.[27] While the difference could suggest that different sites are used, it should be borne in mind that positive charge is accumulated on the protein on lowering the pH. This could force the rate for positively charged $[Co(phen)_3]^{3+}$ to decrease faster than for negatively charged $[Fe(CN)_6]^{3-}$, and lead to an apparently higher pK_a for $[Co(phen)_3]^{3+}$ and an apparently lower pK_a for the $[Fe(CN)_6]^{3-}$ determination. Likewise, His-59 pK_a estimates for *S. obliquus* PC by three different methods, NMR, and oxidation by $[Fe(CN)_6]^{3-}$ and $[Co(phen)_3]^{3+}$, give three different values, namely 7.8, 7.6 and 8.2 respectively,[16] also in line with such electrostatic effects.

The PC midpoint potentials determined in this study agree with previous reports.[27,40] The *A. variabilis* PC midpoint potentials are somewhat lower than for PC from algae and higher plants (~370 mV),[11] reflecting the lower overall protein charge stabilization of the oxidized state. There is a small but significant midpoint potential decrease with increasing pH. This is probably due to removal of positive charge on His-59. This stabilizes the higher-charged Cu(II) state by an amount ($\approx$ 10 mV) in line with observations for other proteins of similar size.[41]

Conclusion

We have shown that PC from *A. variabilis*, unlike PC from other sources, is unlikely to exploit two ET pathways and seems to react predominantly at the north site with positively charged reaction partners. This would emphasize the importance of local charges on protein surfaces in determining reaction sites.

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