TRANSIENT HOMODIMER INTERACTIONS STUDIED USING THE ELECTRON SELF-EXCHANGE REACTION

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Transient homodimer protein interactions have been investigated by analyzing the influence of ionic strength (NaCl) on the electron self-exchange (the bimolecular reaction whereby the two oxidation states of a redox protein interconvert) rate constant ($k_{ese}$) of four plastocyanins. The $k_{ese}$ values for the plastocyanins from spinach, Dryopteris crassirhizoma (a fern) and the green alga Ulva pertusa, which possess acidic patches of varying size and locations, increase 190-, 29- and 21-fold respectively at elevated ionic strength ($I = 2.03$ M). In contrast, the $k_{ese}$ for the almost neutral cyanobacterial plastocyanin from Anabaena variabilis exhibits very little dependence on ionic strength. The temperature dependence of the $k_{ese}$ for spinach plastocyanin ($I = 0.28$ M) provides evidence for poor packing at the homodimer interface. Representative structures of the transient homodimers involved in electron self exchange, which are consistent with fits of the ionic strength dependence of $k_{ese}$ to van Leeuwen theory, have been obtained from protein modeling and docking simulations. The Coulombic energy of the docked homodimers follows the order spinach $>$ D. crassirhizoma $>$ U. pertusa $>$ A. variabilis, which matches that of the overall influence of ionic strength on $k_{ese}$. Analysis of the homodimer structures indicates that poor packing and high planarity are features of the interface which favor transient interactions. The physiologically relevant Mg$^{2+}$ ion has a much more pronounced influence on the $k_{ese}$ of spinach plastocyanin, which along with the known properties of the thylakoid lumen suggests a biological role for electron self exchange.

Electron self exchange (ESE) is a simple reaction exhibited by redox proteins which provides a measure of their ET capabilities. An essential step in ESE is homodimer formation giving rise to transient protein interactions on the sub-millisecond timescale. In order to investigate the features of transient protein-protein interactions involved in ET we have studied the influence of ionic strength on the ESE reactivity of a single family of redox metalloproteins. For this purpose we have chosen the plastocyanins (PCus), which are cupredoxins involved in photosynthetic ET, as their surface features vary considerably depending upon the organism from...
which the protein is obtained, yet their active site structures are almost identical (8).

Plastocyanin is one of the best structurally characterized ET metalloproteins and possesses a β-barrel structure with the type 1 copper ion buried approximately 6 Å beneath the protein surface (Fig. 1A) (9-13). Higher plant PCUs have two distinct surface features (9,11), namely the hydrophobic patch surrounding the exposed His87 ligand, and the acidic patch which is more distant from the copper site (Fig. 1). In the PCu from the fern Dryopteris crassirhizoma (seedless, vascular plant) the acidic region is relocated and surrounds the hydrophobic patch (Fig. 1) (13). In green algal PCUs the acidic patch is more diffuse (12), whilst in the cyanobacterial PCUs it is non-existent (Fig. 1) (10). The charged and hydrophobic patches of PCu are utilized in the interaction with both physiological ET partners; cytochrome $f$ (cyt $f$ of the cyt $b_6 f$ complex) and photosystem I (PSI) (5,15-21). It has been shown that the structure of the interface between PCu and cyt $f$ is organism dependent (5,19).

Herein we study the influence of ionic strength (NaCl) on the ESE rate constants of spinach (higher plant), D. crassirhizoma, Ulva pertusa (green alga) and Anabaena variabilis (cyanobacterium) PCUs. We also investigate the influence of the physiologically-relevant Mg$^{2+}$ ion and temperature on the ESE reactivity of spinach PCu, the most highly charged protein studied. Protein modeling and docking simulations have been used to obtain representative structures of the transient homodimers formed. These models highlight the features of the protein-protein interface which enable transient interactions.

**MATERIALS AND METHODS**

**Protein Samples** - The spinach PCu gene was over-expressed in E. coli TG1 and the protein was isolated and purified as described previously (8). PCu from A. variabilis was isolated and purified according to a published method (22). Purity ratios for PCu(II), corresponding to single bands on 15% SDS-PAGE gels were; $A_{278}/A_{597} \leq 1.1$ for spinach, $A_{278}/A_{595} \leq 2.0$ for U. pertusa, $A_{278}/A_{597} \leq 1.2$ for A. variabilis and $A_{278}/A_{590} \leq 1.5$ for D. crassirhizoma. Protein concentrations were determined from the following molar absorption coefficients of PCu$^{II}$ at the wavelength given in parenthesis: $4700 \text{ M}^{-1}\text{cm}^{-1}$ (597 nm) for spinach (20), $4500 \text{ M}^{-1}\text{cm}^{-1}$ (597 nm) for A. variabilis (22), $4900 \text{ M}^{-1}\text{cm}^{-1}$ (595 nm) for U. pertusa (20) and $4700 \text{ M}^{-1}\text{cm}^{-1}$ (590 nm) for D. crassirhizoma (20).

Samples with a total protein concentration of 1.0 to 2.5 mM were used for ESE rate constant measurements and were typically prepared in 10 mM phosphate buffer pH* 8.0 (99.9 % D$_2$O) containing the required concentrations of NaCl or MgCl$_2$. The pH* (pH uncorrected for the deuterium isotope effect) of the buffer was adjusted prior to the addition of salt. The U. pertusa PCu sample with an ionic strength of 0.10 M was prepared in 35 mM phosphate buffer pH* 8.0. The ESE rate constant of A. variabilis PCu was measured in 73 mM phosphate buffer (99.9 % D$_2$O) pH* 6.2 ($\lambda = 0.10$ M) and in 10 mM phosphate buffer (99.9 % D$_2$O) pH* 6.2 plus the appropriate amount of NaCl. The concentration of oxidized protein [PCu$^{II}$] in the ESE samples was determined as described previously (8). The temperature dependence of the ESE rate constant of spinach PCu was studied in 10 mM phosphate buffer (99.9 % D$_2$O) pH* 8.0 containing 250 mM NaCl, at 10 °C, 25 °C, 38 °C and 48 °C.

**NMR Spectroscopy** - $^1$H NMR spectra were acquired on a JEOL Lambda 500 spectrometer at 25 °C as described previously (8). Spin-lattice relaxation times ($T_1$) were determined using a standard inversion recovery pulse sequence (8). Spin-spin relaxation times ($T_2$) were derived from the peak width at half-height using $\nu_1/2 = (\pi T_2)^{-1}$. ESE rate constants determined from the slopes of plots of $T_1^{-1}$ and $T_2^{-1}$ values against [PCu$^{II}$] provide $k_1$ and $k_2$ respectively, based on the theory described elsewhere (8,22).

**Protein Structures and Models** - The coordinates of protein structures were retrieved from the Protein Quaternary Structure (PQS) server (23). Models of PCu homodimers were built by alignment with the crystallographic dimer of Silene pratensis PCu (1byo) (24) and van der Waals clashes between interface side chains were removed by energy minimization in GROMOS (25) (250 steps of steepest descent, in vacuum).
**Protein Docking** - Docking simulations were performed using ClusPro (26) (available at http://nrc.bu.edu/cluster/). A constrained, rigid body docking was implemented in DOT (27), yielding 20,000 candidate geometries ranked according to geometric complementarity. Constraints were applied to the Cα, Ce and Ne atoms of the exposed His ligand such that homodimers involving these atoms were scored more favorably during the geometric matching step. The desolvation and electrostatic energies were independently calculated for each orientation and 2000 structures with the lowest desolvation free energies (500) and the lowest electrostatic energies (1500) were retained. A symmetric docking protocol was then implemented, which retains only those structures having two-fold symmetry. These solutions were then subjected to a pair-wise, hierarchical clustering process, in which docking orientations were ranked according to those having the highest number of neighbors within a cluster radius of ≤9 Å Cα rmsd. In order to remove side chain clashes a van der Waals energy minimization of the docked structures was performed in CHARMM (28). Docking simulations were carried out with the PCu crystal structures from S. pratensis (1byo, 24), spinach (1ag6, 11), D. crassirhizoma (1kdi, 13) and U. pertusa (1iu, 12). In the case of A. variabilis PCu, model 10 from the NMR structure (1nin, 10) was used.

**Interface Analysis** - Interface areas, defined as the solvent accessible surface area (ASA) of the free protein minus the ASA of the protein in the homodimer, were calculated using NACCESS (29) (probe radius = 1.4 Å). Interface hydrogen bonds, the gap volume index and shape (29) (probe radius = 1.4 Å). Interface hydrogen bonds, the gap volume index and shape complementarity were calculated using HBPLUS (30), SURFNET (31) and SC (32), respectively. Coulombic electrostatic energies were calculated for formal charges only, as assigned in GRASP (33). In addition, the thiol group of the copper-coordinating Cys residue was given a charge of -1, while the copper ions were assigned as CuI and CuII. The calculation was implemented between all charges on each of the monomers using a dielectric constant of 80.

**RESULTS**

**Ionic Strength Dependence of the ESE Rate Constant** - The influence of increasing [PCuII] and ionic strength (using NaCl) on the inversion recovery experiments of spinach PCuI is demonstrated in Supplemental Fig. S1. Plots of $T_1^{-1}$ against [PCuII] for His resonances in spinach, D. crassirhizoma, U. pertusa, and A. variabilis PCus at various ionic strengths (NaCl) are shown in Supplemental Figures S2, S3, S4 and S5, respectively. The ESE rate constants ($k_{ese}$) obtained from the average of $k_1$ values (which are less prone to errors than $k_2$ values) of at least two resonances, are plotted as a function of ionic strength in Fig. 2A and the low and high ionic strength values are summarized in Table I. At the lowest ionic strength ($I = 0.03$ M) the ESE of spinach PCu is extremely slow ($k_{ese} = 4.8 \times 10^2$ M$^{-1}$s$^{-1}$). As the ionic strength is raised, the $k_{ese}$ increases and at $I = 2.03$ M, the value of $9.1 \times 10^4$ M$^{-1}$s$^{-1}$ is 190-fold larger. Similar trends for the ionic strength dependence of $k_{ese}$ are observed for D. crassirhizoma and U. pertusa PCus (see Fig. 2A), both of which exhibit significantly larger $k_{ese}$ values than spinach PCu at low $I$ (Table I). The overall accelerating effect of increasing ionic strength on $k_{ese}$ is 29- and 21-fold for D. crassirhizoma and U. pertusa PCus, respectively. In contrast to the other PCus, the $k_{ese}$ for the A. variabilis protein exhibits very little dependence on ionic strength (see Table I and Fig. 2A).

The influence of ionic strength on $k_{ese}$ of the PCus has been analyzed using van Leeuwen theory (35,36), which considers monopole-monomole, monopole-dipole and dipole-dipole interactions involved in protein-protein association, using equations 1-4.

$$\ln\left(\frac{k_{ese}}{k_{ini}}\right) = -(Z_{ox}Z_{red} + (ZD)(1 + \kappa r) + (DD)(1 + \kappa r)^2)\left(q^2/4\pi\varepsilon_0k_BT\rho Tr\right)f(\kappa)$$ (1)

$$ZD = (Z_{ox}D_{\text{red}} + Z_{red}D_{\text{ox}})/qr$$ (2)

$$DD = D_{\text{ox}}D_{\text{red}}(qr)^2$$ (3)

$$f(\kappa) = (1 - \exp(-\kappa r))/\kappa r(1 + \kappa r/2)$$ (4)

where $Z_{ox}$ and $Z_{red}$ are the net charges on PCuII and PCuI respectively, $D_{\text{red}}$ and $D_{\text{ox}}$ are the
components of the dipole moments through the site of electron transfer, \( r \) is the sum of the radii of the two proteins (31.0 Å for PCu ESE), \( \kappa = 0.329(T^{1/2}) \) Å⁻¹, \( k_{\text{ese}} \) is the ESE rate constant at a given ionic strength (I) and \( k_{\text{inf}} \) is the rate constant at infinite I. The symbols \( q, k_B, \epsilon_0, \epsilon \) and \( T \) are the elementary charge (1.6 × 10⁻¹⁹ C), Boltzmann’s constant, \( (1.3807 \times 10^{-23} \text{ JK}^{-1}) \), the permittivity constant (8.85418 × 10⁻¹² \( \text{C}^2\text{N}^{-1}\text{m}^{-2} \)), the static dielectric constant (80) and the absolute temperature respectively. Knowing the charges and radii of PCuII and PCuI the experimental data can be fit (see Fig. 2B) to give \( D_{\text{ox}}, D_{\text{red}} \) and \( k_{\text{inf}} \) values of -141 D, -159 D and \( 1.5 \times 10^5 \text{ M}^{-1}\text{s}^{-1} \), respectively for spinach PCu. Fitting of the data for the other proteins is less precise due to the limited number of data points, but the \( k_{\text{inf}} \) values are informative (see Table I).

The influence of increasing concentration of the physiologically-relevant Mg²⁺ cation on the ESE rate constant of spinach PCu has also been investigated. Plots of \( T_{1}^{-1} \) against [PCuII] for the His87 C resonance of spinach PCu at different MgCl₂ concentrations are shown in Supplemental Fig. S6 and the \( k_{\text{ese}} \) values are compared to those in which the ionic strength was altered by the addition of NaCl in Fig. 2A. The \( k_{\text{ese}} \) increases 133-fold from \( 4.8 \times 10^2 \text{ M}^{-1}\text{s}^{-1} \) at \( I = 0.03 \text{ M} \) (10 mM phosphate) to \( 6.4 \times 10^4 \text{ M}^{-1}\text{s}^{-1} \) at \( I = 1.91 \text{ M} \) (10 mM phosphate plus 0.63 M MgCl₂).

To obtain further information about the interactions involved in homodimer formation the temperature dependence of the ESE reactivity of spinach PCu has been studied at \( I = 0.28 \text{ M} \). Plots of \( T_{1}^{-1} \) against [PCuII] for the His87 Cδ¹H resonance of spinach PCu at different temperatures are shown in Supplemental Fig. S7. Under these conditions \( k_{\text{ese}} \) increases from \( 5.8 \times 10^3 \text{ M}^{-1}\text{s}^{-1} \) at 10 °C to \( 6.0 \times 10^4 \text{ M}^{-1}\text{s}^{-1} \) at 48 °C. The data were fit (see Fig. 3) to the Eyring equation (5) which provides the activation parameters for this reaction.

\[
k_{\text{ese}} = (k_B T/h) \exp(-\Delta H^\ddagger/RT + \Delta S^\ddagger/R)
\]  

In equation (5), \( h \) is Planck’s constant, \( R \) is the gas constant, \( \Delta H^\ddagger \) is the activation enthalpy and \( \Delta S^\ddagger \) is the activation entropy. Values of \( \Delta H^\ddagger = 44 \text{ kJmol}^{-1} \) and \( \Delta S^\ddagger = -16 \text{ Jmol}^{-1}\text{K}^{-1} \) are obtained for the ESE reaction of spinach PCu (giving a \( \Delta G^\ddagger \) of 49 kJmol⁻¹ at 298 K).

**Analysis of Cupredoxin Structures** - The PQS server (23) provides co-ordinates of likely quaternary states for protein structures determined by X-ray crystallography. Among the cupredoxins, azurin frequently crystallizes as a dimer or as a tetramer, in which the interfaces are formed between the hydrophobic patches of adjacent monomers (37). Auracyanin, an azurin-like protein, forms similar crystallographic dimers (38). Crystal dimers have also been observed for rusticyanin (39) and a hydrophobic patch mutant (Pro94Phe) of amicyanin (40). A survey of the PCu structures in the PQS database reveals several that contain two or more molecules in the asymmetric unit. The *S. pratensis* (24) protein is of particular interest since it forms a crystallographic dimer similar to those found in azurin, auracyanin, rusticyanin and amicyanin (Table II lists the features of the interfaces in these dimers). A Cu-Cu distance of 14 Å, compatible with fast ET, is observed in the *S. pratensis* PCu dimer. There is a small contact area involved in dimer formation and the orientation of the monomers minimizes repulsion between the acidic patches (see Fig. 4), resulting in a Coulombic electrostatic energy of ~50 kJ mol⁻¹.

**Models of PCu Homodimers** - To obtain a first approximation of the homodimer interfaces involved in ESE, models based on the crystallographic dimer of *S. pratensis* PCu (1byo) (24) were built. The interface properties of these models are summarized in Table II with the overall features for spinach, *U. pertusa* and *A. variabilis* proteins similar to *S. pratensis* PCu. In all cases, the contact surfaces are small, hydrophobic and poorly packed, and the interfaces are extremely planar. Notably, the electrostatic energy of the PCu dimers decreases dramatically from spinach to *U. pertusa* to *A. variabilis*. In terms of sequence and structure *D. crassirhizoma* PCu is the most divergent member of the family (41). The large structural differences around the exposed His ligand in this PCu are reflected in the altered character of its homodimer interface, which is 150 Å² larger than in the *S. pratensis* protein. Moreover, the side chains of Glu8, Glu34, Glu68 and Asp69 form directly
opposing clusters in the dimer, resulting in unfavorable Coulombic repulsion (Table II). It can be concluded that the *D. crassirhizoma* PCu homodimer is poorly represented by the model based on the *S. pratensis* structure.

**Protein Docking** - Docking simulations were used to obtain further information about the structure of the transient homodimers formed during ESE. The ClusPro protocol was used as it has been successful in docking PCu and cyt *f* (21). As the conserved hydrophobic patch of the cupredoxins (41) is known to be involved in protein interactions (17,19-21,42,43) including ESE (44,45) a constrained approach has been used in which a favorable bias was introduced for docking orientations involving the exposed His ligand.

Docking results (hits) involving the hydrophobic patch and having a Cu-Cu distance of \( \leq 15 \) Å were considered representative of productive binding (see Table III). Five docking orientations were found for spinach PCu with contact areas ranging from 420-570 Å\(^2\), with an average content of 60 % non-polar (carbon) atoms. With the exception of the top-ranking orientation, the interfaces produced are typical of what is expected for this type of interaction (compare Tables II and III). In particular, the geometric complementarity is low, while the atoms involved in the interface have a highly planar arrangement.

Despite sharing 60 % sequence identity with spinach PCu, only three positive docking hits were found for the *U. pertusa* protein. This may be a result of the amino acid deletions around the upper acidic patch of *U. pertusa* PCu (Fig. 1). The fourth ranked complex involves only two of the loops in the vicinity of the hydrophobic patch (residues 10-14 and 85-90) in an “edge-on” interaction (whereas usually all four loops are involved in a “head-on” association). This gives rise to an uncharacteristically high rmsd for the interface planarity and a larger than expected contact area. The complexes ranked 7 and 10 are more similar to the hits found for spinach PCu, except that the geometric complementarity is higher. In the case of *A. variabilis* PCu, the docking simulations gave rise to five hits involving the hydrophobic patch of the protein, four of which have Cu-Cu distances \( \leq 15 \) Å. The highly planar interfaces of the *A. variabilis* PCu homodimers are larger (520-690 Å\(^2\)) and more hydrophobic (average \( f_{NP} 79 \%) than in the spinach and *U. pertusa* proteins (Table III).

The constraints applied to the exposed His ligand, were insufficient to produce docking orientations involving the hydrophobic patches of *D. crassirhizoma* PCu. Presumably, Coulombic repulsions between the acidic residues around the hydrophobic patch increase the difficulty of finding low-energy docking orientations for this protein. When additional constraints were applied to the exposed \( C^{82}, C^{e1} \) and \( C^{e2} \) atoms of Phe12 (homologous to Leu12 and adjacent to His87, which is important for the interaction with both cyt *f* and PSI) a single docking result involving the hydrophobic patches was obtained. The interface of this dimer, while similar in terms of planarity and packing to those of the other PCus, is the least hydrophobic (Table III). It is interesting that in the cases of spinach, *U. pertusa* and *A. variabilis* PCus, the calculated Coulombic electrostatic energy of the modeled homodimers (based on the *S. pratensis* PCu structure) and the complexes obtained from the docking simulations are remarkably similar (compare Tables II and III). In contrast, the Coulombic repulsion for *D. crassirhizoma* PCu is approximately halved in the docking orientation with respect to the modeled homodimer.

**DISCUSSION**

**ESE and Protein Association** - Protein-protein association rates are typically of the order of \( 10^5-10^6 \) M\(^{-1}\)s\(^{-1}\) with an upper limit of \( \sim 10^9 \) M\(^{-1}\)s\(^{-1}\) for interactions between oppositely charged proteins (46). The ESE reaction requires almost identical molecules to associate. Therefore Coulombic repulsion makes this an unfavorable process for charged proteins. This is the case for spinach PCu where the large acidic patch (Fig. 1) severely impedes homodimer formation at low \( I \) (Table I). The larger \( k_{ese} \) values for *D. crassirhizoma* and *U. pertusa* PCus at low \( I \) indicate that their acidic patches have a diminished influence on ESE. As the ionic strength is increased, Debye screening reduces the
provide comparative information about factors affecting $k_{eq}$ such as relative Cu-Cu distances, and it is interesting to note that the $D_{Cu-Cu}$ value is largest in the A. variabilis docked complexes consistent with a smaller $k_{eq}$.

Physical Basis for Transient Interactions - Complexes of ET proteins have interfaces with particularly poor geometric complementary (6). Poorly packed interfaces have fewer intermolecular interactions, thus favoring a high $k_{off}$. Furthermore, a low geometric complementarity implies that association of the protein surfaces does not yield the maximum exclusion of surface water molecules, and thus the hydrophobic effect is diminished with respect to a tightly packed interface. The temperature dependence of the ESE reactivity of spinach PCu (Fig. 3) yields $\Delta H^\ddagger$ and $\Delta S^\ddagger$ values of 44 kJmol$^{-1}$ and -16 Jmol$^{-1}$K$^{-1}$, respectively. The $\Delta H^\ddagger$ value for spinach PCu is consistent with the electrostatic repulsion which has to be overcome for ESE encounter complex formation. The fact that the reaction is also disfavored on the grounds of activation entropy suggests that displacement of water molecules from the protein interface is insufficient to counteract the loss of translational and rotational freedom upon protein-protein association. Thus the experimental data provides evidence of poor packing in the interface.

The arrangement of the interface atoms in the cupredoxin crystal dimers is highly planar (the atoms have a low rmsd from the plane which intersects the interface, Table II). This is especially true for the S. pratensis PCu dimer, with an rmsd close to the van der Waals radii. Such planar interfaces were reproduced in the docking simulations (Table III). A survey of the PQS server (23) reveals several other redox proteins that form crystal dimers with properties similar to ET complexes (see Supplemental Table SI). In all of these structures the interfaces are small, poorly packed and highly planar. Interface planarity contributes further to the decrease in the hydrophobic effect since it has been shown that concave and convex surfaces of equal but opposite curvature interact more strongly than two planar surfaces (33). A recent study (4) supports the idea that highly planar interfaces favor low affinity interactions. An average planarity of 3.8 Å was found amongst a group of stable homodimers. In contrast, transient homodimers had an average planarity of 2.0 Å. High planarity is consistent with poor packing since it means that the interface is defined by the extremities of the protein surfaces, which approach each other to the minimal distance necessary for desolvation. Unlike the protein interior or the interfaces found in obligate protein complexes, there is little or no “inter-digitation” of the side chains. Therefore, as the planarity of an interface increases, both desolvation and intermolecular contacts decrease enabling faster dissociation rates, which are imperative for transient protein interactions.

Physiological Role for ESE - Although frequently observed, transient homodimers do not always have an obvious biological role (4,7). However, the ESE reaction of PCu has potential physiological relevance. During photosynthesis, the environments of the cyt $b_{6}f$ complex and PSI (both membrane bound) are reducing and oxidizing respectively. This will give rise to a concentration gradient of PCuI and PCuII, which functions as an electron transporter between the two complexes. As PCuI diffuses down the concentration gradient in the vicinity of cyt $f$ it will encounter other molecules of PCuI and an increasing concentration of PCuII, which can accept electrons for subsequent delivery to PSI. This process of continuous ESE can be compared to proton transfer in water, which occurs via a network of hydrogen bonded molecules, rather than by the net displacement of one molecule bearing the proton. Several features of the photosynthetic apparatus favor this hypothesis. PCu is one of the most abundant proteins in the thylakoid (51,52) and exists as a pool of both the CuII and CuI forms (53). Furthermore, during high flux rates the PCu concentration increases approximately 10-fold in response to the increased electron flow (54). The high protein concentration of the thylakoid lumen (> 20 mg/mL) (51) and the constricted volume promotes transient interactions via crowding effects thus enabling ESE (3). The ionic strength within the thylakoid lumen is estimated to be about 0.1 – 0.2 M, thus facilitating ESE by Debye screening. As well as general screening at high ionic strength, Coulombic repulsion between
acidic PCu molecules will be diminished further by the presence in the thylakoid lumen of Mg\(^{2+}\) ions at approximately mM concentrations (55), which have been implicated in catalysis of the oxidation of PCu\(^1\) by PSI (56). The results presented in Fig. 2A demonstrate that ESE is dramatically enhanced in an acidic PCu even at low concentrations of Mg\(^{2+}\) (the addition of 10 mM MgCl\(_2\) increases \(k_{ese}\) approximately 30-fold). Therefore, under physiological conditions the ESE reactivity is expected to reach a value of \(\sim 10^5\) M\(^{-1}\)s\(^{-1}\) regardless of the surface charge properties (or distribution) of the PCu. Similar conclusions have been made about the in vitro reactivity of PCu and cyt \(f\) from different organisms at physiological ionic strength (57) and electrostatics seem to be less important for this interaction in vivo (58). Taken together all these facts suggest the possibility of ESE facilitating inter-complex ET during photosynthesis.

CONCLUSIONS

The analysis of the ionic strength dependence of the ESE reactivity for PCus confirms that homodimer formation occurs via the conserved hydrophobic patch. PCu homodimers, generated by modeling and docking simulations, provide a structural framework for rationalizing the relative influence of ionic strength on \(k_{ese}\). The interfaces of the ESE homodimers have low complementarity and high planarity, features which are essential for transient protein interactions. The accelerating effect of physiological concentrations of the Mg\(^{2+}\) ion, plus the known attributes of the photosynthetic machinery indicates a potential biological role for ESE.

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REFERENCES


FIGURE LEGENDS

Fig. 1. A, The structure of spinach PCu (PDB code 1ag6) (11). The coordinating side-chains, the acidic patch residues and the position of the hydrophobic patch are indicated. The acidic patch is made up of the upper (E59, E60 and D61) and lower (D42, E43, D44 and E45) regions. B, Sequence alignment of the PCus from S. pratensis, spinach, U. pertusa, A. variabilis, and D. crassirhizoma obtained using DbClustal (14). Fully conserved residues, including the four copper ligands (in purple), are marked with an asterisk. Those residues which contribute to the top of the molecule (as represented in A) and the hydrophobic patch are enclosed in boxes. Charged residues, which contribute to the acidic patches (plant and algal PCus) or which are located near the hydrophobic patch are red (acidic) or blue (basic). C, The surface properties of PCus from spinach (1ag6) (11), D. crassirhizoma (1kdj) (13), U. pertusa (1iuz) (12) and A. variabilis (1nin) (10) in which the exposed imidazole ring of His87 is shown in purple and the surrounding hydrophobic patch is yellow. The acidic and basic residues are red and dark blue, respectively, polar residues are cyan and Tyr83 is green.

Fig. 2. A, Ionic strength dependence (25 °C) of log $k_{\text{ese}}$ for spinach (●), U. pertusa (■), D. crassirhizoma (▲) and A. variabilis (▲) PCus. The ionic strength was adjusted by the addition of NaCl to 10 mM phosphate buffer (99.9 % D2O) pH* 8.0 for spinach, U. pertusa and D. crassirhizoma PCus and at pH* 6.2 for A. variabilis PCu. For the $k_{\text{ese}}$ determination of U. pertusa PCu at $I$ = 0.10 M, 35 mM phosphate buffer pH* 8.0 was used and 73 mM phosphate buffer (pH* 6.2) was utilized for the A. variabilis PCu measurement at $I$ = 0.10 M. The $k_{\text{ese}}$ value for D. crassirhizoma PCu at $I$ = 0.10 M was taken from ref. 34 (36 mM phosphate buffer at pH* 7.9). Also included is the ionic strength dependence (25 °C) of log $k_{\text{ese}}$ for spinach PCu in 10 mM phosphate buffer (99.9 % D2O) at pH* 8.0 with added MgCl₂ (○). B, Ionic strength dependence (25 °C) of ln $k_{\text{ese}}$ for spinach PCu (using the $k_1$ data for the His37 Cδ2H resonance only). The solid line is the fit of the data to van Leeuwen theory (35) which yields values of -141 D, -159 D and $1.5 \times 10^5$ M⁻¹s⁻¹ for $D_{\text{ox}}^\prime$, $D_{\text{red}}^\prime$, and $k_{\text{inf}}$ respectively. Best fits were obtained when the ionic strength was calculated on the basis of the small ion concentration plus the charge on the protein multiplied by the protein concentration.

Fig. 3. Eyring plot of the ESE rate constant of spinach PCu in 10 mM phosphate buffer (99.9 % D2O) at pH* 8.0 plus 250 mM NaCl ($I$ = 0.28 M).

Fig. 4. The molecular surface and electrostatic properties of the S. pratensis PCu (24) crystal dimer (1byo). Images were created with a color ramp for positive (blue) or negative (red) surface potentials saturating at 10 kT. The surface potentials were calculated for formal charges at $A$, 0 and $B$, 0.1 M ionic strength using the program GRASP (33).
TABLE I

*Electron self-exchange rate constants of the PCUs*

<table>
<thead>
<tr>
<th>Plastocyanin</th>
<th>Net Charge</th>
<th>( k_{\text{ese}} \times 10^4 ) (M(^{-1})s(^{-1})) Low I</th>
<th>( k_{\text{ese}} \times 10^4 ) (M(^{-1})s(^{-1})) High I</th>
<th>( k_{\text{inf}} \times 10^4 ) (M(^{-1})s(^{-1}))</th>
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<td>–9</td>
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<td>9.1(^d)</td>
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<tr>
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<td>1.7(^c)</td>
<td>35(^d)</td>
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<tr>
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<td>0.12(^c)</td>
<td>3.5(^d)</td>
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<tr>
<td>A. variabilis</td>
<td>+1</td>
<td>17(^c)</td>
<td>34(^f)</td>
<td>53</td>
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\(^a\) Net charge as calculated for the reduced protein (PCu\(^i\)) at neutral pH. \(^b\) \( k_{\text{inf}} \) obtained from fits of the ionic strength dependence of \( k_{\text{ese}} \) to van Leeuwen theory (35) (see Fig. 2B). \(^c\) \( I = 0.03 \) M. \(^d\) \( I = 2.03 \) M. \(^e\) \( I = 0.01 \) M. \(^f\) \( I = 2.01 \) M.
### TABLE II
Interface Properties of Cupredoxin Crystal Dimers and PCu Dimer Models

<table>
<thead>
<tr>
<th>Protein</th>
<th>PDB</th>
<th>Size (Å²)</th>
<th>( f_{NP} )</th>
<th>Packing ( \text{GV} )</th>
<th>Planarity (rmsd)</th>
<th>( E_{\text{Coulomb}} ) (kJ/mol)</th>
<th>( D_{\text{Cu-Cu}} ) (Å)</th>
<th>Bridging groups</th>
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\( a \) The area of the monomer surface which becomes solvent inaccessible in the homodimer. \( b \) Fraction of the interface composed of non-polar (carbon) atoms. \( c \) The gap volume (GV) index (32) is a measure of geometric complementarity in the interface. \( d \) An alternative indication of packing given by the shape complementarity (SC) (33). \( e \) Defined as the rmsd of interface atoms (in Å) from the best-fit plane through the interface. \( f \) Hydrogen bond mediators between the exposed His ligands. \( g \) Not applicable.
<table>
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<tr>
<th>Protein</th>
<th>Rank No.</th>
<th>Size (Å²)</th>
<th>f&lt;sub&gt;NP&lt;/sub&gt;</th>
<th>GV</th>
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<th>E&lt;sub&gt;Coulomb&lt;/sub&gt; (kJ/mol)</th>
<th>D&lt;sub&gt;Cu-Cu&lt;/sub&gt; (Å)</th>
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<sup>a</sup> A constrained, symmetric docking approach was used, see Materials and Methods. <sup>b</sup> In the case of *D. crassirhizoma* PCu it was necessary to apply additional constraints using the side chain of Phe12.
Figure 1

A

B

C

Spinach  U. pertusa  D. crassirhizoma  A. variabilis

16
Figure 2
Figure 3
Figure 4
SUPPLEMENTAL MATERIAL

Transient Homodimer Interactions Studied Using the Electron Self Exchange Reaction

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**TABLE SI**

*Interface Properties of Crystallographic Dimers of Redox Proteins*

<table>
<thead>
<tr>
<th>Protein</th>
<th>PDB</th>
<th>Size (Å²)</th>
<th>( f_{NP} )</th>
<th>Packing</th>
<th>Planarity (rmsd)</th>
<th>( D_{ET} ) (Å)</th>
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</table>

* a The area of monomer surface which becomes solvent inaccessible in the homodimer.  
  b Fraction of the interface composed of non-polar atoms.  
  c The gap volume (GV) index is a measure of geometric complementarity in the interface.  
  d An alternative indication of packing given by the shape complementarity (SC).  
  e Defined as the rmsd of interface atoms from the best fit plane through the interface.  
  f Distance between the metal centres.  
  g This structure has a dimer arrangement involving contacts between the two haem edges.  
  h Improperly folded cytochrome c₅₅₂ from Thermus thermophilus.  
  i The interface in the crystal dimer of cyt c₆ from Scenedesmus obliquus is homologous to that used for the interaction of A. variabilis cyt c₆ with cyt f.
Figure S1. Selected spectra from inversion recovery experiments on spinach PCu(I) at 25 °C in 10 mM phosphate buffer (99.9 % D₂O) at pH* 8.0 showing the influence of [PCu(II)] and ionic strength on the relaxation rates of the His37 C⁶²H (7.64 ppm in A and B, and 7.77 ppm in C and D) and the His87 C⁶¹H (7.70 ppm in A and B, and 7.87 ppm in C and D) resonances. The spectra shown in A and B were obtained at $I = 0.07$ M, with 15 μM and 210 μM of [PCu(II)], respectively. The spectra in C and D were acquired at $I = 2.03$ M, with 19 μM and 132 μM [PCu(II)], respectively. The interpulse delays (τ₀) used in the inversion recovery experiments are shown.

(A) $I = 0.07$ M
[PCu(II)] = 15 μM  τ₀ (s)

(B) $I = 0.07$ M
[PCu(II)] = 210 μM  τ₀ (s)

(C) $I = 2.03$ M
[PCu(II)] = 19 μM  τ₀ (s)

(D) $I = 2.03$ M
[PCu(II)] = 132 μM  τ₀ (s)
*Figure S2.* Plots (25 °C) of $T_1^{-1}$ against [PCu$^{II}$] for the His87 C$^{\varepsilon1}$H resonance of spinach PCu$^{I}$ in 10 mM phosphate buffer (99.9 % D$_2$O) pH* 8.0 at the following NaCl concentrations: 0 M, $I = 0.03$ M (●); 0.50 M, $I = 0.53$ M (■); 1.20 M, $I = 1.23$ M (▲) and 2.00 M, $I = 2.03$ M (×).
Figure S3. Plots (25 °C) of $T_1^{-1}$ against $[\text{PCu}^{II}]$ for the His87 C$^{52}$H resonance of
*D. crassirhizoma* PCu in 10 mM phosphate buffer (99.9 % D$_2$O) pH* 8.0 at the
following NaCl concentrations: 0 M, $I = 0.03$ M (●); 0.50 M, $I = 0.53$ M (■) and
2.00 M, $I = 2.03$ M (▲).
Figure S4. Plots (25 °C) of $T_1^{-1}$ against [PCu$^{II}$] for the His37 C$^{52}$H resonance of *U. pertusa* PCu$^1$ in 10 mM phosphate buffer (99.9 % D$_2$O) pH* 8.0 at the following NaCl concentrations: 0 M, $I = 0.03$ M (●); 0.50 M, $I = 0.53$ M (▲) and 2.00 M, $I = 2.03$ M (△). Also included are data at $I = 0.10$ M (■) which were measured in 35 mM phosphate buffer pH* 8.0 (99.9 % D$_2$O).
Figure S5. Plots (25 °C) of $T_1^{-1}$ against $[\text{PCu}^{ll}]$ for the His37 C$^{32}$H resonance of *A. variabilis* PCu$^I$ in 10 mM phosphate buffer (99.9 % D$_2$O) pH* 6.2 at the following NaCl concentrations: 0 M, $I = 0.01$ M (○) and 0.50 M, $I = 0.51$ M (▲). Also included are data at $I = 0.10$ M (■) which were measured in 73 mM phosphate buffer pH* 6.2 (99.9 % D$_2$O).
Figure S6. Plots (25 °C) of $T_{1}^{-1}$ against [PCu$^{II}$] for the His87 C$^{6+}$H resonance of spinach PCu$^{I}$ in 10 mM phosphate buffer (99.9 % D$_2$O) pH* 8.0 at the following MgCl$_2$ concentrations: 0 M, $I = 0.03$ M (○); 0.01 M, $I = 0.07$ M (■); 0.16 M, $I = 0.52$M (▲) and 0.63 M, $I = 1.91$ M (×).
**Figure S7.** Plots of $T_1^{-1}$ against [PCu$^{II}$] for the His87 C$^{61}$H resonance of spinach PCu$^I$ in 10 mM phosphate buffer (99.9 % D$_2$O) pH$^*$ 8.0 plus 250 mM NaCl ($I = 0.28$ M) at 10 °C (●), 25 °C (■), 38 °C (▲) and 48 °C (×).