Effects of interface mutations on association modes and electron-transfer rates between proteins

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Although bonding networks determine electron-transfer (ET) rates within proteins, the mechanism by which structure and dynamics influence ET across protein interfaces is not well understood. Measurements of photochemically induced ET and subsequent charge recombination between Zn-porphyrin-substituted cytochrome *c* peroxidase and cytochrome *c* in single crystals correlate reactivity with defined structures for different association modes of the redox partners. Structures and ET rates in crystals are consistent with tryptophan oxidation mediating charge recombination reactions. Conservative mutations at the interface can drastically affect how the proteins orient and dispose redox centers. Whereas some configurations are ET inactive, the wild-type complex exhibits the fastest recombination rate. Other association modes generate ET rates that do not correlate with predictions based on cofactor separations or simple bonding pathways. Inhibition of photoinduced ET at <273 K indicates gating by small-amplitude dynamics, even within the crystal. Thus, different associations achieve states of similar reactivity, and within those states conformational fluctuations enable interprotein ET.

cytochrome | protein dynamics | protein-protein interaction | electron tunneling

Many long-range electron-transfer (ET) reactions in biology occur across transient protein-protein interfaces. Reaction rates depend on factors that control both electron tunneling and conformational dynamics coupled to protein association processes (1–3). As such, interprotein ET is sensitive to structure and dynamics at the interface (1, 4–11). Residue substitution (achieved either by use of protein homologs, site-directed mutants, or computations) has been a popular and powerful approach for probing how interface composition influences interprotein ET (7, 10–14). Nevertheless, effects of residue variation on interface structure are not often known.

The natural redox partners yeast cytochrome *c* peroxidase (CcP) and yeast cytochrome *c* (yCc), whose structure as a complex was first determined in 1992 (15) and later as a covalent complex (16), have served as a paradigm for studying interprotein ET reactions (8, 17). Hoffman and colleagues (8) have exploited ZnCcP substitution to photoactivate ET reactions and examine the effects of many structural and chemical perturbations on interprotein ET. In this system, the ZnCcP triplet state ($^3\text{ZnCcP}$) reduces Fe(III)Cc, and then back ET recombines the charge separation (Fig. 1). Recently, it has been demonstrated that a Trp-191 $\rightarrow$ Phe CcP variant has much slower ET back-rates (k_{et}) than wild-type (WT) CcP in the 1:1 complex with yCc (18). Thus, electron hopping through Trp-191 may accelerate the recombination reaction (Fig. 1), in analogy to the natural reaction between CcP compound I and Fe(II)Cc (19). Nevertheless, much slower ET back-rates in the complex between CcP and hCc compared with yCc, both in solution (20) and in crystals (21), indicate that ET across the protein-protein interface limits the overall rate. Because of their close proximity, Zn(II)porphyrin $^{+}$ and Trp $^{+}$ likely equilibrate rapidly compared with the interprotein ET step. Consistent with fast equilibration between Zn(II)porphyrin $^{+}$ and Trp $^{+}$, reduction of Zn(II)porphyrin $^{+}$ in crystals depends on the contact with the Cc donor (21) and produces much slower interprotein ET than that observed between

CcFe(II) and Trp $^{+}$ of compound I (19). Thus, when comparing relative ET rates for different CcP:Cc complexes, ET to an acceptor center that includes both the Zn-porphyrin $^{+}$ and Trp-191 $^{+}$ must be considered (Fig. 1).

To better reconcile structural perturbations with reactivity, we have measured photochemically initiated ET reactions between Zn-porphyrin-substituted CcP (ZnCcP) and site-directed mutants of yCc in single crystals and determined structures of the complexes by x-ray diffraction. Whereas substitutions of Cc Phe-82 to either Trp (CcW) or Ser (CcS) largely retain the interaction with ZnCcP observed for the WT Cc, substitutions to both Tyr (CcY) and Ile (CcI) change how the proteins associate. These structurally distinct complexes, as well as an additional configuration provided by horse Cc (hCc) in complex with yeast ZnCcP complex (21), all have similar ET reactivity despite considerably different donor-to-acceptor distances and intervening structures. The ET properties of the four Phe-82 Cc variants with CcP contrast with that of the WT complex and that of the F82Y/K72S yCc double mutant, which exhibit much higher and much lower reactivity, respectively.

Materials and Methods

Protein Mutagenesis, Expression, and Purification. Mutants of yCc were constructed by overlap extension PCR in pBTR1 [a bacterial overexpression vector for yeast *iso-1*-cytochrome Cys-102 $\rightarrow$ Ser, which will be referred to as WT (22, 23)]. Cells were expressed in *Escherichia coli* BL-21(DE3) with 50 mg δ -aminolevulinic acid to improve heme incorporation. Protein was purified as described in refs. 21–23, dialyzed against two changes of 10 mM ammonium acetate at 4°C, and concentrated to 0.5 mM ($\epsilon_{416} = 129 \text{ mM}^{-1}\text{cm}^{-1}$).

Crystallization and X-Ray Data Collection. Crystals of Fe(III)yCc:ZnCcP grew from 10- μl drops (either hanging or sitting) made from equal volumes of reservoir and 0.5 mM 1:1 complex. The protein complex was initially oxidized with potassium ferricyanide and dialyzed extensively against water before crystallization. Crystallization drops were equilibrated against 500 μl of reservoir containing 15–20% poly(ethylene glycol) (molecular weight 3,350) and 150–200 mM NaCl. Addition of *N*-octyl- β -D-glucoside (1–4 mM), 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (1–2 mM), or adenosine-5-triphosphate disodium salt (10 mM) facilitated formation of diffraction-quality crystals. Crystals of F82W and F82S mutant complexes belong to space group $P2_1$ with unit cell dimensions $a = 44 \text{ \AA}$, $b = 118 \text{ \AA}$, $c = 88 \text{ \AA}$, and $\gamma = 104^\circ$ with two molecules of the ZnCcP:yCc complex per asymmetric unit (21). However, crystals of F82Y and F82I mutant complexes belong to space group $P2_12_12_1$ with unit cell dimensions $a = 44 \text{ \AA}$, $b = 52 \text{ \AA}$, $c =$

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Abbreviations: ET, electron transfer; Cc, cytochrome *c*; CcP, Cc peroxidase; hCc, horse Cc; yCc, yeast Cc; ZnCcP, Zn-porphyrin-substituted CcP.

Data deposition: The atomic coordinates have been deposited in the Protein Data Bank, www.pdb.org (PDB ID codes 2B10, 2B11, 2B07, and 2B12).

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Fig. 1. Reactions initiated by photoinduced ET between ZnCcP and Fe(III) yCc.

183 Å. High-resolution diffraction data were collected at the Cornell High Energy Synchrotron Source, beamline A1, and the National Synchrotron Light Source, beamline X-25. Temperature was controlled by an Oxford Cryodevice, and 15% (vol/vol) ethylene glycol cryoprotectant was used when necessary. All diffraction data (see Table 2, which is published as supporting information on the PNAS web site) were indexed, integrated, and scaled by using HKL 2000 (24).

Crystallographic Structure Determinations. Initial models for refinement were obtained by molecular replacement [AMORE (25)]; the probes consisted of WT ZnCcP:yCc (Protein Data Bank ID code 1U74) for complexes of the F82W Cc and F82S Cc mutants and ZnCcP alone for complexes of the F82Y Cc, F82I Cc, and F82Y/K72S Cc mutants. In the latter cases, yCc was placed manually in electron-density maps. Rigid-body, positional, and thermal factor refinement was implemented with CNS (26), amidst rounds of manual rebuilding, Zn-protoporphyrin incorporation, and water placement with XFIT (27). TLS parameters for the translation, liberation, and screw-rotation displacements of a pseudorigid body were refined in REFMAC (28) to generate the final models (Table 2). All residues of four structures have good stereochemistry with no disfavored backbone dihedral angles. In all of the reported structures, the crystal lattices are close-packed, and no significant electron density remains unaccounted for.

Crystal Spectroscopy. Crystal ($\approx 100 \times 100 \times 10 \mu\text{m}^3$) were grown in an anaerobic chamber, mounted, and sealed inside $1 \times 1\text{-mm}$ quartz capillaries to prevent oxidative photodegradation of ZnCcP. Transient absorption spectroscopy was carried out with a custom-built microspectrophotometer, as described in ref. 21. Kinetic traces were fit to exponential-decay processes by nonlinear least-squares (ORIGIN 7, Microcal, Amherst, MA). Although signals were followed for both intermediates [Fe(II)yCc at 520 nm and ZnCcP⁺ at 680 nm], because of better signal-to-noise ratio, the progress curves at 680 nm were used to describe the rate constants. Data are presented from a representative experiment, although rate constants are given as averages from multiple (more than three) crystals. Crystals were reduced to Fe(II)Cc by titration with increasing amounts of dithionite to evaluate natural ³ZnCcP decay constants. Formation of the charge-separated intermediate, ZnCcP⁺, was fitted to the function $\Delta A(t) = [\beta \sum f_i (\exp(-k_i t) - \exp(-k_{eb} t)) / (k_{eb} - k_i)] + A_3 \exp(-k_3 t)$, where $i = 1, 2$; k_{eb} is the intracomplex Fe(II)Cc to ZnCcP⁺ ET rate constant for phase i ; f_i is the fractional weight of each kinetic phase; and A_3 accounts for changes in triplet state absorption (29). If ³ZnCcP transfers electrons with a rate constant k_e to an Fe(III)Cc bound, then the factor β is given by $\beta = k_e \Delta \epsilon_\lambda [^3\text{ZnCcP}(0)]$, where k_e is the forward ET rate constant, $\Delta \epsilon_\lambda$ is the difference in extinction coefficients of the intermediate and ground state at wavelength λ , and $[^3\text{ZnCcP}(0)]$ is the initial concentration of ³ZnCcP produced by the laser photolysis. Owing to the larger $\Delta \epsilon_{680}$ value for Zn-porphyrin⁺ compared

to ³Zn-porphyrin (30), the ³ZnCcP contribution to $\Delta A(t)$ was small ($<1.5\%$ as judged by fitted A_3 values). Inclusion of a third kinetic component to recombination reaction ($i = 3$) does not significantly improve the data fits.

Temperature Dependence of Crystal ET. Temperature was regulated by adjusting the flow of cold nitrogen stream from boiling liquid nitrogen with Cryostat (MSC, The Woodlands, TX) and read with a thermocouple mounted on a goniometer (Hampton Research, Riverside, CA) at the crystal position. Measurements were obtained at 10–20°C temperature intervals beginning at -178°C . During a measurement, the temperature was maintained to within $\pm 5^\circ\text{C}$. Single protein crystals were soaked in cryoprotectant [ethylene glycol/glycerol 15% (vol/vol)] and mounted in nylon loops (Hampton Research). Diffraction quality was maintained for crystals subjected to laser pulses of $<1 \text{ mJ}$.

Isothermal Titration Calorimetry. All titrations were carried out by using a VP-ITC titration calorimeter (Microcal) at 25°C . For each titration, 2.0 mM given Phe-82 yCc mutant was titrated with 0.1 mM ZnCcP. Before titration, samples were dialyzed extensively against 50 mM potassium phosphate (pH 7.0).

Electrochemistry. Protein-film voltammetry on gold electrodes modified by carboxylate- and alcohol-terminated alkane thiol mixed self-assembled monolayers was used to evaluate the redox properties of the yCc mutants. See *Supporting Materials and Methods* and Table 3, which are published as supporting information on the PNAS web site, for details and results.

Pathways Calculations. ET coupling between the redox centers of ZnCcP and yCc were examined with HARLEM (31) based on a bonding network model (32).

Results and Discussion

Structures and Redox Properties of ZnCcP in Complex with yCc Phe-82 Mutants. Highly conserved Cc Phe-82 lies adjacent to the heme and contributes 13.0 Å^2 of buried surface area to the interface with CcP. Mutations at Phe-82 modestly affect yCc stability, structure, and redox potential (33–35) and reduce rates of back ET from Fe(II)Cc to ZnCcP⁺ in comparison with WT Cc (12). We mutated Phe-82 of yCc to Tyr, Trp, Ser, and Ile and determined the crystallographic structures of all four variants in complex with yeast CcP (Table 2). The resolution of the structures ($2.3\text{--}3.0 \text{ Å}$) and large anisotropic B-factors associated with the Cc molecules indicate that some of the complexes are not highly ordered (Table 2). Nevertheless, $F_o - F_c$ omit maps confirm the positions of each heme, and thus the relative molecular orientations in each complex are well determined (see Fig. 5, which is published as supporting information on the PNAS web site). Consistent with previous reports (33), these mutations produce only minor perturbations of Cc potential and apparent reorganization energy when analyzed by protein-film voltammetry (Table 3); however, in some cases the binding affinity to ZnCcP decreases by $\approx 10\text{--}100$ fold (Table 1). Surprisingly, whereas CcW and CcS maintain the same contact with CcP as the WT ($1,208 \text{ Å}^2$ buried surface area), CcY and CcI bind CcP through a completely different interface (803 Å^2). Relative to the WT Cc:CcP complex, CcY(I) rotates by $\approx 90^\circ$ and translates by 8 Å (Fig. 2A and B). In the WT complex, Cc binds CcP with regions that surround the Cc heme and involve loops preceding and following the His-18 and Met-80 axial heme ligands, respectively (Fig. 2B). In the altered complex, only the Cc loop preceding Met-80 (residues 75–84) interacts with CcP (primarily on the 193–209 loop and 226–229 β -hairpin; Fig. 2B). It is not obvious why the CcY(S) variants prefer the altered binding interaction, although substitutions at residue 82 do influence the order of the immediate C-terminal loop that would interact with CcP in the WT orientation. Moreover, the new orientation generates a much longer donor-to-acceptor separations

Table 1. Interprotein ET kinetics and binding coefficients between ZnCcP and yCc WT or Phe-82 mutants

Cc	$k_{\text{et}}, * \text{ s}^{-1}$	$k_{\text{eb}}^1 (f^1), * \text{ s}^{-1}$	$k_{\text{eb}}^2 (f^2), * \text{ s}^{-1}$	$\frac{H_{\text{AB}}^2/H_{\text{AB(WT)M-to-M}}^2}{(H_{\text{AB}}^2/H_{\text{AB(WT)T-to-H}}^2)^{\dagger}}$	Donor–acceptor distance, Å			$K_{\text{D}}, \mu\text{M}$
					Metal-to-metal	Edge-to-edge	Trp-to-heme	
Yeast	230	>6,000	—	1 (1)	26.5	15.6	13.6	1.4 [¶]
F82W	310	5,800 (0.92)	60 (0.8)	1 (0.4)	26.4	15.6	14.0	5.0
F82Y	290	4,000 (0.90)	70 (0.10)	10^{-4} (10^{-6}) 10^{-2} (10^{-3}) [§]	29.4	22.8	19.6	20.0
		4,300 (0.92) [‡]	21 (0.06) [‡]					
F82I	140	2,100 (0.81)	40 (0.19)	10^{-4} (10^{-5}) 10^{-1} (0.04) [§]	29.8	22.6	19.4	100.0
		1,900 (0.89) [‡]	19 (0.05) [‡]					
F82S	200	2,000 (0.78)	70 (0.22)	1 (0.9)	26.3	15.4	13.5	10.0
		2,500 (0.72) [‡]	35 (0.12) [‡]					
Horse heart	80	1,700 (0.82)	30 (0.18)	10^{-3} (10^{-6})	29.5	18.8	18.6	12.0 [¶]
Tuna heart	320	2,000	—	10^2 (NA)	24.1	12.7	NA	NA

*Errors in rate constants: $k_e = \pm 30 \text{ s}^{-1}$; $k_e^1 = \pm 500 \text{ s}^{-1}$; $k_e^2 = \pm 30 \text{ s}^{-1}$. Values in parentheses represent the contributions of the two kinetic phases.

[†]M-to-M and T-to-H indicate metal-to-metal and Trp-191-to-heme, respectively. Values in parentheses are for T-to-H calculations.

[‡]Solution data (12).

[§]Pathway calculations performed after manually adding hydrogen bonds between Lys-72 and (Met-78 and Asn-205).

[†]Ref. 29.

^{||}Interprotein ET between ZnCc and Fe(III)Cc in crystals (38).

than in the WT complex (Table 1). We assume that the asymmetric units represent the interactions that dominate ET between molecules in the lattice; all other cofactor separations in the crystals are at least 8 Å larger than those discussed.

ET Kinetics in Crystals. The ET kinetics and relative reactivity of all four Cc Phe-82 mutants (Table 1) agree remarkably well with previous solution studies of the same variants (12). Thus, the crystal associations likely represent the 1:1 complexes formed in solution. Because $k_{\text{eb}} \gg k_{\text{e}}$ (Fig. 1), it has been difficult to unambiguously resolve a transient charge-separated intermediate for the WT ZnCcP:yCc complex in solution (29) and crystals (21). However, in crystals of CcW and CcS, a subtly altered interface slows back ET to within an observable regime (Fig. 3). In these variants, ZnCcP⁺ and Fe(II)Cc form at the rate expected for ³ZnCcP quenching and disappears with multiphasic kinetic phases, whose major components are indeed rapid (Fig. 3 and Table 1). Notably, k_{eb} has decreased compared with WT Cc in the CcW complex even though the substitution induces only small changes of side-chain and solvent molecule positions at the interface.

The ET rate constant between Fe(II)Cc and Trp-191⁺ of CcP compound I in the WT complex [$2 \times 10^6 \text{ s}^{-1}$ (19)] significantly exceeds the largest components of k_{cb} observed for these mutant crystal complexes ($2\text{--}4 \times 10^3 \text{ s}^{-1}$). However, if ZnCcP⁺ and Trp-191⁺ equilibrate rapidly in the back reaction,[†] the ratio of [Trp⁺] to [Zn-porphyrin⁺] will downweight the effective rate constant for ET to Trp-191⁺ from Fe(II)Cc.[‡] Comparison of k_{cb}^1 for ZnCcP in complex with the Phe-82 mutants (Table 1) to the value for ET to compound I suggests that only $\approx 0.1\%$ of the radical is localized at Trp-191⁺. It follows that an $\approx 180 \text{ mV}$ difference in the redox potentials separates Trp⁺ and Zn-porphyrin⁺, which is consistent with previous estimates of these and related potentials (29, 36, 37). Nonetheless, if k_{cb}^1 represents a pure ET tunneling rate in all of these complexes, a much greater difference in this quantity would be predicted by the structures of the CcW(S) and CcY(I) complexes (Table 1).

As seen in solution studies of these systems (12), all of the mutant

complexes have a variable slow phase ($k_{\text{cb}}^2 = 30\text{--}70\text{ s}^{-1}$, $f_2 = 0.08\text{--}0.22$) associated with the back ET process (Table 1). This slow phase could reflect direct Fe(II) to Zn-porphyrin⁺ ET, as seen in the CcP W191F mutant (18); however, short-circuit through Trp-191⁺ should greatly limit porphyrin–porphyrin ET, provided the porphyrin and indolyl radicals rapidly interconvert relative to the interfacial ET reaction. Alternatively, a subset of complexes has reduced ET reactivity in the crystal due to altered conformations. Interestingly, ET in CcS has the largest contribution from the slow phase ($f_2 = 0.22$) and also shows alternate associations within the asymmetric unit of the crystal (Fig. 2C). The two unique CcS complexes differ in their interface structure. Whereas one complex is well-ordered and has the WT association mode, difference electron-density maps reveal that the Cc heme and interface regions have shifted by $\approx 2.0\text{ \AA}$ in the second complex (Fig. 2C). The second Cc molecule also has poor electron density in the peripheral regions, further suggesting variable positioning in the crystal. Thus, the crystal tolerates small rigid-body motions of the two redox partners, and the mutant where this behavior is most evident (CcS) also exhibits the largest contribution from slow back ET. Understanding the effects of these modest conformational changes on ET reactivity will likely require detailed computational studies.

Although the altered binding modes of CcY and CcI complexes generate cofactor-to-cofactor separations that are 4–5 Å greater than the WT binding mode, the kinetics of ZnCcP⁺ and Fe(II) Cc decay parallel those of CcW and CcS (Fig. 3). Distance considerations or pathway calculations predict that electronic coupling between the cofactors in the two Cc association modes should be very different (Table 1). However, coupling in these systems could substantially increase if a tunneling pathway includes Lys-72, which is positioned to bridge a gap in the interface by hydrogen bonding to both proteins (Fig. 3). In the CcY structure, the average position of Lys-72 puts these interactions out of typical hydrogen-bonding range (3.4 and 3.6 Å, respectively), but in the CcI complex, multiple conformations for this residue are able to hydrogen bond with each side of the interface (see Fig. 6, which is published as supporting information on the PNAS web site). Hence, dynamics may allow sampling of these hydrogen bonds and increased donor-to-acceptor coupling in the CcY(I) complexes.

The F82Y, K72S Cc Double Mutant Decouples ET with ZnCcP. We further investigated the importance of Lys-72 in the CcY complex by mutating this residue to Arg, Gln, and Ser. In crystals of the double mutants, the forward $^3\text{ZnCcP}$ quenching rate decreases, with the most dramatic effect from K72S, where the $^3\text{ZnCcP}$ lifetime is essentially that of unquenched $^3\text{ZnCcP}$ ($k_p = 123 \text{ s}^{-1}$).

[†]In support of redox participation by Trp-191 in the recombination reaction, the structure of the CcP W191F mutant in complex with yCc has no significant structural differences compared with the WT (data not shown).

[‡]For the case of a rapidly equilibrating Zn-porphyrin⁺, Trp-191⁺ pair, in the back ET reaction with Fe(II)Ct, both species will decay together with an observed rate constant (k_{eb}) that reflects back ET to the Zn-porphyrin⁺ ($k_{\text{eb}}^{\text{Zn}}$) and back ET to Trp-191⁺ (k_{eb}^{W}) weighted by the equilibrium ratio (f_{W}), i.e., $k_{\text{eb}} = (k_{\text{eb}}^{\text{Zn}}) + f_{\text{W}}(k_{\text{eb}}^{\text{W}})$. Because Fe(II) to Zn-porphyrin⁺ ET is slow in the CcP W191F mutant ($\approx 70 \text{ s}^{-1}$) (18), ET to Trp-191⁺ should dominate the observed rate constant.

Fig. 2. Complexes of ZnCcP with various Cc mutants have different association modes. (A) Representative ribbon diagrams for ZnCcP bound to Cc variants. From left to right: yCc WT, F82W, and F82S mutants; yCc F82Y and F82I mutants; horse Cc; yCc K72S/F82Y. (B) Relative positioning and interface structures for the ZnCcP:F82Y yCc (Left) and ZnCcP:yCc F82I (Right) complexes. Cc (gray ribbons, top) rotates 90° and translates 8 Å in the F82I mutant generating altered heme orientations (yellow bonds), interprotein contacts (black and orange side chains), and intervening solvent structure (red spheres) with CcP (yellow, below) compared with the F82W complex. (C) Superposition of ZnCcP from the two ZnCcP:F82S yCc complexes (red and blue) indicate variability in Cc positioning within the asymmetric unit.

Crystals containing F82Y/K72S Cc diffract poorly and only provide a 4.3-Å resolution structure for the complex with ZnCcP (Table 2). In the asymmetric unit, these crystals contain two molecules of ZnCcP and one partially disordered Cc. Nevertheless, electron density for the heme region and constraints of crystal packing allow a reasonable positioning of the molecule. Clearly, the association mode of Cc with ZnCcP has again been drastically altered by the additional substitution at Lys-72 (Fig. 2A). In fact, lattice contacts prevent an association of Cc with CcP analogous to WT yCc or the CcY(I) variants. In agreement with no ET quenching of $^3\text{ZnCcP}$ by F82Y/K72S Cc, all Zn-porphyrin-to-Fe-heme distances in the crystal (>39 Å) exceed the ET active configurations found in the other structures.

Distance Dependence of the Recombination Rates. If the ZnCcP:Cc association mode places the cofactors too far apart, as in the K72S mutant, there is no ET between the proteins. In contrast, when the proteins closely associate, as in the WT complex, k_{cb} increases to a value that prevents resolution of an intermediate. Inside these extremes, the yCc F82 single variants have intermediate values for k_{cb} that appear relatively insensitive to changes in the structure of

the complex (Table 1). Furthermore, the hCc:ZnCcP complex also has k_{cb}^1 similar to the yCcW(S):ZnCcP complexes, despite a much longer distance of separation between cofactors and commensurately lower predicted electronic coupling (Table 1). Additionally, rate constants for forward and back interprotein ET rates in a tuna ZnCc:Fe(III)Cc mixed crystal system are again similar to the F82 variants (38), even though structural considerations predict that they should be much larger (Table 1). Thus, static structures and simple bonding models cannot easily explain the relative reactivities of these various complexes.

Temperature Dependence of Forward ET in ZnCcP:yCc WT. Temperature dependence of the WT yCc:ZnCcP forward ET rates in crystals indicate that protein and/or solvent motion gate the reaction. The quenching of ZnCcP by ET to Fe(III)Cc diminishes at <270 K in the crystal; within the error of our measurements, the decay curves appear monoexponential throughout this range (Fig. 4). By 170 K, the quenching rate reaches that of the Fe(II) Cc crystals or crystals of ZnCcP alone, indicating no ET (Fig. 4). This result somewhat contrasts the solution behavior (38), which also shows marked temperature dependence of the quenching constant; however,

Fig. 3. Kinetics of interprotein ET reactions with crystals of ZnCcP:yCc. (A) Kinetic progress curves for the formation and decay of ZnCcP^+ in crystals of Fe(III) yCc mutants as monitored at 680 nm by transient absorption spectroscopy. See Table 1 for fit parameters. (B) Progress curves following formation and decay of $^2\text{ZnCcP}$ (475 nm), Fe(II)Cc (520 nm), and ZnCcP^+ (680 nm) in single crystals of ZnCcP: F82Y yCc.

quenching becomes nonexponential in a lower transition range that centers at 230 K. Comparisons of crystal structures at 100, 170, and 298 K show no noticeable differences at the resolutions of the structures (data not shown). Thus, the active states for forward ET are not likely represented time-averaged crystal structures and rather result from small-amplitude dynamic conversions, perhaps involving coupled protein, solvent, or proton rearrangements (1). At cryogenic temperatures, intraprotein ET rates in the microsecond or shorter time range do not diminish and can even increase (40, 41). Thus, fast ET within proteins does not appear to fall subject to the same dynamic constraints as some interfacial ET reactions.

Structural Perturbation and Gating for Interfacial ET. Insensitivity of ET rates to donor–acceptor separation also has been observed for Cc and blue-copper protein azurin absorbed or attached to gold electrodes by means of chemical linkers of variable length. At short distances (<17 Å) ET rates between the metallo-cofactors and the electrode surface plateau. This behavior has been attributed to a number of mechanisms: (*i*) dynamic modes of the solvent or protein interior that damp motion along the ET reaction coordinate (so-called “quantum friction”) (42); (*ii*) electrode-derived electric field effects that influence proton rearrangements (43); and (*iii*) larger amplitude gating processes that produce transitions between active vs. inactive states (44–46). In the electrochemical systems, ET

Fig. 4. Temperature dependence of forward ET in ZnCpP:yCp WT crystals. The lifetime of $^3\text{ZnCpP}$ (monitored at 475 nm) increases with decreasing temperature until $^3\text{ZnCpP}$ is nearly unquenched. (*Inset*) k_{et} in one crystal of ZnCpP:yCp WT as temperature is raised from 100 to 298 K.

rates plateau in the same range that we observe for the ZnCcP:Cc complexes (200–5,000 s⁻¹) (14, 42, 44, 45). The CcP:Cc crystal systems show that leveling behavior is an intrinsic property of interfacial protein ET and not unique to electrodes. At longer distances of separation however, ET reactions between Cc variants and carboxylic acid-terminated self-assembled monolayers do show marked sensitivity to the interactions that mate the protein to the electrode (14). In particular, substitutions of Lys-13 of rat Cc reduce ET rates by 5 orders of magnitude (14). Furthermore, cross-linked yCc at the CcP primary WT interface shows much higher ET reactivity than yCc cross-linked to the opposing back face of CcP, despite similar presumed cofactor separations (47). In our crystal systems, changes in orientation that should have such a dramatic change on the ET reactions [e.g., CcY(I) vs. CcW(S)], do not; yet very small changes in structure [CcW(S) vs. Cc WT] do slow the back rates to within an observable regime. Thus, electrochemical, solution, and crystal systems indicate that certain association modes are highly reactive, perhaps because they lock the system into well-coupled structures. Outside of these defined states, interfacial ET may not be particularly sensitive to changes in association because of limiting dynamics.

For a solvent-damping mechanism (42) to explain the equivalent ET reactivity of the CcW(S) and CcY(I) crystal systems, the effective relaxation time for solvent/protein modes to decouple the system would have to exceed 10 μ s, assuming the donor–acceptor coupling is $<0.1 \text{ cm}^{-1}$ (as is likely in the ZnCcP:Cc complexes).⁸ The source of such a slow process that would be common to the

[§]In the overdamped regime (49, 50) the ET rate constant k_e can be expressed as

$$k_{\text{et}} = \frac{4\pi^2 (|H_{\text{AB}}|^2) \exp(-E_{\text{A}} / k_{\text{B}}T)}{h (1 + g) \sqrt{4\pi\lambda k_{\text{B}}T}},$$

where H_{AB} is the electronic coupling between donor and acceptor, E_A is the activation energy along the reaction coordinate, λ is the reorganization energy and

$$g = \frac{4\pi^2(|H_{AB}|^2)\tau_{\text{eff}}}{h\lambda},$$

with τ_{eff} being the characteristic relaxation time of the reaction coordinate. The adiabaticity parameter g is the ratio between the time the reaction coordinate spends in the crossing region to the time for an electron to transfer from donor to acceptor. Thus, if H_{AB} is on the order of 0.1 cm^{-1} and λ is on the order of 0.8 eV , rates will be damped by relaxation times in the 100-ns range (42); however, if $H_{AB} = 0.01 \text{ cm}^{-1}$, a number more appropriate for coupling beyond the Cc surface (51), damping motions by this mechanism must have effective relaxation times of $10 \text{ }\mu\text{s}$ or longer.

various association modes found in the crystals is not obvious; although the decreased forward ET rates at <273 K do indicate that motion in the crystal can limit rates. Additionally, differential coupling of the extended Fe(II) heme donor orbital to the acceptor site on CcP also may mitigate structural effects. Molecular orbital calculations on hCc indicate that the heme iron-centered HOMO, which accepts and donates electrons, delocalizes over protein regions relatively distant to the cofactor (48). Thus, coupling of ZnCcP electronic states with this HOMO may compensate changes in the orientation and separation of Cc. However, studies by Gray and colleagues (3, 14) on Ru-modified Cc (3) and Cc coupled to electrodes via self-assembled monolayers (14) indicate that ET from the heme to the Cc surface reflects the detailed structure of bonding pathways and interfacial contacts (3).

Conclusions

These studies demonstrate that transient protein-protein interactions have high sensitivity to small structural changes at interfaces. Remarkably, addition of a hydroxyl group to Phe-82, a residue on the periphery of the WT CcP contact region, completely disrupts the favored association mode. These results give caution to interpreting reactivity data of protein variants with even subtly altered

surface properties. The insensitivity of k_{cb} to distance and cofactor orientation in some variants likely reflects limiting dynamic processes, although changes in electronic coupling due to different cofactor orientations cannot as yet be ruled out. The slower forward rates, which give monoexponential kinetics through the transition temperature range, are gated by dynamic processes that allow rapid sampling but rare conversion to active states. As the crystal fixes the general association mode (but allows some structural variability), it follows that the active states do not structurally differ substantially from the inactive states. This hypothesis could explain why the variants that maintain the WT yCc:CcP association mode have significantly slower back ET rates than the WT complex. The alternate association modes observed for the CcS complex may represent the extent of such conformational motion. Presumably, the WT complex limits conversion to unreactive structures, because its charge recombination rate exceeds that of all other variants.

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