Protein Dynamics Enhance Electronic Coupling in Electron Transfer Complexes*

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Electron-transferring flavoproteins (ETFs) from human and Paracoccus denitrificans have been analyzed by small angle x-ray scattering, showing that neither molecule exists in a rigid conformation in solution. Both ETFs sample a range of conformations corresponding to a large rotation of domain II with respect to domains I and III. A model of the human ETF-medium chain acyl-CoA dehydrogenase complex, consistent with x-ray scattering data, indicates that optimal electron transfer requires domain II of ETF to rotate by ~30 to 50° toward domain I relative to its position in the x-ray structure. Domain motion establishes a new “robust engineering principle” for electron transfer complexes, tolerating multiple configurations of the complex while retaining efficient electron transfer.

Many biological electron transfer reactions (e.g. defense against toxic compounds and pathogens, the generation of signaling molecules, and the production of energy-rich compounds) rely on the coexistence of protein-protein complexes in dissociation equilibrium with their constitutive reactants. The mechanism of electron transfer between weakly associating electron transfer partners has been the focus of intensive research activity in recent years (1, 2). An in-depth understanding of these reactions requires knowledge of the role of protein dynamics in complex assembly and the geometries of the complex that are compatible with interprotein electron transfer (eT). 1 A role for interdomain flexibility has been demonstrated in eT both within a single molecule (3–5) and between molecules (6).

Electron-transferring flavoproteins (ETFs) act as physiological electron carriers between degradative enzymes in bacteria and mitochondria and their respective membrane-bound electron transfer pathways (7). All ETFs are heterodimeric, and all possess one equivalent of noncovalently bound flavin adenine dinucleotide (FAD), with the exception of ETF from Megasphaera elsdenii, which contains two equivalents of noncovalently bound FAD (8). ETFs from human, pig, Paracoccus denitrificans, and Methylophilus methylotrophus also contain one equivalent of AMP per dimer; this AMP facilitates reconstitution of the holo-ETF from the guanidine-denatured protein (9, 10).

Human and P. denitrificans ETFs share 54% sequence identity and belong to the category of housekeeping ETFs (11), oxidizing fatty acids and some amino acids. The catalytic and redox properties of the two proteins are very similar (12, 13). Crystal structures have been determined for both ETFs at resolutions of 2.1 and 2.6 Å, respectively (14, 15). These reveal that the overall fold for both proteins is identical with the exception of a single loop region. The two subunits comprise three domains, domain I (the N-terminal region of the α-subunit), domain II (the C-terminal region of the α-subunit and a small C-terminal region of the β-subunit), and domain III (the majority of the β-subunit). Both proteins have a Y-shaped structure, with domains I and III forming a shallow bowl in which domain II rests. The FAD cofactor lies in a cleft between domains II and III, with most of the FAD molecule interacting with domain II.

Human ETF is the electron acceptor for nine physiological partners (16). It has been modeled previously in complex with one of these partners, medium chain acyl-CoA dehydrogenase (MCAD), by docking the crystal structures of the two proteins as rigid bodies (14). In this model complex, the distance between the redox centers of the proteins (FAD in ETF to FAD in MCAD) was 19.5 Å, and it was suggested that this could be reduced by 3–4 Å if changes in the conformation of one or both of the proteins were to occur on complex formation (14). However, this is still larger than the expected maximum distance for electron transfer of ~14 Å on the basis of the principle of “robust engineering” (17).

The dynamics of Methylophilus methylotrophus (sp. W3A1) ETF have been studied previously by small angle x-ray scattering (SAXS) (18). This study revealed that domain II does not have a well defined position relative to domains I and III but samples a range of conformations corresponding to rotation relative to domains I and III. W3A1 ETF is a highly specific electron carrier that accepts electrons from only one enzyme, trimethylamine dehydrogenase (TMADH) (19). This ETF shares 31 and 33% sequence identity with human and P. denitrificans ETFs, respectively. Binding of W3A1 ETF to its physiological redox partner, TMADH, is thought to involve the
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Fig. 1. Experimental SAXS results for solutions of oxidized human and P. denitrificans ETFs and semiquinone (reduced; this is more stable in the semiquinone than oxidized form, which shows identical SAXS characteristics) M. methylotrophus (sp. W_{A4}) ETF. The scattering curves of all three ETFs are represented by a combination of low and high concentration data for the low angle and outermost scattering region, respectively. Error bars are based on counting statistics (for clarity only a third of the experimental data points are displayed). The calculated distance distribution functions are shown for all three ETFs (see inset). I(s) and p(r) functions for the three proteins have been normalized such that I(s) and the area under p(r) are scaled to unity. The fit (solid line) to the experimental SAXS curves represents the scattering from the restored shape of human ETF (see Fig. 3). Deviations between the fit representing the uniform molecular envelope and experiment for s > 0.045 Å⁻¹ are attributable to internal structural inhomogeneities that become more pronounced at higher scattering angles.

formation of a number of distinct metastable states (20) rather than a single eT complex with unique configuration. In the TMADH/W_{A4} ETF system, the intrinsic rate of eT is much faster than the experimentally observed rate, and it is thought that binding and/or reorganization of ETF on the surface of P. denitrificans (anionic semiquinone; this is more stable than the oxidized form, and this is more pronounced at higher scattering angles.

Experimental Procedures

Expression and Purification—Human and P. denitrificans ETFs were expressed and purified as described previously (Refs. 21 and 13, respectively).

Sample Preparation and X-ray Scattering Data Collection—Oxidized human and P. denitrificans ETFs were used in scattering experiments after gel filtration and dilution (for concentration-dependent scattering experiments). Both proteins were prepared in the AMP-bound form; the semiquinone form of either protein was not used because this is unstable in air and therefore inaccessible to SAXS studies. UV-visible absorption spectra were recorded before and after x-ray exposure for samples to ensure that no x-ray-dependent redox change occurred. This also allowed the concentration of ETF samples to be determined using the absorption at 438 nm (human ETF, ε_{438} = 13,300 M⁻¹ cm⁻¹ (21); P. denitrificans ETF, ε_{438} = 13,600 M⁻¹ cm⁻¹ (13); W_{A4} ETF, ε_{438} = 11,300 M⁻¹ cm⁻¹ (18)). The scattering from W_{A4} ETF in its reduced (anionic semiquinone; this is more stable than the oxidized form and this is more pronounced at higher scattering angles.

Fig. 2. Simulated x-ray scattering profiles for molecular models of human (A) and P. denitrificans (B) ETFs with domain II rotated 0° (red), 25° (green), and 50° (blue) toward domain I relative to the respective crystal structure. These have been fitted against the experimental scattering curve for the corresponding ETF (the two experimental curves are those from Fig. 1). The simulations for human and P. denitrificans ETFs resulted in Rg values of 25.1, 25.2, and 25.5 Å and 24.6, 24.8, and 25.1 Å using the crystal structure, 25° and 50° domain rotation models, respectively.

Gives identical scattering (18°) form was extended compared with our previous study (18) and allowed a straightforward comparison with the other ETFs. SAXS experiments were performed with protein concentrations between 0.3 and 19 mg/ml. X-ray solution scattering data were collected in two sessions with the low-angle scattering camera on station 2.1 (22) at the Synchrotron Radiation Source (Daresbury, UK) using a position-sensitive multiview proportional counter (23). At the sample-to-detector distance of 2.25 m (1.25 m) and the x-ray wavelength of λ = 1.54 Å, a momentum transfer interval of 0.002 Å⁻¹ ≤ s ≤ 0.050 Å⁻¹ (0.005 Å⁻¹ ≤ s ≤ 0.088 Å⁻¹) was covered. The modulus of the momentum transfer is defined as s = 2 sin θ/λ, where 2θ is the scattering angle. The s-range was calibrated using an oriented specimen of wet rat tail collagen (based on a diffraction spacing of 670 Å). The samples were contained in a brass cell holding a Teflon ring sandwiched by two mica windows that define the sample volume of 120 μl and a thickness of 1.5 mm. The brass cell was maintained at 4 °C during data acquisition. The buffer and sample were measured in alteration, each in frames of 60 s (amounting to a total measuring time between 5 and 45 min depending on sample concentration and changes monitored on-line during experiments).

Interpretation of X-ray Scattering Data—Reduction and analysis of scattering data was performed as described previously (24). The radius of gyration Rg, forward scattering intensity I0, and the intraparticle distance distribution function p(r) were calculated from the experimental scattering data using the indirect Fourier transform method as implemented in the program GNOM (25). Relative Ic values (c = sample concentration) give the relative molecular weight of the protein
also represents the molecular envelope of
lope structure for human ETF superimposed over its crystal
underlined by the scattering pattern simulations.

samples when referenced against a suitable standard (bovine serum albumin was used with a known molecular mass of 66 kDa). The maximum molecular dimension \(D_{\text{max}}\) of the particle can be evaluated because of the characteristic of \(p(s)\). The volume \(V\) of the particle can be calculated from the Porod invariant (26) and a correction factor taking into account the limited experimental scattering range (27).

The multipole expansion method proposed by Stuhrmann (28) and developed by Svergun et al. (29) was used to obtain the molecular shape of human ETF. The smoothed scattering profile of reduced human ETF was fitted \textit{ab initio} by the scattering from an envelope function starting from an ellipsoidal initial approximation (consistent with the experimental \(R_g\) and \(D_{\text{max}}\) values). The molecular shape was characterized with spherical harmonics using 19 free parameters (fourth-order harmonics), which is acceptable given the minor differences compared with the shape obtained for third-order harmonics (10 free parameters) and considering the information content in the data used. The x-ray scattering curve at higher angles (starting approximately from \(s = 0.04\) Å\(^{-1}\)) contains significant contributions from the internal molecular structure. Consequently, there are deviations between the experimental profile and the curve resulting from the shape fit for higher scattering angles (see Fig. 1). Only models based on crystallographic information (i.e., that take into account the molecular inhomogeneities) allow exploitation of the full experimental data range.

Scattering data simulations were carried out using the x-ray structures of human (14) and \(P. \text{denitrificans}\) (15) ETFs in their crystal conformations and with domain II rotated by 25 and 50 ° with respect to domains I and III. Parameters and scattering curves were computed from the model coordinates using the program CRYSSOL (30), which also considers the hydration shell of the solvated protein.

**Protein-Protein Docking**—The crystal structure of human ETF (Ref. 14; PDB (31) accession code 1efv) was docked into the crystal structure of the human MCAD homodimer (Ref. 32; PDB accession code 1egd) (crystal structures of only the oxidized forms of human ETF and human MCAD are available) using the protein-protein docking program GRAMM (33). Ten complexes were produced and analyzed using InsightII (InsightII, MSI, San Diego, CA) to select the most appropriate docking. Our criteria for selecting the most reasonable docking were that the ETF did not overlap with the acyl-CoA binding site on MCAD and the calculated \(eT\) rate (Table I) was a maximum. To produce potential “\(eT\) active” conformations of ETF, the flexible linkers between domains I and II (residues 205–220) and domains II and III (222–233) were aligned along the pseudo 2-fold axis using InsightII. Domain II (residues 209–231 and 222–255) was rotated by 10 ° toward domain I. Domains I (residues 20–204) and III (residues 24–228) were kept rigid. This conformation of human ETF was then docked into MCAD as before. The procedure was repeated for 20, 30, 40, 50, and 60 ° of rotation of domain II with respect to domains I and III.

**eT Rates**—eT from MCAD to ETF involves two sequential one-elec-

**Fig. 3. Three orientations of the calculated molecular envelope structure for human ETF superimposed over its crystal structure.** Because the scattering results for human and \(P. \text{denitrificans}\) ETFs are virtually identical, the restored shape of human ETF also represents the molecular envelope of \(P. \text{denitrificans}\) ETF. The shape displays the excellent fit between the globular base of the envelope structure and domains I and III but highlights the conformational shape displays the excellent fit between the globular base of the envelope and domains I and III but highlights the conformational shape displays the excellent fit between the globular base of the envelope structure. Consequently, there are deviations between the experimental profile and the curve resulting from the shape fit for higher scattering angles (see Fig. 1). Only models based on crystallographic information (i.e., that take into account the molecular inhomogeneities) allow exploitation of the full experimental data range.

**Fig. 4. Dockings of human ETF (brown) onto MCAD (blue).** The backbone of each protein is shown schematically, the positions of the FADs are shown in black space-filling representations, and the flavins used in calculating \(eT\) rates from MCAD to ETF are encircled in red (MCAD contains a pseudo 2-fold axis, therefore \(eT\) is equally likely to the FAD in either chain A or B; in all cases, the second flavin in MCAD, related by the pseudo 2-fold axis, is much further away than the flavin to which \(eT\) is indicated). Each panel illustrates the docking onto MCAD (the orientation of MCAD is identical in all panels). A, ETF with domain II rotated 0 ° toward domain I with respect to the crystal structure of human ETF with the flavins in the four subunits of the MCAD tetramer labeled; B, ETF with domain II rotated 10 ° toward domain I; C, ETF with domain II rotated 20 ° toward domain I; D, ETF with domain II rotated 30 ° toward domain I; E, ETF with domain II rotated 40 ° toward domain I; F, ETF with domain II rotated 50 ° toward domain I; G, ETF with domain II rotated 60 ° toward domain I.
tron steps (34, 35). One molecule of ETF is reduced from the oxidized to semiquinone form by MCAD, which in turn is oxidized from the hydroquinone to semiquinone form. Subsequently a second molecule of ETF is reduced from the oxidized to semiquinone form by MCAD, which in turn is oxidized from the hydroquinone to oxidized ETF (36). Analysis of the low angle scattering range (s ≤ 0.01 Å⁻¹) confirmed that human and *P. denitrificans* ETFs also behave as heterodimers with molecular masses of ~60 kDa each without forming higher oligomers. In this context, it is interesting to note that unlike human and *P. denitrificans* ETFs, *W3A1 ETF* exhibits a broad peak in the very low angle range (corresponding to a spacing of about 250 Å) at concentrations higher than 15 mg/ml (data not shown). This indicates that the scattering data is already affected by the protein distribution in the solution (i.e., the so-called solution structure factor) and implies that *W3A1 ETF* interactions at these protein concentrations are dominated by a repulsive force, a feature that may notably influence the crystallization behavior of this protein. The p(r) function for both human and *P. denitrificans* ETFs (see Fig. 1, inset) possesses a characteristic shoulder at 36 Å, which (as with *W3A1 ETF*) indicates that both proteins have a diffuse Y-shaped conformation with distinct domain features as opposed to a compact globular structure. The latter would possess a pair distribution function without distinctive features, for instance the p(r) function of the compact iron-bound conformation of transferrin (24). Small differences in the p(r) function at higher intraparticle distances (between 50 and 70 Å) reflect the above-mentioned increased flexibility of *W3A1 ETF* compared with the other two ETFs.

**Simulated X-ray Scattering Profiles**—The x-ray scattering data for human and *P. denitrificans* ETFs have been solved previously (14, 15). Using these atomic coordinates, simulated x-ray scattering profiles were generated for solvated structures of both human and *P. denitrificans* ETFs and fitted against the experimental data for each protein, respectively (Fig. 2). The experimental scattering profile for human ETF does not correspond...
adequately to the simulated profile generated from the crystal structure of human ETF. Significant differences in the scattering interval 0.025 Å⁻¹ ≤ s ≤ 0.045 Å⁻¹ result in a fit with \( \chi^2 = 3.31 \). Similarly, the experimental scattering profile for \( P. denitrificans \) ETF does not correspond adequately to the simulated profile generated from the crystal structure of \( P. denitrificans \) ETF (\( \chi^2 = 2.90 \)). Consequently, it is clear that in both cases the crystallographic model does not satisfactorily describe the molecular structure in solution. It was hypothesized that human and \( P. denitrificans \) ETFs in solution may possess a static conformation that differs substantially from that suggested by their respective crystal structures. Both human and \( P. denitrificans \) ETFs possess two polypeptide loop regions, between domains I and II and domains II and III, which may, as suggested for \( W_2A_1 \) ETF (39) (see above), act as a flexible “hinge” to allow the rotation by up to \(-50^\circ\) of domain II with respect to domains I and III. Therefore, molecular models were produced of human and \( P. denitrificans \) ETFs in which domain II was rotated about this hinge with respect to domains I and III, treating these two domains as a rigid body. Because these ETFs possess a pseudo 2-fold axis of symmetry about the interface of domains I and III, a conformational model of ETF in which domain II is rotated in the direction of domain I generates an essentially identical simulated SAXS profile to that generated from a model in which domain II is rotated in the direction of domain III. Hence, four conformational models were designed: human ETF with domain II rotated by \( 25^\circ \) in the direction of domain I, human ETF with domain II rotated by \( 50^\circ \) in the direction of domain I, \( P. denitrificans \) ETF with domain II rotated by \( 25^\circ \) in the direction of domain I, and \( P. denitrificans \) ETF with domain II rotated by \( 50^\circ \) in the direction of domain I. Simulated SAXS profiles were generated for all these molecular models of ETF; the experimental scattering profile for human ETF does not correspond well to any of the simulated profiles generated from the conformational models of human ETF even though the fits improve significantly compared with the crystallographic model. For SAXS simulations generated from models of human ETF in which domain II was rotated by \( 25^\circ \) or \( 50^\circ \), respectively, \( \chi^2 \) values of 2.32 and 2.00 were obtained for fits to the experimental data. Similarly, the simulated scattering profiles generated from the conformational models of \( P. denitrificans \) ETF do not entirely match the experimental profiles generated from the conformational models of \( P. denitrificans \) ETF. For SAXS simulations generated from models of \( P. denitrificans \) ETF in which domain II was rotated by \( 25^\circ \) or \( 50^\circ \) (a larger number of rotations were not generated because no single conformation will fit the molecular envelope; see below), respectively, \( \chi^2 \) values of 2.31 and 2.32 were obtained for fits to the experimental data. The fact that none of the simulated SAXS profiles for these structures/models can be unambiguously fitted to the experimental SAXS data suggests that neither the structure of human ETF nor the \( P. denitrificans \) ETF can be represented by a static conformational model, as illustrated by their molecular envelope structures (Fig. 3). This substantiates the conclusion that both proteins sample a range of different conformations in solution. We note that because of the assumption of a uniform density inside the molecular envelope, the presented shape gives only an average static picture of this flexible protein in solution.

Nevertheless, because of the size of domain II, its conformation will influence the overall shape of the molecule significantly. This can be seen in the form of a spread out space for this domain sitting above domains I and III. The latter are more tightly connected to each other, and changes in their conformational position are therefore less likely. In contrast, small sections of the polypeptide such as loop regions (e.g. of domain III; Fig. 3) are much more flexible compared with the compact domain core and can therefore appear outside of the shape envelope.

**Intrinsic Electron Transfer Rates**—We suggested previously that rotation in the \( W_2A_1 \) ETF of domain II by \(-50^\circ \) (with respect to its position relative to domains I and III in the crystal structure of human ETF) was required for eT from its redox partner TMADH (18, 39). The SAXS data presented above suggests that such large conformational changes could also occur in human ETF upon complex formation with MCAD. To test this hypothesis, we have assumed that the conformation of MCAD remains essentially unchanged during complex formation (our future SAXS studies will investigate the validity of this assumption further). This enabled us to model the MCAD-human ETF complex (Fig. 4) and calculate the intrinsic eT rates (Table I). The method we have adopted for calculating eT rates (17) considers the density of the protein packing between redox centers without regard to specific “pathways.” An alternative approach (e.g. see Refs. 40 and 41) regards eT as occurring via well defined pathways (e.g. via specific bonds and through space jumps). We have used this pathways approach in other studies to identify key amino acids involved in eT (e.g. see Ref. 42), but we feel that experimental evidence favors an approach that utilizes the protein-packing density. Nevertheless, the pathways approach could have proved useful for identifying such residues in the current study if only a single binding mode (and therefore only a single eT pathway) was likely. However, this is not the case because our results (see below) suggest that efficient eT can occur when domain II is rotated by anywhere between \(-30^\circ \) and \( 50^\circ \) toward domain I, resulting in a large number of potential eT pathways (and therefore a large number of different residues likely to be involved in different eT “routes”) because of the large number of different binding modes.

Our calculations suggest that domain II has to be rotated by \(-50^\circ \) toward domain I to maximize electronic coupling between the two redox centers. For a robust eT system, the distance between the redox centers should be less than \(-14 \text{ Å} \) (17). Therefore, a robust and productive eT complex will likely be formed when domain II of human ETF is rotated by between \(-30^\circ \) and \(-60^\circ \) (Table I) toward domain I. However, a rotation of \(-60^\circ \) can be ruled out because this results in severe steric overlap between domains I and II, suggesting that rotations of between \(-30^\circ \) and \(-50^\circ \) are required for a productive eT complex. Implicit in the above discussion is the concept of a two-step “collision plus eT transfer” process. However, it is likely that eT occurs not from a single bound conformation of ETF but from an “ensemble” of thermodynamically metastable complexes (i.e. this comprises both a range of conformations of ETF and a range of orientations of each conformation in the eT complex). Within the ensemble of MCAD-ETF structures, a range of intrinsic eT rates (e.g. see Table I) will be present, most of which will likely be much faster than the observed rates of eT in stopped-flow experiments. By utilizing an ensemble of structures in which the intrinsic eT rate far exceeds the observed rate, the system does not rely on adopting a unique geometry for the productive eT complex and all the conformational sampling and thermodynamic restrictions that this imposes. Most experimentally observed and physiological interprotein eT rates are in the range of \( 10^{-9} -10^{-8} \text{ s}^{-1} \), i.e. much slower than the values calculated when domain II is rotated by between \(-30^\circ \) and \( 50^\circ \) with respect to domains I and III (Table I). In this limit where the intrinsic rate of eT is much greater than the observed rate, given the efficient coupling between redox centers, the observed eT rate can be maintained from a number of different eT complex geometries. This is yet another
example of a robust engineering principle and builds on the idea that naturally occurring mutations can be accommodated in a reox protein without affecting significantly the eT rate (17).

Conclusion—The small angle x-ray scattering studies are consistent with the finding that human and P. denitrificans ETFs sample a large range of conformations in solution. This is supported by modeling studies that illustrate that a significant change in conformation of ETF (domain II rotating by between ~30 and 50° toward domain I from its position in the respective crystal structures) creates a new regime in which the intrinsic eT rate is elevated well above typical values observed in physiological eT complexes ([10^4–10^5 s^{-1}]). This regime introduces a new robust engineering principle, relaxing the specificity required between MCAD and ETF without compromising the observed eT rates.

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