Intramolecular Electron Transfer Kinetics of a Synthetic Flavocytochrome *c*

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The kinetics of long-range electron transfer have been shown to be dependent on three factors: the thermodynamic driving force of the reaction ($-\Delta G°$), electronic coupling between the electron donor and acceptor centers (H_{ab}), and nuclear reorganization (λ).[1] Rate constants for long-range, non-adiabatic electron transfer reactions can be described by the following expression (eq 1):[1]

$$k_{et} = (4\pi^2/h)(1/(4\pi\lambda RT)^{1/2})(H_{ab})^2 \exp(-(\Delta G° + \lambda)^2/4\lambda RT) \quad (1)$$

Kinetics experiments with modified cytochromes *c* have evaluated the effects of changing driving force and electronic coupling extensively through use of various Ru and Co complexes and differing intramolecular electron transfer pathways.[2]

We have developed a new technique to study intramolecular electron transfer reactions of proteins that involves the covalent attachment of a flavin moiety to the protein surface. Although flavins have been used extensively by Tollin, Cusanovich, and their colleagues as electron donors for investigation of intermolecular electron transfer reactions,[3] and by Kaiser and colleagues as protein-modifying agents for the production of novel enzyme adducts,[4] flavins have not been used as protein-modifying reagents for introduction of electron donor sites in the study of intramolecular electron transfer reactions. The use of flavins as an alternative to transition metal complexes in such studies permits, in principle, the assessment of the contribution of the unique attributes of flavins to the kinetics of electron transfer in naturally-occurring flavocytochromes. The current study demonstrates the usefulness of this approach in providing insight into the contribution of flavin nuclear reorganization to the rate of intramolecular electron transfer reactions.

To achieve site-specific modification, the His39Cys variant of *Saccharomyces cerevisiae iso*-1-cytochrome *c* was prepared by standard methods.[5] The flavin 7α-(bromoacetyl)-10-methylisoalloxazine was synthesized[4a] and used to alkylate Cys39.[6] HPLC tryptic peptide maps were consistent with flavin attachment at this site.[7] The electronic absorption spectrum of the modified protein corresponds to the sum of the spectra for the unreacted flavin and cytochrome *c*, and the circular dichroism spectra of the unmodified and modified proteins are superimposable. These observations indicate that little or no change in the structure of the cytochrome is induced by the modification reaction.[8]

The reduction potentials[9] of the flavin and heme centers of the modified protein are similar to those of the unmodified cytochrome variant (E_m values (vs SHE) are as follows (pH 7.0, sodium phosphate buffer, μ = 0.1 M, 298 K): His39Cys, 0.259(2) V; free flavin, $fl_{ox}/fl_{red} = -0.110(2)$ V, fl_{ox}/semiquinone radical = −0.140(7) V; flavin−His39Cys, Fe(III)/Fe(II) = 0.257(2) V, $fl_{ox}/fl_{red} = -0.115(2)$ V). The dependence of the potentials of the modified cytochrome on pH (pH 5.5−8.0) defined the dependence of thermodynamic driving force ($-\Delta G°$) for intramolecular electron transfer on pH. The pH dependence of the midpoint potentials was used to investigate the effects of substituting a cysteinyl residue for His39, which has been identified previously as the single titratable group, the pK_a of which is dependent on the oxidation state of the heme iron.[10] The pK_a values for this residue in the oxidized (pK_o) and reduced (pK_r) states of the wild-type cytochrome are 6.6 and 7.0, respectively, while for the His39Cys variant the corresponding values are 7.5 and 7.9. These results indicate that in the variant, the pK_a of Cys39 is oxidation-state dependent. This conclusion is substantiated by the finding that after modification of the variant with the flavin, the reduction potential of the heme iron is independent of pH.

Laser flash photolysis was employed[11] to study the electron transfer kinetics of the flavin-modified cytochrome from pH

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(6) Ferricytochrome *c* His39Cys (sodium phosphate buffer, pH 7.2, 20 mM) was mixed with a 5-fold molar excess of 7α-(bromoacetyl)-10-methylisoalloxazine that was dissolved in Me_2SO and incubated at room temperature for 18 h. A 5-fold molar excess of cysteine was then added to remove the Cys39 thiol C-4A adduct. The modified cytochrome was eluted over a Sephadex G-25 gel filtration column to remove excess flavin and oxidized to generate the flavin ferricytochrome *c* species. Residual unmodified protein (<10%) was removed by ion exchange chromatography (Mono-S HR 10/10 cation exchange column, Pharmacia).

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(8) The intensity of fluorescence emission ($\lambda_{excitation}$ = 425 nm) associated with the flavin was significantly quenched (94%) following attachment to the protein as expected from proximity of the flavin to the sulfur atom of Cys39 (Penzer, G. R.; Radda, G. K. *Q. Rev. Chem. Soc.* **1967**, *21*, 43−65). The $\lambda_{emission}$ for the attached flavin exhibited a bathochromic shift of 6 nm relative to that of the corresponding unreacted flavin.

(9) All electrochemical measurements were performed by direct electrochemistry at a modified gold electrode with equipment and procedures described previously (Rafferty, S. P.; Pearce, L. L.; Barker, P. D.; Guillemette, J. G.; Kay, C. M.; Smith, M.; Mauk, A. G. *Biochemistry* **1990**, *29*, 9365−9369) except for studies of the flavin semiquinone which involved spectroelectrochemical (Reid, L. S.; Taniguchi, V. T.; Gray, H. B.; Mauk, A. G. *J. Am. Chem. Soc.* **1982**, *104*, 7516−7519) and numerical (Michaels, L. *J. Biol. Chem.* **1932**, *96*, 703−715. Draper, R. D.; Ingraham, L. L. *Arch. Biochem. Biophys.* **1968**, *125*, 802−808) methods described elsewhere.

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(11) The flash photolysis spectrophotometer was comprised of an optical bench and optical components obtained from OLIS (Bogart Georgia) and a Phase-R DL1020 dye laser that was operated under computer control (Blue Moon Technical Services, Vancouver). The laser was operated with an ethanol solution of Coumarin 440 (Exciton, Inc.). Sample solutions were placed into a modified 10 mm cuvette and deaerated thoroughly with a vacuum line. Protein reduction was monitored at 550 nm, and kinetic data were analyzed with the program Scientist (version 2.0, MicroMath, Orem, UT). Electron transfer rate constants were independent of the concentration of the modified protein (50−150 μM).

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Table 1. Parameters for Electron Transfer from the Flavin Semiquinone Radical to the Heme Iron of the Flavin-Modified Yeast *iso*-1-Cytochrome *c* His39Cys Variant[a]

pH	T (K)	$-\Delta G°$ (eV)	k_{et} (s^{-1})	ln k_{et}
5.56	298	0.302	4.00×10^2	6.00
6.00	298	0.336	6.40×10^2	6.46
6.94	298	0.382	1.15×10^3	7.05
7.00	284	0.390	8.04×10^2	6.69
	288		1.16×10^3	7.05
	293		1.18×10^3	7.07
	298		1.29×10^3	7.16
	304		1.32×10^3	7.18
	313		1.64×10^3	7.40
7.26	298	0.400	2.17×10^3	7.68
7.96	298	0.417	2.70×10^3	7.90

[a] Sodium phosphate buffer, $\mu = 0.1$ M.

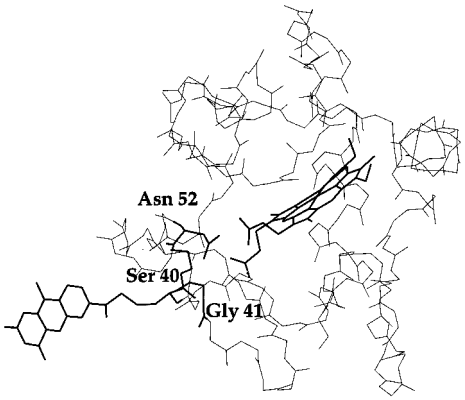

Figure 1. Minimum-energy structure of flavin-modified yeast *iso*-1-cytochrome *c*. Residues involved in the "best" electron transfer pathway, identified as described in the text, are highlighted by dark lines.

5.5 to 8.0[12] and from 284 to 313 K (sodium phosphate buffer, $\mu = 0.1$ M) (Table 1, Figure 2). Interpretation of these results was initiated by calculation of energy-minimized structural models for the His39Cys variant and the corresponding flavin-modified protein (Figure 1).[13] In the resulting model, the flavin is oriented away from the protein surface, into the surrounding solvent. The electronic coupling between the flavin-modified Cys39 residue and the heme center was calculated with the artificial intelligence (AI) superexchange method.[14] The amino acid residues selected by the AI search are Ser40*, Gly41*, Asn52*, Val57, Leu58, and Trp59 (residues labeled with an asterisk constitute the "best" path (Figure 1)). Using only these amino acid residues as the part of the protein-mediating electron transfer, the electronic coupling (H_{ab}) is calculated to be 0.034 cm^{-1}.[15] Similar kinetic analysis[14] of intramolecular electron transfer in *Candida krusei* Ru(bpy)$_2$-(His39) ferricytochrome

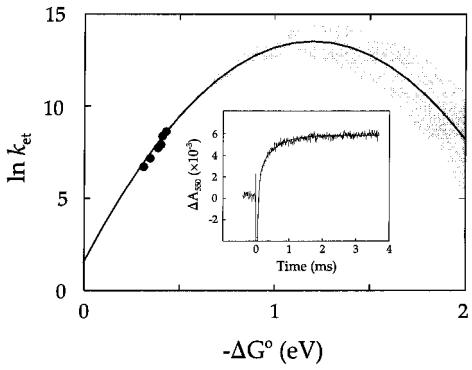

Figure 2. Dependence of k_{et} on $-\Delta G°$ for the reduction of cytochrome *c* by the covalently-bound flavin. The solid line is the best fit to eq 1. The shaded area represents the uncertainty in the fit when using the range of values for λ obtained from the temperature dependence data. A representative absorption transient and fit are shown in the inset.

c[2d] produced a value for H_{ab} of 0.11 cm^{-1}. At present, it is difficult to provide a definitive explanation for the apparent difference in electronic coupling exhibited in the flavin-modified and ruthenium-modified proteins. Nevertheless, the attachment of the new electron donor to a Cys residue as opposed to a His residue is undoubtedly a major contributory factor.

The reorganization energy of the system (λ) can be estimated[16] from the temperature dependence of the electron transfer rate constant (Table 1) to yield a value of 1.2(1) eV.[17] With λ fixed within this value range, the experimental thermodynamic driving force dependent electron transfer rate constants were fitted by least squares analysis to eq 1 to generate a plot of ln k_{et} vs $-\Delta G°$ (Figure 2). The experimental estimate for the electronic coupling associated with the electron transfer process obtained in this manner is 0.04(3) cm^{-1}, which is in good agreement with the theoretical prediction.

From the value of 0.5 eV for the reorganization energy of the protein component of this system that was derived from analysis of the self-exchange reaction of cytochrome c,[1] it is possible to determine that the reorganization energy for the flavin donor center is 0.7(1) eV. This high value for λ can be explained by the fact that the energy required to distort the equilibrium nuclear geometries of organic molecules, such as flavins, is increased in aqueous media.[18] The present work demonstrates an alterative technique for the study of intramolecular electron transfer processes within protein structures that uses a novel donor state and provides the first experimentally-based estimate for reorganization energy of a flavin in an intramolecular electron transfer reaction.

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(12) The major cytochrome and flavin semiquinone components are assumed to be independent of pH under the conditions employed in these measurements.

(13) The structures of the His39Cys variant and the corresponding flavin-modified derivative were derived from the structure of yeast *iso*-1-cytochrome *c* (Louie, G. V.; Brayer, G. D. *J. Mol. Biol.* **1990**, *214*, 527−555) by side chain substitution and molecular mechanics energy minimization with INSIGHT (Version 2.2.0; Biosym, San Diego, CA).

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(15) The exact calculated value is uncertain, since it is obtained from an approximate theory, namely the extended Hückel method, and further assumes that the protein structure and the electronic coupling are independent of temperature.

(16) Assuming that the weak dependence of $T^{-1/2}$ on temperature can be neglected[1] and that the protein structure and H_{ab} are temperature independent, a plot of ln k vs T^{-1} has a slope of $-(\Delta G + \lambda)^2/4\lambda k_B$.

(17) For ruthenium-modified cytochromes, λ for the system is estimated to be 0.8 eV,[1c,14] therefore the donor contribution to λ would be ~0.3 eV in such cases.

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