

Interprotein Electron Transfer

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I. Introduction

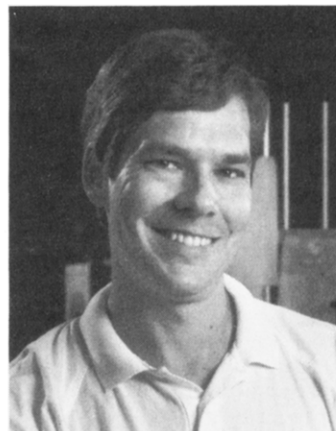
Within the last decade, the general understanding of electron transfer reactions has been dramatically altered by the confluence of theory and experiment. Experiments have confirmed some surprising fundamental predictions. As a first example, it is now well established¹⁻¹⁰ that electron-transfer reactions can occur at reasonable rates even when the reactants are separated far beyond "collisional" distances. Under optimal circumstances, rates of 10^6 s^{-1} can be obtained even when the electron donor and electron acceptor are separated by ca. 10 Å of intervening solvent.¹ If the reactants are directly linked, even by an "insulating" aliphatic chain, higher rates can be sustained.¹³ Such "long-distance" electron-transfer reactions are ubiquitous in biology.

As a second example, it is now established that the redox reactions can have an optimal free energy.¹³ As the reaction free energy increases, the electron-transfer rate increases correspondingly, reaching a maximum rate when ΔG equals the "reorganization energy" (vide infra). Further increases in ΔG lead to a decrease in rate; the region $\Delta G > \lambda$ is thus referred to as the "inverted region". Although this "inverted" effect was long controversial, it is now well established.^{1-10,13}

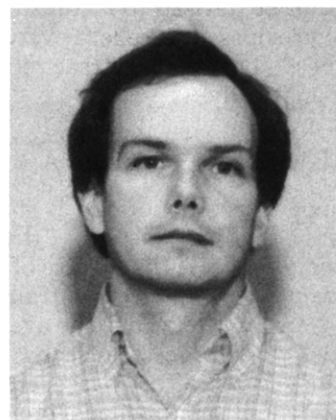
As a result of such advances, we can now reliably estimate the electron-transfer rate constant for a given couple to the nearest order of magnitude.

In the following review, we will argue that this level of understanding holds true not only for small molecule electron transfer but also for redox reactions involving proteins.

These advances in understanding have not gone unnoticed, and reviews in this field have reached the level of a major cottage industry.¹⁻¹⁷ We therefore will take a different tack in this review, using a few examples to



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illustrate the general trends observed in biological electron-transfer reactions.

II. Overview of Electron-Transfer Theory¹¹

Although electron-transfer theory will be more thoroughly developed elsewhere in this volume, a cursory overview, applicable to interprotein electron transfer, will be useful for subsequent discussion.

Electron transfer between proteins is facilitated by formation of a complex of the reacting proteins prior to the electron transfer. Conformational changes in the

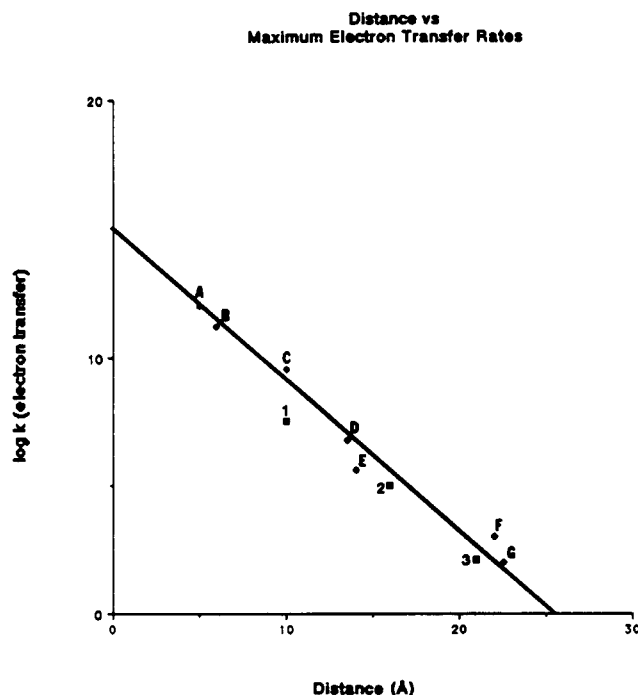
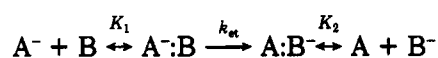


Figure 1. Distance dependence of the maximum electron transfer rate ($\Delta G^\circ = \lambda$) (adapted from ref 104) for several systems. Dutton uses 18 different systems to determine the line on this plot; several of his systems are indicated by \blacklozenge : (A) bacteriochlorophyll to bacteriopheophytin (*Rhodospirillum rubrum*); (B) iridium to pyridinium; (C) bacteriopheophytin to quinone A (*R. rubrum*); (D) cyt *c* to His Ru; (E) quinone A to quinone B (*R. rubrum*); (F) myoglobin to His Ru; (G) quinone A to quinone B (*R. rubrum*). Interprotein systems covered in this review are indicated by \blacksquare : (1) hemoglobin; (2) cyt *c* to CcP; (3) cyt *c* to cyt *b₅*.

proteins may also occur before electron transfer. Kinetic schemes can be devised to include such complications. Kostic provides an excellent overview of these different schemes.⁵ This simplest reaction can be best illustrated schematically:



Here, A is the electron donor and B is the electron acceptor, K_1 is the binding constant of the reacting or "productive" complex, K_2 is the inverse of the binding constant for the products of electron transfer, and k_{et} is the electron transfer rate. Thus the overall reaction actually includes three processes, each optimized by different factors. Much effort has been directed at analyzing the structural and functional properties of the reactant and product proteins and precursor electron transfer complexes.

For any first-order reaction:

$$k = Ae^{-(\Delta G^\circ/RT)}$$

The rate is determined by a prefactor, A, and the activation energy, ΔG° . In the context of biological electron transfer, A is not a simple collision frequency, but instead is an electronic term that measures electronic wave function (orbital overlap) between the electron donor and the electron acceptor within the protein-protein complex. At optimal overlap (approximately van der Waals contact), $A = \text{ca. } 10^{13} \text{ s}^{-1}$, but decreases exponentially with distance, $A \propto e^{-\beta R}$. The damping factor, β , depends upon the properties of the intervening medium. For a wide range of systems, β has

been empirically determined as $1.2 \pm 0.3 \text{ \AA}^{-1}$, as best summarized in Figure 1 (adapted from Dutton¹⁰⁴).

ΔG° is the activation energy of the reaction. Although, in principle ΔG° should be quite complex, in practice, $\Delta G^\circ = (\Delta G^\circ - \lambda)^2/4\lambda$. λ is the "reorganization" energy alluded to above. It includes all of the redox-dependent nuclear motion in the chromophore, λ_i , and the associated charge-dependent changes in the surrounding medium, λ_s . For biological electron transfer, the term λ_s can include not only solvent repolarization but also repolarization of the protein medium around the redox-active site and, perhaps, the interface between the donor protein and the acceptor protein. A key insight of the theory is that the activation energy is related to the thermodynamic driving force for the reaction, ΔG , and to the reorganization energy, λ , of the medium. This is demonstrated in Figure 2. At small driving forces, (a) the activation energy will be proportional to the thermodynamic free energy until an activationless situation occurs at $\Delta G^\circ = \lambda$, (b) one prediction is that at very high driving forces, in the "inverted" region discussed above, decreased reaction rates will be seen (c), and although corrections which include quantization of the relevant modes can be included, they appear to be beyond the level of sophistication necessary to describe most biological reactions. Thus the driving force of the reaction depends largely on the redox center, and the reorganization energy depends on the entire structure; the two are somewhat separable variables. Thus, the equation of interest in Marcus theory is

$$k_{et} = Ae^{-\beta R}e^{-[(\Delta G^\circ - \lambda)^2/4\lambda]/kT}$$

This theory has been of great utility in understanding small-molecule charge-transfer reactions. The major successes of the theory are (1) the quantitative prediction of rate constants between nonidentical redox partners when self-exchange rates and free energy change are known, (2) the relationship between activation energy (ΔG°) and standard free energy of the reaction (ΔG°),¹³ (3) the description of nonspecific solvent effects on the rate constant, (4) the relationship between the self-exchange reaction in solution and the heterogeneous electron transfer between reactant and electrode, (5) the calculation of rate constants from molecular size, bond length changes, vibrational frequencies, and solvent properties, and (6) the inverted region, where reaction rates slow as the driving force increases.¹⁴ The dependence of the electron-transfer rate constant upon donor-acceptor distance,¹⁵ orientation,^{16,17} and electronic coupling have also been explored. Successful separation of these variables is difficult.

The Marcus theory forms the backdrop for our discussion of electron transfer between proteins.

III. Biological Electron Transfer

Because electron transfer can take place over "large distances" in a confined area (the cell), with redox potentials spanning 2 V, it is surprising that the cell doesn't come to equilibrium very rapidly, i.e. "short out". Molecular recognition helps ensure the physiological specificity of redox proteins. The problem of "short circuits" is circumvented by sequestering the reactive centers in a nonconductive polypeptide coat,

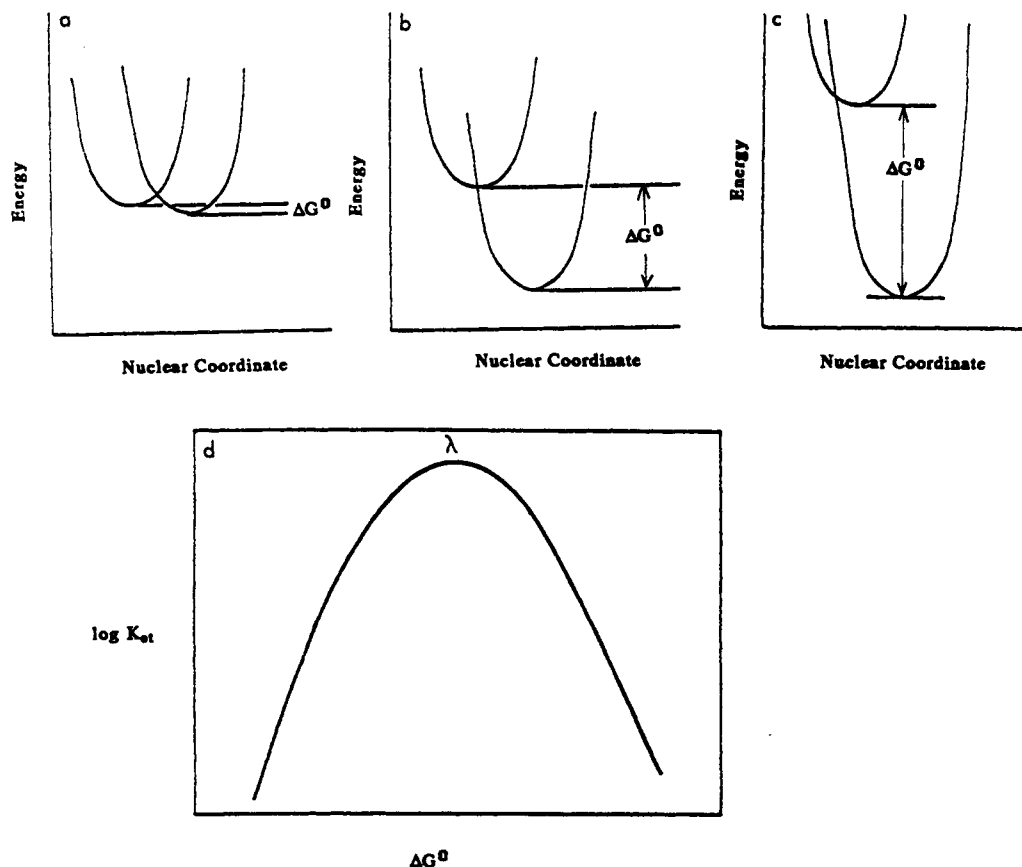


Figure 2. The three regimes for electron transfer: (a) $\Delta G^\circ < \lambda$; (b) $\Delta G^\circ = \lambda$, where the maximum electron-transfer rate is obtained; (c) $\Delta G^\circ > \lambda$, with increasing driving force an increasing activation energy is observed; and (d) a plot of ΔG° vs K_{et} , predicted by the theory.

just thick enough to make βR large enough to prevent random interactions, while maintaining a stable conformation with surface properties that allow binding of only the correct partner(s). Thus, the entire protein is not necessarily required for electron-transfer function but provides reaction specificity to an otherwise indiscriminate redox center.

A simple "back of the envelope" calculation can illustrate the effect of (transient) binding between proteins. (Only a small envelope is required.) Consider the case of the relatively reactive protein, ferryl cytochrome *c* peroxidase. The reactive ferryl group (Fe(IV)O) has a reduction potential of ca. 1.0 V. It is therefore capable of oxidizing many cellular components, yet it seems to react rather specifically with cytochrome *c*. For example, at identical concentrations of the specific partner, cytochrome *c* (II), and a non-specific reagent, Fe(CN)_6^{4-} , CcP will react only with cyt *c* (II). This is because, even though the second-order rate constant for reaction with Fe(CN)_6^{4-} is high ($10^6 \text{ M}^{-1} \text{ s}^{-1}$), at micromolar concentration, the effective pseudo-first-order rate is only 1 per second. By contrast, given a binding constant for the CcP:cyt *c* complex of ca. 10^7 M^{-1} , even at 10^{-6} M cyt *c*, all of the cyt *c* will be bound. Using eq 1, with $\Delta G = 0.8 \text{ V}$ and $\lambda = 1.2 \text{ V}$ and $R = 16 \text{ \AA}$, we predict a rate of electron transfer of ca. 10^3 s^{-1} . Thus the cytochrome, by binding, obtains a 10^3 advantage over the purely collisional reaction, and specificity is assured. (Of course, when Fe(CN)_6^{4-} is 1 M, CcP would "short circuit", but if a cell were 1 M in Fe(CN)_6^{2-} , then other problems would be more pressing....) This simple argument provides a

possible guide to understanding the dynamics of protein to protein complex formation. Assume that the protein complex formation is diffusion controlled (which is roughly correct); if the reaction is to occur while the protein is bound, then the residence time must be (just slightly) longer than the mean reaction time. Obviously, there is no advantage to binding longer than necessary: if the "off" rate becomes very slow, this will limit the rate of turnover of the system. Thus, taking $k_{et} = 10^3 \text{ s}^{-1} \geq k_{off}$, we expect the off rate to be ca. 10^3 s^{-1} . We thereby predict an optimal binding constant $K = k_{on}/k_{off} = 10^{10} \text{ M}^{-1} \text{ s}^{-1}/10^3 \text{ s}^{-1} = 10^7 \text{ M}^{-1}$ which quite nicely predicts the measured value for the system.

IV. Basic Approaches to Study

The structure of each individual protein reactant can, in best cases, be deduced from crystal structures. Where this information is not available, as for most protein-protein complexes, models of the electron transfer complexes, based on docking of crystal structures,¹⁸⁻²⁶ are important starting points for predicting binding behavior and electron transfer rates, and therefore for formulation of experiments.

Such models have been tested by a variety of methods which probe the recognition and binding between the protein pairs, including UV-visible spectroscopy, infrared spectroscopy, NMR spectroscopy, time-resolved and steady-state fluorescence quenching, chemical modification, and mutagenesis.²⁷⁻³³

The kinetics of electron transfer has been studied using a variety of methods, depending on the time scale

required. Physiological reactions lend themselves well to steady-state (seconds–minutes), stopped-flow (milliseconds–seconds), and laser-flash photolysis (microseconds–milliseconds) kinetic studies.

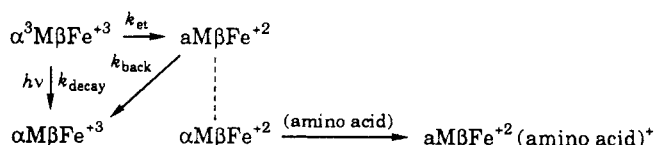
The final technique is particularly suitable for studying electron transfer in preformed protein complexes. Laser-flash photolysis has often been used to probe the reaction, using photoinducible electron-transfer agents or photochemical initiators for the reaction. These agents fall into three classes: flavin semiquinones and other small molecules, ruthenium–amine complexes attached to the residue of interest at the protein surface, and for heme proteins, to initiate electron transfer, iron substitution by metals with a long-lived triplet state.^{4,34–36} These various approaches are detailed elsewhere.⁴

With this background, we consider, on a case study basis, reactions of some individual protein complexes.

A. Hemoglobin

Electron transfer between two redox active sites in a single protein is the simplest regime for studying biological electron transfer, since the distances and angles between redox centers can be well defined. An excellent example of this type of system is hemoglobin. This tetrameric protein can be considered a dimer of dimers; two α and two β subunits arranged as $[\alpha\beta]_2$. Each subunit contains a ferric heme, and the structure of the protein with several ligands is known. This unique juxtaposition of insulated hemes provides an opportunity to determine electron-transfer rates in a system which contains well-defined distances and geometries. These subunits can be separated and the hemes in one type of subunit, either α or β , can be replaced by another protoporphyrin IX molecule containing another metal, e.g., a closed-shell dication like Zn, Mg, or metal-free H_2 . When the subunits are mixed again, a deoxy or T type tetramer reforms. These hybrid hemoglobins are designated as $(\alpha M\beta Fe)_2$ or $(\alpha Fe\beta M)_2$, depending upon where the metal was substituted. These have been characterized by several methods.^{25,37} The hemes are arranged in the protein as shown in Figure 3.

Hoffman pioneered the flash photolysis of long-lived triplet excited states in metal-substituted hemoglobin, with particular reference to subunit to subunit charge transfer. The reaction



has been explored for Zn, Mg, and H_2 for this system.^{38–47} The reorganization energy of the system was estimated to be 2.1 eV from studies of the temperature dependence of the reaction. Because the vinyl groups of the porphyrins in the α and β subunits are oriented toward each other, the distance dependence was explored by substituting deuterioporphyrin into the subunits, thereby increasing the distance between the nearest π carbons from 16 to 17 Å. This change led to a temperature-independent decrease in k_{et} by a factor of 2.8,^{45a} demonstrating the distance contribution to electron transfer.

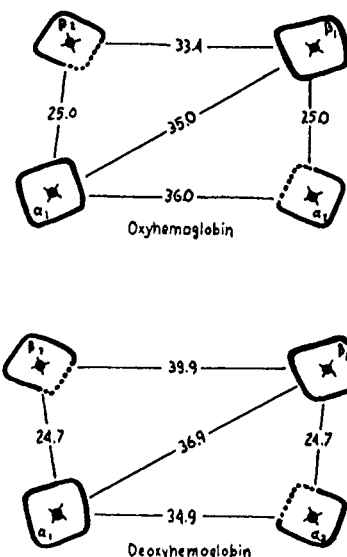


Figure 3. Arrangements of hemes within oxyhemoglobin and deoxyhemoglobin. Fe–Fe distances are shown in angstroms. Note that not all hemes are coplanar.

B. Cytochrome c

Cytochrome *c* (cyt *c*) has been a particularly popular protein because of its stability and availability. Of the electron-transfer proteins, more is known about cyt *c* than any other redox protein.⁴⁸ Cytochrome *c* is an ubiquitous protein among eucaryotes; the complete sequence of over 100 eucaryotic cyt *c*'s have been reported. The tertiary structures of several different cytochromes have been determined by crystallography.⁴⁹ Numerous studies, exploring structural, physical, enzymatic, and genetic properties of cyt *c*,^{50,51} have been reported. Large patches of positive charge near the solvent-exposed heme edge of cyt *c* facilitate interaction with several redox partners. In mitochondria, it is the penultimate electron-transfer agent in the mitochondrial electron-transport chain, shuttling electrons between the membrane bound cytochrome *c* reductase and cytochrome *c* oxidase. In yeast, it has other physiological partners, including cytochrome *b*₂ (lactate dehydrogenase), and cytochrome *c* peroxidase. Because of its charge and size it has been used to study electron transfer with many nonphysiological (plastocyanin, cytochrome *b*₅) as well as physiological (cyt *c* oxidase, cyt *c* peroxidase, cytochrome *b*₂) partners.

1. Cytochrome *c*/Cytochrome *b*₅ Complex

Cytochrome *b*₅ (cyt *b*₅) is a membrane-bound protein abundant in the liver and involved in stearyl Co-A desaturation^{52–54} as well as in cytochrome P-450 reduction.^{53–55} The second form is found in red blood cells, where it functions in the reduction of adventitiously oxidized (met)hemoglobin.^{56,57} Although the direct interaction of cyt *c* and cyt *b*₅ is unlikely in vivo, they react readily in vitro via a strongly bound complex. Thus, the cyt *c*/cyt *b*₅ complex served as an early model for testing fundamental aspects of protein to protein electron transfer. The active portion of the protein, which includes the heme prosthetic group, is about 95 amino acids. The cyt *c*/cyt *b*₅ system was the first where computer graphics were applied to the problem of redox protein interaction.²⁶ By matching oppositely

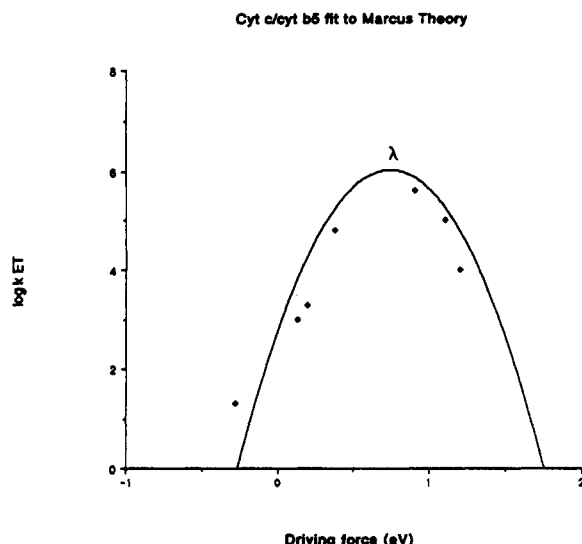
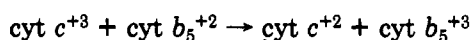


Figure 4. Plot of ΔG vs k_{et} for the cyt b_5 /cyt c system. The λ indicated is 0.8 eV.

charged residues while avoiding steric crowding near the exposed heme edges of each protein, with the heme edges nearly coplanar, a set of complementary charges in the correct steric configuration could be arranged so that the heme edges were only 8 Å apart and Fe centers only 17 Å apart. The model predicts that the Lys residues 13, 27, 79, and 72 interact with the Glu 48, Glu 44, Asp 60, and a heme propionate, respectively. This theoretical model has since been refined using molecular dynamics methods.^{25,58} These later models include the original complex and several others, which may be more favored for electron transfer than the original complex. Visible difference⁵⁹ and fluorescence⁶⁰ spectroscopy were used to determine binding affinity of the complex. The binding constant [$2 \times 10^7 \text{ M}^{-1}$ at pH 7 and ionic strength (μ) = 0.02] decreases as ionic strength increases and is driven by an increase in entropy. NMR,⁶¹ high-pressure studies,⁶² and mutagenesis⁶³ have been employed to map the binding faces of the complex.

In experimental studies, both chemical modification and NMR show that 5–6 lysines (Lys 13, 25, 27, 72, and 79)⁶⁴ in cyt c are important in the electrostatic binding of cyt c to cyt b_5 . Esterification of the heme propionates hinders the electron transfer between the partners and favors a set of complexes which are different from the unesterified system. Esterification of the other anionic residues decreases the stability of the complex as well. Cross-linking with carbodiimide as well as high-resolution NMR indicate that there are several static complexes. The heme Fe–heme Fe distance has been estimated, by fluorescence quenching, to be 17 Å.⁶⁵ The reaction



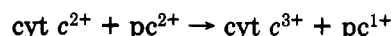
can be explored by combining several methods and provides an exemplary fit to the theory. The observed electron-transfer rate appears to be well described by Marcus' theory where rate increases as ΔG increases, reaching a maximum as $\Delta G = \lambda = 0.08 \text{ eV}$ (Figure 4). The observed rate of 1600 s^{-1} for the native system is well below the maximum possible rate. The key lessons learned from this system was that protein to protein electron transfer can be rather rapid, but still involves large reorganization ($\lambda = 0.8 \text{ eV}$).

TABLE I

reductant	oxidant	ΔE° (V)	K_{et} (s^{-1})	ref
pc ⁺¹	cyt c^{+3}	-0.1	87.3	66
cyt c^{+2}	pc ⁺²	0.1	4800 + 600	66
cyt c^{+2}	pc ⁺²	0.1	1050 + 150	69
cyt $c+c$ (covalent)	pc ⁺²	0.14	<0.2	69

2. Cytochrome c /Plastocyanin Complex

Plastocyanin (pc) is a blue copper protein found in plants. The plastocyanin crystal structure is known for both Cu(I) and Cu(II) oxidation states. The function of the protein is to transfer electrons in the photosynthetic cycle to cytochrome f . Unfortunately, cytochrome f is rather poorly characterized, in part because it is both large and membrane bound. Thus, cyt c is often used in place of the cytochrome f , since both are similar in surface charge and E° .⁶⁶ The analogue of the physiological reaction is



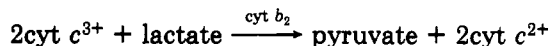
Kinetic studies indicate that the pc binds near the exposed heme edge of cyt c through the acidic segments 42–45 and Tyr 83⁶⁷ on plastocyanin to the basic lysine patch of cyt c . Docking simulations estimate that the (pc)Cu–(cyt c)Fe distance in the complex is ca. 18 Å, and the distance between the heme edge and the copper thiolate ligand is about 12 Å. NMR experiments, focusing on the heme methyl resonances of cyt c indicate a 1:1 complex is formed and that the binding constant is $1 \times 10^3 \text{ M}^{-1}$ at 10 mM (μ) at pH 7.5 and that there is rapid exchange between the free and bound pc in the complex.

The two proteins have been cross-linked by carbodiimide,⁶⁸ forming four species. The bonds formed were groups around the heme edge of the cyt c and the acidic region of plastocyanin, as suggested in simulated docking experiments. These cross-linked complexes were studied by several spectroscopic methods, and no perturbation of their active sites were found. However electron transfer was very slow between covalently attached proteins, suggesting that some reorientation of the protein surface is necessary for electron transfer. This interfacial motion within a protein–protein complex to obtain efficient electron transfer is apparently rather common in biology, as the next two cases show.

Because the heme and the copper spectra do not overlap in the visible region of the spectrum, the system is well suited for kinetic studies. Studies reported in Table I show differences in electron-transfer rates, depending upon conditions.

3. Cytochrome b_2 /Cytochrome c Complex

Cytochrome b_2 [bakers' yeast L-(+)-lactate dehydrogenase; flavocytochrome b_2 ; yeast L-lactate: cytochrome c oxidoreductase] is a low-spin ($s = 1/2$) tetrameric enzyme of about 235 000 molecular weight, located in the intermembrane space of the mitochondria. Cytochrome b_2 catalyzes the transfer of reducing equivalents from L-lactate to cytochrome c by means of its prosthetic groups, flavin mononucleotide (FMN) and heme, in the reaction



The crystal structure at 3.0-Å resolution of intact cyt b_2 has been published.²⁰ This protein contains two do-

TABLE II

reductant	oxidant	ΔE° (V)	K_{et} (s^{-1})
cyt b_2 + 2	yeast cyt c^{+3}	0.2	570
cyt b_2 + 2	horse cyt c^{+3}	0.2	200 ± 80
horse 3H_2 cyt c	cyt b_2^{+3}	0.4	700 ± 100
horse 3Zn cyt c	cyt b_2^{+3}	0.8	600 ± 200

mainly: a flavin domain, which abstracts H atoms from lactate to effect oxidation, and a cytochrome domain, which provides the necessary oxidizing equivalents (via cyt c) to regenerate the flavin cofactor. The flavin ring and heme group are separated by 16 Å center-to-center in each monomer, and the planes are inclined by 17° toward each other. The flavodehydrogenase domain is involved not only in the reaction with lactate, but also in modulating the dynamics of electron transfer between the bc cytochrome domain and cyt c .⁷⁰⁻⁷²

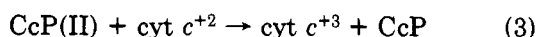
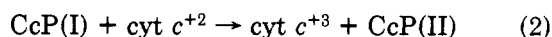
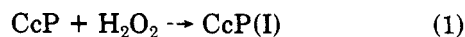
Several investigations of the electron transfer behavior of this protein couple have been reported.⁷³ Cyt c , Zn cyt c , and metal-free cyt c all form complexes with cyt b_2 with essentially equal affinity. Rate constants for the complexes are shown in Table II.⁸⁰

In contrast to the cases discussed in Table II, the reaction rate is independent of reaction free energy.⁷⁶⁻⁷⁹ Such free energy independence is a likely indicator that the rate-determining step is not an electron transfer (where $k_{et} \propto \Delta G$), but instead the electron transfer is "gated".^{12,80} This is not an isolated case of strong coupling of conformational change and electron transfer. Cytochrome c oxidase is not treated explicitly in this review due to limited knowledge of its structure. However, it is known that cyt c oxidase induces a conformational change and polarity change on cyt c before electron transfer and that this conformational change is strongly coupled to electron transfer.⁸¹ As a third example, the pc :cyt c system just discussed shows a free energy dependence at low ΔG , where electron transfer is rate limiting. At high ΔG the "surface diffusion" at the pc :cyt c interface can become the slowest step.

4. Cytochrome c /Cytochrome c Peroxidase Complex

Of all of the protein to protein electron-transfer systems which have been studied to date, probably the best characterized is the reaction between cytochrome c and cytochrome c peroxidase (CcP). The cyt c /CcP system is a particularly attractive model for studying biological electron transfer. Both proteins are stable and are readily isolated in pure form. High-resolution structures are known for both proteins in oxidized and reduced forms,^{82,83} and detailed static and dynamic models of the interaction have been proposed.⁸⁴⁻⁸⁶ These proteins form an electron-transfer complex which is purported to be involved in yeast physiology as detailed below. Finally, both proteins have been cloned,^{87,88} opening the possibility for site-directed mutagenesis as a tool to probe specific interactions.

Cytochrome c peroxidase catalyzes the oxidation of cytochrome c by H_2O_2 as shown in the following unbalanced equations:



The product, called CcP(I) is referred to as compound

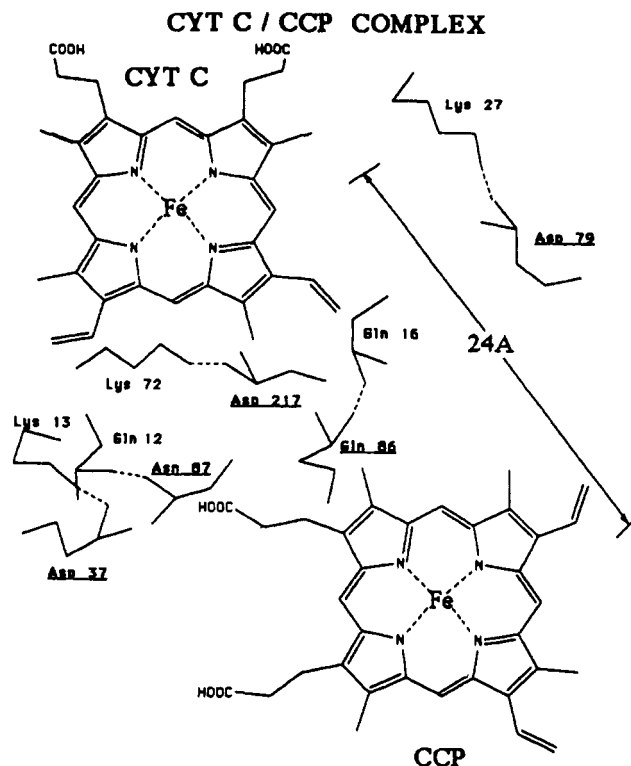


Figure 5. The original model for the cyt c /CcP complex, based on docking of tuna cyt c and CcP crystal structures. CcP residues are underlined (adapted from ref 84).

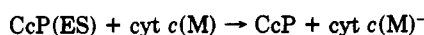
I or is often designated "ES", contains an oxyferryl (Fe(IV)O) heme and an amino acid cation radical. The first reduction yields the semireduced compound II, an equilibrium mixture of the oxyferryl and cation radical species. The radical is extensively delocalized over an aromatic network, focused on Trp 191. The kinetic scheme implied by eqs 1-3 is rather complicated.⁸⁹ The limiting rate constant for reaction 2 is construed as the intracomplex electron-transfer rate.

In early studies, the interaction between cyt c and CcP was interpreted in terms of a geometrically well-defined electron-transfer complex.^{24,90} This proposed complex, shown in the Figure 5, was based on the docking of the individual crystal structures of tuna cyt c and CcP by optimizing electrostatic interactions. The optimization by computer graphics places the heme groups nearly coplanar, with the heme edges ca. 18 Å apart and the Fe atoms ca. 24 Å apart. The primary interaction domain on cyt c is localized around the exposed heme edge, near Lys 13 and the heme pyrrole II. Other residues implicated in the model are Lys 27, 72, 79, 86, and 87, which surround the exposed heme edge. The peroxidase has complementary aspartates at positions 37, 79, 217, and 34 that form hydrogen bonds ("salt bridges") with the cyt c lysines. An electron-transfer pathway, including His 181 of the CcP, hydrogen bonded to the heme propionate, and Phe 82 on the surface of cyt c , close to the heme. Like the cyt b_2 model, this work prompted a great deal of research to test the model.

Early binding studies and chemical modification experiments broadly support the model but suggest that a broader domain than originally suggested might be involved in binding.

Site-directed mutagenesis has been used to investigate the roles of individual amino acids in protein in-

TABLE III



metal	ΔE° (V)	k_{obs} (s^{-1})
Fe(II)	0.9	800
Zn(II)	0.35	0.2
H ₂	0.05	0.05

teraction. Early work focused on the interaction of the Asp 37, 79, and 217; residues implicated by Poulos and Kraut as having complementary charge and steric interactions with corresponding cyt *c* residues. Stepwise mutagenesis of these residues on the surface of CcP and the corresponding residues on cyt *c* have probed this interaction.

The results, while broadly consistent with the Poulos and Kraut model, are best explained if there is not a single rigid complex. Such a flexible interaction motif is consistent with more recent and more complex docking experiments. Brownian dynamics calculations suggest a complex that allows multiple binding sites, with the possibility of restricted or two-dimensional diffusion after binding.⁸⁶ CocrySTALLIZATION of CcP and cyt *c* yielded X-ray diffraction patterns indicating that the cyt *c* may occupy several sites on CcP, but is disordered in the crystal.⁹¹ Finally, there evidence for a distribution of heme-heme distances in the complex and reactant (ferrous cyt *c*) and product (ferric cyt *c*) have different equilibrium binding characteristics.^{92,93}

Physical measurements on mutant forms of the proteins indicate that cyt *c* uses at least two different binding patches which closely correspond to those implicated by Northrup's Brownian dynamics studies⁸⁶ and, furthermore, that the reduced and oxidized species have different affinities for these subdomains of the general binding region. The reduced cyt *c* appears to localize around Asp 37 and Asp 79, while the oxidized cyt *c* localizes around Asp 217 and Asp 37. This implies that the couple may utilize interfacial motion (a restricted dimensional diffusion) to arrive at the "productive complex" for the electron transfer. While this motion occurs rapidly at room temperature, Hoffman and co-workers have postulated that this motion may be slowed enough at 250 K to make this motion the rate-determining step for electron transfer: the redox reaction becomes "gated" by the interfacial conformational change. At 220 K, no electron transfer is observed, indicating that the complex is trapped in a nonproductive conformation(s).⁹⁴

The reorganization energy for this couple has been estimated by both temperature-dependence measurements and by free energy perturbation methods (Table III).⁷⁶

Both methods suggest a reorganization energy of about 1.4 eV. If interfacial motion is indeed linked to electron transfer, as these studies imply, then the thermally activated motion will contribute to the apparent reorganization energy for the reaction.

In this context it has been observed that complexes which cannot undergo Brownian diffusion cannot transfer electrons well.⁹⁵ If the electrostatic interactions were identical for both product and reactant, low ionic strength, which favors strongest binding, should also favor fastest electron-transfer rates. The opposite is observed. When the ionic strength is increased, with concomitant decrease in binding, electron-transfer rates

increase, as expected if surface diffusion is necessary for efficient electron transfer.

In like manner, those derivatives that bind less well (e.g., Asp 37 Lys) give lower apparent reorganization energies for electron transfer (ca. 1.0 eV). This suggests that we have opened a new path for electron transfer or, in this context, lessened the interfacial reorganization necessary for electron transfer. Redox-linked interfacial dynamics provide a simple and natural explanation for the relatively large reorganization energy ($\lambda = 1.5$ V)⁷⁶ observed in the cyt *c*/CcP complex and related complexes.

Why should biological electron transfer involve this rather messy sequence of nonspecific binding, interfacial diffusion, and subsequent electron transfer? Why not just bind to a single (optimal) domain in the first place? A simple analysis suggests that the "messy" solution may be the best. The probability of obtaining a precise alignment of small (charges) groups in random collisions of large protein surfaces is very low. [Given a surface area of ca. 200 Å² and a reactive area of 10 Å², few collisions (5%) would be productive; if three such points had to form simultaneously, the probability becomes vanishingly small (0.05).^{3]} By contrast, if the complexes can be "electrostatically guided" as Margolias suggests,⁹⁶ into a general binding domain, then virtually all collisions will produce an initial complex. Since subsequent redistribution is fast compared to electron transfer, the high degree of reaction specificity is assured by this "patchwise" recognition mechanism.

V. Conclusions

We have discussed several protein complexes, focusing on the cyt *c*:CcP complex, in terms of Marcus theory and interprotein electron transfer. These results lead to the following conclusions and speculations:

While this work is still in its infancy, results indicate that Marcus theory, in its simplest (classical) form, provides a reasonable description for protein to protein electron-transfer reactions. As other reviewers have pointed out,^{4,35,36,97,101} certain generalizations emerge from such work.

In cases where electron transfer is the rate-determining step, the correlation between driving force for interprotein electron transfer is good. Most interprotein electron-transfer systems do not exhibit Marcus "inverted" region kinetics. This likely reflects the rather high Marcus reorganization energies, which may be preventing us from preparing a system with a large enough driving force. Alternatively, "surface diffusion", or some other conformational change may be rate limiting at high ΔG values, so that the reaction becomes "gated".

A general distance dependence has also been demonstrated for protein to protein systems. However, in an interprotein complex, it is difficult to know the distance between reacting centers, because of the lack of "lock and key" interactions. Furthermore, it is not clear that a "straight-line distance" provides the best electronic overlap. For example, Beratan¹⁰² has proposed that through-bond effects can dominate electronic coupling in proteins. Thus the dependence of rate on distance or pathway is still best studied by intraprotein electron transfer, like the hemoglobin case, protein-derivatized electrodes,¹⁰³ or small molecules

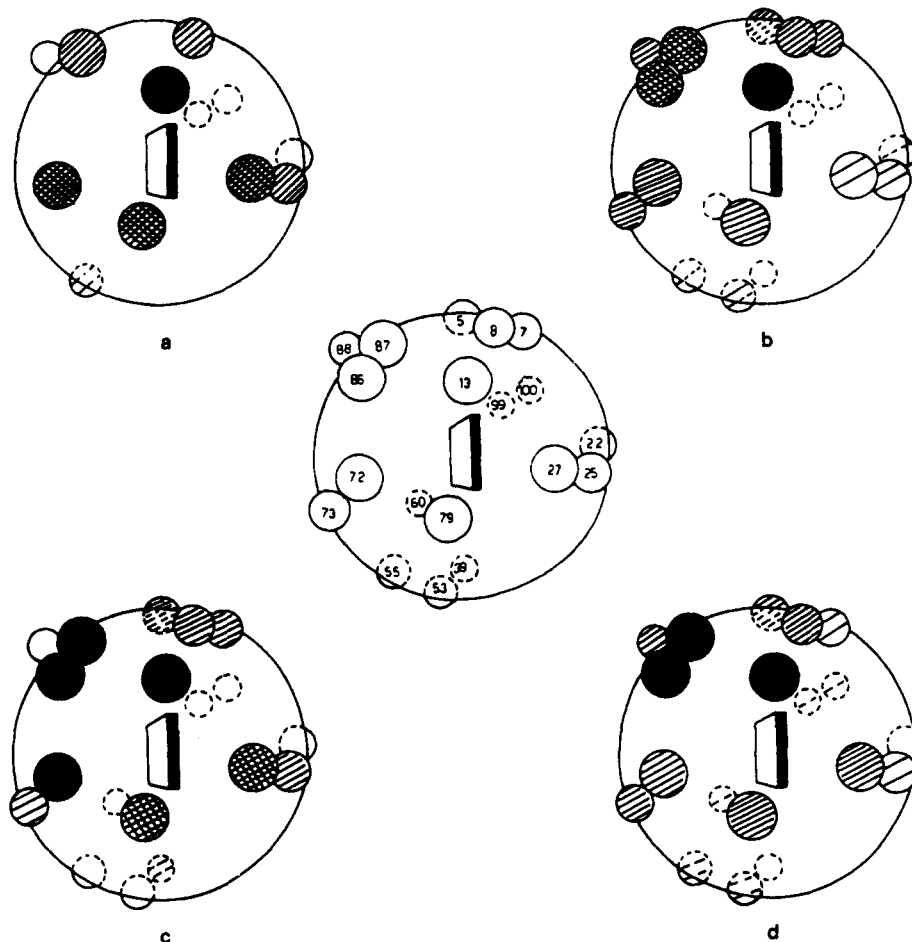


Figure 6. Representation of cyt *c* residues important in the interaction with cyt *b*₅ (a), cyt *c* oxidase (b), cyt *c* peroxidase (c), and cyt *c* reductase (d) (adapted from ref 64). The degree of shading indicates the relative importance of each residue. Dashed outlines indicate residues located on the face opposite the exposed heme crevice.

covalently attached to the protein.¹⁰² With that caveat, the general similarity for the distance dependence of protein-protein electron transfer to that for small molecules was noted in the first studies of the cyt *c*/cyt *b*₅ system, and recently systematized by Dutton.¹⁰⁴ It is amusing to note that those systems treated here (except for the "gated" reactions) fit well with Dutton's generalizations (Figure 1).

Peculiar to proteins is the problem of recognition. Unlike the small molecules originally used to formulate the electron-transfer theory, proteins recognize and bind their redox partners. This recognition, although useful to the cell, remains difficult to model.

In this context, the cell has used a generalized mode of electrostatic binding to its advantage. Certain proteins (e.g., cyt *c*) can recognize several physiological partners (e.g., cyt *c* peroxidase, cyt *c* reductase, cyt *c* oxidase, cyt *b*₂) as well as nonphysiological ones (cyt *b*₅, plastocyanin). Figure 6⁶⁴ illustrates the relative importance of certain cyt *c* residues in interacting with electron-transfer partners. Binding domains differ for each partner.

A corollary to this observation is that cyt *c* and perhaps many other proteins are not, and cannot be, optimized for all partners. In vivo, a typical rate (10^3 s^{-1}) is required. Anything faster, or much slower is unnecessary and often undesirable, causing saturation or depletion of materials necessary to the cells survival. Since this rate corresponds to the mean residence time

of the cyt *c* on CcP, one can speculate that the cell has engineered recognition to facilitate electron transfer at that rate.^{1,103}

A remaining thorny problem in protein electron transfer is understanding the protein matrix itself. What kind of a solvent is a protein? How does it conduct electrons? What is tunneling like, and what pathways are used by an electron traveling through a protein? How can λ be calculated with the plethora of data available on the simpler systems? How can an interface reorganize, if it does? These problems lie in the area of protein chemistry and are dealt with, in part, in the review by Gray in this volume.

Although much remains to be done in this area, data from the past decade indicate that many of the questions left unanswered here may be printed in textbooks a decade from now.

VI. References

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