Simulation of Electron-Transfer Self-Exchange in Cytochromes *c* and b_5[†]

Sedonia M. Andrew, Kathryn A. Thomasson, and Scott H. Northrup*

Contribution from the Department of Chemistry, Tennessee Technological University, Cookeville, Tennessee 38505

Received August 26, 1992

Abstract: Brownian dynamics (BD) has been employed to simulate the kinetics of the electron-transfer self-exchange reactions of trypsin-solubilized bovine liver cytochrome b_5 (*cytb5*) and horse heart cytochrome *c* (*cytc*). A structurally robust BD method simulating diffusional docking and electron transfer was employed to compute bimolecular rate constants, which were then compared with those obtained experimentally. BD provides a detailed description of the collision stage of the process, determined by the actual atomic scale irregularity of the proteins (steric factors) and the mutual electrostatic interactions. A realistic two-parameter model of the electron-transfer unimolecular rate constant was employed which is exponentially varying over donor–acceptor distance. The BD theory successfully reproduces the ionic strength dependence of the reaction. A slightly better fit was obtained than that afforded by van Leeuwen theory, with only two adjustable parameters. By fitting the BD-generated rate constants to the experimental curve and using Marcus theory, we extracted a reorganization energy λ and distance decay factor β for both self-exchange reactions. Values obtained were λ = 1.06 and 0.69 eV for the *cytb5* and *cytc* systems, respectively, and β = 0.9 Å^{-1} was obtained for both systems. For the first time, BD was used in the limit where reaction is activation-controlled rather than diffusion-controlled. This was facilitated by a model that embodies an explicit coupling between the diffusion and chemical dynamics. In the activation-controlled regime the Brownian algorithm efficiently generates a Boltzmann distribution of docked conformers. A direct calculation of the entropy cost of forming docked complexes was performed by tallying the potential of mean force versus heme–heme distance.

Introduction

Electron-transfer reactions of metalloproteins play a fundamental role in many important biological processes.[1] The kinetics of electron transfer (ET) between metalloproteins are controlled by a large number of physical factors.[2] The initial stage of reaction involves translational and rotational diffusion to form associated precursor complexes, which is in turn influenced by electrostatic and steric forces between proteins communicated through the intervening dielectric medium. The second stage of reaction involves the intrinsic ET event from preformed complexes, which depends on the mutual orientation and separation of donor and acceptors at docking, thermodynamic driving force, reorganization energy, and the composition of the salient pathways for ET. The unraveling of each role among the variety of factors is an important goal in the complete understanding of the regulation of ET in biological systems.

Numerous self-exchange reactions have been studied in order to understand ET in inorganic complexes[3] and metalloproteins.[4] The study of self-exchange kinetics simplifies the interpretation of the intrinsic ET rate, since the thermodynamic driving force is zero, and the reorganization energy becomes the predominant energetic term in the Marcus theoretical rate constant for ET.[2d] Recently, the ET self-exchange kinetics of tryptically solubilized bovine liver cytochrome b_5 (*cytb5*) and horse heart cytochrome *c* (*cytc*) were studied[4] as a prototypical metalloprotein system. These proteins are easily isolated and amenable to site-directed mutagenesis.[5] These systems are also accessible to rigorous atomic scale theoretical modeling since the three-dimensional structure has been characterized to high resolution by Mathews and co-workers (*cytb5*)[6a,b] and by Brayer and co-workers (*cytc*), respectively.[6c]

In this paper we will compare the results of these experimentally obtained rate constants[4] with predictions of a structurally robust Brownian dynamics (BD) method simulating diffusional docking and ET.[7] In our model the BD of two whole proteins rotating and diffusing in their mutual electrostatic force field is explicitly coupled with the intrinsic ET dynamics of the docked complexes.[7,8] A realistic model of the ET event embodied in an intrinsic unimolecular rate constant is used which is exponentially varying over donor–acceptor distance. The BD method quantitatively predicts bimolecular rate constants as a function of environmental variables such as ionic strength, pH, and temperature, and fitting to experiment can be made by adjusting the parameters controlling the model intrinsic ET rate constant.[7b,8a]

* Author to whom correspondence should be addressed.

† Abbreviations used: ET = electron transfer, BD = Brownian dynamics, PMF = potential of mean force, *cytb5* = trypsin-solubilized bovine liver cytochrome b_5, *cytc* = horse heart cytochrome *c*.

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Table I. Experimental[4] and BD-Simulated Bimolecular Rate Constants k (M^{-1} s^{-1}) for Electron Self-Exchange in *cytb5* and *cytc* as a Function of Ionic Strength μ[a]

	cytb5		*cytc*	
μ (M)	expt	theory	expt	theory
0.1	2.6×10^3	$1.46 (\pm 0.25) \times 10^3$		
0.12			5.4×10^3	$4.32 (\pm 1.06) \times 10^3$
0.3	4.6×10^3	$6.62 (\pm 0.77) \times 10^3$	1.6×10^4	$2.02 (\pm 0.41) \times 10^4$
0.5			2.8×10^4	$3.12 (\pm 0.44) \times 10^4$
0.6	1.6×10^4	$1.93 (\pm 0.36) \times 10^4$		
0.8			4.2×10^4	$4.23 (\pm 1.06) \times 10^4$
1.0	2.8×10^4	$3.00 (\pm 0.36) \times 10^4$	5.0×10^4	$4.68 (\pm 1.06) \times 10^4$
1.5	4.5×10^4	$4.12 (\pm 0.52) \times 10^4$	5.9×10^4	$5.80 (\pm 0.68) \times 10^4$

[a] T = 25 °C, pH = 7.0. The theoretical values are those using the best-fit value of the adjustable parameters $k_{et}° = 3.43 \times 10^8$ s^{-1} (*cytb5*) and 1.18×10^{10} s^{-1} (*cytc*) and $\beta = 0.9$ $Å^{-1}$ (both systems).

The BD simulation method described here has already been used successfully to predict bimolecular rate constants for reactions of other cytochrome couples. These include *cytc* and yeast cytochrome *c* peroxidase,[7,9] horse and yeast ferricytochrome *c* with *cytb5*,[7,8a] and self-exchange in *Pseudomonas aeruginosa* cytochrome c_{551}.[8b]

Theoretical Background

Previous experimental studies of Dixon *et al.*[4] have obtained bimolecular rate constants for self-exchange in the *cytb5* and *cytc* systems as a function of ionic strength. These data are given in Table I. In their work, the Marcus theory[2d] of the ET rate constant was used to correlate the data for the purpose of extraction of the reorganization energy. We discuss here their theoretical analysis and how it compares with our own more detailed analysis. Their expression of the ET rate constant is of the form

$$k_{et}^{eq} = SK_a\nu_n\kappa_{el}\exp(-\Delta G_r/k_BT) \quad (1)$$

which has a number of factors needing to be estimated for both proteins. First, the factor S is a steric factor based on the fractional surface exposure of the heme group. In actuality, the computed heme fractional surface exposure was multiplied by 5 to account for the fact that ET can take place from a variety of distances and orientations. In our BD simulation, however, this steric factor is replaced by explicitly monitoring the heme–heme distance and computing a fractional reaction probability at every Brownian step.

The quantity K_a is the association constant, estimated by Dixon *et al.* by calculating effective volume over which reaction occurs along the reaction coordinate, multiplied by w_r, an electrostatic work term. The association constant depends upon the range of internuclear separations contributing to reaction, the effective spherical diameter of protein *cytb5*, and the work factor w_r, the energy to bring proteins together.

The combined quantity SK_a then is the equilibrium constant for formation of the ET complex. Thus, eq 1 is an equilibrium theory of the rate constant in that a spatial equilibrium of reactant pairs is assumed. In our BD theory this was supplanted by an explicit simulation of the collision probability for forming the complex. Instead of beginning with an equilibrium approximation for the rate constant, we make no specific approximation other than assuming that species come into docked arrangement by a translational and rotational diffusion mechanism. We do not assume diffusion control either, since the diffusion dynamics are explicitly coupled in our model to the reactive ET dynamics through an intrinsic unimolecular ET rate constant k_{et}. If this rate is inherently slow compared to diffusion into a docked ET complex, then our BD algorithm simply will supply an equilibrium population of docked complexes sampled by the distance-dependent exponential reactivity function. The work factor is a quantity for which we can estimate an average over many possible ET docked complexes that we generate.

The quantity $\nu_n\kappa_{el}$ in eq 1 is an ET efficiency factor, where ν_n is the nuclear frequency factor estimated as 10^{13} s^{-1} and κ_{el} is the probability of electron tunneling once the nuclear transition state has been formed, and is estimated as an exponentially varying function of heme–heme distance:

$$\kappa_{el} = \exp(-\beta(d - d_0)) \quad (2)$$

In the work of Dixon *et al.*, the quantity β (assumed to equal 0.9 $Å^{-1}$) is the distance decay factor for ET, d is the distance of closest approach of hemes in a docked ET complex, and d_0 is the minimum possible heme–heme distance of two isolated hemes, at which $\kappa_{el} = 1$. Finally, the term ΔG_r is the free energy of activation. In the absence of driving force, it is simply related to the reorganization energy by the formula $\Delta G_r = \lambda/4$.

Simulation Method

The BD method of simulating the translational and rotational diffusion of two whole proteins has been thoroughly described in recent articles,[7-10] and so we provide an abbreviated sketch here. Atomic scale modeling of *cytb5* and *cytc* began with the crystallographic coordinates of Mathews *et al.*[6a,b] and Brayer *et al.*,[6c] respectively. The oxidized and reduced structures were assumed for our purposes to be identical. The protonation state of each titratable amino acid residue was estimated by performing a Tanford–Kirkwood calculation with static-accessibility modification.[11] Thus each residue was assigned a net charge on the basis of its protein environment, pH, ionic strength, and temperature. The net charges at pH = 7.0, μ = 0.1 M, and T = 25 °C on the oxidized and reduced forms are estimated to be −7.2e and −8.2e for *cytb5*, respectively, and +7.0e and +6.0e for *cytc*.

In BD, the Brownian motion of two interacting proteins in a solvent is simulated by a series of small displacements governed by the diffusion equation with forces using the Ermak and McCammon algorithm.[12] The connection of BD trajectory statistics with bimolecular rate constants is made by separating the problem into centrosymmetric diffusion to a starting surface b from outside treated analytically and diffusion in a complicated force field inside b, where BD simulation provides the description.[13] Trajectories of diffusing species are begun at random orientations from a separation $r = b$ = 65 Å, outside the region of asymmetric Coulombic forces, and are truncated at an outer spherical surface c = 200 Å. As many as 25 000 trajectories are required to obtain statistically significant values of the probability p of eventual association of pairs into favorable geometries for reaction prior to ultimate separation to distance $r = c$. We may study the influence of a whole range of different ET reactivities simultaneously in a single simulation, monitoring the survival of the trajectory with respect to a parallel set of survival probabilities w_i which correspond to a parallel set of reaction criteria i. The reaction probability p_i for reactive criterion i is then $p_i = 1 - w_i$.

In our BD scheme for reaction between two proteins, we typically choose one of the proteins of a reacting pair to be designated protein I, or the target protein. All motion is monitored in the reference frame chosen to rotate and translate along with protein I. Thus, while rotations and translations of both proteins are computed, rotations of the first protein show up as a translation of protein II in the reference frame fixed upon protein I.

Excluded-volume interactions between proteins were handled by storing a cubic spatial exclusion grid of 1.0-Å resolution about protein I to define its excluded volume. All surface atoms of protein II were tested against this grid after each Brownian step to check for atomic overlaps, and steps which violate the excluded region were repeated.

The electrostatic forces between proteins were treated by iterating the finite-difference numerical solution of the linearized Poisson–Boltzmann equation on a cubic lattice by the Warwicker and Watson[14] method as adapted by Klapper *et al.*[15] The electrostatic potential field surrounding each isolated protein was computed, each represented as an irregularly shaped cavity of low dielectric constant (ϵ = 4) and zero internal ionic strength and having fixed imbedded charges in the crystallographic configuration. Surrounding the protein cavity is a continuum dielectric

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with $\epsilon = 78.3$, representative of bulk water and having appropriate ionic strengths. The direct force between the two proteins and the torque operating on protein II was determined at each time-step by placing the protein II array of test charges into the field around the protein I cavity and, consulting the stored grid of forces, performing a summation over all protein II charges.

The above procedure allows for treatment of rotation of protein II in a field of torque generated by protein I. We also included the rotation of protein I in the field of protein II by consulting the inner and outer force lattices around protein II. These lattices rotate in rigid-body rotation with protein II. A dipolar pair of charges were included on protein I to serve as test charges, which interact with the field around protein II, and were used to compute the approximate torque on protein I in the field of protein II. This feature of two rotating proteins is essential in treating protein pairs which are comparably sized.

Of special importance in this study is the means of treating the ET event for juxtaposed proteins. We used the *exponential reactivity model* originally employed in our study of the *cytc–cytb5* reaction.[8a] The intrinsic spatially dependent ET rate constant $k_{et}(d)$ is given as a function of donor–acceptor distance d by

$$k_{et}(d) = k_{et}^{\circ} \exp(-\beta(d - d_0)) \quad (3)$$

This measures reactivity as a function of distance d between the π systems of the two porphyrins. We do not interpret this as a through-space model of the ET. Instead, this function defines the degree of reactivity on the exposed surface of the proteins on the basis of the distance from the heme edge. The distance d is computed at every Brownian step as the shortest distance between any atom in the heme atom set (CHA, CHB, CHC, CHD) chosen to represent the periphery of the porphyrin system on protein I to the same set on protein II. The term d_0 is a constant shift factor (=5 Å according to the manner we specify the heme edge) to account for the distance of closest approach of this set of atoms in a perfect edge-on arrangement. The preexponential factor k_{et}° is the ET rate constant when porphyrins are in direct contact edge-on. The quantity β is the ET distance decay factor and is of considerable interest.[2c] Thus, we have basically a two-parameter model for the intrinsic ET unimolecular rate constant in our study. All fitting to experiment was performed by adjusting both parameters k_{et}° and β.

Once the preexponential factor k_{et}° was determined by fit to experimental ionic strength dependence, we used the Marcus expression[2d]

$$k_{et}^{\circ} = \nu_n \exp[-(\Delta G_{el}^{\circ} + \lambda)^2/4\lambda k_B T] \quad (4)$$

to estimate the reorganization energy λ, knowing the redox free energy ΔG_{el}° is zero for self-exchange and assuming $\nu_n = 10^{13}$ s^{-1}.

In BD, the intrinsic rate constant $k_{et}(d)$ is dynamically coupled to diffusional dynamics as follows. The spatially dependent probability $P(d)$ that the reactant pair survive a given Brownian step Δt without ET is $P(d) = \exp(-k_{et}(d)\,\Delta t)$. This probability is multiplicative throughout the trajectory, finally giving the escape probability for that trajectory.

BD simulations were performed at experimental conditions of pH = 7.0, T = 25 °C, and several ionic strengths using the program *bdtirm* (Brownian dynamics of two irregular rotating macromolecules) written at Tennessee Tech. Simulations each required approximately 20 hours to run on a Personal IRIS 4D/30G. Statistical uncertainties in the rate constants were computed by dividing the simulations into 10 equal segments.

Results

Table I and Figure 1 show the theoretical and experimental ionic strength dependence of the bimolecular rate constant for ET using the combination of parameters β and k_{et}° giving the best fit of BD theory to experiment. Note that the rate increases with ionic strength, as the dielectric screening increasingly shields the electrostatic repulsion between like-charged reactants. The ionic strength dependence for both the *cytb5* and *cytc* self-exchanges is optimally reproduced by the BD model using values of $k_{et}^{\circ} = 3.43 \times 10^8$ s^{-1} and $\beta = 0.9$ Å^{-1} (*cytb5*) and $k_{et}^{\circ} = 1.18 \times 10^{10}$ s^{-1} and $\beta = 0.9$ Å^{-1} (*cytc*). The experimental data for *cytc* were of higher quality than those for *cytb5*, enabling a more unambiguous determination of the best-fit value of β. For *cytb5*, fitting was obtained using β values ranging all the way from 0.8 to 1.1 Å^{-1}, depending upon the inclusion or exclusion of the relatively more uncertain low ionic strength data values. Using

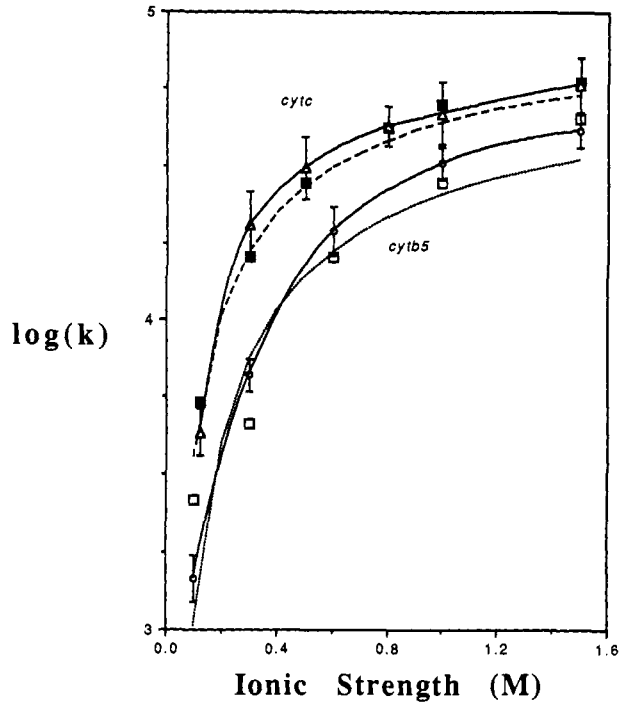

Figure 1. Ionic strength dependence of the bimolecular rate constants k for self-exchange reactions of *cytb5* and *cytc*. Experimental values[4] for *cytb5* (□) and *cytc* (■) are compared to BD-simulated values for *cytb5* (○) and *cytc* (△) and van Leeuwen theory for *cytb5* (···) and *cytc* (– – –). Best-fit parameters for the BD and van Leeuwen theories are given in text and in ref 4, respectively.

eq 4 and our optimum value of k_{et}°, we may then extract an estimate of the reorganization energy $\lambda = 1.06$ eV for *cytb5* and 0.69 eV for *cytc*. These values correspond with the estimates 1.2 and 0.7 eV made by Dixon *et al.*[4] but are obtained with fewer estimated parameters, since the diffusion–collision part of the problem is treated explicitly by our theory and no estimate need be made of steric factors, the association constant, and heme exposure. Also shown in Figure 1 is the best-fit theoretical curve given by the van Leeuwen equation,[16] with parameters specified in ref 4, including the net monopole charges of the proteins, the components of the dipole moments throughout the exposed heme edge, the sum of spherical radii of the two partners, and the rate constant at infinite ionic strength. Although the ionic strength dependence it predicts is comparable, the BD theory appears to give a slightly better fit at higher ionic strengths and with fewer parameters.

Our results determined definitively whether the reaction rate is diffusion-controlled, diffusion-influenced, or activation-controlled (i.e., controlled by the intrinsic ET step). This was determined in two different fashions. One way was by observing the variation of the net bimolecular rate constant k with increasing k_{et}°, the preexponential factor of the ET rate constant. Increasing k_{et}° accelerated the chemical step while the diffusive rate was held constant. The rate constant should increase linearly with k_{et}° as long as the rate is activation-controlled and should eventually saturate as the diffusion limit is reached. This was found to be the case, as seen in Table II, which gives the results for a *cytb5* run at μ =1.5 M. The rate constant is found to be quite linear with k_{et}° values up to 10^{11} s^{-1}, which includes the region of the optimum value of k_{et}° (=3.43×10^8 s^{-1}). Thus the reaction is in the activation-controlled limit at appropriate values of k_{et}°, with no influence on the net *dynamics* arising from the diffusion stage. We verified this finding by an additional simulation in which the viscosity of the medium was doubled. This was accomplished simply by cutting in half the translational

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Table II. BD-Simulated Bimolecular Rate Constants for *cytb5* Self-Exchange as a Function of the ET Preexponential Factor $k_{et}°$ To Determine Degree of Diffusion Control[a]

	k (M^{-1} s^{-1})	
$k_{et}°$ (s^{-1})	double viscosity	normal viscosity
10^8	1.2×10^4	1.2×10^4
10^9	1.2×10^5	1.2×10^5
10^{10}	1.2×10^6	1.2×10^6
10^{11}	1.1×10^7	1.2×10^7
10^{12}	8.7×10^7	1.0×10^8
10^{13}	3.3×10^8	4.7×10^8

[a] μ = 1.5 M, T = 25 °C, pH = 7. Comparison is made between a simulation at normal water viscosity and one at double the viscosity.

Table III. Reorganization Energies λ Obtained by BD Using Various Choices of β, the Distance Parameter for ET[a]

	β	optimum $k_{et}°$ (s^{-1})	λ (eV)
cytb5	0.8	1.2×10^8	1.17
	0.9	$\mathbf{3.4 \times 10^8}$	**1.06**
	1.0	9.2×10^8	0.96
	1.1	2.3×10^9	0.86
cytc	0.8	3.7×10^8	0.81
	0.9	$\mathbf{1.2 \times 10^{10}}$	**0.69**
	1.0	3.9×10^{10}	0.57
	1.1	1.2×10^{11}	0.45

[a] T = 25 °C, pH = 7. The "optimum $k_{et}°$" is the value which brings BD into agreement with experiment for that particular choice of β. Then, λ is derived from $k_{et}°$ using Marcus theory. Best fit is in bold.

and rotational diffusion coefficients and repeating the study at μ = 1.5 M. As shown in Table II, we obtained virtually identical rate constants (within simulation uncertainty) at double the viscosity when using $k_{et}°$ values in the appropriate range. As $k_{et}°$ increases, the rate of the high-viscosity run saturates to a lower rate, as expected. This latter simulation also provides a nice check of the accuracy of the BD algorithm.

We also studied the sensitivity of our estimation of the reorganization energy to the selection of the ET parameters, particularly the choice of β, the distance decay parameter. We collected rate constants for β = 0.8, 0.9, 1.0, and 1.1 $Å^{-1}$. Table III shows the optimum $k_{et}°$ value which brings BD into agreement with experiment at μ = 1.5 M at different choices of β. Note that as β ranges extensively from 0.8 up to 1.1 $Å^{-1}$, which spans most estimates for this quantity, the value extracted for the reorganization energy varies from 1.17 down to 0.86 eV for *cytb5* and from 0.81 down to 0.45 eV for *cytc*.

Since the reaction is activation-controlled rather than diffusion-controlled, one may ask what the point is of running BD calculations if the equilibrium properties should suffice. While it is certainly true that equilibrium properties are sufficient in this case, that does not mitigate the necessity to do a computational average of the intrinsic reactivity function over the entire spectrum of energetically accessible docking conformers. That is precisely what the Brownian algorithm accomplishes in this limit. As such, BD is more efficient for generating docked conformers than a simple Monte Carlo algorithm in which trial selections are unguided by energetic preferences. In the BD method applied to an equilibrium case, the Brownian dynamics simply plays the role of supplying the equilibrium distribution of docked ET complexes. In fact, by tallying from BD a pair distribution ρ as a function of the reaction coordinate for the association stage of reaction (the heme–heme distance d), we obtained a potential of mean force (PMF), $\omega(d)$, which is an orientationally preaveraged free energy profile of protein–protein association. The reaction coordinate space was divided up into bins of 1.0-Å thickness, and the total residence time in each bin was tallied and normalized to obtain $\rho(d)$. The observed distribution was related to the effective free energy or PMF according to the equation

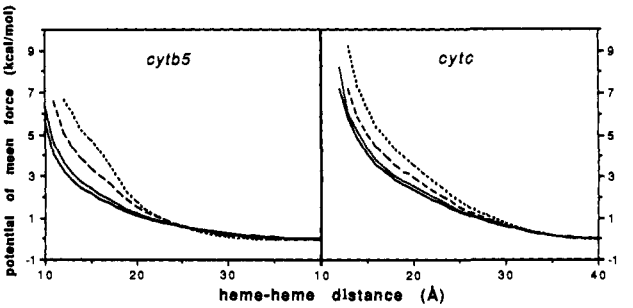

Figure 2. Potentials of mean force derived from BD-simulation of *cytb5* and *cytc* self-exchange as a function of reaction coordinate, the heme–heme distance. Curves represent various values of the ionic strength, μ: 0.1 (- - -); 0.3 (– – –); 1.5 (···); zero-force case (—).

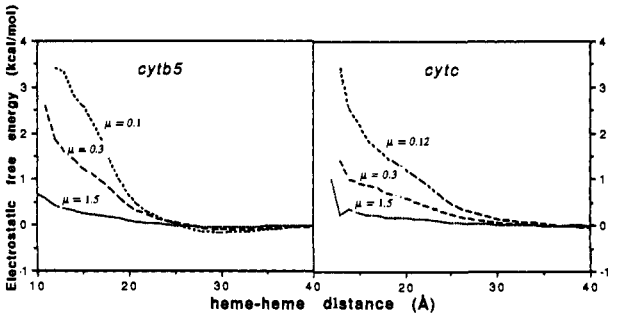

Figure 3. Orientationally averaged electrostatic component of free energies derived from BD-simulation of *cytb5* and *cytc* self-exchange as a function of reaction coordinate, the heme–heme distance. Curves represent various values of the ionic strength as indicated on the figure.

$$\rho(d) = \rho(\infty) \exp(-\omega(d)/k_B T) \qquad (5)$$

where the term $\rho(\infty)$ was set such that $\omega(\infty) = 0$. The PMF profiles are shown in Figure 2. The free energy of association of pairs is uphill throughout the entire approach due to two contributing factors. First, a repulsive mean electrostatic effect is present. Second, there is a loss of entropy as the proteins are forced to adopt increasingly restrictive orientations in order to achieve shorter heme–heme distances. The entropic portion is simply the PMF for the zero-force case, which is the solid curve in Figure 2. We thus observe an entropy cost of +5.6 kcal/mol for *cytb5* and +7.2 kcal/mol for *cytc* for bringing the reactants into juxtaposition. The entropy of heme–heme docking in *cytc* is slightly higher for *cytc* than for *cytb5* because of the more buried nature of the heme group in the former. Thus, for *cytc* more restrictive docking geometries are required to bring the hemes into close proximity. By subtracting this entropic effect from the other PMF curves, as shown in Figure 3, we isolated the orientationally averaged electrostatic energy of the association process. This term is uphill in energy at every ionic strength and is steepest at lowest ionic strength, as expected. The electrostatic work of forming the *cytb5* and *cytc* self-exchange ET complexes at μ = 0.1 M is +3.4 kcal/mol for both (fortuitously), which is quite close to the estimates of +3.1 and +2.7 kcal/mol of Dixon *et al.* extracted from the van Leeuwen equation fit. There is a slight attractive well around 30 Å in the *cytb5* reaction which may be artifactual but could represent pairs which are slightly attractive at collision separation but oriented improperly for ET. This was not observed in the *cytc* reaction.

For the *cytb5* and *cytc* reactions, respectively, the minimum observed heme–heme distances obtained in our simulations of *rigid-body* docking were 9.1 and 12.1 Å, with angles between the heme normals of 18 and 30°. These are compared to 7.5 and 8.9 Å calculated by Dixon *et al.* after energy minimization of the coplanar docking arrangement. Our simulations do not systematically search for the minimum distance and angle, and so they are representative searches and not exhaustive searches. The

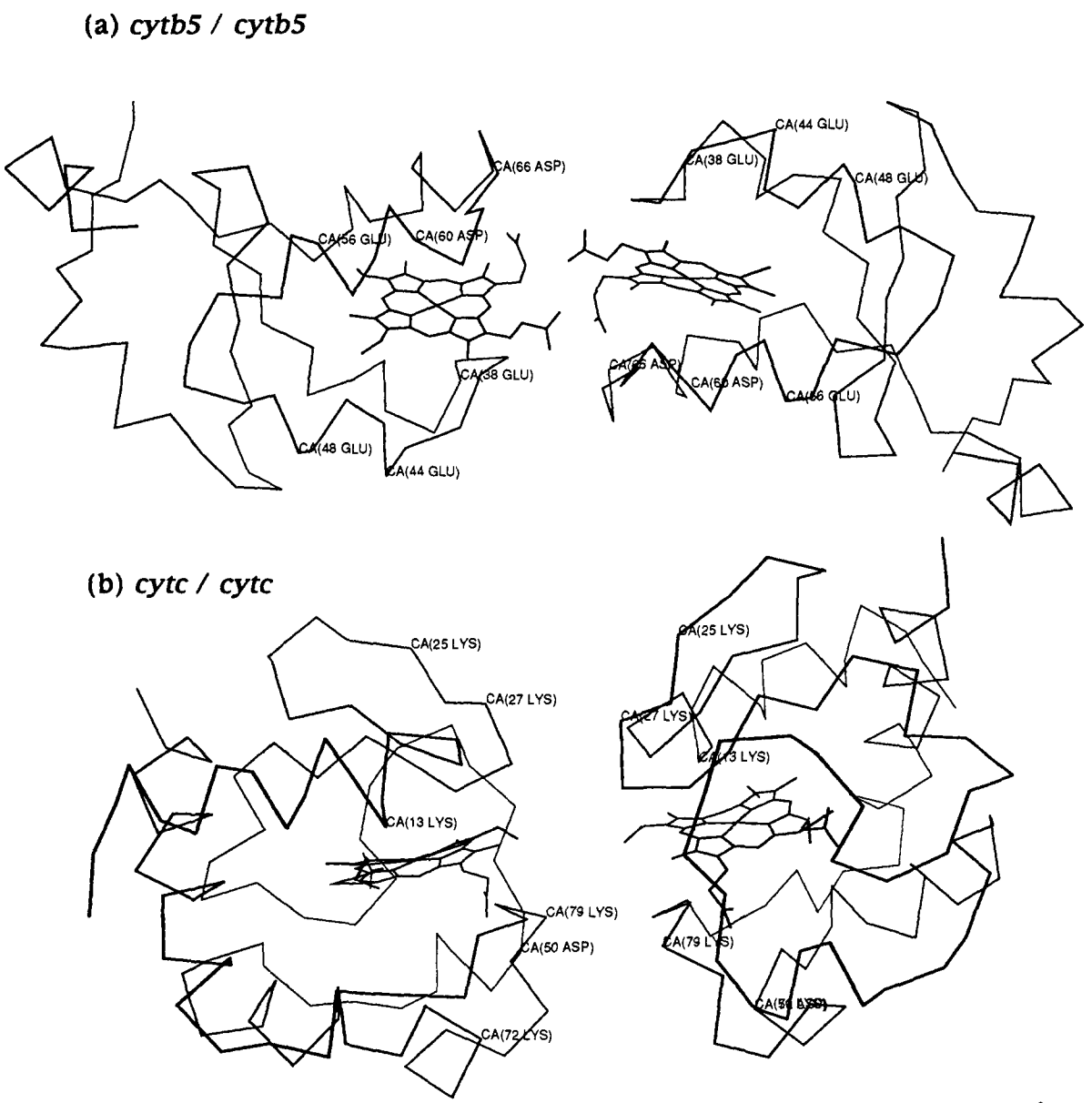

Figure 4. (a) MidasPlus (UCSF) rendering of the C_α skeleton and heme group of an intimate *cytb5* self-exchange complex (d = 9.4 Å; ψ = 46°) of relatively low electrostatic energy (+1.5 kcal/mol at μ = 0.6 M). (b) Intimate *cytc* self-exchange complex (d = 12.1 Å; ψ = 30°) of relatively low electrostatic energy (+0.25 kcal/mol at μ = 1.0 M).

complexes depicted in Figure 4 are representative intimate self-exchange complexes for each system having also a low electrostatic repulsive energy relative to other complexes.

Figure 5 shows a correlation plot between the minimum obtained heme–heme distance in each reactive trajectory and the heme–heme coplanar angle obtained in *cytb5* complexes for the simulation in the absence of electrostatic forces. We observed no obvious pattern of correlation, indicating that a spectrum of protein–protein docked complexes exists having arbitrary orientations around the protein–protein axis which essentially minimize the ET distance. Thus the formation of the complexes involves a loss of all but one of the orientational degrees of freedom needed to specify the mutual orientation, leaving a degree of rotational freedom around the protein–protein axis.

Discussion

For the first time, BD was used in this study in the limit where reaction is activation-controlled rather than diffusion-controlled or -influenced. One may wonder how bimolecular processes with rate constants as low as 10^3–10^4 M^{-1} s^{-1} can be simulated successfully using a diffusion model of docking, since reactions this slow should be well out of the so-called "diffusion-controlled" regime. First, let us point out that the rate of diffusional docking of two neutral proteins into a single precise fit occurs on the order of 10^6 M^{-1} s^{-1}, and yet the process is still purely diffusional, as demonstrated by Northrup and Erickson.[17] This is 3 orders of magnitude slower than the Smoluchowski diffusion-controlled reaction between two isotropically reactive spheres. A reaction rate between two highly charged proteins of the same sign should be even slower than 10^6 M^{-1} s^{-1}, without invoking any activation barrier to the chemical step itself, so long as the diffusion stage is understood to take place on a long-ranged repulsive field of force. Thus the diffusion rate may still influence the rates of bimolecular processes even slower than 10^6 M^{-1} s^{-1}. On the other hand, the presence of a significant activation barrier to the ET event at juxtaposition could render the reaction activation-controlled. In this case, the Brownian association algorithm still provides a valid description of the concentration of docked precursor complexes present in an *equilibrium* spatial distribution and is an effective means of determining that distribution when

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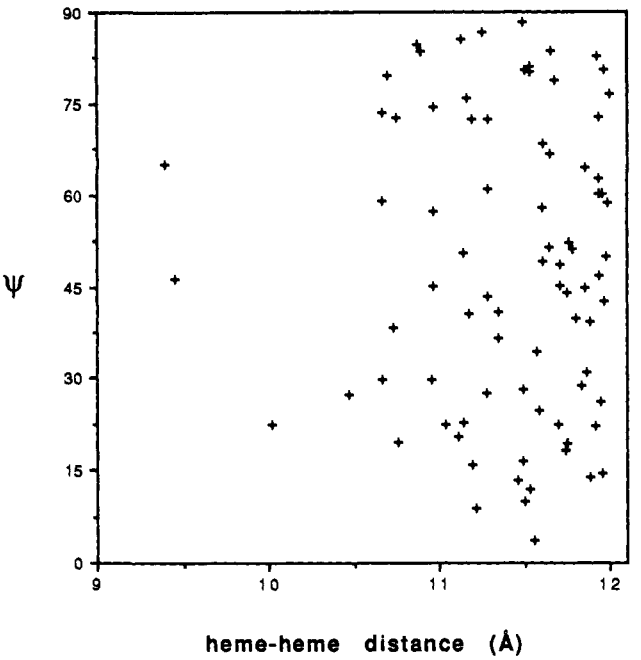

Figure 5. Analysis of docked ET complexes of *cytb5* from the $\mu = 0.6$ M simulation demonstrating the absence of correlation of heme–heme distance with heme plane mutual orientation angle ψ.

a complicated interaction potential and geometry are considered. This is a consequence of the fact that our model embodies an explicit coupling between the diffusion and chemical dynamics which should be valid in both the diffusion limit and the chemical activation-controlled limit. In the present study, we did in fact determine that the self-exchange reaction dynamics are completely controlled by the ET step and not by the diffusion dynamics. Variations in the viscosity of the medium produced no effect on the simulated rate constants.

The BD theory successfully reproduced the ionic strength dependence of the reaction. A slightly better fit was obtained than that afforded by van Leeuwen theory, with fewer adjustable parameters. By fitting the BD-generated rate constants to the experimental curve, we were able to extract a reorganization energy estimate which we believe to be a more rigorous estimate than that of Dixon *et al.* Furthermore, we obtained a best-fit value of the distance decay parameter, which turned out to be $\beta = 0.9$ Å^{-1} for both proteins, in agreement with the value $\beta = 0.91$ Å^{-1} determined by Gray and Malmstrom[18] by fitting ET distances and rates for five ruthenated heme metalloproteins. Since BD provides a more detailed description of the collision stage of the process, determined by the actual atomic scale irregularity of the proteins (steric factors) and the mutual electrostatic interactions, no estimates of the association constant and heme exposure are required, and so we have fewer parameters to contend with than the model employed by Dixon *et al.* to make estimates of the reorganization energy.

Our simulations quantitatively demonstrated several factors involved in these ET reactions. For example, why does *cytc* exchange more rapidly than *cytb5*? First of all, the electrostatic repulsions in these systems are quite comparable and so do not account for this effect. The smaller reorganization energy of the *cytc* system plays the dominant role. This factor far outweighs the steric effects which are as follows: (i) the slight entropic advantage of the *cytb5* reaction arising from its more exposed heme group and (ii) the greater ET efficiency in *cytb5* due to its shorter accessible heme–heme distance.

To our knowledge, this is the first publication of the free energy or PMF of association of whole proteins as a function of reaction coordinate with rigorous molecular shapes and electrostatic field employed. A direct calculation of the entropy cost of forming ET complexes is performed, showing the entropy loss of restricting the associating pair to one degree of freedom, the rotation around the protein–protein bond.

The utility of the BD method for simulation of ET between metalloproteins will increase as more detailed modeling of the intrinsic electron-transfer step is incorporated. For instance, simple exponential distance decay models are probably inadequate, and as such the parameters λ and β should perhaps be viewed as effective quantities useful for correlating the behavior of a wide range of metalloprotein ET kinetic data. A more complete knowledge of the variable reactivity of the protein surfaces is being made available by analysis of bonding pathways,[19] and these theories could be included in our modeling by special reactivity functions. We have already demonstrated that effects of site-directed mutations and chemical modifications can be predicted by BD simulation.[7b] We have further shown[9a,b] in the context of the cytochrome *c*/cytochrome *c* peroxidase reaction that a spectrum of docked complexes is likely to contribute to the dynamical behavior of ET rather than a single optimized geometry complex. The exciting new finding by Pelletier and Kraut[20] that cytochrome *c*/cytochrome *c* peroxidase binds in a highly specific complex in the crystal does not preclude the existence of a spectrum of suboptimal complexes in solution which contribute to electron transfer. Furthermore, the conditions of crystallization may be quite different from the physiological ionic strength environment of the kinetic studies we have been simulating.

Acknowledgment. This work was made possible by Grant GM34248 from the National Institutes of Health, by Grant 24159-B6 from the Petroleum Research Fund, administered by the American Chemical Society, and by the Camille and Henry Dreyfus Scholar/Fellow Program for Undergraduate Institutions. S.H.N. is a Camille and Henry Dreyfus Scholar; K.A.T. is a Camille and Henry Dreyfus Fellow. We thank Dr. James B. Matthew for providing the Tanford–Kirkwood computer program, Dr. F. Scott Mathews for sending the *cytb5* coordinates, and Dr. Gary Brayer for sending the *cytc* coordinates. We thank the experts, Profs. Dabney Dixon and Grant Mauk, for many useful discussions and Christine Northrup for secretarial support and encouragement.

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