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Cyclic Changes in Active Site Polarization and Dynamics Drive the ‘Ping-pong’ Kinetics in NRH:Quinone Oxidoreductase 2: An Insight from QM/MM Simulations

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Abstract

Quinone reductases belong to the family of flavin-dependent oxidoreductases. With the redox active cofactor, flavin adenine dinucleotide, quinone reductases are known to utilize a ‘ping-pong’ kinetic mechanism during catalysis in which a hydride is bounced back and forth between flavin and its two substrates. However, the continuation of this catalytic cycle requires product displacement steps, where the product of one redox half-cycle is displaced by the substrate of the next half-cycle. Using improved hybrid quantum mechanical/molecular mechanical simulations, both the catalytic hydride transfer and the product displacement reactions were studied in NRH:quinone oxidoreductase 2. Initially, the self-consistent charge-density functional tight binding theory was used to describe flavin ring and the substrate atoms, while embedded in the molecular mechanically-treated solvated active site. Then, for each step of the catalytic cycle, a further improvement of energetics was made using density functional theory-based corrections. The present study showcases an integrated interplay of solvation, protonation, and protein matrix-induced polarization as the driving force behind the thermodynamic wheel of the ‘ping-pong’ kinetics. Reported here is the first-principles model of the ‘ping-pong’ kinetics that portrays how cyclic changes in the active site polarization and dynamics govern the oscillatory hydride transfer and product displacement in this enzyme.

Graphical Abstract

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Supporting Information

Additional data for gas-phase studies, the simulation of product displacement reaction, pK_a calculation, PMF correction, and convergence of MD simulations are available. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Keywords

Double displacement reactions; flavoenzyme; hydride transfer reactions; ‘ping-pong’ kinetics; Kohn-Sham density functional theory; quantum mechanical/molecular mechanical calculations; quinone oxidoreductase 2; self-consistent charge density functional tight-binding theory

INTRODUCTION

NRH: quinone oxidoreductase 2 (NQO2) and its paralog NADPH: quinone oxidoreductase 1 (NQO1) are flavin-dependent cellular defense enzymes,^{1–6} which catalyze the reduction of a wide variety of quinone derivatives,^{7–10} including melatonin, menadione (vitamin K3), and estrogen quinones.^{5, 11–14} Using the flavin adenine dinucleotide (FAD) cofactor, they reduce quinones to hydroquinones in an obligatory $2e^-/H^+$ (i.e. hydride) transfer process.^{14–16} The dimethylisoalloxazine ring (flavin) of FAD oscillates between two oxidation states allowing the active site to facilitate the opposing hydride transfer reactions catalyzed with a single set of active site residues.^{15–20} In these enzymes, the general catalysis follows a ‘ping-pong’ mechanism, where one substrate binds to the active site and serves as a hydride donor to the flavin during the reduction half-cycle. The exit of the product is followed by binding of another substrate (in the oxidative half-cycle) serving as a hydride acceptor (Scheme 1).^{14, 16} The changes in the active site environment due to the change in flavin’s redox states has been proposed to facilitate the substrate shuttling in ‘ping-pong’ kinetics.^{2, 18}

Many recent studies suggest a link between quinone reductases and cancer biochemistry.^{1, 3–4, 6, 21–26} Earlier studies have shown that the estrogen quinones, implicated in several types of cancer,⁴ are catalytically reduced by NQO2 in cells. Additionally, since NQO2 is overexpressed in cancer cells, these enzymes were used as targets for anti-cancer prodrug therapy.²⁷ In particular, NQO2 is an ideal prodrug target as it also exhibits enhanced nitro-reductase activity compared to its paralog, NQO1.^{25, 28–29} Furthermore, NQO2 was found to act as a flavin redox switch in the regulation of p53 – a tumor suppressor protein.^{2, 30} The binding and subsequent stabilization of p53 is dependent on the redox state of the enzyme; the donor-to-FAD hydride transfer reaction is considered to be the key mechanistic step that enables NQO2 to modulate the p53 level in cells.

The relative stability of flavin’s oxidation states plays a central role in the redox switch function of the NQO2.^{1, 31} The charge separation caused by each redox step in NQO2 is expected to promote significant changes in the active site environment. Therefore, a molecular-level analysis of the ‘ping-pong’ kinetics could provide important insight into the mechanistic features of the redox switch function of NQO2. In addition, it could help designing inhibitors targeting a specific redox state of the enzyme. As a sequel to our previous study of the interplay of redox chemistry of flavin and NQO2 enzymology,^{17–20, 32} herein presented is an investigation of the geometric and energetic changes of the ‘ping-pong’ kinetics. As illustrated in Scheme 1, the hydride donor, N-methyldihydronicotinamide (abbreviated here after as NMH), and the hydride acceptor *p*-benzoquinone (abbreviated here after as PBQ) were used in this study. The catalytic hydride transfer steps, as well as the product displacement steps, were investigated using a combination of electronic structure

calculations and improved hybrid quantum mechanical/molecular mechanical (QM/MM) simulations.

THEORY & METHODS

The energetics of the four steps of the “ping-pong” kinetics involving the two catalytic hydride transfer reactions (Scheme 1, steps a and c) and the two product displacements (Scheme 1, steps b and d) were calculated using the hydride donor (NMH, shown in blue) and the acceptor (PBQ, shown in red) molecules. For the gas-phase and aqueous system calculations, 7, 8, 10-trimethylisalloxazine (or lumiflavin) was used instead of FAD. Electronic structure calculations were carried out at the level of Kohn-Sham density functional theory (DFT)³³ and self-consistent charge-density functional tight-binding protocol with dispersion correction (abbreviated hereafter as SCC-DFTB-D).^{34–38} DFT calculations were carried out using the program Gaussian 09.³⁹ Based on our previous studies on the accuracy of the computed flavin-aromatic interaction energies,¹⁹ these calculations employed the Minnesota hybrid functional M06–2X^{40–41} and 6–31+G(d,p) basis set.⁴² The previous study also demonstrated that the same basis set could be used for predicting the hydride transfer barrier height of the redox reactions between flavin and a stacked aromatic substrate. Solvation effects were modeled implicitly with continuum solvation using the Polarizable Continuum Model (PCM).⁴³ In parallel, hybrid QM/MM simulations of both the aqueous and enzyme systems were carried out using the CHARMM⁴⁴ program suite. The QM-region atoms were treated with SCC-DFTB-D protocol. Atoms in the MM region were modeled by CHARMM all-atom force field⁴⁵ and CHARMM27 parameters with grid-based cross-term energy map corrections for protein backbone atoms.⁴⁶ Visual Molecular Dynamics (VMD)⁴⁷ was used to visualize and edit molecular structures. The dipole moment (μ) of a molecule and surfaces with electrostatic potentials were visualized using Gabedit.⁴⁸

Setting Up Solvated Active Site for Simulations.

For NQO2-bound flavin, the atomic coordinates were obtained from the protein data bank⁴⁹ (PDB code: 1ZX1). Both enzyme-bound and enzyme-free systems were built using methods described earlier.^{17–19} Briefly, the HBUILD module of CHARMM was used to add hydrogen atoms. Ionic amino acid residues were maintained in a protonation state corresponding to pH 7. The protonation state of histidine residues were determined based on the local environment. Location of the proton on either or both N ϵ and N δ atoms of a certain imidazole moiety was determined by considering the possible hydrogen-bonding network surrounding that residue, which was subsequently verified by computing the pK_a using Propka.^{50–51} A geometric center was defined by taking the statistical mean of the flavin ring atoms and was solvated by placing it in a water sphere of 30 Å radius. All residues beyond this were deleted. Atoms falling within 24 Å, but not defined in the QM region, were treated with Newtonian dynamics. Langevin dynamics were employed in the 24–30 Å region, with increasing frictional forces as the circumferential boundary is approached.⁵² The three-point-charge TIP3P model⁵³ was used to treat water molecules. Non-bonded interactions were truncated using a switching function between 11 and 12 Å. Bond lengths and bond angles of water molecules were constrained by the SHAKE algorithm.⁵⁴ In all MD

simulations, a time step of 1 fs was used in the leapfrog Verlet algorithm for integration.
 52, 55 Before free energy simulations, all structures were equilibrated with 500 ps steps of
 MD simulations following QM/MM protocols used earlier with flavoenzyme systems.
 17–18, 56

QM/MM Partitioning.

The partitioning scheme of the solvated enzyme-cofactor-substrate is illustrated in Scheme 2. Atoms of the flavin ring and the substrate/product were treated using approximate quantum mechanics at the level of SCC-DFTB-D.^{34–36} The remaining atoms (-ribityl-ADP group of flavin, enzyme, ions, and solvent) were modeled by classical mechanics using the CHARMM27 force fields.^{45, 57} To simulate the enzymatic-solvent interactions, stochastic boundary conditions were utilized.⁵⁸ The link atom method was used to define the QM/MM boundary,⁵⁹ where a dummy atom was used between the QM frontier atom C1' and MM boundary atom C2' of the FAD (Scheme 2). The link atom is constrained to the coordinates of both the C1' and C2' atoms to remove the extra degree of freedom during optimization and molecular dynamics (MD) simulations.⁶⁰ The energetics of such a hybrid QM/MM system can be derived from the Hamiltonian operator as shown in eq. 1

$$E_{\text{Total}} = \langle \Psi^0 | \hat{H}_{\text{QM}} + \hat{H}_{\text{QMMM}} | \Psi^0 \rangle + E(\text{MM}) \quad (1)$$

where $\hat{H}_{\text{QM}}$ is the Hamiltonian of the QM region, $\hat{H}_{\text{QMMM}}$ is the interaction Hamiltonian for the QM/MM boundary region, and Ψ^0 is the converged electronic self-consistent-field wave function. In general, the effective Hamiltonian of the QM subsystem computes the electrostatic (Coulomb) contributions to the QM/MM interactions, while such contributions due to van der Waals and bonded interactions are included classically along with the MM subsystem's energy, $E(\text{MM})$. Simplifying eq. 1 yields

$$E_{\text{Total}} = E_{\text{g}}^0(\text{QM}) + \Delta E(\text{QM/MM}) + E(\text{MM}) \quad (2)$$

where the first two quantities, obtained from the converged wave function Ψ^0 are the gas-phase energy of the QM region and the change in the energy to bring the QM system from the gas-phase state to the state immersed in the MM surroundings, respectively. The sum of the first two terms in eq. 2, therefore, primarily accounts for the change in the energy due to a chemical process in the active site and was used in subsequent free energy calculations during catalysis and product displacement reactions.

Quantum Correction to Free Energies.

The SCC-DFTB-D/MM-computed energy (described above) was corrected by incorporating quantum effects, which is discussed below. The protocol is illustrated in Scheme 3 and provides necessary corrections for free energies (*vide infra*). Based on a benchmark study on the dispersive interactions in flavin-aromatic system, published earlier from our group,¹⁹ the SCC-DFTB-D-computed energies were corrected using the improved Minnesota density functional M06–2X in a two-step correction protocol. In the first step, the Born

Oppenheimer potential energies were computed on a small model of the QM subsystem as shown in Scheme 2. For each step of the reaction, a high-level correction (HLC) term was computed from the difference in the potential energies of reaction calculated by the two methods:

$$\text{HLC} = \Delta E_{\text{DFT}} - \Delta E_{\text{SCC-DFTB-D}} \quad (3)$$

where E indicates the change in potential energies of the process under consideration. The potential energy of a chemical system using DFT was computed from the geometrically optimized conformation with the SCF-converged wavefunction. In contrast, for the SCC-DFTB-D calculations, the ensemble-averaged potential energies were used from a 100 ps gas-phase MD simulation. In the second step, the differences in the DFT-computed zero-point energies (ΔZPE) for the two states were also included in the correction, so that the overall correction for the free energy (*vide infra*) is given by

$$\Delta\Delta G_{\text{corr}} = \text{HLC} + \Delta\text{ZPE} \quad (4)$$

Therefore, the free energy of the step X, calculated by the DFT-corrected SCC-DFTB-D/MM scheme (Scheme 3) is given by,

$$\Delta G_X^0 = \Delta G_{\text{uncorr}}^0 + \Delta\Delta G_{\text{corr}} \quad (5)$$

where $\Delta G_{\text{uncorr}}^0$ is the uncorrected free energy change of a reaction step under consideration.

Free Energy of the Hydride Transfer Reactions.

The energetics of the catalytic hydride transfer reactions involving the donor NMH and the acceptor PBQ (Scheme 1, reactions a and c) were studied using explicit solvation treatment. The Gibbs free energy change was computed from the plot of potentials of mean force (PMF)⁶¹ as a function of the reaction coordinate. As shown in Figure 1, the reaction coordinate is defined as

$$\xi = r_{\text{broken}} - r_{\text{formed}} \quad (6)$$

where r_{broken} and r_{formed} are the inter-nuclear distances between atoms involved in bond breaking and forming, respectively. The complete range of the reaction coordinate (ξ) was divided into segments of 0.4 Å. In each of these windows, independent simulations were run using the umbrella sampling technique.⁶² In this method, biased conformational sampling is accomplished by means of applying a harmonic biasing potential, which acts as a restraining force centered at the mid-point (ξ_0) of the given simulation window. The biasing potential was chosen based on a trial-and-error method, so that the population generated in a specific window produce a Gaussian distribution around ξ_0 . These simulations yielded histograms of

conformations along the reaction coordinate and their energies, which are then collected and analyzed by the Weighted Histogram Analysis Method⁶³ to produce a plot of the PMF.

As discussed earlier, the quasi-classical PMF was corrected using eq. 5. In order to incorporate corrections mentioned earlier, to the SCC-DFTB-D/MM-derived PMF, a simple statistical normalization procedure was applied to the original data to scale up/down the PMF (see supporting materials for details), so that the original range of the data, with its minimum and maximum, would be rescaled to the corrected values determined by eq. 5.

Free Energy of the Proton Transfer Reactions.

The free energy of a proton transfer reaction to the NQO2-bound hydroquinone was calculated using a thermodynamic integration (TI)⁶⁴ based protocol published by Bartholow et al.⁶⁵ Following a thermodynamic diagram (Figure S2), the protonation of the NQO2-bound flavin-PBQH⁻ can be expressed by a combination of three steps (Table S2, eq. S3 in the footnote): a desolvation of the proton, a conversion of the gas-phase proton to a dummy atom that is attached to the anion, and a conversion of the dummy atom to a proton (Figure S1). The Gibbs free energy of desolvation of the proton is known and is equal to 264.0 kcal/mol.⁶⁶ The second step is the annihilation (opposite to the formation) of a gas-phase proton and its Gibbs free energy is known to be equal to 6.28 kcal/mol.⁶⁷ The Gibbs free energy of the third step is obtained by the standard extension of TI,^{64, 68} where the electrostatic component of the bond between the dummy atom and the rest of the QM-treated region was slowly generated in a stepwise manner^{17, 56, 65, 68} over several windows, where independent MD simulations were carried out. The free energy from TI was determined from the simulated data, by calculating the ensemble-averaged potential energy fluctuations with respect to the perturbation parameter for each window and then integrating the change over the entire perturbation range.^{17, 56, 65}

Free Energy of the Product Displacement Reactions.

The energy changes due to product displacement as depicted in Scheme 1 (steps b and d) were computed following Schemes 3. Investigation of the Gibbs free energy of product displacement was conducted in two stages. In the first stage, the free energies of product displacement were investigated in gas and aqueous phases (Scheme 4, the top half section) using DFT and SCC-DFTB-D. Subsequently, the free energy calculations were extended to the enzyme environment by using DFT-corrected SCC-DFTB-D/MM (Scheme 4, the bottom half section).⁶⁹

As illustrated in the thermodynamic diagram of Scheme 4, $\Delta\Delta G_{\text{disp}}^0(g)$ in the central reaction is the gas-phase Gibbs free energy change for the displacement of flavin-bound ligand 1 (S1) by the ligand 2 (S2). Fl is our generic short-hand representation of flavin; F and FH⁻ being the oxidized and the reduced forms, respectively. The $\Delta\Delta G_{\text{disp}}^0(aq)$ in the top reaction of Scheme 4 is the Gibbs free energy change for the displacement reaction in the aqueous state and can be represented as

$$\Delta\Delta G_{\text{disp}}^0(aq) = \Delta\Delta G_{\text{disp}}^0(g) + \Delta\Delta G_S(S2 \rightarrow S1, aq) + \Delta\Delta G_S(\text{Fl}:::S1 \rightarrow \text{Fl}:::S2, aq) \quad (7)$$

where $G_S(S2 \rightarrow S1, \text{H}_2\text{O})$ is the difference of the hydration free energies of S1 and S2 and is equal to $G_S(S2) - G_S(S1)$. Similarly, the quantity $G(\text{Fl}:::S1 \rightarrow \text{Fl}:::S2, aq)$ corresponds to the difference of the hydration free energies of the flavin-bound S1 and flavin-bound S2 and is given by $G(\text{Fl}:::S2, aq) - G(\text{Fl}:::S1, aq)$.

The lower-half of the Scheme 4 exhibits an analogous thermodynamic diagram for the enzymatic product displacement reaction and the free energy difference can be computed using eq. 8

$$\Delta\Delta G_{\text{disp}}^0(enz) = \Delta\Delta G_{\text{disp}}^0(g) + \Delta\Delta G_S(S2 \rightarrow S1, aq) + \Delta\Delta G_S(\text{Fl}:::S1 \rightarrow \text{Fl}:::S2, enz) \quad (8)$$

One can notice that the last quantity in the RHS of eq. 8 is analogous to that in eq. 7, where the aqueous surrounding is replaced by the protein matrix of the NQO2 active site. Thus, the quantity $G(\text{Fl}:::S1 \rightarrow \text{Fl}:::S2, enz)$ is equal to $G(\text{Fl}:::S2, enz) - G(\text{Fl}:::S1, enz)$, which corresponds to the difference in the interaction free energies of the two ligands, when bound on the surface of the flavin ring located at the NQO2 active site.

For each product displacement reaction, the successor complex (SC) of a catalytic cycle was simulated for additional length of time. To ensure equilibration of the active site after the hydride transfer reactions, the simulations were carried out with two alternate starting orientations of the product state (see supporting information). In each case, 2-ns MD simulations were carried out and the energetics were computed from the average of two simulations.

Dipole Moment Calculations.

Conformations were obtained from the MD simulations and atoms of the QM site were used for DFT-based single-point electronic structure calculations. Since flavin bending could change the dipole moment (μ), both bent and planar geometries of flavin were used. A total of 20 conformations were chosen for each of the precursor complex (PC), the transition state (TS), and SC of the reaction. Each set contained 10 conformations where the flavin ring was found to be bent in the NQO2 active site. The remaining 10 conformations had flavin in a planar geometry. Orbital populations were analyzed with natural bond orbital analysis⁷⁰ with the use of the Gaussian 09 program.³⁹

Analysis of Electrostatics.

The effect of the partial charge of a certain residue can be determined from the QM/MM interaction energy. The stabilization due to a certain polar residue was determined by annihilating the partial charges of its atoms and calculating the difference in the QM/MM interaction energies.^{17–18, 65} as in eq. 9:

$$\Delta E_{\text{elec}} = E_{\text{elec}}^0 - E_{\text{elec}}^{\delta} \quad (9)$$

Where E_{elec}^{δ} and E_{elec}^0 are the interaction energies, before and after the neutralization of the given amino acid residue's charge, respectively. Thus, more positive value of E_{elec} signifies that the residue favored the particular redox state of the flavin in the active site. Furthermore, the change in the E_{elec} values between PC and SC is a measure of active site polarization as the enzyme cycles through the entire 'ping-pong' cycle (Scheme 1). The computed energy was averaged over an ensemble of population from 500 ps MD simulation.

RESULTS AND DISCUSSION

The 'ping-pong' kinetics of NQO2 was probed by studying the hydride transfer and product displacement reactions. Initially, the gas-phase calculation is discussed, which provides the corrections for the energetics involved in the catalytic and product displacement steps of the 'ping-pong' kinetic mechanism (Scheme 1). The two enzymatic hydride transfer reactions are described next followed by an analysis of the role of the active site in stabilizing PC, TS, and SC. Finally, the product displacement reaction steps and the role of the active site polarization in the overall 'ping-pong' kinetics of NQO2 are elucidated.

DFT-corrected SCC-DFTB-D/MM Scheme.

The SCC-DFTB-D/MM-computed energies following the scheme described in eq. 2 is quasi-classical in nature, as it fails to account for the zero-point energy and incompletely address the dynamic correlational effect for the electrons of quantum subsystem atoms. As discussed in the method section and illustrated in Scheme 3, this issue was addressed by including the zero-point energies computed at a high-level theory and accounting for the difference in the Born-Oppenheimer energies (eq. 3–5). For high-level theory, DFT with the functional M06–2X was used, as our previous study demonstrated that this functional provided an accurate estimate of the binding free energy of the flavin-aromatic stacking complexes.¹⁹ Thus, these correctional quantities were calculated for small models of the 'ping-pong' reaction steps (Scheme 1, reactions a–d) of NQO2 and are given in Table S1.

For the two hydride-transfer catalytic reactions, shown in eq. 10, these corrections were computed on the PC, TS, and SC conformations of the QM sub-system atoms in the gas-phase (Table S1).

The calculations show that both hydride transfer reactions are favorable in the gas-phase (Table S1) with SCC-DFTB-D overestimating the energy of the both catalytic reaction by ~3 kcal/mol. However, SCC-DFTB-D underestimates the barrier height in both catalytic process by 3–5 kcal/mol.

Similarly, for product displacement reactions, the correction was calculated using QM subsystem atoms, given in eq. 11 (Table S1):

The first product displacement reaction (eq. 11a) was found to be significantly endothermic – both DFT and SCC-DFTB-D-computed energies for the reaction were found to be ~80 kcal/mol (Table S1). In contrast, the substitution of flavin-bound reduced anionic hydroquinone by nicotinamide (11b) was found to be less unfavorable (Table S1). The proton addition to the flavin-bound anionic hydroquinone (PBQH^-) was found to be highly favorable in the gas-phase (Table S1). Thus, the study of the product displacement reaction was carried out with neutral hydroquinone (i.e. PBQH_2) by NMH (Table S1). The corrections for the SCC-DFTB-D-computed energy of product displacement reactions are in the range of 2–5 kcal/mol (Table S1).

Energetics of the Hydride Transfer Reactions.

The effect of the enzyme environment was probed by simulating each hydride transfer reaction (Scheme 1, reactions a and c) in aqueous environment as well as in a solvated enzyme active site and their energetics were compared (Table 1). As illustrated in Figure 1, a reaction coordinate was defined from the difference of lengths of the bonds being broken and formed following eq. 6. The free energy was calculated as a PMF along the reaction path using the DFT-corrected SCC-DFTB-D/MM scheme (Scheme 3) as discussed in the free energy correction section. The details of the PMF correction is given in the supporting information section (Figure S2).

Hydride-transfer from NMH to flavin.—The standard Gibbs free energy of enzymatic flavin reduction by NMH (Scheme 1, reaction a) was found to be only –2.6 kcal/mol (Figure 2), identical to that obtained for the aqueous system (Table 1). This shows that the enzyme environment does not provide additional stability of SC. The comparison of the Gibbs free energy of activation ($G^\ddagger(aq)$) reveals a substantial reduction (by ~3 kcal/mol) of the barrier height in NQO2 active site, when compared to the aqueous system (Figure 2, Table 1). The theoretically determined barrier heights are 15.0 and 17.8 kcal/mol for NQO2 and water (Table 1), respectively. The results are quite consistent with the experimentally observed rate constants, which correspond to estimated barrier heights of 14.2 and 16.7 kcal/mol, in enzyme¹⁴ and water,⁷¹ respectively.

Hydride-transfer from flavin to PBQ.—In contrast to the NMH-to-flavin hydride transfer reaction, the flavin-to-PBQ hydride transfer (Scheme 1, reaction c) offers more stability for SC; the standard Gibbs free energies of the reaction in aqueous and enzymatic systems were -14.0 and -19.6 kcal/mol, respectively (Figure 2, Table 1). This indicates that the NQO2 active site stabilizes SC by more than 5 kcal/mol as compared to the aqueous environment. Likewise, the role of the enzyme matrix is evident from the 3.0 kcal/mol lowering of the activation barrier ($G^\ddagger(aq)$). The enzymatic hydride transfer reaction has a notably low Gibbs activation free energy (4.4 kcal/mol, Table 1).

There are no available experimental data for the kinetics of the PBQ reduction step, however, the observed energetics of the two hydride transfer reactions indicates that the activation of flavin by the donor substrate NMH (Scheme 1, reaction a) will be considerably slower and therefore, the rate determining step in the ‘ping-pong’ kinetic mechanism.

Energetics of Proton Transfer Reaction of PBQH⁻.

Anionic hydroquinones have high pK_a values⁷² and therefore the flavin bound anionic benzohydroquinone (F:::PBQH⁻) produced during the flavin oxidative half-cycle (Scheme 1, reactions c), is expected to be protonated readily in NQO2 active site. The gas-phase Born-Oppenheimer potential energies calculations (Table S1) show high proton affinity for the flavin-bound anionic hydroquinone (~ -335 kcal/mol). The higher negative potential energy is indicative of a higher pK_a for the protonated neutral hydroquinone species. The free energy for adding a proton on the NQO2-bound anionic hydroquinone, $\Delta G_{\text{prot}}^0(\text{enz})$, was determined by thermodynamic integration^{17, 65} using the DFT-corrected SCC-DFTB-D/MM scheme (Scheme 3). The corrected free energy was determined to be -12.5 kcal/mol, which corresponds to a pK_a of 9.1 (Table S2, eq. S4 in the footnote) for the flavin-bound neutral hydroquinone (F:::PBQH₂) at the active site of NQO2. The theoretically determined value is approximately 2 units less than the experimentally observed pK_a of 10.9 for the aqueous anionic hydroquinone.⁷²

Energetics of the Product Displacement Reactions.

The product displacement reactions (Scheme 1, reactions b and d) were studied in water and the NQO2 active site following the thermodynamic diagram in Scheme 4. Results with various components of the energetics for the 2-ns replicate simulations are given in Table S3. The convergence was ascertained by plotting the difference of the energies of the QM subsystem from the final cumulative average (Figure S3).

Displacement of NQO2-bound NM⁺ by PBQ.—In water, the displacement of the product, NM⁺ from the reduced active site by PBQ (Scheme 1, reaction b) is slightly thermodynamically favorable with the free energies of reaction of ~ -1 kcal/mol (Table 2). However, as expected, this change becomes thermodynamically favorable in the presence of the enzyme (Table 3) – the Gibbs free energy of the product displacement was determined to be equal to -33.0 kcal/mol. The binding of the menadione (a derivative of PBQ) to the NQO2 active site pocket can be estimated from the experimentally determined Michaelis constant¹⁴ and accounts for ~ -7 kcal/mol. Thus, the present calculations show that NQO2 active site has a significant role in the displacement of NM⁺ by PBQ.

Displacement of NQO2-bound PBQH₂ by NMH.—The computed Gibbs free energy of the aqueous phase displacement of PBQH₂ by NMH (Scheme 1, reaction d) was ~ -1.5 kcal/mol (Table 2) indicating that the reaction is barely spontaneous in water. Within the NQO2 active site environment, the reaction becomes favorable and the calculated free energy change was determined to be -12.4 kcal/mol (Table 3). The experimental value of only the NMH binding to NQO2 active site is available and is ~ -6 kcal/mol¹⁴ and the computation reveals that the thermodynamic favorability of the displacement reaction was primarily driven by stronger interaction between NMH and NQO2 protein matrix (Table 3). However, if the protonation free energy of -12.5 kcal/mol (Table 3) for the NQO2-bound PBQH⁻ is considered (*vide supra*), the free energy of the product displacement reaction was calculated to be equal to -24.9 kcal/mol (Scheme 5). This observation shows that the protonation plays an important role in the continuation of the ‘ping-pong’ mechanism.

Changes in the Hydrogen Bonding Interactions.

To explore the conformational changes associated with the catalytic reduction process, hydrogen bonding interactions were analyzed for both hydride transfer reactions. The summary of the important active site hydrogen bonding interactions for the PC, TS, and SC are given in Table 4.

Reduction of flavin by NMH (hydride donor).—The stacking of the donor substrate on the flavin ring and the key interactions in the TS are shown in Figure 3a. As depicted in Figure 3a, during the hydride transfer to the flavin ring, NMH assumes nearly a planar position over the centroid of the pyrazine – the central ring of the flavin ring system. The backbone amide hydrogens of Gly149, Trp105, and Phe106 tether the flavin ring through strong H-bond interactions with O2, N5, and O4 atoms, respectively (Table 4). The two interactions, namely, Phe106:HN $\cdots$ FAD:O4 and NMH:HN12 $\cdots$ Gly149:O (Figure 3a) appear to become stronger in the TS, as the H-bond distances decrease to ~ 2 and 2.25 Å, respectively (Table 4).

Reduction of the PBQ (hydride acceptor) by flavin.—In contrast to the hydride donor NMH, PBQ is bound over the pyrimidine ring with respect to the isoalloxazine ring of FAD (Figure 3b). Much like the NMH-containing system, the flavin ring atoms O2, N5, and O4 are anchored by the amide protons of Gly149, Trp105, and Phe106, respectively (Figure 3b, Table 4). The Tyr155 plays a key role in the reaction as the phenolic hydroxyl group stabilizes the distal oxygen of PBQ by serving as a H-bond donor (Figure 3b). The variations of Phe106:HN $\cdots$ FAD:O4 interaction distances become minimum at the TS suggesting that the residue Phe106 stabilizes similarly to the flavin reduction step by NMH.

Dipole Moment Variations.

The electron density on the flavin ring (stacked with donor/acceptor) alters during both hydride transfer steps as a result of the transfer of the charged hydride ion. The dipole moment (μ) of the QM core i.e. the donor/acceptor-bound flavin ring is expected to exhibit the change more succinctly and therefore, its variation was scrutinized along the reaction coordinate of both hydride transfer reactions following procedures described in theory and methods section.

In confirmation of the above hypothesis, a significant increase of μ was noted for the SC of both reactions (blue arrow in Figure 4, Table 5). For the SC (i.e. the complex $\text{FH}^-::\text{NM}^+$), μ was found to be about 24–28 D, which is ~13 D higher as compared to that of the PC (i.e. $\text{F}::\text{NMH}$) (Table 5). The bent structure in $\text{FH}^-::\text{NM}^+$ produced a slightly lower (by ~4 D) value of μ in the SC, (Table 5), when compared to its planar analog. Similarly, a marked two-fold increase of the computed μ was seen with the PBQ-bound system (Table 5) upon completion of the hydride transfer reaction. The physical manifestation of this change can be seen in Figure 4, where the accumulation of negative charge over the flavin's uridyl and pyrazine ring is evident from the appearance of the brighter red color on the iso-electron density surface.

In summary, the present study shows that μ of the QM center increased after the completion of both hydride transfer reactions. Furthermore, in both catalytic processes, μ was found to be directed parallel to the major axis of the flavin, which is along the O2 to C8 direction (Scheme 2, Figure 4). Therefore, it can be speculated that after the completion of hydride transfer reactions, the active site would become significantly more polarized to accommodate the increasingly polar QM region. The increased active site polarization would accompany a redistribution of water molecules, especially, along the major axis of the flavin ring. The evidence for this hypothesis is presented in the following section.

Redistribution of Water.

As the catalytic hydride transfer step is associated with increased QM site polarization, the water molecules are expected to redistribute during each catalytic/product displacement process. To explore the distribution of water molecules, a radial distribution function (RDF) was calculated to examine the change in probability density of water extending radially from the O2, N5 or C8 atom of the flavin ring (Figure 5).

The hydride transfer reaction between flavin and the donor NMH exhibits a significant increase in the RDF at 2 Å from O2 indicating water accumulation along this end of the flavin ring (green line, Figure 5a). A small increase in the RDF at around 3 Å from the N5 atom was also noted. This peak also moves ~0.4 Å closer to N5 in comparison to the PC (depicted by the blue line), which is consistent with the increased electronic charge on this atom due to its participation in the hydride transfer reaction. As the reaction proceeds, the C8-centered RDF shows a small increase in the peak at 4 Å and the generation of a second peak at 7 Å indicating water accumulation close to that atom (Figure 5a). The effect can be visualized in the increased dot density close to flavin's O2 and C8 atoms (Figure 5c).

The reduction of the PBQ shows a much stronger trend, as expected by its roughly horizontal μ (see dipole moment variations discussed in the results section). The O2-centered RDF shows a small increase in the aqueous probability density beginning at 2 Å, although the emergence of two new peaks at 4 and 5 Å provides stronger evidence of water accumulation around this atom as the reaction progresses from PC to SC (blue to green line Figure 5b). The N5-centered RDF does not show a noticeable change. In contrast, a sharp change was observed in the C8-centered RDF plot. As the active site became more polarized in the SC, the RDF at 5 Å almost doubles in intensity (Figure 5b). Similar to the case of the

NMH reaction, the visualization confirms increased water accumulation along the major axis of the flavin, which is parallel to the direction of μ (Figure 5c).

Alteration of Active Site Electrostatics.

As described earlier, μ of the QM site was found to increase during the formation of SC in both catalytic steps but decrease during the product displacement reaction (Table 5). Furthermore, the increased active site polarization during catalysis is evident from the realignment of water molecules along the direction of μ of the QM site, as discussed earlier in the water RDF calculations (*vide supra*). Therefore, the active site that stabilizes the QM core of the SC during a catalytic cycle would be more polarized than the active site for the PC of the next cycle (Scheme 5). This further suggests that significant depolarization of the active site ought to occur as the product is being displaced by the new substrate after the half-cycle. At the end of the complete ‘ping-pong’ cycle, the active site needs to return into its initial configuration. Therefore, the active site response in the first half-cycle would be followed by an opposing response during the next half-cycle. In particular, the geometric and electrostatic effects of the charged residues surrounding the QM center would exhibit an opposite but cyclic behavior.

Following this hypothesis, six charged residues close to the flavin moiety in the active site (Figure 6a) were neutralized and the energetic changes were studied (Table 6) following a procedure similar to what was used in our earlier work.¹⁷ In particular, the computed residue-specific electrostatic stabilization energy E_{elec} (Table 6) for all four reactive states shown in Scheme 1 were plotted against the distances measured from their C α to the flavin center of mass (COM). The observed changes are shown with arrows labeled with the specific reaction step illustrated in Scheme 1. The vertical component of the arrow shows the relative electrostatic stabilization of the QM site by the residue, while the movement of the C α residue moving towards or away from the flavin COM is represented by the horizontal component. Thus, an arrow directed upward and right indicates that the partial charge of the residue favored the reaction, while moving further from the QM site. The overall ‘ping-pong’ cycle, indeed exhibits a cyclic behavior in terms of geometric and electrostatic responses during the catalytic and product displacement reactions (Figure 6).

As illustrated in Figure 6b–d, all negatively charged residues were observed to resist the product displacement reaction – the conversion of $\text{FH}^-::\text{NM}^+(\text{enz})$ to $\text{FH}^-::\text{PBQ}(\text{enz})$ (Scheme 1, reaction b). This is illustrated in the downward direction of the arrows designated for reaction b). As expected, these negatively charged residues were found to stabilize the $\text{FH}^-::\text{NM}^+(\text{enz})$ more than the $\text{FH}^-::\text{PBQ}(\text{enz})$. Glu193 and Asp117 produced the sharpest changes in energy and are represented by almost vertical arrows in Figure 6b–6d.

A reverse effect due to the negatively charged residues was observed in the other product displacement reaction – the conversion of $\text{F}::\text{NMH}$ to $\text{F}::\text{PBQH}^-$ (Scheme 1, reaction d). As evident in Figure 6b–6d, these charged residues were found to favor the product displacement. The negative charges of these residues favored the enzyme-bound neutral $\text{F}::\text{NMH}$ complex more than the negatively charged $\text{F}::\text{PBQH}^-$ complex. The Glu193 (Figure 6b) and Asp117 (Figure 6c) resulted in a sharp change in the energy, which is

quantitatively similar to the other product displacement (Scheme 1, reaction b). In both product displacement reactions, the positively charged residue, Arg165, produced an opposite effect to those of the negatively charged residues, as expected.

Taken together, the catalysis and product displacement led to an overall oscillatory electrostatic change during the ‘ping-pong’ kinetics (Figure 6b–6d). It is generally difficult to characterize the effects of polarization of the active site and their effect on catalysis experimentally. In citrate synthase, the effect of polarization has been demonstrated experimentally,^{73–74} and attributed with theory,⁷⁵ to the stabilization of the intermediate. Increased polarization of the active site has also been shown to drive catalysis in another flavoenzyme, thymidylate synthetase (ThyX).⁷⁶ In the case of NQO2, the polarization of the active site oscillates as the enzyme shuttles between the donor- and the acceptor-bound conformations.

Oscillatory Collective Protein Dynamics.

The collective (essential) dynamics⁷⁷ of the protein was extracted by examining the principal component of the backbone fluctuations using the program CARMA⁷⁸ following a procedure published earlier from our lab.^{18, 79} The analysis of the collective dynamics of active site and its surrounding structural elements display a contrasting motion of the four regions identified in our earlier study with the holoenzyme,¹⁸ that exhibited major thermal fluctuations. These regions, highlighted in Figure 7 are as follows: I, helix-loop motif (191–217); II, loop (149–163); III, loop (125–137); and IV, helix-loop (55–78). The changes in region II, III and IV are more prominent as apparent in the backbone fluctuation in the left and right panels in Figure 7. As indicated by the red arrows, a part of the region II moves farther from the QM site for NMH to flavin hydride transfer reaction (i.e. reaction a in Scheme 1). An opposing nature of dynamics is evident from the observed changes during the flavin-to-PBQ hydride transfer reaction (i.e. reaction c in Scheme 1). This corresponds well with the geometric changes of the residue Asp163, observed in the analysis of electrostatics (Table 6, Figure 6d). The essential dynamics in region III also exhibits contrasting motions going from reaction a to c in Scheme 1 (Figure 7). As shown by the green arrows, the loop region moves towards the QM site (for reaction a), but moves away from the same during reaction c. Similarly, for region IV, the purple arrows show that the helix-loop moves in and out in an opposite manner for reactions a and c, respectively. This was also corroborated by the opposing geometric changes of Glu70 in Figure 6b. The opposing motions in regions II, III, and IV indicate that the two hydride transfer reactions produce contrasting coupled dynamics.

CONCLUSIONS

The energetics, geometric changes, and the role of active site polarization associated with the ‘ping-pong’ (double displacement) kinetics (Scheme 1) in the NQO2 active site were investigated using hybrid QM/MM simulations. The Gibbs free energy of the NMH-to-flavin hydride transfer reaction (Scheme 1, reaction a) was found to be identical (–2.6 kcal/mol) in both water and the NQO2 active site. The Gibbs free energy of activation for the enzyme-catalyzed reaction was determined to be 15.0 kcal/mol (Table 1), which is consistent with

the experimentally observed barrier¹⁴ of 14.2 kcal/mol. Compared to the aqueous system, the activation free energy was found to be ~ 3 kcal/mol less in the enzyme system. The reduction of flavin by the hydride donor (Scheme 1, reaction a) is considered to be the key activation step for enabling NQO2 to act as a redox switch for the stabilization of the tumor suppressor protein p53,^{2, 30} and the present study finds that the NQO2 active site enhances the catalysis by 10⁴-fold.

A significant role of the NQO2 active site on the catalytic reduction of quinone (Scheme 1, reaction c) is also evident from the low barrier height, which was calculated to be only 4.4 kcal/mol (3 kcal/mol less than the barrier in water). In addition, the enzymatic reaction is highly favorable with a free energy of reaction of -19.6 kcal/mol (Table 1). This higher value of free energy indicates that the anionic hydroquinone is well stabilized in the active site environment. This also demonstrates that the acceptor quinone is kinetically unstable and readily converts to the hydroquinone state, quite consistent with the obligatory 2e⁻ reduction of quinones by this enzyme. Assuming the product displacements are much faster, the present study predicts that the activation of flavin by the donor NMH (Scheme 1, reaction a) is the rate determining step for the entire 'ping-pong' kinetics.

Furthermore, while the product displacement reactions (Scheme 1, reactions b and d) are barely favorable in water (Table 2), both are highly favorable in the NQO2 active site (Table 3). The Gibbs free energy change for the displacement reaction of NM⁺ by PBQ is -33.0 kcal/mol, the large thermodynamic favorability is presumably due to solvation of positively charged NM⁺ upon release in water. The Gibbs free energy change computed for the substitution reaction in PBQH₂ by NMH is -12.4 kcal/mol (Table 3). In summary, these computations reveal significant thermodynamic advantage for both catalytic and product displacement reactions (Scheme 5).

The role of NQO2 in cancer is still evolving and the 'ping-pong' mechanism is involved in two antitumorigenic functions: the reduction of the estrogen quinones and the stabilization of tumor suppressor protein, p53.⁴ One key finding from the present result is the role protonation plays in the 'ping-pong' kinetics. The protonation of the NQO2-bound anionic hydroquinone was found to contribute an additional -12.5 kcal/mol and thus appears to have a major contribution in the displacement of the PBQH⁻ by NMH (Scheme 5, Table 3). Previous studies found that the binding of p53 by NQO2 is NMH dependent, which suggests that the hydride transfer to the flavin is a prerequisite for the binding event. The present study finds that the protonation-deprotonation equilibria in the cell could play an important role in the redox switching function of NQO2. In particular, one might claim, within the realm of thermodynamic principle, that the protonation of the anionic hydroquinone could lead the release of the product and subsequent binding of the donor-substrate, NMH. Since the NMH-to flavin hydride transfer is the necessary step before binding to the p53, the protonation could tilt the equilibrium in favor of the NQO2-p53 complexation.

Finally, in the present study, the oscillatory effect of the active site electrostatics was found to greatly influence the 'ping-pong' kinetics in NQO2. During the hydride transfer reactions, the SC was found to be more polarized than the PC state. This is evident not only from the evolution of μ (for the QM site) but also from the water accumulation along the major axis

of the flavin ring after the completion of both catalytic reactions (Table 4, Figures 4 and 5). Further scrutiny of the role of several charged residues demonstrated that the geometric and energetic changes during one half-cycle is reversed back in the next half-cycle (Figure 6). In particular, the changes in catalytic and product displacement steps maintain a cyclic pattern. The cyclic nature of active changes is also corroborated by the essential dynamics analysis of the two catalytic steps, which documents the evolution of contrasting coupled dynamics encompassing the structural elements surrounding the active site (Figure 7). The ‘ping-pong’ mechanism is common in biological chemistry^{80–81} and for the first time, the theoretical analysis was able to capture the molecular details on how the oscillating active site polarization plays a mediatory role in the substrate shuttling in NQO2.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS USED

MD

molecular dynamics

MM

molecular mechanical

NMH

N-methyldihydronicotinamide

NQO2

NRH: quinone oxidoreductase 2

PBQ

p-benzoquinone

PC

precursor complex

QM

quantum mechanical

QM/MM

quantum mechanical/molecular mechanical

self-consistent charge-density functional tight-binding with dispersion correction**SC**

successor complex

TS

transition state

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Figure 1.

The reaction coordinates of the two hydride transfer reactions involving 7, 8 dimethylisoalloxazine (flavin) as defined in eq. 6: a) from NMH to flavin ring and b) flavin to PBQ. The color of the reduced and oxidized systems are coded with blue and red, respectively. The 'R' attached to the flavin ring is a methyl group for aqueous systems and represents a –ribityl-ADP moiety for the enzymatic reactions.

Figure 2.

Corrected potentials of mean force (PMF) for the two hydride transfer processes catalyzed in both enzyme-bound (solid line) and aqueous (dashed line) states: a) NMH to oxidized flavin and b) reduced flavin to PBQ. For each catalytic reaction, the reaction coordinate ξ is calculated using eq. 6 and illustrated in Figure 1.

Figure 3. Hydrogen bonding interactions for the transition state structures observed in the two hydride transfer reactions. The substrates are stacked on top of the tricyclic isoalloxazine ring: a) the hydride donor substrate, NMH and b) the hydride acceptor substrate, PBQ. Selected interactions with the surrounding side chains are highlighted using purple broken lines.

Figure 4.

Iso-electron density surfaces of the PC and SC for the hydride transfer reactions: NMH to oxidized flavin and the reduced flavin to PBQ. Each surface is color coded based on their natural bond orbital-computed partial charges: red for negative charges and green for positive charges. In each structure, the dipole moment, μ is shown from the center of each complex by a blue arrow.

Figure 5.

The radial distribution function (RDF) of water around the QM site for the PC (blue line) and the SC (green line) in the catalytic hydride transfer reactions involving a) NMH and b) PBQ. Dotted illustrations in c) indicates the accumulation of water molecules during around the QM region. The O2, N5, and C8 atoms of the NQO2-bound flavin were chosen to compute the RDFs. Data from 500 ps dynamics simulation was used for all calculations and visualizations.

Figure 6.

Computed energetic and geometric changes of selected charged residues in the catalysis and product displacement reactions of ‘ping-pong’ cycle as shown in Scheme 1 (reactions a–d). The E_{elec} for each charged residue, computed using eq. 9, averaged over 500 ps data, provides a measure of the electrostatic stabilization energy of the QM region defined in Scheme 2. The E_{elec} values were plotted against the change in the C_{α} -flavin COM distances. Charged residues in a) are labeled by the same color as the arrows that represent the change in geometric and energetics for the specific process in Scheme 1. A positive value (i.e. upward arrow) of E_{elec} indicates that the residue favored the specific reaction step. An arrow pointing to the right signifies that the C_{α} atom moved further away from the QM region.

Figure 7.

The essential dynamics of the NQO2 active site during the hydride transfer reactions. The left and right panel shows the principal component of motions during the hydride transfer reactions involving NMH and PBQ, respectively. A cartoon representation of the holo-enzyme is shown in the middle, the four dynamic regions identified in our earlier studies are highlighted: helix (191–217) of subunit A as blue, loop (149–165) of subunit A as red, loop (125–137) of subunit B as green, and loop (55–78) of subunit B as purple. The arrows of the dynamic regions in the left and right panels are color coded to match with the structural elements of the holo-enzyme (central panel). The conformational changes in the left and right panels are indicated by superimposed backbones shown in tubes: the initial conformation in red and the final one in blue.

Scheme 1.

The 'ping-pong' kinetics in NQO2 comprises two redox half cycles, each of which consists of a catalytic hydride transfer step (steps a or c) and a product displacement step (steps b or d). The first half cycle starts with step a), during which a hydride (shown in green color) is transferred from the NMH (in blue) to the neutral flavin. This is followed by a product displacement step b), when the oxidized product is displaced by PBQ (in red). In the next half cycle, during step c), a hydride is transferred from the reduced flavin to the PBQ. Subsequently, in step d) the protonation (the proton shown in purple) of the anionic hydroquinone and subsequent displacement of the hydroquinone by NMH occurs, thereby completing the cycle.

Scheme 2.

The computational setup for the hybrid QM/MM simulation. The flavin ring of the FAD and the substrates (shown within the elliptical boundary) are inside the QM region, while embedded into a 30 Å spherical region of solvated NQO2 active site. A link-atom shown by 'L' acts as the boundary between the QM and MM regions.

Scheme 3.

The protocol used in the study for incorporating quantum corrections to the free energies of reaction for the steps of ‘ping-pong’ kinetics. The free energy obtained in the SCC-DFTB-D/MM was corrected using two corrections derived from the study of small model systems comprising only QM atoms: i) a high-level correction (HLC), obtained from the difference in the DFT and SCC-DFTB-D-computed Born-Oppenheimer potential energies for each model systems; ii) the difference in the DFT-computed zero-point energy corrections (Δ ZPE), for each step.

Scheme 4.

The thermodynamic scheme employed for the product displacement reactions. The reaction at the center represent the gas-phase product displacement, while those at the top and bottom show the displacement processes in the aqueous and enzyme environments, respectively. ‘Fl’ is the short-hand notation of the flavin ring, while the ligand S1 is displaced by the ligand S2, in each case. The oxidation states of the flavin as well as the ligands depend on the specific half-cycle of the ‘ping-pong’ kinetics being considered. Such complexes are indicated in the right bottom corner; reduced flavin binds to the oxidized form of nicotinamide and PBQ, while neutral flavin binds to the hydroquinone and NMH.

Scheme 5.

The free energies obtained in this study for the catalytic and product displacement steps of the 'ping-pong' kinetics. The red and blue shades indicate the oxidized and reduced states of the flavin-bound active site, respectively. The purple glow indicates a more polarized active site as observed for the product state of both hydride transfer reactions.

Table 1.

Standard Gibbs free energies of reaction and activation for the catalytic hydride transfer reactions in NQO2 and water (in parenthesis). Free energies were calculated following DFT-corrected SCC-DFTB-D/MM scheme (Scheme 3). All values are given in kcal/mol.

Reaction	Free Energy Components	G^0	$G^\ddagger$
$F:::NMH(enz) \rightarrow FH^-:::NM^+(enz)$	G_{uncorr}	-1.0 (-1.0)	12.5 (15.3)
	HLC	-2.6	5.3
	ZPE	1.0	-2.8
	$G^{\ddagger}$	-2.6(-2.6)	15.0 (17.8)
	G_{uncorr}	-16.6 (-11.0)	5.4 (8.0)
$FH^-:::PBQ(enz) \rightarrow F:::PBQH^-(enz)$	HLC	-2.9	1.4
	ZPE	-0.1	-2.4
	$G^{\ddagger}$	-19.6 (-14.0)	4.4 (7.0)

^aCalculated using eq. 5

Table 2.

Various components of the Gibbs free energies in kcal/mol for the product displacement reactions in gas and aqueous environments following Scheme 4. The solvation free energy differences are given by G_s .

Reaction	G^0	
	SCC-DFTB-D	DFT
PBQ(aq) + FH ⁻ :::NM ⁺ (aq) → NM ⁺ (aq) + FH ⁻ :::PBQ(aq)		
$\Delta\Delta G_{\text{disp}}^0(g)$	77.6	78.3
$G_s(\text{PBQ} \rightarrow \text{NM}^+, \text{aq})$	-49.6	-49.4
$G_s(\text{FH}^-:::\text{NM}^+ \rightarrow \text{FH}^-:::\text{PBQ}, \text{aq})$	-26.7	-29.8
G_{corr}^a	-2.4	---
$\Delta\Delta G_{\text{disp}}^0(\text{aq})^b$	-1.1	-1.0
NMH(aq) + F:::PBQH ₂ (aq) → PBQH ₂ (aq) + F:::NMH(aq)		
$\Delta\Delta G_{\text{disp}}^0(g)$	-1.0	1.7
$G_s(\text{NMH} \rightarrow \text{PBQH}_2, \text{aq})$	2.3	2.5
$G_s(\text{F}:::\text{PBQH}_2 \rightarrow \text{F}:::\text{NMH}, \text{aq})$	-7.8	-5.7
G_{corr}^a	5.2	---
$\Delta\Delta G_{\text{disp}}^0(\text{aq})^b$	-1.3	-1.5

^aCalculated using eq. 4

^bCalculated using eq. 7

Table 3.

Various component of the Gibbs free energies (in kcal/mol) of product displacement as shown in Scheme 4. The Gibbs free energy difference of solvation in water is given by G_S , while in enzyme this notation refers to the difference of the interaction free energies (see Table S2).

Reaction	G^0
$\text{PBQ}(aq) + \text{FH}^{-}::\text{NM}^{+}(enz) \rightarrow \text{NM}^{+}(aq) + \text{FH}^{-}::\text{PBQ}(enz)$	
$\Delta\Delta G_{\text{disp}}^0(g)$	77.6
$G_S(\text{PBQ} \rightarrow \text{NM}^{+}, aq)$	-49.6
$G_S(\text{FH}^{-}::\text{NM}^{+} \rightarrow \text{FH}^{-}::\text{PBQ}, enz)$	-58.6 ^a
G_{corr}^b	-2.4
$\Delta\Delta G_{\text{disp}}^0(en z)^c$	-33.0
$\text{H}^{+}(aq) + \text{F}::\text{PBQH}^{-}(enz) \rightarrow \text{F}::\text{PBQH}_2(en z)$	
$\Delta G_{\text{TI}}^0^d$	-18.0
G_{corr}	5.5
$\Delta G_{\text{prot}}^0(en z)^e$	-12.5
$\text{p}K_a = 9.1^f$	
$\text{NMH}(aq) + \text{F}::\text{PBQH}_2(aq) \rightarrow \text{PBQH}_2(aq) + \text{F}::\text{NMH}(aq)$	
$\Delta\Delta G_{\text{disp}}^0(g)$	-1.0
$G_S(\text{NMH} \rightarrow \text{PBQH}_2, aq)$	2.3
$G_S(\text{F}::\text{PBQH}_2 \rightarrow \text{F}::\text{NMH}, enz)$	-18.9 ^a
G_{corr}^b	5.2
$\Delta\Delta G_{\text{disp}}^0(en z)^c$	-12.4

^aCalculated from the replicate simulation (Table S2)

^bCalculated using eq. 4

^cCalculated using eq. 8

^dObtained from thermodynamic integration as shown in Figure S1

^eVarious components of the Gibbs free energy for the protonation is detailed in Table S2

^fCalculated using eq. S4

Table 4.

Hydrogen bonding (in Å) as measured in the PC, the TS, and the SC for the two hydride transfer reactions described in Scheme 1.

Reaction	Donor ... Acceptor	PC	TS	SC
$F \cdots NMH(enz) \rightarrow FH^- \cdots NM^+(enz)$	Gly149:HN...FAD:O2	2.15	2.93	1.80
	Trp105:HN...FAD:N5	2.08	2.27	2.17
	Phe106:HN...FAD:O4	2.03	1.99	2.06
	NMH:HN12...Gly149:O	3.38	2.24	3.79
$FH^- \cdots PBQ(enz) \rightarrow F \cdots PBQH^-(enz)$	Gly149:HN...FAD:O2	1.85	2.20	1.82
	Trp105:HN...FAD:N5	2.37	2.28	2.22
	Tyr155:HH...PBQ:O4	1.72	2.41	1.72
	Phe106:HN...FAD:O4	1.95	1.73	1.80

Table 5.

The dipole moment (μ , in Debyes) of the QM-core (Figure 1) for the two hydride transfer reactions calculated with DFT using the M06-2X functional with a 6-31+G(d,p) basis set. For each reaction, μ was averaged over 10 conformations for PC, TS, and SC. The ‘bent’ and ‘planar’ indicate the butterfly conformational shift of the flavin ring.

Reaction	PC		TS		SC	
	Bent	Planar	Bent	Planar	Bent	Planar
F:::NMH $\rightarrow$ FH:::NM ⁺	14.9 $\pm$ 0.5	15.2 $\pm$ 0.7	23.1 $\pm$ 1.0	22.0 $\pm$ 0.4	24.4 $\pm$ 1.1	28.4 $\pm$ 2.0
FH:::PBQ $\rightarrow$ F:::PBQH ⁻	11.9 $\pm$ 0.6	11.8 $\pm$ 0.6	16.6 $\pm$ 1.5	16.8 $\pm$ 0.9	23.5 $\pm$ 1.0	24.5 $\pm$ 0.8

Table 6.

Changes in the residue-specific electrostatic stabilization energies E_{elec} (eq. 9, in kcal/mol) computed for the two hydride transfer reactions. For each reaction, a difference was calculated by $E_{\text{elec}}(\text{product}) -$

$E_{\text{elec}}(\text{reactant})$ for selected residues and averaged over an ensemble of 300 conformations from a MD simulation trajectory. A positive value implies stabilization of the product state of these reactions.

Reactions	Glu193	Glu70	Asp117	Asp127	Asp163	Arg165
F ⁻ ::NMH → FH ⁻ ::NM ⁺	7.6	6.2	-5.3	-3.2	1.8	2.0
FH ⁻ ::PBQ → F ⁻ ::PBQH ⁻	7.9	0.4	-1.1	-3.9	-21.7	-0.4