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Accurate Quantum Mechanical/Molecular Mechanical Calculations of Reduction Potentials in Azurin Variants

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Abstract

Understanding the regulation mechanism and molecular determinants of the reduction potential of metalloprotein is a major challenge. An ab initio quantum mechanical/molecular mechanical (QM/MM) method combining the minimum free energy path (MFEP) and fractional number of electron (FNE) approaches has been developed in our group to simulate the redox processes of large systems. The FNE scheme provides an efficient unique description for the redox process, while the MFEP method provides improved conformational sampling on complex environments such as protein in the QM/MM calculations. The reduction potentials of wild-type and seven mutants of azurin, a type 1 copper metalloprotein, were simulated with the QM/MM-MFEP+FNE approach in this paper. A range of 350 mV for the variations of the reduction potentials of these

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ASSOCIATED CONTENT

Supporting Information

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Table S1 for the coordination bond lengths between Cu and ligands in azurins. Table S2 for the comparison between optimized and experimental Cu-ligand bond lengths. Figure S1 for the convergence of the relative free energies and QM geometries during QM/MM-MFEP. Figure S2 for the optimized geometries of the active sites in azurins. Figure S3 for the difference between the QM ESP charges and the MM point charges of the side-chain atoms in the mutation sites. Figure S4 for the electron density of HOMOs of azurins on the reduced states (PDF)

The authors declare no competing financial interest.

azurin proteins was reproduced faithfully with relative errors around 20 mV. The correlation between structural interactions and reduction potentials observed in simulations provides in-depth insight into the regulation of reduction potentials, which potentially can also be very useful to the engineering of metalloprotein-based electrocatalysts in artificial photosynthesis. The excellent accuracy and efficiency of the QM/MM-MFEP+FNE approach demonstrate the potential for simulations of many electron transfer processes in condensed phases and biochemical systems.

INTRODUCTION

Electron transfer (ET) is a fundamental reaction in a variety of important biochemical processes such as photosynthesis and respiration.^{1–5} Knowledge of the electrochemical properties of ET reactions is critical for understanding the ET mechanisms and improving the performance of the related molecular processes. High-level accurate quantum mechanical calculation as well as molecular simulation can contribute greatly in deciphering structure and function relationship of the ET processes with the atomic and electronic details.^{6–10}

Azurin, a small blue copper protein with 128 residues, is one of the most prevalent metal complexes serving as electron shuttles to mediate various ET reactions in biological metabolisms.^{11–14} Azurin features a type 1 (T1) copper redox center, which is characterized by a 5-fold coordination with a trigonal-bipyramidal structure, including the strong equatorial coordination of His-Cys-His and the weaker coordination of Met-Gly in axial positions.^{15,16} Different from aqueous copper ions with varying coordination numbers between oxidation states, the copper center in azurin maintains stable 5-fold coordination in both Cu(I/II) oxidation states. The stable coordination in azurin leads to much smaller reorganization energy (0.7–0.8 eV)^{17–19} compared with that in aqueous solution (2.1 eV),²⁰ facilitating much higher ET kinetic rate in the protein.^{3,15} The hydrophobic copper-binding loop region also controls the outer-sphere reorganization energy through the accessibility to the solvent of the active site and the dynamics of the whole protein.²¹ The thermodynamic driving force of the ET processes, on the other hand, is determined by the reduction potentials covering a broad range with different mutations in azurin.^{22–26} Many molecular determinants relevant to the reduction potential variability in azurin have been discussed. For example, Marshall et al. reported that two properties, hydrophobicity related to the mutation on Met121 and hydrogen-bonding in the inner and outer coordination spheres, can influence the reduction potentials over a 700 mV range.²⁷ Hadt et al. investigated the covalent and nonlocal electrostatic contributions to reduction potentials and proposed that the mutations in the second coordination sphere can modulate hydrogen bonds and protein-derived dipoles close to the copper binding site.²⁸ Furthermore, a combination of various long-range interactions, such as perturbations in electrostatic field and the dynamic interplay between the loop regions of protein and the copper center, should be also responsible for the reduction potential changes.^{22,24,29,30} The sensitivity of the reduction potential to mutations makes the T1 copper proteins attractive in biochemical, biotechnological and bioengineering fields such as the design for new tunable electrocatalysts in artificial photosynthesis and enzymatic biofuel cells.²⁷ However, quantitative and accurate prediction of the delicate

dependence of reduction potential on mutations of metalloproteins raises a great challenge to both methodologies and computational resources.^{31–34}

In order to determine redox properties of a metalloprotein accurately, it is crucial to address both the electronic structure of the active redox center and contributions of the environment. Treating the outer-sphere contribution with implicit solvent models eliminates the necessity for sampling the conformational space of the environment and thus greatly reduces the computational cost.^{35–39} Recently, Fowler et al. predicted the reduction potentials of azurin mutants using a combination of continuum electrostatics and density functional theory (DFT).⁴⁰ The experimental values of the relative reduction potentials of 30 mutants were reproduced accurately within a mean absolute deviation of 24 mV. However, the computational model was system-dependent on the hydrophobic and hydrogen bond interactions at the active site, and an empirical hydrophobicity factor should be introduced as a correction term for some mutants. Sinicropi studied the reduction potentials of wild-type, nickel-substituted, and two mutated azurins using DFT combined with a conductor-like screening model. The experimental changes on the redox properties were at least qualitatively reproduced.⁴¹ Nevertheless, homogeneous implicit models are not applicable to describing the explicit contributions of out-sphere residue mutations in metalloproteins, where heterogeneous site-specific residue polarization and hydrogen bonding are important.⁴² The explicit inclusion of the surrounding residues and water molecules is usually necessary in order to accurately characterize the distinct chemical environment and structures of the redox centers in metalloproteins. Treating both the active site and the surroundings using quantum mechanical method such as DFT-based molecular dynamics (MD)^{20,43} is possible but extremely time-consuming. In addition, the uncertainty from finite system size effects and the challenges for DFT models lead to an error for reduction potentials about 200 mV.^{44,45} The combined quantum mechanical and molecular mechanical (QM/MM) method, first developed by Warshel and Levitt, provides an accurate and computationally efficient tool to describe chemical and biological systems in a complex environment.^{46–54} During the past decade, Yang and co-workers developed a series of QM/MM approaches to calculate oxidation free energies and reorganization energies.^{55–57} Several QM/MM calculations on the reduction properties of copper protein systems have been also reported in the past few years.^{19,29,58–60} Even with the classical MM force field models for the outer-sphere and solvents, the quality of the conformational sampling on the whole system remains a key issue to validate the results because the expensive QM calculation is required at every step during direct QM/MM MD. Some alternative techniques were proposed to reduce the computational cost, for example, refitting MM point charges in the classical model to capture the polarization effect from both protein and solvents,⁶¹ performing MD sampling with classical force field followed by QM evaluations on the active center from partial trajectories,^{29,60} decoupling of the QM and MM regions,^{49,62} or using semiempirical QM/MM models.⁶³ More rigorous approaches can be applied to describe the influence of the environment on the reduction properties of active site accurately, such as QM/MM free energy perturbation (QM/MM-FEP),⁶⁴ QM/MM thermodynamic cycle perturbation,^{65,66} MD perturbation matrix method,^{29,67} QM/MM adiabatic approximation,⁶⁸ and QM/MM minimum free-energy path (QM/MM-MFEP) method in which the geometry of the active site was minimized on the potential of mean

force (PMF) surface of QM coordinates rather than the potential energy surface.^{69,70} We further combined QM/MM-MFEP with the fractional number of electron (FNE) method to study redox reactions.^{71,72} Because the direct QM/MM MD sampling is replaced by iterative optimizations on the active site, the combined QM/MM-MFEP and FNE method is an excellent approach to calculate protein reduction potentials with a good balance between computational accuracy and efficiency.

In the present paper, we used the combined QM/MM-MFEP and FNE method,⁷¹ denoted as QM/MM-MFEP+FNE, to study the redox properties of eight azurins, including the wild-type *Pseudomonas aeruginosa* azurin, six single site mutants (M44K, N47L, N47S, M121Q, M121L, and M121Oxm) and one double site mutant (N47S+M121L). Here Oxm in M121Oxm denotes an unnatural amino acid as oxomethionine. The accuracy and efficiency of our computations demonstrate the promising applications of the QM/MM-MFEP+FNE approach to the study of electron transfer in complex biochemical systems.

THEORY

The QM/MM-MFEP method describes the thermodynamics of the entire molecular system with the PMF of the QM subsystem as

$$A(\mathbf{r}_{\text{QM}}) = -\beta^{-1} \ln \left[\int e^{-\beta E(\mathbf{r}_{\text{QM}}, \mathbf{r}_{\text{MM}})} d\mathbf{r}_{\text{MM}} \right] \quad (1)$$

and its gradient with respect to the QM degrees of freedom is

$$\frac{\partial A(\mathbf{r}_{\text{QM}})}{\partial \mathbf{r}_{\text{QM}}} = \left\langle \frac{\partial E(\mathbf{r}_{\text{QM}}, \mathbf{r}_{\text{MM}})}{\partial \mathbf{r}_{\text{QM}}} \right\rangle_{\{\mathbf{r}_{\text{MM}}\}} \quad (2)$$

where $A(\mathbf{r}_{\text{QM}})$ is the PMF of the QM subsystem, $\mathbf{r}_{\text{QM}}$ and $\mathbf{r}_{\text{MM}}$ are the coordinates of the QM and MM subsystem, respectively, $E(\mathbf{r}_{\text{QM}}, \mathbf{r}_{\text{MM}})$ is the total QM/MM potential energy of the whole system, $\beta = 1/k_{\text{B}} T$ is the inverse temperature, and the angular bracket denotes an average over the sampled MM ensemble as $\{\mathbf{r}_{\text{MM}}\}$. The mutual dependence of geometric and electronic structures of the QM subsystem and the conformations of the MM subsystem is solved in a self-consistent manner through iteratively performing the geometry optimization of the QM subsystem and the conformational sampling of the MM subsystem. More detailed procedure of the QM/MM-MFEP method was discussed in our earlier work.^{70,71} Once the optimized geometries of the QM subsystem on both reduced and oxidized states are obtained, we then perform an interpolation of the QM structures and use the FNE as the order parameter to connect the two redox states smoothly. Here each intermediate state is represented by the linearly interpolated geometry and is assigned with the corresponding FNE. DFT calculation on each redox or intermediate state is performed with a fixed occupation number of the highest occupied molecular orbital (HOMO) to be the corresponding FNE, in which a self-consistent field procedure is employed without constraint on other variables including all the molecular orbitals. Then MD sampling of the

MM subsystem with a fixed QM subsystem is performed at each state for FEP calculations, and the overall oxidation free energy is calculated as the summation over the free energy change in each sampling interval as follows:

$$\Delta A^{\text{abs}} = \sum_{i=0}^{N-1} \Delta A(\eta_i \rightarrow \eta_{i+1}) \quad (3)$$

Where

$$\Delta A(\eta_i \rightarrow \eta_{i+1}) = -\beta^{-1} \ln \left[\left\langle e^{-\beta \Delta E(\eta_i \rightarrow \eta_{i+1})} \right\rangle_{\eta_i} \right] \quad (4)$$

N is the total number of states sampled, i denotes the i th intermediate state varying from $i=0$ as the reactant (reduced) state to $i=N$ as the product (oxidized) state, η_i is the order parameter with the interpolated QM geometry and the corresponding FNE at state i , $E(\eta_i \rightarrow \eta_{i+1})$ is the difference of the QM/MM potential energies between two neighboring states, and the angular bracket with η_i denotes the ensemble average on the i th intermediate state, which is generated from MD sampling on the MM subsystem with a fixed QM geometry and the corresponding FNE. Finally, we converted the oxidation free energy obtained to the absolute reduction potential using the relationship $A^{\text{abs}} = nFE^{\text{abs}}$, where n is the number of electrons transferred in the process and F is the Faraday constant. Since the experimental values were measured relative to the standard hydrogen electrode (SHE), the absolute SHE potential should be subtracted from the calculated absolute reduction potential using the relationship $E^0 = E^{\text{abs}} - E^{\text{SHE}}$ to obtain the standard reduction potential to compare with experimental results.

The outer-sphere reorganization energy can be estimated based on the linear response approximation and QM/MM calculations as^{73,74}

$$\lambda_{\text{out}} = \frac{1}{2} \left[\left\langle \Delta E_{\text{QM/MM}} \right\rangle_{\text{Red}} - \left\langle \Delta E_{\text{QM/MM}} \right\rangle_{\text{Ox}} \right] \quad (5)$$

Where

$$\Delta E_{\text{QM/MM}} = E_{\text{QM/MM}}^{\text{Ox}} - E_{\text{QM/MM}}^{\text{Red}} \quad (6)$$

$E_{\text{QM/MM}}^{\text{Red}}$ and $E_{\text{QM/MM}}^{\text{Ox}}$ are the QM/MM interactions in the reduced and oxidized states, respectively, and the angular brackets denote the ensemble average on the two redox states. The QM/MM electrostatic interactions can be obtained using MM point charges and QM ESP fitted charges.

COMPUTATIONAL DETAILS

The initial structure of the WT azurin was built from the 4AZU entry of the Protein Data Bank (PDB) with the X-ray coordinates resolved at 1.9 Å in a tetramer unit.⁷⁵ Only one monomer is considered in the present simulation with adjusted conformation and protonation using MolProbity.⁷⁶ The values of pK_a for residues were estimated using PROPKA3.^{77,78} His46 and His117 are in neutral state with protons on the $N_{\epsilon 2}$ atoms. The sulfur atom in Cys112 is deprotonated. His35 and His83 are assumed to be deprotonated with the estimated values of pK_a as 6.1 and 5.3, respectively. Lys44 in M44K is assumed to be protonated with an estimated pK_a of 10.9. The first coordination shell of T1 copper in azurin is composed by five residues: Gly45, His46, His117, Cys112, and Met121. In order to capture the effects of the mutations accurately, two additional residues that would be mutated, Met44 and Asn47, were also treated in the QM subsystem. The structures of the WT azurin and the corresponding QM region are shown in Figure 1. The initial structures of the mutant variants were constructed based on the wild-type geometry.

The azurins with the size of about 40 Å in length were solvated in a cubic water box of 90 Å × 90 Å × 90 Å, containing 23 104 water molecules represented using TIP3P model.⁷⁹ The BLYP exchange-correlation functional^{80,81} with LanL2DZ effective core potential basis set for copper⁸² and cc-pVDZ basis set for other QM atoms⁸³ was selected as the QM model. The rest part of the protein and the QM/MM vdW interactions were described using CHARMM22 force field.⁸⁴ Periodic boundary condition was used in the MD simulation and particle mesh Ewald method⁸⁵ was employed for long-range electrostatic interactions of the MM subsystem. The cutoff distance for nonbonded interactions was set as 20 Å, which was found to be enough to generate convergent results for systems with net charge varying between 0 and -1 previously.⁷¹ The pseudobond method with improved electrostatic parameters^{86,87} was used to treat the QM/MM boundary. In the iterative sequential optimization procedure, 160 ps MD sampling under NVT condition at 300 K was performed to build the fixed MM ensemble in each cycle. After the geometry optimizations on the reduced and oxidized states were completed, a linear interpolation was performed to connect the geometries of two states of the active site (as described by QM) for each protein, and the corresponding FNE was assigned to each interpolated state to calculate the free energy difference using FEP (see the protocol in our previous work⁷¹). The whole reaction progress was divided into 10 intervals, with 0.1 increment in the FNE. The FEP calculations should be repeated several times in order to enhance the accuracy and also to evaluate the sampling convergence of the simulations. For each protein, 8 FEP calculations, each with an 160 ps MD sampling to generate an independent MM ensemble with the same QM geometries, were performed to obtain the final free energy difference with the standard deviation. All simulations were implemented using the program SIGMA^{88,89} combined with a modified version of the GAUSSIAN 03 program⁹⁰ for DFT calculations with FNE.

RESULTS AND DISCUSSION

Geometries of Active Sites in Azurins.

Geometry optimizations were performed on the QM subsystems of WT azurin and the mutants in both reduced and oxidized states using the QM/MM-MFEP approach. The

convergence of relative free energies and the changes in the root of mean squared displacements (RMSD) of the structures are reported in Figure S1. The free energies of all systems converged to plateaus after 160–240 QM optimization steps in total (that is, 8–12 iteration cycles), showing a much lower computational cost on DFT calculations compared with direct QM/MM MD. It should be noted that the Cu-ligand bond lengths achieve their convergence more quickly than the relative free energies and the RMSD of the QM subsystem. At the last two iteration cycles, the values of these bond lengths are almost unchanged. The geometry differences between reduced and oxidized states are not significant for azurin and its mutants (see Figure S2), suggesting small inner-sphere contributions to the reorganization energies.⁹¹ The RMSD of the heavy atoms in all azurin proteins between the reduced and oxidized states are less than 0.1 Å. The main conformational change between the two redox states is the small variation in the distances between the copper ion and the five coordination ligands. As shown in Table 1 and Table S1, the coordination distances slightly shorten when the copper ions are oxidized because of the stronger interactions between the Cu(II) and the electron rich ligands, and the axial coordination bonds are more flexible than those in the equatorial position for all azurin proteins. The small geometry changes during the redox process allow us to perform a linear interpolation of the geometries for the intermediate FNE states so that we can smoothly connect the two redox states without generating severely strained structures or large energetic perturbations. The comparisons between our calculated Cu-ligand bond lengths and the available experimental results on the wild-type and N47S+M121L variants^{16,27,75} are shown in Table S2. First, the difference on all Cu-ligand bond lengths between the reduced and oxidized states in the wild type was reproduced well using QM/MM-MFEP. Second, the RMSDs of the heavy atoms in the binding center including the copper atom and five inner-sphere ligands between the optimized and experimental structures are 0.15–0.45 Å. Finally, the calculated Cu–N bond lengths are consistent with the experimental data, while the Cu–O bond length in Gly45 of N47S+M121L, the Cu–S bond length in Met121 of the wild type, and the Cu–S length in Cys112 of N47S+M121L were overestimated. On one hand, the computational errors, especially the limitation on the DFT treatment of open shell and transition metal systems,^{44,92} should be responsible for the structural deviations. On the other hand, the different conditions between the crystallography experiments and room temperature simulations should be considered carefully in the reproduction of protein structures.⁵⁸

Reduction Potentials of Azurins.

The calculation results of the reduction potentials of the azurin mutants are reported and compared with experimental measurements in Table 2. The absolute oxidation free energy $\Delta A_{\text{MFEP}}^{\text{abs}}$ for each protein was obtained following eqs 3 and 4. Eight independent FEP calculations were performed, and the computational results were averaged to generate the final value of oxidation free energy with the standard deviation for each protein. The absolute SHE potential is currently uncertain with a range from 4.20 to 4.84 V,⁵⁵ so the calculated standard reduction potential of the WT azurin is 0.56–1.20 V. In this paper, the value of E^{SHE} was selected as 4.28 V,⁹³ leading to the calculated result as 1.12 V. In comparison with the experimental value of about 0.3 V (0.265–0.341 V),^{23–25,27} our

prediction was over-estimated by 0.8 V. It has been known that the computational results of free energy difference with a change on the net charge are very sensitive to the simulation protocols.^{44,94,95} For example, the physical interface between the real system and the outside vacuum does not exist under periodic boundary conditions. The artificial periodicity may introduce the spurious finite size effects and uncertain electrostatic potential shifts in particular to the systems with a net charge. It is more complex that these errors depend not only on the redox metal or protein systems but also on the details of solvent models. On the other hand, the relative oxidation free energies are observed to be robust under various approximations on different descriptions on the surrounding environment.^{55,57,71} In order to study the effects of mutations, the relative reduction potential of each mutant was calculated using the reference of the WT azurin. As presented in the comparison of relative reduction potentials to experiments in Figure 2, our calculations generated relative reduction potentials with consistently low errors around 20 mV. The excellent accuracy can be mainly attributed to three factors.

First, the systems studied presently are single and double site mutants of azurin, which only involve the change in one to two residues near the active site. As shown in Figure S2, all of the seven mutants maintain very similar geometries of both the T1 copper sites and the overall protein structures, indicating the rigidity of the azurin proteins during the mutation and redox cycling. No significant conformational change is observed in the simulated process, which is supported by the experimental structural characterization of the mutants using UV-visible, electron spin resonance, and NMR spectroscopy.^{23–25} The geometric similarity among the systems significantly reduces the systematic error when we compare the relative reduction potentials.

Second, a large active site containing both the mutation residues and the T1 copper center was selected as the QM subsystem in our simulation, which requires efficient QM calculations without the loss of accuracy. The QM/MM-MFEP method developed in our group is one of the most appropriate computational tools for this goal. On one hand, direct QM/MM MD sampling beyond one nanosecond on such system with about 100 QM atoms is very expensive if not impossible. On the other hand, the inclusion of the mutation site in the QM calculations is crucial in order to capture the effects of mutated residues such as the Coulomb interactions, polarizations and complete responses to the changes in nuclear and electronic configurations because the accuracy of the conventional nonpolarizable force field on transition metal-containing systems is usually limited.⁹² For example, the QM ESP charges of the mutated groups were compared with CHARMM22 force field in Figure S3, which illustrate the significant difference. Although one can recalibrate the electrostatic parameters according to QM methods and adapt the parameters into the classical models to simulate redox processes, an appropriate charge scheme for deriving the partial charges, which plays an important role on redox calculations of proteins, is still under research.⁶¹ No such extra effort is required in our QM/MM-MFEP+FNE approach.

Third, the protein structures are solvated in a large water box under periodic boundary condition to obtain accurate long-range electrostatic interactions between explicit solvent molecules and redox center, which is vital to the reduction potentials and other energetic properties.^{55–57} In addition, the MM subsystem were sampled using MD for relatively long

time scale (1280 ps) to achieve better convergence. Combining the rigorous QM calculations of the active site at the highest level of accuracy we can afford with converged MD samplings of the MM subsystem at the nanosecond time scale, this approach eliminates the dependence of reduction potential on a specific protein conformation. Thus, the simulation results of the azurins using QM/MM-MFEP+FNE agreed with the experimental measurements with an excellent accuracy.

Understanding Reduction Potential Shifts.

The seven different mutants studied here involve three mutation sites, Met121, Met44, and Asn47, expanding the shifts of the reduction potential over a range of 350 mV. Although Met121 is considered as the inner-sphere ligand, the S_δ atom of Met121 binding to the copper ion was observed to be in a loose manner at a relatively large distance as 3.0 Å, making the coordination with little covalent character.^{16,25} The S_δ and O_{δ1} of Met44 and Asn47 in the outer-sphere are even further away from the copper center with the distance larger than 5.0 Å, incapable to form any direct covalent bond to the copper ion. Therefore, the major contribution from the mutations on the three sites originates from the nonbonded effects, mostly the electrostatic interactions. In the visualization of electron density of the redox centers shown in Figure S4, the localized HOMOs on copper and three equatorial ligands (His46, Cys112, and His117) suggest that none of the axial Met121 or outer-sphere residue as Met44 or Asn47 has significant covalent bonding with the copper ion, indicating that the various mutations impose mainly electrostatic influence perturbing the electronic structures of the T1 copper center. Without dramatic change on the covalent interaction between the mutated residue and the copper, one can expect that the effects of these mutations may be explored more efficiently at the beginning of the studies on redox proteins using classical models, such as a reparametrized MM force field⁶¹ or even a continuum electrostatics model with some empirical corrections.⁴⁰

We categorized the seven mutants into three types. The first type involves inner-sphere mutations on the residue Met121 at the axial ligand position, which is known to stabilize the Cu(I/II) ion.⁹⁶ The relationship between the hydrophobicity of the axial ligand (residue 121) and the reduction potential shifts has been observed experimentally,²⁷ which is consistent with our calculations. The two inner-sphere mutations M121Oxm and M121Q cause downshifts of the reduction potentials because they provide stronger electron donors that stabilize the oxidized state; that is, O_δ in Oxm and O_ε/N_ε in Gln compared with S_δ in Met in the axial ligand coordination. On the contrary, the lack of an electron donor in M121L makes the copper binding interaction weaker, which is more sensitive to the redox process and causes the upshift. The second type is the mutation to a residue with net charge in the outer sphere. The change on net charge in out-sphere residues can influence the redox properties on the copper binding site in different ways. The hydrogen bond between His35 and Met44, which affects the distance between His35 and the copper and thus contributed to the reduction potential,²⁹ can be disturbed by the protonated Lys44 in M44K mutation. Lys44 is partially exposed to the solvent in M44K mutant and may form a salt bridge with a nearby Asp11. Nevertheless, the hydrogen bond and salt bridge related to residue 44 are more than 6.0 Å away from the copper, posing only a small influence on the electrostatic field near the redox center and causing an upshift smaller than 100 mV in the reduction potential of the M44K

mutant. The third type involves the outer-sphere mutations between residues with the same net charge but different polarities. Based on the MM force field, Asn47 in the WT azurin has a strong polarity with the dipole moment of 7.4 D, while the Ser and Leu both have weaker polarities with dipole moments of 5.4 and 3.1 D, respectively. The less polar residues modulate the environmental electrostatic contributions to reduction potentials.²⁸ The rigidity of the copper binding site and the loop regions of the protein, which can be affected by hydrogen bonding interactions, was also found to be changed in N47S mutant.^{27,29} In addition, both Met44 and Asn47 have large contributions to the fluctuation of the electrostatic potential on the redox center, tuning reduction potentials through the potential fluctuation modes on the whole protein.³⁰ Interestingly, the 250 mV upshift of the reduction potential of the double mutant N47S+M121L is approximately the sum of the up-shifts we obtained in the single mutants N47S (140 mV) and M121L (120 mV). This indicates that the effects of individual mutations on different aspects are additive, confirming the observations in both experimental and theoretical works reported previously.^{27,40} The dominant classical effects and the additive structural features suggest that rational tuning of the reduction potentials of T1 copper proteins as a single protein redox ruler is possible through the mutagenesis on axial ligand in the inner-sphere and on residues with different polarities or net charges in the outer-sphere.

It has been observed in experiments that the mutants of azurins span a much wider range of reduction potentials from -1 to 1 V.²⁶ Our calculations explored only a smaller range of reduction potentials from -100 to 250 mV. We concentrate our present study on type 1 copper proteins and leave the study on other variants such as type zero that are constructed without thiolate ligation^{97,98} as our future work. The accurate and efficient QM/MM calculation on some other variants is still challenging because of some limitations on our current QM/MM-MFEP+FNE scheme. First, the perturbation on the key His-Cys-His residues, e.g., the C112D+M121L mutate,⁹⁷ results in dramatic changes on the electronic and geometric structures of the copper binding site and thus introduces uncertainty to protein simulations when relative reduction potentials are studied. It may also require a sophisticated DFT functional and the optimal basis set choice for accurate ab initio QM calculations to describe new behaviors in chemistry. Second, the multiple site mutants such as HPaz²⁶ indicate a much larger size of the QM subsystem and much more expensive QM/MM computations. Furthermore, the inclusion of several out-sphere mutant residues in the QM subsystem always leads to multiple QM regions that are at a large distance and surrounded by an MM environment represented as background point charges. The combination with advanced computational techniques such as the density-fragment interaction approach^{56,99,100} or the double QM/MM method^{101–104} is necessary to address the issue.

Reorganization Energies of Azurins.

It has been observed that the inner-sphere contribution to the reorganization energy is about 0.10 – 0.15 eV for WT azurin, which is only a small component of the overall reorganization energy.^{58,105} This is also consistent with our observation that the structural changes of the active sites of azurins are smaller than 0.1 Å in RMSD during the redox processes. Although direct calculations of the inner-sphere reorganization energies are not available in the current

implementation of QM/MM-MFEP method, the outer-sphere contribution, which is the major part of the total reorganization energy for our systems, can be estimated from QM/MM interactions using eqs 5 and 6. The calculation results of λ_{out} were reported in Table 1. In order to make direct comparison to experiments, some empirical corrections for the finite system size effect and nonpolarizable solvent model should be further considered. First, even though a large water box of $90 \text{ \AA} \times 90 \text{ \AA} \times 90 \text{ \AA}$ was used in our simulations, the reorganization energies were roughly underestimated by about 0.1 eV because of the finite system size effect.^{106,107} Second, the outer-sphere reorganization is known to be overestimated using a nonpolarizable TIP3P water model,^{49,107} which requires a rescaling coefficient of 0.6 in our calculations.⁵⁶ Finally, the inner-sphere corrections of 0.1 eV was added to obtain the final estimation of the total reorganization energies. As listed in Table 1, the total reorganization energy of WT azurin was estimated to be 0.7 eV, which agrees well with experimental values of 0.55–0.80 eV.^{17,18,108}

CONCLUSION

In this work, we have investigated the redox properties of T1 copper metalloproteins using the QM/MM-MFEP+FNE method developed in our group, which accurately and efficiently describes the electronic structures of the ET center and the atomic contributions of the heterogeneous environment. The MD sampling generated well converged ensembles of the protein conformations and eliminated the dependence of the results on specific protein or solvent configuration. Based on the ensembles, the optimization of the active site on the PMF avoids the expensive direct QM/MM MD simulations thus enables us to efficiently handle large active sites of more than 100 atoms using DFT method with large basis sets. In the simulations of WT azurin and its seven different mutants, our method accurately reproduced the experimental measurements of the relative reduction potentials for three different types of mutations with the errors around 20 mV, providing an efficient approach to study the reduction potential dependence on the mutations in metalloproteins. Our results showed that the structural effects introduced by mutations in axial ligand and outer-sphere sites are mainly electrostatic and additive, suggesting that the rational tuning of reduction potentials of metalloproteins is possible in the design of electrocatalysts for oxygen reduction and water oxidation in artificial photosynthesis. The outer-sphere reorganization energy, which contributes the major component to the total reorganization energy of each azurin protein, was captured by the MM ensemble sampled in the MD simulations and reconstructed based on the QM/MM interactions. Since the conformational sampling of the QM subsystem is avoided, this method as currently implemented cannot directly compute the inner-sphere contribution to the overall reorganization energy. In the case of azurins, the active sites undergo only subtle changes in the redox processes and the inner-sphere contributions were observed to be small. In other situations where inner-sphere geometry changes are more significant during redox processes, conformational sampling of QM subsystem is still necessary to address the inner-sphere components of the reorganization energies.

Our computational approach needs input of the protein structure but requires no *ad hoc* parameters. All the electronic structure and the protein and solvent configuration responses are captured with first-principles QM/MM method, so are all the electrostatic interactions

and ligand coordination. Therefore, it is highly transferable among different systems. Built on the basis of well-established MM force fields and rigorous ab initio QM methods, our QM/MM-MFEP+FNE approach, as demonstrated here, should provide a platform generally applicable to the studies of electron transfer problems in metalloproteins and other complex biochemical systems.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Structures of WT azurin from X-ray crystallography. (A) Ribbon diagram of WT azurin with active site highlighted as bond diagram. (B) QM region of WT azurin, including the active site with copper ion shown in orange, five residues in the first coordination shell (45, 46, 112, 117, and 121), and the two additional mutation residues (44 and 47).

Figure 2.

Comparison of the calculated and experimental relative differences of the reduction potentials of azurins with the reference of WT azurin set to be zero. Error bars are the standard deviations of multiple independent FEP calculations, which represent the magnitude of the statistical errors in the simulations.

Table 1.

Outer-Sphere Reorganization Energies (eV) and Variations in Coordination Bond Lengths (Å) of Azurins in Simulations

mutant	$\Delta\bar{x}_{\text{eq}}^a$	$\Delta\bar{x}_{\text{ax}}^b$	λ_{out}^c	λ^d
WT	0.05	0.16	0.9	0.7
M44K	0.07	0.18	0.8	0.6
N47L	0.06	0.13	1.0	0.8
N47S	0.07	0.15	1.1	0.8
M121Q	0.05	0.14	0.8	0.6
M121L	0.05	0.18	0.7	0.6
M121Oxm	0.05	0.07	0.7	0.6
N47S+M121L	0.10	0.17	0.8	0.6

^a Average variations in equatorial coordination bonds.

^b Average variations in axial coordination bonds.

^c Outer-sphere reorganization energies calculated from QM/MM interactions.

^d Total reorganization energies estimated with corrections for finite system size effects, nonpolarizable solvent, and inner-sphere contributions.

Table 2. Comparison of the Reduction Potentials (V) of WT Azurin and Its Mutants Obtained from Experiments and Simulations

mutant	$\Delta A_{\text{MFEP}}^{\text{abs}}$	a	E_{MFEP}^0	b	E_{Expt}^0	c	d	ΔE_{MFEP}	ΔE_{Expt}^0	error
WT	5.40		1.12		0.265–0.341 ^{e,h}					
M44K	5.48		1.20		0.371 (0.315) ^e		0.08	0.056	0.056	0.02
N47L	5.57		1.29		0.396 (0.286) ^f		0.17	0.110	0.110	0.06
N47S	5.54		1.26		0.396 (0.265) ^g		0.14	0.131	0.131	0.01
M121Q	5.33		1.05		0.190 (0.265) ^g		–0.07	–0.075	–0.075	0.01
M121L	5.52		1.24		0.358 (0.265) ^g		0.12	0.093	0.093	0.03
M121Oxm	5.30		1.02		0.222 (0.341) ^h		–0.10	–0.119	–0.119	0.02
N47S+M121L	5.65		1.37		0.496 (0.265) ^g		0.25	0.231	0.231	0.02

^a Absolute oxidation free energies (eV) using QM/MM-MFEP+FNE.

^b Standard reduction potentials estimated based on the absolute oxidation free energies with respect to that of the standard hydrogen electrode (4.28 V).⁹³

^c Experimental values of standard reduction potentials with the values of WT azurin listed as references in parentheses. Because the experimental values are reported in different works, the values of WT azurin are slightly different. The values from the same work were always used together to calculate the relative reduction potentials ΔE_{Expt}^0 .

^d The values for N47L and the corresponding WT were obtained from the experimental data on *Alcaligenes Denitrificans* azurin. Other values were from *Pseudomonas aeruginosa* azurin.

^e Reference 24.

^f Reference 23.

^g Reference 27.

^h Reference 25.