THEORETICAL STUDIES OF
IRON-SULFUR ELECTRON
TRANSFER PROTEINS

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To the Faculty of Washington State University:

The members of Committee appointed to examine the thesis of ELIZABETH
ASTILL DOLAN find it satisfactory and recommend that it be accepted.

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Chair

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THEORETICAL STUDIES OF
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Abstract

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Electron transfer proteins transfer electrons through the electron transport chains necessary for respiration, photosynthesis, and nitrogen fixation, and are thus ubiquitous to all life. These reactions utilize oxidation-reduction chemistry, where the free energy of the reaction can be related to the reduction potential of each metal redox site in the chain. The high efficiency of these reactions, thought to be ~70% under physiological conditions, is partially due to the ability of the environment around the redox site to affect the reduction potential. The environment surrounding the redox site includes the aqueous solvent as well as the protein matrix. Thus, the protein matrix of an electron transfer protein can affect the reduction potential and the rate of the reaction.

In this work, we focus on a single class of electron transfer proteins, namely, the iron-sulfur proteins, which appear in a wide range of biological reactions. Classical molecular mechanical computational methods are used to analyze the structure and energetics of two types of iron-sulfur electron transfer proteins, namely, rubredoxins and ferredoxins. Here, two levels of interaction between the protein matrix and the reduction potential are explored. First, the protein matrix can create large differences in reduction potential (on the order of 1 V) due to the inhomogeneous protein partially surrounding the redox site. Second, specific residues can be used to fine-tune the reduction potential
(on the order of 100 mV). Three different questions about the role of protein structure in influencing electron transfer are studied here. First, how does the entire protein matrix structure influence electron transfer reactions? Second, how do specific amino acids affect the reduction potential of the redox site? Finally, what are the thermodynamic properties of an electron transfer reaction, and how does the protein matrix contribute to these properties
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACKNOWLEDGMENTS</td>
<td>iii</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>iv</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>viii</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>ix</td>
</tr>
<tr>
<td><strong>CHAPTER</strong></td>
<td></td>
</tr>
<tr>
<td>1. INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>References</td>
<td>8</td>
</tr>
<tr>
<td>2. PROTEIN CONTROL OF ELECTRON TRANSFER RATES VIA POLARIZATION: MOLECULAR DYNAMICS STUDIES OF RUBREDOXIN</td>
<td>14</td>
</tr>
<tr>
<td>Introduction</td>
<td>17</td>
</tr>
<tr>
<td>Methods</td>
<td>19</td>
</tr>
<tr>
<td>Results</td>
<td>24</td>
</tr>
<tr>
<td>Conclusion</td>
<td>30</td>
</tr>
<tr>
<td>References</td>
<td>31</td>
</tr>
<tr>
<td>3. THE EFFECT OF MUTATIONS AT THE CYSX POSITION ON THE REDUCTION POTENTIAL OF FERREDOXIN: A MOLECULAR DYNAMICS STUDY</td>
<td>39</td>
</tr>
<tr>
<td>Introduction</td>
<td>41</td>
</tr>
<tr>
<td>Methods</td>
<td>45</td>
</tr>
<tr>
<td>Results</td>
<td>49</td>
</tr>
<tr>
<td>Conclusion</td>
<td>55</td>
</tr>
<tr>
<td>References</td>
<td>58</td>
</tr>
</tbody>
</table>
4. PROTEIN CONTROL OF ELECTRON TRANSFER IN
FERREDOXIN: A MOLECULAR DYNAMICS STUDY..............................70
Introduction.......................................................................................................72
Methods.............................................................................................................75
Results...............................................................................................................79
Conclusion.........................................................................................................82
References.........................................................................................................84
5. CONCLUSIONS..............................................................................................96
## LIST OF TABLES

2-1 Averages and fluctuations of the environmental potential ........................................34
2-2 Measures of linearity and dielectric response ..........................................................35
2-3 Decomposition of polarization .................................................................................35
3-1 Calculation of the root mean square deviation (RMSD) of $C_\alpha$ ..................62
3-2 Angles and distances of residues at the Cys$^\alpha$ position in the average MD structures ..........................................................63
3-3 Measures of the electrostatic potential between the residue at the Cys$^\alpha$ position and the redox site relative to alanine ......................................................64
4-1 Norm of the residuals for parabolic least squares fit to $\Delta G^\alpha$ data ..............88
4-2 Thermodynamic parameters for the electron transfer reaction with a calculated driving force ..........................................................................................88
4-3 Thermodynamic parameters for the electron transfer reaction assuming the driving force is zero .................................................................88
LIST OF FIGURES

2-1 A schematic representation of potential energy curves for the electron transfer reaction D+A→D^+A^- .................................................................37

2-2 A schematic representation of the effects of the environment of the electron acceptor for an electron transfer reaction .............................................38

3-1 Ribbon diagram of Peptostreptococcus asaccharolyticus ferredoxin........66

3-2 Superposition of cysteine, serine and alanine at the Cys^3 position from crystal structures (a), MD simulations at residue 22 in Pa Fd (b), and MD simulations at residue 50 in Pa Fd (c) .................................................68

3-3 Polar plots of histograms for the three conformations of both cysteine (a) and serine (b). over the 1 ns MD simulation .........................69

4-1 Ribbon diagram of Clostridium acidurici ferredoxin .................................................................90

4-2 A schematic representation of potential energy curves for the electron transfer reaction D+A→D^+A^- .................................................................91

4-3 Data plotted from histograms calculated into free energy surfaces .................................................................................................................92

4-4 Data plotted from histograms into free energy surfaces with the product state displaced by the driving force (ΔG°) .................................94
Dedication

Goodbye, and thanks for all the fish.
CHAPTER ONE

INTRODUCTION

In 1789, Antoine Lavoisier, the father of modern chemistry, elucidated the role of oxygen in biological combustion and wrote that “respiration is nothing but a slow combustion of carbon and hydrogen, which is entirely similar to that which occurs in a lamp or lighted candle...”. In biological energetics, the consumption of carbon and hydrogen, and the less common consumption of nitrogen, is catalyzed by intracellular enzymes. In these processes, the transport of electrons occurs through the sequential oxidation-reduction of a series of redox centers, resulting in the concentration of protons on one side of the intracellular membrane. This process creates an electrochemical gradient, which stores cellular energy. Therefore, electron transfer proteins are used to manipulate and direct the energy needs of all organisms, and are ubiquitous to all life. The electron transfer process is highly efficient, thought to be ~70% under physiological conditions in active mitochondria (Voet and Voet, 1995). In comparison, the energy efficiency of a typical automobile engine is less than 30%.

The high efficiency of biological electron transfer reactions is of great interest, both for applications in bioengineering and for a greater understanding of basic scientific principles. The protein itself can influence the electron transport process in a variety of ways. The protein matrix can act as a scaffold on which a redox site can be constructed, so that the redox site is limited to specific conformations and orientations, which affect the energetics of electron transfer. The protein can also create binding sites for specific electron transfer partners and thus direct the flow of electrons as is in the mitochondrial and photosynthetic electron transport chains. Finally, and perhaps most importantly, the protein can alter the environment around the redox site, which can affect the kinetic properties of electron transport, including the reaction rate and the driving force.
Therefore, understanding the structural factors that influence the rate of the reaction as well as the reduction potential is of great interest.

The reduction potential, $E$, describes the affinity that an atom or molecule has for an electron in solution, and can thus be related to both the driving force and rates for electron transfer processes. The effect of the protein on the reduction potential can be demonstrated in that identical redox sites in different proteins can have vastly different reduction potentials, even within families of related proteins (Cammack, 1992). Understanding the relationship between the structure of electron transfer proteins and their ability to regulate the reduction potential of the redox reaction is thus of great interest.

In this work, we focus on a single class of electron transfer proteins, namely, the iron-sulfur proteins. Iron-sulfur proteins are an important class of electron transfer proteins because they appear in a wide range of biological reactions, and are ubiquitous in nature (Armstrong, 1982; Cammack, 1992; Matsubara and Saeki, 1992). Here, classical molecular mechanical computational methods are used to analyze the structure and energetics of two types of iron-sulfur electron transfer proteins, namely, rubredoxins and ferredoxins. These are small (MW 6-12 kDa) bacterial proteins, which act as general electron transfer proteins. The rubredoxins have a simple redox site composed of a single iron ligated to the protein matrix by four cysteinyl residues. Rubredoxins have reduction potentials that generally lie between –50 and 6 mV (Adams, 1992; Lovenberg and Sobel, 1965; Moura et al., 1979; Sieker et al., 1986) and have redox sites with a $-1/-2$ redox couple. Ferredoxins have a more complex redox site comprised of a [4Fe-4S] cubane structure ligated to the protein matrix by four cysteinyl ligands, and a redox couple of $-2/-3$ with reduction potentials between –650 and 0 mV (Cammack, 1992).

All of the approaches in this work are based on the principle that the reduction potential can be related to the electrostatic interactions between the environment (which includes the protein and the aqueous solution) and the redox site. More specifically, the
free energy of reduction, $\Delta G$ is related to the reduction potential, $\mathcal{E}$, by the well known Nernst equation

$$\Delta nF \mathcal{E} = \Delta G = \Delta E + \Delta PV - T \Delta S$$

(1)

where $F$ is Faraday’s constant, $n$ is the number of electrons transferred in the reaction, $\Delta E$ is the change in energy, $\Delta PV$ is the change in system pressure and volume, $T$ is the absolute temperature, and $\Delta S$ is the change in entropy. Therefore, the reduction potential measures the energy of a change in charge. This energy contains both classical and quantum components, where

$$\Delta E = \Delta E_{classical} + \Delta E_{quantum}$$

(2)

The classical component is mainly electrostatic and thus can be calculated via classical Coulombic electrostatics. Electron transfer is influenced by the protein matrix on two levels. First, the matrix can create large differences (on the order of 1 V) due to the inhomogeneous protein partially surrounding the redox site. Second, specific residues can be used to fine-tune the reduction potential (on the order of 100 mV). Three different questions about the role of protein structure in influencing electron transfer are studied here. First, how does the entire protein matrix structure influence electron transfer reactions? Second, how do specific amino acids affect the reduction potential of the redox site? Finally, what are the properties of an electron transfer reaction, and how does the protein matrix contribute to these properties?

**Structural Basis for the Reduction Potential**

The first set of studies focuses on the nature of the protein matrix that surrounds the redox site, and how this matrix influences the electron transfer properties of the protein. Several studies have shown that the reduction potentials of electron transfer proteins are considerably different from their redox site analogs (Harbury et al., 1965; Lane et al., 1977; Hill et al., 1977; Ueyama et al., 1985; Werth et al., 1989), with differences as large as 800 mV seen between a redox site analog and *Pyrococcus furiosus* rubredoxin (Lane et
Moreover, homologous proteins from different species can have different redox potentials with ranges that span over 400 mV (Moura et al., 1979; Meyer et al., 1983).

Many theoretical studies have attempted to understand the molecular basis for reduction potentials in proteins with a variety of methods (Kassner and Yang, 1973; Moore, 1983; Rees, 1985; Moore et al., 1986; Rogers et al., 1985; Gunner et al., 1996; Gane et al., 1995; Churg and Warshel, 1986; Cutler et al., 1989; Langen et al., 1992a; Langen et al., 1992b; Jensen et al., 1994; Shenoy and Ichiye, 1993; Yelle et al., 1995; Swartz et al., 1996; Swartz, 1996; Dolan et al., 2003). Molecular dynamics simulations have an advantage over other methods such as Poisson electrostatic calculations or the protein dipoles Langevin dipoles (PDLP) method because the atoms and molecules are allowed to move and thus the influence of structural changes can be studied.

An obvious way that an electron transfer protein can alter the environment around a redox site is by excluding solvent, or water, from the redox site, thus providing a low dielectric environment. However, the question arises as to how the protein provides sufficient driving force to allow fast electron transfer while providing this low dielectric environment. The answer may lie in the observation that many proteins appear to have highly polarized environments for their redox sites (Yelle et al., 1995; Swartz et al., 1996; Gunner et al., 1996; Gunner et al., 2000), which would influence the reduction potential and thus the driving force. It has not been demonstrated whether the degree of polarization is simply the amount expected by introducing a charge equivalent to that of the redox site into a protein matrix that has no intrinsic polarization without the redox site. If the degree of polarization is greater than expected this would indicate that the protein is organized to be hyper-polarized around the redox site. Hyper-polarization would result in a better electron acceptor than a redox site analog in a hypothetical solvent of dielectric response equivalent to that of the protein. In Chapter 2, we have found that rubredoxin is highly polarized around the redox site; more so than could be
expected based on the net charge of the redox site. The redox site in these proteins is therefore better able to accept an electron from a donor site than a redox site analog in a solvent with a dielectric constant equivalent to that of the protein. Here, measures of deviations of the degree of polarization, or environmental electrostatic potential, from a simple linear dielectric response are proposed. In addition, a decomposition of the polarization is proposed to describe the apparent deviations from linearity, in which the polarization is divided into a “permanent” component that is independent of the redox site charge and a dielectric component that linearly responds to the charge. The nonlinearity measures and the decomposition were calculated for Clostridium pasteurianum rubredoxin from molecular dynamics simulations. The results presented here demonstrate that rubredoxin has a positive polarization of the protein environment around the redox site created mainly by the polar backbone. In addition, rubredoxin appears to have a permanent component to this polarization persisting even as the charge of the site goes to zero. Finally, rubredoxin has a relatively constant dielectric response.

Predicting Changes in the Reduction Potential

Small changes in the reduction potential of the redox site can be made by the specific sequence of a given protein. Specifically, the reduction potential of an electron transfer protein can be fine-tuned through single site mutations, thus improving the efficiency of the electron transfer reaction. In Chapter 3, we investigate a sequence position that appears to affect the reduction potential by about 50 to 200 mV, depending on whether a cysteine, alanine, or serine is present. In previous work, this so-called sequence determinant, which we refer to as the Cys\(^+\) position (Beck et al., 2000; Xie et al., 2000; Xie and Ichiye, 2000), was found in a series of ferredoxins. In this previous investigation, it was postulated that this cysteine formed a putative hydrogen bond with the redox site sulfurs in ferredoxin, which stabilized a conformation that contributed to a lower reduction potential than if an alanine were in the same position. Additionally,
serine, although similar to cysteine in many ways, has a different dipole orientation and therefore forms putative hydrogen bonds with different sulfurs in the redox site of ferredoxin. This results in a higher electrostatic interaction between serine and the redox site and thus a higher reduction potential when compared to alanine. In Chapter 3, molecular dynamics simulations are used to test these predictions by mutating the native residues (cysteine and alanine) at the two Cys positions in Peptostreptococcus asaccharolyticus ferredoxin (Pa Fd) and calculating behavior of the protein when cysteine, alanine, and serine are alternatively present. The results of the electrostatic interactions indicate that by changing between these three residues, the reduction potential can be predictably changed by 50 to 200 mV in either direction.

The Molecular Basis for Electron Transfer Rates
Proteins in the electron transport chain transfer electrons over large distances (>10 Å) at very fast rates (>10⁶ s⁻¹)(Bertini et al., 1992; Kyritsis et al., 1997; Voet and Voet, 1995). This rate, k, of an electron transfer reaction is given by

\[ k = C \exp \left( \frac{D_G^\ddagger}{RT} \right) \]  

where \( C \) is the transmission coefficient (the averaged transition probability for electron transfer), \( C \) is the collision frequency, and \( D_G^\ddagger \) is the free energy of activation (Marcus and Sutin, 1985). The framework for current theories of electron transfer rates was developed by Marcus (Marcus and Sutin, 1985) and Levich (Levich, 1966). Marcus theory assumes that the free energy as a function of a given reaction coordinate is parabolic, which leads to a simple relationship between the activation free energy \( D_G^\ddagger \), the solvent reorganization, \( \Delta \), and the driving force, \( D_G^\circ \), where,

\[ D_G^\ddagger = \frac{\left( \Delta + D_G^\circ \right)^2}{4 \Delta} \]  

The reorganization energy, \( \Delta \), is the energy released when the solvent relaxes from the equilibrium configuration of the reactants to the equilibrium configuration of the
products, as the charge changes from the reactant state to the product state. Therefore, this term describes the environment of the redox site, which includes the protein matrix. Thus, it is of importance to understand how the environment of a protein influences the rate of electron transfer.

Warshel and co-workers have developed methods for the calculation of free energy curves from computer simulations (Warshel, 1982; Churg et al., 1983; Hwang, 1987; Creighton et al., 1988; King, 1990). Similar methods have been used by others to study various systems (Kuharski et al., 1988; Marchi et al., 1993; Yelle and Ichiye, 1997; Yelle and Ichiye, 1999; Yelle, 1996). Ferredoxins typically have two redox sites in each protein separated by a distance of ~12 Å, and have been shown to transfer electrons between these two sites at high rates ($10^6$-$10^7$ s$^{-1}$) (Kyritsis et al., 1997). Studying the transfer of electrons between these two sites is advantageous because the donor and acceptor are already bound, therefore effects of orientation do not need to be considered (Cusanovich, 1991; Mauk, 1999), and the distance between the donor and acceptor complex is known.

In Chapter 4, we investigate the transfer characteristics of *Clostridium acidurici* ferredoxin (Ca Fd), a well-characterized protein with a high-resolution crystal structure available in the Protein Data Bank (Dauter et al., 1997). The free energy curves of the reaction are calculated using the electrostatic potential energy from molecular dynamics simulations as the reaction coordinate, and the activation energy, reorganization energy, and driving forces for the reaction are investigated. Ca Fd is known to transfer electrons at high rates (Kyritsis et al., 1997); however, the reduction potentials of the redox sites are almost identical (~420 mV) (Backes et al., 1991). This work helps to explain how the protein maintains this high rate of transfer with similar reduction potentials, which is of great interest in understanding electron transfer properties in general.
References


CHAPTER TWO

Protein Control of Electron Transfer Rates Via Polarization:
Molecular Dynamics Studies of Rubredoxin

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ATTRIBUTION

The additional authors contributed to this work in the following manner. Robert B. Yelle and Brian W. Beck developed much of the initial theory, and performed some previous molecular dynamics simulations. Justin T. Fischer helped perform some of the molecular dynamics simulations for this work.
ABSTRACT

The protein matrix of an electron transfer protein creates an electrostatic environment for its redox site, which influences its electron transfer properties. Our studies of Fe-S proteins indicate that the protein is highly polarized around the redox site. Here, measures of deviations of the degree of polarization, or environmental electrostatic potential, from a simple linear dielectric response are proposed. In addition, a decomposition of the polarization is proposed here to describe the apparent deviations from linearity, in which the polarization is divided into a “permanent” component that is independent of the redox site charge and a dielectric component that linearly responds to the charge. The nonlinearity measures and the decomposition were calculated for Clostridium pasteurianum rubredoxin from molecular dynamics simulations. The polarization in rubredoxin is greater than expected from linear response theory, which implies it is a better electron acceptor than a redox site analog in a solvent with a dielectric constant equivalent to that of the protein. In addition, the polarization in rubredoxin is described well by a permanent polarization plus a linear response component. This permanent polarization allows the protein matrix to create a favorable driving force with a low activation barrier for accepting electrons. The results here also suggest that the reduction potential of rubredoxin is determined mainly by the backbone and not the side chains, and that the redox site charge of rubredoxin may help to direct its folding.
INTRODUCTION

One of the most intriguing questions in the study of biological electron transfer is how proteins are able to facilitate the long-range electron transfer that occurs in biological systems. Obvious ways are by having recognition sites for specific donors and/or acceptors and by forming complexes, which are both ways of facilitating the optimal formation of the donor-acceptor system. Once the donor-acceptor complex is formed, both the electronic coupling and the nuclear reorganization of the redox site and its environment are important (Devault, 1980; Moser et al., 1992; Gray and Ellis Jr., 1994). Focusing on the latter, the protein provides a certain electrostatic environment for the redox site and changes in the protein matrix can affect the driving force of an electron-transfer reaction (Ichiye, 1999). Although the driving force for the electron transfer reaction can be adjusted by using different co-factors, the substitution of different metals or co-factors does not appear to be a simple way for nature to change reduction potentials. Marcus theory (Marcus and Sutin, 1985) for electron transfer assumes that the activation energy for the electron transfer process is dependent on the energy required to reorganize the environment surrounding the electron donor and acceptor between the reactant and the product states (Fig. 1). This energy is due in large part to the polarization of the environment (i.e., the protein matrix and solvent) around the donor and acceptor redox sites caused by the electrostatic interaction between the redox site charges and the environment.

The environmental response of the protein matrix surrounding the protein redox sites can be compared with the environmental response of the solvent surrounding simple ions. In the case of simple ions in solution, the solvent can freely reorganize around the ions upon electron transfer between two ions, which is a dielectric response that depends on both the polarity of the solvent molecules and the degree of coupling between the molecules. The large reorganization in a high dielectric solvent such as water gives rise to a large activation energy (Fig. 2A), whereas the small reorganization in a low
dielectric solvent gives rise to a small activation energy (Fig. 2 B). On the other hand, the protein matrix around the redox site in a protein cannot reorganize freely due to the restraints of the backbone (Fig. 2 C). Calculations also indicate a relatively low reorganization energy of the protein for rubredoxin (Ichiye et al., 1995; Yelle et al., 1995), cytochrome c (Churg et al., 1983) and the photosynthetic reaction center (Creighton et al., 1988). This implies that the protein is a low dielectric medium. Thus, an electron transfer protein can enhance electron transfer by providing a low dielectric environment for the donor-acceptor complex, which reduces the reorganization energy and thus the activation energy for electron transfer. However, in the case of simple ions in solution, the large solvation energy of a high dielectric solvent such as water can provide a strong driving force for the reaction (Fig. 2 A), whereas a low dielectric solvent provides a much weaker driving force (Fig. 2 B). Since the protein matrix is a low dielectric medium, the question arises as to how the protein provides sufficient driving force to allow fast electron transfer. The answer may lie in the observation that many proteins appear to have highly polarized environments for their redox sites (Yelle et al., 1995; Swartz et al., 1996; Gunner et al., 1996; Gunner et al., 2000), which would influence the reduction potential and thus the driving force. It has not been demonstrated whether the degree of polarization is simply the amount expected by introducing a charge equivalent to that of the redox site into a protein matrix that has no intrinsic polarization without the redox site. If the degree of polarization is greater than expected this would indicate that the protein is organized to be hyper-polarized around the redox site. Hyper-polarization would result in a better electron acceptor than a redox site analog in a hypothetical solvent of dielectric response equivalent to that of the protein.

The comparison of proteins to simple ions in solution is especially useful because the polarization energy for a simple ion in a dielectric continuum can be simply described by the Born solvation energy. This linear response theory assumes no permanent polarization. Furthermore, relationships can be made between the polarization, the
fluctuations in the polarization, and the dielectric response (Yelle and Ichiye, 1997). Thus, deviations from the Born linear response picture are indications that the protein matrix has more complex behavior than a simple dielectric continuum.

In this work, equations are developed that describe deviations of the polarization response of any media, including a protein, from a linear response, which are based on the Born model for a simple ion in a dielectric continuum. First, measures are developed that indicate apparent deviations from linear response. These measures are used to determine the degree of the apparent deviations in the protein rubredoxin using molecular dynamics simulations. Next, since the simplest explanation of a deviation from a simple Born linear response is the addition of a permanent polarization, a decomposition of the polarization into a permanent and a dielectric component is also developed. This decomposition is used to determine the degree of permanent polarization in rubredoxin from the molecular dynamics simulations.

**METHODS**

**Theory**

Here, the energetics of a simple ion in a dielectric continuum followed by that of a protein in solution are reviewed first. Next, the measures of nonlinearity followed by the decomposition are defined.

The energetics of an ion of charge $q$ and radius $R$ in a dielectric continuum with dielectric constant $\epsilon$ is described by the Born solvation free energy

$$G_q = \frac{q^2}{2R} \left( \frac{1}{\epsilon} - \frac{1}{\epsilon_0} \right)$$

(1)

which is due to the polarization of the solvent environment by the charge of the ion. Furthermore, it can be shown that the average solvation energy $\langle V_q \rangle$ is given by

$$\langle V_q \rangle^{(LR)} = \frac{q^2}{R} \left( \frac{1}{\epsilon} - \frac{1}{\epsilon_0} \right)$$

(2a)

and the average solvation potential $\langle f_q \rangle$ is given by (Hyun, 1996)
\[
\langle q_i^{(LR)} \rangle = \frac{q}{R} \sum_{j=env} \frac{1}{r_{ij}}
\]  

(2b)

Thus, the Born model is a linear response model, as indicated by the superscript “(LR).” Finally, assuming linear response at temperature \( T \) with \( \frac{1}{[\varepsilon]} = 1/k_BT \) where \( k_B \) is Boltzmann’s constant, the relationship between the fluctuations in the solvation energy and the dielectric constant \( \varepsilon \) is given by (Yelle and Ichiye, 1997; Ichiye, 1996)

\[
\langle \delta V^2_{q}^{(LR)} \rangle = \frac{q^2}{R} \sum_{j=env} \frac{1}{r_{ij}}
\]  

(3a)

where \( \delta V = V_{redox} - V_{env} \) and the relationship between the fluctuations in the solvation potential and the dielectric constant is given by (Yelle and Ichiye, 1997)

\[
\langle \delta \varepsilon_q^{(LR)} \rangle = \frac{1}{R} \sum_{j=env} \frac{1}{r_{ij}}
\]  

(3b)

where \( \delta \varepsilon = \varepsilon_{redox} - \varepsilon_{env} \). Thus, in the linear response model, the averages and the fluctuations are not independent quantities and can be related by Eq. 2 and 3.

The energetics of polarization can also be defined when the entire system is described at a molecular level. The redox site can be defined as multiple atoms or sites that all undergo a change in charge upon oxidation or reduction. Also, the environment, which can consist of solvent alone or protein (for example) plus solvent, is also defined in terms of individual atoms or sites. For such cases, an environmental potential energy \( V_q \) is defined as

\[
V_q = \sum_{i=redox \ site} \sum_{j=env} \frac{q_i q_j}{r_{ij}}
\]  

(4a)

and an environmental potential \( \varepsilon \) is defined as

\[
\varepsilon V_q = \frac{1}{n} \sum_{i=redox \ site} \sum_{j=env} \frac{q_i q_j}{r_{ij}}
\]  

(4b)

where \( r_{ij} \) is the distance between atoms \( i \) and \( j \), \( q_i \) are the changes in charge of atom \( i \) as the redox site is reduced, \( n \) is the number of electrons in the reduction (i.e., \( n=1 \) for a one-electron reduction) and \( e \) is the magnitude of the electron charge. The summation over \( i \) is carried out over atoms of the redox site and that over \( j \) is carried out over atoms of the rest of the environment. To get average environmental potential energies \( \langle V_q \rangle \) and
average environmental potentials \( \mathcal{U}_\alpha \) the quantities in Eq. 4a and 4b can be calculated from molecular dynamics simulations and then averaged over time or from Monte Carlo simulations and then averaged over configurations.

Here, three measures of the linearity of the response of the environment are defined. These measures utilize the averages and fluctuations of the environmental potential (Eq. 4b) or potential energy (Eq. 4a) from molecular dynamics or Monte Carlo simulations. Moreover, since these numbers can also be predicted from the Born model (Eq. 2a,b and 3a,b), this allows comparison to the “ideal” case of an ion as a simple linear response solvent with an equivalent \( \mathcal{U} \) assuming that \( R \) is constant. Only the environmental potential and not the environmental potential energy is examined here because of the direct relevance to Marcus theory; results for the environmental potential energy are very similar.

First, a measure of whether the degree of polarization of the charge is consistent with the dielectric response is defined here by

\[
\mathcal{D}_q = \frac{\langle f_q \rangle}{\mathcal{Q} \langle \mathcal{Q} \rangle}
\]  

From Eq. 2b and 3b, \( \mathcal{D}_q < 1 \) implies that the polarization is less than expected for a medium with a dielectric response constant with the fluctuations, while \( \mathcal{D}_q > 1 \) implies that it is more than expected. The second two measures provide a means of understanding deviations of \( \mathcal{D}_q \) from 1. A measure of the apparent linearity of the polarization response of a medium is defined here by comparing the environmental potentials for two different charge states \( q \) and \( q' \)

\[
\mathcal{D}_{qq'}^{(1)} = \frac{\langle f_{q'} \rangle}{\langle f_q \rangle}
\]  

From Eq. 2b, if \( |q| < |q'| \) \( \mathcal{D}_{qq'}^{(1)} < 1 \) implies that the polarization is increasing slower than a purely linear response, while \( \mathcal{D}_{qq'}^{(1)} > 1 \) implies that it is increasing faster than a purely linear response. In addition, a measure of the consistency of the dielectric response is
defined here by comparing the fluctuations in the environmental potentials for two
different charge states $q$ and $q'$

$$\mathcal{D}_{qq'}^{(2)} = \frac{\langle \mathcal{D}^2_q \rangle}{\langle \mathcal{D}_q^2 \rangle}$$

(7)

From Eq. 3b, if $|q|>|q'|$, $\mathcal{D}_{qq'}^{(2)} < 1$ implies that the dielectric response is decreasing with
charge, while $\mathcal{D}_{qq'}^{(2)} > 1$ implies that is increasing with charge.

A decomposition of the polarization is also proposed based on a prediction of the
physical basis of the polarization. A polarization greater that expected from the
fluctuations ($\mathcal{D}_q > 1$) can arise in more than one way. For instance, it may be caused by a
permanent polarization that exists even at $q=0$, with a constant dielectric response ($\mathcal{D}_{qq'}^{(1)}$
$< 1$ and $\mathcal{D}_{qq'}^{(2)} > 1$). In the opposite extreme, it may be caused by a decreasing dielectric
response with charge (as might happen as a dielectric saturates) but otherwise linear
polarization ($\mathcal{D}_{qq'}^{(1)} > 1$ and $\mathcal{D}_{qq'}^{(2)} < 1$). Assuming the former, i.e., that there is a permanent
polarization that persists even as the charge $q\rightarrow 0$ in addition to the polarization due to the
dielectric response of the media, the environmental potential may be written as

$$\langle \mathcal{D}_q \rangle = \mathcal{D}_{\text{perm}} - q \mathcal{D}^2_q$$

(8)

where the constant $\mathcal{D}_{\text{perm}}$ is the permanent polarization and the constant $\mathcal{D}^2_q$ is the
dielectric response factor. In the case of linear response for an ion in a dielectric
continuum, $\mathcal{D}_{\text{perm}} = 0$ and $\mathcal{D}^2_q = (1-1/\varepsilon)/R$ by analogy with Eq. 3b.

Given data for $\mathcal{D}_q$ and $\mathcal{D}^2_q$ it is possible to solve for $\mathcal{D}_{\text{perm}}$ and $\mathcal{D}^2_q$ in a variety
of ways. First, they may be solved by a linear regression of Eq. 8. Since this involves
only data for $\mathcal{D}_q$ this will be referred to as the polarization method. Second, they may
be solved by the assumption that $\mathcal{D}^2_q$ is determined by the fluctuations, so that

$$\mathcal{D}^2_q = \frac{1}{N_{c \text{ charges}}} \langle \mathcal{D}^2_q \rangle$$

(9)

$$\mathcal{D}_{\text{perm}} = \mathcal{D}_q \langle \mathcal{D}_q \rangle + q \mathcal{D}^2_q$$

(10)
where \( N_c \) is the number of charge states simulated and \( q \) is summed over the charges of each state simulated. Since this involves both data for \( f \) and \( Df \), this will be referred to as the fluctuation method.

**Molecular Dynamics Simulations**

Molecular dynamics simulations were performed for rubredoxin with a net charge on the redox site \([\text{Fe(SR)}_4]\) of 0, -1 and –2, which will henceforth be referred to as \([\text{1Fe}]^{0,1,-2}\), respectively. Coordinates for the oxidized rubredoxin structure from Cp rubredoxin were obtained from the Brookhaven Protein Data Bank (5RXN). For the \([\text{1Fe}]^{2}\), \([\text{1Fe}]^{1}\) and \([\text{1Fe}]^{0}\) rubredoxins, a total of 1835, 1836 and 1837 waters, respectively, were used to solvate the protein, with the addition of 5 Cl\(^-\) and 16, 15 and 14 Na\(^+\) counterions, respectively, to neutralize the system. All simulations were performed using CHARMM25 (Brooks et al., 1983) with a potential energy function that combines parameters from CHARMM19 (Brooks et al., 1983) with the TIP3 water model (Jorgensen, 1981) plus additional parameters for the \([\text{1Fe}]^{0,1,-2}\) site (Yelle et al., 1995) and the Na\(^+\) and Cl\(^-\) counter-ions (Hyun and Ichiye, 1997). The partial charges for the \([\text{1Fe}]^{0,1}\) sites are from fits to electrostatic potentials from electronic structure calculations (Mouesca et al., 1994). The partial charges for the \([\text{1Fe}]^{0}\) site were obtained by subtracting the difference in the partial charges between the \([\text{1Fe}]^{1}\) and \([\text{1Fe}]^{2}\) sites from the \([\text{1Fe}]^{1}\) site such that the net charge was zero, while the remainder of the energy parameters were the same as the \([\text{1Fe}]^{1}\) site. No explicit electronic polarization was included because the partial charges have been parameterized for the condensed phase environment, allowing the simulations to account implicitly for this feature. All non-polar hydrogens were treated via the extended atom model as part of the heavy atom to which they are attached, and all bonds containing hydrogen were held at their equilibrium bond lengths using the SHAKE algorithm (Rychaert et al., 1977).
The simulations were carried out in the microcanonical ensemble with a target temperature of 300 K. The simulations were carried out using the particle mesh ewald (PME) summation algorithm (Feller, et al., 1996). Cubic boundary conditions of 54 Å x 54 Å x 54 Å were utilized, with grid spacings equal to 1, a $\beta$-spline coefficient equal to 6, and a $\gamma$ value of 0.34. No atomic polarizability was included and a dielectric constant of one was used throughout the simulations. The time step was 1 fs and the total length of each simulation was 1.16 ns. The last 1 ns of data was analyzed. The reported quantities $\|f\|$ and $\|Df^2\|$ were calculated for blocks of 50 ps by averaging $f$ and $|Df|^2$ from coordinates taken at 10 fs intervals and then averaging a total of 20 blocks to yield a total average and a standard deviation for each. The measures of linearity and dielectric response, as well as the decomposition, were calculated from the reported $\|f\|$ and $\|Df^2\|$. Errors in the measures and decomposition were calculated from the reported standard deviations of $\|f\|$ and $\|Df^2\|$ using standard methods of error propagation.

RESULTS

The averages and fluctuations of the environmental potential, $\|f\|$ and $\|Df^2\|$ respectively, were calculated from the molecular dynamics simulations of rubredoxin (Rd) in the $[1Fe]^{0,1-2}$ states for the protein backbone; the protein polar groups, which are the backbone and polar side chains; all polar groups in the system, which include the protein polar groups and solvent; and the entire system (Table 2-1). The polarization of the polar environment is significant even for $[1Fe]^0$-Rd. For all of the simulations, the similarity of $\|f\|$ for the protein backbone and the protein polar groups indicates that the contribution from the polar side chains is small and so the polarization environment is created largely by the backbone. Moreover, the larger value of $\|Df^2\|$ for all polar groups in the system versus the entire system indicates that the contribution of the solvent is correlated with that of the counter-ions and the charged side chains. In the specific case
of the charged side chains, it is important to note that in Cp Rd there are no residues that are titratable at physiological pH (e.g. His). The other charged groups (such as Lys) that occur in Cp Rd are on the surface and solvated, and are thus less likely to be affected by changes in the redox state of the iron. Thus, the contributions of all polar groups in the system will not subsequently be reported independently.

The average value and fluctuations of the environmental potential of the redox site was calculated. The average and fluctuations of the polarization of just the backbone or the backbone plus polar side chains are quite similar. However, when water, charged side chains, and counter-ions are included in addition to the protein, the average value and fluctuations in polarization increase.

The deviations from the linear response and dielectric response models are given by $\xi_{qq'}^{(1)}$, $\xi_{qq'}^{(2)}$ and $\xi_f$ (Table 2-2). The values of $\xi_{qq'}^{(1)}$ indicate that the increase in polarization as the charge changes between $[1\text{Fe}]^{1-}$-Rd and $[1\text{Fe}]^{2-}$-Rd is significantly smaller than expected from linear response. The increase for the backbone and all protein polar groups is much less than linear, while the entire system is somewhat more linear. The values of $\xi_{qq'}^{(2)}$ for the backbone and all protein polar groups indicate that the dielectric response increases as the charge changes between $[1\text{Fe}]^{0}$-Rd and $[1\text{Fe}]^{1-}$-Rd but that the dielectric response decreases as the charge changes between $[1\text{Fe}]^{1-}$-Rd and $[1\text{Fe}]^{2-}$-Rd. This observation indicates a saturation of the response of the protein with increasing field. However, even given the possible changes in the dielectric response, $\xi_f$ indicates that the polarization of the polar part of the protein is larger than expected from the values of the fluctuations while the polarization of the entire system is consistent with the fluctuations.

Finally, the decomposition of the protein into a permanent and dielectric component, $\xi_{perm}$ and $\xi_f$, respectively, was performed (Table 2-3). All of the results are quite similar using either the polarization method, which is a linear regression of the average polarization data using Eq. 8, or the fluctuation method, which utilizes both the
averages and fluctuations of the polarizations in Eq. 9 and 10. Specifically, for the protein polar groups, the results using the polarization method clearly indicate a $\Delta_{perm}$ of 36 kcal/mol and a $\Delta_{fl}$ of 23 kcal/mol, while the results using the fluctuation method give a $\Delta_{perm}$ of 33 kcal/mol and a $\Delta_{fl}$ of 27 kcal/mol. Therefore, the results using the two alternative methods are consistent with each other and with the degree of polarization when $q=0$; i.e., $\Pi_{q=0}=33$ kcal/mol and $\Pi_{q=0}^2=27$ kcal/mol. Interestingly, the results for the total system are similar for $\Pi_{q=0}$ and only slightly larger for $\Pi_{q=0}^2$, and are consistent between the two alternative methods and the degree of polarization when $q=0$.

**DISCUSSION**

The results presented here for the protein backbone and for all protein polar groups clearly indicate that the protein environment creates a polarization potential that is larger than expected from a simple linear response model. Moreover, they indicate that the protein environment creates a polarization that is greater than expected from the fluctuations in the polarization, which implies that the polarization is greater than expected from the dielectric response properties. Although there are many possible explanations for this, including many different nonlinear effects, the large nonzero $\Pi_{q=0}$ from the protein polar groups in the [1Fe]$^0$-Rd simulation indicates a permanent polarization. Also, the similarity of $\Pi_{q=0}$ for the protein polar groups to that of the entire system when the redox site is uncharged (Table 2-1) indicates that the permanent polarization is in the protein. The results for the decomposition into a simple permanent plus linear dielectric component are consistent with this explanation. These results are also consistent with preliminary results for the [4Fe-4S] ferredoxins and High Potential Iron-Sulfur Protein (HiPIPs) (Beck, 1997; Ichiye, 1999). In what follows the implications of these results for electron transfer and folding are discussed.
**Implications for reduction potentials**

The results here indicate that the permanent polarization of the protein apparently is due mainly to the backbone, since little difference is seen between results for just the backbone versus the backbone plus side chains. This is consistent with experimental and theoretical observations that homologous proteins with the same redox site generally have similar reduction potentials, but that specific side chains may cause slight changes (Ichiye, 1999). These observations have led to the picture that the overall three-dimensional fold of the backbone determines the gross value of the reduction potential due to the sequence dependent positions of the backbone polar groups while the side chains can tune the reduction potential, much as the fold determines the basic function of a variety of proteins while the side chains can modify the function.

**Implications for electron transfer**

The overall model of a permanent plus linear dielectric polarization has important implications for understanding the electron transfer properties of these proteins. The overall free energy for an electron transfer reaction may be written as

\[
\mathbb{G}^\gamma = G_{D^+A^0} - G_{D^0A^+} = \mathbb{G}_o^\gamma - \mathbb{G}_{perm}
\]

(11)

where acceptor \(A\) has a permanent polarization \(\mathbb{G}_{perm}\) (assumed positive here) and \(\mathbb{G}_o^\gamma\) is the free energy due to the dielectric response (Fig. 1). Therefore, the larger the \(\mathbb{G}_{perm}\), the more favorable is the reaction. Another important factor for an electron transfer reaction is the activation energy, which may be written as

\[
\mathbb{G}^\gamma = (\mathbb{G}_o^\gamma + \mathbb{G}^\gamma)^2 / 4 \mathbb{G}
\]

(12)

where

\[
\mathbb{G} = \mathbb{G}\{r_{D^+A^0}; q_{D+A^0}\} - \mathbb{G}\{r_{D+A^0}; q_{D+A^0}\}
\]

(13)

is the environmental reorganization energy (Fig. 1), and where \(r\) and \(q\) denote the set of all coordinates and charges for the system indicated in the subscript. Since \(\mathbb{G}\) is
independent of $L_{perm}$, $L = L_0$ where the subscript "D" indicates that it is solely due to the dielectric response. The activation energy may thus be written as

$$DG^\ddag = \left( L_0 + L_0^G \cdot L_{perm} \right)^2$$

Therefore, the larger the permanent polarization (as long as $L_{perm} < L_0^G + L_{perm}$), the smaller the activation energy. Eq. 11 and 14 together imply that a large permanent polarization can increase the favorable driving force while also increasing the reaction rate for the reaction.

The physical interpretation of the above can be made by assuming a Born-type model for the solvation of $D$ and $A$ independently, with the radii of $D$ and $A$ independent of charge state,

$$DG_0^G = \frac{1}{2} \left[ \frac{2q_D + 1}{R_D} + \frac{2q_A + 1}{R_A} \right]$$

so that

$$L_0 = \frac{q_D}{R_D} - \frac{q_A}{R_A}$$

and

$$DG^\ddag = \frac{1}{4} \left[ \frac{2R_D}{R_D} + \frac{1}{2R_A} \right] L_{perm}$$

First, consider the case of no permanent polarization, $L_{perm} = 0$, such as for two free ions $A$ and $D$ in solution with $q_A \leq 0$ and $q_D \geq 0$. The free energy of the reaction $DG_0^G$ will decrease with increasing $L_0$ so that the reaction is favored in a high $L_0$ such as in water, over a low dielectric $L_0$ such as found inside a protein. However, the environmental reorganization $L_0$ will increase with the increasing $L_0$ so that the rate of the reaction is slower in a high $L_0$ over a low dielectric $L_0$ (Fig. 1 A or Fig. 1 B).

Next, consider the case of a permanent polarization $L_{perm} > 0$ with a low dielectric $L_0$ such as for $A$ and $D$ in a protein with $q_A \leq 0$ and $q_D \geq 1$. The free energy $DG^G$ will
decrease with increasing $\Delta_{perm}$ so that the reaction is favored by the permanent polarization over a reaction in the same low dielectric without a permanent polarization. However, $\Delta G^\ddagger$ will also decrease with increasing $\Delta_{perm}$ so that the rate of the reaction is even faster with a permanent polarization than a reaction in the same low dielectric without a permanent polarization, which is already fast compared to a high dielectric. Thus, the permanent polarization can help overcome the low driving force found in the low relative to high dielectric media without sacrificing the low reorganization energy of the low relative to high dielectric media and actually enhance it over the low dielectric rate.

**Implications for protein folding**

These findings also lead to speculations as to how the permanent polarization arises. One possibility is that it is inherently built into the amino acid sequence of the protein. Another possibility is that the polarization arises during folding around the redox site. In this model, the charged redox site forms at a stage sometime before the protein is fully folded. At this point, which maybe be a molten globule state, the protein will behave more like a liquid, such as N-methylacetamide ($\epsilon=179$, $\mu=4.0$ D) or formamide ($\epsilon=109$, $\mu=3.73$ D), than in the fully folded form, where the dielectric fluctuations are restrained by hydrogen bonds, etc., giving rise to a lower dielectric.

In this higher dielectric state, the protein would polarize more than it would based on the folded protein dielectric value. Then, hydrogen bonds would form that lock in this polarization and lower the dielectric. This model supposes formation of the metal site before complete folding. It would be of interest to examine the polarization of metal proteins that can fold without the prosthetic group and/or have large redox sites such as hemes, since the study here and elsewhere have been done on Fe-S proteins (Beck, 1997; Ichiye, 1999), which have relatively small redox sites.
CONCLUSIONS

The results presented here demonstrate that rubredoxin has a positive polarization of the protein environment around the redox site created mainly by the polar backbone, that rubredoxin has a permanent component to this polarization persisting even as the charge of the site goes to zero, and that rubredoxin has a relatively constant dielectric response.

One implication of these results is that the reduction potential of rubredoxin is determined mainly by the backbone and not the side chains. A second implication of these results is that rubredoxin is a good electron acceptor because it has both a low dielectric environment, which keeps the activation energy low, and a permanent polarization, which increases the driving force relative to simple dielectric solvents with no permanent polarization. A final implication is that the redox site charge might help to direct the folding of this protein.

ACKNOWLEDGMENTS

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REFERENCES


TABLE 2-1  Averages and fluctuations of the environmental potential

<table>
<thead>
<tr>
<th>Charge</th>
<th>Polar Backbone</th>
<th>Polar Protein</th>
<th>Polar All</th>
<th>Total Polar</th>
<th>Polar Backbone</th>
<th>Polar Protein</th>
<th>Polar All</th>
<th>Total Polar</th>
</tr>
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<tr>
<td>0</td>
<td>30±3</td>
<td>33±4</td>
<td>57±10</td>
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<td>26±6</td>
<td>27±5</td>
<td>84±24</td>
<td>71±12</td>
</tr>
<tr>
<td>-1</td>
<td>63±1</td>
<td>67±1</td>
<td>116±11</td>
<td>114±4</td>
<td>36±7</td>
<td>37±8</td>
<td>173±138</td>
<td>123±28</td>
</tr>
<tr>
<td>-2</td>
<td>78±2</td>
<td>80±2</td>
<td>159±14</td>
<td>181±13</td>
<td>20±5</td>
<td>22±5</td>
<td>161±92</td>
<td>138±46</td>
</tr>
</tbody>
</table>
TABLE 2-2 Measures of linearity and dielectric response

<table>
<thead>
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<th>Measure</th>
<th>Polar Backbone</th>
<th>Polar Protein</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\phi_{(-2)}$</td>
<td>0.62±0.01</td>
<td>0.59±0.01</td>
<td>0.80±0.01</td>
</tr>
<tr>
<td>$\phi_{(-1)}$</td>
<td>1.40±0.02</td>
<td>1.37±0.08</td>
<td>1.72±0.09</td>
</tr>
<tr>
<td>$\phi_{(-1)}$</td>
<td>0.55±0.02</td>
<td>0.59±0.01</td>
<td>1.12±0.06</td>
</tr>
<tr>
<td>$\phi_{1}$</td>
<td>1.75±0.33</td>
<td>1.81±0.37</td>
<td>0.93±0.17</td>
</tr>
<tr>
<td>$\phi_{2}$</td>
<td>1.98±0.49</td>
<td>1.85±0.33</td>
<td>0.66±0.17</td>
</tr>
</tbody>
</table>

TABLE 2-3 Decomposition of polarization

<table>
<thead>
<tr>
<th>Method</th>
<th>$\phi_{perm}$ (kcal/mol)</th>
<th>$\phi_{2}$ (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Polar Backbone</td>
<td>Polar Protein</td>
</tr>
<tr>
<td>Polarization</td>
<td>33± 7</td>
<td>36± 6</td>
</tr>
<tr>
<td>Fluctuations</td>
<td>30± 3</td>
<td>33± 4</td>
</tr>
</tbody>
</table>
FIGURES
FIGURE 2-1 A schematic representation of potential energy curves for the electron transfer reaction $D + A \rightarrow D^+ + A^-$, where $D$ indicates the donor and $A$ indicates the acceptor, $\Delta G^*$ is the driving force, $\Delta G^\ddagger$ is the activation energy barrier, and $\lambda$ is the reorganization energy.
FIGURE 2-2 A schematic representation of the effects of the environment of the electron acceptor for an electron transfer reaction. The charge is represented by an open circle and the surrounding dipoles are represented by arrows pointing in the direction of their orientation for three types of transfer environments: (A) a simple solvent with a high dielectric, where the reorganization is high, as is the driving force for the reaction; (B) a simple solvent with low dielectric, where the reorganization is low, as is the driving force of the reaction; and (C) a protein, where the reorganization is low due to constraints of the backbone, while the driving force is high.
CHAPTER THREE

The Effect of Mutations at the Cys \( ^3 \) Position on the Reduction Potential of Ferredoxin:
A Molecular Dynamics Study

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ABSTRACT  Electron transport proteins transfer electrons using oxidation-reduction reactions, for which the redox potential is a measure of the driving force. [4Fe-4S] ferredoxins are a class of electron transfer proteins that are found in a wide variety of electron transport chains, including the photosynthetic pathway. Based on the crystal structures of eleven ferredoxins, we previously predicted that a semi-conserved, non-ligating cysteine decreases the reduction potential of ferredoxin relative to an alanine in the same sequence position due to the change in electrostatics from an outward shift of the backbone. We also predicted that a serine in this position increases the reduction potential relative to an alanine due to a change in electrostatics from an inward shift of the backbone and the polar OH group. We proposed that the conformations of both cysteine and serine are stabilized by hydrogen bonds formed with the redox site; however, difference in their dipole vectors lead to the differences in their conformation. The difference in the reduction potential due to a cysteine versus an alanine has been confirmed by site-specific mutation experiments, but the difference due to serine versus alanine has not been confirmed. Here, molecular dynamics computer simulations indicate that the site-specific mutations to cysteine, alanine, and serine will adopt the predicted conformation and electrostatics, although secondary mutations may sometimes be necessary.
INTRODUCTION

Electron transfer proteins serve a vital function in a wide variety of metabolic reactions. The bacterial [4Fe-4S] ferredoxins (Fd) are a group of low molecular weight (6 to 12 kDa) electron transport proteins that are found in a wide range of biological functions, including nitrogen fixation (Gao-Sheridan et al., 1998), proton transfer (Chen et al., 2000), and reduction-oxidation reactions in the cytoplasm (Moulis and Davasse, 1995; Breese and Fuchs, 1998; Boll and Fuchs, 1998). These proteins use oxidation-reduction chemistry to transfer electrons across their respective redox clusters with a high efficiency (~70% (Voet and Voet, 1990)). High resolution (<2.3 Å) x-ray crystal or NMR solution structures of four one-cluster and seven two-cluster ferredoxins, plus a number of mutant ferredoxins, have been solved and are available in the Brookhaven Protein Data Bank (PDB) (Bernstein et al., 1977). These proteins are characterized by an overall \( \text{abbabb} \) fold motif containing either one or two cubane-like [4Fe-4S] clusters, in which the basic structure consists of two iron sulfur clusters and a quasi two-fold symmetry of the backbone (Figure 3-1) (Adman et al., 1973). Variations of this basic structure include deletion of the cluster at the second cubane site, substitution of a [3Fe-4S] cluster at the first cubane site, and additional secondary structural elements. The relevant redox couple for ferredoxin is \( \text{Fe}_4\text{S}_4(\text{SR})_4^{2-/3-} \) (or \( [4\text{Fe}-4\text{S}]^{2+/1+} \) for the cubane), which has characteristic potentials from –645 to 0 mV (Cammack, 1992).

The iron atoms in the redox cluster of ferredoxin are ligated to the rest of the protein by cysteinyl residues. The ligation pattern of a given [4Fe-4S] cluster is generally \( \text{Cys}^1\text{-}X_2\text{-Cys}^2\text{-}X_2\text{-Cys}^3\text{-}X_n\text{-Cys}^4 \), where superscripts indicate the four ligands of one cluster, \( X \) is any residue, and \( n \) indicates that the fourth redox site ligand is far removed (either upstream or downstream) in sequence from the first three ligands (Matsubara and Saeki, 1992). The two-cluster ferredoxins have two of these ligation patterns, which can alternatively be described by the sequence motif \( \text{Cys}^1\text{-}X_2\text{-Cys}^2\text{-}X_2\text{-Cys}^3\text{-}X\text{-Cys}^{4\dagger} \), where the superscript \( 4\dagger \) indicates a ligand of a different cluster than the unprimed superscripts. Therefore, the first three cysteines of one motif and the last cysteine of the other motif ligate each [4Fe-4S] site.

Identification of environmental factors that affect the redox properties of metal clusters in proteins of great interest because the driving force of an electron transfer
reaction is determined by the reduction potentials of the donor and the acceptor sites. Reduction potentials are apparently strongly dependent on the protein environment around the redox site, resulting in different reduction potentials in different proteins for the same cluster. The electrostatic energy is one of the largest contributors to the change in energy upon reduction, and the backbone with the polar side chain contribution has been successfully used to correlate differences in protein reduction potentials (Swartz et al., 1996a; Stephens et al., 1996).

Previously, we have successfully predicted shifts in reduction potential on the order of 50 mV due to single site amino acid substitutions in rubredoxin (Swartz et al., 1996b). In that case, it was noted that the reduction potential of rubredoxin was altered by approximately 50 mV depending on whether residue 44 was a valine or an alanine. Energetic analysis of high-resolution x-ray crystal structures indicated that this difference in reduction potential was the consequence of a 1 Å shift in the backbone position due to the difference in size of the side chains of valine and alanine. Energy minimization and molecular dynamics calculations were then used to simulate a mutation from valine to alanine, which supported the backbone shift and the change in electrostatics (Swartz et al., 1996a). Finally, experimental mutations and crystal structures confirmed the backbone shift and the change in reduction potential (Eidsness et al., 1999). The same prediction strategy is used here, where differences noted in amino acid sequences are coupled with structural data and molecular dynamics simulations to predict changes in the reduction potential.

Also in previous work (Beck et al., 2001), we noted that some ferredoxins contained a semi-conserved, non-ligating cysteine that was four residues C-terminal to the fourth ligand (Cys$^4$) of either cluster in addition to the highly conserved cysteiny1 ligands. This residue position will henceforth be referred to as the Cys$^x$ position. Of the eleven x-ray crystal or NMR solution structures of ferredoxins in the PDB at that time, four different two-cluster ferredoxins and a single one-cluster ferredoxin were found to have the extra cysteine. The remainder of the sites had alanines at the Cys$^x$ position, with the exception of one serine. By examination of the experimental structures, the extra cysteine was found to have the same structure with respect to the nearest cluster, with the sulfur atom positioned very close to a redox site sulfur in all five cases. The calculations
using these experimental structures indicated that a cysteine rather than an alanine led to a shift in the backbone, which increased the electrostatic potential at the redox site by another 60 mV over the alanine value. This result qualitatively agrees with the experimentally observed shift in reduction potential for the C24A mutation in *Azotobacter vinelandii* (*AvI*) ferredoxin and the A53C mutation in *Pyrococcus furiosus* (*Pf*) ferredoxin. Furthermore, we proposed that the extra cysteine forms an SH⋯S hydrogen bond with the redox site sulfur, thus stabilizing the requisite conformation. Although sulfurs are generally considered weak hydrogen bond donors and acceptors (Voet and Voet, 1990), our *ab initio* unrestrained Hartree-Fock and density function theory quantum mechanical calculations of the interactions of [Fe(SCH$_3$)$_4$]$^{1/2-}$ with H$_2$S indicate that Fe-S sulfurs are better hydrogen bond acceptors than non-metal-ligated sulfurs (Beck, 1997; Beck et al., 1999a). A further indication is that numerous NH⋯S hydrogen bonds involving Fe-ligated sulfurs have been identified in Fe-S proteins and also appear to have some relevance to redox properties (Adman et al., 1975; Backes et al., 1991). Tritium and deuterium exchange studies of ferredoxins also indicate that the stability of these NH⋯S bonds is similar to that of NH⋯O bonds found in proteins (Crespi et al., 1974; Hong and Rabinowitz, 1970).

Since the PDB only contained ferredoxins from eleven different organisms, sequence data was also examined for the prevalence of different residue types at the Cys$^x$ position. A sequence analysis of over 100 homologous ferredoxins shows that the Cys$^x$ position is highly conserved for either alanine or cysteine, with a few serines and valines. Including both sites, ~59% are alanines, ~27% are cysteines, ~8% are valines, and ~4% are serines (Fajardo and Ichiye, unpublished). The relative homogeneity in the sequence, i.e. either cysteine or alanine, indicates that there may be a functional significance in the identity of this residue. Additionally, the occurrence of valines and especially serines, which are found in two thermophilic ferredoxins and two photosynthetic bacterial and *nif*-associated ferredoxins, may be an indication of differences in function associated with the change in amino acid type.

The structure and energetics of different residue types at the Cys$^x$ position have also been examined (Xie et al., unpublished). The analysis of the experimental structure
of *Bacillus thermoproteolyticus* (Bt) ferredoxin indicates that a serine at the Cys\(^x\) position has a different conformation than cysteine. This is apparently due to with the difference in the orientation of the dipole moment in cysteine versus serine, which results in the OH group of serine and the SH group of cysteine hydrogen bonding to different sulfurs of the redox site. Hence, the backbone of serine is closer to the redox site than the backbone of cysteine, although both residue conformations are stabilized by their hydrogen bonds to the redox site. Moreover, the change upon reduction of the electrostatic interaction energy for the serine side chain is about \(-50\) mV, with little change in the backbone relative to alanine, which implies that the reduction potential for a serine should be higher than alanine, and even higher than cysteine. This is consistent with the experimental result that Bt Fd has a higher reduction potential (-280 mV) than other ferredoxins with alanines or cysteines at the Cys\(^x\) position such as Pa Fd (-427 mV). The structural analysis indicates a similarity in the structure near the Cys\(^x\) position regardless of the identity of the Cys\(^x\) residue, suggesting that this position is a prime candidate for site-directed mutations to test the energetics. However, there are currently no serine/alanine mutants.

Based on these observations, mutating the residue in the Cys\(^x\) position between cysteine, alanine and serine may be predicted to change the reduction potential. In other words, within an otherwise identical ferredoxin, a serine at the Cys\(^x\) position should result in a higher reduction potential than an alanine, and a cysteine should result in a lower reduction potential than an alanine. However, the Cys\(^x\) residue must adopt the conformation seen in the homologous structures and also must not grossly perturb its environment for the predicted change in reduction potential. Molecular dynamics simulations provide a means of addressing whether it is likely for a mutant to satisfy these constraints.

The research presented here is a molecular dynamics study of mutations at the Cys\(^x\) position in *Peptostreptococcus asaccharolyticus* (Pa) Fd. Of the ferredoxins with experimental structures, Pa Fd was chosen for several reasons even though there are mutational studies with both structures and reduction potentials for AvI Fd. First, Pa is smaller than AvI, making it better suited for multiple molecular dynamics simulations, and is well characterized. Additionally, AvI has one [3Fe-4S] site and one [4Fe-4S] site,
whereas Pa has two pseudo-symmetric [4Fe-4S] sites, providing two semi-equivalent mutational positions. Finally, Pa Fd appears to be an example of the most basic ferredoxin structure. Here, the structures and energetics of alanine, cysteine and serine residues at the Cys position near both clusters were studied.

The goal was to investigate whether geometries and energetics for mutant residues at the Cys position can be predicted based on the geometries and energetics of the amino acid type of the mutant in homologous proteins. Molecular dynamics simulations were performed for mutant structures based on the wild type crystal structures of Pa Fd, where different amino acids were introduced into the Cys position near each cubane. The computational mutant structures were then compared with crystal structures of homologous ferredoxins.

**METHODS**

**Molecular dynamics simulations**

All molecular dynamics simulations were carried out using CHARMM (v25a1) (Brooks et al., 1983) in the microcanonical ensemble at a target temperature of 300 ± 5 K. The Verlet algorithm was used to propagate the dynamics using a time step of 0.001 ps. All bonds containing hydrogen were held to their equilibrium bond lengths using the SHAKE algorithm (Rychaert et al., 1977). Long-range forces were switched smoothly to zero using an atom-based force-switch with a switching region of 10.0 to 14.0 Å (Steinbach and Brooks, 1994). Non-bonded and image lists were updated heuristically using a cutoff distance of 15 Å. No atomic polarizability was included and a dielectric constant of 1 was used throughout the simulations.

The potential energy parameters used in these calculations were the CHARMM19 force field (Brooks et al., 1983), which uses the extended atom model for non-polar hydrogens. In particular, it uses an extended atom model for cysteiny1 sulphhydrils, therefore preventing the representation of SH⋯S hydrogen bonds. Thus, all parameters for the sulfur and the attached hydrogen of the explicitly protonated cysteiny1 side chain
were taken from the all-atom CHARMM22 force field (MacKerell Jr. et al., 1992), with the additional parameters for the X—CH2E—S—X dihedral angle of \( k = 0.5 \) kcal/mol and \( n = 3 \). The energy parameters for waters were those from the TIP3P model (Jorgensen et al., 1983). Parameters for the [4Fe-4S] site were previously determined (Beck et al., 1999). \( \text{Na}^+ \) and \( \text{Cl}^- \) van der Waals parameters were derived from those developed by Pettit and Rossky (Pettitt and Rossky, 1986) and are given elsewhere (Hyun and Ichiye, 1997a; Hyun and Ichiye, 1997b).

Initial coordinates for oxidized Pa Fd consist of the protein from the crystal structure with CHARMM25 (Brooks et al., 1983) generated hydrogen positions for all polar hydrogens, as well as 89 crystal waters. The 2 Å crystal structure of Pa Fd (Backes et al., 1991) was obtained from Elinor Adman at the University of Washington and is used instead of the older PDB entry (1FDX). The initial system for oxidized Pa Fd was generated as follows. The protein was solvated by placing it in a pre-equilibrated truncated octahedral water box of dimensions 45.5 Å x 45.5 Å x 45.5 Å, containing 1574 TIP3P water molecules (\( \rho_{H2O} = 1.0 \) g/ml). All solvent waters within 2.6 Å of any non-hydrogen protein atom or crystal water oxygen were then deleted, resulting in 1248 total waters. Next, the solvent was relaxed slightly by 50 steps of steepest descent energy minimization followed by 2.0 ps of molecular dynamics with Gaussian assignment of velocities every 0.2 ps in which only the solvent was allowed to move and the protein remained fixed. Next, counter-ions were added by replacing a water molecule with an ion near each charged group (a sodium ion for the negatively charged side chains, the C-terminus, and the redox clusters, and a chlorine ion for the positively charged side chains and the N-terminus) of the protein to make the system net neutral. Finally, the solvent environment was equilibrated by fixing the protein while the counter-ion and solvent velocities were propagated for 60 ps, during which time the velocities were scaled every 0.2 ps to a target temperature of 300 ± 5 K. The final box contained 465 protein atoms, 12 \( \text{Na}^+ \), 2 \( \text{Cl}^- \), and 1234 water molecules for a total of 4181 atoms.
Mutations were made using the molecular modeling program QUANTA. The coordinates of the mutant side chains were those of the equivalent atoms in the wild-type crystal structure as far as possible, with any new atom positions generated using the standard geometries in the QUANTA program. Thus, the initial conformation for all of the mutants was the same. In the case of the proline to isoleucine mutation, an additional 50 steps of steepest descent energy minimization with all atoms outside a 10 Å radius centered on the carbon of the isoleucine held fixed preceded the rest of the calculations because of extremely unfavorable interactions. The systems at this point will be referred to as the “starting” systems.

For the molecular dynamics calculations, 50 steps of steepest descents energy minimization was performed on each starting system to remove unfavorable interactions. Following this, all constraints were released and all particles were propagated for 10 ps, during which time the velocities were scaled to a target temperature of 300 ± 5 K. Finally, the system was allowed to evolve during which the system was not further perturbed. The total propagation time for the native structure of Pa Fd, the C22A mutation, and the C22S mutation was 1.2 ns, where only the final 1 ns of data was used. The structures for mutations at or near residue 50 were propagated for 200 ps where only the final 100 ps was used, except for the P51I mutation which was propagated for 220 ps for better equilibration, with only the final 100 ps used. Data was collected every 0.01 ps.

**Electrostatic energy calculations**

To assess the relative contribution of the different types of residues present at the Cys position, the contribution of different protein residues to the energetics of the redox sites in the Pa structure were evaluated via calculations of the electrostatic interaction energies. The relationship between the standard free energy change upon reduction, \( \Delta G \), and the reduction potential, \( E \), is given by the Nernst equation

\[
\Delta nF \varepsilon = \Delta G = \Delta E + PV \ln T \ln S
\]

(1)
where $F$ is Faraday’s constant, $n$ is the number of electrons transferred, $\Delta E$ is the change in energy, $\Delta PV$ is the change in system pressure and volume, $T$ is the absolute temperature, and $\Delta S$ is the change in entropy. The electrostatic potential is one of the largest contributors to the change in energy upon reduction and has been successfully used to correlate differences in protein and solvent structure with variations in protein reduction potentials (Stephens et al., 1996; Swartz et al., 1996a; Yelle et al., 1995).

Based on previous work (Swartz et al., 1996b; Beck et al., 2001),

$$ \varepsilon_2 - \varepsilon_1 \neq f_2 - f_1 $$

where, $\varepsilon_i$ and $f_i$ refer to the reduction potential and the electrostatic potential, respectively, of protein $i$ in the oxidized state. Although the protein will relax upon reduction, the degree of relaxation for a set of non-polar residues will be small in magnitude and similar to each otherso that the contribution of relaxation to a difference in reduction potential will be small (Beck et al., 2001). However, the contribution may be somewhat larger between alanine and serine or cysteine.

The change in charge upon reduction is delocalized over several atoms, and a delocalized $\Delta$ can be defined as

$$ n \Delta = n \sum_{k=\text{protein}} \Delta(k) $$

$$ \Delta = \sum_i \sum_{j \neq i} \frac{q_i q_j}{r_{ij}} $$

where $j$ represents atoms in the redox site, $i$ represents atoms interacting with the redox site, $q_i$ or $q_j$ are the charges of atoms $i$ or $j$, $\Delta(k)$ is the electrostatic potential of residue $k$, $\Delta q_i$ is the change in charge of atom $i$ upon reduction, $q_j$ is the charge of atom $j$, and $r_{ij}$ is the distance from atom $i$ to atom $j$. A dielectric constant of 1.0 with no cutoffs was used. The use of such a simple sum allows the contribution of backbone, side chain atoms, and solvent to be separated. Therefore, only the electrostatics of amino acid residues at the Cys position are compared here.

The electrostatic potential was calculated in two ways. First, the average structure was obtained by averaging the structures from the complete dynamics data then the electrostatic potential of this structure was calculated. This method is equivalent to calculating the electrostatic potential of a crystal structure, which does not include the
dynamics fluctuations of the protein that occur while a reduction potential is being measured experimentally. Second, the average potential of the system over the entire trajectory was calculated. This should more closely mimic the electrostatic contributions in the experimental system.

**Residency times**
The relative occupancies of different conformations about dihedral angles were determined by the following method. The dihedral angle at each time step \( t \) of the trajectory was calculated at each time step \( t \) of the trajectory. A Gaussian curve was constructed for each conformation from histograms of equally spaced bins by calculating a best fit parabolic function using a least squares fit. The standard deviation of this curve was calculated using the standard method. If the angle at time step \( t \) and the angle at the previous time step \( t-1 \) resided within the standard deviation of the same gaussian curve \( l \), then \( n \) of the integer \( n_m \) increased by 1. If the angle at time step \( t \) and the angle at the previous time step \( t-1 \) did not reside within the standard deviation of the same gaussian distribution \( l \), the \( n \) value of the integer \( n_m \) was not increased. When the angle at \( t \) and the angle at \( t-1 \) again occupied the standard deviation of the same gaussian distribution \( l \), \( m \) of the integer \( n_m \) increased by 1 and \( n \) of the integer began counting, starting over from a value of 1. Therefore, \( m \) is the number of times the \( \gamma \) angle resided consecutively within the standard deviation of a given gaussian distribution \( l \), and \( n \) is the number of time steps it resided in \( l \) consecutively. Thus, the residency times were calculated by taking the average of the integers \( n_m \) for each distribution \( l \).

**RESULTS**
Pa Fd, due to its pseudo-symmetric structure, has two Cys positions: one located at cubane 1 (A50) and another located at cubane 2 (C22). Molecular dynamics simulations of Pa Fd with three different residues (cysteine, alanine and serine) at the two Cys positions were performed to examine the changes in conformation and electrostatic potential at the redox. Additionally, residue 51 was mutated from a proline to an isoleucine. Therefore, a total of eight separate systems were simulated: wild-type, C22A, C22S, A50C, A50S, P51I, A50C + P51I, and A50S + P51I. All simulations were initially
calculated for a total of 100 ps to test the predictions. While the simulations at residue 22 quickly adopted the predicted conformations and energetics, the simulations at residue 50 did not. Therefore, the simulations examining residue 22 were extended to a total time of 1.2 ns, including equilibration, to provide a more robust analysis of the statistics. However, the simulations examining residue 50 were only extended to a total of 200 ps, including equilibration. This will be discussed in further detail below. In the following section, the results from the calculations will be compared to the conformations and energetic trends observed in crystal structures with the corresponding amino acid at the Cys\textsuperscript{x} position.

Structure
The average structures from the molecular dynamics simulations of the wild-type and mutant proteins agree with each other and the crystal structure, as shown by the small root mean square (RMS) deviations of the backbone (Table 3-1). The structure of the residue at the Cys\textsuperscript{x} position is characterized by $D_R$, which is the distance between the amide N of the Cys\textsuperscript{x} residue and the S\textsuperscript{4g} of the redox site, as well as the backbone and side chain dihedral angles of the Cys\textsuperscript{x} residue (Table 3-2). The conformation of the cysteine and the alanine at the two Cys\textsuperscript{x} positions shows no significant change between the crystal structure, the average structure, and the average conformation of the cysteine or alanine, respectively, at the Cys\textsuperscript{x} position calculated from the known crystal structures of all ferredoxins (Table 3-2). The Cys\textsuperscript{x} position will be discussed in detail below.

The MD average structure of the wild-type cysteine at residue 22 remains quite close to the experimental x-ray structure of the cysteine residue in both wild-type Pa Fd (Table 3-2) and also cysteines in general throughout the ferredoxins of known structure (Cys xtal ave in Table 3-2). There are slight shifts in the average dihedral angles so that when comparing x-ray versus MD, there is a 10° decrease in $\phi$, a 10° increase in $\psi$, and a 44° decrease in $\chi_2$, which are most likely systematic shifts due to an inexact potential energy function.

The C22A mutant structure is within the range predicted by other alanines at the Cys\textsuperscript{x} position in ferredoxins of known structure (Ala xtal ave in Table 3-2) in terms of $\phi$.
and \( \theta \), while \( \theta R_1 \) differs by 0.11 Å, which is slightly more than expected based on the average of the homologous proteins (Figure 3-2b).

Finally, the C22S mutant structure is quite close to what was found in the Bt Fd x-ray structure (which has a serine at residue 65), but with a 14° increase in \( \theta \). Quite remarkably, the average \( \theta \) and \( \varphi \) angles are in good agreement with those found in the Bt Fd structure, even though the initial coordinates of the simulation were those of the cysteine, which has significantly different \( \theta \) and \( \varphi \) angles (Figure 3-2b).

Because cysteine and serine adopt conformations that include hydrogen bonds to two sulfurs each in the redox cluster as well as no hydrogen bonds to any of the sulfurs, the \( \varphi \) dihedral angles of cysteine and serine at this position were analyzed statistically using the summation of three Gaussian curves. Residence times were then calculated about the standard deviation for each \( \theta \) angle over the entire 1 ns to determine the relative stability of each of these conformations.

Cysteine was found to fluctuate around three \( \varphi \) dihedral angles at approximately 64° (53% occupancy, 2.3 ps residence time), 150° (35% occupancy, 6.0 ps residence time) and -58° (22% occupancy, 3.1 ps residence time), corresponding to hydrogen bonds with \( S_g^4 \), nothing, and \( S_{*2}^* \), respectively. Serine was found to fluctuate around three \( \varphi \) dihedral angles at approximately 98° (55% occupancy, 2.7 ps residence time), 153° (43% occupancy, 2.3 ps residence time) and -45° (2% occupancy, 0.1 ps residence time), corresponding to hydrogen bonds with \( S_g^4 \), nothing, and \( S_{*3}^* \), respectively.

The structure of residue 50 at the Cys⁵ position near cluster 1 has also been characterized (Table 3-2). In these calculations, a short simulation was performed (200 ps). The MD average structure of the wild-type alanine remains quite close to the experimental structure of the alanine in the wild-type Pa Fd (Table 3-2). Again, there are slight shifts in the dihedral angles when comparing the experimental crystal structure to the molecular dynamics (Table 3-2). However, the A50C mutant structure is in poor agreement with other cysteines at the Cys⁵ position in ferredoxins of known structure (Figure 3-2b, Table 3-2), with both the backbone dihedrals and \( \theta R_1 \) closer to the original alanine values, and a completely different \( \theta \) and \( \varphi \). The deviations when comparing the averaged MD structure of A50C with that of the averaged MD structure of C22 indicate that these differences go beyond a systematic shift.
On the other hand, the A50S mutant is in reasonable agreement with the experimental Bt Fd S65 structure, except that $\theta$ is $-71^\circ$ for the MD average and $-179^\circ$ in the crystal structure. This difference is larger than what is seen in the MD of the C22S mutant. The origin of the poor results for the cysteine appears related to be the presence of a proline at residue 51 following the Cys$^x$ position at residue 50. Analyzing the sequence of the homologous ferredoxins, it was noted that most of the ferredoxins have an isoleucine at this position, and that none of the ferredoxins with a cysteine at the Cys$^x$ position have a proline at the Cys$^x$+1 position. In fact, the only instances of a proline at the Cys$^x$+1 position occur when there is an alanine at the Cys$^x$ position close to the C-terminus. Presumably, this proline is constraining the backbone, decreasing the flexibility of the C-terminus. Therefore, the backbone conformation necessary for the hydrogen bonding conformations of cysteine and serine might be hindered by this proline.

To determine whether the proline at the Cys$^x$+1 position was constraining the backbone at the Cys$^x$ position, residue 51 in Pa Fd was mutated from a proline to an isoleucine. Interestingly, the A50/P51I single mutant exhibits a somewhat larger distance from the redox site than predicted, where $R_1=3.71$ Å. Additionally, the structure of the A50C/P51I double mutant is not consistent with the cysteines at the Cys$^x$ position in the other homologous ferredoxins, and looks only slightly similar to the MD structure of the wild-type C22 of Pa Fd (Figure 3-2b). However, the structure of A50S/P51I is similar to the serine at the Cys$^x$ position in Bt Fd.

**Energetics**

The energetics of the native and mutant Pa Fd have been calculated as described in the Methods section. For the native structure, it is evident when comparing the crystal structure and the average dynamics structure that there is a small change in the electrostatic potential due to movement of the backbone and the side chain for the cysteine at residue 22 (Table 3-2 and 3-3). This is not surprising as relaxation of the protein from the crystal structure takes place. We predict that when comparing an alanine to a cysteine at the Cys$^x$ position, alanine will have a smaller electrostatic potential contribution than cysteine. For the mutation C22A, the trend in the electrostatic
contribution is consistent with the results from the crystal structure as well as the experimentally measured reduction potential, where alanine is seen to provide a more stabilized contribution than cysteine (Table 3-2 and 3-3).

For A50, the changes in potential between the crystal structure and the average structure are minimal. For the mutation A50C, the calculated potential contribution appears completely inconsistent with the previously noted trends (Table 3-3); instead, cysteine has a higher electrostatic potential contribution than alanine at residue 50. We propose that the putative hydrogen bond is unable to interact with the redox site as previously demonstrated, due to the presence of a proline at the Cys$^{\pm}$+1 position, as was described in the structure section. With the additional mutation of P51I, the electrostatic potential shows a dramatic change, ultimately agreeing with the prediction.

For the mutations at the Cys$^{\pm}$ position at residue 22 to a serine, we propose that the electrostatic contribution will provide a net increase in the overall reduction potential due to the different orientation of the serine dipole compared with the net cysteine dipole. This difference in orientation causes the OH···S hydrogen bond to occur with different sulfurs in the redox site, providing a negative electrostatic interaction. The results of the molecular dynamics simulations done in this work agree with the predictions we made in previous work, (Xie et al., unpublished) where the reduction potential increases with the presence of a serine at the Cys$^{\pm}$ position when compared to alanine, and the conformation is very similar to the predicted conformation found in the only known structure containing a serine at the Cys$^{\pm}$ position (Bt Fd) (Table 3-2 and 3-3).

The electrostatic potential at the redox site due to the Cys$^{\pm}$ residue was calculated by finding the average potential over the simulation and by finding the potential of the average structure. By comparing these two methods, it may be possible to account for variations found in crystal structure calculations compared with the measured results, which are on the order of 50 mV. The changes in the numerical values are relatively low (~2 to 20 mV) when comparing the difference for the cysteine residue at the Cys$^{\pm}$ position and also for the alanine residue at the Cys$^{\pm}$ position. However, a significant change (over 50 mV at times) is observed for the case where serine is present at the Cys$^{\pm}$ position. Upon examination, it appears that this change can be accounted for in the orientation of the new hydrogen bond formed (it’s definitely related to the average
orientation, which has to due with the fluctuations, but I don’t think that we can say whether it has to do with the fluctuations themselves...)between the serine and the redox site. Like cysteine, serine appears to have two conformations that include hydrogen bonds, and one without. However, it appears that the fluctuations between two of these states are rapid and common when compared to cysteine (Figure 3-3).

**DISCUSSION**

The results will be discussed in terms of the two goals mentioned in the introduction. In terms of the first goal, the molecular dynamics simulations indicate that the geometries and energetics of mutations at the Cys\(^x\) position can be predicted by the geometries and energetics of residues with the same amino acid type that occur natively at the Cys\(^x\) position in homologous ferredoxins. For PaFd, this holds for residue 22 but not as well for residue 50 as discussed further below. Especially compelling is the fact that serine adopts the same conformation in the simulation as found in the crystal structure of Bt Fd, even though the starting conformation was that of the cysteine. These results imply that the conformation of the Cys\(^x\) residue is relatively independent of the environment, although the results are dependent on the potential energy parameters. However, in a somewhat circular argument, the fact that the Cys\(^x\) residues adopt the conformation predicted by the homologous proteins as opposed to completely new conformations indicates that the potential energy parameters are reasonable. Of course, experimental verification is necessary; however, these results are positive indicators of the success of the predictions based on the previous homolgous protein studies (Xie et al., unpublished; Beck and Ichiye, 1999b).

As previously mentioned, a trend has been noted to exist between the electrostatic contribution of cysteine, serine, and alanine at the Cys\(^x\) positions. Cysteine is shown to lower the electrostatic potential by adopting a conformation stabilized by putative hydrogen bonds to the redox site (Beck et al., 2001) while serine increases the electrostatic potential by a similar mechanism. Alanine, on the other hand, can affect the electrostatic potential by moving in closer to the redox site and increasing its backbone contribution to the electrostatics of the redox site. Upon the mutation of C22S, the total electrostatic contribution to the redox site from the side chain changes by approximately
150 mV, from 87 mV for cysteine to 244 mV for serine. This is a significant shift, and is in the same direction and magnitude as predicted by analysis of the crystal structure of Bt Fd.

The simulations for mutations at residue 22 were extended to 1 ns, while those for residue 50 were not. The latter were not extended because the preliminary results indicated that residue 50 was not a good site for mutations that would adopt the desired conformations. First, it should be noted that the mutations made at residue 50 did adopt a stable and equilibrated conformation reasonably fast time (~50 ps), even though this was not the predicted conformation. The mutations at residue 22 adopted the predicted conformation very quickly (~10 ps), and so a longer simulation time might not affect the average conformation of the structure. Additionally, the simulations of 200 ps did not demonstrate any change in this equilibrium position over time. Second, there is a proline at the Cys\(^{x} + 1\) position at residue 50 and not at residue 22. The proline constrains the backbone in such a way that there is not enough flexibility necessary for the cysteine to adopt the predicted conformation. However, removing the proline does not completely solve this problem, as the short terminal tail (with a length of only 4 amino acids, and present in all 8 known sequences with a proline at this position) becomes too flexible and apparently “floats away.” The proline may be present in sequences with such short tails in order to constrain the N-terminus. Therefore, rather than extend the simulation of the mutations at residue 50, a better choice may be to test this second site in ferredoxins with an alanine at the Cys\(^{x}\) position, where the Cys\(^{x}+1\) position is not a proline.

A larger perspective is that the failure of the mutant A50C to adopt the conformation and energetic trend predicted by the homologous protein analysis indicates a further utility of the molecular dynamics simulations. The simulations point out a possible “failure” for the prediction, but the further simulations of the double mutant A50C/P51I indicate that the “failure” can be partially remedied. Furthermore, the deviations of the single mutant A50/P51I from the wild-type A50 indicates that the proline at the Cys\(^{x}+1\) position near the C-terminus may help an alanine at the Cys\(^{x}\) position preserve a conformation more like that found on average for other alanines at the Cys\(^{x}\) position. Again, these results require experimental verification, but the simulations provide a better predictive framework within which to make mutations. Additionally, the
failure of A50C to completely adopt its predicted conformation, along with the subsequent mutation at P51I (which remedied the problem) is reflected in the energetics. Without the proper conformation, the energetic contribution of the cysteine at residue 50 is 248 mV, much greater than its symmetric cysteine at the other site, at 87 mV. However, with the P51I mutation, the energetic contribution decreases to 135 mV, much closer to the predicted value and less than that of alanine at residue 50 (217 mV).

CONCLUSIONS
A conformational and energetic trend can be predicted when a cysteine, alanine, and serine are present at the $\text{Cys}^\alpha$ position in ferredoxin. At site 2, cysteine is shown to decrease the reduction potential of the redox site when compared to alanine, and serine is shown to increase the reduction potential of the redox site when compared to alanine. The structure of the residue at this site is self-consistent and predictable as well. This energetic trend has also been shown to exist at site 1 when consideration is taken for the short N-terminal tail found to be present in Pa Fd, as well as several other ferredoxins. In this case, a proline present at the $\text{Cys}^\alpha+1$ position, postulated to stabilize the short terminal tail, also constrains the backbone so that cysteine cannot move away from the redox site. However, when mutated to an isoleucine, the energetic trend is predictable, although the structure takes a second conformation.

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Table 3-1: Calculation of the root mean square deviation (RMSD) of Cys residue

<table>
<thead>
<tr>
<th>Cys(^a) residue</th>
<th>W(_{\text{twd}}) vs MD (Å(^2))</th>
<th>W(_{\text{MD}}) vs MD (Å(^2))</th>
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<tr>
<td>C22/A50 (wt)</td>
<td>1.00</td>
<td>N/A</td>
</tr>
<tr>
<td>C22A/A50</td>
<td>1.13</td>
<td>0.65</td>
</tr>
<tr>
<td>C22S/A50</td>
<td>0.85</td>
<td>0.53</td>
</tr>
<tr>
<td>C22/A50C</td>
<td>1.07</td>
<td>0.90</td>
</tr>
<tr>
<td>C22/A50C+P51I</td>
<td>0.89</td>
<td>0.84</td>
</tr>
<tr>
<td>C22/A50S</td>
<td>0.72</td>
<td>0.74</td>
</tr>
<tr>
<td>C22/A50S+P51I</td>
<td>1.08</td>
<td>0.79</td>
</tr>
<tr>
<td>C22/A50+P51I</td>
<td>1.33</td>
<td>0.89</td>
</tr>
</tbody>
</table>
Table 3-2: Angles and distances of residues at the Cys$^\text{x}$ position in the average MD structures.

<table>
<thead>
<tr>
<th>Cys$^\text{x}$ residue</th>
<th>Residue position</th>
<th>$\theta$ (°)</th>
<th>$\phi$ (°)</th>
<th>$\psi$ (°)</th>
<th>$\delta$ (°)</th>
<th>$R_1$ (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cys</td>
<td>C22 (wt xtal)</td>
<td>147</td>
<td>121</td>
<td>61</td>
<td>156</td>
<td>3.44</td>
</tr>
<tr>
<td></td>
<td>C22 (wt)$^\ddagger$</td>
<td>137</td>
<td>132</td>
<td>55</td>
<td>112</td>
<td>3.65</td>
</tr>
<tr>
<td></td>
<td>A50C</td>
<td>83</td>
<td>143</td>
<td>-71</td>
<td>-131</td>
<td>3.02</td>
</tr>
<tr>
<td></td>
<td>A50C+P51I</td>
<td>152</td>
<td>122</td>
<td>-42</td>
<td>84</td>
<td>3.38</td>
</tr>
<tr>
<td>Ala</td>
<td>A50 (wt xtal)</td>
<td>91</td>
<td>160</td>
<td>N/A</td>
<td>N/A</td>
<td>3.43</td>
</tr>
<tr>
<td></td>
<td>Ala xtal ave</td>
<td>109 ± 21</td>
<td>124 ± 87</td>
<td>N/A</td>
<td>N/A</td>
<td>3.40 ± 0.08</td>
</tr>
<tr>
<td></td>
<td>C22A</td>
<td>125</td>
<td>162</td>
<td>N/A</td>
<td>N/A</td>
<td>3.51</td>
</tr>
<tr>
<td></td>
<td>A50</td>
<td>98</td>
<td>157</td>
<td>N/A</td>
<td>N/A</td>
<td>3.35</td>
</tr>
<tr>
<td></td>
<td>A50 + P51I</td>
<td>91</td>
<td>163</td>
<td>N/A</td>
<td>N/A</td>
<td>3.71</td>
</tr>
<tr>
<td>Ser</td>
<td>S65 (wt Bt xtal)</td>
<td>102</td>
<td>141</td>
<td>-31</td>
<td>-179</td>
<td>3.12</td>
</tr>
<tr>
<td></td>
<td>C22S</td>
<td>100</td>
<td>155</td>
<td>-38</td>
<td>142</td>
<td>3.51</td>
</tr>
<tr>
<td></td>
<td>A50S</td>
<td>112</td>
<td>147</td>
<td>-49</td>
<td>-71</td>
<td>3.38</td>
</tr>
<tr>
<td></td>
<td>A50S + P51I</td>
<td>72</td>
<td>175</td>
<td>-55</td>
<td>116</td>
<td>3.33</td>
</tr>
</tbody>
</table>
Table 3-3: Measures of the electrostatic potential between the residue at the Cys<sup>x</sup> position and the redox site relative to alanine.

<table>
<thead>
<tr>
<th>Cys&lt;sup&gt;x&lt;/sup&gt; residue</th>
<th>Residue position</th>
<th>PSC</th>
<th>BB</th>
<th>PSC + BB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cys</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C22 (xtal)</td>
<td></td>
<td>-104</td>
<td>-96</td>
<td>-200</td>
</tr>
<tr>
<td>Cys Ave</td>
<td></td>
<td>-100 ± 13</td>
<td>-66 ± 31</td>
<td>-170 ± 29</td>
</tr>
<tr>
<td>&lt;E(r)&gt;</td>
<td>C22</td>
<td>-39</td>
<td>-183</td>
<td>-122</td>
</tr>
<tr>
<td></td>
<td>A50C</td>
<td>30</td>
<td>0</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>A50C + P51I</td>
<td>-30</td>
<td>-52</td>
<td>-78</td>
</tr>
<tr>
<td>E(&lt;r&gt;)</td>
<td>C22</td>
<td>-61</td>
<td>-70</td>
<td>-130</td>
</tr>
<tr>
<td></td>
<td>A50C</td>
<td>44</td>
<td>30</td>
<td>70</td>
</tr>
<tr>
<td></td>
<td>A50C + P51I</td>
<td>22</td>
<td>-87</td>
<td>-65</td>
</tr>
<tr>
<td>Ser</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S65 (Bt xtal)</td>
<td></td>
<td>30</td>
<td>-9</td>
<td>22</td>
</tr>
<tr>
<td>&lt;E(r)&gt;</td>
<td>C22S</td>
<td>96</td>
<td>-17</td>
<td>78</td>
</tr>
<tr>
<td></td>
<td>A50S</td>
<td>57</td>
<td>4</td>
<td>57</td>
</tr>
<tr>
<td></td>
<td>A50S + P51I</td>
<td>35</td>
<td>4</td>
<td>31</td>
</tr>
<tr>
<td>E(&lt;r&gt;)</td>
<td>C22S</td>
<td>126</td>
<td>-9</td>
<td>91</td>
</tr>
<tr>
<td></td>
<td>A50S</td>
<td>-91</td>
<td>35</td>
<td>-52</td>
</tr>
<tr>
<td></td>
<td>A50S + P51I</td>
<td>135</td>
<td>-35</td>
<td>96</td>
</tr>
</tbody>
</table>
Figure 3-1: Ribbon diagram of Peptostreptococcus asaccharolyticus ferredoxin containing an `abbabb` pseudo-symmetric backbone fold. Two [4Fe-4S] cubane redox sites are ligated with cysteinyl ligands to the protein matrix.
Figure 3-2: a. Superposition of the crystal structures of Pa Fd cysteine at the Cys\(^x\) position (residue 22), Bt Fd serine (residue 65) and Pa Fd alanine at the Cys\(^x\) position (residue 50) colored in yellow, red and blue, respectively. The redox site has sulfurs colored yellow and irons colored green. The conformation of the side chain is significantly different between the cysteine and the serine, even though both of these amino acids are polar. This difference is due to the different direction of the dipole moment of the residue, such that the putative hydrogen bond fluctuates between different sulfur groups of the redox site for cysteine than for serine. This leads to two differing conformations and thus two different electrostatic interactions.

b. Superposition of the average structures over 1 ns MD trajectories for three residues at the Cys\(^x\) position at residue 22, where the native cysteine is yellow, the serine mutation is red, and the alanine mutation is blue. The redox site has sulfurs in yellow and iron in green. Although these three amino acids were started in the same conformation, after equilibration cysteine and serine have very different conformations, as seen in the crystal structures of Figure 3-2a.

c. Superposition of the average structures over 1 ns MD trajectories for cysteine at the Cys\(^x\) position. The native cysteine at residue 22 is shown in yellow. The cysteine at residue 50 was mutated from an alanine, and does not assume the predicted conformation. Upon a second mutation at residue 51 (the Cys\(^x+1\) position) the backbone moves closer to the redox site, however, the side chain still does not assume the predicted conformation.
Figure 3-3: a. A polar plot of histograms for the three conformations of $\square_a$ angle of C22 during 1 ns run of MD shows the relative occupation of each angle over the lifetime of the MD simulation.

b. A polar plot of histograms for the three conformations of $\square_a$ angle of C22S during 1 ns run of MD shows the relative occupation of each angle over the lifetime of the MD simulation.
CHAPTER 4

Protein Control of Electron Transfer in Ferredoxin:
A Molecular Dynamics Study

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Washington State University
Pullman, Washington 99164-4660
ABSTRACT  Electron transport proteins transfer electrons using oxidation-reduction reactions in a variety of important biological pathways. An important question about these proteins is how the environment of the redox site affects their electron transfer properties. These environmental factors include the accessibility of solvent to the redox site and the orientation of backbone and side chain peptide dipoles around the redox site. An important framework for understanding these factors is Marcus theory, in which the activation energy of an electron transfer reaction is related to the polarization of the environment. Here, we investigate the [4Fe-4S] ferredoxins, which are a class of iron-sulfur electron transfer proteins found in a wide variety of electron transport chains, including the photosynthetic pathway. The basic structure of ferredoxin contains two redox sites, and intramolecular electron transfer between the two sites has been experimentally shown to occur. The nuclear polarization is calculated from molecular dynamics simulations of ferredoxins, and is then used to calculate free energy curves for the intramolecular electron transfer reaction. The contributions of backbone, side chains, and solvent to the driving force and activation energy for electron transfer are calculated separately. It is found that a driving force exists between the two sites, although this is most likely due to the need for better statistics, as further evidenced by the non-parabolic character of the free energy surfaces. Furthermore, the rates are found to be significantly lower than those found using experimental methods. However, the fact that these characteristics can be calculated for this transfer reaction is the first step in a further analysis of how specific amino acids can affect the transfer properties of intramolecular transport in ferredoxins, and in general, redox reactions in biological energetics.
INTRODUCTION

Electron transfer proteins serve a vital role in the transport and utilization of cellular energy. They are ubiquitous to all life and are found in the pathways of cellular respiration, photosynthesis, and nitrogen fixation. These proteins use oxidation-reduction chemistry to transfer an electron from a donor site to an acceptor site and thus can be described by theories for electron transfer (Marcus and Sutin, 1985; Levich, 1966), in which the environment is assumed to affect the rate of an electron transfer reaction. Specifically, in Marcus theory, the polarization of the environment is assumed to respond linearly to changes in charge, which means that the environmental free energy functions are quadratic or parabolic, with the same curvature.

Computer simulations are now an important means of studying electron transfer systems. Free energy curves for electron transfer reactions have been calculated from computer simulations using methods pioneered by Warshel and co-workers (Warshel, 1982; Churg et al., 1983; Hwang, 1987; King, 1990). For example, the exchange between two benzene-like solutes (Hwang, 1987), ferric-ferrous self-exchange (Kuharski et al., 1988; Chandler and Kuharski, 1988; Yelle and Ichiye, 1997), rubredoxin self-exchange (Yelle, 1996; Yelle and Ichiye, 2000), and the photosynthetic reaction center (Marchi et al., 1993) have all been studied using these methods. In these studies, the assumption of a quadratic free energy curve was reasonable, although a non-linear response leading to non-parabolic free energy curves becomes apparent under certain conditions, such as the self-exchange of electrons for small ions (Yelle and Ichiye, 1997).

Electron transfer reactions are highly efficient in biological systems, transferring electrons over relatively large distances and with very fast rates (Voet and Voet, 1995). For example, electrons can be transferred between reduced hemes over distances of 10 to 20 Å at physiologically significant rates (Voet and Voet, 1995). The means by which electron transfer proteins control these reactions is of great interest, both for basic understanding of these systems and also for bioengineering applications. Specifically, an
important question is how the protein is able to provide an environment that gives rise to small activation energies. While mutations along the entire pathway between the two sites may affect the electronic coupling, individual mutations close to the redox site do not affect the electronic coupling significantly (Kyritsis et al., 1997). However, mutations close to the redox sites have been shown to affect the properties of electron transfer, and thus, are more likely to affect $G^\ddagger$ due to the contributions of the reorganization energy. Although a significant amount of work has been performed on electron transfer between ruthenium-modified histidines and natural redox centers in proteins (Bechtold et al., 1986; Yocom et al., 1982), our focus is on electron transfer between natural sites in proteins. Ferredoxins with two redox sites are known to transfer electrons between the sites (Blanchard et al., 1993) and the transfer is fast on the NMR time scale ($k > 10^4$ s$^{-1}$) (Bertini et al., 1992).

The bacterial [4Fe-4S] ferredoxins (Fd) are a group of low molecular weight (6 to 12 kDa) electron transport proteins that are found in a wide range of biological functions, including nitrogen fixation (Gao-Sheridan et al., 1998), proton transfer (Chen et al., 2000), and reduction-oxidation reactions in the cytoplasm (Moulis and Davasse, 1995; Breese and Fuchs, 1998; Boll and Fuchs, 1998). Ferredoxins are small, ranging in size between 6-12 kDa (55-110 aa), and consisting of a $\alpha\beta\alpha\beta$ fold motif containing either one or two cubane-like [4Fe-4S] clusters, where the basic structure consists of two iron sulfur clusters and a quasi two-fold symmetry of the backbone (Figure 4-1) (Adman et al., 1973). Variations of this basic structure include deletion of the cluster at the second cubane site, substitution of a [3Fe-4S] cluster at the first cubane site, and additional secondary structure such as an extra $\alpha$-helix. The relevant redox couple for ferredoxin is $[\text{Fe}_4\text{S}_4(\text{SR})_4]^{2-}/^{3-}$, which has characteristic potentials from $-645$ to $0$ mV (Cammack, 1992). High resolution (<2.3 Å) x-ray crystal or NMR solution structures of four one-cluster and seven two-cluster ferredoxins, plus a number of mutant ferredoxins, have
been solved and are available in the Brookhaven Protein Data Bank (PDB) (Bernstein et al., 1977). Additionally, the sequences of approximately 120 species are available.

The iron atoms in the redox cluster of ferredoxin are ligated to the rest of the protein by cysteiny1 residues. The ligation pattern of a given [4Fe-4S] cluster is generally Cys\(^1\) -X\(_2\)-Cys\(^2\) -X\(_2\)-Cys\(^3\) -X\(_n\)-Cys\(^4\), where superscripts indicate the four ligands of one cluster, X is any residue, and \(n\) indicates that the fourth redox site ligand is far removed (either upstream or downstream) in sequence from the first three ligands (Matsubara and Saeki, 1992). The two-cluster ferredoxins have two of these ligation patterns, which can alternatively be described by the sequence motif Cys\(^1\) -X\(_2\)-Cys\(^2\) -X\(_2\)-Cys\(^3\) -X-Cys\(^4\)\(^¢\), where the superscript \(4\)\(^¢\) indicates a ligand of a different cluster than the unprimed superscripts. Therefore, the first three cysteines of one motif and the last cysteine of the other motif ligate each [4Fe-4S] site.

Here, the intramolecular transfer reaction of ferredoxin is studied, which is advantageous for several reasons. First, the donor and the acceptor are bound and therefore no orientation effects need to be considered (Cusanovich, 1991; Mauk, 1999). Second, the distance between the donor and acceptor is known (approximately 12 Å). Third, ferredoxins are well characterized, with intramolecular transfer rates on the order of \(10^6\) - \(10^7\) s\(^{-1}\) and where theoretical electron pathways can be calculated (Gray and Winkler, 1996). Finally, computational studies have identified sequence determinants of the reduction potential (Beck and Ichiye, 1999b). Specifically, *Clostridium acidurici* ferredoxin (Ca Fd) is studied in this work. Ca Fd is an example of the simplest form of ferredoxin (Fajardo and Ichiye, to be published), and a high-resolution crystal structure (0.94Å) is available in the Protein Data Bank (Dauter et al., 1997). The protein is very symmetric and thus the redox sites are in relatively similar environments with similar reduction potentials of approximately -420 mV (Backes et al., 1991). The similar reduction potentials indicate that the driving force should be relatively small; however, transfer has been shown to be fast on the NMR time scale and theoretical electron
transfer pathways have been calculated ($k > 10^4 \text{ s}^{-1}$) (Kyritsis et al., 1997). (Cusanovich, 1991; Mauk, 1999; Dauter et al., 1997; Kyritsis et al., 1997).

In this work, molecular dynamics simulations of Ca ferredoxin are performed with different oxidation states of the redox sites. The nuclear polarization of the protein around the redox site is calculated from the trajectories and is then used to calculate the free energy curve, driving force, and activation energy for the electron transfer reaction.

METHODS

Theory

Consider the following reaction,

$$D^m + A^n \rightarrow D^{m+1} + A^{n+1}$$ (1)

where a donor ($D$) transfers an electron to an acceptor ($A$). In ferredoxin, the donor and the acceptor are the two respective iron-sulfur clusters, such that

$$[4Fe\square 4S(SR)_4]^{3+}[4Fe\square 4S(SR)_4]^{2+} \rightarrow [4Fe\square 4S(SR)_4]^{2+}[4Fe\square 4S(SR)_4]^{3+}$$ (2a)

or

$$D^{3+} A^{2+} \rightarrow D^{2+} A^{3+}$$ (2b)

The letters $R$ and $P$ will refer to the reactants and products of Eqs. 2, and $\square G_a(X)$ is the value of the free energy when the environment has configuration $X$ and the donor and acceptor are in state $a$; i.e., $a = R, P$. Marcus theory assumes that the free energy as a function of a given reaction coordinate is parabolic, as shown in Figure 4-2, where $\square G_R(X)$ is the free energy curve of the reactants, $\square G_P(X)$ is the free energy curve of the products, $\square G^\ddagger$ is the activation barrier, $\square G^\circ$ is the driving force, and $\square$ is the reorganization energy (Marcus and Sutin, 1985). This leads to the well-known relation

$$\square G^\circ = \left(\frac{\square + \square G^\ddagger}{4\square}\right)^2$$ (3)
where the activation barrier is shown to be a function of the driving force and the reorganization energy.

The free energy curves are constructed using the molecular version of Marcus theory, as developed by Warshel and co-workers (Warshel, 1982; Churg et al., 1983; Hwang, 1987; King, 1990). This method calculates the free energy curves $R$ and $P$ from the ratio of the probability of being at $X$ on the reaction coordinate versus the probability of being at the minimum,

\[ DG_R(X) = k_B T \ln[P_R(X)] \]

\[ DG_P(X) = k_B T \ln[P_P(X)] + DG' \]

where $P(X)$ is the probability of $X$, $k_B$ is Boltzmann’s constant, and $T$ is the temperature. The functions $P(X)$ can be obtained from simulation by histograms of $X$. However, $X$ must be defined explicitly.

We define the reaction coordinate $X$ to be the differences in the electrostatic potential energy between state $R$ and state $P$, following the method by Warshel and co-workers (Parson et al., 1998), such that

\[ V(r) = V_P(r) - V_R(r) \]

and

\[ V_a = \sum_{a_i} \sum_{\alpha R} \left( q_{a_i} q_j \right) / r_{ij} \]

where $a = R,P$, $a' = R,P$, $V_a$ is the electrostatic energy, $q_{a_i}$ are the partial charges on the respective redox site, $q_j$ are the partial charges on the atoms in the environment, and $r_{ij}$ is the distance. Thus, combining Eq. 4a and 4b with Eq. 5a, the relationship between the free energy surfaces and the reaction coordinate defined as the difference in the electrostatic energy between states $R$ and $P$ is

\[ DG_R(\square V) = k_B T \ln[P_R(\square V(r_R))] \]

\[ DG_P(\square V) = k_B \ln[P_P(\square V(r_P))] + DG' \]
where $r_R$ are the distances from the molecular dynamics trajectories of the reactant state, and $r_P$ are the distances from the molecular dynamics trajectories of the product state. The driving force, $\Delta G^\circ$, is given by the relation

$$\Delta G^\circ = k_B T \ln \left( \exp \frac{1}{k_B T} \sum_{R} V_R \right) \tag{7}$$

**Molecular Dynamics Simulations**

Molecular dynamics simulations were carried out using the molecular mechanics package CHARMM27b1 (Brooks et al., 1983), with CHARMM19 parameters (Brooks et al., 1983) with additional parameters for the iron-sulfur redox site as described elsewhere (Beck et al., 1999a). The simulations were carried out in the microcanonical ensemble with a target temperature of 300 K, using the particle mesh Ewald (PME) summation algorithm (Feller et al., 1996). The time step was 1 fs. Cubic boundary conditions of 45 Å x 45 Å x 45 Å were utilized, with a grid spacing of 0.9375 Å, a $\Gamma$-spline coefficient equal to 6, and a $\Gamma$ value of 0.34. All non-polar hydrogens were treated via the extended atom model as part of the heavy atom to which they are attached, and all bonds containing hydrogen were held at their equilibrium bond lengths using the SHAKE algorithm (Rychaert et al., 1977). No atomic polarizability was included and a dielectric constant of one was used throughout the simulations.

The high-resolution crystal structure (0.94 Å) of the fully oxidized structure for Ca Fd was obtained from the Brookhaven Protein Data Bank (Bernstein et al., 1977) (2FDN) (Dauter et al., 1997). In what follows, the donor (D) refers to the site ligated by residues 8, 11, 14 and 47 and the acceptor (A) refers to the site ligated by residues 37, 40, 43, and 18. Two forms of the protein were simulated, $D^3A^2-$ and $D^2A^3-$, which utilized the fully oxidized structure as starting structures but with the appropriate oxidized and reduced potential energy parameters for each site. The protein was completely solvated in a 45 Å x 45 Å x 45 Å box of pre-equilibrated TIP3 (Jorgensen, 1981) water. All
solvent waters within 2.6 Å of any non-hydrogen protein atom or crystal water oxygen were then deleted, resulting in 2782 total waters. Next, the solvent was relaxed slightly by 50 steps of steepest descent energy minimization followed by 2.0 ps of molecular dynamics with Gaussian assignment of velocities every 0.2 ps in which only the solvent was allowed to move and the protein remained fixed. Next, counter-ions [(Pettitt and Rossky, 1986; Hyun and Ichiye, 1997a; Hyun and Ichiye, 1997b) were added by replacing a water molecule with an ion near each charged group (a sodium ion for the negatively charged side chains, the C-terminus, and the redox clusters, and a chlorine ion for the positively charged side chains and the N-terminus) of the protein to make the system net neutral. Thus, the final system consisted of 2765 TIP3 waters, 15 Na+ ions, 2 Cl- ions, and the protein. The solvent environment was equilibrated by fixing the protein while the counter-ion and solvent velocities were propagated for 60 ps, during which time the velocities were scaled every 0.2 ps to a target temperature of 300 ± 5 K. Finally, the entire system was equilibrated, where velocities were assigned to the entire system according to a Gaussian distribution every 200 fs. Following this assignment, the velocities were allowed to scale every 200 fs if the temperature exceeded 300 K ± 5 K until there was no scaling for at least 5 ps. The amount of scaling was approximately 10 ps for both systems. The system was then allowed to run unrestrained for 600 ps. The last 500 ps of data were analyzed.

**Construction of Free Energy Curves**

The electrostatic potential energy at each time step in the trajectory data was calculated using Coulomb’s Law. Using the commercial package MATLAB, differences in the electrostatic potential energy \( \Delta V \) were then calculated. Then, histograms were constructed based on equally spaced energetic values, and the free energy curve for the two systems \( D^3A^2^- \) and \( D^2A^3^- \) calculated via Eq. 6a and 6b. Finally, the free energy
curves from the histograms were fit by least-squares to parabolic functions. The driving force for the electron transfer reaction was calculated from Eq. 7.

RESULTS

Using the difference in the electrostatic potential energy as the reaction coordinate, the parabolic free energy surface of an intramolecular electron transfer reaction was calculated for Ca Fd, where $[4Fe-4S(SR)_4]^3/2$ is referred to as the reactant state, and $[4Fe-4S(SR)_4]^2/2$ is referred to as the product state (Figure 4-3c). The free energy surfaces for two separate contributions, the backbone and polar side chains of the protein (Figure 4-3a), and the solvent, counter-ions, and charged side chains of the protein (Figure 4-3b) were also calculated. The fit to a parabola for the backbone and polar side chains of the protein results in a norm of the residuals of 0.30 kcal/mol for the reactants and 0.25 kcal/mol for the products (Table 4-1). Since the norm of the residuals for the solvent, counter-ions, and charged side chains is 0.37 kcal/mol and 0.65 kcal/mol for the reactants and products, respectively, it appears that the curves for the aqueous solution are less well represented by parabolas than curves for the polar groups in the protein. For the total system, the norm of the residuals is 0.29 kcal/mol and 0.36 kcal/mol for the reactants and the products, respectively.

The various parameters describing the free energy curves were also calculated. The driving force, $\Delta G^\circ$, was calculated from the reaction coordinate (Table 4-2a) using the method of Warshel and co-workers as described in Eq. 7. For the total system, $\Delta G^\circ = -7.9$ kcal/mol. For the separate components, $\Delta G^\circ = -9.3$ kcal/mol for the backbone and polar side chains, and $\Delta G^\circ = -16.0$ kcal/mol for the solvent, counter-ions, and charged side chains. The activation energy was then calculated by finding the intercept of the parabolic functions where the product surface was displaced by the driving force (Table 4-2a). For the total system, $\Delta G^\ddagger = 0.5$ kcal/mol, while for only the protein backbone and polar side chains, $\Delta G^\ddagger = 0.6$ kcal/mol, and for the solvent plus the counter-ions and
charged side chains, $\Delta G^\ddagger = 3.6$ kcal/mol. The reorganization energy, $\mathcal{R}$, could be calculated from the activation energy and the driving force by using the Marcus relation from Eq. 3 (Table 4-2a). For the total system, $\mathcal{R} = 2.2$ kcal/mol but when the backbone and polar side chains are considered separately, $\mathcal{R} = 5.5$ kcal/mol, and when the solvent, counter-ions, and charged side chains are considered separately, $\mathcal{R} = 6.4$ kcal/mol.

Since the calculation of the driving force is probably the least accurate number reported and could benefit from longer simulations, the activation and reorganization energies were also calculated assuming a zero driving force. For the total system, $\Delta G^\ddagger = 0.6$ kcal/mol, while for only the protein backbone and polar side chains, $\Delta G^\ddagger = 1.2$ kcal/mol, and for the solvent plus the counter-ions and charged side chains, $\Delta G^\ddagger = 0.3$ kcal/mol. For the total system, $\mathcal{R} = 2.2$ kcal/mol; for the backbone and polar side chains, $\mathcal{R} = 4.9$ kcal/mol, and for the solvent, counter-ions and charged side chains, $\mathcal{R} = 1.0$ kcal/mol.

The rate of electron transfer, $k$, was calculated using estimated constants (Kyristis et al., 1997) with and without the inclusion of the calculated driving force. For the case where the calculated driving force was included, $k = 1.1 \times 10^{-3}$ s$^{-1}$ for the total system, $k = 8.3 \times 10^{-2}$ s$^{-1}$ for the polar protein groups, and $k = 8.7 \times 10^{-3}$ s$^{-1}$ for the solvent, counter-ions and charged side chains. When the driving force was considered to be zero, $k = 6.7 \times 10^{-3}$ s$^{-1}$ for the total system, $k = 6.3 \times 10^{-3}$ s$^{-1}$ for the protein polar groups, and $k = 1.3 \times 10^{-3}$ s$^{-1}$ for the solvent, counter-ions and charged side chains.

**Discussion**

Ca Fd is known to transfer electrons between the two [4Fe-4S] redox sites, and the intramolecular electron transfer characteristics can be calculated using MD simulations. However, the accuracy of a parabolic free energy surface is questionable, given the high error in the parabolic fit to the data calculated by the norm of the residuals, especially when considering the aqueous solution, in both the contribution of just solvent, counter-
ions, and charged side chains as well as the total system. The free energy curves of the protein backbone and polar side chains seem to be more reasonably fit to a parabolic function. These results indicate that the polar protein groups respond more linearly to the transfer of an electron from one redox site to the other.

The fact that both redox sites in Ca Fd have similar reduction potentials of ~420 mV, and yet intramolecular transfer between these two sites occurs with a high rate (on the order of $10^6$ s$^{-1}$), gives credibility to the results here that the activation energy between the two sites is low. However, the results here also indicate a driving force between the two redox sites, which is inconsistent with the similar reduction potentials found in experimental work. Indeed, a significant amount of this driving force appears to be contributed by the protein backbone and polar side chains (Table 4-2a), and not just the aqueous solution and counterions (which would simply indicate a lack of equilibration.) Overall, these inconsistencies indicate a need for longer simulation times, which would yield more accurate statistics.

The activation energy and reorganization energy for the entire system are relatively low, indicating that the electron transfer should be fast. The separate contributions of the polar part of the protein and the aqueous solution plus charged side chains are not additive since there is obviously coupling between these contributions. However, the activation energy and reorganization energy due to the protein backbone and polar side chains alone are very low (Table 4-2a), which is consistent with the fact that the protein backbone is constrained and cannot reorganize freely upon transfer of an electron between redox sites. Likewise, the activation energy and reorganization energy due to the aqueous solution are higher, which is consistent with the ability for the solvent and counter-ions to freely reorganize upon transfer of the electron from one redox site to the other.

Finally, the rates of electron transfer are several orders of magnitude lower than that of the experimental measurements, where the calculations give rates on the order of
10^{-3} \text{ s}^{-1}, and experimental rates are approximately 6 \times 10^6 \text{ s}^{-1}. The fact that these rates are significantly lower than the experimental rate is a further indication of the need for longer simulation times. However, it is important to note that the ability to calculate the rate of transfer allows for experimental verification of the calculations, and thus can be used as a benchmark for future computational work.

The most important aspect of this work is that it has been demonstrated here that the properties for intramolecular electron transport can be calculated using molecular dynamics simulations. In previous work, a specific residue position referred to as the “Cys\textsuperscript{x}” position was shown to predictably shift the reduction potential of ferredoxin (Dolan & Ichiye, unpublished). The calculation of the characteristics of the electron transfer reaction using mutations at the Cys\textsuperscript{x} position will lead to a further understanding of fundamental principles of electron transfer proteins, specifically, what role the protein matrix has in manipulating the reaction.

**CONCLUSIONS**

The results presented here show that the electron transfer properties of Ca Fd can be calculated using MD simulations, although better statistics from longer simulations is warranted. However, two important results are apparent from these calculations. First, a driving force apparently exists between the two [4Fe-4S] redox sites in Ca Fd, even though the measured redox potentials of the sites are highly similar (\sim 420 \text{ mV}). This indicates a need for increased sampling provided by an increase in the simulation time. Second, the calculated rate of transfer is significantly lower, on the order of $10^{-3} \text{ s}^{-1}$, than the measured rate of transfer between the redox sites in Ca Fd, which is on the order of $10^6 \text{ s}^{-1}$. Finally, the fact that the properties of the intramolecular electron transfer reaction in ferredoxin can be calculated will allow the future investigation of how specific amino acids affect the transfer properties, specifically that of the Cys\textsuperscript{x} position discussed in Chapter 3.
ACKNOWLEDGMENTS

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REFERENCES


Table 4-1: Norm of the residuals for parabolic least squares fit to $\Delta G^\circ$ data

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<th>BB+PSC</th>
<th>SOLV+CI+CSC</th>
<th>Total</th>
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<tr>
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<tr>
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Table 4-2a: Transfer parameters for the electron transfer reaction

<table>
<thead>
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</tr>
</thead>
<tbody>
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Table 4-2b: Transfer parameters for the electron transfer reaction assuming the driving force is zero

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</tr>
</thead>
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<td>0.0</td>
</tr>
<tr>
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<tr>
<td>$\Delta$(kcal/mol)</td>
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<td>2.2</td>
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<tr>
<td>$k$(s$^{-1}$)</td>
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<td>1.3E-3</td>
<td>6.7E-3</td>
</tr>
</tbody>
</table>
FIGURE 4-1 Ribbon diagram of *Clostridium acidurici* ferredoxin containing an 8-strand pseudo-symmetric backbone fold.
FIGURE 4-2 A schematic representation of potential energy curves for the electron transfer reaction $D+A \rightarrow D^+ + A^-$, where $D$ indicates the donor and $A$ indicates the acceptor, $\Delta G^\circ$ is the driving force, $\Delta G^\ddagger$ is the activation energy barrier, and $\lambda$ is the reorganization energy.
Free energy surfaces for 1 e- transfer plus best fit parabolas of BB+PSC

Free energy surfaces for 1 e- transfer plus best fit parabolas of SOLV+CSC+c

Free energy surfaces for 1 e- transfer plus best fit parabolas of total
FIGURE 4-3 Data plotted from histograms into free energy surfaces, where open circles indicate the data from the reactant state and crosses indicate the data from the product state. Parabolic fits using a least-squares fitting routine are indicated by a solid line for the reactant state and a dashed line for the product state. The free energy data is furthermore delineated into three components where (A) is the contribution from the protein backbone and polar side chains, (B) is the contribution from the solvent, counterions and charged side chains, and (C) is the contribution of the total system.
FIGURE 4-4  Data plotted from histograms into free energy surfaces with the product state displaced by the driving force ($\Delta G^\circ$), where open circles indicate the data from the reactant state and crosses indicate the data from the product state. Parabolic fits using a least-squares fitting routine are indicated by a solid line for the reactant state and a dashed line for the product state. The free energy data is furthermore delineated into three components where (A) is the contribution from the protein backbone and polar side chains, (B) is the contribution from the solvent, counter-ions and charged side chains, and (C) is the contribution of the total system.
CHAPTER FIVE

CONCLUSIONS

In this work, the role of the protein matrix in influencing the reduction potential of electron transfer proteins was examined using classical molecular dynamics computer simulations. Iron-sulfur proteins, a class of electron transfer proteins found ubiquitously throughout living organisms, were used to calculate the electrostatic potential between the redox site and the protein environment, where the electrostatic potential can be related to the reduction potential. Specifically, calculations using Cp Rd indicated that a permanent polarization is associated with the backbone fold of this electron transfer protein, such that the redox site is a more favorable electron acceptor. Additionally, mutations at a single residue position, referred to as the Cys\textsuperscript{x} position, were shown to predictably shift the reduction potential in Pa Fd, where cysteine was found to decrease the reduction potential relative to alanine, and serine was found to increase the reduction potential relative to alanine. Finally, the characteristics of the intramolecular electron transfer process of Ca Fd were calculated through the calculation of the parabolic free energy surfaces of the reactants and the products. The fast rate associated with this transfer was linked to the low activation energy of the reaction, and a driving force appeared to exist for the reaction, although the reduction potential of the two sites is nearly identical.