Pressure-Controlled Voltammetry of a Redox Protein: An Experimental Approach to Probing the Internal Protein Dielectric Constant?

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Equilibrium reduction potentials of recombinant Pyrococcus furiosus *rubredoxin, a low molecular weight redox protein, are determined as a function of pressure directly at a pyrolytic graphite electrode using cyclic voltammetry. The observed changes in reduction potentials are discussed in terms of protein and solvent electrostatic interactions.*

Electron transport is a fundamental process by which virtually all organisms obtain energy. For this reason, biochemists are interested in electrochemically characterizing proteins that make up electron transport pathways. They are also interested in understanding at a molecular level how a protein's structure influences its electron transfer function. In this study, the electron transfer protein rubredoxin **(F1)** from the deep-sea microorganism *Pyrococcus furiosus* was electrochemically characterized. Formal equilibrium reduction potentials (E°´s), which represent an important aspect in the thermodynamic analysis of biological electron transfer reactions, were measured as a function of pressure. We are interested in determining and rationalizing how electron transfer equilibrium properties are influenced by pressure. In an earlier study presented in this journal, it was suggested that temperature-controlled electrochemical studies provide useful information concerning electrostatic interaction energies in proteins (1). We suggest that such information can also be obtained from pressure-controlled electrochemical studies.

Reduction potentials at ambient pressures have been measured for many electron transfer proteins using a variety of voltammetric methods and various modified and unmodified solid electrode surfaces (2). Advantages of direct electrochemical methods include low cost, rapidity and nondestructive nature of the measurements, and small sample volume and low concentration requirements. In order to further our understanding of biological electron transfer reactions, an electrochemical cell was specifically designed to measure equilibrium reduction potentials as a function of pressure **(F2)**. Pressure-controlled electrochemical experiments provide important information concerning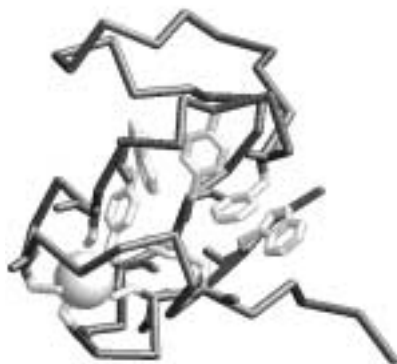 the role of protein/solvent interactions on electron transfer, as well as information concerning changes in electrostatic interaction energies of electron transport proteins.

While pressure-dependent reduction potentials have been reported for an electron transfer protein (3), few studies have been done to understand these observed changes at a molecular level (4, 5). It has long been recognized that electrostatic interactions are an important determinant of enzymatic and electron transfer reactivity. In simple terms, coulombic interaction energies, which contribute to the magnitude of the reduction potential, are directly related to the magnitude of charge and inversely related to the distance between charges and the screening of the medium which separates the charges. Coulombic interaction energies between two charged particles i and j in a homogenous medium can be calculated as follows:
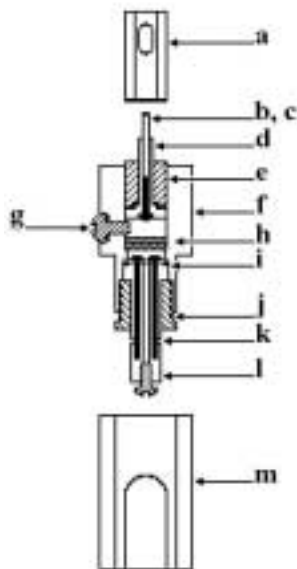
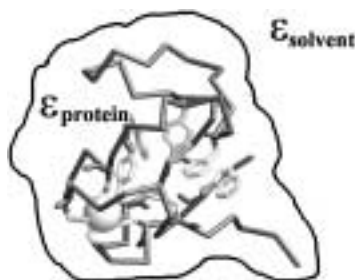$$E = q_i q_j / \varepsilon_{ij} r_{ij} \qquad \textbf{(E1)}$$

F1

Cartoon of Pyrococcus furiosus rubredoxin illustrating peptide backbone (black), aromatic and cysteinyl side-chains (grey), and non-heme iron atom.
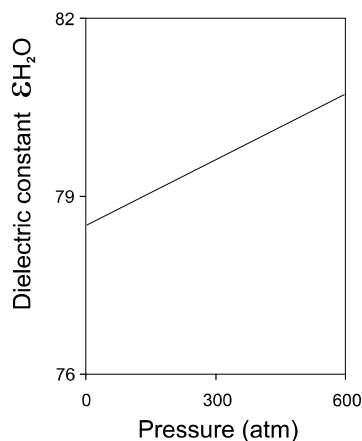
F2

Cross section view of pressure-controlled electrochemical cell indicating upper jacking component (a); stainless steel rod (b); pyrolytic graphite working electrode (c, illustrated in black); upper Delrin sheath (d); stainless steel piston (e); cell body (f); sample port (g); stainless steel frits (h); stainless steel retaining ring (i); threaded brass fitting (j); Ag/AgCl reference and counter electrodes (k, illustrated in black); lower Delrin sheath (l); and lower jacking component (m). (Modified from ref. 4.)

F3

Cartoon representation of Pyrococcus furiosus rubredoxin illustrating the distinct spatial regions with a low dielectric constant (ε protein) and high dielectric constant (ε solvent).

F4

Dielectric constant of water as a function of pressure (linear regression of experimental values listed in ref. 12).

where q is the charge, ε is the dielectric constant, and r is the distance between the two charges. Electrostatic interaction energies are much more difficult to determine for biomolecules that typically contain thousands of partially charged atoms that are immersed in media with different dielectric constants.

Macroscopic continuum models, which are essentially based on **E1**, have been used to successfully reproduce experimental changes in reduction potentials of proteins due to site-specific mutations (6). These relatively sophisticated models allow for the dielectric constant and charge density to vary in space (7-9). The protein is mapped onto a three-dimensional grid. The distances between atoms (r) are determined from X-ray crystal structures, and atomic partial charges (q) are assigned to individual atoms based on molecular mechanical simulations. The dielectric constant of both the protein ($\varepsilon_{protein}$) and the solvent ($\varepsilon_{solvent}$) are assigned to specific regions in space as defined by the structure of the protein, and this is illustrated schematically in **F3**. The solvent dielectric constant has been previously determined to vary slightly as a function of pressure (**F4**) (10). In contrast, the protein dielectric constant is not known; however, it is believed to be low (8, 9). This assumption is based on the belief that in the presence of an electric field, the reorientation of protein dipoles is highly restricted.

As illustrated in **F3**, the interior of rubredoxin contains a number of tightly packed aromatic residues. It

has been previously and generally assumed that the interior of all proteins is an "oil drop" with a very low dielectric constant. A value of the internal protein dielectric constant ($\varepsilon_{protein}$) for all proteins has been estimated to be between 2 and 4 based on dielectric measurements of polyamides (8,9). In this study, we present an indirect method for determining a reasonable value for the internal protein dielectric constant through simulations of the pressure-dependent reduction potentials.
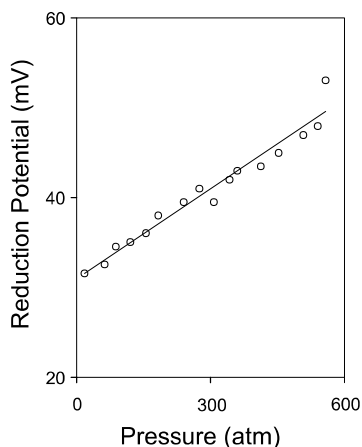
A novel application of the continuum macroscopic model is used to help rationalize the role of pressure on equilibrium reduction potentials. We simulate the observed changes in reduction potential as a function of pressure by varying the dielectric constant of both the protein and the solvent. These calculations are based on the previous assumption that the distance between charges (r) and the magnitude of charges (q) remains essentially pressure-independent (4, 5). The dielectric constants of the solvent are assigned appropriate values as illustrated in **F4**, and the internal protein dielectric constant is changed in order to reproduce the experimental results. Our group has successfully reproduced experimental changes in reduction potential as a function of temperature using a similar approach (11).
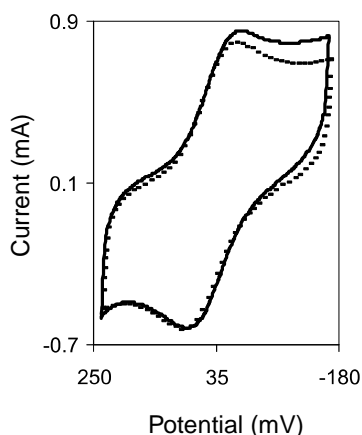
Procedures

Recombinant *P. furiosus* rubredoxin was isolated as previously described (12). A 2.0 ml solution containing 0.5 mM recombinant *P. furiosus* rubredoxin in 25 mM phosphate, pH 6.7, and 0.05 M $MgCl_2$ as an electrode promoter in a stoppered 8 ml vial was made anaerobic by cycling between a vacuum and nitrogen gas. The sample and reference solution (0.100 M NaCl) were then transferred under anaerobic conditions using an anaerobic glove box into the appropriate sample compartment of the electrochemical cell, avoiding the introduction of gas bubbles.

The cross-sectional view of the pressure-controlled stainless steel electrochemical cell is shown in **F2**. This electrochemical cell was originally designed and tested to maintain an anaerobic sample at pressures from 1 to 600 atm (4). In this study, the original sample port was replaced with a tiny bolt to insure no leaking occurred at high pressure. A vise purchased from a local hardware store was used instead of a hydraulic jack to minimize problems with drifting in the pressure measurements. The entire electrochemical cell assembly as illustrated in **F2** was placed within a vise; a load was applied and monitored via a load cell as previously described (4). The applied pressure was calculated by dividing the load by the known surface area of the piston and converted to units of atmospheres.

Reduction potentials were determined by direct electrochemistry, a method that entails the unmediated,

interfacial reduction and re-oxidation of a redox molecule at an electrode surface. At least two cyclic voltammograms were recorded at each pressure utilizing a BAS CV-50W potentiostat. Cyclic voltammograms were determined to be reversible at scan rates below 30 mV/sec, and scan rates from 5-30 mV/sec were used throughout the study. Reduction potentials from each cyclic voltammogram were determined from the midpoint between the anodic and cathodic peak currents. The pressure-dependency of the reduction potential of the Ag/AgCl reference electrode was previously determined to be -0.003 mV/atm (13). All reduction potentials were normalized for pressure and reported versus SHE as previously described (4). Simulations of reduction potentials as a function of pressure were made using the macroscopic continuum model, Delphi (Columbia University, 1989), using appropriate values of the dielectric constant according to previously described methodology (11).

Results and Discussion

The reduction potential of *P. furiosus* rubredoxin was determined to increase 0.033 mV/atm as illustrated in **F5**. It is noted that the reduction potential of the Ag/AgCl reference electrode was previously determined to change -0.003 mV/atm (13). Representative cyclic voltammograms of rubredoxin at two different pressures are illustrated in **F6**. These data indicate that the protein is easier to reduce as the pressure increases. At a macroscopic level, the variation in the reduction potentials reflects the changes in electrostatic interaction energies resulting from changes in pressure. The stability of each oxidation state is dependent on the ability of protein/solvent interactions to neutralize excess charge as function of pressure. For instance, if solvent molecules reorient around an electron transfer center to more efficiently solvate excess charge at high pressure, this oxidation state would

be favored under those conditions. It is expected that the screening of charges would be more efficient at higher pressures, and this more efficient screening is reflected by a higher dielectric constant under those conditions. Notably, the solvent dielectric constant increases as pressure increases, as shown in **F4**.

At low pressure, the solvent dielectric constant is lower, and therefore the solvent is likely to be less efficient at solvating excess charge. Thus, the oxidation state of the protein with the least amount of excess charge is more likely to be the most stable at low pressures. Indeed, the data indicate that the oxidized state of the electron transfer center of rubredoxin, $[FeS_4]^{-1}$, is more stable than the reduced state, $[FeS_4]^{-2}$, at lower pressures. Moreover, as pressure is decreased, the ordering or shielding of the solvent molecules around a charged electron transfer center of the protein decreases and hence charged atoms are "felt" more strongly near the electron transfer center. It would be expected that at low pressure, it would require more work to bring an electron to an electron transfer center that is surrounded by atoms with a net negative charge. The observation that it is harder to reduce rubredoxin at low pressure is consistent with the fact that the protein is known to be very acidic (14).

The dielectric constant of water increases, but does not change significantly as a function of pressure from 1-600 atm ($\Delta\varepsilon_{solvent} < 4\%$, see **F4**). It has been previously assumed that the dielectric constant of a protein is low ($\varepsilon_{protein} < 4$) based on dielectric measurements of polyamides and since the reorientation of protein dipoles in response to an electric field is expected to be highly restricted (8,9). The dielectric constant of a protein would be expected to be even less pressure-dependent than that of a polar solvent such as water (i.e., $\Delta\varepsilon_{protein} << 4\%$ from 1-600 atm). Therefore, it is likely that the internal protein dielectric constant ($\varepsilon_{protein}$) is essentially

pressure-independent from 1-600 atm. Using a pressure-independent internal protein dielectric constant, the electrostatic model Delphi can reproduce the pressure-dependent reduction potential of rubredoxin as illustrated in **F5**. However, $\varepsilon_{protein} \approx 5$ was required to reproduce the experimental changes. The pressure-dependent reduction potential could not be reproduced using $\varepsilon_{protein} \leq 4$. Low values of the internal protein dielectric constant overestimated the experimental changes.

The value of the internal protein dielectric constant ($\varepsilon_{protein}$) is not known; it is not readily measured, and it has been previously assumed to be low ($\varepsilon_{protein} \leq 4$) (8,9). In contrast, we have previously presented an indirect method for estimating the value of the internal protein dielectric constant using electrostatic simulations of temperature-controlled electrochemical studies (11). The internal protein dielectric constants for a variety of electron transfer proteins were estimated to be large ($\varepsilon_{protein} > 4$). In our previous study (11), both the dielectric constant of the solvent ($\varepsilon_{solvent}$) and protein ($\varepsilon_{protein}$) were assumed to change with temperature. The internal protein dielectric constant was changed in order to reproduce the experimental change in reduction potential of the protein as a function of temperature.

In this pressure-controlled electrochemical study, it was assumed that the internal protein dielectric constant is essentially pressure-independent (see discussion above). Thus, the change in reduction potential of the protein as a function of pressure is believed to result from changes in the dielectric constant of the solvent. The value of the internal protein dielectric constant is then estimated based on electrostatic simulations that reproduce the experimental change in reduction potential as a function of pressure. The higher internal protein dielectric constant ($\varepsilon_{protein} > 4$) necessary to reproduce the experimental change in reduction potential of rubredoxin as a function of pressure is consistent with our findings in earlier temperature-controlled electrochemical studies of rubredoxin and other redox proteins (11). Pressure-controlled electrochemical studies may prove to be another useful method for estimating the value of the internal dielectric constant of a protein. Future electrochemical studies may show that the dielectric constant of a protein is higher than previously assumed (8,9), and that it may vary from protein to protein.

An important consequence of changes in solvent-solute interactions induced by pressure is that the electrostatic environment of a redox center is perturbed. These changes alter the electrostatic interaction energy between atoms in a protein, which determines the reduction potential. Our group has previously noted the relationship between the slopes of pressure/reduction potential profiles (as well as temperature/reduction potential profiles) and the solvent dielectric constant for cytochrome c (4). The stability of each oxidation state is dependent on the ability of protein/solvent interactions to neutralize excess charge as a function of pressure. At low pressure, the solvent and protein dielectric constants are lower, and therefore both the solvent and protein are likely to be less efficient at solvating excess charge. The change in the amount of work required to bring an electron to the electron transfer center, through the perturbation of the dielectric constant of the medium, is reflected in the experimentally measured formal equilibrium reduction potential (E°´s).

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