# Probing Protein Electrostatic Interactions by Direct Electrochemistry

*Qiandong Zeng, Donald M. Kurtz, and Robert A. Scott Center for Metalloenzyme Studies, University of Georgia, Athens, GA 30602*

*Laura L. Popovich and Eugene T. Smith* Department of Chemistry, Florida Tech, Melbourne, FL 32901*

** Corresponding author. Phone: (407) 768-8000 Ext. 7310, Fax: (407) 984-8461, E-mail: smithg@fit.edu*

*Equilibrium reduction potentials of recombinant* Clostridium pasteurianum *rubredoxin, a low molecular weight redox protein, are determined directly at a pyrolytic graphite electrode using cyclic voltammetry. The changes in reduction potential, due to site-mutations containing charged amino acid side chains near the electron transfer site, have been determined. These changes are discussed in terms of electrostatic interactions.*

Direct electrochemical methods for the study of electron transfer proteins has been used extensively for examining equilibrium properties of biomolecules (1). This technique entails the unmediated potentiometric equilibration between a protein and electrode surface. Its growing popularity has been attributed to low cost, rapidity of measurements, and minimal sample volume and concentration requirements. In this study, direct electrochemistry is utilized to probe the relationship between equilibrium electron transfer properties and protein structure. Specifically, we examine by direct electrochemistry the role of charged amino acid side chains in close proximity to the redox site of a bacterial electron transfer protein, *Clostridium pasteurianum* rubredoxin. Site-directed mutagenesis was used to generate recombinant proteins with altered surface charges. Rubredoxin serves as an excellent model system for probing the influence of protein structure on electron transfer, because it is relatively small (about 55 amino acids), it contains a relatively uncomplicated nonheme iron electron transfer site, and its tertiary structure is known. In this study, direct electrochemistry was used to determine the effect of replacing a neutral amino acid near the electron transfer site (valine-8) with a negatively charged (aspartic acid) and a positively charged (arginine) amino acid side chain.

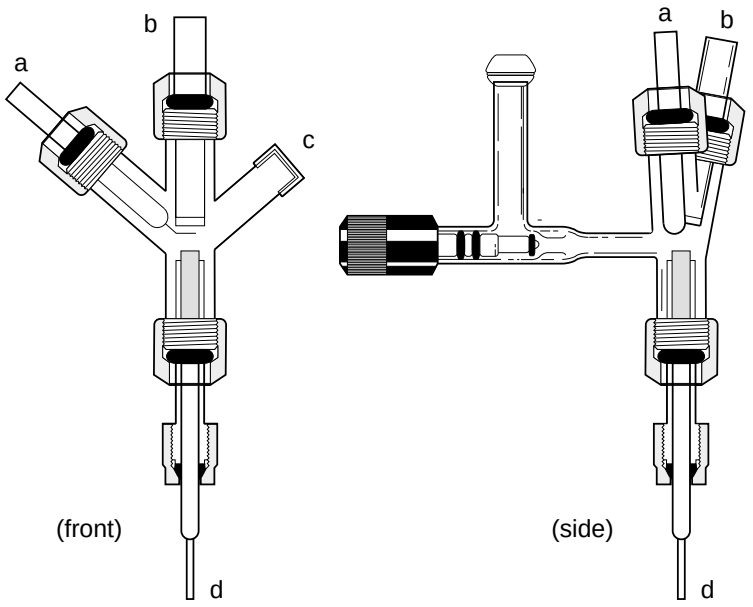
## Procedures

The microelectrochemical cell shown in ***F1*** (the operation of which is described in ref. 3) is composed of a pyrolytic graphite working electrode, platinum counter electrode, and Ag/AgCl reference electrode. Prior to each experiment, the pyrolytic graphite electrode was polished in figure eights across a 60 µm aluminum oxide polishing wheel (Buehler, Lake Bluff, II) moistened with distilled water. The electrode was then rinsed with distilled water, and residual graphite was removed with lint free tissue. The assembled electrochemical cell was flushed with nitrogen to remove oxygen.

Generation of the site-directed mutants and their purification have been described (2). Rubredoxin with the native sequence, a valine-8 to a negatively charged aspartatic acid (V8D) variant, and a valine-8 to a positively charged arginine (V8R) variant were examined. The location of valine-8 relative to the non-heme iron electron transfer site is illustrated in ***F2***. A 10-20 µL anaerobic sample of approximately 100 µM protein and 20 mM phosphate, pH 7, in a Hamilton gas tight syringe was inserted through the cell septum and injected in the cell to form a drop between the working and reference electrode. Next, 2 µL of a supporting electrolyte, 1 M $MgCl_2$, was anaerobically added to this droplet. Cyclic voltammograms of rubredoxins were recorded util-

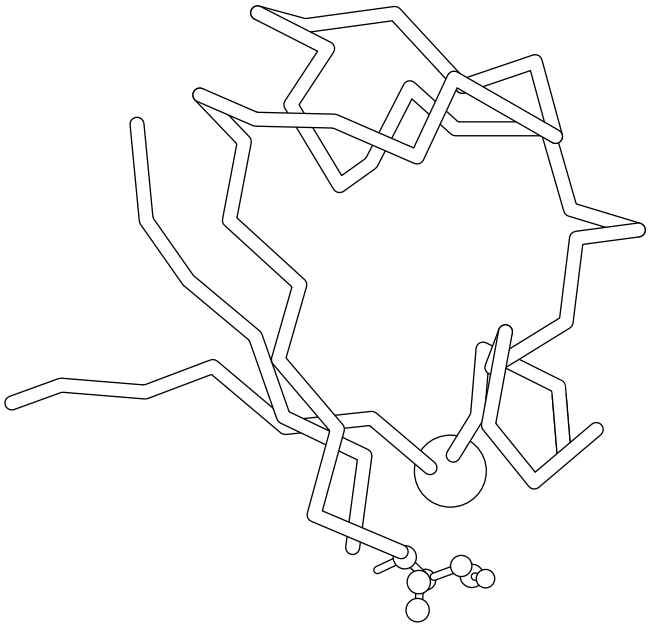
**F1**

*Cross section view of microelectrochemical cell depicting: (a) pyrolytic graphite electrode, (b) platinum counter electrode, (c) Ag/AgCl reference electrode, and (d) septum (adapted from ref. 1).*



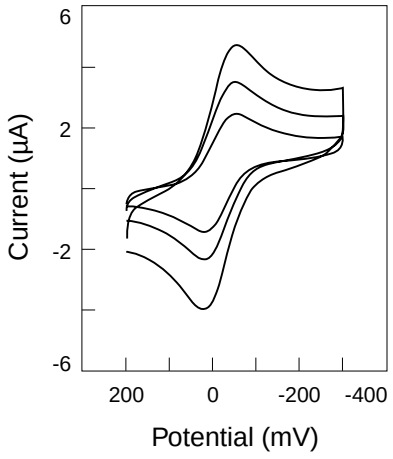

**F2**

*Illustrations of C. pasteurianum rubredoxin backbone, valine-8 as ball-and-stick, and non-heme as spacefill representation.*



**F3**

*Cyclic voltammograms of 100 μM recombinant valine-8 to arginine (V8R) rubredoxin ($E^{o'}$ = -15 mV) in 20 mM phosphate, pH7 and 0.1 M $MgCl_2$. Scan rate was 5, 10, and 20 mV/sec. in order of increasing current.*




izing a CV-50W BAS voltammetric analyzer from 2 - 200 mV/sec. All reduction potentials are reported versus NHE.

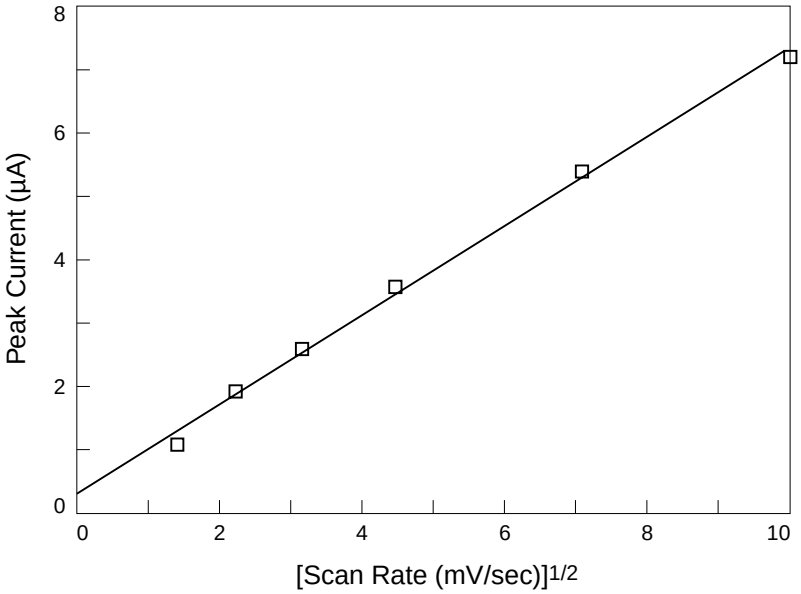
### *Results and Discussion*

Voltammograms of recombinant V8R *C. pasteurianum* rubredoxin at various scan rates are shown in ***F3***. It was determined that electron transfer between the electrode and protein was rapid and essentially reversible based on the potential difference between the cathodic and anodic peak currents ($\Delta E_{pc,pa}$ < 70 mV). The direct relationship between peak current versus square root of the scan rate is illustrated in ***F4***. The reduction potentials were determined to be: wild type, $E^{o'}$ = -55 mV; V8R, -15 mV; and V8D, -28 mV (2).

It has long been recognized that electrostatic interactions are an important determinant of protein reactivity. In many cases, these interactions can be rationalized in simple coulombic terms (4). Placement of a negatively charged residue near an electron transfer center, for example, typically results in the lowering of the reduction potential. More work is required to bring an electron to the electron transfer site due to electrostatic repulsion. In contrast, placement of a positively charged residue near the electron transfer center typically stabilizes the reduced electron transfer center, and results in an increase in the reduction potential. Notably, the change in reduction potential due to the substitution of a positively charged residue near the electron transfer center of rubredoxin (V8R) increased its reduction potential by 40 mV. However, in contrast to expectations based on simple coulombic interactions, the placement of a negatively charged residue in this same position also resulted in an increase in reduction potential of rubredoxin by 27 mV. Although the positive change in reduction potential for a negatively charged residue is less than that for the positively

**F4**

*Cathodic peak current of recombinant valine-8 to arginine (V8R) rubredoxin versus square root of scan rate.*




charged residue, it is clear that the net charge of amino acid side chains alone can not be used to explain the magnitude and direction of change in its equilibrium reduction potential. It has been proposed (2) that solvent exposure of the electron transfer site may have been perturbed by these mutations and further analysis of these results awaits the structural determination of the recombinant proteins.

Although the role of electrostatic interactions on equilibrium electron transfer reactions is not fully understood, it is clear that direct electrochemical studies provide a powerful and convenient method for the measurement of important thermodynamic parameters. Additional structural information of recombinant proteins should provide insight as to the molecular basis for the observed changes in reduction potential.

### Acknowledgment

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### References

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