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LETTERS

Reorganization Energies of the Individual Copper Centers in Dissimilatory Nitrite Reductases: Modulation and Control of Internal Electron Transfer

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Knowledge of the parameters controlling the electron transfer reactivity of the redox centers in copper proteins remains elusive despite the wealth of data accumulated over the years. Here, we present the first experimentally based estimate of the reorganization energies of copper(II/I) ions bound to sites which confer on them unique functional properties: type 1 (T1) and type 2 (T2). The former functions as an electron mediator in a wide range of electron carriers and enzymes, while type 2 serves primarily in direct interaction with substrates. Comparing the kinetics of intramolecular electron transfer in copper containing nitrite reductases (CuNiRs), the reorganization energy of the former center is now shown to depend on the symmetry of the coordination sphere, and thus, the more asymmetric flattened tetrahedral T1 site of a green CuNiR gives rise to a smaller reorganization energy (0.6 eV) when compared with the distorted tetrahedral geometry of a blue CuNiR T1 center (0.7−0.8 eV). It is noteworthy that it is still larger than that of the binuclear (purple) Cu_A center, which was found to be 0.4 eV when inserted in a mutated azurin. The tetragonal distortion, possibly arising from small shifts in the loop carrying the Met ligand in the blue and green enzymes, emphasizes the subtle, yet important, role of the protein structure in determining its reactivity.

The parameters controlling the electron transfer (ET) reactivity of the redox centers in copper proteins are still under debate despite the wealth of data accumulated over the years. Here, we present the first experimentally based estimate of the reorganization energies of the T1 and T2 copper sites involved in intramolecular ET in copper containing nitrite reductases (CuNiRs). This highly conserved family of bacterial enzymes catalyzes the one-electron reduction of nitrite to nitric oxide as part of the geo-biological nitrogen cycle:[1]

$$NO_2^- + e^- + 2H^+ \rightarrow NO + H_2O$$

Three-dimensional structures have been determined for NiRs from several sources, and the overall structures are very similar.[2] The proteins are shown to be homotrimers with a molecular mass of 109 kDa and with two copper ions per monomer, which constitutes the catalytic unit. One copper ion is bound to a T1 site, while the second is bound to a T2 site. The T1Cu(II) site

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TABLE 1: Kinetic and Equilibrium Data for the Internal ET in CuNiRs (pH 7.0, 5 mM Phosphate, 10 mM 1-Methylnicotinamide, 0.1 M *tert*-Butanol)

protein	k_{298}/s^{-1}	$-\Delta G°$/eV	$\Delta G^{\neq}$/eV	$\Delta S^{\neq}$/meV/T	λ_{tot}/eV	λ_{T1}/eV
blue *Ax*NiR[a]	450 ± 30	−0.009	0.32	−1.31 ± 0.11	1.26 ± 0.08	0.77 ± 0.05[b]
green *Ac*NiR	1030 ± 80	−0.019	0.30	−1.09 ± 0.09	1.16 ± 0.07	0.57 ± 0.07

[a] Reference 7. [b] References 12 and 13.

exhibits a strong absorption in the visible region, while the T2 center does not contribute significantly. Small differences in the ligand geometry of the T1 site, evident from the 3-D structures of *Alcaligenes xylosoxidans* nitrite reductase (*Ax*NiR) and *Achromobacter cycloclastes* nitrite reductase (*Ac*NiR), determine whether the protein appears blue (as in *Ax*NiR, $\epsilon_{595} \sim 6300$ M^{-1} cm^{-1})[3] or green when an additional strong absorption at lower wavelength is present (as in *Ac*NiR, $\epsilon_{458} \sim 4800$ M^{-1} cm^{-1}).[4] These differences also result in the blue NiRs exhibiting an axial electron paramagnetic resonance (EPR) signal, as seen in plastocyanin and azurin, in contrast to the rhombic EPR signal of the green NiRs. The T1 site serves as the electron uptake site from azurin or pseudoazurin, while binding and reduction of nitrite takes place at the T2 site. Thus, the internal T1 → T2 ET is an essential part of the catalytic cycle, and the T1Cu(I) to T2Cu(II) ET in NiRs isolated from different bacteria has been a subject of earlier studies.[5−8] Previously, the reorganization energy of the T2 copper site was derived from studies of this ET reaction in *Ax*NiR.[8] To resolve the impact of subtle structural differences in the copper sites on their reorganization energies, we have now extended these studies to the intramolecular ET in the green enzyme isolated from *Achromobacter cycloclastes* IAM 1013.[9] The kinetics were studied by pulse radiolysis over a temperature range from 4 to 40 °C where the T1Cu(II) was reduced by 1-methylnicotinamide radicals in an essentially diffusion-controlled process followed by an intramolecular ET equilibration between the two copper centers.

The semiclassical Marcus theory for nonadiabatic intramolecular ET reactions predicts that the rates are governed by the driving force of the reaction ($-\Delta G°$), the nuclear reorganization energy (λ_{tot}), the distance separating the electron donor (D) and acceptor (A), and the electronic coupling (H_{DA}) between D and A at the transition state.[10]

$$k = \frac{2\pi}{\hbar}\frac{{H_{DA}}^2}{(4\pi\lambda_{tot}RT)^{1/2}}\,e^{-(\Delta G°+\lambda_{tot})^2/4\lambda_{tot}RT} \qquad (1)$$

The ET pathway in both blue and green NiRs is very short, consisting of the T1Cu ligand Cys136 and the neighboring His135 ligand of T2Cu (*Ax*NiR numbering), altogether 11 covalent bonds corresponding to a 1.26 nm separation of the two Cu ions (Figure 1). Rate constants and driving forces for internal ET of the green *Ac*NiR, determined from the temperature dependence of the reaction in this study, are given in Table 1 together with those of the blue *Ax*NiR published earlier.[7] It is noteworthy that the green enzyme exhibits a higher rate constant than the blue enzyme despite a lower driving force ($-\Delta G°$) in the former protein. Thus, the enhanced rate may be rationalized by differences in reorganization energies. It is further interesting that the activation entropy has a smaller magnitude in the green CuNiR as compared with the blue protein. Since the activation entropy includes an electronic term,[10] it means that electron tunneling to the green copper center is slightly more advantageous. Gray and co-workers have determined the rates of bond-mediated electron tunneling in modified iron−sulfur proteins, one where the electron donor and the electron acceptor are separated by a Cys-His bridge, as found in CuNiRs.[11] The observed rates are essentially coupling-limited; that is, $k_{max} \sim 2 \times 10^7$ to 2×10^8 s^{-1}.

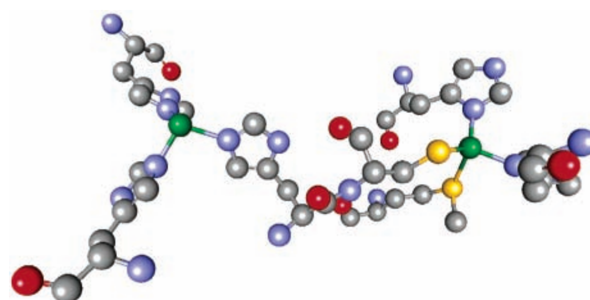

Figure 1. Calculated ET pathway between the T1 (right) and T2 (left) copper ions in *Ac*NiR. The figure includes the coordination spheres of the two centers. The coordinates were taken from the Protein Data Bank, ID no.1NDT.

The reorganization energy of intramolecular ET in the green *Ac*NiR can now be calculated using eq 1 above and gives $\lambda_{tot} = 1.16 \pm 0.07$ eV. We earlier determined the rate of intramolecular ET in the blue *Ax*NiR[7] (see Table 1) for which we find $\lambda_{tot} = 1.26 \pm 0.08$ eV.

Gray et al. previously found the reorganization energy of the T1 copper center to vary from 0.72 eV in plastocyanin[12] to 0.82 eV in azurin.[13] Comparison of the 3-D structures of the blue sites of azurin, plastocyanin, and *Ax*NiR shows similar geometries and metal ligand distances. Thus, we can safely assume a reorganization energy of the T1 center in the blue *Ax*NiR in the same range, and we calculate the reorganization energy of the T2 copper center from the relation $\lambda_{tot} = \lambda_{T1}/2 + \lambda_{T2}/2$ and find $\lambda_{T2} = 1.75$ eV. The reorganization energy of this copper site is thus much larger than that calculated for T1. This is also expected since the T2 center is solvent accessible, as it is involved in nitrite binding, reduction, protonation, and product release. Nonetheless, the reorganization energy calculated here for the T2 copper center is still below the values quoted for low molecular weight copper complexes. Thus, for $Cu(phen)_2^{2+/+}$, the reorganization energy has been determined to be 2.4 eV.[14]

Although the T1 center of the green *Ac*NiR exhibits the "classic" coordination sphere of a T1 center with an axial S(Met), the distortion of the site weakens the Cu−S(Cys) bond, as demonstrated by the spectral features with a decrease in the dominant S(Cys)π → Cu(II) charge transfer intensity together with a more significant S(Cys)σ → Cu(II) intensity[15] which causes an increased absorption around 450 nm, changing the color to green.

Since the structures of the T2 domains of *Ac*NiR and *Ax*NiR are essentially identical,[2] we expect the reorganization energies, λ_{T2} values, to be equal in the two proteins, and from the λ_{tot} value determined for the distorted green copper center in *Ac*NiR, we calculate $\lambda_{T1} = 0.57 \pm 0.07$ eV. Thus, the more asymmetric flattened tetrahedral T1 site of the green CuNiR gives rise to a lower reorganization energy when compared with the distorted tetrahedral geometry of this site in blue NiRs. It is noteworthy that its value is still larger than that of the binuclear (purple)

Cu_A center, which was found to be 0.4 eV when inserted in a mutated azurin.[16]

In conclusion, the tetragonal distortion, possibly arising from small shifts in the loop carrying the Met ligand in the blue and green enzymes, emphasizes the subtle, yet important, role of the protein structure in determining not only the geometric and electronic structure of the T1 site but also its reactivity. Further studies on the impact of structure on driving force and reorganization energy in proteins is clearly needed, and we are presently studying intramolecular ET in a series of green CuNiR mutants.

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