Met144Ala mutation of the copper-containing nitrite reductase from *Alcaligenes xylosoxidans* reverses the intramolecular electron transfer

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Abstract Pulse radiolysis has been employed to investigate the intramolecular electron transfer (ET) between the type 1 (T1) and type 2 (T2) copper sites in the Met144Ala *Alcaligenes xylosoxidans* nitrite reductase (*Ax*CuNiR) mutant. This mutation increases the reduction potential of the T1 copper center. Kinetic results suggest that the change in driving force has a dramatic influence on the reactivity: The T2Cu(II) is initially reduced followed by ET to T1Cu(II). The activation parameters have been determined and are compared with those of the wild-type (WT) *Ax*CuNiR. The reorganization energy of the T2 site in the latter enzyme was calculated to be 1.6 ± 0.2 eV which is two-fold larger than that of the T1 copper center in the WT protein.

Key words: Pulse radiolysis; Driving force; Reorganization energy

1. Introduction

Intramolecular electron transfer (ET) between type 1 (T1) and type 2 (T2) copper sites is part of the catalytic cycle of many copper-containing redox enzymes such as ascorbate oxidase, ceruloplasmin, and copper nitrite reductase. These reactions are thus of considerable interest as they potentially can provide insights into the evolution of selected ET pathways; in particular, how ET pathways has developed over time in order to optimize and control the catalytic processes. With the increase in the number of known high-resolution three-dimensional structures of copper-containing redox enzymes, studies of the structure/reactivity relationship have become feasible and indeed many have been carried out during the last decade [1]. Among the more interesting enzymes is the copper-containing dissimilatory nitrite reductase (CuNiR) which has been isolated from different bacterial sources [2,3]. These enzymes are homotrimers of 109 kDa molecular mass with two copper ions per monomer, which constitutes the catalytic unit. One copper ion is bound to a T1 site while the second is found in a T2 site. The enzyme catalyzes the one-electron reduction of nitrite to nitric oxide:

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

The T1 site serves as the electron uptake site from azurin or pseudoazurin [2]. Binding and reduction of the substrate, nitrite ions, takes place at the T2 site. Hence, the internal T1 → T2 ET is an essential part of the catalytic cycle. In fact, it has been suggested that the rate of ET from T1 to T2 is controlled by changes in the T2 potential induced upon nitrite binding [4,5]. Thus, perturbations of the reduction potential of the two redox centers (i.e. driving force of the reaction) are expected to control both rates and direction of the internal ET.

Recently, several mutants of the blue *Alcaligenes xylosoxidans* CuNiRs have been produced, and in one of these the weaker T1 ligand, Met144, has been substituted by a non-ligating alanine, and the three-dimensional structure of the mutated enzyme has been determined at 2.2 Å resolution [6]. This mutant still maintains 30% activity relative to the wild-type (WT) enzyme. The reduction potential of T1 was found to have increased from 240 to 314 mV upon substituting Met144 with Ala [6], while no change in the T2 potential (230 mV) is expected since the structure is the same as that of the WT. Thus, a significant change in the intramolecular ET reactivity is expected to take place in the M144A mutant.

Previous pulse radiolysis studies have demonstrated that a relatively fast intramolecular ET between T1 and T2 is induced in the WT *A. xylosoxidans* CuNiR (*Ax*NiR) following initial bimolecular reduction of T1Cu(II) even in the absence of substrate with observed rate constants in the range from 450 to 1400 s^{-1} [7,8]. Here we show that changing the driving force by the above mutation can have a dramatic influence on the kinetics of intramolecular ET in this enzyme. Indeed, in the M144A mutant with the elevated T1 reduction potential, a bimolecular direct reduction of T1Cu(II) is not observed. Rather, it is T2Cu(II) which is apparently reduced by the CO_2^- or 1-methylnicotinamide (1-MNA)* radicals, after which a reverse ET takes place, i.e. in the direction of T1Cu(II).

2. Materials and methods

Cloning and overexpression of the gene encoding the NiR protein was performed as reported previously [9]. The site-directed mutagenesis, purification and characterization of the *A. xylosoxidans* M144A CuNiR mutant have been described earlier [6]. Both T1 and T2 sites were fully occupied with copper(II) as determined by integration of the electron paramagnetic resonance (EPR) spectrum and metal analysis [6].

All chemicals were of analytical grade and used without further purification. Milli-Q water was used throughout the studies. For the production of CO_2^- radicals nitrous oxide-saturated solutions of 100

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mM sodium formate, 10 mM sodium phosphate, pH 7.0 were used in one series of experiments. Following the pulse, equivalent amounts of hydrated electrons, e^-_{aq}, and OH radicals are formed within a fraction of a microsecond. Under the above conditions e^-_{aq} reacts with N_2O to produce N_2 and an additional equivalent of OH radicals. OH radicals react with the formate ions to yield CO_2^-, which then becomes electron donor to the enzyme. In another series of experiments NMNA radicals were employed as reductant. They were produced by pulse radiolysis in Ar-saturated solutions of 10 mM 1-MNA, 10 mM phosphate, pH7, using 0.1 M *tert*-butanol as scavenger for the OH radicals.

Pulse radiolysis experiments were carried out using the Varian V-7715 linear accelerator of the Hebrew University in Jerusalem. Electrons accelerated to 5 MeV were used with pulse lengths in the range from 0.2 to 1.2 μs, equivalent to ~1–6 μM CO_2^- radical anions or neutral 1-MNA* radicals. All optical measurements were carried out at 593 nm (T1Cu(II) absorption, with $\varepsilon_{593} = 4300\ M^{-1}\ cm^{-1}$) under purified argon at a pressure slightly in excess of 1 atm. Each kinetic run was repeated at least four times. The observed time-dependent absorption changes were fitted to a sum of exponentials using a non-linear least squares program written in MATLAB®. The temperature of the reaction solutions was controlled by a thermostatting system, and continuously monitored by a thermocouple attached to the cuvette.

3. Results and discussion

When solutions of M144A are subjected to pulses of accelerated electrons in the media described in Section 2, the strongly reducing 1-MNA* or CO_2^- radicals are formed within fractions of a microsecond. The redox state of the enzyme was monitored at 593 nm where T2Cu(II) has no significant contribution to the absorption spectrum. Only one reduction phase was observed throughout the examined concentration range of the M144A mutant (15–35 μM). A typical kinetic trace following T1Cu(II) reduction is illustrated in Fig. 1. The absorbance at 593 nm was found to decay in a single exponential mode. At 298 K and pH 7.0 the rate constant was found to be $k_{298} = 440\ s^{-1}$ and was independent of the reducing radical and protein concentration. Hence, we assign this reduction to an intramolecular process. From the reported reduction potentials of the two copper centers [6] an equilibrium constant $K_{298} = 0.04$ is calculated for the internal electron equilibration:

$$\mathrm{T1Cu(I)T2Cu(II)} \rightleftarrows \mathrm{T1Cu(II)T2Cu(I)}$$

i.e. the internal ET from T1 to T2 is highly disadvantageous, in line with the observation that only intramolecular reduction of T1Cu(II) by T2Cu(I) is monitored. Similar reverse ET has been observed in an earlier stopped-flow study of WT CuNiR when dithionite was used as reductant, where, in addition to the direct reduction of T1Cu(II), a minor reduction of T1 arose from an intramolecular ET from T2Cu(I) to T1Cu(II) [4]. In order to determine the activation parameters of the internal ET equilibration the reaction was studied over a temperature range from 5 to 41°C (cf. Fig. 2). The results of these experiments are summarized in Table 1 together with the activation parameters for WT CuNiR determined in an earlier study [8].

It is noteworthy that contrary to the reactivity of WT Cu*Ax*NiR, no direct T1Cu(II) reduction by CO_2^- or 1-MNA* radicals is observed in the present experiments. The crystallographic model of M144A shows that the T1 site is more protected from solvent compared with the WT CuNiR, since the T1 copper ion is significantly disordered and has moved 0.3 Å away from the cavity, together with the ligating His139 residue [6]. His139 is essential for the bimolecular reduction of T1Cu(II) by its physiological electron donors, since it has been shown that a His139Ala mutant is non-reactive with the natural reducing substrates, azurin or pseudoazurin [10]. The direct reaction of the T2Cu(II) site by dithionite/MV was shown to be responsible for the reduction of the *A. xylosoxidans* His139Ala mutant.

The semi-classical Marcus theory for non-adiabatic intramolecular ET reactions predicts that the rates are governed by the standard free energy of reaction (ΔG^0), the nuclear reor-

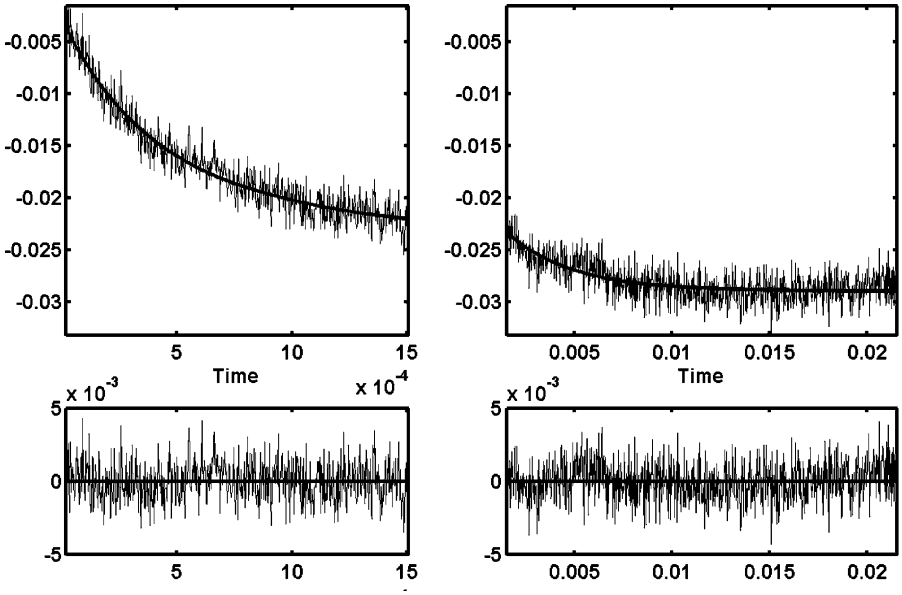

Fig. 1. Time-resolved absorbance changes at 593 nm in *Ax*CuNiR following a pulse. The reaction was carried out at 25.1°C under anaerobic conditions. The reaction mixture contained 11 μM enzyme in 10 mM phosphate buffer, 100 mM formate at pH 7.0. The optical path length was 12.3 cm. Pulse width 0.5 μs. Upper panels: Ordinates indicate absorbance changes (in Abs) at two different time scales (abscissas), both in seconds. The two traces represent one continuous decay which was fitted to one exponent only. Lower panels illustrate the residuals of the fitting.

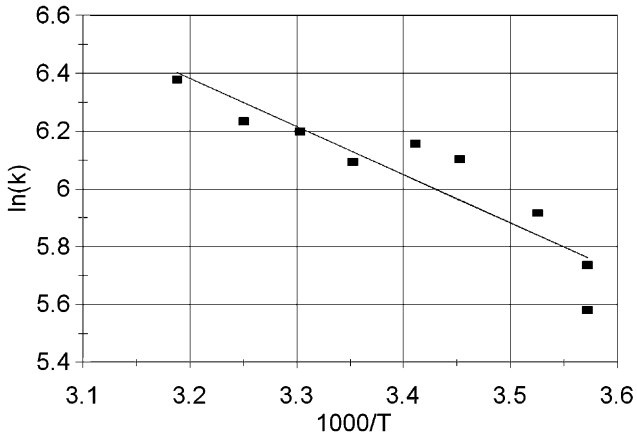

Fig. 2. Temperature dependence of the intramolecular ET reaction between the T2Cu(I) center and the T1Cu(II) center. Each point represents the average rate constant calculated from at least 10 time-resolved spectral changes as shown in Fig. 1.

ganization energy (λ), the distance separating electron donor (D) and acceptor (A) and the electronic coupling (H_{DA}) between D and A at the transition state [11]:

$$k = \frac{2\pi}{\hbar}\frac{H_{DA}^2}{(4\pi\lambda RT)^{1/2}}e^{-(\Delta G^0+\lambda)^2/4\lambda RT} \quad (1)$$

The electronic coupling energy, H_{DA}, is expected to decay exponentially with the distance separating D and A as:

$$H_{DA} = H_{DA}^0 e^{-(\beta/2)(r-r_0)} \quad (2)$$

The crystal structures of several different CuNiRs [6,12–14] show that the two copper sites are 12.5 Å apart and connected by two neighboring amino acid residues in the sequence, a histidine coordinated to the T2 copper ion and a cysteine, which is ligated to the T1 copper ion. This provides a relatively short, direct ET pathway consisting of 11 covalent bonds corresponding to a sigma path length of $\sigma_l = 15.4$ Å (cf. Fig. 3). Gray and coworkers have determined rates of bond-mediated electron tunneling in modified iron–sulfur proteins [15], one where the electron donor and acceptor are separated by a Cys–His bridge identical to that of CuNiR. Their observed rates are essentially coupling limited, i.e. $k_{max} \sim 10^8$ s^{-1}. However, we find a rate constant of 450 s^{-1} for the intramolecular ET in WT CuNiR with a driving force of -0.01 eV. The reorganization energy of this reaction can now be calculated using Eq. 1 above. From an estimated 5–50 ns coupling limited tunneling time we can further estimate the error range in the calculated reorganization energy, i.e. $\lambda = 1.2 \pm 0.1$ eV. Gray et al. have previously determined the reorganization energy of the T1 copper center to be 0.82 eV [16]. The T1Cu atom of the NiRs has $(His)_2CysMet$ ligation in a distorted trigonal planar geometry with the methionine residue as a weak axial ligand, similar to the T1 center of azurins, except that a second weak axial carbonyl ligand (at

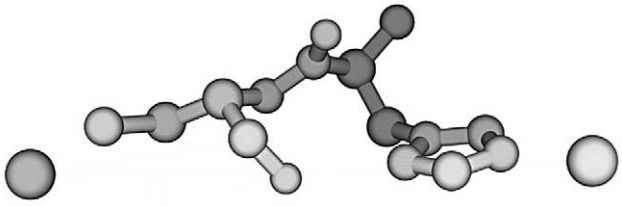

Fig. 3. Calculated ET pathway between T1 and T2 in *Ax*CuNiR. The coordinates were taken from The Protein Databank, ID # 1NDT.

2.7 Å) present in azurins, is missing [2,3]. The solvent-exposed histidine Cu ligand, thought to mediate ET to the center, is present in both protein families. Depending on the organism from which they are isolated, the T1Cu centers of CuNiRs give rise to a blue (as in *Ax*NiR) or a green color depending on the relative intensities of absorption bands at 450 and 600 nm. Despite these differences, comparison of the crystallographic structures of the blue sites of azurin and *Ax*NiR shows them to have very similar geometry and metal ligand distances. Thus, assuming a similar reorganization energy of the T1 center in the blue WT Cu*Ax*NiR, we can now calculate the reorganization energy of the T2 copper center from the relation $\lambda_{tot} = \lambda_{T1}/2 + \lambda_{T2}/2$ and find $\lambda_{T2} = 1.6 \pm 0.2$ eV. The reorganization energy of this copper site is thus much larger than that calculated for T1. This is also expected since the latter center is buried inside the protein some 6 Å below the Connoly surface of the molecule and is isolated from solvent while the nitrite binding T2 center is accessible to the solvent via a 13 Å deep hydrophobic channel [6]. Nevertheless, the reorganization energy calculated here for the T2 copper center is below values quoted for simple copper complexes. Thus, for $Cu(phen)_2^{2+/+}$ the reorganization energy has been determined to be 2.4 eV [17].

Comparative EPR, extended X-ray absorption fine structure (EXAFS) and ultraviolet (UV)-visible spectroscopy of reduced and oxidized *Ax*NiR have shown that reduction of the enzyme by ascorbate/phenazine methosulfate in the absence of nitrite results in the loss of the water bound to the T2Cu ion of the oxidized protein [18]. This change in coordination of the T2Cu ion from the near tetrahedral $(His)_3$–H_2O site to a trigonal $(His)_3$ site on reduction would make a significant contribution to both the reduction potential and the difference in reorganization energy of this site when compared with the T1Cu, where only minor changes in geometry are associated with change in redox state [19,20].

In the case of *Ax*NiR it has been shown from Cu difference EXAFS that binding of the competitive inhibitor azide is not observed when the T2Cu ion is reduced, consistent with the observation that the enzyme is inactivated by reduction in the absence of substrate [18]. The decreased affinity of the reduced T2 site for nitrite is also apparent in an X-ray crystallographic study of AfNiR, that showed a low occupancy of nitrite in

Table 1
Rate constants and activation parameters for the internal electron equilibration in *Ax*CuNiR

	k_{298} (s^{-1})	k_f (s^{-1})	k_b (s^{-1})	$\Delta H^{\neq}$ (kJ/mol)	$\Delta S^{\neq}$ (J/K/mol)	E^0 (T1) (mV)	E^0 (T2) (mV)
WT	450	185	265	167	−145	240	230
M144A	440	15	425	114	−156	314	230

Data for the WT protein are taken from [8].

crystals of reduced enzyme soaked with this substrate [21]. Thus, during NiR turnover nitrite must bind to a T2Cu(II) ion before ET from the reduced T1 center occurs. The nitrite-induced gating of ET during catalysis has been suggested to involve the longer linkage between the two Cu centers formed by amino acid residues His89 (a T1Cu ligand) and the T2Cu ligand His94 (*Ax*NiR numbering) [18], a loop which includes hydrogen bonding to an aspartic acid residue (*Ax*Asp92).

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