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The intramolecular electron transfer between copper sites of nitrite reductase: a comparison with ascorbate oxidase

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Abstract The intramolecular electron transfer (ET) between the type 1 Cu(I) and the type 2 Cu(II) sites of *Alcaligenes xylosoxidans* dissimilatory nitrite reductase (AxNiR) has been studied in order to compare it with the analogous process taking place in ascorbate oxidase (AO). This internal process is induced following reduction of the type 1 Cu(II) by radicals produced by pulse radiolysis. The reversible ET reaction proceeds with a rate constant $k_{\mathrm{ET}} = k_{1\to2}+k_{2\to1}$ of 450 ± 30 s^{-1} at pH 7.0 and 298 K. The equilibrium constant K was determined to be 0.7 at 298 K from which the individual rate constants for the forward and backward reactions were calculated to be: $k_{1\to2} = 185 \pm 12$ s^{-1} and $k_{2\to1}$265 ± 18 s^{-1}. The temperature dependence of K allowed us to determine the $\Delta H°$ value of the ET equilibrium to be 12.1 kJ mol^{-1}. Measurements of the temperature dependence of the ET process yielded the following activation parameters: forward reaction, $\Delta H^{\neq} = 22.7 \pm 3.4$ kJ mol^{-1} and $\Delta S^{\neq} = -126 \pm 11$ J K^{-1} mol^{-1}; backward reaction, $\Delta H^{\neq} = 10.6 \pm 1.7$ kJ mol^{-1} and $\Delta S^{\neq} = -164 \pm 15$ J K^{-1} mol^{-1}. X-ray crystallographic studies of NiRs suggest that the most probable ET pathway linking the two copper sites consists of Cys^{136}, which provides the thiolate ligand to the type 1 copper ion, and the adjacent His^{135} residue with its imidazole being one of the ligands to the type 2 Cu ion. This pathway is essentially identical to that operating between the type 1 Cu(I) and the trinuclear copper centre in ascorbate oxidase, and the characteristics of the internal ET processes of these enzymes are compared. The data are consistent with the faster ET observed in nitrite reductase arising from a more advantageous entropy of activation when compared with ascorbate oxidase.

Key words: Activation parameter for electron transfer; Nitrite reductase; Pulse radiolysis; *Alcaligenes xylosoxidans*

1. Introduction

One extensively employed approach to the study of the parameters controlling the rate of electron transfer within proteins is the introduction of an additional redox centre into electron mediating proteins, e.g. *c*-type cytochromes or azurins [1]. In a different approach [2], the single disulfide bridge present in the latter protein has been used as a redox centre, though its natural role is most probably structural. These studies of relatively small, electron mediating proteins have allowed the effects of distance and the chemical properties of the polypeptide separating electron donors and acceptors to be studied in a systematic manner. Such studies have demonstrated the involvement of this matrix in mediating electron transfer as well as indicated a distinct role for its conformation [2,3].

In parallel, intramolecular electron transfer processes which are part of catalytic cycles of redox enzymes have also been investigated. Protein structural elements involved in catalytic activity are assumed to have evolved from selective evolutionary pressure and therefore may reveal features optimized for the chemical processes. One such reaction step involved in catalysis is the electron transfer between the type 1 (T1) Cu(I) of ascorbate oxidase and its dioxygen reduction site, the trinuclear copper centre. This system has attracted considerable attention because the 1.8-Å resolution structure of this enzyme clearly suggested that a covalent bonding pathway links the reaction partners over a distance of 12.5 Å [4,5]. The internal ET has been studied independently by three different groups and found to have several noteworthy features [6–8]. Although the reaction is characterized by rather slow rate constants, the activation enthalpy of the process is exceptionally low [7]. In addition, two or three distinct phases characterize the time course of this first order process. These two unusual features were rationalized, at least in part, by the low driving force of the process and the possible existence of multiple states of the trinuclear centre, respectively [7].

More recently, the three dimensional structures of the copper-containing dissimilatory nitrite reductase (NiR) isolated from *Achromobacter cycloclastes* (AcNiR, pdb ref. 1NRD) [9], *Alcaligenes faecalis* S-6 (AfNiR, pdb ref. 1AFN) [10] and *Alcaligenes xylosoxidans* (AxNiR, pdb ref. 1NDS, R1IDSSF) [11] have been determined. These enzymes have a high degree of amino acid sequence homology and their three dimensional structures are very similar showing a ~0.7-Å r.m.s. deviation of the C_α positions between AxNiR and the other enzymes [11]. They are trimers of 109 kDa with 6 Cu ions per trimer and with two copper binding sites per catalytic unit, one a T1 Cu centre and the other a type 2 (T2) Cu centre. The T1 centre is more than 5 Å from the Connolly surface of the protein and the T2 site is in an intersubunit interface, at the bottom of a hydrophobic pocket, some 14 Å from the protein surface. The closest non-liganding solvent molecule in this pocket is some 6 Å away from the T2 Cu(II) ion.

The consensus view of the mechanism of Cu-containing NiRs when catalysing the reaction

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Abbreviations: AcNiR, AfNiR, AxNiR, AxgNiR, the Cu-containing dissimilatory nitrite reductases from *Achromobacter cycloclastes*, *Alcaligenes faecalis* S-6, *Alcaligenes xylosoxidans* (NCIMB 11015) and *Alcaligenes xylosoxidans* (GIFU 1051), respectively; AO, ascorbate oxidase; T1, T2 and T3, type 1, type 2 and type 3 Cu centres, respectively; ET, electron transfer; NMNA, *N*-methylnicotinamide

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

is that the T1 Cu(II) site functions in electron uptake and transfer to the T2 Cu(II) sites at which NO_2^- binding and reduction occurs. Significantly, the 3-D structures of the three Cu NiRs which have been determined show the two sites to be 12.5 Å apart and connected by a covalent bonding very similar to that linking the T1 and type 3 (T3) copper sites in AO, namely the adjacent residues in the sequence His^{135} and Cys^{136} provide coordinating ligands to the copper ions of the T2 and T1 sites, respectively [9–11]. This striking similarity of linkage between the redox centres in these enzymes and the structurally simpler nature of the electron acceptor in NiR, i.e. being a T2 Cu(II) centre, makes NiR an interesting system for studying intramolecular ET. Indeed, the T1 Cu(I) to T2 Cu(II) ET in NiRs isolated from three different bacteria has recently been observed [12,13]. This called for characterisation of the internal ET process in terms of the activation barriers for this reaction in NiR, to enable comparison with the analogous process taking place in AO. In the present paper we report the first determination of these parameters for any Cu-containing NiR, together with the ET rates of the forward and back reaction between the two Cu centres.

2. Materials and methods

The copper containing nitrite reductase was isolated from *Alcaligenes xylosoxidans* (NCIMB 11015) and purified according to reported protocols [14]. Stock solutions of the enzyme (14 mg/ml) had the specific activity of 176 μmol of nitrite reduced/min/mg of protein (pH 7.0 and 23°C) and a 280/593 nm absorption ratio of 12. The Cu content was 6.2 ± 0.2 (mean ± S.E.M.) Cu atoms per trimer of which approx. 50% were T1 and 50% T2 centres determined by integration of the EPR spectrum. Argon saturated solutions of oxidised AxNiR (2–18 μM) in aqueous 10-mM phosphate buffer, pH 7.0, containing 100 mM *tert*-butanol and 1 mM *N*-methylnicotinamide (analytical grade, Sigma) were employed in some of the pulse radiolysis experiments. In another series of experiments, N_2O saturated solutions of AxNiR (18 μM) in 100 mM sodium formate with 10 mM sodium phosphate, pH 7.0, were used. In the former solutions, the primary $OH^\bullet$ radicals produced by the radiation pulse are scavenged by the *tert*-butanol, while the hydrated electrons (e_{aq}^-) reduce the NMNA to its radical-ion state which then donates electrons to NiR. In the latter solutions, e_{aq}^- reacts with N_2O to produce N_2 and an additional equivalent of $OH^\bullet$ radicals, both of which react with the formate ions to yield two equivalents of the CO_2^- radical which then functions as the electron donor to the enzyme.

Pulse radiolysis experiments were carried out with 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 ions. The temperature of the solution was controlled by a thermostatting system and continuously monitored by a thermocouple attached to the cuvette [15]. All experiments were carried out under pseudo-first-order conditions with an excess of AxNiR redox sites over the concentration of reducing radicals. The kinetic traces were analysed by a non-linear curve fitting programme and statistical analyses performed using the Corel Quattro Pro 7 advanced regression tool.

3. Results and discussion

3.1. Reduction of the type 1 Cu(II) site by N-methylnicotinamide or CO_2^- radicals

Solutions of oxidized AxNiR (as isolated) were subjected to pulses of accelerated electrons (5 MeV) which, in the presence of the above solutes, led to the production of the reducing *N*-methylnicotinamide ($NMNA^-$) or CO_2^- radicals, respectively. The changes in the redox state of the enzyme were monitored at 593 nm which is the maximum absorption of the T1 Cu(II) site ($\varepsilon_{593} = 4300\ M^{-1}\ cm^{-1}$). In agreement with the earlier observations of Suzuki et al. for the reactivity of AcNiR, AxgNiR and AxnNiR [12,13], a direct bimolecular reduction of the T1 Cu(II) site by $NMNA^-$ radicals was observed with a second order rate constant of $k_{298} = (5 \pm 1) \times 10^8\ M^{-1}\ s^{-1}$. We found this rate to be the same, within experimental error, when CO_2^- radicals were used as the electron donor. These results demonstrate that the T1 Cu(II) centre of AxNiR is the primary electron accepting site using both $NMNA^-$ and CO_2^- radicals, with a reaction rate close to, or at the diffusion controlled limit, since it is very similar to that observed for bimolecular reduction of the T1 Cu(II) in azurin by CO_2^- radicals [15].

3.2. Internal electron transfer between the type 1 and type 2 Cu sites of AxNiR

Following the rapid reduction of the T1 Cu(II) centre, a slow reoxidation process takes place which is clearly unimolecular, since the rate was independent of pulse width (i.e. reductant concentration) and the [AxNiR] over the range 2–18 μM. This process was therefore assigned to be an intramolecular ET from T1 Cu(I) to the T2 Cu(II). The observed rate constant at 298 K and pH 7.0 was $450 \pm 30\ s^{-1}$. This value of observed rate constant for the electron transfer is the sum of the forward ($k_{1\rightarrow2}$) and backward ($k_{2\rightarrow1}$) reactions for ET between the T1 and T2 Cu sites:

$$\text{T1 Cu(I)T2 Cu(II)} \underset{(k_{2\rightarrow1})}{\overset{(k_{1\rightarrow2})}{\rightleftharpoons}} \text{T1 Cu(II)T2 Cu(I)}.$$

As illustrated in Fig. 1 the extent of reoxidation of the T1 Cu(I) as a result of the intramolecular ET is only partial. Assuming a redox equilibrium has been established between the two centres, the amplitude of the reoxidation enables the equilibrium constant for the ET reaction to be determined and $K = 0.7$ at 298°C was calculated for this process. Using this value, the individual rate constants of the forward and back reactions were calculated to be $k_{1\rightarrow2} = 185 \pm 12\ s^{-1}$ and $k_{2\rightarrow1} = 265 \pm 18\ s^{-1}$. The temperature dependence of K allowed us to determine a value for ΔH° of the ET equilibrium to be 12.1 kJ mol^{-1}.

In an earlier study Suzuki et al. [12,13] compared the intramolecular ET between the T1 and T2 copper centres in AcNiR (a green NiR), AxnNiR and AxgNiR (both blue NiRs). They reported observed rate constants (the sum of the forward and back reactions) for these enzymes to be 1400 s^{-1}, 1900 s^{-1} and 1800 s^{-1}, respectively, at pH 7.0. The relatively small differences in these ET rates between the green and the blue NiRs may arise from the differences in ligand geometry of the T1 Cu(II) centres which determine the spectroscopic properties as well as the redox potential of these sites of NiRs. A direct comparison of our kinetic data for AxNiR with those of Suzuki et al. [13] is not possible as the temperature at which the latter rates were determined was not reported.

3.3. Redox potentials and activation parameters for the internal electron transfer reaction

As shown in Fig. 1, the amplitude of the increase in absorbance associated with the reoxidation of the T1 Cu(I) centre

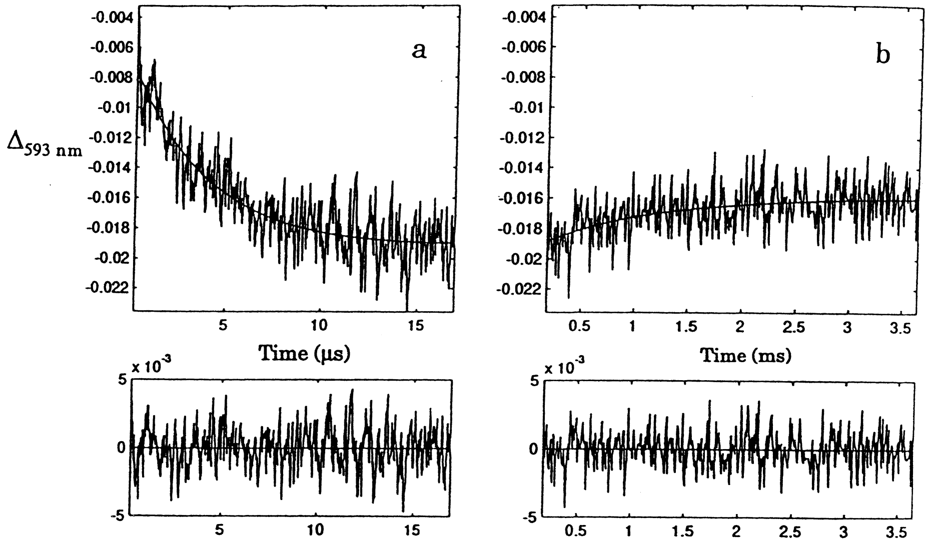

Fig. 1. Time resolved absorbance changes in AxNiR at 593 nm following pulse radiolysis. The reaction was carried out at 35.6°C under an atmosphere of argon. The reaction mixture contained 11.8 μM enzyme in 10 mM phosphate buffer, pH 7.0; AxNiR, 10 μM; *tert*-butanol, 100 mM; and *N*-methylnicotinamide, 1 mM. The reaction progress curves show (a) the reduction of the type 1 Cu(II) centres by the NMNA$^-$ radical; (b) the subsequent partial reoxidation of the type 1 centres as electrons are transferred to the type 2 Cu(II) centres. The optical pathlength was 12.3 cm. The lower panels show the residuals of the theoretical fits to the data.

during the intramolecular ET from T1 Cu(I) to T2 Cu(II) does not correspond to the complete reoxidation of the T1 site. A similar observation was made in the earlier pulse radiolysis studies [7,12,13]. Since the midpoint potential of T1 Cu(II)/Cu(I) for AxNiR has been reported to be 240 mV at 298 K [12] one may calculate a midpoint potential of T2 Cu(II)/Cu(I) from the above ET equilibrium to be 230 mV. This means that no driving force is available for the ET, in fact the T1-T2 ET is an 'uphill' process. In addition, other factors may cause the slow ET rate, namely changes taking place in the T2 Cu(II) site following reduction; for example, our recent EXAFS data for AxNiR [16] indicate that the water molecule coordinated to the T2 Cu(II) centre in the oxidized protein is lost following reduction. This change in ligation on reduction has also been observed in a crystallographic study of AfNiR [17]. Loss of water from the coordination sphere of the T2 Cu(II) ion, or the exchange of the proton resolved by ENDOR studies of the T2 Cu centre of AxNiR [18], cause changes in the coordination sphere of this centre and increase the reorganization energy of the ET process and slow it down.

In order to determine the activation parameters for the internal ET process in AxNiR we studied the reaction over the temperature range from 0.8 to 49°C and determined the rate constants of the internal ET. An Arrhenius plot of data accumulated from three independent series of experiments using either NMNA$^-$ or CO$_2^-$ radicals is shown in Fig. 2. (It is noteworthy that N$_2$O present in all experiments where CO$_2^-$ is the reductant does not affect the ET process.) The activation parameters were determined to be: forward reaction, $\Delta H^{\Delta} = 22.7 \pm 3.4$ kJ mol^{-1} and $\Delta S^{\neq} = -126 \pm 11$ J K^{-1} mol^{-1}; backward reaction, $\Delta H^{\neq} = 10.6 \pm 1.7$ kJ mol^{-1} and $\Delta S^{\neq} = -164 \pm 15$ J K^{-1} mol^{-1}.

3.4. Comparison with ascorbate oxidase

In ascorbate oxidase (AO), an analogous intramolecular ET takes place between the electron uptake site, T1 Cu(I) and the trinuclear T2/T3 centre, where O$_2$ binds and becomes reduced. Here the process, however, is at least biphasic with rate constants for the reversible ET process at 298 K of 200 s^{-1} and 2 s^{-1}, respectively, and an equilibrium constant of 4. The three dimensional structure of AO in both oxidised and reduced states has been determined at 1.9 Å resolution [4,5]. Since the crystallographic structures were determined at pH 7.0, we have also carried out our current kinetic studies at this pH which is far from the optimum for AxNiR activity of pH

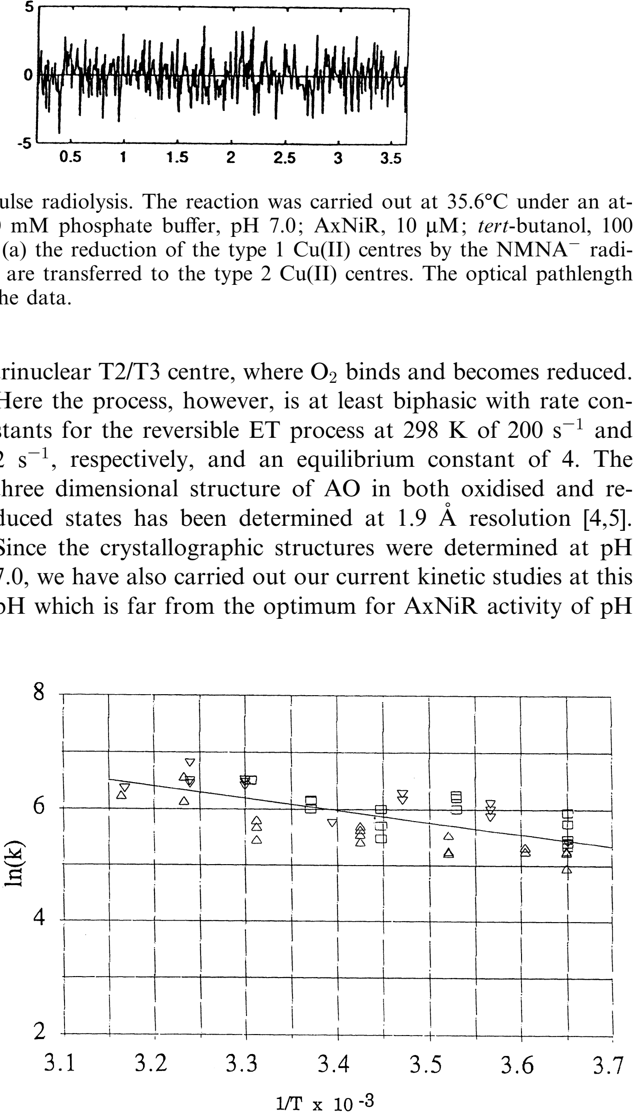

Fig. 2. An Arrhenius plot for the temperature dependence of the intramolecular electron transfer reaction between the type 1 Cu centre and type 2 Cu centres of AxNiR. Data obtained from three independent series of experiments are presented: △, 5.2 and 10.4 μM AxNiR in the reaction mixture described above for Fig. 1; ▽, 11.8 μM AxNiR in the same reaction mixture; □, 18 μM AxNiR in N$_2$O saturated solutions of Na formate (100 mM) and Na phosphate (10 mM), pH 7.0. In the former series of experiments the reductant is the *N*-methylnicotinamide radical, while in the last series the CO$_2^-$ radical anion is used as the reductant. All experiments were performed at pH 7.0. Each point represents one individual experiment as presented in Fig. 1. The drawn line is fit to the linear least squares analysis of all the data.

5.2. However, preliminary results obtained at pH 5.0 yield a very similar rate constant for the internal ET process of AxNiR to that determined at pH 7.0. As stressed above, a comparison of these 3-D structures shows the shortest pathways for ET between the electron donor and acceptor sites to be essentially identical in AO and NiR. In the latter, a cysteine thiolate is coordinated to the T1 copper ion and its neighbouring residue in the sequence, a histidine imidazole, is coordinated to the T2 copper ion. In AO two histidine imidazoles flank the cysteine, each binding to one of the copper ions constituting the T3 site. In both enzymes the distance between the two centres is 12.5 Å. The activation parameters for the faster phase of intramolecular ET in AO are: $\Delta H^{\neq} = 9.1 \pm 1.1$ kJ mol^{-1} and $\Delta S^{\neq} = -170 \pm 9$ J K^{-1} mol^{-1} [7]. It is of considerable interest to compare these values with those determined in the present study for the forward ET process in NiR: $\Delta H^{\neq} = 22.7 \pm 3.4$ kJ mol^{-1} and $\Delta S^{\neq} = -126 \pm 11$ J K^{-1} mol^{-1}. In both enzymes the activation enthalpies are relatively low. The reason for the internal ET proceeding with a faster rate constant in NiR is clearly the more advantageous entropy of activation. Because the entropy of activation includes an electronic factor, namely the transmission coefficient (degree of nonadiabaticity), the cause of the rather slow rates in both enzymes might be due to a combination of the low driving force and the changes taking place in the structures of the respective electron accepting sites in NiR and AO.

The observation that the T1 Cu(I) to T2 Cu(II) ET proceeds in a single phase in NiRs lends some support to the rationale proposed for the multiple phases observed in the T1 Cu(I) to T3 Cu(II) ET in AO; namely that more than a single species may exist in the trinuclear centre. The rather slow rates observed in all NiRs examined so far, are still compatible with their specific activities. At pH 7.0 which is essentially at the lowest end of the pH activity profile of AxNiR [19], a specific activity of ca. 240 μmol of NO_2^- reduced by a mg protein per min or about 145 mol per active centre per second, some 3-fold slower than the internal ET in the absence of substrate. This is consistent with the observation that the presence of substrate (NO_2^-) slows down the internal ET rate [13]. Thus we propose that structural changes at the T2 Cu(II) site occurring during catalytic turnover of NiR are responsible for gating the internal ET.

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