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Evidence for Substrate Activation of Electron Transfer from Methylamine Dehydrogenase to Amicyanin

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It has been previously established that electron transfer (ET) from methylamine dehydrogenase (MADH) to amicyanin may be true ET or gated ET, depending upon the redox form of MADH.[1] It has also been shown that MADH possesses two sites in close proximity to the tryptophan tryptophylquinone (TTQ) cofactor which can bind monovalent cations[2] and that the gated ET from the substrate-reduced aminoquinol (N-quinol) form of MADH is dependent on the presence of monovalent cations.[3] This study demonstrates that the gated ET reaction may be converted to a slower true ET reaction by removal of monovalent cations and that the protonated methylammonium substrate may perform the role previously attributed to monovalent cations in regulating the rate and mechanism of ET from MADH.

It is possible to study electron transfer from several different redox forms of MADH to amicyanin.[4] Because TTQ[5] is a two-electron carrier and the type I copper protein, amicyanin,[6] is a one-electron carrier, two sequential oxidations of fully reduced TTQ by amicyanins are required to completely reoxidize MADH. Nonphysiologic O-quinol and O-semiquinone forms of MADH may be generated by reduction by dithionite.[7] The product of the reduction of MADH by the substrate amine is an N-quinol which retains the covalently bound substrate-derived amino group after release of the aldehyde product[8] (Figure 1). An N-semiquinone is the product of the first one-electron oxidation of the N-quinol.[9] The reactions of the O-quinol, O-semiquinone, and N-semiquinone forms of MADH with amicyanin are true ET reactions which exhibit a predictable dependence on $\Delta G°$.[1] Analysis of the $\Delta G°$-dependence of k_{ET} for these reactions yielded values of reorganization energy (λ) and electronic coupling (H_{AB}) that were identical to those obtained from analysis of the temperature dependencies of k_{ET} for these reactions. These analyses also predicted an ET distance that closely matched that seen in the crystal structure[10] of the protein complex. In contrast, analysis of the temperature dependence of the gated ET reaction from N-quinol MADH to amicyanin yielded unreasonable values of λ and H_{AB} and a negative value for ET distance[11] (Table 1).

The ET reaction from N-quinol MADH to amicyanin was studied in HEPES buffer adjusted to pH 7.5 with $Ca(OH)_2$,without any added monovalent cations. The rate of this reaction is much less than the rate that is observed in the presence of monovalent cations, which becomes too fast to measure at saturating concentrations of K^+ or NH_4^+.[3] The dependence of this rate on temperature (T) from 15 to 40 °C was determined (Figure 2), and these data were analyzed by ET theory[12] (eqs 1 and 2). The terms not defined

$$k_{ET} = [4\pi^2 H_{AB}{}^2/h(4\pi\lambda RT)^{0.5}] \exp[-(\Delta G° + \lambda)^2/4\lambda RT] \quad (1)$$

$$k_{ET} = k_o \exp[-\beta(r - r_o)] \exp[-(\Delta G° + \lambda)^2/4\lambda RT] \quad (2)$$

previously in the text are Planck's constant (h), the gas constant (R), and the characteristic frequency of the nuclei (k_o). In these calculations, a $\beta = 1.0$ was used. The values of λ, H_{AB}, and ET distance (r) which were obtained are consistent with this being a true ET reaction and are nearly identical to values which have previously been obtained for the true ET transfer reaction from dithionite-reduced O-quinol MADH to amicyanin[13] (Table 1).

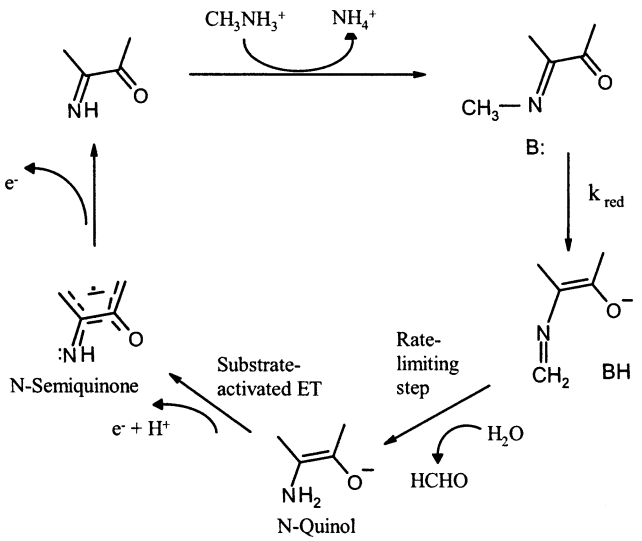

Figure 1. Steady-state reaction mechanism of MADH. Only the reactive portion of TTQ is shown. B is an active-site base. Once the steady state is achieved, the release of the ammonia product is coupled to the binding of the next substrate molecule to TTQ.[4] Amicyanin is the electron acceptor.

Table 1. Electron Transfer from MADH to Amicyanin

redox form (buffer)	λ (eV)	H_{AB} (cm^{-1})	r (Å)
O-quinol (K-phosphate)[a]	2.4 ± 0.1	12 ± 4	9.5 ± 0.8
N-quinol (Ca-HEPES)	2.3 ± 0.1	3 ± 2	12.6 ± 1.2
N-quinol (K-phosphate)[b]	3.5 ± 0.1	23 000	−4.9

[a] Data taken from ref 13. [b] Data taken from ref 3.

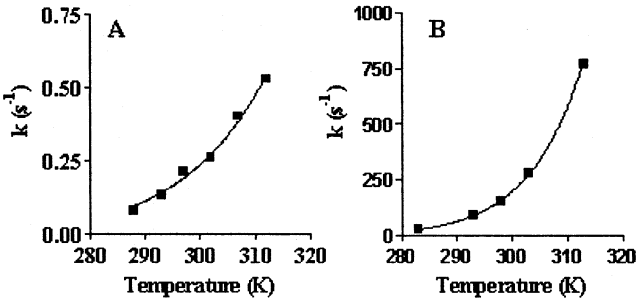

Figure 2. Temperature dependence of the rate of the reaction of N-quinol MADH with oxidized amicyanin in 10 mM Ca-HEPES (A) and 10 mM potassium phosphate + 0.2 M KCl (B). The data in B were taken from ref 11. Lines are fits of the data to eq 1 and 2, which are superimposable.

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Table 2. Kinetic Parameters for Methylamine-Dependent Reactions

Methylamine-Dependent Amicyanin Reduction (Steady-State)

buffer	K_m (M)	k_{cat} (s^{-1})
K-phosphate[a]	6.4×10^{-6}	48
Ca-HEPES	1.3×10^{-2}	31

Methylamine-Dependent TTQ Reduction (Single-Turnover)

buffer	K_d (M)	k_{red} (s^{-1})
K-phosphate[a]	1.3×10^{-5}	275
Ca-HEPES	1.4×10^{-4}	275

[a] Data taken from ref 16. Methods are also described in this reference.

The k_{ET} for the reaction between N-quinol MADH and amicyanin in the absence of monovalent cations is much less than the k_{cat} which had been observed for methylamine-dependent amicyanin reduction by MADH in the steady state.[14] The steady-state assay is usually performed in potassium phosphate buffer. As such, the steady-state reaction was examined in HEPES buffer in the absence of added monovalent cations (Table 2). The k_{cat} for the reaction was similar in the presence and absence of monovalent cations. However, the K_m for methylamine was approximately 1000-fold greater in the absence of monovalent cations. To determine whether this reflected a decreased affinity for methylamine at the catalytic substrate-binding site, the rate of methylamine-dependent TTQ reduction was directly measured by stopped-flow spectroscopy[14] (Table 2). The rate constant for TTQ reduction by methylamine was identical in the presence and absence of monovalent cations. The K_d value for methylamine determined from the concentration dependence of the reaction rate is somewhat greater in the absence of monovalent cations, but still 100-fold less than the K_m for methylamine in the steady-state reaction in the absence of monovalent cations (Table 2).

These results may be explained within the context of a two binding-site model. Two potential cation binding sites in close proximity to TTQ have been identified in the crystal structure of MADH.[2,15] In the proximal cation-binding site nearest to TTQ, several carbonyl groups point toward the cation. In contrast, the distal cation-binding site involves primarily cation$-\pi$ interactions with the aromatic side chains of βTyr119 and αPhe55 in the *Paracoccus denitrificans* enzyme. Monovalent cation binding to the proximal site is required to facilitate the proton-transfer reaction that gates ET from N-quinol MADH.[15] Monovalent cation binding to the distal site causes spectral perturbations of TTQ,[15] but has not yet been assigned a functional role. Under standard assay conditions, in the presence of added cations, the rate-limiting step in the steady-state reaction has been shown to be the release of the formaldehyde product from the enzyme$-$product complex[16] (Figure 1). The K_m value for methylamine in this assay is similar to the K_d value for binding to the substrate binding site that is obtained in single turnover studies. At this pH (7.5), the substrate will be essentially all present as the protonated methylammonium ion ($pK_a = 10.7$). When the steady-state reaction is performed in the absence of cations, then the slow true ET reaction from N-quinol MADH becomes the rate-limiting step. The reaction rate increases with increasing substrate because methylammonium is binding to the proximal site and acting as a cation to facilitate the gated, but faster, ET reaction. The K_m value for methylamine under these conditions reflects the binding to the regulatory proximal site, not the catalytic substrate-binding site which prepares the substrate for nucleophilic attack of TTQ.

Several years ago, on the basis of spectroscopic titrations of cation binding, Kuusk and McIntire[17] proposed that MADH possessed two cation-binding sites which could be distinguished by different affinities for several monovalent cations. They suggested that methylammonium binding to one of these sites was a prelude to reacting with TTQ. The data obtained here are consistent with that proposal. Combined with results of previous mutagenesis studies,[15] these data indicate that the distal binding site is likely the substrate-binding site. As stated earlier, this binding site was shown to be responsible for cation-dependent spectral perturbations. It now can be assigned a functional role as the site which binds and likely deprotonates the cationic methylammonium substrate prior to reaction with TTQ. The proximal site is a regulatory site which binds cations to facilitate ET. It has never been clear what the relevance, if any, of such a mode of regulation might be. Cations such as Na^+ and K^+ do not seem to be sensible regulators for a process which occurs in the periplasmic space of a bacterium. These data suggest that the effects of monovalent cations in vitro mimic the effects of methylammonium in vivo and that the substrate is actually also serving as an activator and regulator of the ET to amicyanin under physiologic conditions.

Several features of this revised picture of the reaction mechanism of MADH are relatively novel. It identifies a regulatory role for the substrate of MADH in its subsequent ET reaction. We have previously described the phenomenon of chemically gated ET,[18] in which a chemical reaction activates a system for ET. While the faster activated ET reaction is gated by the slower chemical reaction, the gated ET rate is still orders of magnitude greater than that of the ungated true ET reaction in the absence of chemical activation. In this case, the ET reaction from the N-quinol MADH is not only chemically gated, but also substrate activated ET. While allosteric regulation of enzyme activity by substrate binding to noncatalytic sites has been reported many times, the mode of regulation of MADH by substrate is different. The noncatalytic binding of substrate also occurs in the active site. The mode of regulation is not via long-range conformational changes but by stabilization of a charged reaction intermediate in the active site.

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