Direct Detection by 15N NMR of the Tryptophan Tryptophylquinone Aminoquinol Reaction Intermediate of Methylamine Dehydrogenase

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Direct Detection by ^{15}N NMR of the Tryptophan Tryptophylquinone Aminoquinol Reaction Intermediate of Methylamine Dehydrogenase

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Redox-active cofactors, derived from post-translational modifications of amino acid side chains, are emerging as critical participants in many enzyme-catalyzed reactions.[2] Amino-acid-derived quinone-containing prosthetic groups,[2d,3] such as tryptophan tryptophylquinone[2c] (TTQ) and 2,4,5-trihydroxyphenylalanine[2b] quinone (TPQ), have been suggested, although not directly shown, to be chemically modified by a substrate during their catalytic cycles. We present ^{15}N NMR data which directly confirms the covalent modification, by substrate nitrogen (N) of TTQ in methylamine dehydrogenase (MADH), and demonstrate that ^{15}N NMR may be used to monitor the fate of substrate ^{15}N throughout the catalytic cycle.

MADH[3] catalyzes the oxidative deamination of primary amines to the corresponding aldehyde, ammonium, and two electrons that are conveyed *in vivo* by sequential one-electron transfers to a type-1 copper protein, amicyanin.[4] These reactions of MADH are mediated by TTQ which is derived by post-translational modification of two tryptophan residues (Figure 1A).[2c,5] Chemical reaction occurs at the C6 carbonyl.[6] It has been postulated that reduction of TTQ by substrate results in a stable aminoquinol intermediate[7] (Figure 1A) by a transaminase type mechanism.[3c,7a] Alternatively, *O*-quinol TTQ with hydroxyl groups at C6 and C7 is generated by titration of MADH with dithionite.[8] Dithionite- and substrate-reduced MADH exhibit different reactivities with amicyanin[7b,c] supporting the idea that substrate N is covalently incorporated[9] into TTQ and released only after reoxidation. This mechanism has been disputed, and differences in these reactivities have alternatively been ascribed to the presence of noncovalently bound product ammonia interacting with the C6 oxygen of the *O*-quinol.[10] Here, we use $CH_3{}^{15}NH_2$ and ^{15}N NMR spectroscopy to observe directly the fate of the substrate-derived ^{15}N.

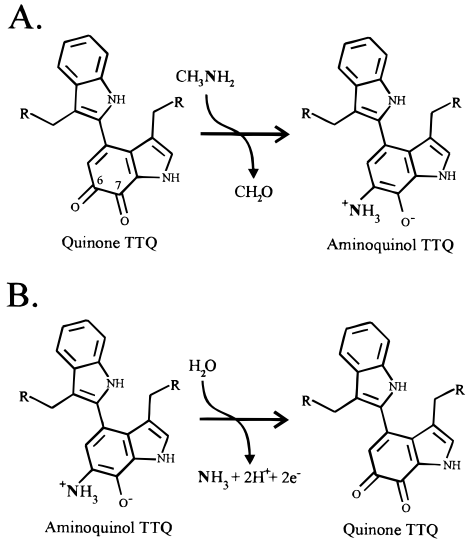

Figure 1. Reductive and oxidative half-reactions of MADH. A. Reduction is hypothesized to result in covalent modification of the C6 carbon of TTQ by substrate-derived N to form the aminoquinol. The protonation state of the aminoquinol is unknown. B. The two-electron oxidation of aminoquinol TTQ is accompanied by hydrolysis of the substrate N and results in a replacement of N by solvent-derived O.

Purification of MADH from *Paracoccus denitrificans* was as previously described.[3b] CH_3NH_3Cl and NH_4Cl (^{15}N enriched to 99%) were purchased from CDN Isotopes and benzamide (^{15}N enriched to 99%) from Cambridge Isotope Laboratories. Experiments were performed in 10 mM potassium phosphate buffer, pH 7.5, plus 200 mM KCl, with 10% v/v D_2O for NMR signal lock at 25 °C. MADH (3 mM) was reacted with 20-fold excess $CH_3{}^{15}NH_3Cl$. No significant loss of MADH activity was observed in conditions mimicking those of the NMR experiments (up to 3 d at 25 °C). Samples were analyzed in 10 mm NMR tubes on a General Electric Omega-400 at 40.55 MHz with proton decoupling at 400.13 MHz. Spectral data were acquired with a width of 20 000 Hz and 128−30000 scans per spectrum and processed using 1.0 Hz line broadening. Chemical shifts (δ) of ^{15}N were referenced to 110.0 ppm for external ^{15}N-labeled benzamide but reported against liquid NH_3 ($\delta = 0.0$ at 40.55 MHz).[11] ^{15}N spectra were collected over a 480 ppm range to assure observation of all N species.

^{15}N NMR relaxation times are long for solutes and short for protein-bound N. Figure 2A shows that $CH_3{}^{15}NH_3Cl$ yields a signal at $\delta = 35$ ppm with a long pulse delay (120 s) which decreases on a short pulse delay (1 s) due to saturation of the slowly relaxing species (data not shown). After reduction of MADH by excess $CH_3{}^{15}NH_3Cl$, two peaks are observed with a 1 s pulse delay (Figure 2B).[12] The upfield signal at $\delta = 35$ ppm exhibits a negative NOE and is characteristic of $CH_3{}^{15}NH_3{}^+$ (Table 1). The new signal observable optimally at a short pulse delay (1 s) appears downfield at $\delta = 54$ ppm. Anilino ^{15}N chemical shifts are downfield by about 25 ppm from alkylamines under similar conditions[13] (Table 1). The signal at $\delta = 54$ ppm is consistent with ^{15}N covalently attached to an

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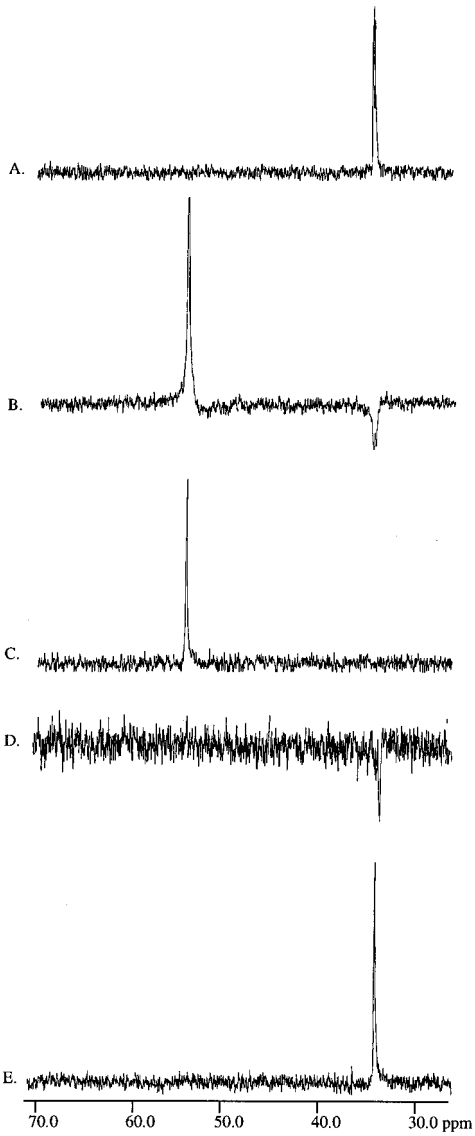

Figure 2. ^{15}N NMR spectra depicting the fate of substrate-derived N during the catalytic cycle of MADH. A. $CH_3{}^{15}NH_3Cl$ in the absence of enzyme (acquisitions per free induction decay [FID] = 128, pulse delay = 120 s, acquisition time = 2.2 s). B. MADH reduced by excess $CH_3{}^{15}NH_3Cl$, (acquisitions per FID = 30 000, pulse delay = 1 s, acquisition time = 2.2 s). C. Substrate-reduced MADH after extensive dialysis (acquisitions per FID = 30 000, pulse delay = 1 s, acquisition time = 2.2 s). D. MADH after reoxidation by excess PES (acquisitions per FID = 5000, pulse delay = 1 s, acquisition time = 2.2 s). E. Sample from D with added $^{15}NH_4Cl$ (acquisitions per FID = 5000, pulse delay = 60 s, acquisition time = 1.6 s).

aromatic ring (i.e., aminoquinol, Figure 1). With a short delay (1.0 s), $\delta = 54$ ppm accumulates, implying the signal relaxes quickly as expected for ^{15}N covalently bound to MADH. Additional verification that $\delta = 54$ ppm is due to nonexchangeable N was obtained from dialysis of the sample (Figure 2C). The negative peak at $\delta = 35$ ppm, attributed to free unreacted $CH_3{}^{15}NH_3{}^+$, is absent, whereas the $\delta = 54$ ppm signal is retained. To verify that $CH_3{}^{15}NH_3{}^+$ was absent and not unobserved due to saturation of a slowly relaxing species, the pulse delay was increased to 60 s. No signal at $\delta = 35$ ppm was detected even after 10 h of spectral acquisition. Thus, the signal at $\delta = 54$ ppm describes ^{15}N derived from a substrate which is covalently bonded to an aromatic system and attached to a protein framework.

(12) Due to their high viscosity, protein samples were somewhat difficult to shim, and observed line widths were variable. Thus, an analytic interpretation of observed line widths is not appropriate, particularly when sample spectra obtained from different runs are compared (e.g., Figures 2B,C). On average, line widths of $\delta = 54$ were 3-fold greater than those of $\delta = 35$ from free $CH_3{}^{15}NH_3{}^+$ and $^{15}NH_4{}^+$ (Figures 2A,D,E). The negative signal at $\delta = 35$ in Figure 2B is noticeably broadened. This suggests that the free $CH_3{}^{15}NH_3{}^+$ may be in slow exchange with protein bound $CH_3{}^{15}NH_3{}^+$.

Table 1. ^{15} NMR Experimental and Reference Chemical Shifts for Model Compounds

compd	concentrations and solvent	δ^{15}N (ppm)[a]	ref
NH_4Cl	saturated in 90% H_2O/10% D_2O	30	15
	1 M in 10% D_2O/90% buffer[b]	35	this work
CH_3NH_3Cl	saturated in 50% D_2O/50% H_2O	33	16
	1 M in 10% D_2O/90% buffer[b]	35	this work
$PhNH_3Cl$[c]	concentrated in 90% H_2O/10% D_2O	55	13b
	40% in 10% D_2O/90% H_2O	60	this work
$PhCONH_2$[d]	saturated in acetone, +10% D_2O	110	this work

[a] Nearest ppm referenced to NH_3(l) where $\delta = 0.0$ (refs 11 and 15). [b] Buffer is 10 mM potassium phosphate in H_2O, pH 7.5 with 200 mM KCl. [c] Natural abundance ^{15}N; all others enriched. [d] External reference in this work.

Immediately after treatment of the reduced MADH sample with a 100-fold excess of the oxidant phenazine ethosulfate[3b] (PES), one observes (Figure 2D) the disappearance of $\delta = 54$ ppm and the emergence of $\delta = 35$ ppm. The $\delta = 35$ ppm signal must be due to a slowly relaxing species (i.e., free rather than protein-bound N), since spectral accumulation occurs optimally at a long (60 s) pulse delay. This is consistent with free product $^{15}NH_4{}^+$ (Table 1). To confirm this, $^{15}NH_4Cl$ was added to the sample which led to an increase in $\delta = 35$ ppm (Figure 2E). Dialysis of the protein results in loss of all ^{15}N signals. The dialysate contains $^{15}NH_4{}^+$, judged by a signal at 35 ppm resolved positively by a long pulse delay (not shown).

Studies with model TPQ compounds suggested that reduced triol preparations may form (alkylamino)resorcinols via a redox cycling mechanism involving condensation of amines with trace amounts of hydroxyquinone in the triol preparations[14] or with cyclohexadienone tautomers. To confirm that the $\delta = 54$ ppm species is not due to covalent modification of *O*-quinol TTQ by excess $CH_3{}^{15}NH_3{}^+$, the experiments described in Figure 2 were repeated with dithionite-reduced MADH under identical conditions. No signal at $\delta = 54$ ppm was observed, only the slow relaxing signal at $\delta = 35$ ppm corresponding to free methylamine.

These data are consistent with and confirm the mechanisms presented in Figure 1, where quinone TTQ is covalently modified by substrate N to form a stable and relevant aminoquinol intermediate. Reoxidation of the aminoquinol to the quinone, results in loss of covalent N as free product $^{15}NH_4{}^+$. These data also demonstrate the utility of ^{15}N NMR spectroscopy as a tool for monitoring the reaction steps of enzymes which catalyze transformations of biological amines.

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