The Nitrogen Atom of Substrate Methylamine Is Incorporated into the Tryptophan Tryptophyl–Semiquinone Catalytic Intermediate in Methylamine Dehydrogenase

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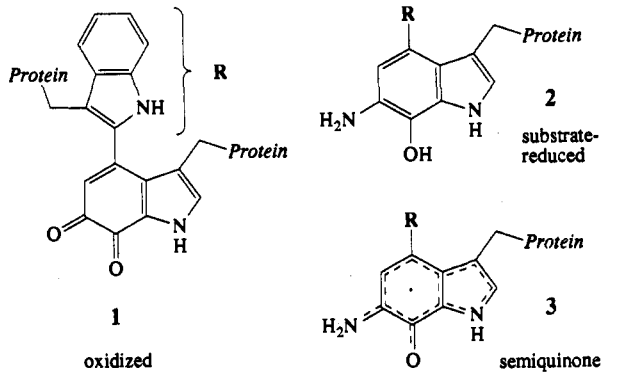
Methylamine dehydrogenase (MADH) from gram-negative bacteria catalyzes the oxidative deamination of methylamine to formaldehyde, ammonia, and two reducing equivalents.[2] MADH is among an emerging class of enzymes that employ redox-active and post-translationally-modified amino acid side chains as covalently-bound catalytic centers.[2,3] The cofactor structure common to MADH from different species has been identified by chemical methods[4,5] and confirmed by X-ray crystallographic analyses[6,7] to be two tryptophan side chains that are cross-linked covalently through their indole rings (see Chart I). One of the 6-membered rings is further modified with an *ortho*-carbonyl structure. Two moles of this tryptophan tryptophylquinone (TTQ, **1**) cofactor are present per mole of MADH. Addition of methylamine to MADH promotes rapid formation of a two-electron, substrate-reduced form of TTQ with concomitant release of formaldehyde.[8-10] By analogy with the reactivity of inhibitors of MADH activity, a nucleophilic attack of the substrate nitrogen atom at one of the carbonyl group carbon atoms has been suggested as an initial step in the reaction pathway, and a structure for substrate-reduced cofactor **2** has been proposed.[11,12] *In vivo*, two single-electron transfers to the blue copper protein amicyanin are required for release of ammonia from substrate-reduced MADH and thus to regenerate the resting enzyme.[2] The incorporation of the substrate nitrogen atom into TTQ has not been demonstrated directly, and its fate during the catalytic cycle is not known. Here, we use the electron spin echo envelope modulation (ESEEM) technique of pulsed-EPR spectroscopy[13,14] to demonstrate that the substrate nitrogen atom is covalently bonded to the predicted semiquinone intermediate **3** derived from substrate-reduced TTQ.

Chart I



The purification of MADH from *Paracoccus denitrificans*[15] and the selective *in vitro* preparation and characterization of various redox states of the TTQ cofactor have been reported.[16,17] Stable formation of the semiquinone intermediate has so far proven untenable in the presence of amicyanin and artificial exogenous electron acceptors.[16] Controlled extraction of a single electron from the substrate-reduced TTQ was achieved by comproportionation of a 1:1 mixture of substrate-reduced and oxidized TTQ cofactor.[17] The enzyme samples used for ESEEM spectroscopy thus contain a 1:1 molar proportion of semiquinone generated by oxidation of substrate-reduced TTQ and by direct reduction of oxidized TTQ.[18]

To reveal the presence of the substrate nitrogen in the semiquinone derived from substrate-reduced MADH, we compare identical samples of enzyme prepared by initial substrate reduction with either ^{14}N-methylamine or ^{15}N-methylamine. Distinct differences are expected in the magnetic interactions between the unpaired electron spin density and the nuclear spin $I = 1$ ^{14}N nucleus relative to those with the $I = {}^1/_2$ ^{15}N nucleus. Figure 1 shows ESEEM spectra obtained from Fourier transformations of the envelope modulation collected for the substrate-reduced MADH samples by using the stimulated echo 90°-τ-90°-T-90° microwave pulse sequence. The ESEEM spectra exhibit a dominant set of modulation components corresponding to the hyperfine and nuclear quadrupole transition frequencies that arise from the indole ring ^{14}N atoms.[19] Two features at 1.5 and 4.3 MHz are observed in the spectra of the ^{14}N-methylamine-reduced sample that are absent in the ^{15}N-methylamine-reduced sample. These results demonstrate magnetic interaction between the substrate nitrogen atom and the unpaired electron spin.

In order to facilitate a more detailed comparison of the spectra, the envelope modulation from the ^{14}N-methylamine- and ^{15}N-methylamine-reduced samples can be divided to attenuate the contributions of nuclei other than the substrate ^{14}N and ^{15}N.[20,21] Figure 1 displays two strong lines in the spectrum obtained from the divided envelopes at 1.5 and 4.3 MHz. The positive signs of these features indicate that they originate from the ^{14}N nucleus of substrate methylamine. The appearance of the ratio spectrum is characteristic of nitrogen coupling near the condition of "exact cancellation" of the ^{14}N nuclear Zeeman and hyperfine frequencies in one electron spin manifold.[23,24] The strong lines respond to

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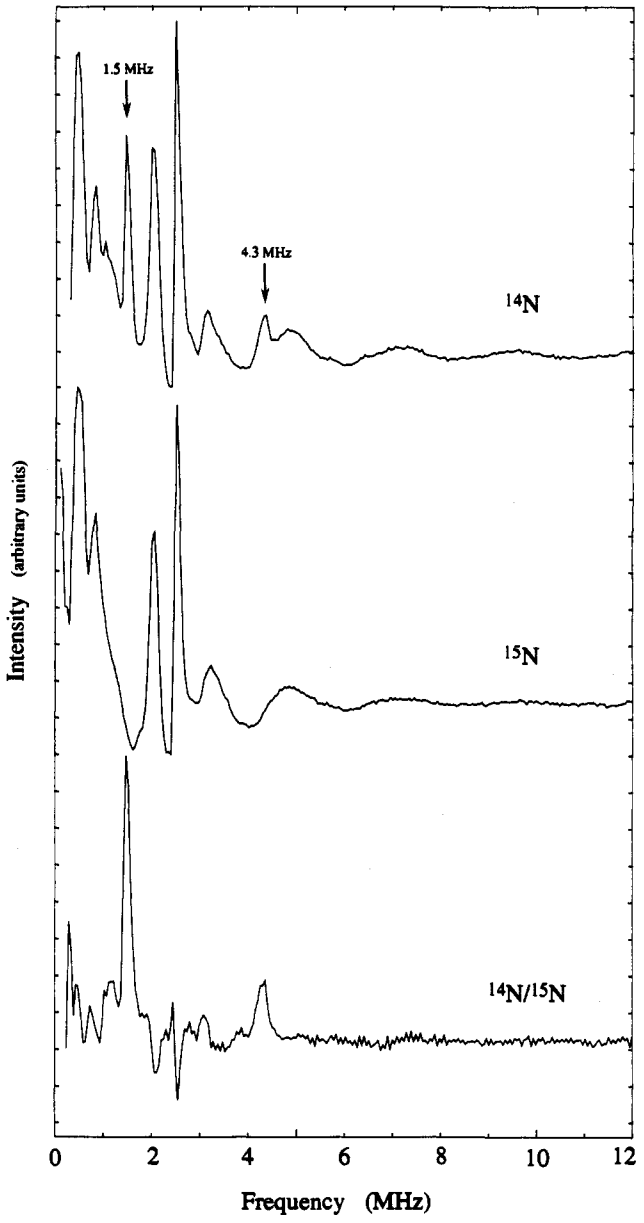

Figure 1. Three-pulse ESEEM spectra of MADH semiquinone derived from ^{14}N and ^{15}N substrate-reduced enzyme samples. Fourier transformation of envelope modulation from ^{14}N-methylamine-reduced sample (top) and ^{15}N-methylamine-reduced sample (middle) and after ^{14}N/^{15}N envelope division (bottom). Conditions: $\tau = 365$ ns; microwave frequency, 8.878 GHz; pulse width, 20 ns; pulse power, 100 W; pulse sequence repetition rate, 6 Hz; magnetic field strength, 0.3213 T; temperature, 4.2 K; enzyme concentration, 2.7×10^{-4} M. The design and construction of the pulsed-EPR spectrometer is essentially as described.[31]

changes in external field strength (0.3150–0.4900 T; 8.8–13.8 GHz) as expected[24] for this coupling regime: the 1.5-MHz peak remains stationary, whereas the 4.3-MHz feature migrates in proportion to twice the free ^{14}N nuclear Zeeman frequency. The relatively small hyperfine coupling of the substrate nitrogen is also consistent with the essentially identical continuous-wave EPR spectra obtained for the ^{14}N and ^{15}N substrate-reduced samples.

The broad feature observed at 4.3 MHz in Figure 1 represents the energy separation between the lowest and highest energy ^{14}N sublevels in the electron spin manifold in which the nuclear Zeeman and hyperfine interactions add. From the position of this feature, the isotropic coupling is estimated to be $|2.2|$ MHz. From the dependence of the breadth of the 4.3-MHz feature on τ values from 140 to 400 ns, we can also estimate that the dipolar coupling strength is ≤ 0.3 MHz. The dominant isotropic contribution to the hyperfine coupling accounts for the absence of strong negative features from the substrate ^{15}N nucleus.[25] These hyperfine parameters differ dramatically from those for substrate ^{14}N incorporated into the topa-semiquinone catalytic intermediate of amine oxidases.[3c]

The magnitude of the ^{14}N isotropic coupling indicates a covalent attachment of the substrate nitrogen to the radical.[26] An interaction between the radical and the substrate nitrogen atom that is mediated by a hydrogen bond is not consistent with the results of electron nuclear double resonance studies, which have demonstrated that the isotropic coupling with protons involved in hydrogen bonds to the oxygen atoms in semiquinones is negligible (<0.2 MHz).[27,28]

The energy level splitting in the electron spin manifold in which the ^{14}N nuclear Zeeman and hyperfine interactions approximately cancel is predominantly controlled by the nuclear quadrupole interaction. Narrow lines in the ESEEM spectra of ^{14}N at or near exact cancellation can reveal the three nuclear quadrupole frequencies ν_0, ν_-, and ν_+.[20,23,24] The appearance of only one strong quadrupole frequency feature at 1.5 MHz in our spectra therefore suggests that ν_- and ν_+ differ by less than 0.2 MHz. This is consistent with a quadrupole coupling constant in the range of 2.0 ± 0.2 MHz and relatively small values for the electric field gradient asymmetry parameter of <0.2.[29] These parameters are comparable with those obtained by pure ^{14}N quadrupole resonance[30] for sp^2-hybridized nitrogen engaged in substantial sharing of its lone-pair electrons.

The substrate nitrogen–TTQ semiquinone adduct demonstrated here provides strong, direct evidence for the incorporation of substrate into the cofactor in the steps preceding radical formation. The results also indicate that the substrate nitrogen is released following or in concert with the transfer of the second electron to amicyanin. Thus, it is clear that return of the cofactor to an oxidized state is required for elimination of ammonia.

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(21) Mims has shown that modulation components from different nuclei that are coupled magnetically to unpaired electron spin density in the same molecule contribute multiplicatively to the total envelope modulation.[22] To first order, division of three-pulse envelope modulation from two radicals that are labeled with different magnetic isotopes but that are otherwise identical therefore attenuates contributions from magnetic nuclei common to each radical and emphasizes contributions from coupling with the isotopes.[20] However, division of three-pulse envelopes gives rise to cross terms that may distort the relative intensities of the quotient lines. Comparison of the relative intensities of the 1.5- and 4.3-MHz ^{14}N features in Figure 1 shows that they differ by <10% before and after envelope division. Therefore, distortion created by envelope division is minor and does not alter our conclusions. Our samples also contain a mixture of two different radicals. We have therefore explored the technique of envelope subtraction by using envelope modulation from dithionite-generated TTQ semiquinine in MADH that is scaled appropriately to remove the additive contribution of this species from envelope modulation from the substrate-reduced samples. Results that are remarkably similar to those in Figure 1B are obtained. This indicates that alteration of the dominant magnetic interaction of the unpaired spin with the indole nitrogen atoms is not significant enough to cause a breakdown in the product rule.

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