Identification of reaction products and intermediates of aromatic-amine dehydrogenase by ^{15}N and ^{13}C NMR

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^{13}C - and ^{15}N -NMR studies of the reaction of aromatic amine dehydrogenase (AADH) with methylamine demonstrated that the products of the reductive half-reaction are an equivalent of formaldehyde hydrate and a reduced aminoquinol form of the tryptophan tryptophylquinone (TTQ) cofactor which contains covalently bound substrate-derived N. These data are consistent with the Ping Pong kinetic mechanism and aminotransferase-type chemical reaction mechanism which have been previously proposed for AADH. Comparison of the ^{15}N -NMR spectra of the aminoquinol TTQ intermediates of AADH and methylamine dehydrogenase (MADH) revealed that the substrate-derived aminoquinol N of AADH and MADH exhibited distinct ^{15}N chemical shifts which are separated by approx. 7 p.p.m. In each

case, the signal for the substrate-derived aminoquinol N appears optimally with short pulse delay and exhibits a relaxation time and chemical shift which are consistent with ^{15}N covalently bound to an aromatic ring (i.e. aminoquinol) which is attached to a rigid protein matrix. The aminoquinol of AADH is less stable against reoxidation than that of MADH. These data suggest that differences in the active-site mediated electrostatic environments of the aminoquinol N in the respective enzymes may influence both the observed ^{15}N chemical shift and the relative reactivities of the TTQ aminoquinols towards oxygen. These data also demonstrate the utility of ^{13}C - and ^{15}N -NMR spectroscopy as a tool for monitoring the intermediates and products of enzyme-catalysed transformations.

INTRODUCTION

Aromatic amine dehydrogenase (AADH) is a quinoprotein which catalyses the oxidative deamination of primary amines to their corresponding aldehydes plus ammonia [1,2]. It possesses the prosthetic group tryptophan tryptophylquinone (TTQ, Figure 1) [3]. We have previously characterized the microscopic rate constants associated with the reductive half-reaction of AADH with a variety of aromatic amines [2] and the kinetic parameters for the reactions of AADH with artificial and natural electron acceptors. On the basis of the results of these steady-state and transient kinetic studies, a mechanism was proposed for the reductive half-reaction of AADH. It was proposed that substrate forms a covalent adduct with TTQ and that the aldehyde product is released from the reduced enzyme prior to reoxidation,

Figure 1 Tryptophan tryptophylquinone

The structure of the TTQ cofactor is shown in its oxidized redox form. In the substrate-reduced form of the enzyme the O at C-6 is displaced by a substrate-derived amino group.

resulting in the formation of a relatively stable aminoquinol intermediate. Similar mechanisms have been proposed for other amine oxidizing quinoproteins, including methylamine dehydrogenase (MADH) [4], which also possesses TTQ [3], plasma amine oxidase [5], which utilizes the topaquinone (TPQ) [6], and lysyl oxidase [7], which utilizes lysine tyrosylquinone (LTQ) [8].

It was previously demonstrated by ^{15}N -NMR spectroscopy that the reaction mechanism of MADH included a stable aminoquinol intermediate with substrate-derived N covalently bound to the C-6 carbon of TTQ [9]. No such studies have been performed for any of the other amine-oxidizing quinoproteins. Furthermore, the identity of the aldehyde reaction product and the order of product release has never been directly addressed by spectroscopic analysis for AADH, or any other of these quinoproteins.

In the present study, the fates of substrate-derived carbon and nitrogen during the reaction with AADH were monitored by ^{13}C and ^{15}N NMR. Reaction products and intermediates are identified. Similarities and significant differences in the properties and reactivities of the aminoquinol TTQ intermediates of AADH and MADH are revealed by these studies.

MATERIALS AND METHODS

AADH was purified from *Alcaligenes faecalis* (I.F.O. 14479) as described previously [1]. MADH was purified from *Paracoccus denitrificans* (A.T.C.C. 13543) as described previously [10]. Protein concentrations were calculated from known absorption coefficients of oxidized and reduced MADH [11] and AADH [1]. Substrate-reduced aminoquinol forms of AADH and MADH were prepared by mixing oxidized AADH or MADH with a 20–100-fold excess of methylamine. Oxidation of reduced methyl-

Abbreviations used: AADH, aromatic-amine dehydrogenase; MADH, methylamine dehydrogenase; TTQ, tryptophan tryptophylquinone; TPQ, topaquinone; LTQ, lysyl tyrosylquinone; PES, phenazine ethosulphate; NOE, nuclear Overhauser effect.

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amine was accomplished by reaction with excess phenazine ethosulphate (PES). When it was necessary to remove excess reactants or non-covalently bound solutes, samples were washed by repeated ultrafiltration over an Amicon XM50 membrane. ^{13}C - and ^{15}N -labelled compounds were purchased from either CDN Isotopes (Pointe-Claire, Quebec, Canada) or Cambridge Isotope Laboratories (Cambridge, MA, U.S.A.). All other reagents were obtained from Sigma.

Static UV-visible absorption spectra of AADH were recorded with a Milton-Roy Spectronic 3000 diode-array spectrophotometer. NMR spectra were recorded with either a General Electric Omega 400 MHz or a Bruker AMX 600 MHz spectrometer in 10 mm or 4 mm NMR tubes at 21 °C.

All ^{13}C -NMR experiments were performed in 10 mM Bistris Propane buffer, pH 7.5, plus 200 mM KCl, with 10 % (v/v) $^2\text{H}_2\text{O}$ for NMR signal lock, unless otherwise stated. ^{13}C -NMR spectra were collected using the 600 MHz NMR spectrometer at an operating frequency of 180.9 MHz with proton (^1H) decoupling at 600.1 MHz. Spectra were collected over a width of 33.3 kHz with 32–1024 transients. The pulse routine used included a 2 s delay and a signal acquisition time of 1 s. Pulses were applied at a 30° angle. The spectral width was 33.3 kHz and all spectra were acquired with 64000 data points. ^{13}C spectra were processed using 1 Hz line-broadening. All chemical shifts (δ) of ^{13}C were referenced against tetramethylsilane ($\delta = 0.0$ at 60.8 MHz).

All ^{15}N -NMR experiments were performed in 10 mM Bistris Propane buffer, pH 7.5, plus 200 mM KCl, with 10 % (v/v) $^2\text{H}_2\text{O}$ for NMR signal lock. The GE Omega 400 instruments operates at a frequency of 40.5 MHz for the ^{15}N nuclei and uses an inverse-gated ^1H decoupling pulse routine. In the 400 MHz spectrometer, ^1H decoupling was applied non-continuously and was active only during signal acquisition. The Bruker AMX-600 spectrometer operates at a frequency of 60.8 MHz with continuous ^1H decoupling at 600.1 MHz. To insure observation of all relevant N species in both 400 and 600 MHz spectrometers, spectral data were collected with a width of 15.0 kHz and 18.5 kHz, respectively. All ^{15}N spectra were collected with 32–1024 transients with a 1–120 s pulse delay with signal acquisition times of 2.2 s at 400 MHz and 0.9 s at 600 MHz. All samples were pulsed at either 45° or 90° angles. Exponential multiplication and Fourier transform with either 1, 5 or 10 Hz line-broadening were utilized for data processing of ^{15}N spectra. All chemical shifts (δ) of ^{15}N were referenced to 110.0 p.p.m. for external ^{15}N -labelled benzamide, but reported against liquid NH_3 ($\delta = 0.0$ at 40.6 MHz or 60.8 MHz) [12]. ^{15}N spectra were collected over a 480 p.p.m. range to assure observation of all N species.

RESULTS AND DISCUSSION

Previous steady-state kinetic analysis indicated that methylamine was a substrate for AADH. The preferred substrates for AADH are substituted phenylethylamines. Methylamine exhibited a K_m approx. 1000-fold greater than phenylethylamine and an approx. 10-fold smaller k_{cat} [2]. Although methylamine is not the preferred substrate for AADH, it was the substrate of choice in the present study for several reasons. An important practical reason is that [^{13}C]methylamine chloride ($^{13}\text{CH}_3\text{NH}_3\text{Cl}$) and [^{15}N]methylamine chloride ($\text{CH}_3^{15}\text{NH}_3\text{Cl}$) are each commercially available, whereas the correspondingly labelled substituted phenylethylamines are not. The use of methylamine also presents the advantage of using a substrate which possesses only a single carbon and single nitrogen. This allows for clear observation of the fates of the single substrate-derived ^{13}C and ^{15}N atoms during the course of experiments, and simplifies the interpretation of the results. Since

Figure 2 Reduction of AADH by methylamine

Absorption spectra of AADH (25 μM) were recorded in 0.05 M potassium phosphate, pH 7.5, at 25 °C before (continuous line) and after (broken line) the addition of methylamine. These spectra are characteristic of the fully oxidized and fully reduced forms of the enzyme [1,2].

one aim of the present study was to compare and contrast the reaction products and intermediates of AADH with those of MADH, the use of the same substrate for both enzymes is also advantageous. The ability of methylamine to reduce AADH is demonstrated in Figure 2. Whereas this redox reaction has been described previously [2], direct determination of the products of the reaction of amines with AADH has not previously been reported. NMR experiments are presented here which address the nature of the intermediates and products which accumulate during reaction of AADH with methylamine.

To examine the fate of the substrate-derived carbon, the steady-state turnover of $^{13}\text{CH}_3\text{NH}_3\text{Cl}$ by AADH was examined with PES present as an oxidant. The fate of the substrate-derived carbon is revealed in Figure 3. ^{13}C -NMR spectra were collected prior to the addition of a catalytic amount of AADH (Figure 3A). The only feature of the resulting spectrum is that of $^{13}\text{CH}_3\text{NH}_3^+$, which exhibits a chemical shift of 27 p.p.m. The ^1H -coupled spectrum of this compound was also collected (Figure 3B). The carbon signal is split into a quartet which is expected for the sp^3 hybridized carbon in $^{13}\text{CH}_3\text{NH}_3^+$. To this solution was added a catalytic amount of AADH. This results in the appearance of a new signal exhibiting a chemical shift of 83 p.p.m. (Figure 3C). The persistence of the signal at 27 p.p.m. is due to the presence of excess methylamine. The appearance of a new signal is expected if some $^{13}\text{CH}_3\text{NH}_3^+$ has been converted into product. In contrast with what is seen for the signal at 27 p.p.m., the ^1H -coupled spectrum of this new species reveals that the carbon responsible for the signal at 83 p.p.m. is split into a triplet (Figure 3D). This indicates that this carbon is bonded to only two H atoms, consistent with that signal representing the oxidized aldehyde product. Undiluted formaldehyde ($^{13}\text{CH}_2\text{O}$) exhibits a chemical shift at 220 p.p.m. (Table 1; [13]) that is far upfield of the signal seen in Figures 3(C) and 3(D). However, formalin-1,1-diol [$^{13}\text{CH}_2(\text{OH})_2$], which is the hydrate of formaldehyde, does exhibit a chemical shift at 83 p.p.m. (Table 1; [13]). The results in Figure 3 indicate that the formaldehyde product is released from the enzyme, since the equilibrium composition of aqueous formaldehyde is shifted nearly exclusively to the formalin diol. Control experiments demonstrated that methylamine is redox-stable in the presence of

Figure 3 ^{13}C -NMR spectra depicting the fate of substrate-derived ^{13}C during the reaction of methylamine with AADH

(A) The ^1H -decoupled ^{13}C -NMR spectrum of the steady-state reaction mixture including $^{13}\text{CH}_3\text{NH}_3\text{Cl}$ and PES in the absence of AADH. (B) The ^1H -coupled ^{13}C -NMR spectrum of the sample in A. (C) The ^1H -decoupled ^{13}C -NMR spectrum of the reaction mixture in (A) after addition of a catalytic amount of AADH. (D) The ^1H -coupled ^{13}C -NMR spectrum of the sample in (C).

Table 1 ^{13}C - and ^{15}N -NMR chemical shifts (δ)

Compound	Concentration (solvent)	δ (p.p.m.)	
		$^{15}\text{N}^*$	$^{13}\text{C}^\dagger$
$^{13}\text{CH}_3\text{NH}_3\text{Cl}$	1 M in 10% $^2\text{H}_2\text{O}$ (90% buffer) ‡		27
$^{13}\text{CH}_2\text{O}$	Undiluted		220
$^{13}\text{CH}_2(\text{OH})_2$ (formalin)	40% in 10% $^2\text{H}_2\text{O}$ (90% buffer) ‡		83
$^{15}\text{NH}_3\text{Cl}$	1 M in 10% $^2\text{H}_2\text{O}$ (90% buffer) ‡	35	
$\text{CH}_3^{15}\text{NH}_3\text{Cl}$	1 M in 10% $^2\text{H}_2\text{O}$ (90% buffer) ‡	35	
$\text{C}_6\text{H}_5\text{NH}_3\text{Cl}$ (anilinium)	40% in 10% $^2\text{H}_2\text{O}$ (90% water)	60	
$\text{C}_6\text{H}_5^{15}\text{NH}_2$ (aniline)	Saturated in C_6H_6 (^{12}H)benzene)	70	
MADH TTQ-aminoquinol	2 mM in 10% $^2\text{H}_2\text{O}$ (90% buffer)	54	
AADH TTQ-aminoquinol	0.5 mM in 10% $^2\text{H}_2\text{O}$ (90% buffer)	61	

* Nearest p.p.m. referenced to NH_3 (liquid), where $\delta = 0.0$.

† Nearest p.p.m. referenced to tetramethylsilane, where $\delta = 0.0$.

‡ Buffer is 10 mM potassium phosphate in water, pH 7.5 with 200 mM KCl.

§ Natural abundance ^{15}N , all others enriched.

|| Buffer was 10 mM Bistris Propane, pH 7.5, with 200 mM KCl.

the oxidant PES (Figures 3A and 3B). AADH, therefore, was responsible for the catalytic reaction. Rapid production of the formaldehyde hydrate was also observed in the presence of stoichiometric amounts of AADH, in the absence of PES (results not shown), consistent with the previously proposed Ping Pong kinetic mechanism for AADH [1] in which the aldehyde product is released prior to reoxidation by the electron acceptor.

A critical question is whether the substrate-derived N of $\text{CH}_3^{15}\text{NH}_3^+$ forms a stable covalent adduct with the TTQ cofactor

of AADH. This was previously demonstrated for the other TTQ-dependent enzyme, MADH, by monitoring that reaction by ^{15}N -NMR spectroscopy [9]. It is important to ascertain whether covalent adduct formation is a common feature of the chemical mechanism of oxidative deamination by TTQ-dependent enzymes. Furthermore, if such an adduct is formed, ^{15}N -NMR provides a powerful probe for examining the similarities and differences in the properties and stability of the adducts formed with TTQ in AADH and MADH.

AADH was incubated with excess $\text{CH}_3^{15}\text{NH}_3\text{Cl}$ and the reaction mixture was analysed by ^{15}N NMR (Figure 4). Figure 4(A) displays the ^{15}N -NMR spectrum of 0.5 M $\text{CH}_3^{15}\text{NH}_3\text{Cl}$ at pH 7.5, in the absence of AADH. The single feature present at 35 p.p.m. accumulates optimally with long pulse delay (60 s) and is, therefore, characteristic of a low-molecular-mass solute. Figure 4(B) displays the resulting spectrum after 12 h spectral acquisition of a sample containing 0.5 mM AADH and 0.5 M $\text{CH}_3^{15}\text{NH}_3\text{Cl}$ with a pulse delay of 1 s. As is evident, two signals are present in the spectrum. The first occurs with a chemical shift of 35 p.p.m. and is characteristic of the alkylammonium N of the excess $\text{CH}_3^{15}\text{NH}_3^+$. The second, at 61 p.p.m., is the result of the reaction with AADH. It is possible to distinguish whether substrate-derived ^{15}N has formed a covalent adduct based on the optimal pulse delay time for observation of the signal [13]. Large molecules, such as proteins, will relax much more quickly than small molecules such as CH_3NH_3^+ and NH_4^+ . If ^{15}N forms a covalent adduct with AADH, we will observe a significant decrease in relaxation time compared with free $\text{CH}_3^{15}\text{NH}_3^+$. This may be easily determined experimentally by varying the pulse time for the NMR measurement. In contrast with the signal at 35 p.p.m., that at 61 p.p.m. is unobservable at longer pulse delay

Figure 4 ^{15}N -NMR spectra depicting the fate of substrate-derived ^{15}N during the catalytic cycle of AADH

(A). The ^1H -decoupled ^{15}N -NMR spectrum of $\text{CH}_3^{15}\text{NH}_3\text{Cl}$ in the absence of AADH. (B). The ^1H -decoupled ^{15}N -NMR spectrum of a mixture of AADH with excess $\text{CH}_3^{15}\text{NH}_3\text{Cl}$ in the absence of PES.

(i.e. 60 s). This observation would be expected if the ^{15}N were covalently bound to a large fast-relaxing molecule such as the protein matrix of AADH. Furthermore, the relative amplitude of the methylamine signal at 35 p.p.m. in Figure 4(B) is lower than expected because that signal is diminished relative to what would be seen with longer pulse delay as a result of saturation at short pulse delay. That these two species exhibit such different relaxation characteristics is consistent with the new signal at 61 p.p.m., which appears optimally with short pulse delay, deriving from the aminoquinol reaction intermediate. Further evidence for the identity of the species responsible for the new signal comes from its relative chemical shift. Model compounds that contain primary amines bound to an aromatic ring have similar chemical shifts which are well separated from those of N which is bonded to aliphatic substituents (Table 1; [14]). That the new signal present in Figure 4(B) has a chemical shift similar to that of aniline confirms that the signal is characteristic of an amino group covalently attached to an aromatic system such as TTQ.

These data may be compared with those obtained previously for the reaction of methylamine with MADH [9]. When $\text{CH}_3^{15}\text{NH}_3\text{Cl}$ was incubated with MADH, ^{15}N -NMR analysis revealed two signals at 35 p.p.m. and 54 p.p.m. Differences in relaxation times for the two signals were also apparent in that study. With short pulse delay, the 35 p.p.m. signal attributable to free $\text{CH}_3^{15}\text{NH}_3\text{Cl}$ exhibited a negative nuclear Overhauser effect (NOE) relative to the peak at 54 p.p.m. as a result of signal saturation [9]. No negative NOE is observed in the current study with AADH, but the diminished signal (discussed above) is consistent with a saturating species of low molecular mass as was observed with MADH. Using the 600 MHz spectrometer we have repeated the previously reported experiments with MADH that were performed with a 400 MHz NMR (results not shown). Two signals with chemical shifts of 35 and 54 p.p.m. were again observed and, as expected, the slow-relaxing signal at 35 p.p.m. does not exhibit a negative NOE but is diminished in amplitude.

A significant difference in the results of the studies of the reactions of AADH and MADH with methylamine is the position of the chemical shift for the substrate-derived aminoquinol N (i.e. 54 p.p.m. in MADH and 61 p.p.m. in AADH). The ^{15}N spectrum of aminoquinol-TTQ in AADH is shifted upfield by 7 p.p.m. with respect to that of the aminoquinol-TTQ observed in MADH. Typically, N chemical shifts collected in 400 and

600 MHz spectrometers are accurate to 0.50 p.p.m. AADH and MADH are believed to contain the same TTQ cofactor. For MADH this has been verified by chemical analysis [3] and X-ray crystallography [15]. For AADH the assignment of TTQ was based on resonance Raman analysis and spectroscopic properties [1]. These results suggest that either the AADH and MADH aminoquinols exhibit different ionization states or that residues at the active site of each enzyme interact strongly, though differently, with the substrate-derived N of the aminoquinol-TTQ intermediate.

Chemical shifts of ^{15}N in the model compound aniline vary with solvent conditions that effect its protonation state and dielectric environment (Table 1). It was not possible to determine directly the protonation state of the aminoquinol in MADH using proton-coupled ^{15}N NMR (results not shown), probably because of rapid exchange of the amine protons. Differences in the dielectric environments of the active sites of MADH and AADH could account for the 7 p.p.m. difference in the chemical shifts of the two aminoquinol signals. The dielectrics of the active sites of enzymes such as MADH and AADH are expected to be controlled by the side chains of the amino acids which comprise the active site. Some significant differences in the active sites of AADH and MADH are expected given their different substrate specificities. Further characterization of this phenomenon must await the determination of a high-resolution crystal structure of AADH for comparison with that now available for MADH [15].

Another significant difference in the aminoquinol intermediates of AADH and MADH is the relative stabilities of the two species. It was previously shown [9] that the aminoquinol of MADH was highly stable in the absence of excess substrate and in the presence of molecular oxygen during the time course of the NMR experiments. With the MADH aminoquinol it was possible to remove excess substrate by buffer exchange by ultrafiltration. Subsequent hydrolysis of the aminoquinol to yield the product ammonia was only accomplished by reoxidation of the aminoquinol by the oxidant PES. In contrast with this, significant reoxidation of AADH occurred during ultrafiltration of the sample to remove excess substrate. Thus it was not possible to remove completely the excess substrate and maintain the AADH aminoquinol redox state for very long periods of time.

Evidence for the relative instability of the AADH aminoquinol was also obtained in NMR experiments in which $\text{CH}_3^{15}\text{NH}_3\text{Cl}$

Figure 5 Relative stabilities of substrate-reduced AADH and MADH to oxidation

Equal concentrations ($60 \mu\text{M}$) of MADH (▼) and AADH (●) were each reduced with methylamine and incubated in 0.2 M potassium phosphate, pH 7.5, at 25°C in the dark. The absorption spectrum of each was periodically recorded and the fraction of each enzyme which was in its reduced redox form was determined from the absorption spectrum of each enzyme. The continuous lines represents fits of each data set to an equation for a single exponential decay. Fits of these data yielded half-times of 56 and 9 h respectively for MADH and AADH.

was incubated with AADH in the absence of PES and the time course of the reaction was monitored. In these experiments, the aminoquinol signal at 61 p.p.m. accumulates within the first 12 h of the run, but begins to disappear over the next 12 h. The disappearance of the 61 p.p.m. signal was accompanied by stabilization of the 35 p.p.m. signal. Stabilization of the 35 p.p.m. signal is most likely due to accumulation of the product ammonia, which corresponds to the loss of aminoquinol signal at 61 p.p.m. The chemical shift for $^{15}\text{NH}_4^+$ is indistinguishable from that of $\text{CH}_3^{15}\text{NH}_3^+$ (Table 1). When the sample was removed from the spectrophotometer after the 24 h run, it exhibited an absorption spectrum which was characteristic of the oxidized form of the enzyme.

To determine the extent to which the reoxidation of substrate-reduced AADH is oxygen-dependent, a redox-stability study was performed. AADH and MADH were each reduced by addition of methylamine, and excess methylamine was removed. The absorption spectra of these samples was monitored with time to determine changes in redox state with time. In the presence of atmospheric oxygen, the redox stability of AADH is much less than that of MADH (Figure 5). The experimentally determined half-times for oxidation were 9 and 56 h respectively for AADH and MADH. (During the present study it was observed that the rate at which the substrate-reduced enzymes oxidized increased in the presence of light. The data reported are for enzymes which were incubated in the dark, since this more closely matched the conditions in the NMR tube during the course of NMR experiments.) The time course for the reoxidation of AADH observed in Figure 5 is consistent with the time course for the loss of covalently bound ^{15}N from substrate-reduced AADH in NMR experiments (discussed above). This is important, because it confirms that the aminoquinol form of AADH is relatively stable in the absence of an oxidant (i.e. N is not being displaced by O in the absence of a redox reaction to generate the reduced

quinol). It also reveals another important distinction between AADH and MADH, namely the reactivity of the aminoquinol forms with oxygen. The ability of AADH and MADH to stabilize the reduced forms of TTQ is an important feature of this class of enzymes. Reduced quinones are typically extremely unstable in the presence of oxygen. Furthermore, other classes of amine-oxidizing quinoproteins which utilize TPQ and LTQ are oxidases which react readily with oxygen. Exactly how the reduced forms of TTQ enzymes are stabilized by the protein environment is not known. It is possible that the same features of AADH which are responsible for the difference in the chemical shift exhibited by the AADH aminoquinol N relative to MADH may also contribute to the differential reactivity with oxygen which is exhibited by these enzymes.

Conclusion

The ^{13}C -NMR and ^{15}N -NMR data obtained in the present studies show that the products of the reductive half-reaction of AADH with methylamine are an equivalent of formaldehyde hydrate and a reduced aminoquinol-TTQ intermediate which contains the substrate-derived N. These data are consistent with the Ping Pong kinetic mechanism [1] and the aminotransferase-type chemical reaction mechanism [2] which have been previously proposed for AADH. The substrate-derived aminoquinol nitrogens of AADH and MADH exhibit ^{15}N chemical shifts which are separated by approx. 7 p.p.m. and may reflect differences in the active-site mediated electrostatic environment of the aminoquinol N. The aminoquinol of AADH is also less stable against reoxidation than that of MADH, suggesting that the active-site environments of the respective enzymes influence the relative reactivity of the aminoquinols. These data also demonstrate the utility of ^{13}C - and ^{15}N -NMR spectroscopy as a tool for monitoring the intermediates and products of enzyme-catalysed transformations.

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REFERENCES

- Govindaraj, S., Eisenstein, E., Jones, L. H., Chistoserdov, A. Y., Sanders-Loehr, J., Davidson, V. L. and Edwards, S. L. (1994) *J. Bacteriol.* **176**, 2922–2929
- Hyun, Y.-L. and Davidson, V. L. (1995) *Biochemistry* **34**, 816–823
- McIntire, W. S., Wemmer, D. E., Chistoserdov, A. Y. and Lidstrom, M. E. (1991) *Science* **252**, 817–824
- Brooks, H. B., Jones, L. H. and Davidson, V. L. (1993) *Biochemistry* **32**, 2725–2729
- Hartmann, C. and Klinman, J. P. (1991) *Biochemistry* **30**, 4605–4611
- Janes, S. M., Mu, D., Wemmer, D., Smith, A. J., Kaur, S., Mautry, D., Burlingame, A. L. and Klinman, J. P. (1990) *Science* **248**, 981–987
- Williamson, P. R. and Kagan, H. M. (1987) *J. Biol. Chem.* **262**, 8196–8201
- Wang, S. X., Mure, M., Katalin, F. M., Burlingame, A. L., Brown, D. E., Dooley, D. M., Smith, A. J., Kagan, H. M. and Klinman, J. P. (1996) *Science* **273**, 1079–1084
- Bishop, G. R., Valente, E. J., Whitehead, T. L., Brown, K. L., Hicks, R. T. and Davidson, V. L. (1996) *J. Am. Chem. Soc.* **118**, 12868–12869
- Davidson, V. L. (1990) *Methods Enzymol.* **188**, 241–246
- Husain, M., Davidson, V. L., Gray, K. A. and Knaff, D. B. (1987) *Biochemistry* **26**, 4139–4143
- Srinivasan, P. R. and Licheter, R. L. (1977) *J. Magn. Reson.* **28**, 277–285
- Levey, G. C. and Nelson, G. L. (1972) *Carbon-13 Nuclear Magnetic Resonance for Organic Chemists*, John Wiley and Sons, New York
- Axenrod, T. and Weider, M. J. (1976) *Org. Magn. Res.* **8**, 350–353
- Chen, L., Matthews, F. S., Davidson, V. L., Huizinga, E., Vellieux, F. M. D. and Hol, W. G. J. (1992) *Proteins: Struct. Funct. Genet.* **14**, 288–299