Resonance Raman Spectroscopy of Methylamine Dehydrogenase from Bacterium W3A1†

William S. McIntire,*,‡ Jennifer L. Bates,§ Doreen E. Brown,§ and David M. Dooley*,§

Molecular Biology Division, Veterans Administration Medical Center, San Francisco, California 94121, Departments of Biochemistry and Biophysics and of Anesthesia, University of California, San Francisco, California 94143, and Department of Chemistry, Amherst College, Amherst, Massachusetts 01002

Received February 21, 1990; Revised Manuscript Received September 12, 1990

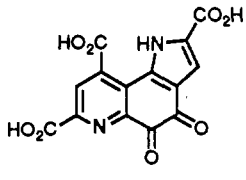
ABSTRACT: Resonance Raman spectroscopy has been used to probe the structure of the covalently bound quinone cofactor in methylamine dehydrogenase from the bacterium W3A1. Spectra were obtained on the phenylhydrazine and 2-pyridylhydrazine derivatives of the native enzyme, on the quinone-containing subunit labeled with phenylhydrazine, and on an active-site peptide also labeled with phenylhydrazine. Comparisons of these spectra to the corresponding spectra of copper-containing amine oxidase derivatives indicate that the quinones in these two classes of quinoproteins are not identical. The resonance Raman spectra of the native enzyme and small subunit have also been measured. $^{16}O/^{18}O$ exchange permitted the carbonyl modes of the quinone to be identified in the resonance Raman spectrum of oxidized methylamine dehydrogenase: a band at 1614 cm^{-1}, together with a shoulder at 1630 cm^{-1}, are assigned as modes containing substantial C=O stretching character. D_2O/H_2O exchange has pronounced effects on the resonance Raman spectrum of the oxidized enzyme, suggesting that the quinone may have numerous hydrogen bonds to the protein or that it is unusually sensitive to the local environment. Resonance Raman spectra of the isolated small subunit, and its phenylhydrazine derivative, are considerably different from the corresponding spectra of the intact protein. An attractive explanation for these observations is that the quinone cofactor in methylamine dehydrogenase from W3A1 is located at the interface between the large and small subunits, as found for the enzyme from *Thiobacillus versutus* [Vellieux, F. M. D., Huitema, F., Groendijk, H., Kalk, K. H., Frank, J. Jzn., Jongejan, J. A., & Duine, J. A. (1989) *EMBO J. 8*, 2171–2178]. Efforts to obtain spectra on the ammonia complex of methylamine dehydrogenase were unsuccessful, owing to the photosensitivity of this form of the enzyme.

Methylamine dehydrogenase is one of a group of enzymes that are known to contain a quinone cofactor. This enzyme and other bacterial quinoproteins, such as methanol dehydrogenase and methylamine oxidase, are involved in C_1 metabolism (Duine & Jongejan, 1989; Duine et al., 1987). Methylamine dehydrogenase catalyzes the oxidation of methylamine to formaldehyde and ammonia, as shown:

$$CH_3NH_3^+ + H_2O \rightarrow HCHO + NH_4^+ + 2H^+ + 2e^-$$

The W3A1 enzyme is a tetramer ($\alpha_2\beta_2$) of two types of subunits with molecular weights of 45000 (α) and 15000 (β); the quinone cofactor is covalently attached to the β subunits.

Methanol dehydrogenases from methylotropic bacteria are known to contain noncovalently bound pyrroloquinoline quinone (PQQ,[1] **1**) (Hartmann & Klinman, 1988; Duine & Jongejan, 1989; Duine et al., 1987), and PQQ was suggested to be the cofactor in methylamine dehydrogenases as well (van der Meer et al., 1987; Husain & Davidson, 1987). However, considerable evidence suggests that the covalently bound quinone in methylamine dehydrogenase is structured differently (DeBeer et al., 1980; Kenney & McIntire, 1983; Ameyama et al., 1984; Vellieux et al., 1989; Vellieux & Hol, 1989). For example, the ENDOR spectrum of the semiquinone form of methylamine dehydrogenase is not identical with that of methanol dehydrogenase (DeBeer et al., 1980); the visible absorption and emission spectra of oxidized, reduced, and semiquinone forms of methylamine dehydrogenase are also substantially different from the corresponding spectra of PQQ and its derivatives (Kenney & McIntire, 1983; Ameyama et al., 1984). Recent X-ray diffraction data on the methylamine dehydrogenase from *Thiobacillus versutus* have been interpreted in terms of a modified form of PQQ, in which the pyridine ring is not closed (Vellieux et al., 1989). Finally, the properties of a cofactor-containing active-site peptide from the methylamine dehydrogenase of bacterium W3A1 are not consistent with unmodified PQQ (McIntire & Stults, 1986).[2] These data may be considered together because there are indications that the methylamine dehydrogenases from *Thiobacillus* and W3A1 are structurally similar. Both have $\alpha_2\beta_2$ composition and identical molecular weights (Kenney & McIntire, 1983; Vellieux et al., 1989). In addition, the se-



1

†This research was supported by the NIH (Grant GM 27659 to D.M.D. and Program Project Grant HL 16251 to W.S.M.), the NSF (Grant DMB-8718741 to W.S.M.), and the Veterans Administration.

* To whom correspondence should be addressed.

‡Veterans Administration Medical Center and University of California.

§Amherst College.

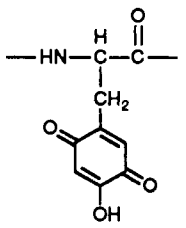
[1] Abbreviations: PQQ, pyrroloquinoline quinone; MADH, methylamine dehydrogenase.

[2] Recent, more detailed studies of the active-site peptides are consistent with this suggestion (W. S. McIntire, unpublished observations).

0006-2960/91/0430-125$02.50/0

quences of the β subunit around the quinone binding site are highly conserved (McIntire & Stultz, 1986; Vellieux et al., 1989). Finally there are spectral similarities, although the data on the *Thiobacillus* enzyme are limited (Kenney & McIntire, 1983; Vellieux et al., 1986).

A covalently bound quinone is also a cofactor of copper-containing amine oxidases, including the methylamine oxidase of *Arthrobacter* P1 (Knowles & Yadav, 1984; Dooley et al., 1990; van Iersal et al., 1986). Previous experimental results suggested that the quinone cofactor in amine oxidases was PQQ or a similar compound (Hartmann & Klinman, 1988; Duine & Jongejan, 1989; Duine et al., 1987; Lobenstein-Verbeek et al., 1984; van der Meer & Duine, 1986; Moog et al., 1986; Knowles et al., 1987; Williamson et al., 1986; Hartmann & Klinman, 1987; Citro et al., 1989; van der Meer et al., 1989). However, recent work by Klinman and co-workers has definitively established that the quinone cofactor in bovine plasma amine oxidase is the oxidized form of 6-hydroxydopa quinone (topa quinone; **2**) (Janes et al., 1990).



2

Comparing the spectral features of copper-containing amine oxidases to those of methylamine dehydrogenases suggests that the quinones in these enzymes are not identical. Furthermore, their reactivities toward ammonia, dithionite, and other small molecules are distinctly different (Kenney & McIntire, 1983; Knowles & Yadav, 1984; Dooley et al., 1990). Collectively, the data suggest that the quinone cofactor in methylamine dehydrogenases is neither unmodified PQQ nor a topa quinone. Clearly much additional work is necessary to definitively elucidate the structure of the covalently bound quinone cofactor in methylamine dehydrogenase.

The quinone moiety in amine oxidases reacts with various hydrazines (e.g., phenylhydrazine, 2,4-dinitrophenylhydrazine, 2-pyridylhydrazine) to give intense visible chromophores (Lobenstein-Verbeek et al., 1984; Moog et al., 1986; Knowles et al., 1987). Resonance Raman spectroscopy of such derivatized amine oxidases has proven to be a useful probe of the cofactor in these enzymes (Moog et al., 1986; Knowles et al., 1987; Williamson et al., 1986). Since hydrazines are also known to react with the quinone cofactor in methylamine dehydrogenase (Kenney & McIntire, 1983; McIntire & Stults, 1986), detailed comparisons between the vibrational spectra of the derivatized cofactors in these two types of quinoproteins appeared feasible. In addition, the spectroscopic and solubility properties of methylamine dehydrogenase suggested that the native enzyme would be an excellent candidate for resonance Raman studies. Our initial results on the oxidized enzyme and an isolated cofactor peptide are reported herein; the spectra establish that different quinone cofactors are present in methylamine dehydrogenase and copper-containing amine oxidases.

Materials and Methods

Methylamine dehydrogenase (MADH) was purified from bacterium W3A1, as previously described (Kenney & McIntire, 1983) and is stored at −20 °C in 25% ethylene glycol. The small amount of reduced MADH in the samples of the oxidized enzyme is reoxidized by incubation with 50 mM $K_3Fe(CN)_6$ overnight at 4 °C. Lysyl oxidase and methylamine oxidase were purified and derivatized as described elsewhere (Williamson et al., 1986; Dooley et al., 1990). Samples in H_2O were buffered with 10 or 50 mM KH_2PO_4 or 50 mM 2-(cyclohexylamino)ethanesulfonic acid (CHES), adjusted to pH 7.0 (phosphate) and 9.3 (CHES) with KOH. Some buffers also contained 250 mM (pH 7.0), 400 mM (pH 7.0), or 200 mM (pH 9.3) $^{14}NH_4Cl$ or $^{15}NH_4Cl$. Centricon-10 and -30 centrifuge concentrators were from Amicon.

Buffers with 50 mM potassium phosphate with or without $^{14}NH_4Cl$ were adjusted to pH 6.50 and 6.65, respectively. A measured volume of each was separately lyophilized and brought back to volume with 99.8% D_2O (Stohler Isotope Chemicals), and the process was repeated. The measured pH, pH*, was 6.68 for both buffers (pD = pH* + 0.4; Schowen, 1977). In a similar manner, 50 mM potassium phosphate buffers ± $^{14}NH_4Cl$, pH 7.0, were prepared in $H_2{}^{18}O$ (97%, Cambridge Isotope Laboratories).

The enzyme samples in H_2O and D_2O were made by concentrating 0.5-mL aliquots of a 12.8 mg/mL MADH solution to ~50 μL in Centricon-30 centrifuge concentrators, followed by dilution to 0.5 mL with the appropriate buffer, and reconcentration to ~50 μL. This cycle was repeated 6 times. In order to ensure that this procedure did not perturb the enzyme structure, and therefore the resonance Raman spectrum, the following control experiment was performed. A sample of MADH was exchanged into D_2O buffer as described above; an aliquot was removed and the remaining sample cycled back into H_2O buffer. The spectrum of the deuterium-exchanged sample was identical with that shown in Figure 7D. No significant differences were observed in the resonance Raman spectra of the aliquot cycled back into H_2O buffer and other samples of MADH in H_2O buffer.

The ^{16}O of the quinone cofactor was replaced by ^{18}O, by first treating 6.4 mg of MADH (≃40 μL) with 50 mM potassium phosphate buffer containing 400 mM NH_4Cl, pH 7.0, in $H_2{}^{18}O$. The resulting solution was concentrated to approximately 40 μL (Centricon-30), another 50 μL of the buffer added, and the process repeated six more times. This results in the displacement of the reactive oxygen of the cofactor by nitrogen due to reversible formation of an iminoquinone. The incorporation of ^{18}O into the cofactor was effected by the same procedure, which now involved cycling with $H_2{}^{18}O$ buffer lacking NH_4Cl.

MADH (12.8 mg/mL) was reacted with 1 mM phenylhydrazine HCl (J. T. Baker Chemical Co.) at pH 7.0 and 23 °C; under these conditions, the color immediately changed from green to red. After 30 min, two samples (pH 7.0 and 9.3) were prepared by cycling six times between ~50 μL and 0.5 mL (Centricon-30) with the appropriate buffers. Since trace amounts of phenylhydrazine and/or potassium ferricyanide increased the photosensitivity of MADH, these were removed from samples by chromatography on a Pharmacia Superose Column 12 HR 10/30 (1 × 30 cm) using 50 mM potassium phosphate and 150 mM KCl, pH 7.2, as the eluant. The 2-hydrazinopyridine derivatives of MADH and *Arthrobacter* P1 methylamine oxidase were prepared by a literature procedure (Collison et al., 1989).

The procedure for preparing the phenylhydrazine-derivatized bispeptidyl derivative is analogous to that used to prepare the semicarbazide derivative (McIntire & Stults, 1986). MADH (59 mg) in 5 mL of 10 mM potassium phosphate, pH 7.2, was treated with 60 μL of 100 mM phenylhydrazine hydrochloride and incubated for 30 min. By use of a Cen-

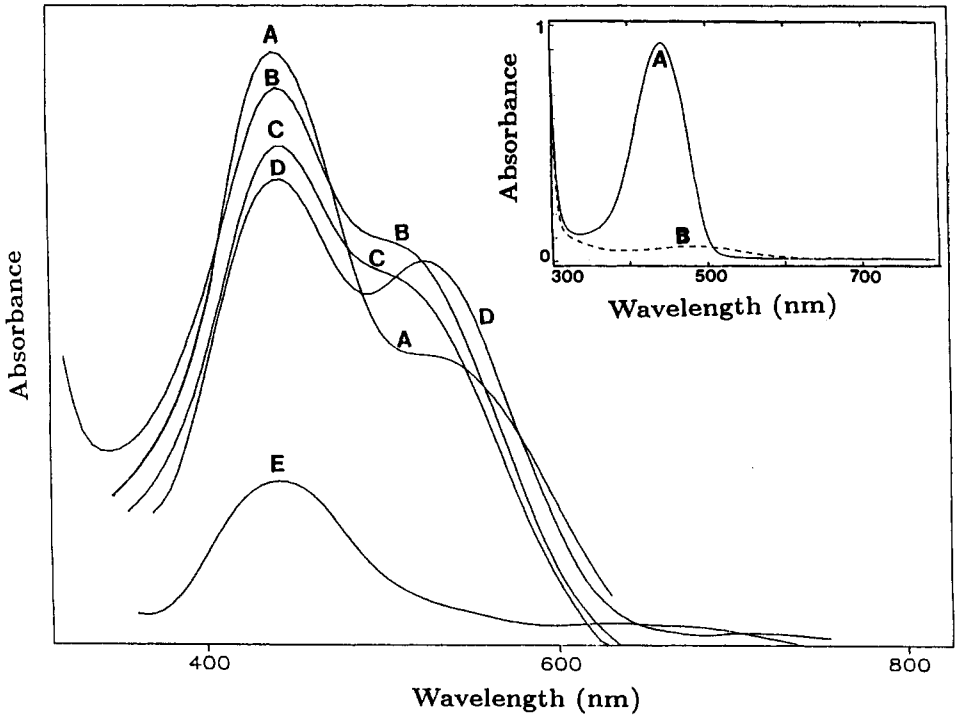


FIGURE 1: Absorption spectra of oxidized MADH and certain derivatives. The absorbance scale is arbitrary. (A) MADH–2-hydrazinopyridine derivative; (B) phenylhydrazone of the MADH cofactor containing peptide; (C) MADH phenylhydrazone at pH 9.2; (D) MADH phenylhydrazone at pH 7.0; (E) oxidized MADH. Inset: Absorption spectra of (A) the phenylhydrazone of *Arthrobacter* P1 methylamine oxidase and (B) oxidized methylamine oxidase.

tricon-30, the enzyme was exchanged into a small volume of 1.44 M Tris-HCl, pH 8.75, which was made 6 M in guanidine hydrochloride (final volume $\simeq$ 4 mL). Under Ar, 20 μL of 2-mercaptoethanol was added, and after $\sim$1 h, 300 mg of sodium iodoacetate was added. After 3 h, the sample was diluted to give a 2.5 M guanidine hydrochloride solution, and the small subunit was obtained by gel filtration and Florisil processing as previously described (McIntire & Stults, 1986). The sample was then digested at 37 °C with Pronase (grade A, CalBiochem Corp.). As dictated by HPLC analysis, the digest was stopped after 12 h and the sample desalted by using a Pharmacia Pep RPC HR 5/5 reverse-phase column. Several purification runs were carried out on the same column by using a 20-min linear gradient from 0 $\rightarrow$ 40% acetonitrile/H_2O containing 0.1% trifluoroacetic acid. A fraction eluting from 18.5 to 21.5 min and containing most of cofactor-containing peptides (70%, 436 nm) was collected for each run. Most non-cofactor-containing peptides eluted from 0 to 15.2 min. The cofactor peptide fraction was treated with aminopeptidase M (Sigma Chemical Co.) at 37 °C for 9.75 h. This was processed in the same manner as the Pronase digest to produce a fraction containing all the cofactor peptides, which eluted between 19 and 22 min on the Pep RPC column. This fraction was further digested with carboxypeptidase Y (Sigma Chemical Co.) at 37 °C for 4.5 h. This fraction was purified on a TSK DEAE 2PW, 0.75 × 7.5 cm column (Toso Haas), with a 60-min linear gradient from 20 $\rightarrow$ 300 mM ammonium phosphate, pH 7.0. The major cofactor peptide peak was detected at 19.29 min (69%, 436 nm). This major fraction was purified to homogeneity on the Pep RPC column (2 $\rightarrow$ 30% acetonitrile/H_2O + 0.1% trifluoroacetic acid in 60 min).

The isolation of the small cofactor-containing subunit was accomplished as follows. Three hundred microliters of 7 M guanidine hydrochloride and 170 mM potassium phosphate, pH 6.10, was added to 12.6 mg of MADH in $\sim$50 mL of 10 mM potassium phosphate, pH 7.0 (the final guanidine hydrochloride concentration was approximately 6 M). After 1 h, 0.49 mL of 10 mM potassium phosphate, pH 6.01, was added. This solution was applied to a 1.5 × 42 cm Bio-Gel A-0.5 (200–400 mesh; Bio-Rad) column equilibrated with 2.5 M guanidine hydrochloride and 200 mM potassium phosphate, pH 7.0. Fractions 45–48 (1.1 mL/fraction) contained the small subunit and were pooled. (The large subunit eluted in fractions 32–38.) The small subunit was dialyzed against 500 mL of 10 mM potassium phosphate, pH 7.0, for 15 h, and again for 12 h. The sample was concentrated to $\sim$100 μL and washed three times by cycling between 100 μL and 2 mL of 10 mM potassium phosphate, pH 7.0, in a Centricon-10. The phenylhydrazine-derivatized small subunit was similarly obtained. Unfortunately, the large subunit could not be isolated as it precipitated in the absence of guanidine hydrochloride.

Resonance Raman spectra were generally obtained by using instrumentation and methods that have been described (Moog et al., 1986). Scattered light from a second laser provided an internal frequency calibration. When necessary, samples were cooled by blowing cold N_2 gas over the sample capillary and holder. Typically, several scans were collected, checked for consistency, and added. Subsequently a linear or second-degree polynomial base line was subtracted. In some cases a standard Savitsky–Golay routine (Savitzky & Golay, 1964) was used to smooth the spectra following subtraction. Only features that were fully reproducible are marked in the figures. The phenylhydrazone of methylamine dehydrogenase was light-sensitive and bleached in the laser beam within a few minutes at ambient or near-ambient temperatures (with laser powers $\geq$25 mW) *unless* excess phenylhydrazine or oxidizing agent was removed by gel filtration.

RESULTS

Resonance Raman Spectra of Methylamine Dehydrogenase Derivatives. Absorption spectra of resting methylamine dehydrogenase, and its derivatives with phenylhydrazine and 2-hydrazinopyridine, are shown in Figure 1. Also shown in

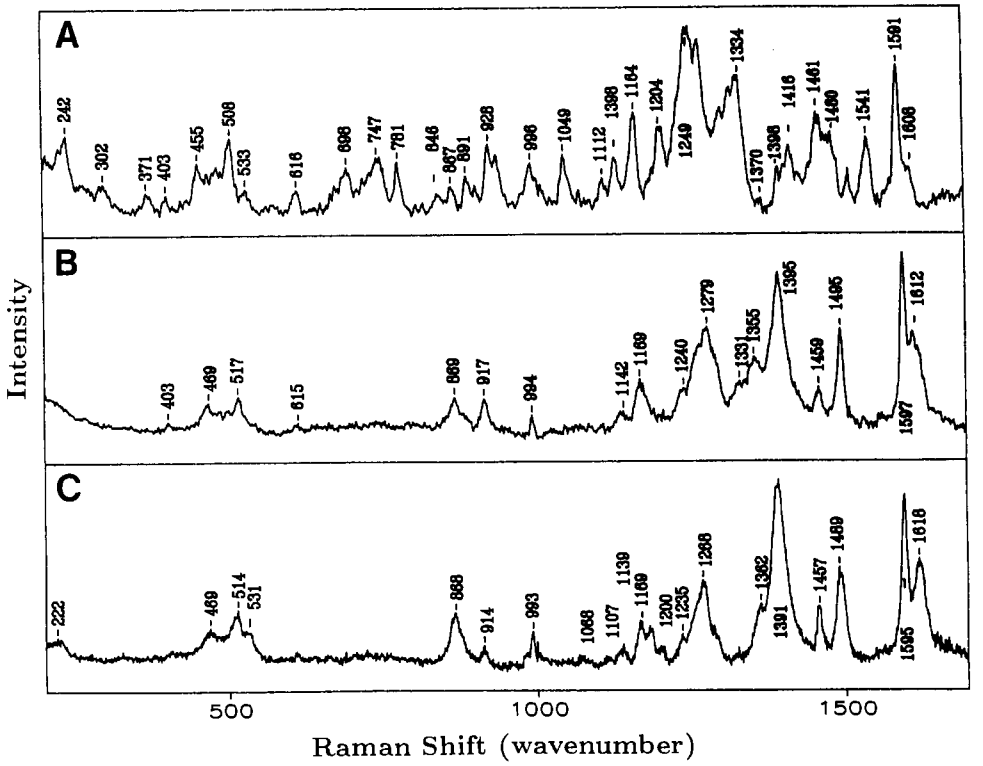


FIGURE 2: Resonance Raman spectra of enzyme phenylhydrazones at pH 7.0. The excitation wavelength was 457.9 nm. (A) Oxidized MADH; (B) oxidized bovine plasma amine oxidase; (C) *Arthrobacter* P1 methylamine oxidase.

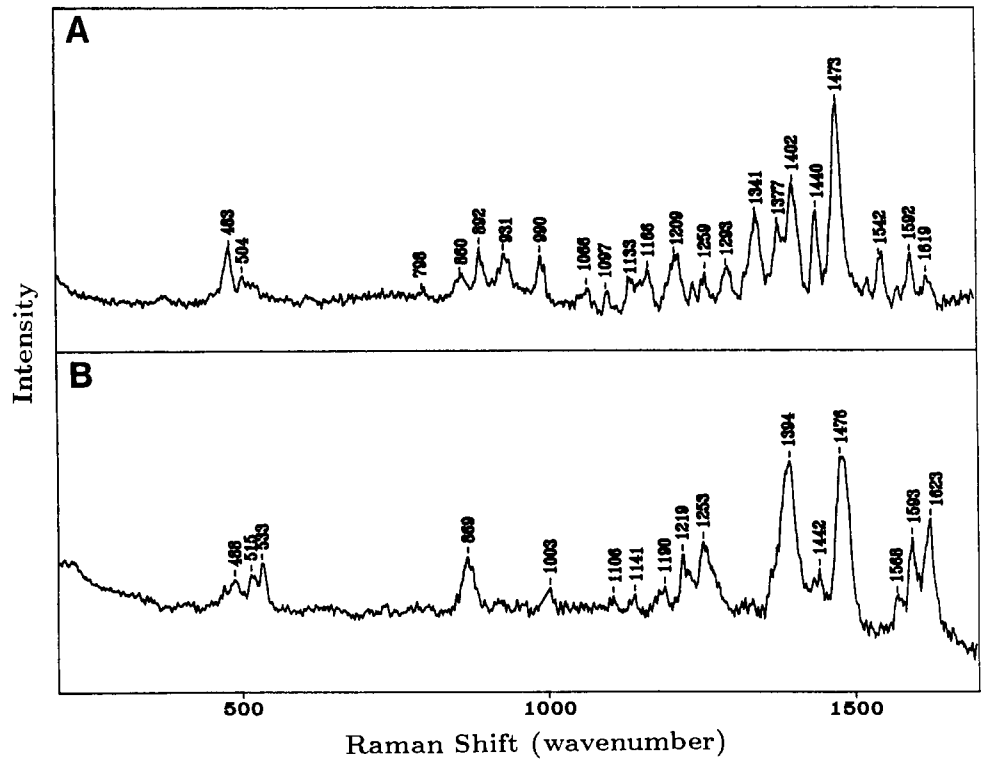


FIGURE 3: Resonance Raman spectra of enzyme–2-hydrazinopyridine derivatives at pH 7.0. Excitation wavelength was 457.9 nm. (A) Oxidized MADH; (B) oxidized *Arthrobacter* P1 methylamine oxidase.

Figure 1 is the absorption spectrum of a phenylhydrazine-labeled cofactor peptide. The absorption spectra of the MADH derivatives differ substantially from those of the amine oxidase adducts (Figure 1, inset; Knowles & Yadav, 1984; Collison et al., 1989). Not surprisingly, the resonance Raman spectra of the methylamine dehydrogenase derivatives are also significantly different from the resonance Raman spectra of the corresponding amine oxidase derivatives (Figures 2–4). The most obvious difference is that the MADH spectra are much more complex. This increased complexity is attributable to differences in the cofactor structures since the contribution from the phenylhydrazine label can be seen to be approximately constant (Table I). Therefore, the simpler spectra of the amine oxidase derivatives may be understandable given that all the structures proposed for the MADH cofactor (McIntire & Stults, 1986; Husain & Davidson, 1987; Vellieux

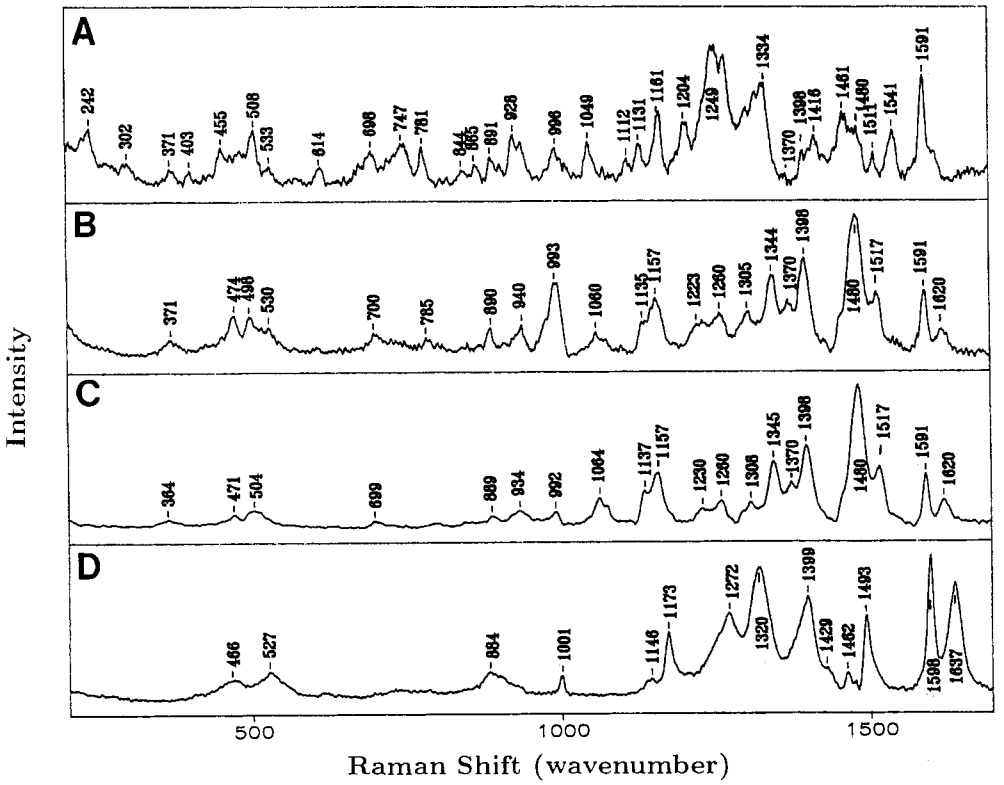


FIGURE 4: Comparison of the resonance Raman spectra of phenylhydrazones (457.9-nm excitation). (A) Oxidized MADH; (B) isolated β subunit of MADH; (C) cofactor-containing peptide of MADH; (D) cofactor-containing peptide of bovine lysyl oxidase.

Table I: Phenyl Modes in the Resonance Raman Spectra of MADH and Amine Oxidase Phenylhydrazones[a]

	trigonal ring "breathing"[b]	ring "breathing"	in-plane ring deformation
MADH	1049	996	614
MADH β subunit	1060	993	614
MADH peptide	1064	992	–[c]
APAO	1068	992	610
BPAO	–[c]	994	615
lysyl oxidase peptide	–[c]	1001	614

[a] Frequencies in cm^{-1}; observed with 457.9-nm excitation. [b] Assignments based on the results reported in Liu et al. (1990). [c] Not observed.

& Hol, 1989) are much more complex than the topa quinone cofactor in amine oxidases. It is important to note that all the resonance Raman spectra of the amine oxidase phenylhydrazones and 2-hydrazinopyridine derivatives are at least closely similar (Knowles et al., 1987),[3] as illustrated by the comparison between bovine plasma amine oxidase and methylamine oxidase (Figure 2). Hence these data strongly suggest that the quinone cofactors in methylamine dehydrogenase and the copper-containing amine oxidases are not the same. Excellent resonance Raman spectra could also be obtained on the phenylhydrazine derivatives of the isolated small (β) subunit from MADH, and a cofactor-containing peptide (Figure 4). The spectra of the β subunit and the peptide are practically identical and differ substantially from that of the intact MADH phenylhydrazone. Further, the MADH spectra in Figure 4 (obtained with 457.9-nm excitation) differ significantly from that of an amine oxidase derivative (see Figure 2) and from the resonance Raman spectrum of a phenylhydrazine-labeled peptide from lysyl oxidase (Figure 4). We emphasize that the latter comparison is not complicated by any potential differences in the microenvironments of the derivatized quinones. The relative intensities of the MADH Raman peaks are markedly dependent on the excitation wavelength (Figure 5), particularly the following groups: (1591, 1620 cm^{-1}); (1480, 1517 cm^{-1}); (1370, 1398 cm^{-1}). Such a dependence upon the excitation wavelength probably reflects the different extent to which particular vibrations are coupled to a given electronic transition within the chromophore. Although we have not examined the excitation profiles of amine oxidase phenylhydrazones in detail, marked changes in relative intensities as a function of excitation wavelength have not been observed for amine oxidase 2,4-dinitrophenylhydrazones.

The absorption spectra of the MADH phenylhydrazone at pH 9.2 and the phenylhydrazone of the isolated small (β) subunit (pH 7.0) are closely similar to the spectrum of the cofactor peptide (pH 7.0) shown in Figure 1. This suggests that raising the pH perturbs the quinone phenylhydrazone in a manner similar to dissociating the subunits. Comparing the resonance Raman spectra provides additional support for the proposal that raising the pH or dissociating the subunits produces similar effects on the phenylhydrazine derivative (Figure 6). It is important to recognize that the resonance Raman spectrum of the intact MADH phenylhydrazone at pH 9.2, while closely resembling the spectrum of the derivatized small subunit at pH 7.0, is *not* identical with that of the small subunit. Nevertheless, it is possible that raising the pH induces subunit dissociation of the MADH phenylhydrazone, but not the underivatized enzyme.

Resonance Raman Spectra of Underivatized Methylamine Dehydrogenase. Resonance Raman spectra of the resting enzyme and the separated small (β) subunit are shown in Figure 7. The S/N in the spectrum of the β subunit is not as good as that of the native enzyme because it is less concentrated. Significant differences are apparent between the spectra of the β subunit and the intact enzyme. For example,

[3] These results have been corroborated by much more extensive data (D. M. Dooley, J. Bates, and M. A. McGuirl, unpublished observations).

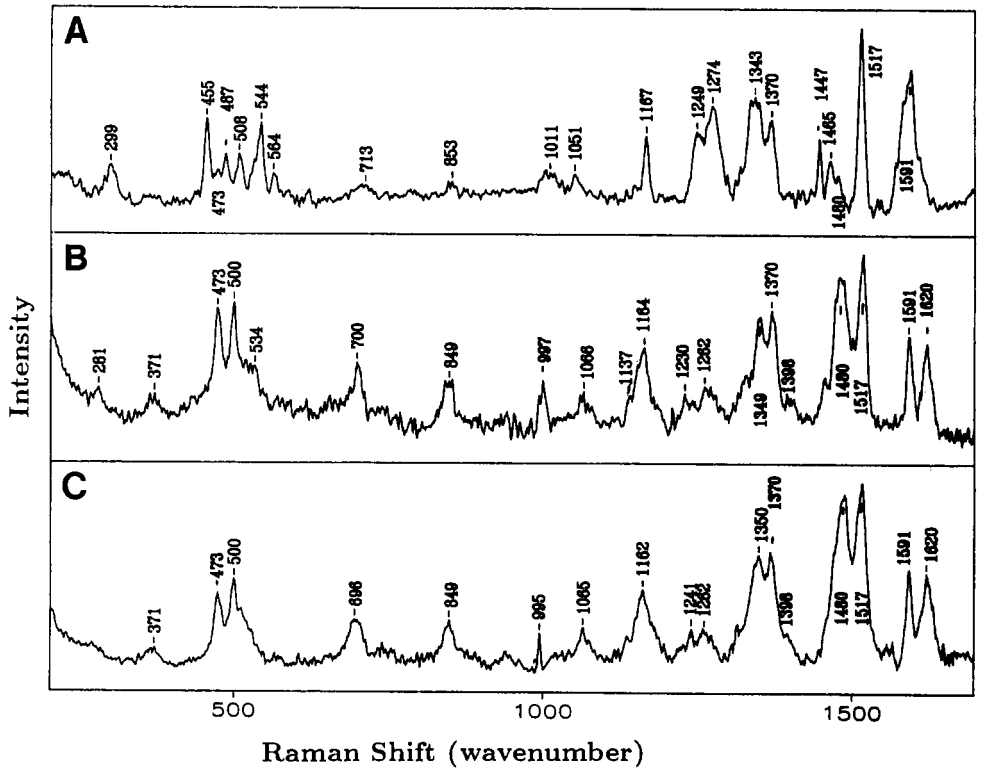


FIGURE 5: Resonance Raman spectra of MADH phenylhydrazones obtained with 514.5-nm excitation. (A) Intact oxidized MADH; (B) isolated β subunit; (C) cofactor-containing peptide.

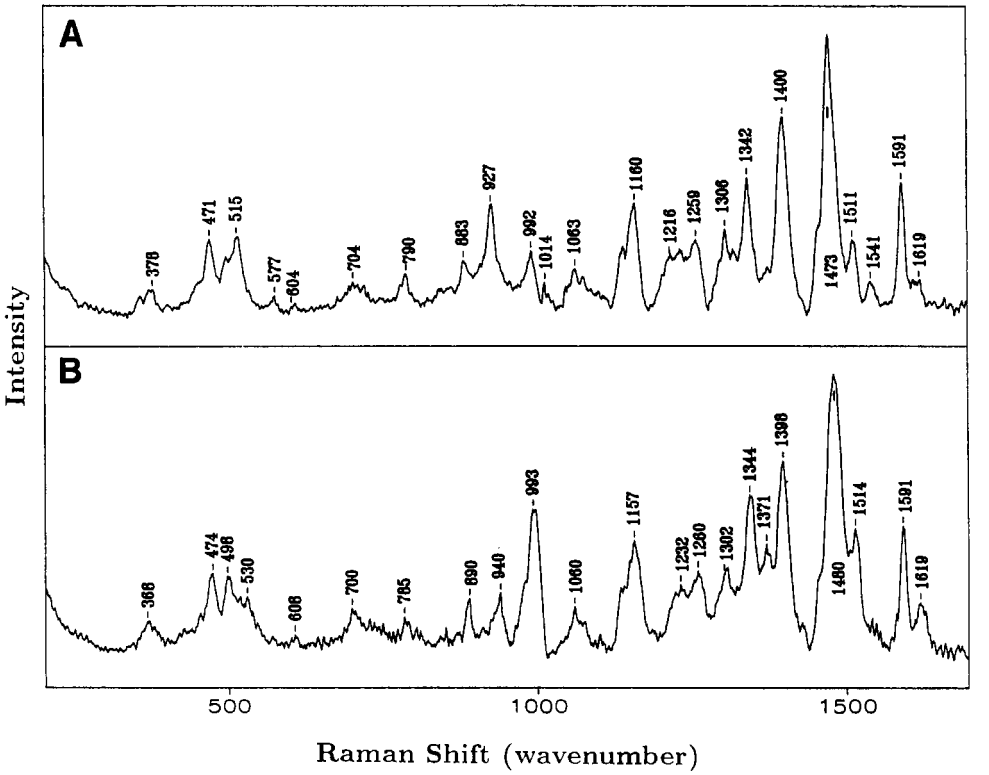


FIGURE 6: Effects of pH vs subunit dissociation on the resonance Raman spectra (457.9-nm excitation) of the MADH phenylhydrazone derivative. (A) MADH at pH 9.2; (B) isolated β subunit.

some intense peaks in the native enzyme spectrum shift to higher frequency following subunit dissociation (1478 → 1498 cm^{-1}; 1558 → 1572 cm^{-1}; 1614 → 1632 cm^{-1}). Two prominent peaks of the native enzyme, at 1059 and 1300 cm^{-1}, are not evident in the spectrum of the β subunit. In remarkable contrast to the behavior of the phenylhydrazine derivative, the spectrum of the native enzyme is identical at pH 7.0 and 9.2.

Also shown in Figure 7 is the spectrum following ^{18}O exchange into the carbonyl groups. Two bands shift significantly to lower frequency upon ^{18}O exchange: 443 cm^{-1} → 433 cm^{-1}; and 1614 cm^{-1} (and shoulder at 1630 cm^{-1}) → 1587 cm^{-1}. Hence these bands must reflect modes involving substantial carbonyl deformation. The high-frequency bands may be plausibly assigned to C=O stretching vibrations. Unshifted

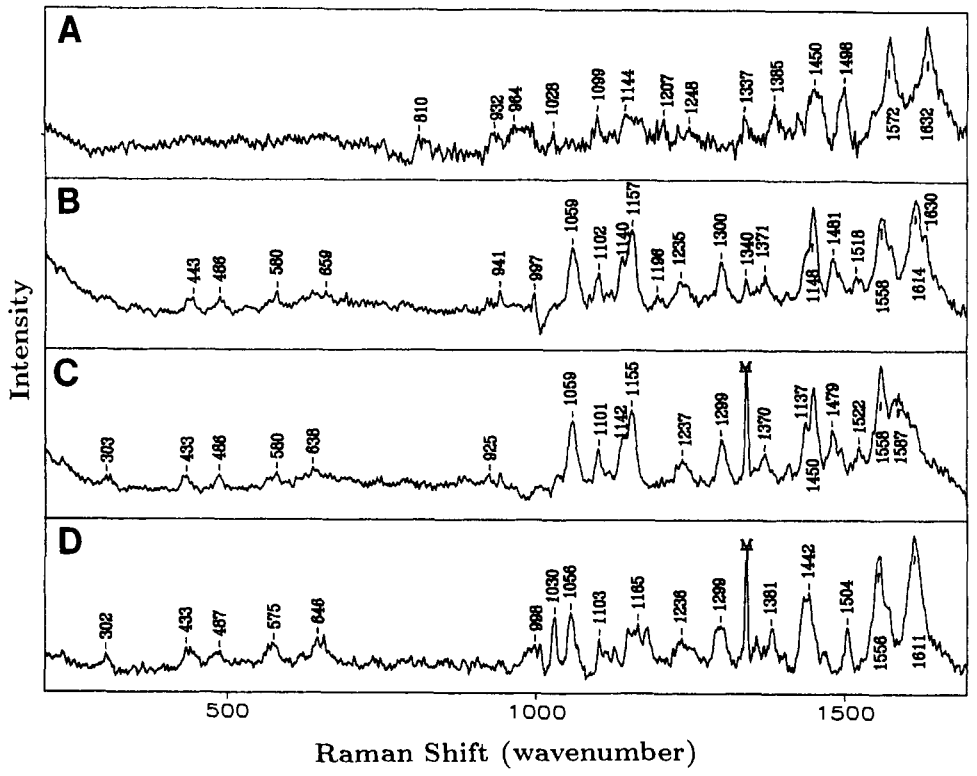


FIGURE 7: Resonance Raman spectra of underivatized MADH obtained with 457.9-nm excitation. (A) Isolated β subunit; (B) native oxidized MADH; (C) native oxidized MADH following exchange with ^{18}O; (D) oxidized MADH in deuterated 10 mM potassium phosphate buffer, pD 7.1. The band marked M is wavelength marker.

bands in the 1450–1600-cm^{-1} region may be indicative of aromatic C=C groups.

In contrast to the highly specific effects of ^{18}O exchange, deuterium exchange produces substantial changes in the resonance Raman spectrum of methylamine dehydrogenase (Figure 7D). Identical and fully reversible deuterium-exchange effects were observed in an entirely independent experiment in which a MADH sample was exchanged into D_2O buffer and then back into H_2O buffer. The observed shifts are too extensive and too large to be attributed *solely* to the perturbation (by deuterium substitution) of hydrogen bonds involving the quinone, although such effects are likely to be present. It might be possible that deuterium exchange induces localized protein conformational changes, thereby altering the environment of the quinone cofactor. Another possibility is that some of the shifts or new features evident in Figure 7D arise from Fermi resonance coupling effects (Shiemke et al., 1986).

Considerable evidence indicates that ammonia reacts with MADH to form a covalent adduct with the quinone cofactor (Kenney & McIntire, 1983). For example, the visible absorption spectra of the native enzyme and its ammonia complex are distinctly different (Kenney & McIntire, 1983). Efforts to obtain the resonance Raman spectrum of the MADH–ammonia complex failed, owing to its photosensitivity. Even brief exposure to the laser resulted in the *irreversible* conversion of the ammonia complex to the uncomplexed form.

Discussion

The most recent interpretation of the electron density map of *T. versutus* methylamine dehydrogenase is not consistent with the presence of authentic PQQ in this enzyme (Vellieux et al., 1989; Vellieux & Hol, 1989). Duine and co-workers propose that a precursor to PQQ ("pro-PQQ") is covalently attached in the active site, which is located at the interface between the large and small subunits. Earlier, one of us (McIntire & Stults, 1986) had proposed that the quinone in methylamine dehydrogenase from W3A1 could not be unmodified PQQ. Whether one compares resonance Raman spectra of the phenylhydrazine or 2-hydrazinopyridine derivatives of the native enzymes (Figures 2 and 3) or the phenylhydrazine derivatives of the isolated peptides from MADH and lysyl oxidase (Figure 4), the same conclusion seems clear: the quinone cofactors in these two types of enzymes are not identical. Given the clear implication of the data presented here that the quinone cofactors in methylamine dehydrogenases and copper-containing amine oxidases are not identical, at least three types of fundamentally different quinoproteins are now known. These are the following: (1) enzymes containing noncovalently bound PQQ, e.g., glucose dehydrogenase (Dokter et al., 1986); (2) enzymes of the methylamine dehydrogenase class containing covalently bound quinones; (3) amine oxidases, which contain topa quinone (Janes et al., 1990). In addition to the resonance Raman data, several other pieces of evidence point to significant differences between the quinone cofactors in methylamine dehydrogenases and amine oxidases. Most notably, the absorption spectra of the native, oxidized enzymes are considerably different, as are the spectra of the phenylhydrazine derivatives. The reactivities of these two classes of enzymes, especially toward ammonia and redox agents, are also distinct. Finally, there are significant differences in the EPR spectra of the semiquinone forms of MADH and the amine oxidases (Kenney & McIntire, 1983; Dooley et al., 1987, 1990). Taken together, the data are not consistent with unmodified PQQ or topa quinone as the cofactor in MADH from W3A1. A key conclusion of this work is that quinoproteins may be more diverse with regard to the identity of the quinone cofactor than previously suspected. We suggest that it may be important to keep this result in mind when investigating PQQ biochemistry and further suggest that care must be taken in the identification of other enzymes as PQQ-containing quinoproteins.

The 2.25-Å resolution X-ray structure of *T. versutus* MADH established that the quinone cofactor is located in a solvent-accessible channel at the interface of the small and large subunits (Vellieux et al., 1989). On the basis of their X-ray data, Vellieux and Hol (1989) have suggested that one quinone is covalently bound to each of the small subunits via an Arg 107 and a Glu 57 residue. Several hydrophobic residues are found in the interface region between the large and small subunits, and these are proposed to play a role in the binding of the subunits (Vellieux et al., 1989). Assuming that the structures are similar, one might therefore expect, and the data presented herein unequivocally show, that the electronic and vibrational states of the quinone (and its phenylhydrazine derivative) of W3A1 MADH are sensitive to subunit dissociation. Furthermore, the differences among the resonance Raman spectra collected under various conditions may reflect changes from the native microenvironment of the quinone in the subunit interface region to some other environment. The spectra (at pH 7.0) of the intact ($\alpha_2\beta_2$) enzyme and its phenylhydrazone are unique; when MADH or its phenylhydrazone are dissociated or digested, very different spectra are observed. Once the subunits are dissociated, the resonance Raman spectra are rather insensitive to further changes in the microenvironment of the quinone or its phenylhydrazone. For example, the phenylhydrazones of the isolated small subunit and the active-site peptide are essentially identical.

At this time, it is difficult to provide an unambiguous molecular interpretation for the effects of dissociation or pH (or both) on the resonance Raman spectra of MADH and its phenylhydrazone. The data presented here and elsewhere do provide several clues that point to specific molecular interactions that may be involved. First, consider the behavior of the native enzyme. The long-wavelength absorption of native MADH evident in Figure 1 is absent in the spectrum of the isolated small subunit. This band may be a charge-transfer transition, possibly from an amino acid residue in the large subunit. Regardless of its origin, the loss of the 700-nm absorption band upon dissociation of the small and large subunits does reflect some electronic structural change that may also be detected in the resonance Raman spectrum. It should be noted that one of the vibrational modes most sensitive to dissociation is the C=O stretching mode, which shifts from 1614 to 1632 cm^{-1} (Figure 7). The resonance Raman spectrum of the MADH phenylhydrazone is also quite sensitive to subunit dissociation. As can be seen in Figure 4, even the ring modes of the phenyl ring (at 614, 996, and 1049 cm^{-1} in the intact enzyme derivative) are affected. Increasing the pH to 9.2 perturbs the resonance Raman spectrum of the MADH phenylhydrazone, *but not the underivatized enzyme*, in a manner similar to dissociating the subunits. The sensitivity of the MADH phenylhydrazone to pH may reflect the presence of the hydrazine functionality in the active site.

The resonance Raman spectra in Figure 7 show that the reactive carbonyl groups of the quinone can be directly observed. This should permit the status of the carbonyl groups to be monitored in other states of the enzyme, and in the presence of physiologically relevant small molecules (e.g., substrate, inhibitors) and proteins (e.g., the redox partner, amicyanin). Our attempts to measure the effects of ammonia on the quinone were frustrated by the novel photosensitivity of the complex. Normally, the formation of the ammonia complex is fully reversible, presumably involving nucleophilic attack on the most reactive carbonyl group to form an imine, which is then subject to hydrolysis. The hydrolysis of this adduct must be stimulated by laser irradiation, either photochemically or via local heating effects. Additional photochemical or thermal events must therefore be responsible for the irreversibility observed in this instance.

Added in Proof

It has recently been determined that the cofactor in MADH from W3A1 is composed of covalently linked indole and indoloquinone moieties derived from two tryptophans in the enzyme (W. S. McIntire, D. E. Wemmer, A. Chistoserdov, and M. E. Lidstrom, manuscript in preparation).

Acknowledgments

We thank Cheryl Cotê and Michele McGuirl for their superb technical assistance. Professor Paul Dorain's help with certain of the Raman experiments is greatly appreciated. We especially appreciate several helpful discussions with Professor Joanne Sanders-Loehr, who suggested the possibility that the methylamine dehydrogenase–ammonia complex was photosensitive.

Registry No. Methylamine dehydrogenase, 60496-14-2.

References

Ameyama, M., Hayashi, U., Matushita, K., Shinagawa, E., & Adachi, O. (1984) *Agric. Biol. Chem. 48*, 561–565.

Citro, G., Verdina, A., Galati, R., Floris, G., Sabatini, S., & Finazzi-Agro, A. (1989) *FEBS Lett. 247*, 201–204.

Collision, D., Knowles, P. F., Mabbs, F. A., Rius, F. X., Singh, I., Dooley, D. M., Cote, C. E., & McGuirl, M. (1989) *Biochem. J. 264*, 663–669.

DeBeer, R., Duine, J. A., Frank, J. Jzn., & Large, P. J. (1980) *Biochim. Biophys. Acta 622*, 370–374.

Dokter, P., Frank, J. Jzn., & Duine, J. A. (1986) *Biochem. J. 239*, 163–167.

Dooley, D. M., McGuirl, M. A., Peisach, J., & McCracken, J. (1987) *FEBS Lett. 214*, 274–278.

Dooley, D. M., McIntire, W. S., McGuirl, M. A., Cote, C. E., & Bates, J. L. (1990) *J. Am. Chem. Soc. 112*, 2782–2789.

Duine, J. A., & Jongejan, J. A. (1989) *Annu. Rev. Biochem. 58*, 403–426.

Duine, J. A., Frank, J. Jzn., & Jongejan, J. A. (1987) *Adv. Enzymol. 59*, 170–212.

Hartmann, C., & Klinman, J. P. (1987) *J. Biol. Chem. 262*, 962–965.

Hartmann, C., & Klinman, J. P. (1988) *BioFactors 1*, 41–49.

Husain, M., & Davidson, V. L. (1987) *J. Bacteriol. 169*, 1712–1717.

Janes, S. M., Mu, D., Wemmer, D., Smith, A. J., Kaur, S., Maltby, D., Burlingame, A. L., & Klinman, J. P. (1990) *Science 248*, 981–987.

Kenney, W. C., & McIntire, W. (1983) *Biochemistry 22*, 3858–3868.

Knowles, P. F., & Yadav, K. D. S. (1984) in *Copper Proteins and Copper Enzymes* (Lontie, R., Ed.) pp 103–129, CRC Press, Boca Raton, FL.

Knowles, P. F., Pandeya, K. B., Ruis, F. X., Spencer, C. M., Moog, R. S., McGuirl, M. A., & Dooley, D. M. (1987) *Biochem. J. 241*, 603–608.

Liu, H.-H., Lin, S. H., & Yu, N.-T. (1990) *Biophys. J. 57* (1), 851–856.

Lobenstein-Verbeek, C. L., Jongejan, J. A., Frank, J., Jzn., & Duine, J. A. (1984) *FEBS Lett. 170*, 305–309.

McIntire, W. S., & Stults, J. T. (1986) *Biochem. Biophys. Res. Commun. 141*, 562–568.

Moog, R. S., McGuirl, M. A., Cote, C. E., & Dooley, D. M. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 8435–8439.

Savitzky, A., & Golay, J. J. E. (1964) *Anal. Chem. 36*, 1627-1639.
Schowen, R. L. (1977) in *Isotope Effects on Enzyme-Catalyzed Reactions* (Cleland, W. W., O'Leary, M. H., & Northrop, D. B., Eds.) pp 64-99, University Park Press, Baltimore.
Shiemke, A. K., Loehr, T. M., & Sanders-Loehr, J. (1986) *J. Am. Chem. Soc. 108*, 2437-2443.
van der Meer, R. A., & Duine, J. A. (1986) *Biochem. J. 239*, 789-791.
van der Meer, R. A., Jongejan, J. A., & Duine, J. A. (1987) *FEBS Lett. 221*, 299-304.
van der Meer, R. A., van Wassenaar, P. D., van Brouwershaven, J. H., & Duine, J. A. (1989) *Biochem. Biophys. Res. Commun. 159*, 726-733.
van Iersal, J., van der Meer, R. A., & Duine, J. A. (1986) *Eur. J. Biochem. 161*, 413-419.
Vellieux, F. M. D., & Hol, W. G. J. (1989) *FEBS Lett. 255*, 460-464.
Vellieux, F. M. D., Frank, J. Jzn., Swarte, M. B. A., Groendijk, H., Duine, J. A., Drenth, J., & Hol, W. G. J. (1986) *Eur. J. Biochem. 154*, 383-386.
Vellieux, F. M. D., Huitema, F., Groendijk, H., Kalk, K. H., Frank, J. Jzn., Jongejan, J. A., Duine, J. A., Petratos, K., Drenth, J., & Hol, W. G. H. (1989) *EMBO J. 8*, 2171-2178.
Williamson, P. R., Moog, R. S., Dooley, D. M., & Kagan, H. (1986) *J. Biol. Chem. 261*, 16302-16305.

Comparison of Various Molecular Forms of Bovine Trypsin: Correlation of Infrared Spectra with X-ray Crystal Structures

Steven J. Prestrelski,*,‡,§ D. Michael Byler,∥,⊥ and Michael N. Liebman#

Department of Physiology and Biophysics, Mount Sinai School of Medicine of the City University of New York, New York, New York 10029, Eastern Regional Research Center, U.S. Department of Agriculture, 600 East Mermaid Lane, Philadelphia, Pennsylvania 19118, and Biotechnology Division, AMOCO Technology Corporation, Naperville, Illinois 60566

Received December 28, 1989; Revised Manuscript Received September 6, 1990

ABSTRACT: Fourier-transform infrared spectroscopy is a valuable method for the study of protein conformation in solution primarily because of the sensitivity to conformation of the amide I band (1700–1620 cm^{-1}) which arises from the backbone C=O stretching vibration. Combined with resolution-enhancement techniques such as derivative spectroscopy and self-deconvolution, plus the application of iterative curve-fitting techniques, this method provides a wealth of information concerning protein secondary structure. Further extraction of conformational information from the amide I band is dependent upon discerning the correlations between specific conformational types and component bands in the amide I region. In this paper, we report spectra–structure correlations derived from conformational perturbations in bovine trypsin which arise from autolytic processing, zymogen activation, and active-site inhibition. IR spectra were collected for the single-chain (β-trypsin) and once-cleaved, double-chain (α-trypsin) forms as well as at various times during the course of autolysis and also for zymogen, trypsinogen, and β-trypsin inhibited with diisopropyl fluorophosphate. Spectral differences among the various molecular forms were interpreted in light of previous biochemical studies of autolysis and the known three-dimensional structures of the zymogen, the active enzyme, and the DIP-inhibited form. Our spectroscopic results from these proteins in D_2O imply that certain loop structures may absorb in the region of 1655 cm^{-1}. Previously, amide I′ infrared bands near 1655 cm^{-1} have been interpreted as arising solely from α-helices. These new data suggest caution in interpreting this band. We have also proposed that regions of protein molecules which are known from crystallographic experiments to be disordered absorb in the 1645 cm^{-1} region and that type II β-turns absorb in the region of 1672–1685 cm^{-1}. Our results also corroborate assignment of the low-frequency component of extended strands to bands below 1636 cm^{-1}. Additionally, the results of multiple measurements have allowed us to estimate the variability present in component band areas calculated by curve fitting the resolution-enhanced IR spectra. We estimate that this approach to data analysis and interpretation is sensitive to changes of 0.01 unit or less in the relative integrated intensities of component bands in spectra whose peaks are well resolved.

The ability of biotechnology and protein engineering to produce new and novel proteins has underscored the need for a simple, reliable probe of protein conformation in a variety of conditions and environments. Currently, X-ray crystallography provides the most detailed information concerning positions of individual atoms in the protein structure. However, the length and complexity of the experiments and analysis, as well as the difficulty in crystallizing many proteins, severely limit the use of this tool. Circular dichroism has been widely used as method for studying protein conformation in solution, but its relative insensitivity to certain protein conformations, such as β-sheets, often results in essentially qualitative results (Johnson, 1988).

Fourier-transform infrared spectroscopy has become recognized as a valuable tool for the examination of protein

* Author to whom correspondence should be addressed.
‡ Present address: Amgen Inc., Amgen Center, 1840 Dehavilland Dr., B-2, Thousand Oaks, CA 91320.
§ Mount Sinai School of Medicine of the City University of New York.
∥ Present address: Philadelphia College of Textiles and Science, Department of Chemistry and Physical Sciences, Schoolhouse Lane and Henry Ave., Philadelphia, PA 19144-3497.
⊥ U.S. Department of Agriculture.
AMOCO Technology Corp.

0006-2960/91/0430-133$02.50/0