Elucidation of the Solution Structure of the *Escherichia coli* Aspartate Aminotransferase-α-Methyl-L-Aspartate Complex by Isotope-Edited Raman Difference Spectroscopy

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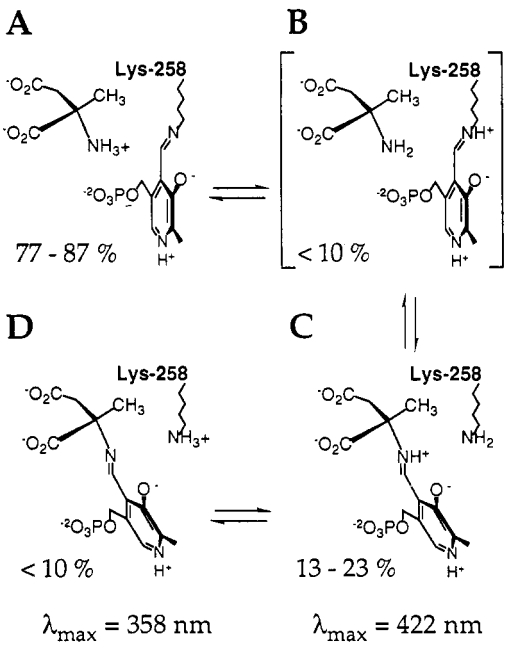
The pyridoxal 5′-phosphate (PLP)-dependent enzyme aspartate aminotransferase (AATase) (EC 2.6.1.1) catalyzes the reversible conversion of L-aspartate and α-ketoglutarate to oxalacetate and L-glutamate.[1,2] The initial reaction of AATase with an amino acid involves conversion of the Schiff base formed from the side chain of active-site Lys-258 and PLP (the "internal aldimine"; Scheme IA,B) to that of the N^{α} atom of the amino acid and the cofactor (the "external aldimine" intermediate; Scheme IC,D). The left- and right-side forms in Scheme I differ only in the placement of a proton. The electronic absorption spectrum of the α-methyl-L-aspartate (αMeAsp)–AATase complex does not distinguish the configuration in IA from that in ID or in structure IB from that in IC. We present evidence obtained from Raman difference spectroscopy that assigns the structures shown in Scheme IA and C as the only significantly populated forms of this complex.

The reaction of AATase with the aspartate analogue αMeAsp, which has an α–methyl group in place of the C_{α} proton, can progress only as far as the external aldimine (Scheme IC,D), as the methyl group, unlike a proton, cannot be extracted from the amino acid.[3] Thus, reaction with this analogue leads to a frozen intermediate on the pathway. The electronic absorption spectrum of the αMeAsp–AATase complex, theoretically including all four species of Scheme I, exhibits pH-independent maxima at 362 and 430 nm in pig cytosolic (pc) AATase,[4] or 358 and 422 nm in *Escherichia coli* (*E. coli*) AATase,[5] implying the existence of two distinct forms.[4] These two peaks do titrate with pH in the unligated internal aldimine form of AATase and have been assigned to the protonated (430 nm) and unprotonated (362 nm) forms of the Schiff base.[6]

While there is consensus that the species responsible for the absorption above 420 nm in the αMeAsp–AATase complex is that given in Scheme IC, the structure of the 358–362-nm absorbing species has not been definitively assigned.[7,8] Recent low-field ^1H NMR studies have been interpreted as favoring the structure shown in Scheme IA.[9]

Scheme I



Non-resonance Raman difference spectroscopy is well suited to the characterization of enzyme–cofactor contacts[10–12] and has been effective, where aided by ^{15}N enrichment of the enzyme, in the identification of atoms involved in the Schiff-base linkage of AATase.[12] Raman studies with ^{15}N-enriched protein have also fully characterized the Schiff-base linkage in visual pigments and in bacteriorhodopsin.[13] The resonance Raman frequency corresponding to the protonated Schiff-base linkage of pcAATase[14] ($\nu_{C=NH^+}$) occurs at 1649 cm^{-1}. This band is found at 1650 cm^{-1} in the non-resonance holoenzyme-minus-apoenzyme Raman difference spectrum of the *E. coli* isozyme (Figure 1a), as confirmed by the presence of a 13-cm^{-1} red shift of this frequency[12] in ^2H_2O.

Curve b of Figure 1 shows the αMeAsp–AATase-minus-AATase Raman difference spectrum. The negative peak at 1640 cm^{-1} is the result of subtraction of the internal aldimine peak of unprotonated, unligated AATase[12] (1639 cm^{-1}) from the Raman spectrum of the αMeAsp–AATase complex. The peaks with positive amplitudes at 1630 and 1655 cm^{-1} in Figure 1b are from the αMeAsp–AATase complex, since αMeAsp alone is Raman silent in this region. The lack of a significant solvent deuterium isotope effect on the peak frequency at 1630 cm^{-1} allows it to be assigned to an unprotonated Schiff base (Figure 1b and c; no negative difference peaks are observed in spectrum 1c since it is

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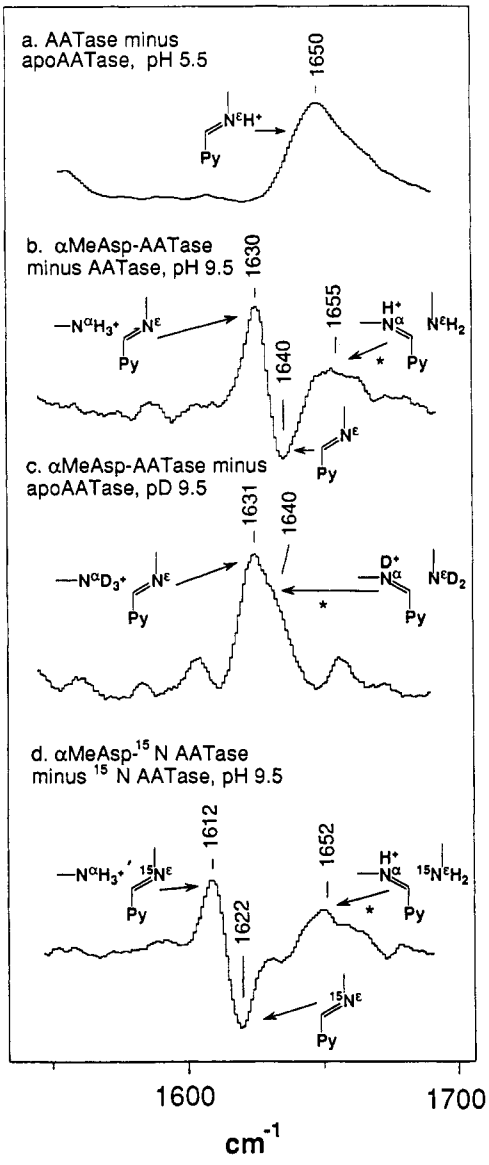

Figure 1. Raman difference spectra of *E. coli* aspartate aminotransferase (AATase) complexes with pyridoxal 5′-phosphate (PLP) and α-methyl-L-aspartate (αMeAsp). The structural assignments in the figure are discussed in the text. The superscripts α and ε indicate the nitrogen substituent of αMeAsp and the ε-N atom of Lys-258, respectively, and Py designates the substituted pyridine ring of the cofactor. An asterisk indicates a tentative assignment. Enzyme concentrations were 2–3 mM, t = 4 °C. Buffer solutions were adjusted to the indicated pH values by addition of NaOH or NaOD. The Raman difference spectra were measured,[11] and AATase samples were prepared and metabolically labeled with ^{15}N, as previously described.[12, 16] (a) AATase-minus-apoAATase Raman difference spectrum at pH 5.5 in 0.2 M sodium acetate. (b) αMeAsp–AATase-minus-AATase Raman difference spectrum at pH 9.5 in 0.2 M 2-[*N*-cyclohexylamino]ethanesulfonic acid (CHES) (Sigma Chemical Co.). The concentration of αMe-DL-Asp (Sigma Chemical Co.) was 100–150 mM; thus, the fraction of AATase bound to αMe-L-Asp was >90%;[5] the D isomer is not recognized by the enzyme.[4] (c) Raman difference spectrum of αMeAsp–AATase-minus-apoAATase at pD 9.5 in 0.2 M CHES in ≥98% ^2H_2O. (d) Same as spectrum b, with the ε-nitrogen atom of active-site Lys-258 and other atoms quantitatively enriched[12,16] with ^{15}N. The 100-mW 568.2-nm laser line from a krypton laser was used, and spectral resolutions were from 6 to 7 cm^{-1}.

based on the apoenzyme.) The peak at 1655 cm^{-1} in Figure 1b is tentatively assigned to $\nu_{C=NH^+}$ of a protonated aldimine by its positional similarity to peaks in the Raman spectra of the protonated Schiff-base form of unligated *E. coli* AATase ($\nu_{C=NH^+}$ = 1650 cm^{-1}, Figure 1a) and of the protonated form of the Schiff base of αMeAsp and PLP in the absence of enzyme ($\nu_{C=NH^+}$ = 1653 cm^{-1}).[15] This assignment is considered tentative due to the low amplitude of the signal relative to the background; however, it is supported by the apparent 15-cm^{-1} shift in this frequency to 1640 cm^{-1} in ^2H_2O (Figure 1b and c).

The negative peak at 1640 cm^{-1} (Figure 1b) shifts 18 cm^{-1} to the red when the N^ϵ atom of Lys-258 is replaced[16] with ^{15}N (Figure 1d), consistent with the fact that it represents the internal aldimine linkage between PLP and protein in unligated AATase. Moreover, $\nu_{C=N}$ of the αMeAsp–AATase complex (1630 cm^{-1}) is shifted 18 cm^{-1} downfield in the ^{15}N-enriched enzyme (Figure 1b and d), proving that it, too, arises from the *internal* aldimine linkage (Scheme IA). By contrast, the insensitivity of $\nu_{C=NH^+}$ of the complex at 1655 cm^{-1} to enzyme ^{15}N substitution (Figure 1b and d) indicates that this peak derives from the external aldimine (Scheme IC).

No peaks are observed in the Raman spectrum of the αMeAsp–^{15}N-AATase-minus-AATase complex (Figure 1d) at 1635–1640 or 1630 cm^{-1}, where the respective protonated internal aldimine (Scheme IB) and unprotonated external aldimine (Scheme ID) bands are expected. Given that the relationships between the amplitudes of the Raman bands of these species and their concentrations are similar to those for the aldimine forms assigned above, and given the experimental noise level, we estimate that the structures shown in Scheme IB and D comprise less than 10% of the total complex population.

These results show that the αMeAsp–AATase complex exists predominantly in two forms (Scheme IA and C), and therefore the 358-nm absorbing species is the Michaelis complex rather than the N^α-unprotonated external aldimine depicted in Scheme ID. The relative concentrations of the complexes shown in Scheme IA and C, estimated by comparison of the UV–visible spectrum of the αMeAsp–AATase complex to those of the protonated and unprotonated forms of unliganded enzyme,[5] are 77–87% and 13–23%, respectively, assuming that the extinction coefficients of the maxima in the complex and the unligated enzyme are similar. The protonated internal aldimine (Scheme IB) is an obligatory intermediate on the pathway to the external aldimine;[2] however, it probably comprises no more than 10% of the total complex population since it is not detected by our methods.

The data presented in this study indicate that the position of the internal–external aldimine equilibrium of the *E. coli* AATase–αMeAsp complex in solution differs from that in crystals, in which only the external aldimine form is observed.[17] Spectrophotometric and X-ray crystallographic studies suggest that similar crystallization-induced shifts in the position of this equilibrium occur in AATase from other sources for complexes with αMeAsp,[8] dicarboxylic inhibitors, and substrates.[18]

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(15) The Raman spectrum of the αMeAsp–AATase complex in the absence of enzyme was obtained using the same buffer and excitation parameters as were used to obtain spectrum a of Figure 1. The concentrations of αMeAsp and PLP were 250 and 25 mM, respectively. The extent of reaction of PLP with αMeAsp under these conditions is estimated to be 86% on the basis of a pH-dependent apparent dissociation constant of 0.042 M for PLP-N^α-L-valine (Metzler, C. M.; Cahill, A.; Metzler, D. E. *J. Am. Chem. Soc.* **1980**, *102*, 6075–6082).

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