Observation of an Aminoacrylate Enzyme Intermediate in the Tryptophan Synthase Reaction by Solid-State NMR

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Tryptophan synthase is an $\alpha_2\beta_2$ tetrameric, 143-kDa enzyme complex which catalyzes the last two steps in the biosynthesis of L-tryptophan.[1] The α subunit catalyzes the cleavage of indole-3-glycerol phosphate (IGP) to indole and glyceraldehyde 3-phosphate; the β subunit catalyzes the condensation of indole with serine to form tryptophan, in a reaction mediated by pyridoxal phosphate (PLP). The first step in the β reaction involves the activation of serine by PLP. This process is thought to involve the generation of an electrophilic aminoacrylate enzyme intermediate. The chemical species involved in this activation process are summarized in Scheme 1. In the absence of serine the PLP is covalently bound to β-Lys87 (**I**). When serine binds, the amino group of serine replaces the ϵ amino group of the lysine, forming an external aldimine species (**II**). Deprotonation of **II** yields a quininoid (**III**), which subsequently is protonated and then loses water to give an aminoacrylate (**IV**). This type of intermediate has been observed in other PLP-utilizing enzymes by a variety of spectral techniques.[2,3] Previous transient kinetic studies have shown that **IV** is formed rapidly.[4] It has been suggested that **IV** may exist in equilibrium with a methyl ketoamine tautomer (**V**) although direct spectral evidence is lacking.[1,6] In the absence of indole, the aminoacrylate is slowly hydrolyzed, most likely through an iminopyruvate, to pyruvate, ammonia, and H_2O. Kinetic studies have also shown that an aminoacrylate at the β site of tryptophan synthase serves as a trigger for the cleavage of IGP at the α site and that there is intersubunit communication between the active sites.[4−6] Indole-3-propanol phosphate (IPP) specifically binds to the α subunit and inhibits the α reaction. It has been suggested that IPP changes the distribution of serine species bound to the β subunit.[7,8]

Although there is evidence for an aminoacrylate by absorbance and fluorescence spectroscopy, no direct observation by solution NMR has been reported.[7,8] X-ray crystallography has been unable to resolve an aminoacrylate due to crystal disorder induced by the addition of L-serine. Solid-state NMR allows selective detection of ligands bound to the enzyme and elimination of signals from the high concentration of serine in solution required to saturate the active sites. The kinetics of formation of the aminoacrylate species (**IV**) indicate that it is formed rapidly and breaks down very slowly. The rate constants for the formation and breakdown of **IV** have been determined in solution, and it has been demonstrated that it is formed 4500-fold faster than it is broken down.[4] Thus, when there is a high concentration of serine in the mother liquor in equilibrium with microcrystals of tryptophan synthase, the aminoacrylate species should be bound at the active site of the β subunit. In this communication we describe the use of L-[3-^{13}C]serine and solid-state NMR detection for the direct observation of the aminoacrylate species (**IV**) in equilbrium with the methyl ketoamine tautomer (**V**) at the enzyme active site.

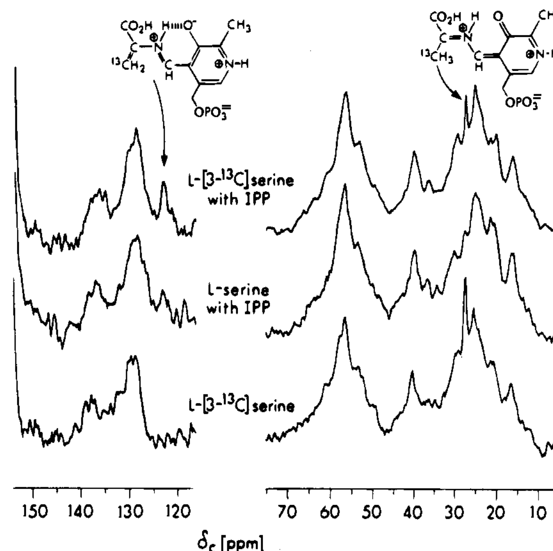

Figure 1. 50-MHz ^{13}C NMR spectra of ~80 mg of α_2-[*ring*-4-^{19}F]-phenylalanine-[*phenol*-4-^{13}C]tyrosine-β_2 tryptophan synthase microcrystals with 5-kHz magic-angle spinning and 80-kHz proton dipolar decoupling. The mother liquor (5 mM Tris, 1 mM EDTA, 1 mM β-mercaptoethanol, 0.1 mM PLP, 5 mM spermine, 30% poly(ethylene glycol) 8000) bathing the microcrystals of enzyme contained (top) 100 mM L-[3-^{13}C]serine and 3 mM IPP, a substrate analog of IGP, which specifically binds to the α subunit; (middle) 100 mM L-serine (unlabeled) and 6 mM IPP; or (bottom) 100 mM L-[3-^{13}C]serine. Two-rotor-cycle CPMAS Hahn echo spectra are shown on the right. Rotor-synchronized CPMAS echo spectra, obtained using a carbon-13 refocusing pulse at the end of each rotor cycle (for 24 cycles) followed by a two-rotor-cycle evolution time with a single Hahn echo refocusing pulse, are shown on the left. Because of differential relaxation, the 26-rotor-cycle spectra are better resolved than the two-rotor-cycle spectra in the vinyl-carbon region. The 28-ppm peak visible in the top and bottom spectra is due to ^{13}C-labeled methyl ketoamine tautomer (V) from reaction of [3-^{13}C]serine with the covalently bound pyridoxal phosphate (PLP) at the β subunit (Scheme 1). The peak at 123 ppm in the top spectrum may indicate a bound [^{13}C]aminoacrylate. The absence of this peak in the bottom spectrum is consistent with enzyme kinetics studies showing that the presence of IPP leads to a redistribution of aminoacrylate species.[7,8] The 2-ms proton to carbon-13 cross-polarization transfer used for all spectra ensures that unbound species are not detected. The intense peak at 156 ppm is due to labeled tyrosine residues.

These studies were performed on a catalytically active, microcrystalline sample of tryptophan synthase $\alpha_2\beta_2$ complex containing isotopic labels in the β subunit from L-[*ring*-4-^{19}F]-phenylalanine and L-[*phenol*-4-^{13}C]tyrosine.[9] The double-labeled tryptophan synthase was prepared to search for conformational changes in the tunnel region[9] based upon the suggestion

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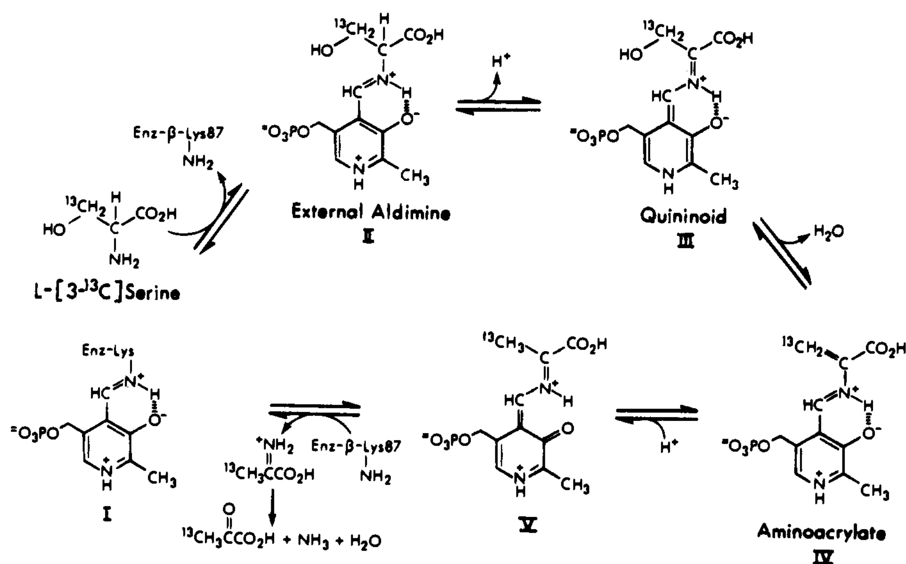
Scheme 1



that β-Phe280 and β-Tyr279 might serve as an allosteric gate which controls opening and closing of the tunnel.[10] The binding of serine and activation to an aminoacrylate may be important in mediating the proposed allosteric regulation.

The ^{13}C NMR spectra in Figure 1 show two regions where resonances for aminoacrylates might be expected. Signals from the natural abundance ^{13}C of the protein and from labeled tyrosine residues (156 ppm) are visible in all spectra. When the microcrystalline sample of tryptophan synthase has been mixed with a solution of L-[3-^{13}C]serine (label chemical shift of 63 ppm), a resonance corresponding to the methyl ketoamine tautomer (**V**) appears at 28 ppm (Figure 1, bottom right) and no resonances due to label appear in the vinyl region (Figure 1, bottom left). It is highly unlikely that pyruvate (methyl carbon shift of 29 ppm) or iminopyruvate gives rise to the 28-ppm resonance because there is no specific affinity of these compounds for tryptophan synthase. If both L-[3-^{13}C]serine and IPP are added to tryptophan synthase, an enhanced resonance due to the aminoacrylate (**IV**) appears in the vinyl region (Figure 1, top left) and the resonance in the methyl region (Figure 1, top right) has reduced intensity. These data indicate that a redistribution of aminoacrylate species has occurred as previously suggested.[7,8] Spectra acquired without IPP and serine present do not show either the 28- or 123-ppm resonances (data not shown). Although the vinyl region is cluttered (Figure 1, middle), the absence of major new peaks in spectra acquired with unlabeled L-serine and IPP provides further evidence that the resonances are due solely to the serine label.

These studies demonstrate for the first time the direct observation of aminoacrylate species bound to the β subunit of tryptophan synthase and the dependence of this binding on a ligand at the α subunit.

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