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Plasticity of the Tryptophan Synthase Active Site Probed by ^{31}P NMR Spectroscopy*

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The functional properties of tryptophan synthase $\alpha_2\beta_2$ complex are modulated by a variety of allosteric effectors, including pH, monovalent cations, and α-subunit ligands. The dynamic properties of the β-active site were probed by ^{31}P NMR spectroscopy of the enzyme-bound coenzyme pyridoxal 5′-phosphate. The ^{31}P NMR signal of the cofactor phosphate of the internal aldimine exhibits a single peak at 3.73 ppm with a line width of 12 Hz. In the presence of saturating concentrations of sodium ions, the ^{31}P signal shifts to 3.97 ppm concomitant with a change in line width to 35 Hz. The latter indicates that sodium ions decrease the conformational flexibility of the coenzyme. In the absence of ions, lowering pH leads to the appearance of a second peak at 4.11 ppm, the intensity of which decreases in the presence of cesium ions. Addition of L-serine in the presence of sodium ions leads to the formation of the external aldimine, the first metastable catalytic intermediate. The ^{31}P signal does not change its position, but a change in line width from 35 to 5 Hz is observed, revealing that this species is characterized by a considerable degree of rotational freedom around the coenzyme C–O bond. In the presence of L-serine and either cesium ions or the allosteric effector indole-3-acetylglycine, the accumulation of the second catalytic intermediate, α-aminoacrylate, is observed. The ^{31}P signal is centered at 3.73 ppm with a line width of 5 Hz, indicating that the phosphate group of the coenzyme in the external aldimine and the α-aminoacrylate exhibits the same flexibility but a slightly different state of ionization. Because the α-aminoacrylate intermediate but not the external aldimine triggers the allosteric signal to the α-subunit, other portions of the β-active site modify their dynamic properties in response to the progress of the catalytic process. A narrow line width was also observed for the quinonoid species formed by nucleophilic attack of indoline to the α-aminoacrylate. The ^{31}P signal moves downfield to 4.2 ppm, indicating a possible change of the ionization state of the phosphate group. Thus, the modification of either the ionization state of the coenzyme phosphate or its flexibility or both are, at least in part, responsible for the conformational events that accompany the catalytic process.

Bacterial tryptophan synthase is a PLP[1]-dependent $\alpha_2\beta_2$ enzyme that catalyzes the final two steps in the biosynthesis of L-tryptophan. α-Subunits cleave indole-3-glycerol phosphate into indole and glyceraldehyde 3-phosphate (α-reaction). Then indole is channeled to the active site of the β-subunit and condensed with PLP-bound α-aminoacrylate, obtained by the β-elimination of water from L-serine, to form L-tryptophan (β-reaction) (see Scheme 1) (1–4, 28–31). The enzyme is allosterically regulated whereby ligands of one subunit alter the function of the other (5–10). In particular, the equilibrium distribution of α-aminoacrylate and the external aldimine is affected by pH, the α-subunit ligand, glycerol 3-phosphate, as well as by temperature (9, 11–13). Monovalent cations affect both conformational and catalytic properties of the tryptophan synthase $\alpha_2\beta_2$ complex. In the presence of either Li^+, Na^+, Cs^+, or NH_4^+, the phosphorescence decay of the unique Trp-177 of the β-subunit, a residue located near the β-active site, is biphasic, and the average lifetime increases, indicating a decrease in flexibility of the N-terminal domain of the β-subunit. A similar effect is caused by glycerol 3-phosphate (14). Cations affect the catalytic activity of the enzyme. In particular, k_{cat} increases in the order $Cs^+ > K^+ > Li^+ > Na^+$. Furthermore, in the absence of cations, at pH 7.9, the predominant species is the α-aminoacrylate absorbing at 350 nm. Cs^+, Rb^+, and Li^+ further stabilize the α-aminoacrylate and cause the formation of a new absorption at about 470 nm, tentatively assigned to a tautomer of the α-aminoacrylate. Na^+ and K^+ stabilize the external aldimine absorbing at 422 nm (15–17). The activation of a large number of enzymes by monovalent cations has been reported (18), but little is known about the structure-function relationship. At present, only very few three-dimensional structures of monovalent cation-dependent enzymes have been solved. Among those are the PLP-dependent dialkylglycine decarboxylase (19, 20), tyrosine ammonia-lyase (21, 22), and tryptophan synthase (23).

Phosphorus NMR spectroscopy has been a very powerful technique to probe the binding site of PLP and the conformational events that take place in catalysis (24–27). In the present study, ^{31}P NMR measurements of $\alpha_2\beta_2$ complex of tryptophan synthase were employed to obtain information about the effects of allosteric compounds on the enzyme conformation as well as on the flexibility of enzyme catalytic intermediates.

MATERIALS AND METHODS

Chemicals—Indole-3-acetylglycine and indoline were obtained from Sigma. All other chemicals were of highest quality commercially available and used without further purification.

Enzyme—Tryptophan synthase from *Salmonella typhimurium* was purified from the *Escherichia coli* strain containing the plasmid encoding the trpA and trpB genes of *S. typhimurium* (13). Experiments were

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[1] The abbreviations used are: PLP, pyridoxal-5′-phosphate; IAG, indole-acetylglycine.

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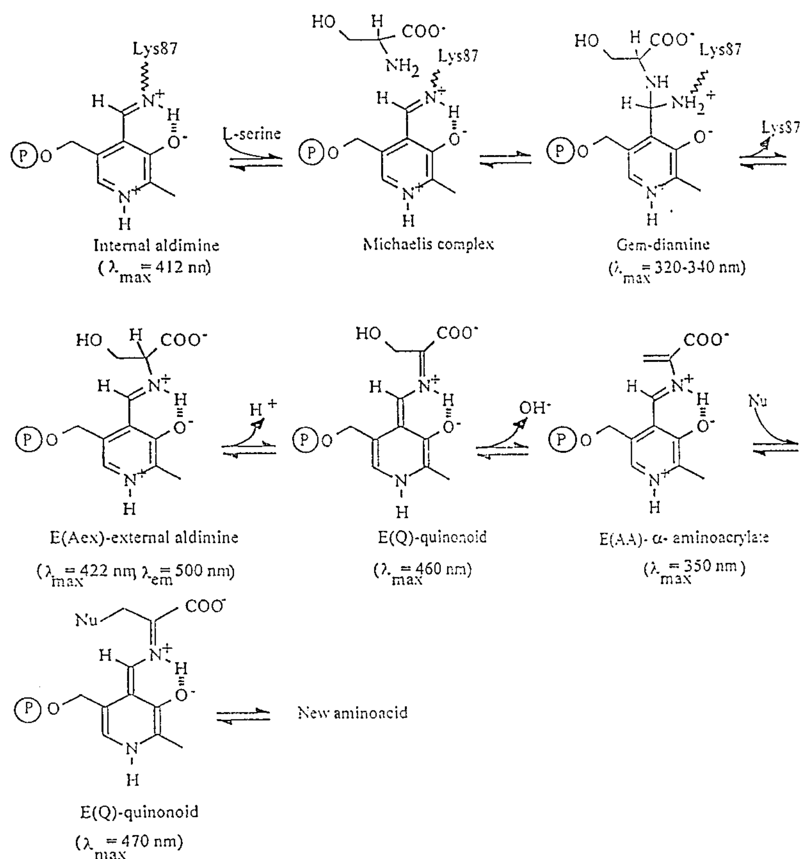

SCHEME 1. **Mechanism of the reaction catalyzed by the β-subunit of the tryptophan $\alpha_2\beta_2$ complex.** Absorption and emission properties are reported below each species (2, 3). *Nu* is indoline.

carried out in 25 mM Bis-Tris-propane buffer adjusted with HCl. Absorption spectra were recorded for all samples before and after the NMR measurements.

NMR Measurements—Fourier-transform ^{31}P NMR spectra were collected at 121.496 MHz on a Bruker 300 MHz SWB superconducting spectrometer using a 10-mm multinuclear probe head with broadband ^{1}H decoupling. The NMR tube contained the sample (2 ml) and $^{2}H_2O$ (0.2 ml) as field/frequency lock and was maintained at 10 ± 0.1 °C using a thermostated liquid nitrogen flow. A spectral width of 2000 Hz was acquired in 8192 data points with a pulse angle of 60 °. The exponential line broadening used prior to Fourier transformation was 10 Hz. Positive chemical shifts in ppm are downfield changes with respect to 85% H_3PO_4. The ^{31}P NMR experiments were performed at high reactant concentrations and 10 °C to assure that the concentration of enzyme intermediates did not change within 2 h and that a good ^{31}P signal with a reasonable signal to noise ratio could be obtained.

RESULTS

^{31}P NMR spectra of the tryptophan synthase $\alpha_2\beta_2$ complex were collected to observe the predominant catalytic species, the internal aldimine, the external aldimine, the α-aminoacrylate, and the quinonoid (Scheme 1). UV-visible absorption spectra were obtained to verify that no changes in the distribution of intermediates occurred during the relatively long time of the NMR experiment (maximally 2 h).

^{31}P NMR Spectra of the Internal Aldimine in the Absence and Presence of Sodium Ions and of the External Aldimine, pH 9.5—The ^{31}P NMR spectrum of the internal aldimine of the $\alpha_2\beta_2$ complex exhibits a resonance at 3.73 ppm with a line width of 12 Hz (Fig. 1*A* and Table I). In the presence of 500 mM sodium ions, the ^{31}P NMR spectrum reveals a single signal at 3.97 ppm with a line broadening of 35 Hz (Fig. 1*B*). No change of the absorbance was observed in the presence and absence of sodium ions. In the presence of sodium ions and L-serine, the reaction catalyzed by the β-subunit of the enzyme leads to the accumulation of the external aldimine. The ^{31}P NMR signal of the external aldimine does not change position but becomes much narrower (5 *versus* 35 Hz) (Fig. 1*C* and Table I).

^{31}P NMR Spectra of the Internal Aldimine in the Absence and Presence of Cesium Ions and of the α-Aminoacrylate, pH 7.3—The ^{31}P NMR spectra of the internal aldimine at pH 7.3 in the absence of ions revealed two signals at 3.71 and 4.11 ppm (Fig. 2*A* and Table I). The two ^{31}P NMR signals are very likely two conformations of the coenzyme. In the presence of 150 mM cesium ions, the signal at 4.11 ppm is diminishing (Fig. 2*B*). In the presence of cesium ions and 400 mM L-serine, the α-aminoacrylate is the predominant species (15). The ^{31}P NMR spectra of the α-aminoacrylate exhibit a signal at 3.73 ppm with a line width of 5 Hz (Fig. 2*C*).

^{31}P NMR of the Internal Aldimine in the Absence and Presence of Indole-3-acetylglycine and of the α-Aminoacrylate—Indole-3-acetylglycine has recently been found to be an allosteric effector,[2] very much like DL-α-glycerol 3-phosphate and indole-3-propanol phosphate. IAG was used in the ^{31}P NMR measurements because it does not contain phosphate groups, and, therefore, its signals do not interfere with those of the coenzyme. The ^{31}P NMR spectrum of the internal aldimine shows at pH 7.6 in the absence of IAG again two signals at 3.73 and 4.11 ppm (data not shown), very similar to those obtained at pH 7.3 (Fig. 2*A*). Upon addition of 5 mM IAG (Fig. 3*A* and Table I), a concentration saturating the α-active site (K_{diss} = 0.12 mM),[2] the signal at 4.11 ppm almost completely disappears, and the signal at 3.73 ppm is predominant (Fig. 3*A*). In the presence of 400 mM L-serine and 5 mM IAG the predominant species is the

[2] A. Marabotti, P. Cozzini, and A. Mozzarelli, manuscript in preparation.

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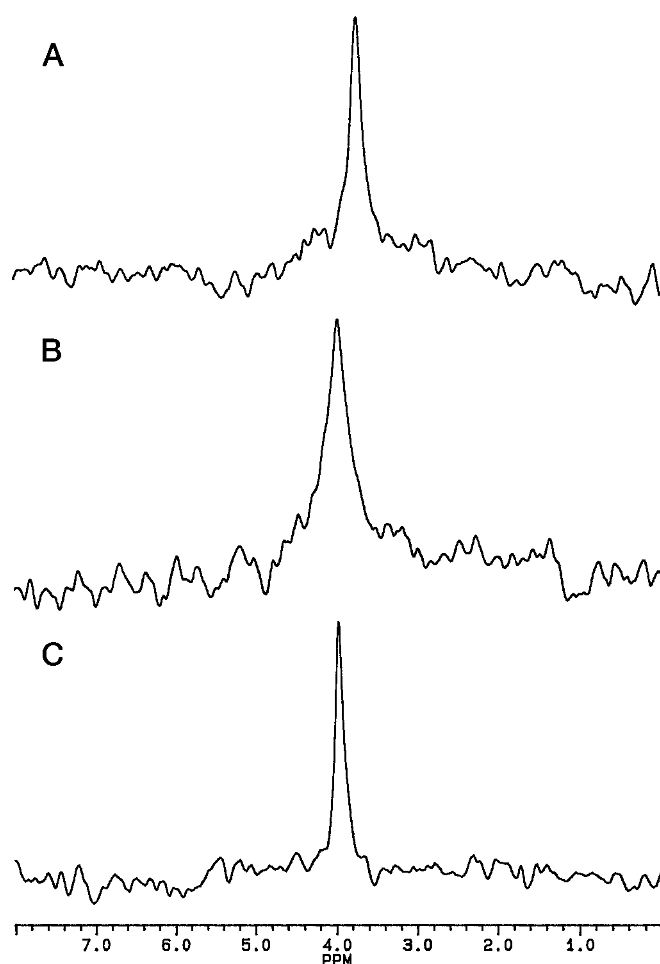

FIG. 1. **^{31}P NMR spectra of tryptophan synthase $\alpha_2\beta_2$ complex at pH 9.5 in the presence of sodium ions and L-serine.** *A*, ^{31}P NMR spectrum of tryptophan synthase in 25 mM Bis-Tris propane buffer, pH 9.5. *B*, spectrum after addition of 500 mM Na^+ to *A*. C, spectrum after addition of 475 mM L-serine to *B*. The protein concentration was 39 mg/ml.

TABLE I
^{31}P chemical shifts of intermediates

Intermediate	Chemical shift	Line width	Relevant figure
	ppm	*Hz*	
Internal aldimine, pH 9.5	3.73	12	1*A*
Internal aldimine (Na^+), pH 9.5	3.97	35	1*B*
External aldimine (Na^+), pH 9.5	3.97	5	1*C*
Internal aldimine, pH 7.3	3.71, 4.11	10, 12	2*A*
Internal aldimine (Cs^+), pH 7.3	3.75, 4.11	10, ND[a]	2*B*
α-Aminoacrylate (Cs^+), pH 7.3	3.72	5	2*C*
Internal aldimine (IAG), pH 7.5	3.74	12	3*A*
α-Aminoacrylate (IAG), pH 7.5	3.65	6	3*B*
Internal aldimine (Na^+), pH 8.0	3.97	35	4*A*
α-Aminoacrylate-External aldimine (Na^+), pH 8.0	3.81	~8	
Quinonoid intermediate (Na^+), pH 8.0	4.2	~10	4*B*

[a] ND, not determined.

α-aminoacrylate. The ^{31}P NMR reveals a single signal at 3.65 ppm with a narrow line width of 5 Hz (Fig. 3*B*), very similar to that obtained for the α-aminoacrylate in the presence of L-serine and cesium ions (Fig. 2*C*).

^{31}P NMR Spectra of the Quinonoid Formed in the Presence of Indoline, L-Serine, and Sodium Ions, pH 8.0—The ^{31}P NMR spectrum of the internal aldimine at pH 8.0 exhibits a signal at 3.97 ppm, similar to that observed at pH 9.5 (Fig. 4*A* and Table I). In the presence of L-serine, sodium ions, and 40 mM indoline, the predominant species is the quinonoid (Scheme 1) (42). The ^{31}P NMR spectrum reveals two signals at 3.81 and 4.20 ppm, indicative of a mixture of the α-aminoacrylate and the external aldimine (3.81 ppm) and the quinonoid intermediates (4.20 ppm) (Fig. 4*B*).

DISCUSSION

^{31}P NMR spectroscopy has been very helpful in studying the environment of the cofactor phosphate of PLP-dependent enzymes and various enzyme intermediates during catalysis. The effects of different amino acids on *O*-acetylserine sulfhydrylase, a PLP-dependent enzyme that catalyzes an α,β-replacement reaction as tryptophan synthase, indicate the formation of different tautomers of enzyme intermediates that can be distinguished by ^{31}P NMR spectroscopy (26). Studies of the effects of K^+ and Na^+ on the ^{31}P NMR spectrum of the PLP phosphate group of D-serine dehydratase have shown that these cations stabilize conformational states, which are different with respect to O–P–O bond angles, and/or hydrogen bonding of the phosphate group of enzyme-bound PLP (32). Different ^{31}P chemical shifts of PLP have been observed in dialkylglycine decarboxylase in the presence of K^+ (active form) and Na^+ (inactive form).[3] These differences have been attributed to changes in torsion angles C4-C5-C5′-OP4 of the PLP aldimine on the basis of the known three-dimensional structure of the decarboxylase (33). The ^{31}P chemical shifts of both enzyme forms are pH-dependent.[3]

The distinctive ^{31}P NMR signals of PLP in the active site of tryptophan synthase obtained in the absence and presence of either sodium or cesium, at pH values between 7.3 and 9.5, with and without the natural substrate L-serine and the α-subunit ligand IAG, indicate that the β-active site exhibits a conformational flexibility finely tuned by bound ligands.

In the absence of cations, ^{31}P NMR signals of PLP of the internal aldimine of tryptophan synthase vary as a function of pH in the range from 7.3 to 9.5 (Figs. 1*A* and 2*A*). The appearance of resonances to lower field when the pH increases has previously been observed for PLP-Schiff base model compounds and for PLP-dependent enzymes (34). Resonances at lower and higher field have been attributed to the existence of the dianionic and the monoanionic state of PLP aldimine, respectively. The interpretation of ^{31}P NMR spectra is still largely empirical. Gorenstein (35) has proposed a correlation between the ^{31}P chemical shift and O–P–O bond angles in phosphates. For a variety of different alkyl phosphates he showed that ionization to the dianionic form decreases the O–P–O bond angle by 3 ° with a concomitant downfield shift of 3–4 ppm, whereas for the monoanion and the free acid chemical shifts and O–P–O bond angles are similar (35). Because the ^{31}P signals of free phosphopyridoxylaspartate show a pH dependence with limiting δ values of 0.75 and 4.3 ppm at low and high pH values, respectively (36), chemical shifts of 3.65, 3.73, 3.97, and 4.11 ppm for PLP aldimine in tryptophan synthase are interpreted as species approaching the status of full dianion. The predominant relaxation mechanisms governing the ^{31}P line width are expected to be dipole-dipole with neighboring protons and a contribution from chemical shift anisotropy. The Stokes-Einstein relationship predicts that τ_c and, therefore, the line width are proportional to the molecular weight. The line width observed for the coenzyme bound to the $\alpha_2\beta_2$ complex of tryptophan synthase is 35 Hz in the presence of Na^+ ions (Fig. 1*B*), markedly smaller than 40–42 Hz, calculated for rigidly bound and solvent-protected PLP in tryptophan synthase.

No change of the absorption and emission properties of the

[3] K. D. Schnackerz and J. W. Keller, submitted for publication.

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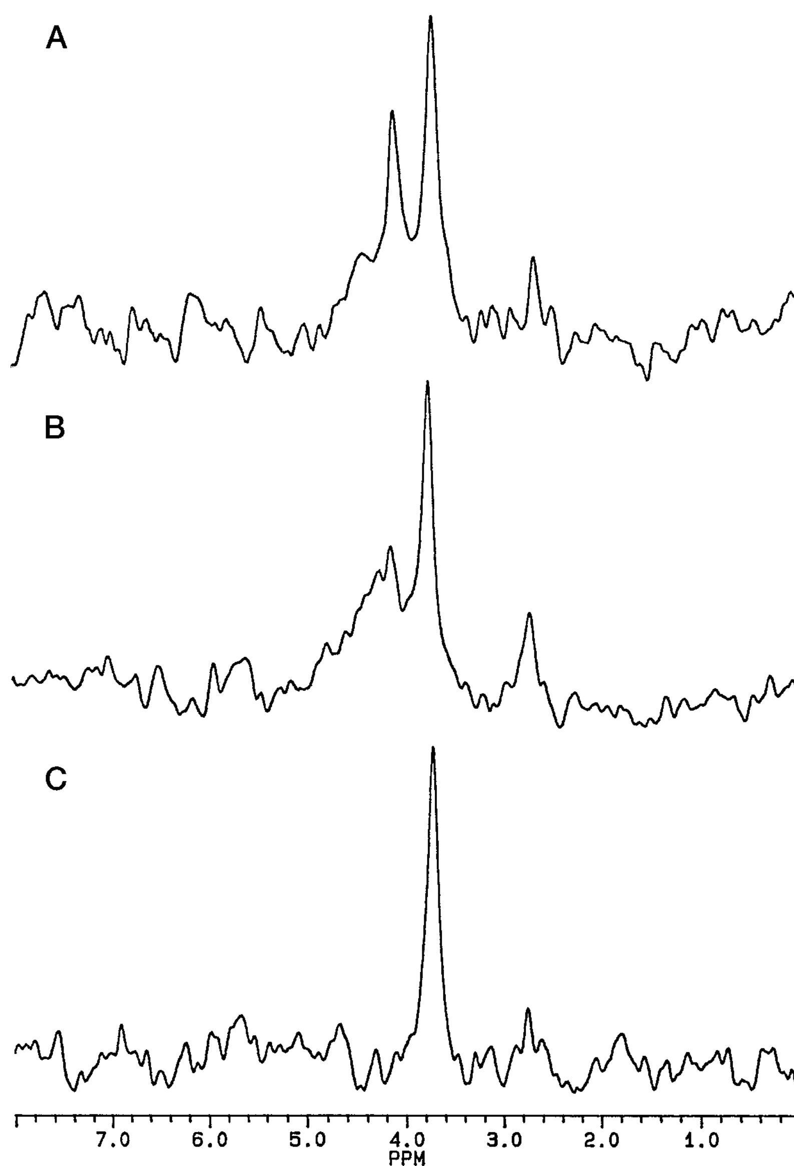

FIG. 2. **^{31}P NMR spectra of tryptophan synthase $\alpha_2\beta_2$ complex at pH 7.1 in the presence of cesium ions and L-serine.** *A*, ^{31}P NMR spectrum of tryptophan synthase in 25 mM Bis-Tris propane buffer, pH 7.3. *B*, spectrum after addition of 150 mM Cs^+ to *A*. *C*, spectrum after addition of 400 mM L-serine to *B*. The protein concentrations were 9.8 (experiments *A* and *B*) and 27.35 mg/ml (experiment *C*). The additional signal around 2.7 ppm is due to free PLP.

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internal aldimine of tryptophan synthase is observed with pH (37). Interestingly, the ^{31}P NMR signal of tryptophan synthase from *E. coli*, dissolved in 0.1 M triethanolamine, 200 mM Na^+ was found to be pH-independent (25). Furthermore, the ^{31}P signal at pH 9.5 in the absence of cations exhibits a 12 Hz line width (Fig. 1*A*), indicating a rather high mobility of the phosphate around the C-O bond of the cofactor. This value is much smaller than that found for the internal aldimine of the *E. coli* enzyme in the presence of 200 mM Na^+ (25–35 Hz). These data suggest that the coenzyme experiences in the two enzyme forms different environment and/or a different rate of interconversion among an ensemble of conformations. In the absence of cations two conformational forms of the internal aldimine can be observed. Cation binding as well as temperature (data not shown) perturb the conformational equilibrium and alter the flexibility of the cofactor phosphate. In particular, sodium ions significantly decrease the mobility of the phosphate group of the internal aldimine reflected by an increase of the line width. The concomitant downfield shift might be the result of a small change in the torsion angle C4-C5-C5′-OP4. The effect of monovalent cations on conformational equilibria of the enzyme has also been probed by characterizing the phosphorescence decay of tryptophan 177 of the β-subunit, the only tryptophan present in tryptophan synthase (14). It was found that the binding of monovalent cations, including cesium and sodium, leads to a significant increase in the species with longer lifetime, independent of the type of cation. These latter results indicate that monovalent cations stabilize a more compact conformation of the β-subunit (14), altering a pre-existing conformational equilibrium (14, 38). However, it should be noted that different techniques reflect the properties of the microenvironment around the probe, the phosphate in the active site being about 20 Å distant from tryptophan 177 of the β-subunit, which is near the α,β interface.

The three-dimensional structure of the tryptophan synthase $\alpha_2\beta_2$ complex has revealed that Na^+, K^+, and Cs^+ bind to the same site in the β-subunit, about 8 Å from the phosphate of PLP but too distant from the active site to play a direct role in catalysis (23). Residues in this region make several contacts with the α-subunit and contribute to the wall that lines the indole tunnel. The number of liganding atoms, which make up the coordination sphere of these atoms, are different and depend upon the size of the cation. The cation binding loop (residues 304–308 of the β-subunit) undergoes a small shift of about 1 Å when Na^+ replaces K^+ or Cs^+, allowing the side chain of Asp-305 of the β-subunit to take up two different orientations. In the Na^+ complex, the carboxylate of Asp-305 forms a salt bridge with ϵ-ammonium group of Lys-167 of the

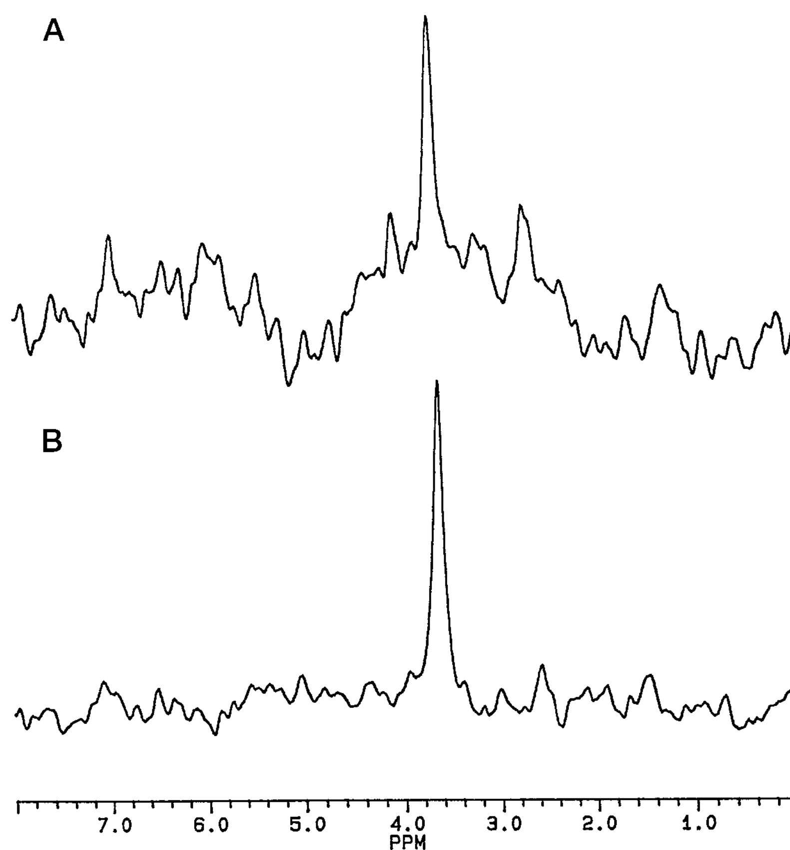

FIG. 3. **^{31}P NMR spectra of tryptophan synthase $\alpha_2\beta_2$ complex at pH 7.5 in the presence of indole-3-acetylglycine and L-serine.** *A*, ^{31}P NMR spectrum of tryptophan synthase in 25 mM Bis-Tris propane buffer, pH 7.5 in the presence of 5 mM IAG. *B*, spectrum after addition of 400 mM L-serine to *A*. The protein concentrations were 9.8 and 30 mg/ml for experiments *A* and *B*, respectively. The additional signal around 2.7 ppm is due to free PLP.

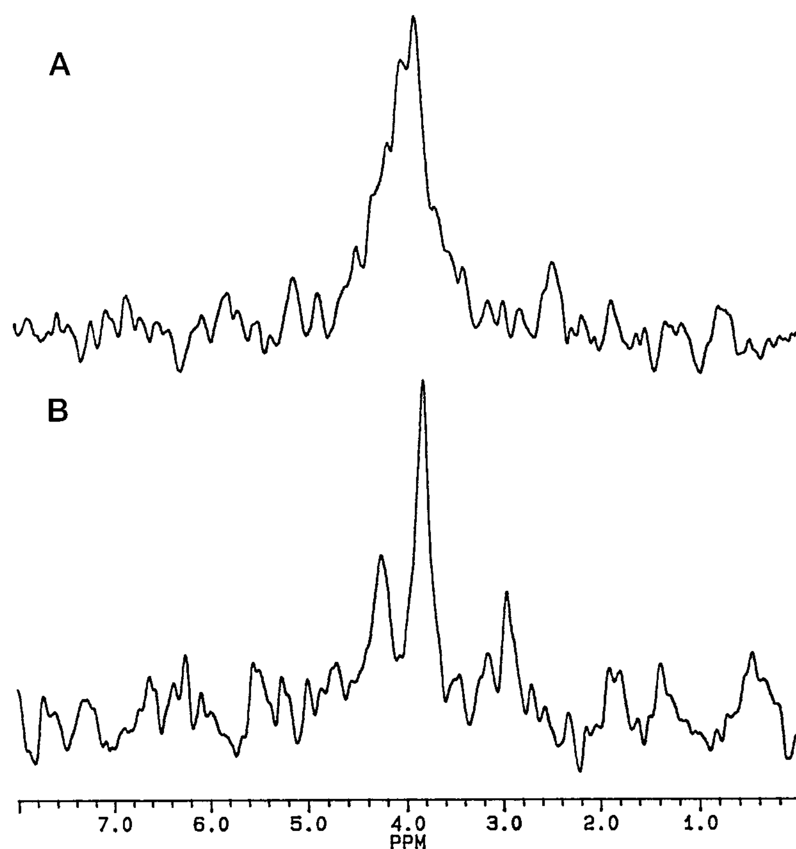

FIG. 4. **^{31}P NMR spectra of tryptophan synthase $\alpha_2\beta_2$ complex at pH 8.0 in the presence of L-serine and indoline.** *A*, ^{31}P NMR spectrum of tryptophan synthase in 25 mM Bis-Tris propane buffer, pH 8.0, in the presence of 500 mM sodium ions. *B*, spectrum after addition of 400 mM L-serine and 40 mM indoline to *A*. The protein concentration was 29.2 mg/ml. The additional upfield signal is due to free PLP.

β-subunit, whereas the K^+ and Cs^+ complexes favor another conformation where Lys-167 of the β-subunit forms a salt bridge with the carboxylate group of Asp-56 of the α-subunit (40). The effect of cations on the enzyme conformation is accompanied by a significant increase in catalytic efficiency of the β-site of tryptophan synthase as documented by the 20–40-fold increase in k_{cat}/K_m (15).

When the internal aldimine reacts with L-serine in the ab-

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sence of the nucleophilic agent indole, external aldimine is preferentially accumulated at high pH in the presence of sodium ions, whereas the α-aminoacrylate accumulates at low pH in the presence of cesium ions. The ^{31}P NMR signals of the external aldimine is characterized by a peak at 3.97 ppm with a line width of 5 Hz (Fig. 1*C*). The internal and external aldimine exhibit in the presence of Na^+ a signal at the same position, but a large difference in line width is observed, indicating that the phosphate of the coenzyme of the external aldimine has a significantly higher flexibility when compared with the internal aldimine. This result is somewhat in disagreement with a suggestion derived from static and time-resolved fluorescence measurements of the internal and external aldimine, which showed that the external aldimine exhibits a very high fluorescence intensity and consistently longer lifetimes with respect to the internal aldimine. Findings were tentatively interpreted as evidence for the external aldimine being endowed with a lower flexibility than the internal aldimine. The three-dimensional structure of the internal and external aldimine show that the transition from one to the other is accompanied by a tilt of the coenzyme ring by about 10 °, keeping the phosphate at the same position (40). No significant difference has been found between the B factors of the phosphate in the internal and external aldimine.[4] However, NMR data more precisely describe changes of the coenzyme flexibility taking place during catalysis.

The α-aminoacrylate exhibits a ^{31}P NMR signal at 3.65 ppm with a line width of 5 Hz, the same as that of external aldimine, indicating that there is no difference in the mobility of the phosphate of the two species (Fig. 2*C versus* Fig. 1*C*). Again, on the basis of the emission properties of the coenzyme, it was suggested that the α-aminoacrylate is more mobile than the external aldimine (37). The three-dimensional structure of the α-aminoacrylate, recently determined (41), indicates that the phosphate tail of the coenzyme does not move with respect to the internal aldimine despite a 20 ° tilt of the PLP ring around the C5-C2 axis. This finding suggests that these catalytic intermediates do not differ in their relative flexibility but in their positions.

When IAG binds to the α-subunit, a change of the distribution conformational is observed, as demonstrated by a change in the ^{31}P NMR signals (Fig. 2*A versus* Fig. 3*A*). This result is in agreement with other spectroscopic data (5, 14). In particular, phosphorescence measurements indicate a tightening of the β-subunit induced by α-subunit ligands (14). When L-serine and IAG are simultaneously present in the absence of monovalent ions, the α-aminoacrylate is the predominant species. Accordingly, the NMR signal is the same as that observed in the presence of L-serine and cesium ions. Thus, the mobility of the phosphate is strongly linked to the type of intermediate present in the active site, independently of the experimental conditions required for its formation. In contrast, the B factor of the phosphate of the coenzyme in the L-serine-indole propanol phosphate-K87T and L-serine-glycerol 3-phosphate-K87T mutant complexes is higher than those observed in the absence of allosteric effectors.[4] The discrepancy might be due to the lower resolution of these structures.

In the catalytic reaction the α-aminoacrylate is attacked by indole to form a labile quinonoid species. A more long-lived quinonoid is obtained by reaction of the α-aminoacrylate with indoline, an indole analog (42). The selective stabilization of this quinonoid is obtained at high pH in the presence of sodium ions. The ^{31}P NMR signal shows a resonance at 3.81 ppm as in the absence of indoline and the appearance of a new resonance at 4.2 ppm with a relatively narrow line width (Fig. 4*B*). Findings suggest that the quinonoid is almost as flexible as the α-aminoacrylate and external aldimine. No structural information is presently available on the quinonoid species.

On the basis of kinetic and spectroscopic data, the catalytic transformation of internal to external aldimine has been proposed to be associated with a conformational transition from an open to a partially open state. The subsequent formation of α-aminoacrylate and the quinonoid species is accompanied by the formation of a closed state of the enzyme (9, 14, 37, 38, 39, 43, 44). Different conformations of the β-subunits send different signals to the α-subunits, thus keeping the activity of the two subunits in phase. The activation of the α-subunits by signals associated to the conformation of the α-aminoacrylate in the β-active site apparently takes place only when sodium ions are present (44). The NMR data provide support to the occurrence of a change in coenzyme mobility in the transformation of the internal to the external aldimine but do not signal a conformational transition from the external aldimine to the α-aminoacrylate and quinonoid species. The phosphate of the coenzyme seems to be a loose anchor of the enzyme-bound PLP complexes. However, changes of the flexibility and the ionization state of the coenzyme phosphate are observed during catalysis. Other regions of the protein in the β-active site and at the α,β-subunit interface change their structural and dynamic properties in response to the presence of either the external aldimine or the α-aminoacrylate to trigger distinct regulatory signals, as clearly evidenced by the three-dimensional structures of the internal aldimine, the external aldimine, and the α-aminoacrylate (40, 41, 45).

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Klaus D. Schnackerz and Andrea Mozzarelli

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