Detection of Open and Closed Conformations of Tryptophan Synthase by $^{15}$N-Heteronuclear Single-Quantum Coherence Nuclear Magnetic Resonance of Bound 1-$^{15}$N-L-Tryptophan*

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1-$^{15}$N-L-Tryptophan (1-$^{15}$N-L-Trp) was synthesized from $^{15}$N-aniline by a Sandmeyer reaction, followed by cyclization to isatin, reduction to indole with LiAlH₄, and condensation of the $^{15}$N-indole with L-serine, catalyzed by tryptophan synthase. 1-$^{15}$N-L-Trp was complexed with wild-type tryptophan synthase and β-subunit mutants, βK87T, βK90/95A, and βE109D, in the absence or presence of the allostery ligands sodium chloride and disodium α-glycerophosphate. The enzyme complexes were observed by $^{15}$N-heteronuclear single-quantum coherence nuclear magnetic resonance ($^{15}$N-HSQC NMR) spectroscopy for the presence of 1-$^{15}$N-L-Trp bound to the β-active site. No $^{15}$N-HSQC signal was detected for 1-$^{15}$N-L-Trp in 10 mM triethanolamine hydrochloride buffer at pH 8. 1-$^{15}$N-L-Trp in the presence of disodium α-glycerophosphate produced a signal twice as intense, suggesting that the equilibrium favors the closed conformation. $^{15}$N-HSQC NMR spectra of βK87T and βE109D mutant Trp synthase with 1-$^{15}$N-L-Trp showed a similar cross peak either in the presence or absence of disodium α-glycerophosphate, indicating the preference for a closed conformation for these mutant proteins. In contrast, the βD305A Trp synthase mutant only showed a $^{15}$N-HSQC signal in the presence of disodium α-glycerophosphate. Thus, this mutant Trp synthase favored an open conformation in the absence of disodium α-glycerophosphate but was able to form a closed conformation in the presence of disodium α-glycerophosphate. Our results demonstrate that the $^{15}$N-HSQC NMR spectra of 1-$^{15}$N-L-Trp bound to Trp synthase can be used to determine the conformational state of mutant forms in solution rapidly. In contrast, UV-visible spectra of wild-type and mutant Trp synthase in the presence of L-Trp with NaCl and/or disodium α-glycerophosphate are more difficult to interpret in terms of altered conformational equilibria.

Bacterial tryptophan (Trp)$^1$ synthase (EC 4.2.1.20) is a pyridoxal 5'-phosphate (PLP)-dependent enzyme that catalyzes the final two steps of the biosynthesis of L-Trp (1–4). The enzyme is an $\alpha_2\beta_2$ complex, consisting of two $\alpha$-subunits bound to a central $\beta_2$-dimer. Each $\alpha$-subunit independently catalyzes the reversible aldol cleavage of indole-3-glycerophosphate to indole and D-glyceroldehyde 3-phosphate (α-reaction, Reaction 1). The $\beta$-subunit catalyzes the conversion of indole and L-serine to L-Trp, with PLP as the cofactor ($\beta$-reaction, Reaction 2). The overall reaction is the combination of the α-reaction and the $\beta$-reaction (αβ-reaction, Reaction 3). Indole is not observed as a free intermediate in the αβ-reaction (5–9), suggesting that indole is not released from the α-subunit of Trp synthase into solution during turnover; hence, Reaction 3 is the physiological reaction of Trp synthase. X-ray crystallographic investigations of Trp synthase from Salmonella typhimurium have revealed a 25–30 Å long interenzyme tunnel linking the $\alpha$- and $\beta$-active sites, through which it has been proposed that indole travels to the $\beta$-active site directly from the α-active site after formation (10–14). Moreover, intermediate complexes of the α- and $\beta$-reactions reciprocally regulate catalysis at the other site in an allosteric manner (11, 15–17). These allosteric interactions coordinate the forward progress of the αβ-reaction.

In the absence of substrate, PLP is bound to βLysS7 in the form of the internal aldimine (E$_A$) (Scheme 1) (18). The reaction of L-Ser at the $\beta$-site, promoted by the presence of indole-3-glycerol phosphate bound at the α-active site, forms an external aldimine (E$_{KA,Ser}$), which is converted to an α-aminoacrylate Schiff’s base (E$_{AA}$) with elimination of water, by the action of βLysS7 as a general base (19). In return, formation of the α-aminoacrylate Schiff’s base with PLP at the $\beta$-site activates the α-reaction (20). D-Glyceroldehyde 3-phosphate bound to the α-site keeps the α-site closed, blocking the release of indole into solution and facilitating transfer of indole from the α- and β-active sites through the interenzyme tunnel. Nucleophilic addition of indole to the α-aminoacrylate intermediate at the $\beta$-site then affords the quinonoid intermediate of L-Trp (E$_Q^\beta$). βGlu109 activates the addition of indole, a weak nucleophile, probably by acting as a general base or H-bond acceptor to the N-H of the reacting indole (21). At this point, βLysS7 acts as a general acid, providing the proton for the conversion of E$_Q^\beta$ to the $\beta$-L-Trp external aldimine (E$_{KA-\beta-Trp}$) (19). Release of α-glyceroldehyde 3-phosphate from the α-site occurs in an open conformation, which then allows release of the L-Trp product from the $\beta$-active site. Nucleophilic attack of βLysS7 on E$_{KA-\beta-Trp}$

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‡ The abbreviations used are: Trp, tryptophan; PLP, pyridoxal 5'-phosphate; GP, glycerophosphate; HSQC, heteronuclear single-quantum coherence; TEA-HCl, triethanolamine hydrochloride.
concomitant with release of L-Trp (19) leads to regeneration of the internal aldimine to begin another catalytic cycle. Evidence of allosteric regulation of Trp synthase comes from both x-ray data (22–25) demonstrating conformational changes associated with bound intermediates and from kinetic and spectroscopic data obtained with wild-type and several mutant forms of Trp synthase, where residues postulated to be associated with allosteric regulation have been mutated to residues bearing less complementary side chains. βAasp305 forms an ion pair with βArg141 in the closed conformation and thus is proposed to help stabilize the closed conformation of Trp synthase (26). Monovalent cations have also been shown to influence the distributions of external aldimine, quinonoid, and aminoaacrylate intermediates (22, 27–29).

In the present study, l-Trp enriched with 15N at the 1-position of the indole ring was synthesized and used as an NMR probe to obtain conformational information on wild-type and several β-site Trp synthase mutants, βK87T, βE109D, and βD305A, in both the absence and presence of Na+, an activating monovalent cation, and an analog of the α-reaction product, disodium α-glycerophosphate (GP). This novel NMR method permits the simple and rapid determination of the conformational state of wild-type and mutant Trp synthase in solution.

**EXPERIMENTAL PROCEDURES**

**Materials**—l-Ser, l-Trp, PLP, and disodium α-GP were obtained from Sigma Chemical Co. or U. S. Biochemical Corp. 15N-Aniline was obtained from Isotec, Inc. Triethanolamine was a product of Fisher Scientific.

**Synthesis of 15N-L-Trp**

15N-Isatin is reduced to indole with LiAlH4 (32). 15N-Isatin (0.350 g, 0.00237 mol) was combined with 6 ml of freshly distilled diethyl ether and cooled to −78 °C. Upon completion of the reaction, the purple mixture was poured over 200 g of crushed ice. The ice quickly melted to reveal a bright orange precipitate, which was collected by filtration to afford 0.412 g (92%) of 15N-isatin.

15N-Indole—Indole is reduced to indole with LiAlH4 (32). 15N-Isatin (0.137 g) was placed in a 100-ml round-bottomed flask with 50 ml of 0.1 M potassium phosphate buffer, pH 7.8. l-Ser, 200 mg, 200 μl of Trp synthase solution (6.7 mg of enzyme), and 15N-indole was isolated from the distillate after concentration by rotary evaporation. The concentrated filtrate was loaded onto a reverse phase column (2.5 x 30 cm; Analtech bonded C18, 35–75 μm). 15N-l-Trp was eluted in 5 to 20% methanol/water to yield 198 mg (83%) of 15N-l-Trp. 15N-Indole, 15N-Isatin, and 15N-Aniline were obtained from Sigma Chemical Co. or U. S. Biochemical Corp. 15N-Aniline was obtained from Isotec, Inc. Triethanolamine was a product of Fisher Scientific.

**Preparation of Trp Synthase**

Wild-type Trp synthase used in chemoenzymatic reactions was expressed from Escherichia coli CB149 cells containing plasmid pSTB7 (18). Cells were grown, collected by centrifugation, and sonicated, and...
the enzyme was purified as described previously (18). All samples of Trp synthase, wild-type and mutant βS7T, βE109D, and βD305A, used in the NMR experiments were purified as described previously (18, 33).

NMR Sample Preparation

Pyridoxal 5'-phosphate (0.5 mg) was added to 1.0 ml of enzyme solution (~40 mg/ml), and it was allowed to stand at room temperature for 1 h. The solution was then applied to a PD-10 (Pharmacia) gel filtration column equilibrated with 10 mM triethanolamine hydrochloride (TEA-HCl), pH 8, eluted with 10 mM TEA-HCl, pH 8, and then concentrated to 0.5 ml by ultrafiltration in a PM-3 Amicon ultrafiltration cell over a YM-30 membrane. The final concentration of Trp synthase was ~75 mg/ml (1 mM). Three or four NMR spectra were then obtained for each enzyme sample. The initial data acquisition was made with 500 μl of enzyme in 10 mM TEA-HCl, pH 8, plus 50 μl of D2O for lock. The subsequent spectra were collected after addition to the sample of 25 μl of a solution containing 0.02 M 15N-L-Trp for the second spectrum, 25 μl of 1 M NaCl for the third spectrum, and addition of 25 μl of 0.5 M disodium α-GP for the fourth spectrum.

NMR Data Collection

NMR data were collected with a Varian Inova 500 spectrometer (499.8 MHz, 1H) with a triple resonance probe. Water signal suppression was done using flip-back pulses (34) and pulse-field gradients (35). Heteronuclear single-quantum coherence (HSQC) experiments (36) were carried out with sensitivity-enhanced, gradient coherence selection (37), and the data were processed with NMRPipe (38).

UV-Visible Spectroscopic Measurements

The UV-visible spectra were obtained with a Cary 1 spectrophotometer. The samples, in a total of 0.6 ml, contained 1 mg/ml of Trp synthase in 10 mM TEA-HCl, pH 8. Spectra were collected of the enzyme alone and together with 1 mM L-Trp and 50 mM NaCl or disodium α-GP from 300 to 550 nm at 25 °C.

RESULTS

Synthesis of 1-15N-L-Trp—The methodology chosen for the synthesis of 1-15N-L-Trp affords a high yield starting from 15N-aniline (Scheme 2). Several total syntheses of 15N-L-Trp have been reported previously in the literature (39–41), and each used the conversion of 15N-indole and L-Ser to 1-15N-L-Trp using Trp synthase, either by reaction in cell cultures modified to express this enzyme in high yield or by the free enzyme in aqueous buffer. However, none of the syntheses reported previously used 15N-aniline, either as a step or starting material, as the source of the label. We considered the various ways to synthesize indole from aniline, e.g., the Fischer indole synthesis, Japp-Klingeman synthesis, and via the Sandmeyer synthesis of isatin, and the latter was chosen for its overall efficiency. A Sandmeyer reaction of 15N-aniline with chloral hydrate and hydroxylamine afforded 15N-isonitrosoacetanilide, which gave 15N-isatin, as a brilliant orange powder, after ring closure with
sulfuric acid. Subsequent reduction of isatin with four equi-
valents of lithium aluminum hydride and purification by steam
distillation and crystallization produced pure 1-13N-indole in
good yield. 15N-L-Trp was synthesized from 1-15N-indole by reac-
tion with one equivalent of L-Ser and a catalytic amount of PLP
using Trp synthase. All steps gave greater than 80% yield, except
for the reduction of isatin to indole, which gave 66% yield.

**HSQC NMR of Trp Synthase—**15N-HSQC NMR measure-
ments were performed on wild-type Trp synthase, K87T,
E109D, and D305A mutants, with 1-15N-L-Trp, with and with-
out a monovalent cation activator, NaCl, and the α-subunit
ligand, disodium α-GP, in 10 mM TEA-HCl, pH 8. Control
experiments showed no detectable 15N-HSQC signal from
1-15N-L-Trp in the presence of wild-type Trp synthase
exhibited a cross peak at 10.2 ppm on the 1H axis and 128.8
ppm on the 15N axis (Fig. 1A). The coupling constant for 15N
and 1H is J_{1H} = 99.8 Hz. Addition of a monovalent cation,
NaCl, resulted in a slight increase in signal intensity (Fig. 1B),
but 50 mM α-GP resulted in a 2-fold increase in peak intensity
(Fig. 1C). In contrast, the mutant enzymes, βK87T (data not
shown) and βE109D (Fig. 2), exhibited HSQC cross peaks with
1-15N-L-Trp that were in equal intensity in the presence or
absence of NaCl or disodium α-GP. For βE109D, the coupling
constant for 15N and 1H is J_{1H} = 97.3 Hz. However, the
βD305A mutant Trp synthase exhibited an HSQC cross peak
with 1-15N-L-Trp only in the presence of disodium α-GP (data
not shown). The results of the 15N-HSQC NMR experiments
are summarized in Table I.

**DISCUSSION**

NMR spectroscopy is a very useful technique for the study of
enzyme structure and function. Whereas x-ray crystallography
is time-intensive and is only capable of providing details of
protein structure in a crystalline sample, observation of pro-
tiens by NMR spectroscopy, with the use of selectively labeled
ligands, is rapid and easy to perform with modern instrumenta-
tion. A problem in the measurement of 15N-NMR spectra in
the past has been the very low sensitivity of the 15N-nucleus,
because of the low resonance frequency of 15N, combined with
the low natural abundance of 15N. The problem of natural
abundance can be overcome by enrichment of the compound of
interest, but the inherently low sensitivity of the 15N nucleus
remains. 15N-HSQC NMR overcomes this problem by observa-
tion of a 15N signal by detecting the attached proton(s) (36).
Because this method observes the 15N nucleus indirectly via
polarization transfer with the attached hydrogens, an N-H
proton must be present, and not exchanging rapidly with the
bulk solvent, for a NMR signal to be seen.

Trp synthase is an ideal enzyme for study by NMR with a
labeled ligand for several reasons. First, Trp synthase has been
the subject of considerable biochemical investigation, and a
number of x-ray crystal structures of the wild-type enzyme,
several mutant forms, and complexes with ligands have been
reported (10, 22–25). Thus, the information provided by NMR
observation of a labeled ligand can be correlated with known
crystallographic structural data. Once structural correlations
are made, the NMR data can provide new information for
systems that lack x-ray structural data. Second, various inter-
mediate complexes of this enzyme allosterically regulate en-
zyme conformation, catalysis, and intermediate stability of the
two active sites, and NMR is a tool that is well-suited for
measuring these subtle conformational and electronic changes.
Indeed, experiments with 19F-NMR of 5-fluoro-L-Trp provided
the first evidence for very slow indole formation from l-Trp by
Trp synthase (42). More recently, NMR studies by McDowell et
al. (43) with β-13C-l-Ser demonstrated direct evidence for an
α-aminoacylate intermediate formed by dehydration of l-Ser
in the β-active site of Trp synthase.

1-Trp bound to the β-active site of Trp synthase was observed
with 15N-HSQC NMR of 1-15N-l-Trp as a cross peak at −129
ppm in the 15N axis and 10.2 ppm in the 1H axis (Fig. 1A). The
observation of this signal is dependent on the slow exchange of
the hydrogen with the solvent, because a signal from 1-15N-l-
Trp is not observed under these conditions in the absence of
Trp synthase. Thus, the observation of the NMR signal in Fig.
1A implies that 1-15N-l-Trp is bound at the β-site and that Trp
N-H is at least partially sheltered from solvent exchange. The
presence of Na+, which can influence the conformational equi-
librium of open and closed conformations, did not have much
effect on the HSQC NMR spectrum (Fig. 1B). The binding of
α-site ligands, disodium α-GP or 1,3-glyceraldehyde 3-phosphate,
results in a closed conformation of the α-site, which is trans-
mittted allosterically to the β-active site and shifts the equilib-
rium at the β-site to favor the closed conformation (11). In
the presence of disodium α-GP, the NMR signal was about twice as
intense (Fig. 1C). This result is consistent with the hypothesis
that the β-active site of Trp synthase with l-Trp bound in is in
equilibrium between open and closed forms in the absence of
disodium α-GP and shifts to a predominantly closed conforma-
tion in the presence of disodium α-GP, with results of the
15N-HSQC experiments with wild-type Trp synthase are sum-
marized in Table I. Although no x-ray structure of Trp synthase
with α-GP bound to the α-site and l-Trp bound to the β-site has
been determined, the structure of Trp synthase with l-glycer-
aldehyde 3-phosphate in the α-site and dihydroxyisotryptophan
bound to the β-site has been determined, and it is in a closed
conformation. The UV-visible spectra of wild-type Trp synthase
with l-Trp present (Fig. 3A) showed little change with
NaCl, but an increase in the content of the quinonoid interme-
diate, absorbing at 476 nm, was seen in the presence of dia-
sonium α-GP, consistent with a closed conformation (11).

The ε-amino side chain of βLys87 binds PLP in the form of the
internal aldime, which undergoes nucleophilic attack by the
incoming substrate, l-Ser, to form the external aldime
(E_{/α-Ser}). Mutation of βLys87 to threonine (K87T) results in an
inactive enzyme, which can form an external aldime (E_{/α-Ser})
but which cannot form an α-aminoacylate intermediate (19).
Addition of ammonia to the K87T l-Ser complex can form an
α-aminoacylate intermediate, suggesting that Lys-87 func-
tions as a general base in the mechanism (20). Furthermore,
addition of l-Trp to βK87T under typical reaction conditions
leads to the slow formation of the l-Trp external aldime.

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1 I. Schlicting, personal communication.
Because the lysine ε-amino group is not present in K87T, this external aldimine is very stable, the internal aldimine cannot form, and hence L-Trp is bound slowly but not released. This property of βK87T has been useful for the study of stable complexes of Trp synthase by x-ray crystallography. Currently, x-ray crystal structures of βK87T Trp synthase have been
solved for various complexes, including l-Ser bound to the β-site, l-Ser bound to the β-site and indole-3-propanol phosphate bound to the α-site, l-Ser bound to the β-site and α-GP bound to the α-site, and of l-Trp bound to the β-site (23). The structure of the βK87T complex with l-Trp is found to be in a closed conformation. Our 15N-HSQC NMR experiments with
**Table I**

| Enzyme         | $^{15}$N-L-Trp $^{+}$NaCl $^{+}$ | $^{+}$GP $^{a}$ |
|----------------|---------------------------------|----------------|
| Wild-type      | $^{+}$                           | $^{+}$         |
| K87T           | $^{+}$                           | ND $^{b}$     |
| E109D          | $^{+}$                           | $^{+}$         |
| D305A          | $^{+}$                           | ND $^{b}$     |

$^{a}$ Addition of 50 mM NaCl.
$^{b}$ Addition of 25 mM disodium $\alpha$-GP.
$^{c}$ ND, not determined.

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1-$^{15}$N-L-Trp are consistent with these x-ray crystallographic results. A strong $^{15}$N signal is observed for 1-$^{15}$N-L-Trp with $\beta$K87T Trp synthase, either with or without the presence of disodium $\alpha$-GP (Table I), as expected, because the closed conformation is seen in the $\beta$K87T-L-Trp crystal structure, even without an $\alpha$-site ligand present.

The second mutant Trp synthase that we examined by $^{15}$N-HSQC NMR was $\beta$E109D. $\beta$Glu109 in the $\beta$-active site is seen in the structure of the $\beta$K87T-L-Trp complex (23) to form a hydrogen bond to the indole N-H of the product. Substitution of this glutamate residue by aspartate reduces the reach and conformational freedom of the carboxylate functionality. Kinetic studies of $\beta$E109D have indicated that allosteric communication between the $\alpha$- and $\beta$-active sites is unaffected by this mutation (21). d-Glyceraldehyde-3-phosphate bound to the $\alpha$-site activates the reaction of $l$-Ser at the $\beta$-site of $\beta$E109D, as it does with the wild-type enzyme. Furthermore, formation of the $\alpha$-aminooxacylate intermediate is unaffected, as well as its allosteric communication to the $\alpha$-site to release glyceraldehyde-3-phosphate. $^{15}$N-HSQC NMR spectra of 1-$^{15}$N-L-Trp with $\beta$E109D Trp synthase revealed a strong signal, with equal intensity either with or without the presence of NaCl or disodium $\alpha$-GP (Fig. 2). This result suggests that the L-Trp external aldimine of $\beta$E109D Trp synthase adopts a predominantly closed conformation, as does the $\beta$K87T mutant Trp synthase. Because formation of the $\alpha$-aminooxacylate intermediate is essentially unaffected by the $\beta$E109D mutation, the critical role of this residue must be catalysis of indole addition to the aminooxacylate (21). Indeed, kinetic experiments reveal an accumulation of indole at the $\beta$-active site for $\beta$E109D Trp synthase, whereas this accumulation does not occur with the wild type, indicating a slower reaction of indole with the aminooxacylate for $\beta$E109D Trp synthase. Furthermore, the $\beta$E109D mutant enzyme demonstrates a marked preference for indoline as opposed to indole as the $\beta$-site nucleophile, perhaps suggesting a change in the steric environment of the active site (21). The N-H coupling constant, $J_{\text{NH}}$, is reduced from 99.8 Hz for wild-type to 97.3 Hz for $\beta$E109D Trp synthase. This is consistent with a stronger hydrogen bond with the N-H of L-Trp for $\beta$E109D Trp synthase, which may be responsible for the apparent increased preference for a closed conformation. The $^{15}$N-HSQC NMR results with $\beta$E109D Trp synthase are summarized in Table I. The UV-visible spectra of $\beta$E109D Trp synthase in the presence of L-Trp did not show much difference with disodium $\alpha$-GP (Fig. 3B) added. There is a small increase in the band at 330 nm with disodium $\alpha$-GP present (21), which could be due to a gem-diamine complex or to an enolimine form of the external aldime. The enolimine form of a PLP Schiff base is generally favored by a hydrophobic environment. In contrast to the NMR data, these UV-visible data are more difficult to interpret in terms of altered conformational equilibria.

The third mutant Trp synthase that we studied by $^{15}$N-HSQC NMR was $\beta$D305A Trp synthase. Unlike the other mutant proteins, $\beta$K87T and $\beta$E109D, residue $\beta$D305 is not located within the $\beta$-active site. The x-ray data of different conformations of Trp synthase suggest that $\beta$Asp305 may be important for allosteric communication between the $\alpha$- and $\beta$-subunits via the monovalent cation binding site (29, 44), because it forms an ion pair with $\beta$Arg141 in the closed conformation. $\beta$D305A Trp synthase has a broader substrate specificity for nucleophiles than wild-type Trp synthase, suggesting that it favors a more open, solvent-accessible active site than wild-type Trp synthase (26). The results of our experiments given in Table I suggest that although the conformational equilibrium in the absence of $\alpha$-ligands indeed preferentially favors the open structure, allosteric communication in $\beta$D305A is similar to that of the wild-type enzyme. The complete absence of a $^{15}$N-HSQC signal for 1-$^{15}$N-L-Trp with $\beta$D305A Trp synthase without disodium $\alpha$-GP is consistent with previous conclusions that the $\beta$D305A protein may adopt a more open conformation than wild-type Trp synthase (26, 29, 44). However, the observation of the $^{15}$N-HSQC cross peak with disodium $\alpha$-GP present implies that the closed conformation of the L-Trp complex can still form, at least partially, when an $\alpha$-ligand is present. This is consistent with the observation that a closed complex with $l$-Ser can be formed by $\beta$D305A Trp synthase in the presence of disodium GP or Cs$^{+}$ but not with Na$^{+}$.
An important concern with x-ray crystallography is whether the conformational state of a protein in solution is the same as that seen in the crystalline state. An advantage of NMR spectroscopy is that it measures the properties of proteins in solution and thus provides direct information on the solution structure of the protein. Our NMR results with wild-type and D305A Trp synthase, for which x-ray crystal structural data are available, are in agreement with the crystal structures. However, no crystal structures have been obtained yet for βE109D and βD305A Trp synthase. The 15N-HSQC NMR results reported herein suggest that the conformational equilibrium of βE109D Trp synthase has a preference for the closed conformation with L-Trp bound, more so than does wild-type Trp synthase, whereas the l-Trp complex of βD305A Trp synthase favors the open conformation more than wild-type Trp synthase.

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