The Tryptophan Synthase $\alpha_2\beta_2$ Complex: Kinetic Studies with a Mutant Enzyme (β K87T) To Provide Evidence for Allosteric Activation by an Aminoacrylate Intermediate

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ABSTRACT: To investigate the mechanism by which the tryptophan synthase β subunit accelerates the cleavage of the indole-3-glycerol phosphate catalyzed by the α subunit (α reaction), kinetic experiments were carried out with wild-type and mutant forms of the $\alpha_2\beta_2$ complex. Previous studies indicate that this activation can be attributed to the conformational changes associated with the formation of a Schiff base between aminoacrylate and pyridoxal phosphate at the β site. To test this hypothesis, we investigated a mutant form of the $\alpha_2\beta_2$ complex having the lysine-87 of its β subunits replaced by threonine. The mutant $\alpha_2\beta_2$ complex (K87T) exhibits normal activity for the α reaction but fails to catalyze formation of L-tryptophan from L-serine and indole (β reaction). However, the mutant enzyme can form a Schiff base intermediate with L-serine at the β site. Using a "chemical rescue" method, we converted K87T L-serine intermediate to an aminoacrylate intermediate. Steady-state kinetic studies reveal that the aminoacrylate derivative exhibits a 7-fold enhancement in k_{cat}/K_m for the α reaction relative to that of the L-serine derivative of the mutant or the wild-type enzyme in the absence of L-serine. Rapid kinetic data show that the aminoacrylate derivative of the mutant enzyme exhibits a 6-fold increase in the rate constant for the indole-3-glycerol phosphate cleavage reaction. In addition, rate constants for the reverse reaction and product release steps are also altered. Together, these changes lead to a decrease in K_m and an increase in k_{cat}. The magnitude of this enhancement is lower than that observed with the wild-type enzyme with saturating L-serine; nevertheless, the results directly demonstrate the postulated allosteric activation in the absence of L-tryptophan formation at the β site.

It is important to understand the structural basis for the allosteric communication between distant sites in enzymes. Tryptophan synthase (EC 4.2.1.20) is an ideal system for investigating this problem. [For reviews, see Miles (1979, 1986, 1991, 1995), Swift and Stewart (1991) and Yanofsky and Crawford (1972).] Crystallographic studies of the tryptophan synthase $\alpha_2\beta_2$ complex from *Salmonella typhimurium* show that the active sites of the α and β subunits are separated by ~30 Å (Hyde *et al.*, 1988). The tryptophan synthase $\alpha_2\beta_2$ complex catalyzes the conversion of indole-3-glycerol phosphate (IGP)[1] and L-serine to D-glyceraldehyde 3-phosphate (G3P) and L-tryptophan, termed the $\alpha\beta$ reaction.

$$\alpha\beta \text{ reaction: } IGP + Ser \rightarrow Trp + G3P + H_2O$$

The $\alpha\beta$ reaction consists of a sequence of two partial reactions catalyzed at the active sites of the α and β subunits. The α subunit catalyzes the reversible cleavage of IGP to indole (IND) and D-glyceraldehyde 3-phosphate, termed the α reaction.

$$\alpha \text{ reaction: } IGP \leftrightarrow IND + G3P$$

The β subunit catalyzes the conversion of IND and L-serine to L-tryptophan, termed the β reaction. The β reaction depends on the coenzyme pyridoxal phosphate (PLP) and proceeds through a series of PLP Schiff base intermediates shown in Scheme 1A.

$$\beta \text{ reaction: } IND + Ser \rightarrow Trp + H_2O$$

The finding that indole does not appear as a free intermediate in solution during turnover of the $\alpha\beta$ reaction suggests that indole formed at the active site of the α subunit migrates to the active site of the β subunit through a channel (Yanofsky & Rachmeler, 1958). The channeling hypothesis is supported by recent kinetic studies (Dunn *et al.*, 1990; Anderson *et al.*, 1991; Brzović *et al.*, 1992; Lane & Kirschner, 1991; Schlichting *et al.*, 1994) and by structural evidence (Hyde *et al.*, 1988). The three-dimensional structure of the tryptophan synthase $\alpha_2\beta_2$ complex reveals that the active sites of the α and β subunits are connected by a ~30 Å long tunnel that is wide enough to permit the intramolecular transfer of indole.

An intriguing feature of the tryptophan synthase $\alpha_2\beta_2$ complex is the functional interaction between the α and β sites. A substrate or substrate analogue that binds to one site affects the properties of the other site. For example,

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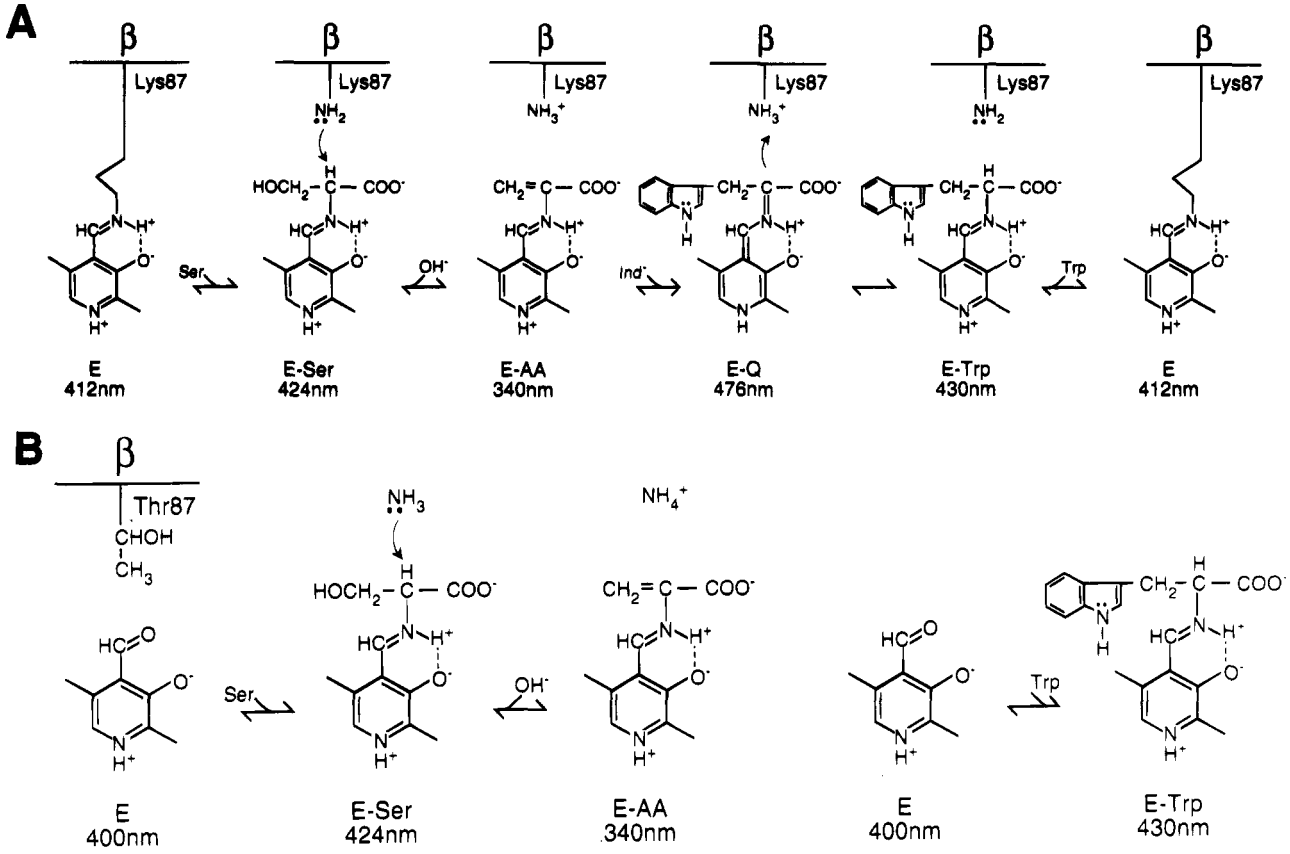
[1] Abbreviations: IGP, indole-3-glycerol phosphate; G3P, D-glyceraldehyde 3-phosphate; IND, indole; PLP, pyridoxal 5′-phosphate; E-AA, PLP Schiff base of aminoacrylate; K87T, β subunit having lysine-87 replaced by threonine.

Scheme 1: Reactions at the Active Site of the β Subunit in the Wild-Type $\alpha_2\beta_2$ Complex (A) and the K87T $\alpha_2\beta_2$ Complex (B)[a]



[a] (A) Wild-type $\alpha_2\beta_2$ complex (E), which contains pyridoxal phosphate bound as an aldimine to the ϵ-amino group of β lysine 87. This complex reacts with serine to form E-Ser. The synthesis of L-tryptophan at the β site proceeds by conversion of E-Ser to the internal aldimine of aminoacrylate (E-AA) followed by addition of indole to form E-Trp, which releases the Trp to regenerate E. (B) K87T $\alpha_2\beta_2$ complex (E) forms an external aldimine with L-serine (E-Ser) or L-tryptophan (E-Trp). E-Ser is converted to E-AA in the presence of a high concentration of NH_3. Thus, NH_3 may partially replace the deleted ϵ-amino group of lysine-87 by accepting the α-proton of L-serine.

the turnover number for IGP cleavage at the α site is 20 times larger in the presence of L-serine ($\alpha\beta$ reaction) than in the absence of L-serine (α reaction). Thus, L-serine or an L-serine derivative at the β site activates IGP cleavage at the α site, apparently over a distance of ~30 Å. It is not possible to use steady-state kinetics to measure the α reaction in the presence of L-serine because the nascent indole is rapidly converted to L-tryptophan by the β reaction. The use of 6-nitroindole-3-glycerol phosphate and of 6-nitroindole, which does not condense with L-serine, shows that L-serine bound to the β site increases the turnover numbers for 6-nitroindole-3-glycerol phosphate and 6-nitroindole (Kirschner *et al.*, 1991).

Rapid kinetic studies under limiting substrate conditions demonstrate a large stimulation of the rate of IGP cleavage (Anderson *et al.*, 1991). These studies provide evidence that formation of the PLP Schiff base of aminoacrylate (E-AA in Scheme 1A) at the β site is associated with a conformational change in one or both subunits that activates IGP cleavage. Studies with L-serine analogues also indicate that E-AA is the chemical signal that triggers the conformational transition that activates the α subunit (Brzović *et al.*, 1992).

To distinguish the effects of the aminoacrylate derivative (E-AA) *per se* from the effects of aminoacrylate turnover, we have used an inactive mutant form of the β subunit in which lysine-87 is replaced by threonine (Lu *et al.*, 1993; Nagata *et al.*, 1989). Lysine-87 forms a Schiff base with PLP in the wild-type enzyme. The mutant $\alpha_2\beta_2$ complex, termed K87T, binds PLP as the free carbonyl form (Scheme 1B) and forms Schiff base intermediates with L-serine, L-tryptophan, and D-tryptophan (Lu *et al.*, 1993). Although the enzyme is totally inactive in reactions catalyzed at the β site, the activity of the α site remains the same. Thus, the effects of the formation of specific derivatives at the β site on the activity at the α subunit can be tested in the absence of turnover at the β site. We have previously reported that addition of a high concentration of ammonia results in partial conversion of the K87T-Ser derivative to an aminoacrylate derivative (K87T-AA) by "chemical rescue" (Lu *et al.*, 1993). Whereas Lys-87 serves as the proton acceptor with the wild-type $\alpha_2\beta_2$ complex (Scheme 1A), ammonia serves as the proton acceptor with the K87T $\alpha_2\beta_2$ (Scheme 1B).

In the present work, we use steady-state and rapid kinetic studies to demonstrate that the K87T-AA $\alpha_2\beta_2$ complex has significantly enhanced activity in the α reaction whereas the K87T-Ser $\alpha_2\beta_2$ complex does not. These results are a direct demonstration of the postulated mechanism of allosteric activation by the aminoacrylate intermediate in the absence of turnover.

MATERIALS AND METHODS

Enzymes. The wild-type (Miles *et al.*, 1989) and K87T (Lu *et al.*, 1993) forms of the tryptophan synthase $\alpha_2\beta_2$ complex from *S. typhimurium* were isolated and purified as described. Buffer B (50 mM sodium *N,N*-bis(2-hydroxyethyl)glycine containing 1 mM EDTA at pH 7.8) was used

for all experiments. The K87T $\alpha_2\beta_2$ complex as isolated contains a low level of tightly bound L-serine (Lu *et al.*, 1993). Serine-free K87T $\alpha_2\beta_2$ complex was prepared for some experiments as described (Lu *et al.*, 1993). Derivatives of the K87T $\alpha_2\beta_2$ complex were prepared by incubating the complex for 24 h at 23 °C with 40 mM L-serine or with 1 mM L- or D-tryptophan (Lu *et al.*, 1993) followed by gel filtration on a PD-10 column (Pharmacia/LKB) in buffer B. The aminoacrylate derivative of the K87T $\alpha_2\beta_2$ complex was prepared by incubating the isolated L-serine derivative with 1 M NH_4Cl in buffer B for 4 h (Lu *et al.*, 1993) followed by gel filtration in buffer B; this derivative was used within 2 h of preparation.

Spectroscopic Methods. Absorption spectra were made using a Hewlett-Packard 8452 diode-array spectrophotometer. Time course measurements at single wavelengths were made using a Cary 118 spectrophotometer.

Chemicals. Unlabeled and ^{14}C-labeled IGP were synthesized enzymatically using tryptophan synthase to catalyze the reverse α reaction with G3P and either indole or [^{14}C]-indole. IGP was isolated by ion exchange chromatography on DEAE-Sephadex-A25 as described (Kawasaki *et al.*, 1987). [^{14}C]Indole (specific activity, 50 mCi/mmol) was purchased from Research Products International Corp. (Mount Prospect, IL). G3P was prepared from the diethyl acetal of DL-G3P (Sigma Chemical Co.) as described by the suppliers.

Enzyme Assays and Steady-State Kinetics. One unit of activity in any reaction is defined as the formation of 0.1 μmol of product in 20 min at 37 °C. The activities of the $\alpha_2\beta_2$ complex in the α reaction and αβ reaction were measured by spectrophotometric assays coupled with D-glyceraldehyde 3-phosphate dehydrogenase (Sigma Chemical Co.) (Creighton, 1970). The reaction conditions for the spectrophotometric assays were also used to determine the rate of conversion of 0.4 mM [^{14}C]IGP (specific activity, 10^5 counts min^{-1} μmol^{-1}) to [^{14}C]indole. Reactions with aliquots of the reaction mixture (0.1 mL) were terminated at the indicated times (0−30 min) with 0.01 mL 1 N NaOH and extracted with 0.5 mL of toluene. Aliquots (0.25 mL) of the toluene layer containing [^{14}C]indole were counted in 1 mL of OptiScint HiSafe (LKB); aliquots (0.05 mL) of the aqueous layer containing [^{14}C]IGP or [^{14}C]tryptophan were counted in 1 mL of OptiPhase HiSafe II (LKB) using a Beckman LS3800 scintillation counter.

Rapid Quench Experiments. The rapid quench experiments were performed at 37 °C using a KinTek Model RQF-3 quench-flow apparatus (KinTek Instruments, University Park, PA) essentially as previously described (Anderson *et al.*, 1991). The reaction was initiated by mixing the enzyme solution (32 μL) with [^{14}C]IGP (32 μL, ~15 300 counts/min). The reaction mixture was then quenched at various times (20 ms to 10 s) with 105−440 μL of 0.6 N KOH and extracted with toluene. Aliquots of the toluene and aqueous layers were counted as described for steady-state kinetic studies.

Data Analysis. Steady-state kinetic data were analyzed using the Enzyme Kinetics program from Trinity Software and CA-Cricket Graph III from Computer Associates. Rapid quench kinetic data were numerically analyzed by solving the differential equations describing the reaction scheme being considered (see Results) using the integrator of the MLAB software package, which utilizes a combined Adams−Gear method (Knott, 1979) (Civilized Software, Inc., Bethesda, MD).

Table 1: Specific Activities of Wild-Type and Mutant $\alpha_2\beta_2$ Complexes in Catalyzing the Conversion of IGP to G3P[a]

	IGP → G3P (units/mg)			IGP → G3P (units/mg)	
$\alpha_2\beta_2$ complex	−Ser	+Ser	$\alpha_2\beta_2$ complex	−Ser	+Ser
wild type	30	620	K87T-L-Trp	20	28
K87T	20	35	K87T-D-Trp	19	28
K87T-L-Ser	32	30	K87T-AA	150	150

[a] K87T complexes with amino acids and aminoacrylate (AA) were prepared as described in Figure 1. Activities were measured as described under Materials and Methods in the presence or absence of 40 mM L-serine.

RESULTS

Effects of Liganded β Subunit on IGP Cleavage. The steady-state rate of IGP cleavage by the wild-type $\alpha_2\beta_2$ complex is increased by 20-fold in the presence of L-serine (Table 1). That is, the rate of the αβ reaction is 20-fold faster than that of the α reaction. Although the faster rate of IGP turnover in the presence of serine suggests that binding of serine to the β site leads to enhancement of the rate of cleavage of IGP at the α site, it is not possible to measure independently the effects of serine on the α reaction at steady state because the indole is rapidly converted to tryptophan (Anderson *et al.*, 1991). To circumvent this problem, we have utilized a mutant form of the $\alpha_2\beta_2$ complex (K87T) (Lu *et al.*, 1993). This enzyme complex consists of two active wild-type α subunits and two completely inactive β subunits which result from the replacement of lysine 87 by threonine. The K87T $\alpha_2\beta_2$ complex forms stable Schiff base intermediates with L-serine, L-tryptophan, and D-tryptophan that can be isolated by gel filtration and have distinctive absorption spectra (Figure 1). The Schiff base of the L-serine complex can be converted to the Schiff base of aminoacrylate by ammonia, which partially replaces the deleted ε-amino group of lysine-87 (Lu *et al.*, 1993) as shown in Scheme 1B. The absorption spectrum of the aminoacrylate derivative of the K87T $\alpha_2\beta_2$ complex (K87T-AA in Figure 1A) is similar to that reported previously (Lu *et al.*, 1993) and to the spectrum observed upon addition of L-serine to the wild-type $\alpha_2\beta_2$ complex (Goldberg *et al.*, 1968; Miles, 1980; Drewe & Dunn, 1985). The spectrum exhibits an absorption maximum near 350 nm and a broad envelope of absorbance that extends out to ~525 nm. These bands probably represent an equilibrium mixture of the PLP Schiff base of L-serine (E-Ser in Scheme 1A), a quinonoid intermediate that results from the removal of the α-proton from E-Ser and is not shown in Scheme 1, and the PLP Schiff base of aminoacrylate (E-AA). Although PLP Schiff base intermediates with aminoacrylate absorb maximally at 450−480 nm in model and enzymatic systems (Cook & Wedding, 1976; Schnackerz *et al.*, 1979), the enolimine tautomer of the aminoacrylate intermediate might absorb at 350 nm (Lane & Kirschner, 1983). Enolimine tautomers of PLP Schiff bases, which have an unprotonated azomethine nitrogen and are favored in hydrophobic environments, absorb at lower wavelengths than ketoenamine tautomers (Heinert & Martell, 1962; Metzler *et al.*, 1980).

Table 1 shows that the K87T $\alpha_2\beta_2$ complex and its Schiff base complexes with L-serine, L-tryptophan, and D-tryptophan

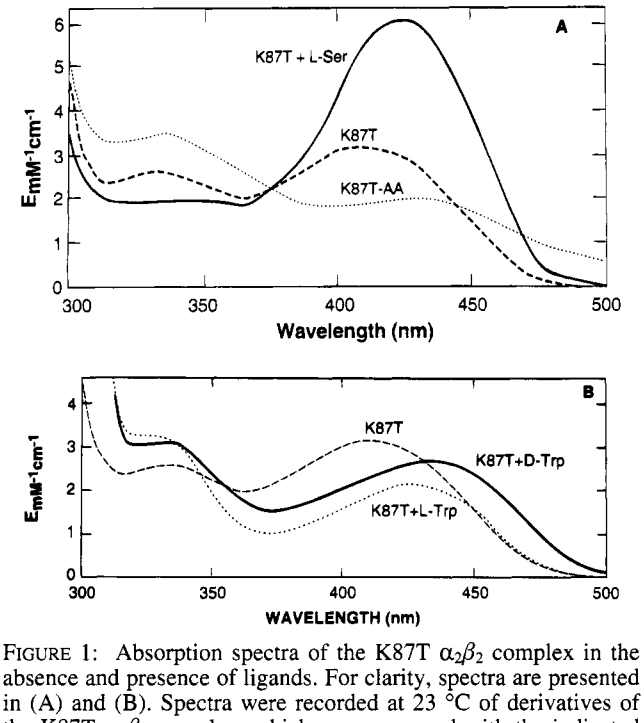


FIGURE 1: Absorption spectra of the K87T $\alpha_2\beta_2$ complex in the absence and presence of ligands. For clarity, spectra are presented in (A) and (B). Spectra were recorded at 23 °C of derivatives of the K87T $\alpha_2\beta_2$ complex, which were prepared with the indicated amino acids and with aminoacrylate (AA) as described in Materials and Methods.

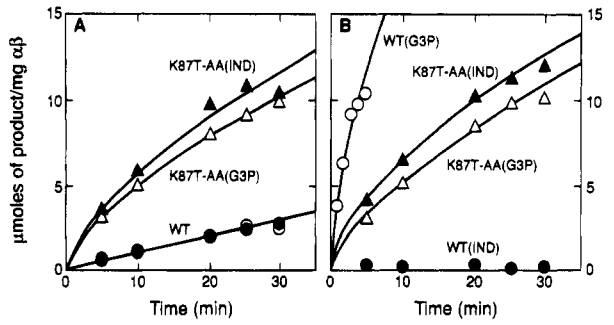


FIGURE 2: Time courses of IGP cleavage catalyzed by the wild-type and mutant $\alpha_2\beta_2$ complexes. Parallel reactions were carried out at 37 °C in the presence (B) or absence (A) of 40 mM L-serine for spectrophotometric assays (G3P) with unlabeled IGP and for the radioactive assays (IND) with [^{14}C]IGP as described in Materials and Methods. Symbols: with the wild-type enzyme and monitoring the formation of G3P (○) or indole (●); K87T-AA complex and monitoring the generation of G3P (△) or indole (▲).

exhibit low specific activities for the α reaction which are similar to that of the wild-type enzyme. These low activities are either unaffected or are increased to a small extent in the presence of L-serine. In contrast, the specific activity of the Schiff base complex with aminoacrylate is 5−7.5-fold higher than that of the K87T $\alpha_2\beta_2$ complex and of the wild-type $\alpha_2\beta_2$ complex. The rate of IGP cleavage catalyzed by K87T-AA is unaffected by the addition of L-serine to the assay mixture.

Figure 2 shows the rates of formation of indole and of G3P catalyzed by the wild-type and K87T-aminoacrylate complexes. In the absence of L-serine (Figure 2A), indole and G3P are formed in approximately equal amounts at each time with each enzyme, as expected for the α reaction. The rate of the formation of each product is 4−5-fold higher for the K87T-aminoacrylate complex than for the wild-type $\alpha_2\beta_2$ complex. In the presence of L-serine (Figure 2B), the wild-type $\alpha_2\beta_2$ complex forms G3P at a rapid rate but accumulates

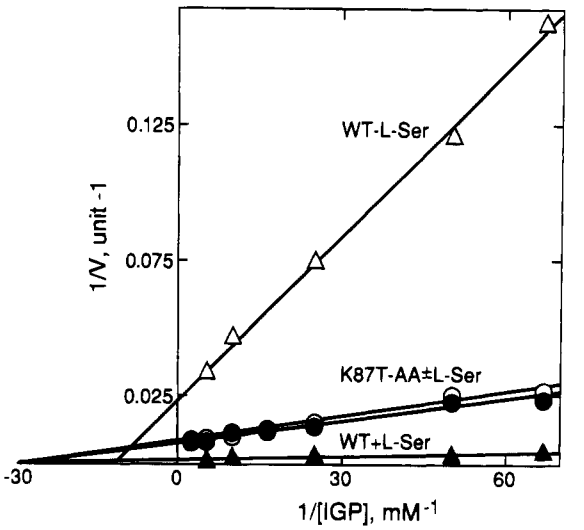


FIGURE 3: Effect of IGP concentration on the rate of the wild-type and mutant $\alpha_2\beta_2$ complexes. Spectrophotometric assays were carried out and analyzed as described in Table 2 in the absence or presence of 40 mM L-serine. Symbols: wild-type enzyme in the absence (△) or presence (▲) of 40 mM L-Ser; K87T-AA in the absence (○) or presence (●) of L-Ser.

Table 2: Steady-State Kinetic Constants of the Wild-Type and Mutant $\alpha_2\beta_2$ Complexes for the IGP Cleavage Reaction[a]

	−L-serine				+L-serine		
$\alpha_2\beta_2$ complex	K_m (IGP) (µM)	k_{cat} (s^{-1})	k_{cat}/K_m (μM^{-1} s^{-1})	rel k_{cat}/K_m	K_m (IGP) (µM)	k_{cat} (s^{-1})	k_{cat}/K_m (μM^{-1} s^{-1})
wild type	90	0.26	0.0029	1.0	30	3.8	0.130
K87T	90	0.17	0.0019	0.7	90	0.3	0.003
K87T-Ser	100	0.19	0.0019	0.7	110	0.24	0.002
K87T-AA	40	0.83	0.0200	6.7	40	0.82	0.020

[a] K87T complexes with amino acids and aminoacrylate (AA) were prepared as described in Figure 1. Activities were measured as described under Materials and Methods; initial rates were determined during the first 1−2 min of the reaction. The concentration of IGP was varied from 0.015 to 0.2 mM in the presence or absence of 40 mM L-serine. Kinetic constants were calculated using Enzyme Kinetics Software by Jacek Stanislowksi (Trinity Software).

no indole. This is expected for the αβ reaction because indole formed from IGP is further converted to L-tryptophan. In contrast, the K87T-aminoacrylate $\alpha_2\beta_2$ complex forms indole and G3P at approximately equal rates, independent of the presence of L-serine, showing that indole is not converted to L-tryptophan.

Figure 3 shows the concentration dependence for the rate of IGP cleavage catalyzed by the wild-type and K87T-aminoacrylate $\alpha_2\beta_2$ complexes in the presence or absence of L-serine. Steady-state kinetic constants derived from these data and analogous data for the K87T $\alpha_2\beta_2$ complex and its L-serine Schiff base are given in Table 2. Addition of L-serine to the wild-type $\alpha_2\beta_2$ complex increases k_{cat} and decreases K_m for IGP as reported previously (Kawasaki *et al.*, 1987). The kinetic constants for the K87T $\alpha_2\beta_2$ complex and its L-serine derivative are closely similar to those of the wild-type enzyme in the absence of L-serine and are not significantly altered by the addition of L-serine. In contrast, the K87T-aminoacrylate complex exhibits a lower K_m for IGP and a higher k_{cat}, thus a higher k_{cat}/K_m value than that of the wild-type $\alpha_2\beta_2$ complex in the absence of L-serine. Thus, the K87T-aminoacrylate complex exhibits to some extent the alterations in steady-state kinetics that are induced

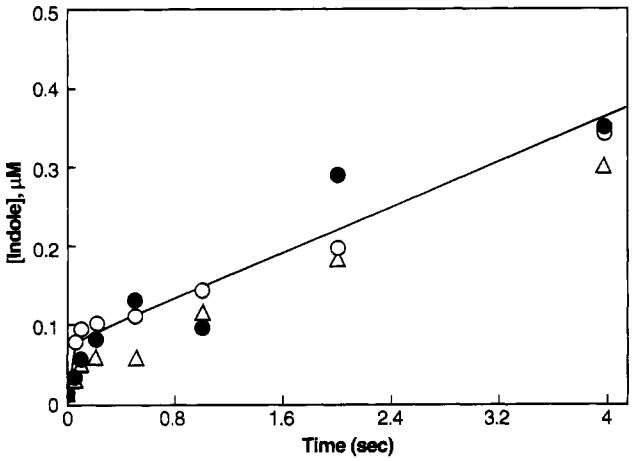


FIGURE 4: Time courses of IGP cleavage catalyzed by the wild-type enzyme in the absence of L-Ser and mutant $\alpha_2\beta_2$ complexes. The reaction was initiated by mixing a solution of enzyme with [^{14}C]IGP. The concentrations after mixing are 20 μM enzymes and 2.4 μM [^{14}C]IGP. The conversion of [^{14}C]IGP to [^{14}C]indole was monitored as described in Materials and Methods. Symbols: wild-type enzyme without added L-Ser (●), K87T (○), and K87T plus L-Ser (△). The solid line was calculated using the scheme shown in reactions 1−4 and the average constants given in Table 3.

in the wild-type $\alpha_2\beta_2$ complex by the addition of L-serine: e.g., lower K_m for IGP and higher k_{cat} and k_{cat}/K_m.

Rapid Kinetic Studies. To investigate the mechanism by which the ligand-bound β subunit facilitates the rate of IGP cleavage catalyzed by the α subunit, rapid quench-flow experiments were carried out. The rate was monitored by the formation of [^{14}C]indole from [^{14}C]IGP. The reaction was initiated by mixing two equal volumes (32 μL) of [^{14}C]-IGP and various forms of enzyme to yield a final concentration of 2.4 μM IGP and 20 μM enzyme. Figure 4 shows the data obtained with the wild-type $\alpha_2\beta_2$ complex in the absence of L-serine, K87T without L-serine, or K87T plus L-serine. In all cases, the indole generation proceeded with an initial rapid step followed by a slow reaction. This suggests that the simplest mechanism required to account for these data is described by eqs 1−4 in which E−IND−

$$E + IGP \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E{-}IGP \quad (1)$$

$$E{-}IGP \underset{k_4}{\overset{k_3}{\rightleftharpoons}} E{-}IND{-}G3P \quad (2)$$

$$E{-}IND{-}G3P \underset{k_6}{\overset{k_5}{\rightleftharpoons}} E{-}G3P + IND \quad (3)$$

$$E{-}G3P \underset{k_8}{\overset{k_7}{\rightleftharpoons}} E + G3P \quad (4)$$

G3P is formed rapidly followed by a slow release of indole. This reaction scheme, as shown below, is similar to that described previously (Anderson *et al.*, 1991). E represents the α subunits of the $\alpha_2\beta_2$, which catalyzes the formation of indole from IGP. Each data set was analyzed as described in Materials and Methods to yield a set of rate constants, k_1 to k_8, given in Table 3. The solid line in Figure 4 was calculated using the average values of the rate constants determined for the wild-type enzyme in the absence of L-Ser, K87T, and K87T plus L-Ser. This calculated curve fits reasonably well with all three sets of data, indicating that

Table 3: Rate Constants Obtained with Numerical Analysis of the Rapid Kinetic Data[a]

	$\alpha_2\beta_2$	K87T	K87T+ L-Ser	av[b]	K87T-AA
k_1 (μM^{-1} s^{-1})	2.3	2.3	2.8	2.5	0.23
k_2 (s^{-1})	292	297	300	296	300
k_3 (s^{-1})	15	29	7	17	105
k_4 (s^{-1})	57	107	45	70	2.4
k_5 (s^{-1})	0.82	0.8	1.12	0.91	1.2
k_6 (μM^{-1} s^{-1})	0.0053	0.009	0.005	0.0064	126
k_7 (s^{-1})	7	7	7	7	5.9
k_8 (μM^{-1} s^{-1})	0.018	0.011	0.019	0.016	1.9×10^{-5}
K_m[c] (μM)	99	100	93	94	41
k_c[d] (s^{-1})	0.17	0.17	0.15	0.17	0.98
K_{eq}[e] (μM)	124	119	120	127	99

[a] Data obtained with a buffer containing 50 mM *N*,*N*-bis(2-hydroxyethyl)glycine/1 mM EDTA, pH 7.8, at 37 °C. The concentrations of IGP and enzyme were 2.4 and 20 μM, respectively. [b] Average rate constants of those obtained with $\alpha_2\beta_2$, K87T, and K87T + L-Ser. The average rate constants were used to calculate K_m, k_c, and K_{eq}. [c] Calculated K_m using eq 5. [d] Calculated k_c using eq 6. [e] Calculated K_{eq} for IGP ⇌ IND + G3P, $K_{eq} = k_1k_3k_5k_7/k_2k_4k_6k_8$.

there is no change in the α reaction with the mutant relative to that of the wild-type enzyme and L-serine fails to activate the α reaction catalyzed by the mutant enzyme. Since the curve-fitting analysis involves the evaluation of eight rate constants, the value so determined for each constant need not be unique. In order to reduce the degrees of freedom, we introduced additional constraints using experimentally determined values of K_{eq}, K_m, and k_c. Based on the reaction scheme shown in eqs 1−4, the expressions of K_m and k_c were derived for the initial rate conditions and are given in eqs 5−6, respectively. Equations 5 and 6 and the expression

$$K_m = \frac{k_7[k_2k_4 + k_5(k_2 + k_3)]}{k_1[k_7(k_3 + k_4) + k_5(k_3 + k_7)]} \quad (5)$$

$$k_c = \frac{k_3k_5k_7}{k_7(k_3 + k_4) + k_5(k_3 + k_7)} \quad (6)$$

for the equilibrium constant based on the reaction scheme shown above were introduced into the rate expressions for the curve-fitting. The calculated K_m, k_c, and K_{eq} are given in Table 3. They are indeed in reasonably good agreement with those determined by the steady-state kinetic method listed in Table 2 and the reported K_{eq} of 120 μM (Weischet & Kirschner, 1976).

The time course for the α reaction catalyzed by K87T-AA was also measured using the quenched-flow method (Figure 5). Like the wild-type enzyme, the time course for the indole generation in this case is also biphasic. Thus, the same reaction scheme (eqs 1−4) was used for the data analysis. The eight rate constants so obtained are given in Table 3. Again, the calculated K_m and k_c are in good agreement with that determined by the steady-state kinetic method shown in Table 2. Furthermore, the value of K_{eq} calculated is 99 μM, which is in reasonably good agreement with a value of 120 μM reported by Weischet and Kirschner (1976). For comparison, we also reanalyzed the quenched-flow data obtained under similar conditions with the wild-type $\alpha_2\beta_2$ complex under saturating levels of L-serine (Anderson *et al.*, 1991). However, the minimum number of reactions required to describe the $\alpha\beta$ reaction under saturating levels of L-Ser is five as depicted in eqs 7−11.

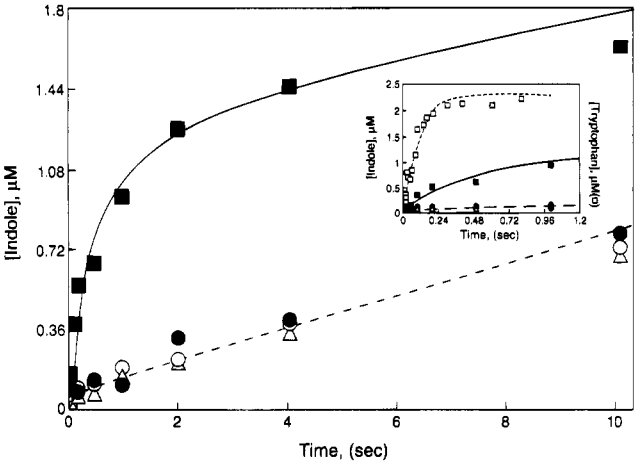


FIGURE 5: Time courses of IGP cleavage catalyzed by K87T-AA. The reaction conditions are the same as those described in Figure 4. The data for the K87T-AA catalyzed reaction are represented by (■). The solid line is the calculated curve using reactions shown in eqs 1−4 and the rate constants given in Table 3. For comparison, the data obtained with the wild-type enzyme without added L-Ser (●), K87T (○), and K87T in the presence of L-Ser (△) are included. The inset shows data for the wild-type enzyme in the presence (□) of 40 mM L-Ser taken from Anderson *et al.* (1991) and the first second of the time courses shown in this figure. The short dashed line in the inset was obtained based on the reaction scheme shown in eqs 7−11 and the rate constants k_9−k_{18} given in the text.

$$\alpha\beta\text{-AA} + \text{IGP} \underset{k_{10}}{\overset{k_9}{\rightleftharpoons}} \alpha\text{−IGP}\beta\text{-AA} \quad (7)$$

$$\alpha\text{−IGP}\beta\text{-AA} \underset{k_{12}}{\overset{k_{11}}{\rightleftharpoons}} \alpha\text{−IND−G3P}\beta\text{-AA} \quad (8)$$

$$\alpha\text{−IND−G3P}\beta\text{-AA} \underset{k_{14}}{\overset{k_{13}}{\rightleftharpoons}} \alpha\text{−G3P}\beta\text{-AA−IND} \quad (9)$$

$$\alpha\text{−G3P}\beta\text{-AA−IND} \underset{k_{16}}{\overset{k_{15}}{\rightleftharpoons}} \alpha\beta\text{−Trp} + \text{G3P} \quad (10)$$

$$\alpha\beta\text{−Trp} \underset{k_{18}}{\overset{k_{17}}{\rightleftharpoons}} \alpha\beta + \text{Trp} \quad (11)$$

where $\alpha\beta$-AA represents the enzyme with aminoacrylate formed at the β subunit, due to its reaction with L-Ser. Analysis of these data for $\alpha\beta$ reaction yields a set of rate constants, namely, $k_9 = 1.2\ \mu M^{-1}\ s^{-1}$, $k_{10} = 191\ s^{-1}$, $k_{11} = 210\ s^{-1}$, $k_{12} = 0.5\ s^{-1}$, $k_{13} = 3500\ s^{-1}$, $k_{14} = 3000\ s^{-1}$, $k_{15} = 766\ s^{-1}$, $k_{16} = 0.1\ \mu M^{-1}\ s^{-1}$, $k_{17} = 7.9\ s^{-1}$, and $k_{18} = 0.1\ \mu M^{-1}\ s^{-1}$. Under initial rate conditions, the expressions of K_m and k_c for this reaction scheme are

$$K_m = [k_{17}[k_{10}(k_{12}k_{14} + k_{12}k_{15} + k_{13}k_{15}) + k_{11}k_{13}(k_{14} + k_{15})]]/ k_9[k_{17}[(k_{11} + k_{12})(k_{14} + k_{15}) + k_{13}(k_{10} + k_{11} + k_{15})] + k_{15}[k_{10}(k_{12} + k_{13}) + k_{11}k_{13}]] \quad (12)$$

$$k_c = k_{11}k_{13}k_{15}k_{17}/[k_{17}[(k_{11} + k_{12})(k_{14} + k_{15}) + k_{13}(k_{10} + k_{11} + k_{15})] + k_{15}[k_{10}(k_{12} + k_{13}) + k_{11}k_{13}]] \quad (13)$$

The validity of these rate constants is supported by the reasonably good agreement between the experimentally determined K_m and k_c, which are 30 μM and 3.8 s^{-1}, respectively, compared to the calculated values of 19.4 μM and 4 s^{-1} obtained using eqs 12 and 13, respectively. The rate constants, k_{13} and k_{14}, for the transfer of IND from the α site to the β site are high, as is expected for an efficient substrate channeling mechanism. The short dashed line in the inset of Figure 5 is the calculated curve based on eqs 7−11 and the rate constants shown above. The relatively higher end point shown in this time course is necessary because the $\alpha\beta$ reaction is known to strongly favor the formation of tryptophan. Together, these data clearly show that covalently linked aminoacrylate at the β site of the mutant enzyme (K87T-AA) accelerates significantly the rate of IGP cleavage. However, this enhancement observed with K87T-AA is still not comparable to that observed with the wild-type $\alpha_2\beta_2$ complex under saturating levels of L-serine.

DISCUSSION

The use of a mutant form of the tryptophan synthase $\alpha_2\beta_2$ complex has allowed us to obtain direct evidence for a postulated mechanism of allosteric activation. Previous studies have suggested that formation of the Schiff base of L-serine or of aminoacrylate at the active site of the β subunit results in activation of IGP cleavage by the α subunit (Anderson *et al.*, 1991; Brzović *et al.*, 1992; Kirschner *et al.*, 1991). This activation is thought to result from a ligand-induced change in the conformation of the β subunit that is transmitted to the α site. A critical test of this postulated mechanism could be achieved if a ligand-induced change in the β subunit could be generated in the absence of turnover at the β site; that is, if the β subunit could be "locked" into an altered conformation. The use of "chemical rescue" to prepare an aminoacrylate derivative from the inactive K87T $\alpha_2\beta_2$ complex has provided material for this test [see Scheme 1B and Lu *et al.* (1993)].

Steady-state kinetic experiments demonstrate that the aminoacrylate derivative of the K87T $\alpha_2\beta_2$ complex exhibits a higher rate with respect to its capacity to catalyze the cleavage of IGP (Figures 2 and 3, Tables 1 and 2) and a reduced K_m for IGP (Figure 3 and Table 2) relative to those of the wild-type enzyme in the absence of L-serine. In contrast, formation of the Schiff base derivatives of L-serine or of L- or D-tryptophan has little effect on the steady-state parameters. These results indicate that formation of the aminoacrylate intermediate results in partial activation of the α subunit in the absence of catalytic turnover at the β site and support the postulated triggering mechanism.

It should be pointed, however, that the activation seen with the aminoacrylate derivative of the K87T $\alpha_2\beta_2$ complex is ~6-fold, whereas activation with the wild-type enzyme by L-serine is ~20-fold. The relatively modest activation of the mutant enzyme may result from the presence of a lower quantity of aminoacrylate in the K87T $\alpha_2\beta_2$ complex, which have been shown to contain ~0.8 mol/mol of $\alpha_2\beta_2$ (Lu *et al.*, 1993)) and from differences in the ligand-induced conformational changes. Several lines of evidence indicate that the structure of K87T $\alpha_2\beta_2$ complex is more rigid than that of the wild-type $\alpha_2\beta_2$ complex. The reactions of the K87T $\alpha_2\beta_2$ complex with amino acids and with hydroxylamine are much slower than those of the wild-type $\alpha_2\beta_2$ complex (Lu *et al.*, 1993), suggesting that the mutant enzyme is more rigid. Comparisons of the crystal structures of the wild-type $\alpha_2\beta_2$ complex (Hyde *et al.*, 1988) with those of the K87T $\alpha_2\beta_2$ complex containing bound L-serine or L-tryptophan (Miles *et al.*, 1994) reveal that several regions

having low electron density in the wild-type enzyme exhibit higher electron density in the mutant enzyme, again suggesting that the mutant enzyme has a more rigid structure.

Rapid kinetic experiments permit more detailed analysis of the effects of intermediates formed at the β site on the rate of the α reaction. The proposed mechanistic scheme, as shown in reactions 1−4, is the simplest scheme required to account for the two-phase time course observed in the quench-flow data. With this reaction scheme, the conformational change step(s) occur(s) following the ligand binding, product release, bond-breaking, and bond-forming reactions, which is well documented for tryptophan synthase [Brzović *et al.* (1992) and reviewed by Miles (1995)], is (are) incorporated into each of the indicated steps, respectively. The proposed scheme is similar to that reported earlier (Anderson *et al.*, 1991; Kirschner *et al.*, 1991). In this earlier work, the activation of indole formation by the reaction of L-serine at the β site is attributed to changes in the rate constant in the forward reaction (Anderson *et al.*, 1991) or both the forward and reverse reactions (Kirschner *et al.*, 1991) of the IGP cleavage step. However, the current data (Table 3) clearly show that both the IGP cleavage step (k_3 and k_4) and the product release steps are affected by the presence of the covalently linked aminoacrylate at the inactive β site of K87T. Reanalysis of the quench-flow data obtained under similar conditions with saturating levels of L-serine (Anderson *et al.*, 1991) shows that the reaction of L-serine at the β site also affects rate constants for the IGP cleavage step, consistent with that observed with K87T-AA.

The individual rate constants obtained for the wild-type $\alpha_2\beta_2$ complex in the absence of L-serine and for the mutant $\alpha_2\beta_2$ complex in the presence or absence of 40 mM L-serine are quite similar. In fact, the time course calculated with the averages of individual rate constants for these three systems fits reasonably well with the experimental data (see Table 3 and Figure 4). This indicates that the α subunit maintains its catalytic integrity in an enzyme complex consisting of two wild-type α subunits and two inactive mutant β subunits. It should be pointed out that the rate constants were obtained using the numerical integration method, without any assumption to curve-fit the experimental data to the proposed reaction scheme. In our case, it requires the evaluation of 8 and 10 rate constants for the α reaction and the $\alpha\beta$ reaction, respectively. Therefore, the values so obtained for each constant need not be unique. To reduce this uncertainty, we used the experimentally determined K_m, k_c, and K_{eq} to provide additional constraints and, thus, limit the degree of freedom. Consequently, the K_m, k_c, and K_{eq} calculated using the rate constants in Table 3 and in the text are in good agreement with those determined by steady-state kinetics and equilibrium measurement. These calculated values are given in Table 3, and the experimental values for K_m and k_c are given in Table 2. The value of the calculated K_{eq} is 124 μM for the wild-type $\alpha_2\beta_2$ in the absence of L-Ser and 99 μM for the K87T-AA, which is in accordance with the reported value of 120 μM (Weischet & Kirschner, 1976). It is interesting to note that the K_d for the $\alpha_2\beta_2$−G3P complex obtained from the ratio of k_7/k_8 is 0.44 mM, which is similar to the value of 0.43 mM reported by Brzović *et al.* (1992) in the presence of 10 mM Trp.

The enhancement in the rate of cleavage of IGP by K87T-AA can be attributed to changes in k_3 and k_4, and the rate constants for release of products. It is interesting to note that the conformational changes induced by the aminoacrylate formation at βK87T cause a large increase in the rate constant for the indole rebinding step (k_6) and a significant decrease in the binding of G3P (k_8). In other words, the aminoacrylate formation in the β subunit stabilizes a conformation in the α subunit, which possesses (i) a higher affinity for the newly formed indole and thus increases its probability to interact with L-serine to form tryptophan and (ii) a lower affinity for the product, G3P, which favors release of G3P from the enzyme. Together these changes, according to eqs 5−6, lead to a decrease in the value of K_m and an increase in k_c. The values of K_m and k_c obtained with the rapid kinetic method, 41 μM for K_m and 0.98 s^{-1} for k_c, are in good agreement with the corresponding values determined by the steady-state method. Similarly, addition of L-serine to the wild-type $\alpha_2\beta_2$ complex also results in changes in the IGP cleavage step, k_{11} and k_{12}, and the G3P release step, k_{15}. According to eqs 12 and 13, changes in these constants will lead to a decrease in K_m and an increase in k_c, which is confirmed by the steady-state kinetic data given in Table 2. The results of this analysis also show the migration of indole between α and β sites proceeds with high rate constants (k_{13} and k_{14}), which is in agreement with that proposed by Anderson *et al.* (1991). This efficient indole channeling pathway is impaired when one replaces a residue lining the tunnel Cys-170 of the β subunit with a bulkier tryptophan residue (Schlichting *et al.*, 1994). The quantitative difference between K87T-AA and the wild-type enzyme with saturating levels of L-serine is mainly derived from the ability of the latter system to further enhance the IGP cleavage step (compare k_3 and k_4 to k_{11} and k_{12}) and the G3P release step (compare k_7 and k_{15}) and the fact that K87T-AA fails to form L-tryptophan.

The occurrence of reciprocal communication between the α and β subunits has been reported by numerous investigators (Anderson *et al.*, 1991; Brzović *et al.*, 1992; Creighton, 1970; Kawasaki *et al.*, 1987; Kirschner *et al.*, 1991; Lane & Kirschner, 1983, 1991) and reviewed recently by Miles (1995). Briefly, the investigators showed that ligands, which bind to the α site, alter reaction kinetics at the β site, which is located ~30 Å away. Likewise, β-site ligands alter the reaction kinetics at the α site. These effects are attributed to ligand-induced conformational changes, which strongly affect the rates of substrate binding and product release. Dunn and co-workers have used their results and those of others to propose a model depicting ligand- or intermediate-induced conformational changes in the $\alpha_2\beta_2$ complex that occur during the course of the $\alpha\beta$ reaction (Brzović *et al.*, 1992). Their model depicts the α and β subunits undergoing conformation changes during catalysis and indicates that each subunit exists in at least two conformational states. These ligand-dependent allosteric interactions, which occur between the heterologous subunits, serve to coordinate the catalytic events at the α and β sites to ensure efficient synthesis of L-tryptophan.

In conclusion, this work illustrates the use of a mutant blocked at a specific reaction step to stabilize a reaction intermediate and to probe the role of this intermediate in intersubunit communication. Our results give direct support to the postulated mechanism of allosteric activation by an aminoacrylate intermediate in the absence of turnover. However, the results do not reveal the effect of the aminoacrylate intermediate on the conformation of the β subunit in the $\alpha_2\beta_2$ complex. Recent crystallographic

analyses at ~2 Å resolution of the L-serine and L-tryptophan intermediates with the K87T $\alpha_2\beta_2$ complex show that the conformation of the β subunit differs from that in the unliganded wild-type $\alpha_2\beta_2$ complex (Miles *et al.*, in press). We hope that future crystallographic analysis of the aminoacrylate derivative of the K87T mutant enzyme will show how formation of this derivative alters the conformation of the β subunit and leads to activation of the α subunit.

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