Interactions between the Subunits of the Tryptophan Synthetase of *Escherichia coli*. Optical Properties of an Intermediate Bound to the $\alpha_2\beta_2$ Complex*

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ABSTRACT: Formation of a tightly associated $\alpha_2\beta_2$ complex of *Escherichia coli* tryptophan synthetase can be demonstrated by the appearance of a new absorption band centered at 468 mμ, provided the solution contains β-mercaptoethanol in addition to L-serine and pyridoxal phosphate. The colored complex which is formed ("the amber complex") has been characterized by its physical and enzymatic properties. The role of β-mercaptoethanol is puzzling. It cannot be replaced by other thiol compounds tried, and although needed to form the amber complex it is not needed for enzymatic activity or for a tight association of the α and β_2 subunits. Breakdown of the amber complex is accompanied by the formation of *S*-(2-hydroxyethyl)cysteine which appears to be an addition product of aminoacrylic acid and mercaptoethanol.

On combination with each other, the α and β_2 subunits of *Escherichia coli* tryptophan synthetase (Crawford and Yanofsky, 1958) become much more efficient catalysts. Each subunit by itself catalyzes a partial reaction, the rate of which is increased 30–100-fold on combination with the complementary subunit (Crawford and Yanofsky, 1958). The complex, the composition of which is $\alpha_2\beta_2$ when fully associated (Goldberg *et al.*, 1966), catalyzes the reaction

$$\text{InGP} + \text{Ser} \xrightarrow[\text{PLP}]{\alpha_2\beta_2} \text{Trp} + \text{PGA} \qquad (3)$$

which is believed to be the physiologically significant one.

The α and β_2 subunits can be separated from each other by the usual methods of protein fractionation and they sediment independently of each other at low concentrations with s_{20} values of 2.7 and 5.1 S, respectively, unless PLP and Ser are either added to the sucrose gradient or preincubated with the subunits; then at low enzyme concentrations a tightly associated $\alpha_2\beta_2$ complex is found which sediments in a zone with $s_{20} = 6.4$ S (Creighton and Yanofsky, 1966). Little is known about the mechanism by which PLP and Ser effect this tight association of α and β_2 subunits.

To aid in studying this problem we looked for an optical property of either the α or β_2 subunit which would change significantly on formation of the $\alpha_2\beta_2$ complex. Such a property could be used to measure the kinetics and equilibria of complex formation in the presence of substrates, products, and analogs. Corresponding studies of the different enzymatic activities of the complex might show whether tight association and the related changes in catalytic properties are connected with the mechanism of enzymatic action of tryptophan synthetase.

We report here the observation of an optical property (a strong absorption band centered at 468 mμ) which appears when the α and β_2 subunits are tightly associated in the presence of PLP, Ser, and β-mercaptoethanol. Formation of the $\alpha_2\beta_2$ complex in the presence of PLP and Ser is a necessary condition, but not a sufficient condition for the appearance of this band; β-mercaptoethanol, which is also needed, is not required either for the tight association or for the enzymatic activity of tryptophan synthetase. A brief study of this situation follows.

Experimental Procedures

Protein Sources. The α protein was kindly supplied to us by Dr. Charles Yanofsky. The β_2 protein was purified from extracts of the $A_2/F'A_2$ strain of *E. coli*, according to the method of Wilson and Crawford (1965).

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[1] Abbreviations used: tryptophan synthetase (TSase), L-serine hydrolase (adding indole) (EC 4.2.1.20); InGP, indole-3-glycerolphosphate; In, indole; PGA, 3-phosphoglyceraldehyde; PLP, pyridoxal phosphate; β-ME, β-mercaptoethanol.

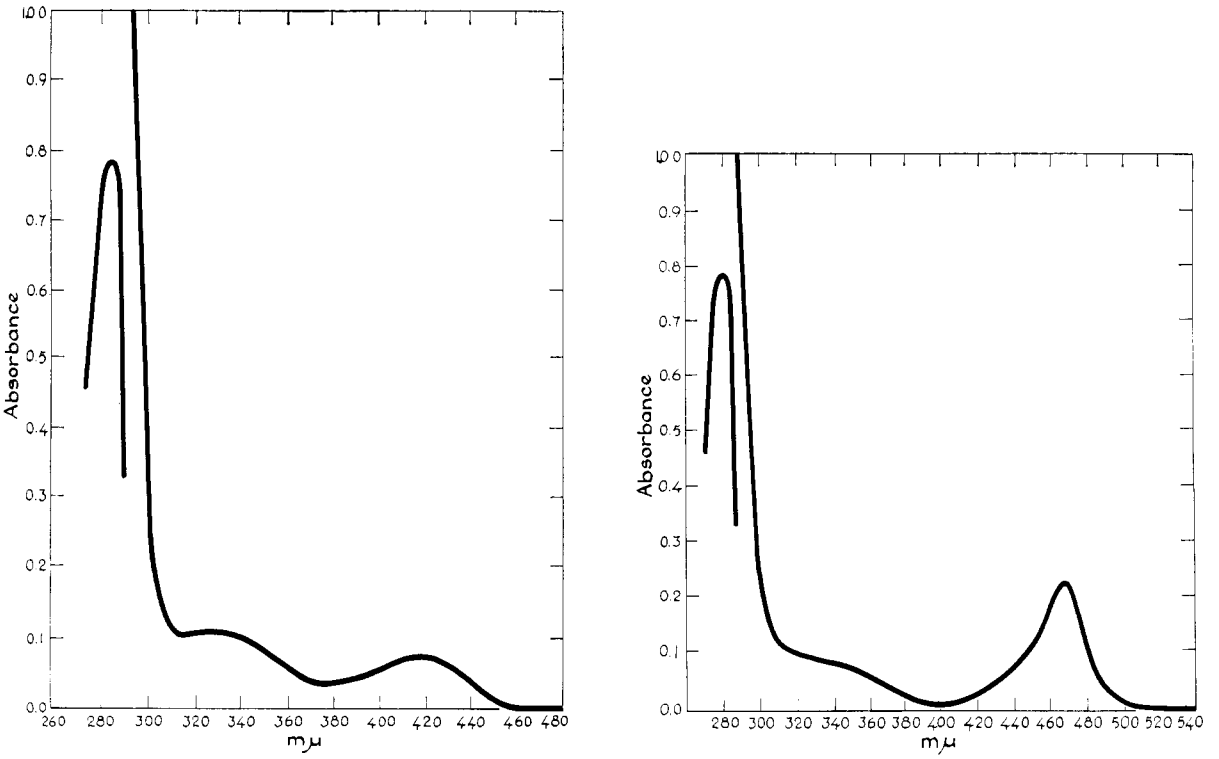


FIGURE 1: Absorption spectrum of the amber complex. Add 1 to the values of A for the inset peak. (a) (left) 2.6 mg of β_2 subunit plus 1.6 mg of α subunit in 1.2 ml of standard buffer. (b) (right) The same containing 0.01 M L-serine.

Enzyme Assays. They were done according to the methods of Smith and Yanofsky (1962).

Buffers. Standard buffer is 0.1 M potassium phosphate (pH 7.8)–0.01 M β-ME and contains 5 μg/ml of PLP. The L-serine concentration of standard buffer containing serine is 0.01 M.

Absorption Spectra. We used a Cary 14 spectrophotometer equipped with a slidewire which gave a full-scale deflection of the pen for an absorbance of 0.1 unit. Measurements were made at room temperature (25 ± 2°) unless otherwise specified.

Ultracentrifugation. A Spinco Model E ultracentrifuge was used. The schlieren optics were focused, following the procedure of LaBar and Baldwin (1962). The absorption optics were modified in the following way. The ultraviolet light source was replaced by a tungsten lamp fitted in a Teflon holder. The bromine filter was removed, and an interference filter with peak transmission at 468 mμ (Baird-Atomic B-1 standard) was fitted just above the light source. The absorbance of this filter at 468 mμ was 0.2. The band width was 4 mμ. The absorption optics were then focused according to the procedure of Schachman *et al.* (1962).

The speed was 59,780 rpm. The temperature was 25°. We used a 4°, 12-mm, Al-filled-epon centrifugation cell, with quartz windows. The schlieren photographs were analyzed with a Gaertner microcomparator, and the absorption photographs with a Joyce-Loebl microdensitometer.

Sucrose Gradient Centrifugations. We followed the Martin and Ames (1961) procedure, using a Spinco Model L centrifuge, with a SW-39 swinging-bucket head. The gradients were spun for 20 hr at 38,000 rpm. Each gradient was collected in approximately 40 fractions. The marker used was fluorescent-labeled bovine plasma albumin, prepared as described by Rinderknecht (1962).

Amino Acid Analysis. A Beckman–Spinco amino acid analyzer was used, modified with long-path cuvets and a slidewire which gives full-scale deflection for an absorbance of 0.1 unit; the volume layered on each column was 1 ml, containing approximately 30 mμ-moles of each amino acid.

Protein Concentrations. The concentration of the α subunit was measured spectrophotometrically, using the specific absorbancy given by Henning *et al.* (1962). To determine the concentration of β_2, we measured the concentration of a stock solution refractometrically as described in a previous paper (Goldberg *et al.*, 1966) and made accurate dilutions from this solution. A spectrophotometric method would be more convenient, and we tried unsuccessfully to develop one. The problem is that solutions of β_2 usually contain PLP, which contributes to the ultraviolet absorption, and the amount of PLP may not be known accurately. Moreover, it appears that there are two modes of binding PLP by β_2, which have different absorption spectra. This is discussed further on.

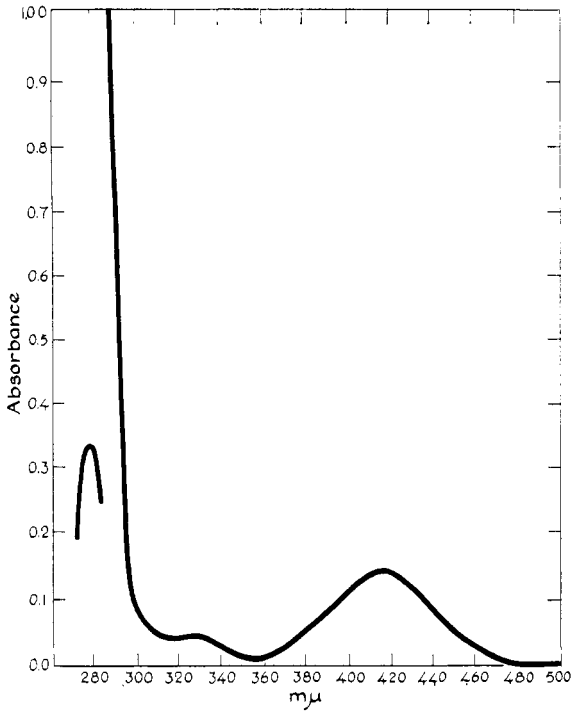


FIGURE 2: Absorption spectrum of a fresh preparation of β_2, in standard buffer. Add 1 to the values of A for the inset peak. Note the large ratio of the absorbancy at 415:330 mμ.

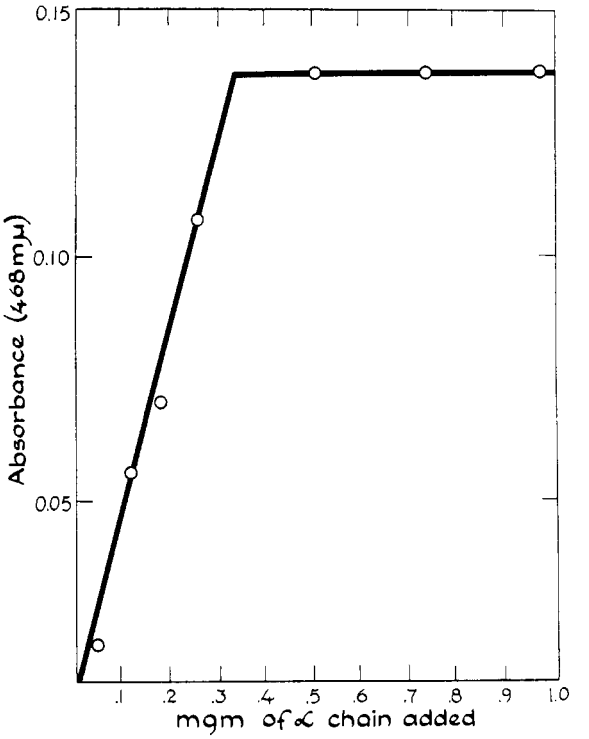


FIGURE 3: Requirement for the α subunit in forming the amber complex. A solution of the β_2 subunit (0.59 mg in 0.75 ml) in standard buffer containing serine is titrated by addition of α subunit in the same buffer.

Results

Formation of a Colored Complex. The absorption spectrum of a mixture of α and β_2 proteins, in the standard buffer (see Buffers), is shown in Figure 1a. The absorption bands at 330 and 415 mμ are characteristic of PLP bound to the enzyme, and are the same as those of PLP bound on the β_2 subunit alone in the same buffer (see below).

Figure 1b shows the spectrum of the same solution as in Figure 1a to which 0.01 M L-serine has been added. The same amount of serine has been added to the reference cuvet.

The comparison of these two spectra shows that the 415-mμ band has disappeared, and a new band at 468 mμ has appeared, whereas the band at 330 mμ remained unchanged. We shall call amber complex the complex which shows the absorption band at 468 mμ.

We were puzzled by the appearance of the two absorption bands at 330 and 415 mμ when PLP is bound to β_2 or $\alpha_2\beta_2$ in the absence of β-ME. Usually pyridoxal-containing enzymes show only one band. (However, two similar bands can be found in the tryptophanase spectra of the *E. coli* enzyme (Newton *et al.*, 1965) or the *Bacillus alvei* enzyme (Hoch and De Moss, 1966).) The following observations suggest that the two bands result from two modes of binding PLP to β_2, and that the 330 band may represent inactive β_2. (1) The ratio of $A_{330}:A_{415}$ is independent of pH between pH 5.8 and 8.8, indicating that the two bands do not correspond to binding by two different ionized forms of the enzyme, in rapid equilibrium with each other. (2) The ratio of $A_{415}:A_{330}$ is comparatively large in a fresh preparation of β_2 (see Figure 2), and decreases on aging as the enzymatic activity decreases. This interpretation of the 330 band would explain why the 415 band disappears, and the 330 band does not, on forming the amber complex. Inactive β_2 subunits may compete for α subunits but do not form the amber complex, and are not active in the indole–tryptophan reaction.

Compounds Required for the Appearance of the 468-mμ Band. The β_2 subunit, in the presence of PLP, serine, and β-ME does not give rise to the band. Neither does the α chain alone in the same conditions. This shows that both the α and β_2 subunits must be present.

Using the procedure described by Crawford and Ito (1964), we prepared the β_2 apoenzyme free of PLP and mixed it with an excess of α subunit, and the standard buffer containing serine. The band did not appear. Upon addition of PLP, it did appear, indicating that PLP is required. L-Serine is required and cannot be replaced by D-serine or by L-cysteine which is, nevertheless, known (Crawford and Ito, 1964) to be a substrate for the TSase.

For reasons which will become obvious later, we also were led to investigate the effect of β-ME, and it

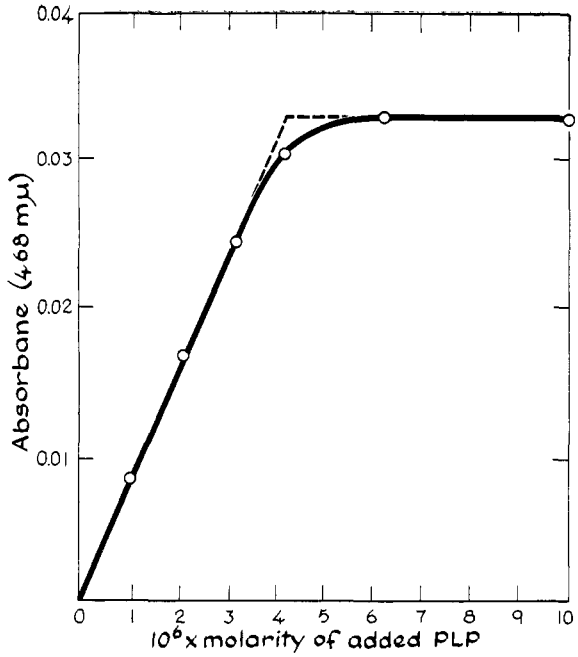


FIGURE 4: Requirement for PLP in forming the amber complex. A solution of β chain (3.9×10^{-6} M in β monomer) with excess α chain, in standard buffer without PLP but containing serine, is titrated against PLP and formation of the amber complex is measured at 468 mμ.

turned out that β-ME is required for the appearance of the band, and cannot be replaced by thioglycolic acid, mercaptoethanolamine, glutathione, or dithioerythritol. The acid and bases used as buffers did not interfere with the appearance of the band.

Stoichiometry of the Amber Complex Formation. Figure 3 shows a titration experiment in which aliquots of a solution of α chain (4.75 mg/ml) were added to 0.750 ml of a solution of β_2 subunit (0.79 mg/ml) in the standard buffer containing serine. For each amount of α chain added, the absorption at 468 mμ was recorded. The saturation was reached when 0.315 mg of α chain was added to 0.580 mg of β_2 subunit. The molecular weight of α is 29,500 (Henning *et al.*, 1962) and of β_2 is 99,500 (Goldberg *et al.*, 1966). Therefore, saturation is reached when 1 mole of α chain has been added to 0.92 mole of β chain.

Next the β_2 apoenzyme was mixed with an excess of α chain in the serine-containing standard buffer without PLP. The concentration of β chain was 3.9×10^{-6} M (calculated as monomer). Increasing amounts of PLP were added and the absorption at 468 mμ was recorded and plotted as a function of PLP concentration. The result is shown in Figure 4; saturation is reached when 1.02 moles of PLP has been added/mole of β chain.

The concentration of serine required to give half of the maximum absorption at 468 mμ, or K', was determined by adding increasing amounts of L-serine to a mixture of α and β_2 subunits in the standard buffer, and measuring the absorbance at 468 mμ. K' was found to be 3×10^{-4} M. It has been shown (see below) that this value is in the same range as the minimum concentration of L-serine required to give full association of α and β_2 as determined by sucrose gradients. In the same way the dependence of the absorbance at 468 mμ on the concentration of β-ME was determined in the standard buffer containing serine; K' for β-ME was found to be 1.3×10^{-2} M.

The dependence of the intensity of the band on pH and ionic strength was studied in the following way. In each experiment, the same volume of different potassium phosphate buffers was added to the $\alpha + \beta_2$ solution so that the final pH and ionic strength of the solution would have the desired values. The PLP concentration was 5 μg/ml and the L-serine concentration was 10^{-3} M. The results are shown on Figure 5a,b. The pH dependence of the intensity of the band at 468 mμ is similar to that of the enzymatic activity of the $\alpha_2\beta_2$ complex in the indole–tryptophan reaction as long as the activity and absorbance measurements are made at the same concentration of L-serine.

Characterization of the Amber Complex. SEDIMENTATION COEFFICIENT. Next we investigated whether the compound responsible for the absorption band is bound to the enzyme. For this purpose, we performed a sedimentation velocity experiment using two different optical systems simultaneously: the schlieren optics, which allowed us to measure the sedimentation coefficient of the protein, and the absorption optics for measuring light transmitted at 468 mμ (as described in Materials and Methods).

The centrifugation was performed in standard buffer containing serine. The protein concentration was approximately 4 mg/ml. The measured sedimentation coefficients were 6.75 S for the protein and 6.78 S for the compound absorbing light at 468 mμ. No attempt was made to extrapolate these values to infinite dilution of protein, but the $s_{20,w}$ value found of 6.35 S (after correction for viscosity, density, and temperature) is in good agreement with the 6.4–S value reported by Creighton and Yanofsky (1966) for the $\alpha_2\beta_2$ complex. We, therefore, concluded that the chromophore responsible for the band at 468 mμ is physically associated with the $\alpha_2\beta_2$ complex.

TIGHT ASSOCIATION. In order to find whether the species absorbing at 468 mμ is a tightly associated complex (*i.e.*, has an $s_{20,w}$ of 6.4 S even at the low enzyme concentrations used in sucrose gradients), we performed three sucrose gradient experiments in the same manner as Creighton and Yanofsky (1966), but in the presence of 0.01 M β-ME (buffer 0.05 M Tris-citrate (pH 7.8) containing 5μg/ml of PLP). The concentrations of L-serine were 5×10^{-5}, 5×10^{-4}, and 10^{-3} M, respectively. When a mixture of α and β_2 subunits was layered on those gradients the sedimentation coefficient of the β_2 subunit was 5.4, 6.35, and 6.4 S, respectively. The minimal L-serine concentration required for $\alpha_2\beta_2$ association is in the same range as that found by Creighton and Yanofsky (1966)

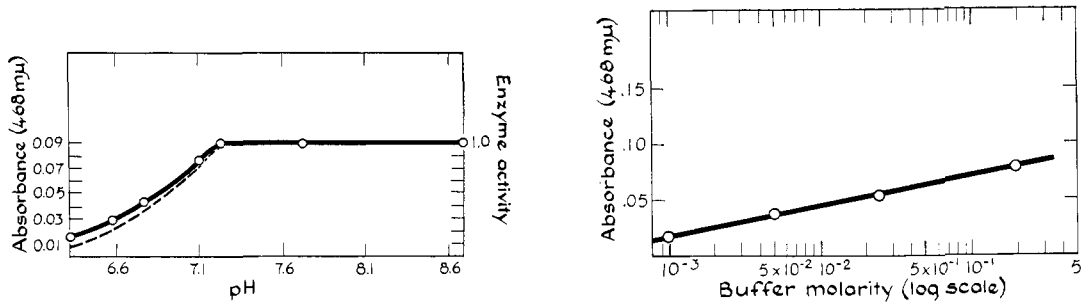


FIGURE 5: Dependence of the 468 band on pH and salt concentration. In both cases the solution contains 0.5 mg/ml of β chain plus excess α chain, in a potassium phosphate buffer containing 0.01 M β-ME, 0.001 M L-serine, and 5 μg/ml of PLP. (a) (left) pH dependence: the phosphate buffer is 0.2 M. The solid line gives the absorbance at 468 mμ and the dotted line gives enzymatic activity in the indole–tryptophan reaction, in arbitrary units. (b) (right) Salt dependence: the phosphate buffer is pH 7.3.

in the absence of β-ME, and also agrees with the K' found for serine in forming the amber complex, in somewhat different conditions. We conclude that the species absorbing at 468 mμ is indeed a tightly associated complex.

ENZYMATIC ACTIVITIES. A solution of β_2 subunit, saturated with α chain, was dialyzed against 0.05 M Tris-chloride (pH 7.8) and 5 μg/ml of PLP. The activity of this solution was measured both in the presence and in the absence of 0.01 M β-ME. No change in activity in the indole–tryptophan reaction was observed upon addition of β-ME to the reaction mixture.

The effect of β-ME on the serine deaminase activity of the $\alpha_2\beta_2$ complex was also investigated. With or without β-ME, the α chain inhibits the serine deaminase activity of the β_2 subunit. We conclude that, with regard to enzymatic activities, the $\alpha_2\beta_2$–PLP_2 complex and the amber complex behave identically.

Location of the Site on the Enzyme Responsible for the 468-mμ Band. The question can be asked whether the formation of the band at 468 mμ is a property of a new site created by the association of $\alpha_2\beta_2$, or was potentially inherent to β_2. By itself the β_2 subunit strongly binds PLP in a specific manner while the α subunit does not; therefore, if the binding site preexists on one subunit, it must be on the β_2 subunit. In the presence of high concentrations of NH_4^+ ions (*i.e.*, 2–3 M) the β_2 subunit is reactivated to nearly 60% of its maximum activity when associated with α (Hatanaka *et al.*, 1962). In the presence of these high concentrations of NH_4^+ ions (and with 0.01 M L-serine, 0.01 M β-ME, and 5 μg/ml of PLP) the β_2 subunit alone gave rise again to a band at 468 mμ; this suggests that the site for this band is inherent to β_2, but must be modified in a way strikingly parallel to the site for enzymatic activity, either by α or by ammonium ions, to be fully efficient. (Precise measurements of the spectrum have not been made because of turbidity. The β_2 subunit is at the point of incipient precipitation in these concentrated NH_4^+ solutions.)

Behavior as a Possible Intermediate in Tryptophan Synthesis. In view of the strong parallel between the presence of the band at 468 mμ and the specific activity of the β_2 subunit, it was asked whether the enzyme–substrate complex responsible for this absorption is an intermediate in the reaction of tryptophan synthesis. However, the observation that β-ME is required for the appearance of the band without being at all required for the enzymatic activity makes it difficult to answer this question clearly. Nevertheless, the following experiments suggest that we are indeed dealing with an intermediate.

(1) The absorption spectrum of a solution containing the $\alpha_2\beta_2$ complex acting on 0.1 M L-serine and 0.01 M indole without β-ME was recorded fast enough (within 1.5 min), that the concentrations of indole remains above 10^{-3} M during measurement of the spectrum, which is shown in Figure 6. It can be seen that, in

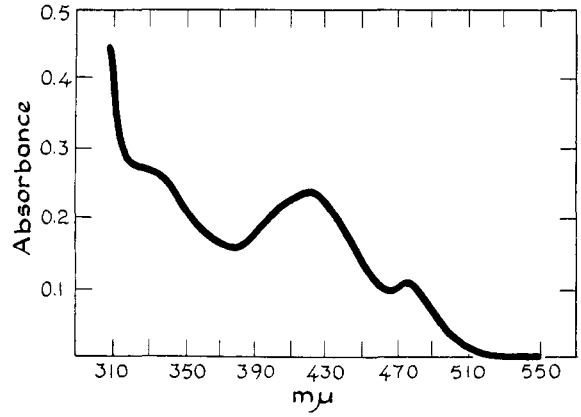


FIGURE 6: Absorption spectrum of the $\alpha_2\beta_2$ complex during the indole–tryptophan reaction. The solution contains 4.35 mg/ml of β chain plus excess α chain in 0.05 M Tris-chloride buffer (pH 7.8), containing 5 mg/ml of PLP and initially 0.1 M L-serine plus 0.01 M indole. This spectrum was recorded within 1.5 min after mixing enzyme and substrates.

addition to the broad absorption band at 415 mμ (PLP in a Schiff base with the enzyme or serine) there is a sharp shoulder at 472 mμ, which one can tentatively interpret as being caused by the same intermediate as with β-ME (and no indole) in a slightly different environment.

(2) When the absorbance of the amber complex is observed in standard buffer containing 10^{-3} M L-serine, the band at 468 mμ disappears with time. After the disappearance of the absorption at 468 mμ, addition of more serine regenerates the band at 468 mμ. In an attempt to learn the fate of the serine, a solution was prepared in the usual way leading to appearance of the band. The absorption was allowed to decay completely, and the solution was then chromatographed on the amino acid analyzer. Only a trace of serine remained and a new amino acid, which was eluted 5 ml before serine on a 55-cm column, was present in the stoichiometric amount predicted from the loss of serine, as calculated by assuming the color constant of the new compound to be the same as that of serine. The fact that β-ME is required to form this amino acid suggested that it might be *S*-(2-hydroxyethyl)-cysteine. The latter amino acid was synthesized chemically, following the procedure of Verderame (1961), and found to migrate on the amino acid analyzer at exactly the same place as the product of the action of tryptophan synthetase on L-serine and β-ME.

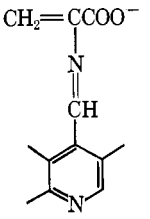
This result suggests that the amber complex is on the pathway to *S*-(2-hydroxyethyl)cysteine and, therefore, is an active complex. Under the same conditions as above, but replacing β-ME by hydrogen sulfide, the $\alpha_2\beta_2$ complex is able to perform the synthesis of L-cysteine. A solution, saturated with H_2S, containing the standard buffer without β-ME, with 10^{-3} M L-serine and 0.7×10^{-5} M $\alpha_2\beta_2$ protein was incubated at 37° for 30 min and an aliquot was analyzed on the amino acid analyzer. In addition to L-serine (the major component) L-cysteine was identified, which was present neither in the solution before incubation nor in a mixture, prepared in the same conditions, but containing 0.7×10^{-5} M β_2 instead of $\alpha_2\beta_2$. We conclude that the $\alpha_2\beta_2$ complex is able to perform the following reaction

$$\text{L-serine} + H_2S \longrightarrow \text{L-cysteine} + H_2O$$

Discussion

Common Properties of the Amber Complex and the Tightly Associated Complex. The amber complex shares most of the known properties of the tightly associated $\alpha_2\beta_2$ complex of Creighton and Yanofsky (1966). Both require PLP and L-serine, in addition to α and β_2 subunits, both have a sedimentation coefficient of $s_{20,w} = 6.4$ S, which corresponds to a subunit composition of $\alpha_2\beta_2$, both require approximately the same minimal concentration of L-serine, and both have the same enzymatic activities. The only differences which have been detected are the 468 absorption band itself, the requirement for β-ME in forming the amber complex, and the formation of a new compound (apparently *S*-(2-hydroxyethyl)cysteine) when the amber complex breaks down.

Probable Nature of the Amber Complex. It has been assumed (*cf.* Snell, 1958) that the mechanism of tryptophan synthesis involves first the formation, on the enzyme surface, of a Schiff base between PLP and L-serine, followed by dehydration to give a double bond between the α and β carbons of serine. The new Schiff base is that between PLP and α-aminoacrylic acid.



The new double bond, conjugated with the PLP resonance system through the Schiff base, can be expected to increase by approximately 50 mμ the wavelength of maximum absorption of the PLP system, raising it from 415 to the neighborhood of 465 mμ.

This suggests that the species absorbing at 468 mμ might be the Schiff base between PLP and α-aminoacrylic acid. A strong argument in favor of this hypothesis is that the formation of *S*-(2-hydroxyethyl)cysteine from serine and β-ME (or of cysteine from serine and H_2S) can hardly be explained by any mechanism which does not involve the intermediate described above. The addition of β-ME (or H_2S) across the $C_\alpha{=}C_\beta$ double bond yields *S*-(2-hydroxyethyl)cysteine (or cysteine); the addition of indole in the same way leads to tryptophan. Tatum and Shemin suggested this mechanism in 1954 using as a main argument the exchange of the proton on the α carbon during the transformation of L-serine into L-tryptophan by an extract of *Neurospora crassa*. If this explanation of the amber complex is correct, then the steady-state concentration of the α-aminoacrylic Schiff base with PLP is very much enhanced upon combination of α and β_2 subunits in the presence of β-ME. It seems however that the important step in the formation of a strong interaction between α and β_2 comes prior to the formation of the amber complex, because the tight association is also found in the absence of β-ME. Formation of the tightly associated complex in the absence of β-ME can be characterized by a different optical method, fluorescence (M. E. Goldberg and L. Stryer, in preparation).

Acknowledgments

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