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Kinetic Spectroscopic Studies of Substrate and Subunit Interactions of Tryptophan Synthetase†

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ABSTRACT: The catalytic properties of the β_2 protein of *Escherichia coli* tryptophan synthetase are markedly altered by the α protein or high concentrations of NH_4^+ ion. The effects of these interactions upon the reaction pathways for the deamination of L-serine and the synthesis of *S*-hydroxyethyl-L-cysteine were investigated by stopped-flow kinetic methods. This study was facilitated by the pyridoxal phosphate prosthetic groups of the β_2 protein, which interact with the substrates to form three spectrally distinct reaction intermediates: the aqua complex fluoresces at 500 nm, the pale species absorbs at 330 nm, and the amber complex absorbs at 470 nm. The results suggest that the aqua, pale, and amber species are sequential intermediates in both the deaminase and synthetase pathways. The α protein and NH_4^+ ion influence the rates of transformations of these species, and thereby alter the catalytic properties of the β_2 protein. Both β_2-catalyzed reactions are rate limited by the disappearance of the aqua species. This step is so greatly accelerated by the $\alpha_2\beta_2$ protein that it no longer limits the rate, and aqua fluorescence appears only transiently before the enzymatic steady state is established. However, the $\alpha_2\beta_2$ protein cannot deaminate L-serine because the pale species that forms with L-serine alone is unreactive. In contrast, the $\alpha_2\beta_2$-catalyzed synthesis of *S*-hydroxyethyl-L-cysteine is rate limited by the disappearance of the amber species. Each of the NH_4^+-β_2-catalyzed reactions is rate limited by two steps. The rate data for these reactions predict turnover numbers which agree with experimental values.

Tryptophan synthetase from *Escherichia coli* is a particularly interesting enzyme because it displays striking effects of subunit interaction upon catalytic activity and specificity (for a review, see Yanofsky and Crawford, 1972). The physiological reaction catalyzed by the fully associated $\alpha_2\beta_2$ enzyme complex (Crawford and Yanofsky, 1958) is the formation of L-tryptophan from indole-3-glycerol phosphate and L-serine (reaction 1, Table I). *In vitro*, the $\alpha_2\beta_2$ enzyme and its dissociated α and β_2 subunits catalyze a variety of reactions (Table I). The $\alpha_2\beta_2$ complex catalyzes reactions 2 and 3 more than tenfold faster than do the isolated subunits (Crawford and Yanofsky, 1958; Miles *et al.*, 1968). Furthermore, the $\alpha_2\beta_2$ complex is a more specific enzyme than the β_2 subunit. The deamination of L-serine (reaction 5) and the transamination of pyridoxal phosphate (reaction 6) by the β_2 subunit are completely inhibited on addition of a stoichiometric amount of α subunit (Crawford and Ito, 1964; Miles *et al.*, 1968).

The elucidation of these subunit interactions is dependent on a detailed knowledge of the reaction pathway. The study of catalytic intermediates is facilitated by the presence of pyridoxal phosphate, which serves as a cofactor in reactions 1 and 3–5 , and as a substrate in reaction 6. The absorption and fluorescence properties of this coenzyme are responsive to substrate and subunit interactions. Three potential intermediates in these enzymatic reactions have been identified in steady-state spectral studies of the pyridoxal phosphate group.

(1) The *aqua* species (Goldberg *et al.*, 1968), which exhibits a strong fluorescence emission at 500 nm, appears when L-serine is added to the β_2 protein. The absorption band of the aqua species is centered at 420 nm.

(2) The 330-nm absorbing species (Miles *et al.*, 1968), which we call the *pale* species, is formed on addition of L-serine to the $\alpha_2\beta_2$ complex.

(3) The *amber* species (Goldberg and Baldwin, 1967), which absorbs maximally at 470 nm, is formed when the $\alpha_2\beta_2$ complex catalyzes synthetase reactions.

Another interesting aspect of this enzyme is that high concentrations of NH_4^+ ion (of the order of 1 M) can substitute for the α subunit in the formation of the amber (Goldberg and Baldwin, 1967) and pale species (York, 1970). NH_4^+ ion also mimics the α subunit in enhancing the synthetase activities of the β_2 subunit (Hatanaka *et al.*, 1962) and in inhibiting the transamination of pyridoxal phosphate (Miles *et al.*, 1968).

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TABLE I: Reactions Catalyzed by Tryptophan Synthetase.

Reaction	Catalyzed by	Reference[a]
(1) Indole-3-glycerol phosphate + L-serine → L-tryptophan + D-glyceraldehyde 3-phosphate + H_2O	$\alpha_2\beta_2$	1
(2) Indole-3-glycerol phosphate ⇄ indole + D-glyceraldehyde 3-phosphate	α or $\alpha_2\beta_2$	1
(3) Indole + L-serine → L-tryptophan + H_2O	β_2 or $\alpha_2\beta_2$	1
(4) 2-Mercaptoethanol + L-serine → *S*-hydroxyethyl-L-cysteine + H_2O	β_2 or $\alpha_2\beta_2$	2
(5) L-Serine → pyruvate + NH_3	β_2	3
(6) Pyridoxal phosphate + L-serine + RSH ⇄ R-*S*-mercaptopyruvate + pyridoxamine phosphate + H_2O	β_2	4

[a] References: (1) Crawford and Yanofsky (1958), (2) Goldberg and Baldwin (1967), (3) Crawford and Ito (1964), (4) Miles *et al.* (1968).

We report here stopped-flow absorption and fluorescence studies of these spectral forms. Our kinetic data suggest that the aqua, pale, and amber species are sequential intermediates in both the deaminase and synthetase pathways. Both the α protein and NH_4^+ ion influence the rates of transformations of these species and thereby alter the catalytic properties of the β_2 protein. Faeder and Hammes (1970, 1971) have investigated the absorption changes occurring during some of these reactions. In these instances, our results are in essential agreement.

Experimental Section

Materials. The β_2 protein, which was purified from extracts of the A2/F′A2 strain of *E. coli* by the method of Wilson and Crawford (1965), was stored in the dark at −15° as an $(NH_4)_2SO_4$ precipitate. Prior to use, it was dialyzed against 0.1 M potassium phosphate buffer (pH 7.8), the standard buffer used throughout this study, to which 38 μM pyridoxal phosphate and 10 mM 2-mercaptoethanol were added. It was then passed quickly over a G-25 Sephadex column equilibrated with standard buffer to remove excess pyridoxal phosphate and 2-mercaptoethanol.

The α protein was kindly provided by Dr. C. Yanofsky. It was purified from the 5927R38 strain (Somerville and Yanofsky, 1965) of *E. coli* according to the method of Henning *et al.* (1962). These preparations were stored frozen at −15° and, prior to use, were also passed over a G-25 Sephadex column equilibrated with standard buffer.

Equivalent concentrations of the α and β_2 subunits were determined by titration of the β_2–Ser[1] complex with α protein, which suppresses the aqua fluorescence (Goldberg *et al.*, 1968). The $\alpha_2\beta_2$ enzyme was reassociated by incubating the β_2 subunit with 1.2 equiv of the α subunit for 15 min at 37° in the reaction buffer (Creighton and Yanofsky, 1966). These conditions were sufficient to completely complex the β_2 subunit.

Protein concentrations were measured by the method of Lowry *et al.* (1951). Since bovine plasma albumin (Armour) was used as standard, the absorbance of the β_2 protein in this assay was multiplied by 0.91 to reduce it to an equivalent weight of albumin (Hathaway *et al.*, 1969). Specific absorbances were then determined: β_2, $\epsilon_{278}^{1\%}$ 6.04; α, $\epsilon_{278}^{1\%}$ 3.96. The concentration of indole was determined from its absorbance at 278 nm in 0.05 N HCl, using ϵ 5.62 × 10^3 M^{-1} cm^{-1} (Andrisano and Vitali, 1957).

The specific activity of each protein in catalyzing reaction 3 was determined in the presence of a threefold excess of the other subunit by the method of Smith and Yanofsky (1962). The specific activities of the β_2 and α proteins used in this study were 1800–2300 and 3000 units/mg, respectively, which are to be compared with previously reported maximum specific activities of 2700 (Wilson and Crawford, 1965) and 5000 units/mg (Creighton and Yanofsky, 1966).

Commercial samples of L-serine (Schwartz), pyridoxal phosphate (Sigma), indole (Fisher), and 2-mercaptoethanol (Matheson, Coleman, and Bell) were used. All other materials were standard reagent grade.

Stopped-Flow Kinetic Studies. A stopped-flow instrument designed by Dr. J. Sturtevant was employed. The flow system and optics have been described in detail (Sturtevant, 1964). This instrument was equipped with two interchangeable optical cells: a 2-cm path length cell for absorption measurements and a 2-mm path length cell for either absorption or fluorescence measurements. The velocity of the pushing carriage was directly measured with a Hewlett-Packard linear transducer (6LV4-N). Flow velocities and dead times for the 2-mm path length cell were then calculated from the dimensions of the flow system. Dead times reported for the more intricate 2-cm path length cell were experimentally determined by following the pseudo-first-order reaction of 2,6-dichloroindophenol sodium salt with excess ascorbic acid at different pushing velocities. These values agreed quite well with those calculated from the dimensions of this cell. Dead times of 5 msec for the 2-mm and 8 msec for the 2-cm path length cells were typical. The chemical signal did not begin to change until 5.2 ± 0.3 msec after the recording was triggered by the cessation of flow. Therefore, the time at which the reactants were mixed is defined as 5.2 msec minus the dead time. The reaction trace was recorded on either a storage oscilliscope (Tektronix Type 564B storage oscilliscope with Type 3A6 dual-trace amplifier and Type 3B4 time base) or a digital analyzer (Hewlett-Packard analog-to-digital converter 5415A and digital processor 5421A). While both methods provided similar resolution of the amplitude of the signal, the time span was tenfold greater with the digital analyzer.

Most of the reactions conformed to first-order kinetics, and the digital analyzer data were fitted to exponential functions by the method of least squares. Two reactions were more complex, but each could be explained by two consecutive first-order processes. For one of these reactions (Figure 2), the rate constants were sufficiently different to permit their independent determination. For the other (Figure 3A), the two processes were observed together in one experiment.

[1] Enzyme–substrate complexes are represented using the following abbreviations: Ser, L-serine; ME, 2-mercaptoethanol. Although the enzyme bears two active sites and binds two molecules of each substrate, the binding of only one is noted for simplicity.

The rate constant for the slower reaction was determined from a least-squares fit of the data at late times to a single exponential function. The logarithm of the difference between the value extrapolated from the slower reaction and that actually observed at early times was plotted *vs.* time to determine the rate constant for the faster reaction. Each first-order rate constant reported is the average of 3–7 experiments. The average deviation is ±2–12% for $k < 100$ sec^{-1}, and ±24% for $k > 100$ sec^{-1}.

Reactions were initiated at 25 ± 0.5° by mixing equal volumes of two reactant solutions prepared with the same reaction buffer. The concentrations reported correspond to those of the mixed solution. Aqua fluorescence was excited at 405 nm with a PEK 112 mercury lamp and the emission isolated either at 500 nm or with a CS3-71 filter which transmitted light of wavelengths above 460 nm. All absorbances have been reduced to a 1-cm path length.

Results

Reactions of the β_2 Protein. The aqua species is formed by addition of L-serine to the β_2 protein (Goldberg *et al.*, 1968). Under these conditions, the enzyme catalyzes the deamination of L-serine (reaction 5). The appearance of the fluorescence emission at 500 nm is first order with $k = 37$ sec^{-1} (Figure 1). Within the precision of the experiment, the rate constant for the appearance of fluorescence is independent of the concentration of L-serine from 0.005 to 0.20 M. Since the lowest concentration tested is still greater than the apparent dissociation constant for L-serine, 2.2×10^{-3} M (York, 1970), these results indicate that the formation of the aqua species is an intramolecular process. The same kinetics are also observed for the increase in absorption at 430 nm, suggesting that this spectral change also arises from the formation of the aqua species. Only half of the fluorescence or absorbance change can be accounted for by extrapolation of this reaction to the time of mixing. The other half of the spectral change apparently occurs during the instrument dead time.

Nanosecond fluorescence measurements showed that the aqua fluorescence has an excited state lifetime of 4.4 nsec (York, 1970). The fluorescence decay over two orders of magnitude of intensity could be completely described by this single lifetime, which suggests that the aqua fluorescence arises from a single enzyme intermediate.

The β_2 protein catalyzes a synthetase rather than a deaminase reaction when both L-serine and 2-mercaptoethanol are present (reaction 4). However, the addition of 0.043 M 2-mercaptoethanol to the β_2 protein prior to mixing with L-serine does not affect the rate or extent of formation of the aqua species.

Reactions of the $\alpha_2\beta_2$ Protein. Steady-state measurements showed that the enzymatically inert $\alpha_2\beta_2$–Ser complex is nonfluorescent, and that it has a lower absorbance at 420 nm and a higher one at 330 nm than the $\alpha_2\beta_2$ protein (Goldberg *et al.*, 1968; Miles *et al.*, 1968). However, our stopped-flow studies reveal that there is a transient increase in the fluorescence intensity at 500 nm when L-serine is mixed with the $\alpha_2\beta_2$ protein. Because the changes in fluorescence intensity at 500 nm are highly specific (Goldberg *et al.*, 1968), this transient form can be identified as the aqua species. It is formed within the instrument dead time and then with $k = 70$ sec^{-1} rapidly disappears.[2] The intensity of fluorescence that disappears in this reaction is similar to that which appears when L-serine reacts with the β_2 protein.

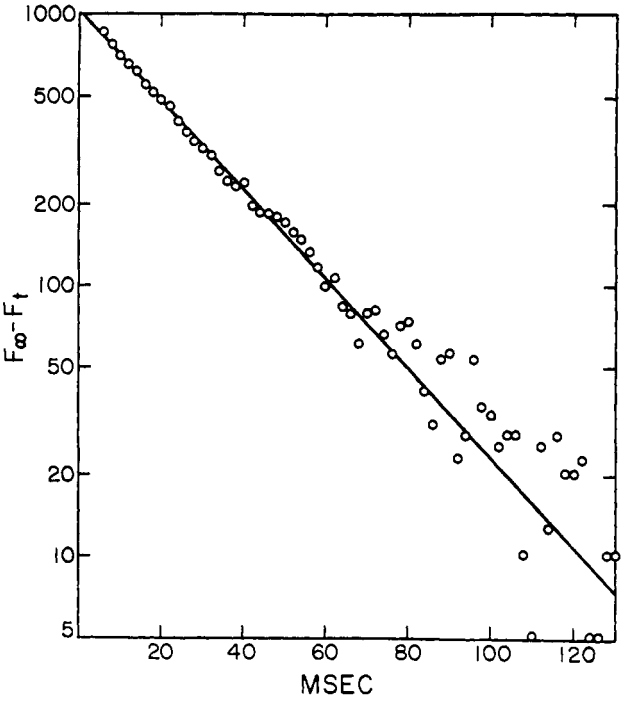

FIGURE 1: Reaction of β_2 protein with L-serine. The appearance of aqua fluorescence is plotted as a first-order reaction for 0.44 mg/ml of β_2 protein reacting with 0.05 M L-serine in standard buffer (pH 7.8). A least-squares fit of the data to the exponential function yields the straight line shown, with $k = 38$ sec^{-1}. The reactants were mixed at +1 msec. Fluorescence intensities (F) in this and other figures have been normalized to represent equivalent experimental conditions and concentrations of β_2 protein. The intensity of fluorescence appearing in this figure has been set to 1000. A similar increase in fluorescence intensity occurred within the instrument dead time.

The addition of 2-mercaptoethanol alters both the enzymatic and spectral properties of the $\alpha_2\beta_2$–Ser complex. The enzyme synthesizes *S*-hydroxyethyl-L-cysteine (reaction 4) and exhibits a strong absorption band at 470 nm, arising from the amber species. In addition, the absorption at 330 nm is lower than that for the $\alpha_2\beta_2$–Ser complex. Nevertheless, the addition of 0.043 M 2-mercaptoethanol to the $\alpha_2\beta_2$ protein prior to mixing with L-serine has virtually no effect on the transient appearance of the aqua species. The aqua fluorescence again appears within the instrument deadtime and disappears with $k = 68$ sec^{-1}. The increase in amber absorbance[3] observed in steady-state spectra occurs with $k = 37$ sec^{-1}.

The $\alpha_2\beta_2$–Ser–indole complex is nonfluorescent and displays an amber absorption band that is only 0.25 as intense as that of the $\alpha_2\beta_2$–Ser–ME complex. Stopped-flow kinetic studies reveal no transient aqua fluorescence when L-serine is added to a mixture of the $\alpha_2\beta_2$ protein and indole. The amber band

[2] Reaction conditions are 0.18 mg/ml of β_2 protein and 1.2 equiv of α protein reacting with 0.05 M L-serine in standard buffer containing 2.5×10^{-5} M pyridoxal phosphate (pH 7.8). If the order of mixing these reactants is altered by adding the β_2 protein to a mixture of the α protein and L-serine, then aqua fluorescence decays at 0.05 this rate as the α and β_2 proteins associate (York, 1970).

[3] Reaction conditions are 0.36 mg/ml of β_2 protein and 1.2 equiv of α protein reacting with 0.05 M L-serine in standard buffer containing 0.043 M 2-mercaptoethanol (pH 7.8).

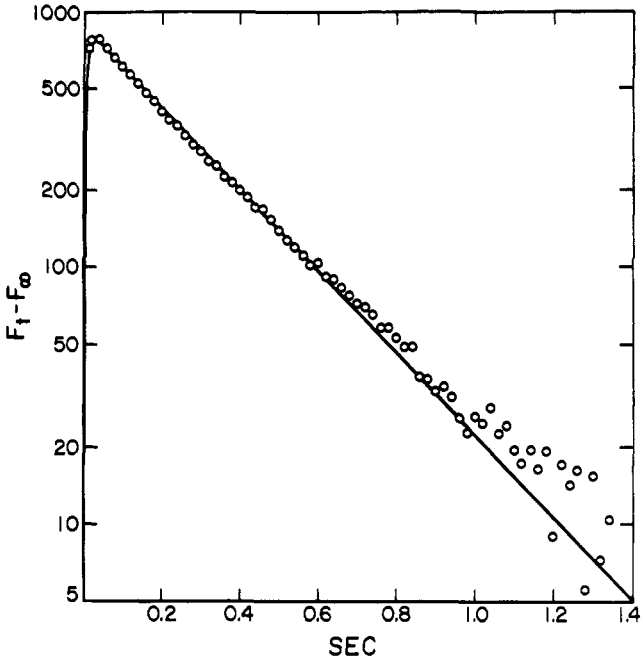

FIGURE 2: Reaction of NH_4^+-β_2 protein with L-serine. The disappearance of aqua fluorescence is plotted as a first-order reaction for 0.19 mg/ml of β_2 protein reacting with 0.05 M L-serine in standard buffer containing 1.5 M NH_4^+ ion (phosphate counterion) and 2.4×10^{-5} M pyridoxal phosphate (pH 7.8). The two earliest points reveal that a rapid increase precedes this decrease. The rate constant for this faster reaction, 220 sec^{-1}, was determined separately by recording the first 30 msec of reaction in detail (not shown). A least-squares fit of the data after 50 msec to the exponential function yields $k = 3.7$ sec^{-1} for the slower reaction. The solid line shown is that expected for these two consecutive first-order reactions.

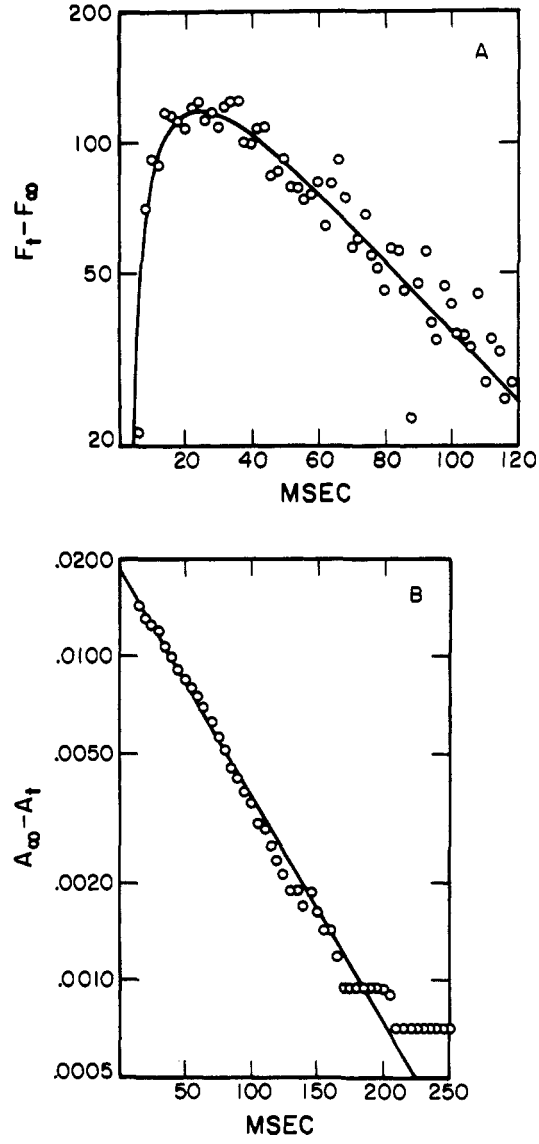

FIGURE 3: Reaction of a mixture of NH_4^+-β_2 protein and 2-mercaptoethanol with L-serine. (A) Aqua fluorescence appears and then disappears as 0.23 mg/ml of β_2 protein reacts with 0.05 M L-serine in standard buffer containing 1.5 M NH_4^+ ion (phosphate counterion), 0.05 M 2-mercaptoethanol, and 2.4×10^{-5} M pyridoxal phosphate (pH 7.8). The reactants were mixed at 0 msec. The data are described by two consecutive first-order reactions. A least-squares fit of the data after 50 msec to the exponential function yields $k = 18.4$ sec^{-1} for the slower reaction. The data prior to 50 msec yield $k = 96$ sec^{-1} for the faster reaction. The solid line shows the fit of the data obtained with these rate constants. (B) The appearance of 470-nm absorbance is plotted as a first-order reaction for 0.22 mg/ml of β_2 protein reacting with 0.05 M L-serine in standard buffer containing 1.5 M NH_4^+ ion (phosphate counterion) and 0.05 M 2-mercaptoethanol (pH 7.8). A least-squares fit of the data to the exponential function yields the straight line shown, with $k = 16.1$ sec^{-1}.

seen in the steady-state spectrum now appears[4] with a rate constant of 19 sec^{-1}.

Reactions of the NH_4^+-β_2 Protein. As mentioned previously, high concentrations of NH_4^+ ion mimic the α protein in stimulating the synthetase activity of the β_2 protein (Crawford and Ito, 1964). This effect is achieved in our studies with solutions containing 1.5 M NH_4^+ ion and phosphate as counterion, adjusted to pH 7.8. To emphasize the altered properties of the β_2 protein under these conditions, we employ the term NH_4^+-β_2 protein. Our steady-state spectroscopic studies (York, 1970) showed that the NH_4^+-β_2 protein has characteristics intermediate to those of the β_2 and $\alpha_2\beta_2$ proteins. NH_4^+ ion decreases the fluorescence of the β_2–Ser complex and promotes the appearance of some amber absorbance within the β_2–Ser–ME complex. Our stopped-flow kinetic studies show that the NH_4^+ ion also alters the reactivity of the β_2 protein, though not so strikingly as does the α protein.

When the NH_4^+-β_2 protein reacts with L-serine, aqua fluorescence rapidly appears and then partially disappears. The first-order rate constant for the appearance of the aqua complex is 220 sec^{-1}, which is faster than with the β_2 protein but slower than with the $\alpha_2\beta_2$ protein. This transient species has an extinction coefficient at 420 nm identical with that of the β_2–Ser aqua complex, further establishing their similarity. One-half of this aqua fluorescence then disappears with a rate constant of 3.6 sec^{-1}, as shown in Figure 2. Simultaneously with these changes in aqua fluorescence, the absorbance at 420 nm increases and then decreases by one-half, while the absorbance at 330 nm first decreases and then increases. It appears that half of the aqua complex initially formed is subsequently converted to the pale complex, which is nonfluorescent and virtually transparent at 420 nm. Thus, the steady state of the NH_4^+-β_2–Ser complex is composed of approximately equal amounts of the aqua and pale species (Table II).

[4] Reaction conditions are 0.18 mg/ml of β_2 protein and 1.2 equiv of α protein reacting with 0.05 M L-serine in standard buffer containing 0.013 M indole (pH 7.8).

TABLE II: Steady-State Proportions of Spectral Intermediates.

Complex	Reaction Catalyzed	Estimated Proportion of		
		Aqua	Pale	Amber
β_2–Ser	Deaminase	1.0	0	0
NH_4^+-β_2–Ser	Deaminase	0.5	0.5	0
$\alpha_2\beta_2$–Ser	None	0	1.0	0
NH_4^+-β_2–Ser–ME	Synthetase	0.85	0	0.15
$\alpha_2\beta_2$–Ser–ME	Synthetase	0	0	1.0

At steady state, the NH_4^+-β_2–Ser–ME complex is 71% as fluorescent as the β_2–Ser aqua complex, and it absorbs only 14% as intensely at 470 nm as does the $\alpha_2\beta_2$–Ser–ME amber complex. Figure 3A shows the change in fluorescence intensity when the NH_4^+-β_2 protein, together with 2-mercaptoethanol, reacts with L-serine. Equal amounts of aqua fluorescence appear and then disappear with rate constants of 125 and 17.7 sec^{-1}, respectively. The magnitude of this change amounts to only 15% of the peak fluorescence intensity. The rest forms within the instrument dead time and then remains at the end of the reaction. Amber absorbance appears as the aqua fluorescence disappears, with a rate constant of 15.8 sec^{-1} (Figure 3B). This accounts for the increase in 470-nm absorbance observed in steady-state spectra. Thus, the aqua species precedes the amber species in both the NH_4^+-β_2- and $\alpha_2\beta_2$-catalyzed syntheses of *S*-hydroxyethyl-L-cysteine.

Transition between Steady States. The steady states of both the NH_4^+-β_2–Ser and the NH_4^+-β_2–Ser–ME complexes are composed of two prominent species (Table II). Approximately equal amounts of aqua and pale species are found for the NH_4^+-β_2–Ser complex, while for the NH_4^+-β_2–Ser–ME complex, 85% is in the aqua form and 15% is in the amber form. The transition between these steady states was investigated by adding 2-mercaptoethanol to the NH_4^+-β_2–Ser complex. During this transition, amber absorbance increases tenfold within the instrument dead time and then decreases with k = 11.8 sec^{-1} (Figure 4A) to the final steady-state level, which is one-third of the transient peak level. The aqua fluorescence also changes, as shown in Figure 4B. The increase of aqua fluorescence is coincident with the decrease in amber absorbance, since k = 13.2 sec^{-1}. Extrapolation to the time of mixing reveals that the aqua fluorescence remains stable during the increase in amber absorbance. The peak absorbance at 470 nm indicates that half of the enzyme is transiently converted to the amber form. Since the concentration of the aqua species remains constant during this phase of the reaction, it seems likely that 2-mercaptoethanol rapidly converts the pale species present in the NH_4^+-β_2–Ser steady state to the amber species.

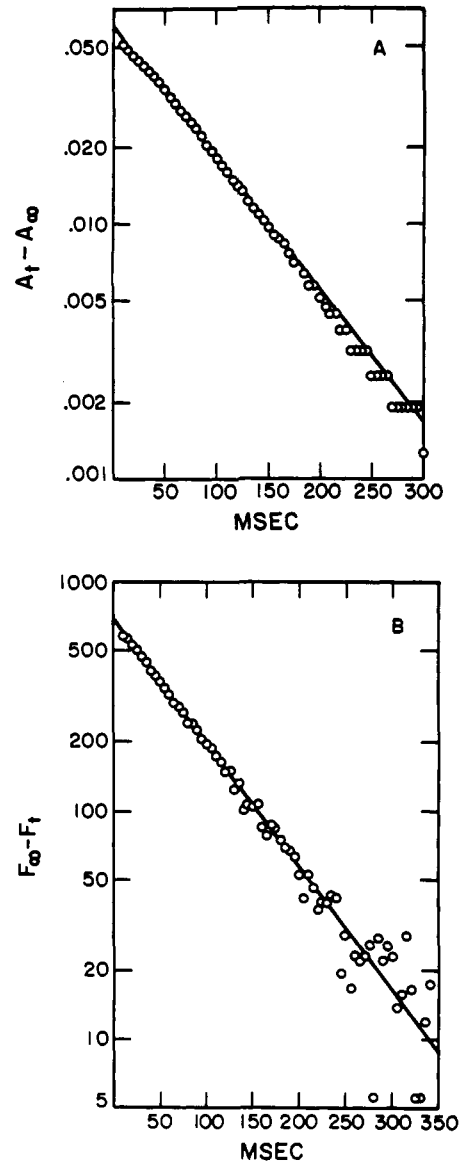

FIGURE 4: Reaction of NH_4^+-β_2–Ser with 2-mercaptoethanol. (A) The disappearance of 470-nm absorbance is plotted as a first-order reaction for 0.22 mg/ml of β_2 protein reacting with 0.05 M 2-mercaptoethanol in standard buffer containing 1.5 M NH_4^+ ion (phosphate counterion) and 0.05 M L-serine (pH 7.8). A least-squares fit of the data to the exponential function yields the straight line shown, with k = 12.0 sec^{-1}. (B) The appearance of aqua fluorescence is plotted as a first-order reaction for 0.23 mg/ml of β_2 protein reacting with 0.05 M 2-mercaptoethanol in standard buffer containing 1.5 M NH_4^+ ion (phosphate counterion), 0.05 M L-serine, and 2.4 × 10^{-5} M pyridoxal phosphate (pH 7.8). A least-squares fit of the data to the exponential function yields the straight line shown, with k = 12.5 sec^{-1}.

Discussion

The aqua, pale, and amber species exhibit several kinetic properties characteristic of enzymatic intermediates. First, they appear more rapidly than the enzymatic products. In its fastest reaction, the $\alpha_2\beta_2$ protein catalyzes the synthesis of L-tryptophan from indole and L-serine with a turnover number of 12 sec^{-1} per active site (Creighton and Yanofsky, 1966), and all three species appear at least this fast. Second, they appear sequentially in an order consistent with the enzymatic mechanism proposed by Miles *et al.* (1968). Third, rate data for these intermediates predict turnover numbers which agree with experimental values (Miles *et al.*, 1968). In these respects, our results corroborate the inferences drawn from steady-state spectroscopic studies by Goldberg and Baldwin (1967), Miles *et al.* (1968), and Goldberg *et al.* (1968), that the aqua, pale, and amber species are indeed intermediates in reactions catalyzed by tryptophan synthetase.

The aqua species, which is the first intermediate observed in all of the enzymatic reactions studied, is characterized by a strong fluorescence at 500 nm with a quantum yield of about

0.15 (Goldberg *et al.*, 1968), an excitation maximum near 420 nm, and an excited state lifetime of 4.4 nsec. Its formation during reaction of the β_2 protein with L-serine is conveniently observed by the stopped-flow method. The reaction rate constant (37 sec^{-1}) is independent of L-serine concentration over a wide range, indicating that this reaction occurs within a β_2–Ser complex. Extrapolation of this reaction to the time of mixing, however, reveals that only one-half of the total increase in aqua fluorescence and 430-nm absorbance occurs at this rate. The other half appears within the instrument dead time. Because fluorescence and absorbance appear in parallel amounts, and because a high degree of specificity is involved in the formation of aqua fluorescence (Goldberg *et al.*, 1968), we favor the interpretation that a unique aqua species is appearing in two distinct stages. This might result from a very rapid establishment of equilibrium between aqua species and enzyme-bound L-serine, followed by a relatively slow isomerization which shifts this equilibrium toward more aqua species.

Faeder and Hammes (1970) also have investigated this reaction, using stopped-flow–temperature-jump as well as stopped-flow techniques to monitor changes in absorbance. They detected two relaxation processes which were both attributed to intramolecular reactions within a β_2–Ser complex. For the slower relaxation process, during which 420-nm absorbance increased, they found $\tau^{-1} = 40$ sec^{-1} at saturating concentrations of L-serine, in excellent agreement with our results. However, the rapid reaction which they detected led to a decrease in 420-nm absorbance, while we infer that a net increase occurs within our instrument dead time. Presumably this initial appearance of aqua species is not accessible even to stopped-flow–temperature-jump techniques.

The aqua species is the only intermediate observed during both synthetase and deaminase reactions catalyzed by the β_2 protein. This would result if both of these reactions were rate limited by aqua disappearance. The presence of α protein or NH_4^+ ion sufficiently increases the rate of this reaction to permit the observation of intermediates following the aqua species.

When either the $\alpha_2\beta_2$ or NH_4^+-β_2 enzymes react with L-serine alone, the aqua fluorescence first increases and then decreases. During the NH_4^+-β_2-catalyzed reaction, the appearance of the pale intermediate is coincident with the disappearance of the aqua intermediate. Although the rate at which the pale species appears in the $\alpha_2\beta_2$-catalyzed reaction was not determined, the aqua species does totally disappear to produce a steady state containing only the pale species. While the spectral properties of the pale species formed in these two reactions are quite similar, these species differ in one striking aspect: the $\alpha_2\beta_2$–Ser complex is enzymatically inert, whereas the NH_4^+-β_2–Ser complex actively undergoes deamination (Crawford and Ito, 1964). The reason for this difference, which makes the $\alpha_2\beta_2$ enzyme a more specific catalyst than either the β_2 or NH_4^+-β_2 enzymes, remains unexplained.

Both the $\alpha_2\beta_2$ and NH_4^+-β_2 proteins behave quite differently during the synthesis of *S*-hydroxyethyl-L-cysteine. In the NH_4^+-β_2-catalyzed reaction, the appearance of the amber intermediate coincides with the disappearance of the aqua intermediate, while in the $\alpha_2\beta_2$-catalyzed reaction, the rate constant at which the amber intermediate appears is only one-half that at which the aqua intermediate disappears. Nevertheless, several observations indirectly suggest that the pale species participates in both of these reactions. (1) The aqua species disappears at the same rate whether the $\alpha_2\beta_2$ protein reacts with L-serine alone or with a mixture of L-serine and 2-mercaptoethanol, which suggests that these reactions share a rate-limiting step. (2) In the ensuing discussion, the rates of the NH_4^+-β_2-catalyzed conversions of aqua to pale species during deamination and aqua to amber species during *S*-hydroxyethyl-L-cysteine synthesis are shown to be identical, which also suggests a common rate-limiting step. (3) The rate-limiting step in common appears to be the formation of the pale intermediate, for when 2-mercaptoethanol is added to the NH_4^+-β_2–Ser complex, the pale species becomes very reactive and is converted to the amber species within the instrument dead time.

The simplest kinetic scheme for tryptophan synthetase-catalyzed reactions, consistent with all of our observations, is presented in Figure 5. The enzyme, whether β_2, $\alpha_2\beta_2$, or NH_4^+-β_2, interacts with its substrates to form an enzyme–substrate complex. Reactions within this complex then sequentially produce the aqua, pale, and amber intermediates. The disappearance of the amber intermediate coincides with the formation of product and the release of free enzyme to reinitiate the reaction. The prominence of any given intermediate in the steady state results from its accumulation before a rate-limiting step. Step 3 is rate limiting in β_2-catalyzed reactions. Steps 2 and 3 are greatly accelerated by the presence of α protein, and the aqua intermediate no longer accumulates. Nevertheless, the $\alpha_2\beta_2$ enzyme cannot deaminate L-serine because step 4 does not proceed. Addition of 2-mercaptoethanol removes this block and step 5 limits the rate of the $\alpha_2\beta_2$-catalyzed synthesis of *S*-hydroxyethyl-L-cysteine.

While these β_2- and $\alpha_2\beta_2$-catalyzed reactions are rate limited by a single step, the NH_4^+-β_2-catalyzed reactions appear to contain two rate-limiting steps because their steady states are each composed of two prominent species (Table II). This difference permits testing of the kinetic scheme shown in Figure 5 by observation of steady-state establishment from opposing directions, a method not possible for either the β_2 or $\alpha_2\beta_2$ proteins. In the reaction of a mixture of the NH_4^+-β_2 protein and 2-mercaptoethanol with L-serine, the concentration of the aqua species is transiently greater than its steady-state level. The concentration of the amber intermediate is transiently elevated during the reaction of the NH_4^+-β_2–Ser complex with 2-mercaptoethanol. In either reaction, the steady state is established with quite similar rate constants, 16.8 sec^{-1} and 12.5 sec^{-1}, respectively.

These two rate constants would be identical if steps 3 and 5 of Figure 5 were much slower than steps 1, 2, and 4. This kinetic scheme would then be formally equivalent to that of two equilibrating species, and the steady state would be established at identical rates starting with either aqua or amber intermediates in excess. The observed rate constant would be equal to the sum of k_3 and k_5, the rate constants for steps 3 and 5. The difference observed in the two rate constants suggests that this simple model is not entirely adequate. Furthermore, both k_3 and k_5 may each contain several rate and equilibrium constants, because the kinetic scheme in Figure 5 neglects reactions more rapid than our stopped-flow studies can detect.

Nevertheless, this model can be used to predict the enzymatic turnover numbers for the NH_4^+-β_2-catalyzed reactions. For the synthesis of *S*-hydroxyethyl-L-cysteine, k_3 = [k_{obsd} (14.6 sec^{-1})][amber fraction (0.15)] = 2.2 sec^{-1}, k_5 = [k_{obsd} (14.6 sec^{-1})][aqua fraction (0.85)] = 12.4 sec^{-1}, and the turnover number = k_3[aqua fraction] = 1.9 sec^{-1}. This calculated turnover number agrees favorably with the experimental val-

TABLE III: Rate Constants for Individual Steps in Reaction Pathway (Sec^{-1}).

Catalyst	k_2	k_3	k_4	k_5
A. Deamination of L-Serine				
β_2	37	[0.5][a]	*b*	*b*
$\alpha_2\beta_2$	>300[c]	70	0	
NH_4^+-β_2	220	3.6 (1.8)[d]	(1.8)[d]	*b*
B. Synthesis of *S*-Hydroxyethyl-L-cysteine				
β_2	37	[0.5][a]	*b*	*b*
$\alpha_2\beta_2$	>300[c]	68	37[e]	[1.7][a]
NH_4^+-β_2	125	17.7, 15.8[f] (2.2)[d]	>300[c]	11.8, 13.2[g] (12.4)[d]

[a] Rate constant in brackets is the turnover number for the reaction at 37° (Miles *et al.*, 1968), placed at the step thought to be rate limiting. [b] Not rate limiting. [c] Reaction occurs within the instrument dead time. [d] Rate constant in parentheses is calculated from the observed rate; see Discussion. [e] Observed rate constant for amber appearance. [f] Observed rate constants for aqua disappearance and amber appearance; assigned to step 3 because step 4 occurs within the instrument dead time. [g] Observed rate constants for amber disappearance and aqua appearance in the reaction of NH_4^+-β_2-Ser with 2-mercaptoethanol.

ues for the $\alpha_2\beta_2$-catalyzed (1.7 sec^{-1}) and β_2-catalyzed (0.5 sec^{-1}) reactions at 37° (Miles *et al.*, 1968). We conclude that the establishment of a true enzymatic steady state, involving product formation, is indeed being observed in our stopped-flow experiments. A similar calculation for the NH_4^+-β_2-catalyzed deamination of L-serine predicts $k_3 = k_4 = 1.8$ sec^{-1} and a turnover number of 0.9 sec^{-1}, similar to the known rate constant for pyruvate formation catalyzed by the β_2 protein at 37°, 0.5 sec^{-1} (Miles *et al.*, 1968).

How are the catalytic properties of the β_2 protein so markedly changed by the α protein and NH_4^+ ion? We have proposed that every reaction proceeds by the same sequence of intermediates (Figure 5). The α protein and NH_4^+ ion do not seem to alter this. Rather, they appear to alter the rates of the individual steps within this pathway. Table III summarizes these rates for the deamination of L-serine and the synthesis of *S*-hydroxyethyl-L-cysteine. The α protein increases the rate of step 2 by more than 8-fold, and of step 3 by about 150-fold. Since step 3 is rate limiting in the absence of α protein, this provides an explanation for how the α protein stimulates this synthetase reaction. The NH_4^+ ion has a lesser effect, increasing the rates of steps 2 and 3 by about fourfold each. The α protein and NH_4^+ ion also differ in their effects on step 4 when only L-serine is present. The NH_4^+-β_2 enzyme catalyzes this step whereas the $\alpha_2\beta_2$ enzyme does not. Yet it is quite possible that the rate of this step is reduced by NH_4^+ ion. Indole is thought to quench the fluorescence of the aqua complex, leaving it otherwise unaltered (York, 1970). Therefore, it is not surprising that no transient fluorescence is observed when a mixture of the $\alpha_2\beta_2$ protein and indole reacts with L-serine. Since amber formation is quite rapid (19 sec^{-1}), it appears that the α protein also accelerates steps 2 and 3 during the synthesis of L-tryptophan.

These results add a new perspective to the subunit interactions occurring between the α and β_2 proteins, and thereby

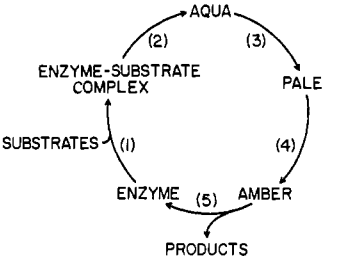

FIGURE 5: Reaction pathway for tryptophan synthetase catalyzed reactions. Step 3 limits the rate of β_2-catalyzed reactions. Whereas step 4 does not proceed in the reaction of $\alpha_2\beta_2$ protein with L-serine alone, step 5 is rate limiting in the $\alpha_2\beta_2$-catalyzed synthesis of *S*-hydroxyethyl-L-cysteine. The rates of the NH_4^+-β_2-catalyzed reactions are limited by two steps: deamination of L-serine by steps 3 and 4, and synthesis of *S*-hydroxyethyl-L-cysteine by steps 3 and 5.

complement the results of Faeder and Hammes (1970, 1971). We have reinterpreted the enzymatic roles of the aqua and amber species in light of these additional findings. Our direct observation of the aqua species reveals that the α protein greatly accelerates both the rate of its formation and the rate of its disappearance. Faeder and Hammes (1971) proposed that the presence of the α protein had little effect on the rate of aqua formation. They also inferred that in the reaction of the $\alpha_2\beta_2$ protein with L-serine and indole, the formation of the amber species must be quite rapid in comparison to the relaxation process observed. We find that the amber species forms with $k = 19$ sec^{-1}, since the increase in amber absorbance during this reaction is similar to that observed in steady-state spectra.

The kinetic scheme presented in Figure 5 is consistent with the enzymatic mechanism proposed by Miles *et al.* (1968). The first step in this mechanism is the formation of a protonated Schiff base between L-serine and pyridoxal phosphate. Such an intermediate is expected to absorb at 420 nm (Morino and Snell, 1967; Miles *et al.*, 1968) and presumably gives rise to aqua fluorescence. The α-hydrogen is then labilized to form an intermediate thought to be the amber species (Miles *et al.*, 1968), followed by β-elimination to form an acrylic acid intermediate. At this point the pathways of the various reactions catalyzed by tryptophan synthetase diverge. Addition of indole or 2-mercaptoethanol to the acrylic intermediate produces L-tryptophan or *S*-hydroxyethyl-L-cysteine, while hydrolysis of this intermediate produces pyruvate. Our kinetic scheme also places the aqua intermediate before the amber intermediate. However, the enzymatic mechanism of Miles *et al.* (1968) does not include the 330-nm absorbing pale species, which in our studies intervenes between the aqua and amber intermediates. This intermediate might correspond to an unprotonated Schiff base (Morino and Snell, 1967).

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