Allosteric Regulation of Tryptophan Synthase: Effects of pH, Temperature, and α-Subunit Ligands on the Equilibrium Distribution of Pyridoxal 5′-Phosphate−L-Serine Intermediates[†]

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ABSTRACT: The equilibrium distribution of catalytic intermediates formed in the reaction of L-serine with the tryptophan synthase $\alpha_2\beta_2$-complex from *Salmonella typhimurium* has been investigated by absorption and fluorescence spectroscopy as a function of pH, temperature, and α-subunit ligands. The novel result of this study is that the equilibrium between the two major catalytic species, the external aldimine and the α-aminoacrylate, is modulated by the ionization of two groups with apparent pK values of 7.8 ± 0.3 and 10.3 ± 0.2. Protonation of these groups stabilizes the α-aminoacrylate Schiff base by an estimated 100-fold with respect to the external aldimine. Furthermore, the formation of the α-aminoacrylate from the external aldimine is an endothermic process. Temperature slightly affects the apparent pK values but remarkably influences the amplitude of the phase associated with the ionization of each group. At 20 °C, each phase accounts for nearly half of the titration. Since the isolated β_2-dimer does not exhibit a pH-dependent distribution of intermediates, the α−β-subunit interactions seem critical to the onset of this functional property of the β-subunit. The modulation of intersubunit interactions by the α-subunit ligands DL-α-glycerol 3-phosphate and phosphate leads to significant changes in the pH-dependent distribution of intermediates. At saturating concentrations of either of these α-subunit ligands, the α-aminoacrylate Schiff base is the predominant species over a wide pH range while the apparent pK values of the groups that control the equilibrium are not significantly affected. The pH-dependent interconversion of catalytic intermediates here reported has not been previously detected because phosphate buffers have usually been employed in the studies of this enzyme. Our findings are discussed in the light of a model in which specific protein conformations are associated with the external aldimine and the α-aminoacrylate Schiff bases, the latter being stabilized by temperature, protons, and α-subunit ligands.

Tryptophan synthase from enterobacteria is an $\alpha_2\beta_2$-bienzyme complex (Yanofsky & Crawford, 1972; Miles, 1979, 1991, 1995). The α-subunits catalyze the cleavage of 3-indole-D-glycerol 3′-phosphate to indole and D-glyceraldehyde 3-phosphate (the α-reaction). Indole is then transferred via an intramolecular tunnel to the pyridoxal 5′-phosphate[1] (PLP)-dependent β-active site (Hyde et al., 1988; Dunn et al., 1987, 1990; Lane & Kirschner, 1991; Kirschner et al., 1991; Anderson et al., 1991; Brzovic et al., 1992a) and there condensed with L-serine (the β-reaction, Scheme 1). In the reaction of the enzyme with L-serine, at least four intermediates are formed: the *gem*-diamine, the external aldimine, the quinonoid, and the α-aminoacrylate (Drewe & Dunn, 1985, 1986).

Catalysis at the α- and β-active sites is modulated by reciprocal interactions within each αβ-protomer (Kawasaki et al., 1987; Houben & Dunn, 1990; Dunn et al., 1987, 1990, 1991, 1994; Lane & Kirschner, 1991; Kirschner et al., 1975, 1991; Anderson et al., 1991; Brzovic et al., 1992a−c, 1993; Leja et al., 1995; Ruvinov et al., 1995a). It has been proposed that these heterotropic interactions switch the α- and β-subunits between conformationally "open" (catalytically inactive) and "closed" (catalytically active) conformations in a coordinated sequence that phases catalytic events at the two sites and makes possible the efficient channeling of indole via the tunnel. The allosteric trigger which activates the α-subunit is the formation of the α-aminoacrylate Schiff base at the β-active site. The allosteric transition is reversed by conversion of the L-Trp quinonoid to the L-Trp external aldimine (Leja et al., 1995).

Luminescence measurements have revealed a change in the dynamic properties of the β-subunit upon binding of a ligand to the α-active site, but no changes at the α-active site upon formation of an external aldimine with L-histidine at the β-active site (Strambini et al., 1992a,b).

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[1] Abbreviations: GP, DL-α-glycerol 3-phosphate; MOPS, 3-(*N*-morpholino)propanesulfonic acid; bicine, *N*,*N*-bis(2-hydroxyethyl)-glycine; MES, 2-(*N*-morpholino)ethanesulfonic acid; MBP, MES−bicine−proline buffer system; PLP, pyridoxal 5′-phosphate; EDTA, ethylenediaminetetraacetic acid.

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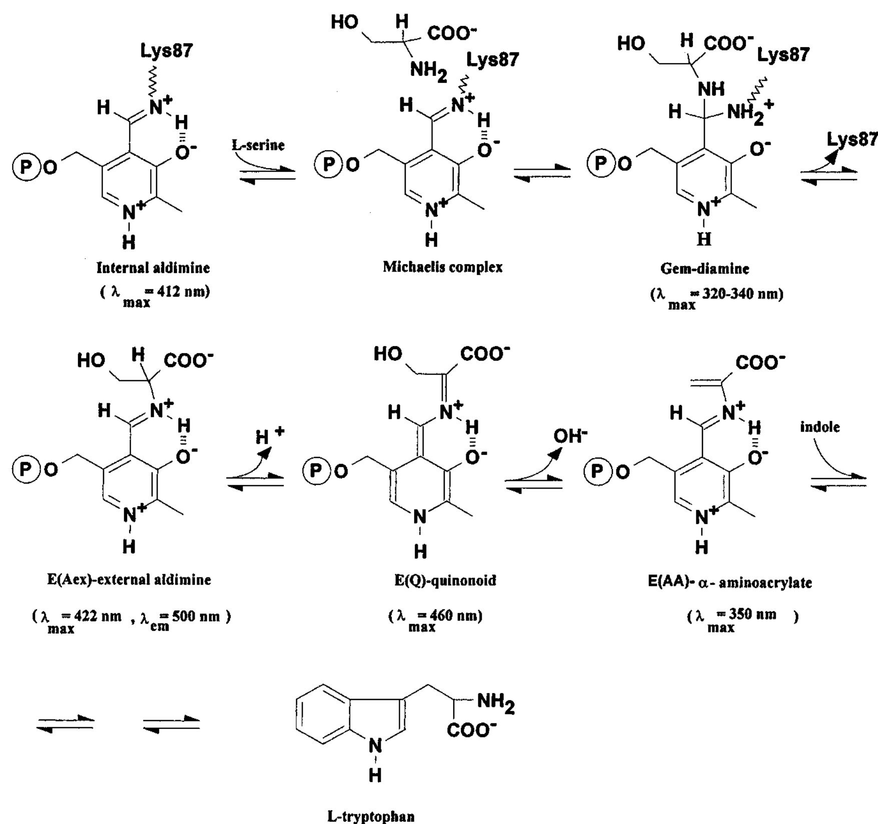
Scheme 1: Enzyme−Substrate Intermediates Formed in the Reaction of Tryptophan Synthase with L-Serine[a]



[a] In the presence of indole, the α-aminoacrylate reacts to form L-tryptophan. The maximum of the absorbance peak, assigned to each intermediate, is shown in parentheses (Miles, 1979, 1991). The α-aminoacrylate exhibits a broad shoulder of absorbance extending to 500 nm (Drewe & Dunn, 1985).

In a study of *Salmonella typhimurium* tryptophan synthase in the crystalline state (Mozzarelli et al., 1989), we observed that the two most abundant intermediates in the reaction between the β-active sites and L-serine are the external aldimine and the α-aminoacrylate Schiff bases and that their relative concentrations are dependent on pH and α-subunit ligands. We confirmed this finding in a preliminary investigation of the enzyme in solution (Mozzarelli et al., 1991). In the present work, we analyze the regulatory effects of pH, temperature, and α-subunit ligands on the basis of a simple model requiring the existence of distinct protein conformations for the two intermediates.

MATERIALS AND METHODS

The $\alpha_2\beta_2$-complex of tryptophan synthase from *S. typhimurium* was purified and assayed according to Ahmed et al. (1987). The enzyme was further purified by high-performance liquid chromatography (HPLC). About 100 mg of the $\alpha_2\beta_2$-complex was applied to a Mono Q HR 10/10 column (Pharmacia) in a Gilson high-pressure liquid chromatography system at 4 °C. The column was washed for 10 min at 2 mL/min with a solution containing 50 mM *N,N*-bis(2-hydroxyethyl)glycine (bicine) and 1 mM EDTA (pH 7.8). The $\alpha_2\beta_2$-complex eluted after about 10 min with a 40 mL linear gradient between the starting buffer and a buffer containing 0.5 M NaCl, at 2 mL/min (we thank Dr. C. C. Hyde for help in this procedure). Isolated α-subunits and β_2-dimers were prepared as previously described (Miles et al., 1987).

L-serine, DL-α-glycerol 3-phosphate (GP), bicine, and *N*-ethylmorpholine were purchased from Sigma. 2-(*N*-morpholino)ethanesulfonic acid (MES), 3-(*N*-morpholino)-propanesulfonic acid (MOPS), and proline were purchased from Fluka. Diethanolamine and EDTA were purchased from Merck. All reagents were of the best available quality and were used without further purification.

pH measurements were carried out with a pHM 83 Radiometer, equipped with a U402-M3 Ingold microelectrode, calibrated with three standard buffers at 20 °C. A three-buffer system was used, containing 50 mM MOPS, 50 mM bicine, 50 mM proline, and 1 mM EDTA (MBP buffer). The pH was raised with sodium hydroxide up to 11.2, and the solution was then back-titrated with HCl to the desired pH value. By this procedure, the ionic strength and the sodium concentration were kept nearly constant over the pH range 6−11. When the MBP buffer included 100 mM L-serine (as in most of the experiments here reported), both the ionic strength and the Na^+ concentration were 0.25 M.

For spectroscopic measurements, a concentrated solution of the enzyme (10−30 mg/mL) in 50 mM bicine and 1 mM EDTA (pH 7.8) was directly diluted in the appropriate buffer. The pH of the final solution was determined in the cuvette after the spectral recording. The fluorescence data were collected by a Perkin-Elmer model MPF3L spectrofluorometer. Absorbance data were collected by a Cary 219 spectrophotometer. The cuvette holders of both instruments were thermostatted, and the temperature was constantly monitored.

In control experiments, apparent half-saturation constants for L-serine were obtained from either absorption (Lane & Kirschner, 1983a) or fluorescence measurements (Goldberg et al., 1968; Kayastha et al., 1991) at various pH values in the absence or in the presence of different GP concentrations. These experiments indicate that 100 mM L-serine saturates the enzyme under all our experimental conditions.

Titration data were fitted to the appropriate equation by a nonlinear least squares procedure, available on SigmaPlot 5.1 software (Jandel Scientific).

RESULTS

pH Dependence of the Distribution of Intermediates Formed in the Reaction of the $\alpha_2\beta_2$-Complex with L-Serine. The absorption spectrum of the native $\alpha_2\beta_2$-complex of tryptophan synthase from *S. typhimurium* was recorded in the pH range 6.9−10.4 and found to be essentially independent of pH. Goldberg and Baldwin (1967) had obtained the same result measuring the spectrum of the *Escherichia coli* tryptophan synthase in the pH range 5.8−8.8.

After reaction of L-serine with pyridoxal 5′-phosphate bound at the β-active sites of tryptophan synthase from *S. typhimurium*, the spectrum of the resulting species is strongly pH-dependent. At 20 °C, the absorption spectra (Figure 1a) indicate that the α-aminoacrylate Schiff base is predominant at low pH, whereas the external aldimine is predominant at high pH.[2] The lack of perfect isosbestic points in spectra recorded at different pH values indicates the presence of minor intermediates, possibly including the *gem*-diamine absorbing at 320−330 nm (Roy et al., 1988).

The pH-dependent interconversion of the external aldimine and the α-aminoacrylate was measured by both absorption and fluorescence spectroscopy (Figure 1b). Although the absorption intensity at 422 nm results from several species (Drewe & Dunn, 1985), the contribution of the external aldimine largely predominates. The external aldimine can also be monitored by measuring the fluorescence at 500 nm (excitation at 420 nm) (Goldberg et al., 1968). Both absorbance and fluorescence changes appear to depend on the ionization of at least two groups, according to the equation

$$A = A_0 + \frac{K_1 \Delta A_1}{[\mathrm{H}^+] + K_1} + \frac{K_2 \Delta A_2}{[\mathrm{H}^+] + K_2} \qquad (1)$$

where A is the spectroscopic signal (absorbance or emission intensity), A_0 is the extrapolated signal at low pH, ΔA_1 and ΔA_2 are the changes in signal amplitude due to the ionization of the first and second groups, respectively, and K_1 and K_2 are the two apparent dissociation constants.

In the case of absorption data, the calculated values for pK_1 and pK_2 are 8.0 ± 0.1 and 10.4 ± 0.1, respectively, and the ratio $R = \Delta A_2/\Delta A_1$ is 0.56; for the fluorescence data, the apparent pK_1 and pK_2 are 7.6 ± 0.1 and 10.2 ± 0.1, respectively, and R is 0.67. The small differences between the two sets of data may reflect changes of the fluorescence quantum yield with pH, in analogy with results obtained on the β_2-dimer (see below).

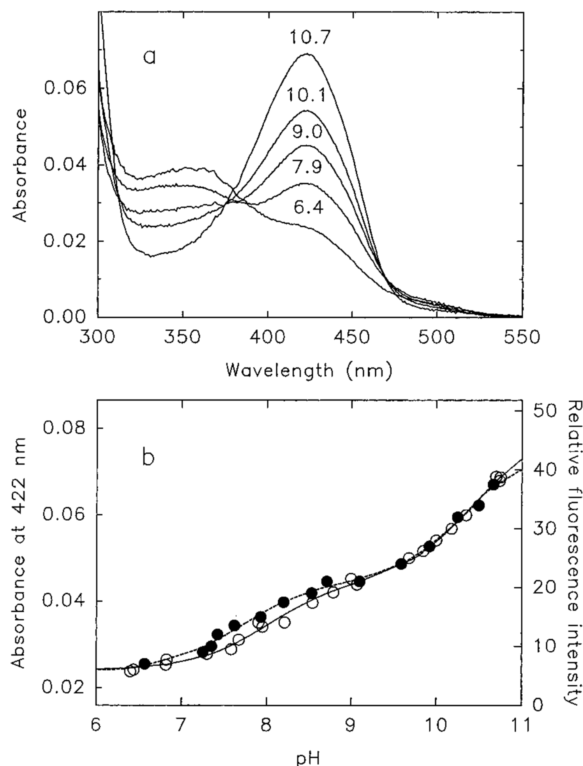


FIGURE 1: Effect of pH on the distribution of intermediates formed in the reaction of L-serine with the tryptophan synthase $\alpha_2\beta_2$-complex. (a) Absorption spectra of tryptophan synthase from *S. typhimurium* in the presence of L-serine at different pH values. The $\alpha_2\beta_2$-complex (0.54 mg/mL) was reacted with 100 mM L-serine in an MBP buffer system, at the pH values shown in the figure, at 20 °C. Each spectrum was recorded within 5 min of the beginning of the reaction to minimize the amount of pyruvate formed by serine deamination (Miles, 1991). (b) Effect of pH on the concentration of the external aldimine. The values of absorbance at 422 nm (○) were obtained from the spectra of the enzyme reacted with L-serine under the experimental conditions reported in part a. The solid line is the least squares fit of the data to eq 1. The calculated pK values are 8.0 ± 0.1 (pK_1) and 10.4 ± 0.1 (pK_2) with a ratio of amplitudes R = 0.56. The fluorescence at λ = 500 nm (λ_{ex} = 420 nm) (●) was measured for a solution containing the enzyme (0.054 mg/mL) and 100 mM L-serine in an MBP buffer system at 20 °C. The dotted line is the least squares fit of the data to eq 1. The calculated parameters are pK_1 = 7.6 ± 0.1 and pK_2 = 10.2 ± 0.1 with a ratio R = 0.67.

The assumption that the ionization of three rather than two residues controls the interconversion of external aldimine and α-aminoacrylate does not lead to a significantly better fitting of the experimental data (root mean square = 0.004 rather than 0.005).

In experiments carried out using different buffers (Mozzarelli et al., 1991) or a different three-buffer system, containing 100 mM MES, 51 mM *N*-ethylmorpholine, and 51 mM diethanolamine (Kuo et al., 1985), we observed similar pK values but different R values, due to differences in the nature and concentration of the monovalent cations present in the reaction mixture (Peracchi et al., 1995).

Reaction of the β_2-Dimer with L-Serine. The isolated β_2-dimer of tryptophan synthase possesses quite different

[2] Dissociation of the $\alpha_2\beta_2$-complex into a mixture of α-subunits and β_2-dimers at high pH could affect the reported pH dependence since the β_2-dimer, in the presence of L-serine, accumulates predominantly the external aldimine species (Goldberg et al., 1968). We demonstrated by gel filtration chromatography of the reaction mixtures that the degree of subunit dissociation is negligible both at pH 6.7 and 9.8. As a further control, the incubation of the L-serine−$\alpha_2\beta_2$-complex with a 2-fold excess of α-subunits decreased the total amount of enzyme-bound external aldimine by less than 10%, both at pH 8.9 and 9.9. Finally, we have verified by activity assays that irreversible enzyme inactivation does not occur upon incubation of the enzyme in MBP buffer for 10 min at pH between 6 and 10.7.

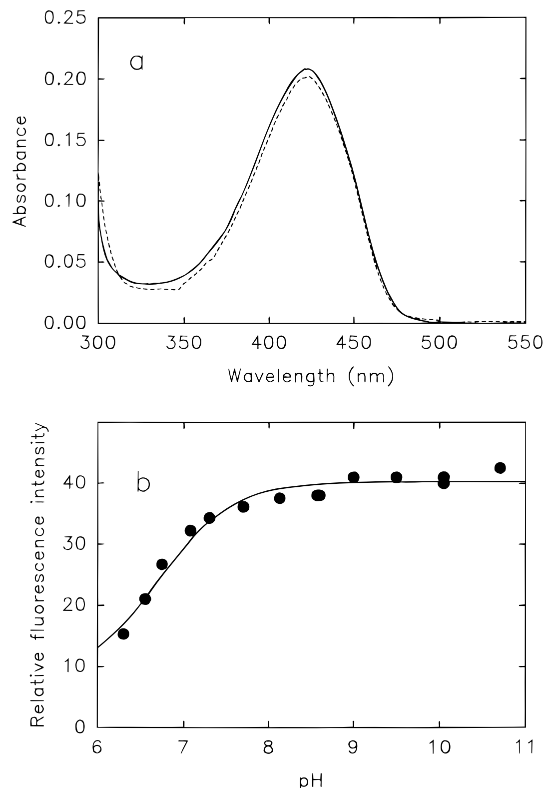


FIGURE 2: Effects of pH on the spectral properties of isolated β_2-dimer in the presence of L-serine. (a) Absorption spectra were recorded for a solution containing isolated β_2-dimer (1.2 mg/mL) in the presence of either 1 M L-serine ($I = 0.4$ M), at pH 6.6 (- - -) or 100 mM L-serine ($I = 0.25$ M), pH 10.0 (—), in MBP buffer, at 10 °C. (b) The fluorescence intensity at $\lambda = 500$ nm ($\lambda_{ex} = 420$ nm) of isolated β_2-dimers in MBP buffer was monitored as a function of increasing concentrations of L-serine, at different pH values. Points reported in the figure are the extrapolated maximal fluorescence, obtained by a nonlinear least squares fitting of the data to an equation for hyperbolic binding. The solid line represents the titration of a single acidic group with p$K = 6.7$.

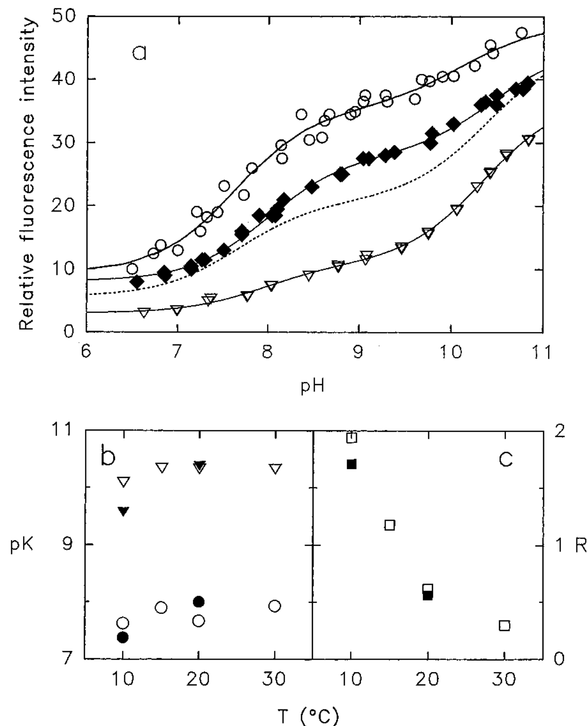


FIGURE 3: Effect of temperature on the pH-dependent distribution of intermediates. (a) The dependence on pH of the amount of the external aldimine was monitored spectrofluorimetrically at 10 °C (○), 15 °C (◆), and 30°C (▽). The dashed curve represents the titration at 20 °C, reported in Figure 1b. (b) The calculated apparent pK values for the first (○) and the second (▽) ionization are reported as a function of temperature. Open and closed symbols refer to fluorescence and absorbance data, respectively. (c) Dependence on the temperature of the ratio of the amplitudes, R, calculated from fluorescence (open symbols) and absorbance (closed symbols) data.

functional features when compared to the $\alpha_2\beta_2$-complex. In particular, it is unable to accumulate the α-aminoacrylate when reacted with L-serine (Goldberg et al., 1968; Miles & McPhie, 1974; Drewe & Dunn, 1985). In fact, the absorption spectra of a solution containing the β_2-dimer saturated with L-serine, collected at either pH 6.6 or 10.0, are nearly identical (Figure 2a). The amount of the α-aminoacrylate present even at low pH is negligible, and the external aldimine is the predominant species over a wide pH range.

The fluorescence of the L-serine−β_2 complex is pH-dependent (Figure 2b) and can be least squares fitted to the ionization of a single acidic residue with an apparent pK of about 6.7. Goldberg et al. (1968) reported a similar pH dependence for the β_2-dimer from *E. coli* and interpreted it as being due to the ionization of a group affecting the fluorescence quantum yield of the external aldimine.

Effect of Temperature on the pH Dependence of the Distribution of Intermediates. The pH dependence of the distribution of intermediates was investigated at four temperatures between 10 and 30 °C by measuring fluorescence changes (Figure 3a). A temperature increase shifts the equilibrium between the external aldimine and the α-aminoacrylate toward the latter species. The temperature has a rather small effect on the calculated apparent pK values (Figure 3b), suggesting an involvement of carboxyl groups in the observed titration (Edsall & Wyman, 1958). On the other hand, the ratio R of the amplitudes of the two phases is strongly affected by temperature (Figure 3c). Control experiments at 10 and 20 °C, using absorption rather than fluorescence to monitor the external aldimine, confirmed the above results (Figure 3b,c), thus excluding major effects of temperature on the fluorescence quantum yield.

Effect of the α-Subunit Ligands GP and Phosphate on the pH-Dependent Distribution of Catalytic Intermediates. The absorption spectra of the L-serine−enzyme complex, recorded at pH 8.5 and 10 °C (Figure 4a) either in the absence or in the presence of increasing concentrations of GP, demonstrate changes in the distribution of intermediates. The amount of external aldimine accumulating at 10 °C was monitored by fluorescence spectroscopy as a function of both pH and GP concentrations (Figure 4b). The least squares fit of these titrations indicates that, while the two apparent pK values seem slightly affected by GP concentration (Figure 4c), the ratio R of the amplitudes of the two phases is strongly dependent on the allosteric effector (Figure 4d). Furthermore, at each defined pH, the fluorescence changes as a function of GP concentration can be fitted to a binding isotherm. The calculated apparent dissociation constant of GP from the L-serine−$\alpha_2\beta_2$-complex depends on pH, exhibiting values of 0.5 mM at pH 7 and 5.6 mM at pH 10.4, respectively (Peracchi, 1994).

Effects on the distribution of intermediates similar to those observed for GP are caused by phosphate (Figure 5). This finding is not surprising, as phosphate has been shown to

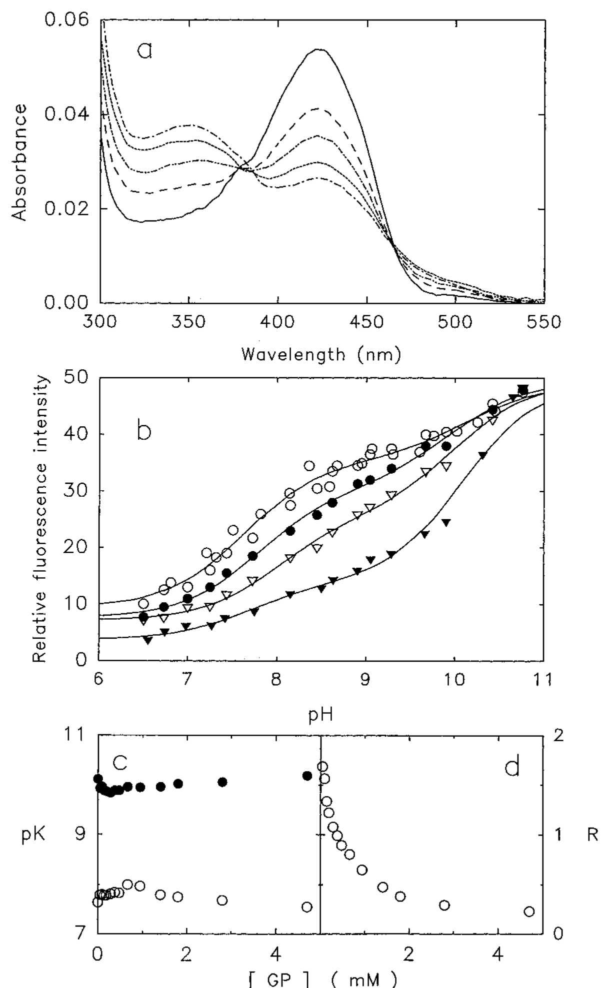


FIGURE 4: Effect of DL-α-glycerol 3-phosphate on the pH-dependent distribution of intermediates formed in the reaction of L-serine with the $\alpha_2\beta_2$-complex. (a) Absorption spectra of a solution containing the enzyme (0.55 mg/mL), 100 mM L-serine, and MBP buffer were recorded in the absence (—) and in the presence of 0.5 mM (— —), 1.2 mM (— - - - —), 4 mM (- - -), and 10 mM (— - - —) GP (pH 8.5) at 10 °C. (b) The dependence on pH of the fluorescence at 500 nm (λ_{ex} = 420 nm) was determined for a solution containing the enzyme (0.054 mg/mL), 100 mM L-serine, and MBP buffer, at 10 °C, in the absence of GP (○) and in the presence of 0.19 mM (●), 0.66 mM (▽), and 2.8 mM (▼) GP. Solid lines are the least squares fits to eq 1. (c) The apparent pK_1 (○) and pK_2 (●) values, determined from the data reported in panel b, are shown as a function of GP concentration. (d) Dependence of the ratio of the amplitudes, *R*, on GP concentration.

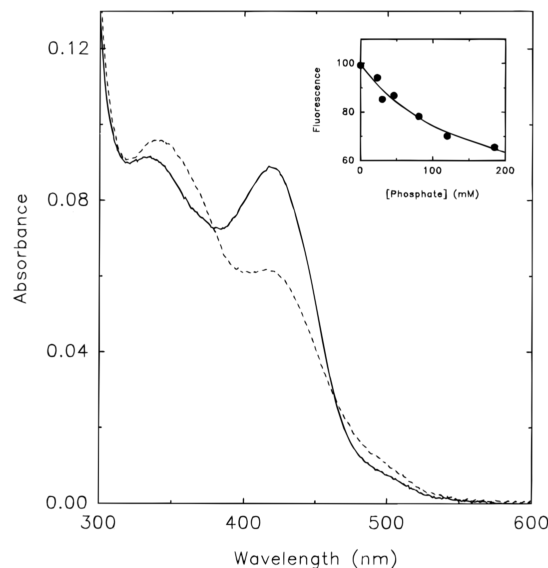


FIGURE 5: Effect of phosphate on the pH-dependent distribution of intermediates formed in the reaction of L-serine with the $\alpha_2\beta_2$-complex. Absorption spectra of a solution containing the enzyme (0.53 mg/mL) and 100 mM L-serine were recorded in MBP buffer (—) (pH 7.9) and in 100 mM potassium phosphate (- - -) (pH 7.8) at 20 °C. The inset is fluorescence change at 500 nm as a function of phosphate concentration. Phosphate was added as sodium salt, and the sodium concentration was kept constant at 0.25 M. The solid line through the data points is a tentative least squares fit to a binding isotherm with a K_{diss} = 160 ± 90 mM.

bind to the α-subunits and to act as an allosteric effector (Dunn et al., 1987, 1990; Houben et al., 1989; Houben & Dunn, 1990). The dissociation constant of phosphate from the L-serine−enzyme complexes is close to the phosphate concentration (100 mM) commonly used to prepare buffer solutions for studies on tryptophan synthase (Figure 5, inset). Under these experimental conditions, a significant accumulation of the α-aminoacrylate Schiff base occurs and the pH dependence of the equilibrium distribution of intermediates can hardly be detected at pH values between 6 and 8.[3]

DISCUSSION

Observing a Quasi-Equilibrium Distribution of True Catalytic Intermediates. In the absence of its second substrate, indole, the reaction catalyzed by the β-subunit of the tryptophan synthase $\alpha_2\beta_2$-complex (Scheme 1) cannot proceed beyond the formation of the α-aminoacrylate and all the catalytic steps occurring until this point are fully reversible (Lane & Kirschner, 1983a,b). Furthermore, the α-aminoacrylate can only be transformed into pyruvate and ammonia via a slow deamination reaction (Miles, 1979). Under the experimental conditions used in this study, the rate constant for this side reaction is negligible compared to both the forward and the reverse rates for the transformation of the external aldimine to the α-aminoacrylate. Therefore, the relative concentrations of the intermediates that accumulate actually reflect the true equilibria between enzyme-bound species, and any effect of pH, temperature, or allosteric ligands must be specifically related to these equilibria.

Relevance of α−β Interactions. The effects of pH on the distribution of β-site intermediates are apparently present only upon interaction of the β-subunits with the α-subunits. In fact, in the isolated β_2-dimer, the external aldimine is the largely predominant intermediate even at pH 6.5 (Figure 2), whereas in the $\alpha_2\beta_2$-tetramer, a substantial amount of α-aminoacrylate is present. Since the β_2-dimer is an active enzyme, the α-aminoacrylate is certainly formed, but its accumulation requires the presence of strong conformational effectors as either 1.5 M ammonium ion (Miles & McPhie, 1974) or 3 M cesium ion (Ruvinov et al., 1995b). Recent

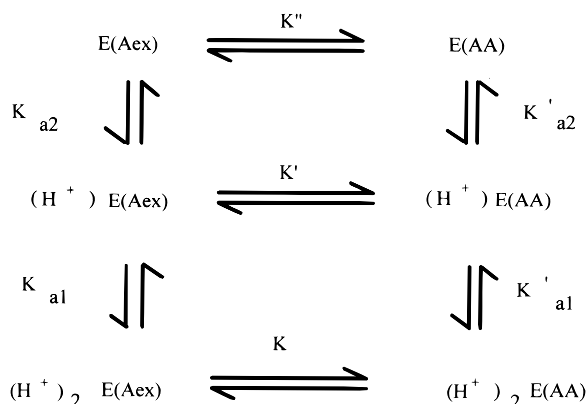
[3] Several studies on the enzyme purified from *E. coli* have been reported. We have monitored the equilibrium distribution of intermediates formed in the reaction of L-serine with this enzyme form and found that two dissociable groups are required to explain the pH dependence of such an equilibrium. While the apparent p*K* values are close to those found for the enzyme from *S. typhimurium,* the ratio of the amplitudes is different. At 20 °C, in the *E. coli* enzyme, less than 20% of the titration is accounted for by the ionization of the more acidic group. Thus, the effect of pH for the *E. coli* enzyme is less likely to be noticed at pH values between 6 and 8.

studies of the $\alpha_2\beta_2$-complex have shown that Na^+ and K^+ favor the formation of the external aldimine (Peracchi et al., 1994, 1995; Dunn et al., 1994; Woehl & Dunn, 1995), whereas Li^+, Rb^+, and Cs^+ favor the α-aminoacrylate (Peracchi et al., 1994, 1995; Ruvinov et al., 1995b). These effects have been explained by a stabilization of alternative conformations of the β-subunits.

The relevance of α−β interactions in the regulation of the distribution of intermediates has recently been confirmed by studies on the β-subunit Lys167Thr mutant (Yang & Miles 1993). The structure of the wild type $\alpha_2\beta_2$-complex shows that Lys167 forms a salt bridge across the intersubunit interface with Asp56 of the α-subunit. Substitution of Lys and Thr leads to the preferential accumulation of the external aldimine and to a reduced effect of GP on the β-site properties.

Two Ionizable Groups Affect the Equilibrium of Intermediates. As shown in Figure 1, the concentration of the external aldimine decreases as pH drops from 10.7 to 6.5, while the concentration of the α-aminoacrylate concomitantly increases. The pH dependence of the interconversion of external aldimine and α-aminoacrylate within the $\alpha_2\beta_2$-complex, reported in Figure 1b, is clearly biphasic and implies the presence of at least two ionizable groups,[4] as formally expressed in Scheme 2:

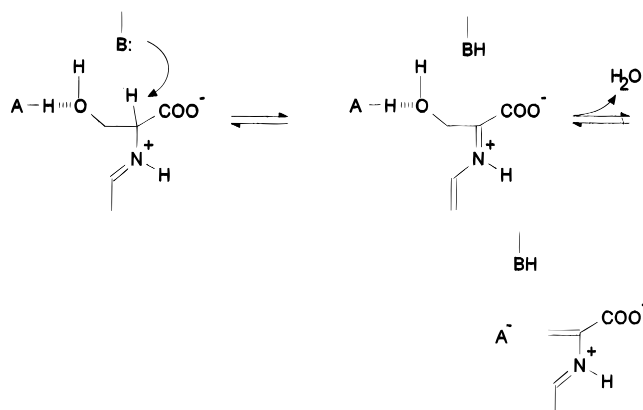
Scheme 2



where K_{a1}, K_{a2} and K'_{a1}, and K'_{a2} are proton dissociation constants of two groups when the enzyme is in the external aldimine [E(A_{ex})] or in the α-aminoacrylate [E(AA)] form, respectively. K, K', and K'' are equilibrium constants for the transformation of the external aldimine in the α-aminoacrylate and a water molecule, at different degrees of protonation of the enzyme. Thus, the calculated values of $pK_1 = 7.8$ and $pK_2 = 10.3$, at 20 °C, are apparent pK values of the ionizable groups, deriving from the combination of these microscopic constants.

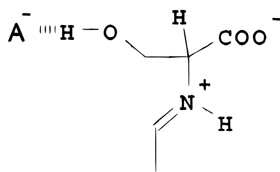
Since protonation favors the accumulation of the α-aminoacrylate derivative, protons must bind more tightly to this species than to the external aldimine. Therefore, the microscopic pK_a values for the ionization of each group must be higher in the enzyme−α-aminoacrylate complex, i.e. $pK_{a1} < pK'_{a1}$ and $pK_{a2} < pK'_{a2}$. The change in the dissociation constants for each group when either the external aldimine or the α-aminoacrylate is present can be estimated from simulations (see below) to be about 10-fold, corresponding to a ΔpK_a of 1.0 unit. Such a change in pK_a suggests a small but significant change in the microenvironment of the groups when the external aldimine is converted to the α-aminoacrylate. It follows that $K = 10^{\Delta(pK'_{a1}-pK_{a1})} \times 10^{\Delta(pK'_{a2}-pK_{a2})} \times K'' = 100K''$; i.e. protonation of both groups stabilizes the α-aminoacrylate by 2 orders of magnitude.

We do not yet know which groups are involved in the observed phenomena. Since pH, *per se*, is not expected to influence the chemical equilibrium between the external aldimine and the α-aminoacrylate Schiff base (see Scheme 1), its effect must arise from changes in the ionization states of residues that, either directly or via stabilization of a particular active site conformation, favor accumulation of one Schiff base or the other . There are obviously a large number of groups that can play this role. Focusing on the possibility that at least one residue may be located within the β-active site, we speculated that it might be the acid catalyst presumably required for the conversion of the external aldimine to the α-aminoacrylate. Potential candidates for such a role include the carboxyl group of Glu109 (Kayastha & Miles, 1991; Brzovic et al., 1992a), Asp305, or Lys87. If a carboxyl residue were the group, as also suggested by the low heat of ionization (Edsall & Wyman, 1958), then our measurements indicate this residue must be assigned an unusually high pK_a value. On the other hand, the lysine residue might have an abnormally low pK_a, as found in other enzymes (Frey et al., 1971; Kokesh & Westheimer, 1971). At low pH (see the Scheme below), the protonated carboxylate could both destabilize the external aldimine via a H-bonding interaction to the oxygen of the L-serine β-hydroxyl that is set up for proton transfer in the elimination step and stabilize the hydrophobic β-CH_2 of the α-aminoacrylate



At high pH, the corresponding carboxylate (as hydrogen bond acceptor) would actually stabilize the external aldimine by making a hydrogen bond to the serine hydroxyl proton and destabilize the α-aminoacrylate by a negative interaction between the charged group and the apolar double bond of the α-aminoacrylate.

[4] A group in a protein can titrate with more than a single pK_a depending on the concomitant presence of neighboring titrating groups. As a consequence, the presence of two pK_a values can reflect either the presence of two residues independently acting on the equilibrium or the ionization of a single residue titrating in two fractions with different pK_a values, due to the vicinity of a second group present in different ionization states (Dixon, 1991, 1992; Dixon et al., 1991). In the present discussion, the two groups are treated independently, but our conclusions would not be modified if the titration of a single group in two fractions were assumed.



The combination of this hydrogen bond and the negative charge would work against elimination. Consequently, as the pH is increased past the pK_a, the external aldimine form would be stabilized relative to the α-aminoacrylate. Attempts to test this hypothesis by carrying out measurements analogous to those reported for the wild type enzyme on the mutant Glu109Ala failed. Since the enzyme accumulated only the external aldimine independently of pH, we were unable to observe any pH dependence of the equilibrium distribution between the external aldimine and the α-aminoacrylate. However, when the Glu109Asp mutant was investigated, only the transition controlled by the group with lower pK was observed in the absence of GP, and the biphasicity of the titration was restored in the presence of GP (Peracchi, 1994). Similar experiments, carried out on a variety of mutants of ionizable residues located near or in the active site, were not rewarding. Due to the known ability of tryptophan synthase to be finely regulated by intra- and intersubunit effectors, it is possible that one or both groups may be located far from the β-active site, near the $\alpha\beta$-interface, or even on the α-subunits. The three-dimensional structures of the external aldimine and α-aminoacrylate might help to identify these ionizable residues.

Effect of Temperature. Independently of the nature and location of the two ionizable groups, it is important to determine whether the proposed thermodynamic scheme is compatible with the observed effects of temperature and of allosteric ligands. We have shown that temperature affects the relative amplitudes of the two phases of the titration curve and hence the relative concentrations of the two Schiff bases (Figure 3). However, the two apparent pK, pK_1 and pK_2, are only slightly affected. Although this finding does not imply that the microscopic pK_a values are temperature-independent, we can assume as a first approximation that the temperature effect is dominated by the temperature dependence of the equilibrium constant between two enzyme conformations. Theoretical titration curves calculated on the basis of our model are shown in Figure 6a. The different curves are built by attributing variable values to K but keeping constant the microscopic pK_a values. Since the relative amplitudes of the two phases of the titration depend on the value of K, it is possible to simulate the experimental data to a good approximation. In fact, the plot of the residuals in Figure 6a does not show systematic deviations from zero, thus indicating that the proposed thermodynamic model seems capable of accommodating the effect of temperature, even with the conservative assumption that the dissociation constants of the two groups are temperature-independent. As the value of K increases with temperature, the conversion of the aldimine to the α-aminoacrylate Schiff base must be an endothermic process.

Effect of the Allosteric Ligand GP. In Figure 4, we show that the presence of variable concentrations of GP, at a given temperature, dramatically changes the pH-dependent distribution of intermediates. GP binding significantly affects the relative amplitudes of the two phases of the titration curve,

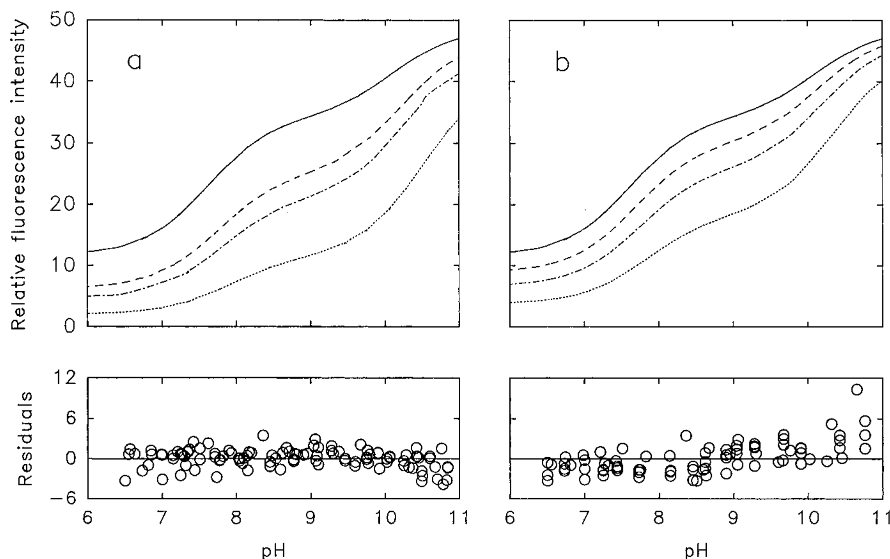


FIGURE 6: Simulation of the pH dependence of the distribution of intermediates as a function of temperature and α-subunit ligands. Curves simulating the dependence of the external aldimine fluorescence on pH were obtained using the following equation derived from the model described in Scheme 2,

$$F = F_{max}([H^+]^2 + [H^+]K_{a2} + K_{a1}K_{a2})/[[H^+]^2(K + 1) + [H^+](K_{a1} + K'_{a1}) + KK'_{a1}K'_{a2} + K_{a1}K_{a2}]$$

where $F_{max} = 50$ is the relative fluorescence intensity of the enzyme solution containing only the external aldimine species. The assigned microscopic pK_a values are pK_{a1} = 7.15, pK'_{a1} = 8.00, pK_{a2} = 9.95, and pK'_{a2} = 11.15. These values were chosen on the basis of a series of simulations on individual sets of data. The equilibrium constant K is equal to $[H_2E(AA)]/[H_2E(A_{ex})]$. The following approximations were applied in deriving the equation: (i) the external aldimine is the only fluorescent species and (ii) its quantum yield is independent of pH, temperature, and GP. (a) The curves are the least squares best fits of the pH-dependent titrations at different temperatures, reported in Figure 3a. Residuals are shown in the bottom panel. The calculated equilibrium constant K is 3.3 ± 0.1 at 10 °C (—), 7.0 ± 0.2 at 15 °C (— —), 9.6 ± 0.3 at 20 °C (— - —), and 23.6 ± 0.3 at 30 °C (- - -). (b) The curves are the least squares best fits of the pH-dependent titrations at different GP concentrations, reported in Figure 4b. Residuals are shown in the bottom panel. The calculated equilibrium constant K is 3.3 ± 0.1 in the absence of GP (—), 4.6 ± 0.2 at 0.19 mM GP (— —), 6.5 ± 0.4 at 0.66 mM GP (— - —), and 12.2 ± 1.0 at 2.8 mM GP (- - -).

while it slightly changes the apparent pK values. Overall, the fact that GP binding, at each pH tested, favors the accumulation of the α-aminoacrylate is consistent with a description of the allosteric behavior of tryptophan synthase (Houben & Dunn, 1990), according to which (a) the conformation of the enzyme molecule is uniquely associated with the chemical nature of the PLP-bound intermediate and (b) allosteric effectors stabilize different reaction intermediates by preferentially binding to their respective conformations. As a consequence, the effect of GP is due to the stabilization of the protein conformation containing the α-aminoacrylate.

In a strict sense, this description predicts that the affinity for protons of both the external aldimine and the α-aminoacrylate should not be affected by GP. However, when a simulation of the data reported in Figure 4 is attempted utilizing the model (Scheme 2) and keeping the pK_a values constant (Figure 6b), the agreement with the experimental data is rather poor (Figure 6b, botton panel). This result suggests that a more complex model is needed to obtain a better fit of the data. A structural model that includes different conformational states of the α- and β-subunits of tryptophan synthase has been recently proposed (Brzovic et al., 1992a−c, 1993; Dunn et al., 1994; Leja et al., 1995; Ruvinov et al., 1995a). In particular, on the basis of kinetic studies carried out at pH 7.8 and 25 °C, it has been suggested that GP binding drives the α-subunits from an open to a closed conformation. If GP affects the protein conformation, then the microscopic ionization constants need no longer be the same as in the absence of GP. Accordingly, the fitting of the data improves significantly, as indicated by a 2-fold decrease of the residuals (data not shown). However, as a result of the interplay between the microscopic proton dissociation constants and the equilibrium constant for the interconversion of the two Schiff bases, the apparent pK values are nearly identical in the presence and in the absence of GP.

CONCLUSIONS

We have shown that in the tryptophan synthase $\alpha_2\beta_2$-complex there is a linkage between proton binding and the conversion of the external aldimine in the α-aminoacrylate Schiff base. Thus, protons act as allosteric effectors in a manner similar to that of α-subunit ligands. This behavior parallels that observed in hemoglobin where protons, as well as 2,3-diphosphoglycerate, favor deoxyhemoglobin over oxyhemoglobin by stabilizing the T over the R quaternary conformation (Perutz, 1989). In spite of the obvious differences between the two proteins, the microscopic origins of the pH dependence of allostery in tryptophan synthase and in hemoglobin might be similar. The pH effects in hemoglobin allostery are due to alteration of microscopic pK_a values resulting from disruption of inter- and intrasubunit salt bridges as the T to R state transition occurs. While we are unable to rule out direct interactions between ionizable residues and the external aldimine or the α-aminoacrylate species, the evidence for the involvement of an intersubunit Asp−Lys salt bridge as a part of the allosteric linkage between α- and β-sites (Yang & Miles, 1993) suggests that alterations of intersubunit interactions may be responsible for the pH effects reported herein.

The pH, temperature, and allosteric ligand-dependent phenomena we have described are maintained by the enzyme in the crystalline state, as first revealed by single crystal polarized absorption microspectrophotometric studies (Mozzarelli et al., 1989; Rossi et al., 1992; Peracchi et al., 1995). These findings indicate that individual catalytic intermediates of tryptophan synthase could be preferentially accumulated in the crystalline state and analyzed by X-ray crystallography to assess the extent of conformational differences between the Schiff bases and to further elucidate the regulatory mechanisms.

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