A proton-magnetic-resonance study of hydrogen-exchange reactions of yeast tryptophan synthase

Christopher J. BAILEY* and J. Paul G. MALTHOUSE†

*Department of Biochemistry, Trinity College, Dublin 2, and †Department of Biochemistry, University College, Belfield, Dublin 4, Ireland

^1H n.m.r. was used to observe tryptophan formation from indole and L-serine, proton exchange at C-2 of L-tryptophan, and proton exchange at C-2 of L-serine, catalysed by yeast tryptophan synthase in the presence of $^2\text{H}_2\text{O}$. Tryptophan synthesis took place with compulsory replacement of C-2 hydrogen by solvent hydrogen. The exponential decay rate ($k_{\text{obs.}}$) of the serine exchange reaction was insensitive to serine concentration in the range 2–20 mM and was used to calculate $k_{\text{cat.}}/K_{\text{m}}$ values. However, $k_{\text{obs.}}$ was very sensitive to pH* values in the range 6.5–8.5 and the data require that the free enzyme is active in the base form resulting from two inseparable ionizations of $\text{p}K_{\text{a}}$ 7.3, and inactive after a third ionization controlled by a $\text{p}K_{\text{a}}$ of 7.5. Initial rates measured by u.v. absorbance and colorimetric procedures were used to calculate kinetic parameters of the tryptophan synthesis reaction. From pH 6.5 to 7, $k_{\text{cat.}}/K_{\text{m}}$ values for L-serine in the tryptophan synthesis and hydrogen exchange reactions were indistinguishable and increased rapidly under the control of two acid–base groups of $\text{p}K_{\text{a}}$ 6.7 and 7.2. Above pH 7, this equivalence breaks down because the exchange reaction alone is responsive to the third $\text{p}K_{\text{a}}$ value of the free enzyme. The pH dependence of the catalytic constant for tryptophan synthesis was qualitatively similar to that of the $k_{\text{obs.}}$ for serine exchange. A mechanism to explain the results is contrasted with recent proposals for the *Escherichia coli* system.

INTRODUCTION

Tryptophan synthase (Miles, 1979) catalyses the final step of tryptophan synthesis, the conversion of indoleglycerolphosphate into tryptophan, by a two-stage process in which the indole product of the first reaction is condensed with serine at another active site. It is one of the simpler multifunctional or multienzyme complexes whose enzyme abilities are not yet adequately explained (Kirschner & Bisswanger, 1976; Keleti, 1984; Srere, 1987). The recent structure determination has confirmed that the two sites are distant in space (Hyde *et al.*, 1988), but has not led to any detailed proposals about the mechanism. In the case of the reaction B:

extensive studies of the *Escherichia coli* enzyme favour a complex mechanism which, after the compulsory initial binding of L-serine, branches so that tryptophan can be formed by reaction of indole with one of two enzyme–serine species (Lane & Kirschner, 1981, 1983*a,b*). These pyridoxal phosphate-dependent reactions are expected to take place with labilization of the proton at the α -carbon of the amino acid substrates. For yeast tryptophan synthase, we have shown that exchanges between $^3\text{H}_2\text{O}$ and L-serine and L-tryptophan are catalysed (Turner *et al.*, 1985). Such reactions are useful for examining the kinetics of reaction B, for they offer a way of observing individual enzyme–substrate interactions in a two-substrate mechanism. In this latter respect, the previous results were not interpretable by simple models. Notable difficulties were the apparent incomplete proton exchange at the α -carbon during tryptophan synthesis and the inequality of $k_{\text{cat.}}/K_{\text{m}}$ values for serine in the hydrogen exchange and tryptophan synthesis reactions.

In order to test previous results, we have re-examined the exchange reactions, using p.m.r. to record continuously the transfer of protons from amino acid to $^2\text{H}_2\text{O}$. The new results

permit a simpler interrelation of reaction B with the serine exchange reaction.

EXPERIMENTAL

Materials

Yeast tryptophan synthase was prepared as described previously (Bailey & Turner, 1983) except that 0.1 mM-dithiothreitol was added to all the buffers used for chromatography. This improved the specific activity to 2.7 units/mg. $^2\text{H}_2\text{O}$ (99.8 atom % ^2H) was from Aldrich Chemical Co. L-[2- ^3H]Serine and L-[2- ^3H]tryptophan were prepared by exhaustive enzyme-catalysed exchange. Reactions were terminated by boiling and the individual amino acids were purified by adsorption on Aminex A5, eluted with ammonia and freeze-dried. Other chemicals were as described previously (Bailey & Turner, 1983).

N.m.r. samples and spectra

Known amounts of buffer salts were freeze-dried and re-constituted in $^2\text{H}_2\text{O}$, and substrates were dissolved in $^2\text{H}_2\text{O}$. All reaction mixtures contained 0.1 mM-pyridoxal phosphate. From pH 5.8 to pH 8.1, 100 mM-phosphate with K^+ counter-ion was used. Above pH 8.1, 100 mM-tetrasodium pyrophosphate, adjusted with KH_2PO_4 , was the buffer mixture. Reactions were started by addition of enzyme (10–20 μl) to buffered substrate solution (1 ml). Accumulation of n.m.r. data began within 4–6 min. pH* values were recorded at the end of the reaction, using a combination electrode standardized in a $^1\text{H}_2\text{O}$ buffer before transfer to $^2\text{H}_2\text{O}$.

Spectra were recorded by using a Bruker WP80 wide-bore spectrometer at 80.0 MHz for ^1H nuclei. Sample size was 1 ml in 5 mm-diameter sample tubes. High-resolution spectra were obtained using 8000 time-domain points, 5.12 s acquisition time and a spectral width of 10 p.p.m. Time-course spectra were

Abbreviation used: pH*, pH-meter reading in $^2\text{H}_2\text{O}$.

* To whom correspondence should be addressed.

recorded using 2000 time-domain points, 1.28 s acquisition time and a spectral width of 10 p.p.m. Most experiments, unless stated otherwise used 99.8 atom % deuterium oxide ($^2\text{H}_2\text{O}$) as a solvent and the residual H^2HO signal was suppressed by pre-saturation for 2 s. Between 100 and 2400 transients were recorded per spectrum. All the n.m.r. spectra were obtained at $22 \pm 2^\circ\text{C}$ over periods of 1–20 days. From enzyme assays of samples recovered after spinning in the spectrometer for 5 days, it was estimated that the rate of activity loss was about 10 % per day. This loss was very slow in comparison with the experimental rate of serine exchange reaction ($t_{1/2} \approx 1\text{h}$), and rates of the serine exchange reaction are not corrected for this effect.

Initial-rate measurements

Reaction B data were fitted to the general equation which explains (Turner *et al.*, 1985) the reaction velocity at a single pH value (see also Fig. 7) as a function of indole (I) and serine (S) concentrations, their respective Michaelis constants $K_{m,B}^I$, $K_{m,B}^S$, the apparent dissociation constant K_s' , the catalytic constant $k_{cat.,B}$ and the total enzyme concentration E_0 :

$$V = k_{cat.,B} E_0 / \left(\frac{K_{m,B}^I K_s'}{IS} + \frac{K_{m,B}^S}{S} + \frac{K_{m,B}^I}{I} + 1 \right) \quad (1)$$

Values of $k_{cat.,B}/K_{m,B}^S$ were estimated from initial velocities at low serine (0.025–0.4 mM) and saturating indole concentrations (200–400 μM) using the u.v. difference assay (Faeder & Hammes,

1970) at 25°C . Under these conditions eqn. (1) can be rearranged to:

$$V/E_0 S \approx k_{cat.,B}/K_{m,B}^S$$

K_s' was estimated by non-linear regression of initial rate data from the range 10–100 μM -indole and 1–10 mM-L-serine to eqn. (1). $k_{cat.,B}$ values at 20°C were based on measurements of initial rate at 0.3 mM-indole and 20 mM-L-serine by the colorimetric procedure (Bailey & Turner, 1983) and extrapolated using eqn. (1) and the measured values of the other kinetic parameters. All rates of tryptophan synthesis were estimated using the same mixtures of buffer ions as in the n.m.r. experiments, except that the solutions were in $^1\text{H}_2\text{O}$.

THEORY

Exchange reactions in the n.m.r. spectrometer were followed to completion except for the exchange reaction of L-tryptophan which was unfinished even after 25 days. The reactions were interpreted in terms of exponential decay so that the concentration of an isotopically labelled substrate (S^*) is related to the total substrate concentration (S_T) and time t , by a first-order decay constant $k_{obs.}$:

$$S^* = S_T \cdot \exp(-k_{obs.} t) \quad (2)$$

For an enzyme-catalysed isotope exchange reaction, in which isotope can only be exchanged into a large excess of unlabelled

Fig. 1. ^1H n.m.r. spectra of indole, L-tryptophan, L-serine, L-[2- ^2H]serine and L-[2- ^2H]tryptophan

The spectra are compared over three ranges of chemical shift. The sample (at pH* 7.2 in 99.8 atom % deuterium oxide, containing 20 mM-phosphate) concentrations, and numbers of accumulations per spectrum were: (a and c) L-tryptophan, 31 mM, 400; (b) indole, 8.5 mM, 400; (d) L-[2- ^2H]tryptophan, 15 mM, 512; (e) L-serine, 20 mM, 100; (f) L-[2- ^2H]serine, 20 mM, 100.

water, the initial rate of exchange from labelled substrate, S^* , is given by:

$$V = k_{\text{cat.,H}} E_0 S^* / (K_{\text{m,H}}^S + S_T) \quad (3)$$

where S_T is the concentration of all forms of substrate and $k_{\text{cat.,H}}$ and $K_{\text{m,H}}^S$ are the catalytic constant and Michaelis constant respectively of the single-substrate hydrogen-exchange reaction of serine.

If only labelled substrate is present at the beginning of the reaction, then the course of the reaction follows

$$S^* = S_T \exp[-k_{\text{cat.,H}} E_0 t / (K_{\text{m,H}}^S + S_T)] \quad (4)$$

From eqns. (2) and (4):

$$k_{\text{obs.}}/E_0 = k_{\text{cat.,H}}/(K_{\text{m,H}}^S + S_T) \quad (5)$$

If $k_{\text{obs.}}$ is independent of S_T then $K_{\text{m,H}}^S \gg S_T$ and

$$k_{\text{obs.}}/E_0 = k_{\text{cat.,H}}/K_{\text{m,H}}^S \quad (6)$$

The pH-dependence of kinetic constants was fitted by non-linear regression (Ralston, 1981) to the equation:

$$k = \tilde{k} / \{1 + K_C/[H^+] + [H^+]/K_B + [H^+]^2/(K_A K_B)\} \quad (7)$$

The three independent acid dissociation constants in the conventional derivation (Tipton & Dixon, 1979) have the relationship:

$$pK_A < pK_B < pK_C \quad (8)$$

Simpler pH-dependent schemes are special cases of eqn. (7).

RESULTS

^1H -n.m.r. spectra of substrates and products

The spectrum of the C-2 and C-3 protons of L-serine is of the ABC type (Fig. 1e), though from pH 2 to 8.9 the chemical shift difference between the C-3 protons is small and the spectrum approximates to an AB₂-type (Ogura *et al.*, 1967). Therefore replacement of the C-2 proton of L-serine by deuterium results in a single signal from the two C-3 protons of L-[2- ^2H]serine (Fig. 1f). The latter signal overlaps with the intense peak 3 in non-deuterated serine (Fig. 1e).

The spectrum of the C-2 and C-3 protons of L-tryptophan is of the ABX type (Fig. 1c). Replacement of the C-2 proton of tryptophan by deuterium results in a spectrum of the AB type (Fig. 1d). Therefore the extent of deuteration of L-tryptophan at C-2 could be followed by the decrease in intensity of the α -CH quartet at 4.0 p.p.m., or the decrease of the β -CH₂ peak at 3.3 p.p.m. (Figs. 1c and 1d).

The C-3' proton of indole was resolved as a doublet at approx. 6.6 p.p.m. (Fig. 1b), which could be used to monitor indole in the presence of tryptophan (Fig. 1a).

Synthesis and hydrogen exchange of L-tryptophan

N.m.r. was used to follow the reactions catalysed by tryptophan synthase with 5 mM-L-serine plus 5 mM-indole in $^2\text{H}_2\text{O}$ at pH 7.16. Synthesis of L-[2- ^2H]tryptophan was observed to take place smoothly (Fig. 2), corresponding to a single exponential process of $t_{1/2} = 465$ min. Concomitantly the C-3' proton signal of indole, and total indole concentration measured by colorimetric analysis, decayed with similar half-lives (417 min and 516 min respectively).

L-[2- ^2H]tryptophan could be produced by compulsory solvent exchange during synthesis, or as a consequence of the fact that most tryptophan is synthesized from the rapidly formed L-[2- ^2H]serine, or by hydrogen-deuterium exchange after synthesis. The rate of the tryptophan exchange reaction, from [2- ^1H] to [2- ^2H]tryptophan at 20 mM-L-tryptophan and

Fig. 2. Synthesis of L-[2- ^2H]tryptophan

The initial reaction mixture contained indole (5 mM), L-serine (5 mM) and tryptophan synthase (0.42 μM) in > 97 atom % deuterium oxide containing 20 mM-phosphate at pH* 7.16. (a) The concentrations of indole (O), L-serine (Δ) and L-[2- ^2H]tryptophan ($\square$) are shown. The calculated amount of L-[2- ^2H]serine is shown by the dashed line. (b) The measured isotopic ratio of tryptophan ($\blacksquare$) is compared to the predicted ratios (dashed lines) from eqns. (9) and (10).

pH* 7.15, corresponded to $t_{1/2} = 3.4 \times 10^4$ min. It is clear that this reaction can only contribute negligibly to the production of [2- ^2H]tryptophan in the experiment of Fig. 2.

The remaining possibilities, that tryptophan is synthesized with compulsory transfer of a solvent proton on to the 2-position or that the synthesis takes place with retention of the proton from the C-2 position of serine, can be distinguished by comparing the measured isotopic ratio of tryptophan ([2- ^1H]/[2- ^2H]) to the predicted values from the alternative schemes (9) and (10):

The progress-curve data from substrate and products were all conveniently modelled as exponential systems, governed by the rate constants k_1 and k_2 , and the relevant differential equations were integrated to give the calculated ratio of [2- ^1H] to [2- ^2H]tryptophan according to the schemes of eqns. (9) and (10). At the end of the reaction, both mechanisms predict a high proportion of the [2- ^2H] form, and it is only in the early stages of reaction that a satisfactory degree of discrimination is achieved (Fig. 2b). No L-[2- ^1H]tryptophan was detected in the tryptophan spectra, which always showed a doublet at 3.4 p.p.m. with the characteristic intensity ratio of L-[2- ^2H]tryptophan. At the earliest time

Fig. 3. N.m.r. spectra during the hydrogen-deuterium exchange reaction of L-serine

Spectrum 1 is a control without enzyme. Spectra 2–10 were recorded consecutively, starting 3 min after the addition of yeast tryptophan synthase ($0.21 \mu\text{M}$) to L-serine (20 mM) in > 98 atom % deuterium oxide buffers with 20 mM-phosphate at $\text{pH}^* 8.09$. An acquisition time of 4.27 s was used with an inter-pulse delay of 2 s during which time the residual H^2HO signal was suppressed by pre-saturation. After 16 dummy scans, 128 transients were recorded per spectrum; the delay between spectra was 10.2 min. Spectrum 11 was recorded 22 h after the start of the reaction.

point examined, it was calculated that the ($^1\text{H}/^2\text{H}$) isotopic ratio was less than 0.2 and at later times the estimations were more precise. The data (Fig. 2b) are inconsistent with reaction pathways that allow any formation of non-exchanged L-tryptophan. It was also impossible to detect L-[2- ^1H]tryptophan production in a similar study at $\text{pH}^* 8.44$. The data thus support the theory that solvent exchange takes place according to eqn. (9).

Hydrogen-deuterium exchange of L-serine

Spectra from a complete reaction of L-serine to L-[2- ^2H]serine are shown in Fig. 3. The signal overlap makes it difficult to estimate deuteroserine concentration at intermediate stages. The rate of the reaction was calculated (Fig. 4) from the loss of signal intensity at peaks 4, 5 and 6 (Fig. 1e). The resulting $k_{\text{obs.}}/E_0$ values ($1.57, 1.44, 1.40 \text{ mM}^{-1} \cdot \text{s}^{-1}$ respectively) were experimentally indistinguishable. Measurements under a number of

Fig. 4. Hydrogen-deuterium exchange of L-serine

Reaction was followed by observing the change in amplitude of peaks 4 (○), 5 (□) and 6 (△). Conditions as in Fig. 3.

Table 1. Exchange rates of the C-2 hydrogen of L-serine

All experiments were carried out in 100 mM-phosphate/0.1 mM-pyridoxal phosphate. Serine exchange was measured in > 97 atom % $^2\text{H}_2\text{O}$; [2- ^2H]serine exchange was measured in 90 atom % H_2O , with residual $^2\text{H}_2\text{O}$ used as an internal lock on the n.m.r. spectrometer.

Reactants initially present	pH^*	Solvent	$k_{\text{obs.}}/E_0$ ($\text{mM}^{-1} \cdot \text{s}^{-1}$)
5 mM-Serine + 5 mM-indole	7.16	$^2\text{H}_2\text{O}$	1.47
20 mM-Serine	7.16	$^2\text{H}_2\text{O}$	1.35
2 mM-Serine	7.17	$^2\text{H}_2\text{O}$	1.67
20 mM-[2- ^2H]Serine	6.91	H_2O	0.80
20 mM-Serine	6.40	$^2\text{H}_2\text{O}$	0.043

conditions are collected in Table 1. The rate of exchange ($k_{\text{obs.}}/E_0$) was independent of serine concentration in the range 2–20 mM and was not perturbed by the presence of saturating levels of indole at $\text{pH}^* 7.17$. The reverse reaction, of proton transfer into L-[2- ^2H]serine, was slower, demonstrating an apparent isotope effect of about 2-fold. However, this conclusion was set at doubt by the observation that a small pH^* change to 6.4 lowered the $k_{\text{obs.}}/E_0$ by more than 30-fold. Accordingly, a systematic study of the effect of pH^* on the enzyme-catalysed exchange at the C-2 proton of L-serine was undertaken.

Effect of pH^* on the rate of hydrogen-deuterium exchange of L-serine

The profile of the $k_{\text{obs.}}/E_0$ -versus-pH profile was bell-shaped (Fig. 5). On the low pH side, the log-log plot had a slope of about +2 and at high pH the slope was -1 . This demonstrated that $k_{\text{obs.}}/E_0$ is dependent on at least three ionizing groups.

Fig. 5. Comparison of the pH-dependences of $k_{\text{cat},B}/K_{m,B}^S$ (○) and $k_{\text{obs.}}/E_0$ (●)

However, because no substantial region of the log-log plot had a slope of +1, the data of Fig. 5 were overdetermined by eqn. (7), and the conclusion was confirmed by data-fitting. A simpler appropriate equation

$$k_{\text{obs}}/E_0 = \tilde{k}/(1 + K_C/[\text{H}^+] + [\text{H}^+]^2/K_{\text{AB}}^2) \quad (11)$$

corresponds to a model in which groups A and B ionize co-operatively, without a monovalent intermediate, at a single pK_{AB} .

The calculated values were $pK_{AB} = 7.3 \pm 0.1$ and $pK_C = 7.5 \pm 0.3$.

Fig. 6. pH-dependence of $k_{cat,B}$ and K'_S

The data for $k_{\text{cat.,B}}$ are fitted to two $\text{p}K_{\text{a}}$ values of 6.3 ($\text{p}K'_{\text{AB}}$) and one value of 7.9 ($\text{p}K'_{\text{C}}$) according to eqn. (11); K_{s} values (O) are fitted arbitrarily.

pH-dependence of reaction B

As L-serine is the compulsory first substrate in reaction B, the pH-dependence of $k_{\text{cat.,B}}/K_{\text{m,B}}^{\text{s}}$ should be identical with that of $k_{\text{cat.,H}}/K_{\text{m,H}}^{\text{s}}$ if the same enzyme-serine complexes are involved in both reactions. Measured values of $k_{\text{cat.,B}}/K_{\text{m,B}}^{\text{s}}$ (Fig. 5) increased markedly in the pH range 6.4–7 and the slope indicated that two ionizations are involved. In the pH range 6.4–7, $k_{\text{cat.,B}}/K_{\text{m,B}}^{\text{s}}$ could be superimposed on to the curve for the n.m.r. parameter $k_{\text{obs.}}/E_0$ of the hydrogen exchange reaction of serine, which has the value $k_{\text{cat.,H}}/K_{\text{m,H}}^{\text{s}}$ [eqn. (6)]. However, above pH 7, $k_{\text{cat.,B}}/K_{\text{m,B}}^{\text{s}}$ reached a constant high value and diverged from $k_{\text{obs.}}/E_0$. The data require that $\text{p}K_{\text{C}}$ does not affect the rate of reaction B. Fitting to eqn. (11) was satisfactory with $\text{p}K_{\text{AB}} = 7.1 \pm 0.1$, but a slightly better fit to the appropriate form of eqn. (7) was given by $\text{p}K_{\text{A}} = 6.7 \pm 0.4$, $\text{p}K_{\text{B}} = 7.2 \pm 0.1$.

The pH-dependencies of $k_{cat,B}$ and K'_s are as shown in Fig. 6. The turnover rate data could be fitted, with a high degree of apparent deviation, to a two- pK_a model (not shown). However, the maximum slope between points was about 1.2 at the acidic end of the pH range, and the three- pK_a solution of eqn. (11) was needed for a satisfactory data-fit $pK'_{AB} = 6.3 \pm 0.1$, $pK'_C = 7.9 \pm 0.1$.

DISCUSSION

The sole product observed by n.m.r. in the reaction of L-serine with indole in $^2\text{H}_2\text{O}$ was L-[2- ^2H]tryptophan. In a previous study of tryptophan synthesis in $^3\text{H}_2\text{O}$ (Turner *et al.*, 1985) the discontinuous assay indicated that the initial product was incompletely exchanged. The observed effect on the specific radioactivity was comparatively small and the result is not confirmed by the more complete n.m.r. experiments. N.m.r. studies of another tryptophan synthase have indicated that partial retention of the proton on the α -carbon atom can occur during tryptophan synthesis (Tsai *et al.*, 1978).

In the pH* range from 6.4–7.0, the equality [terms defined in eqns. (1) and (3)]

$$k_{\text{cat},\text{H}} K_{\text{m},\text{H}}^{\text{S}} = k_{\text{cat},\text{B}} / K_{\text{m},\text{B}}^{\text{S}} \quad (12)$$

holds (Fig. 5), which is as expected when serine is the only substrate (exchange reaction) or the compulsory first substrate, as in reaction B (Turner *et al.*, 1985). Above $\text{pH}^* 7.5$, eqn. (12) breaks down and this transformation cannot be accommodated by simple mechanisms. The $k_{\text{cat.}}/K_{\text{m}}$ values correspond to the rate constant for reaction of free enzyme with initial substrate (Cleland, 1975) and therefore at alkaline pH (Fig. 5) some enzyme-serine complexes that are intermediates or dead ends in

Fig. 7. Possible mechanism for hydrogen exchange and tryptophan synthesis

The reactions involving serine (S), indole (I) and tryptophan (W) are shown. The gem-diamine, external aldimine, quinoid and amino-acrylate complexes of enzyme-serine are indicated by the numerals I-IV respectively. The broken lines indicate steps that are affected by the presence of indole.

proton exchange react with indole. The data require that a kinetic constant, pK_c , governs the conversion of yeast tryptophan synthase to a form that catalyses serine exchange at negligible rate in the absence of indole.

Fig. 7 shows a scheme that explains the pH-dependence of k_{cat}/K_m and k_{cat} in conventional terms (Tipton & Dixon, 1979) and interrelates hydrogen exchange and reaction B by the known chemical intermediates (Miles, 1979; Drewe & Dunn, 1985; Mozarelli *et al.*, 1989).

Below $pH^* 7$, it is proposed that mechanisms above the bottom row of Fig. 7 dictate the rates of the reactions. The isomerizations between ESH(I, II) and ESH(III), and the conversion of ESH(IV) to ESH(III), must proceed faster than $k_{cat,H}$ which is greater than $k_{cat,B}$ (at $pH^* 7.0$, $k_{cat,H} > 30 \text{ s}^{-1}$, $k_{cat,B} = 1.2 \text{ s}^{-1}$). Hence tryptophan synthesis by the first row of Fig. 7 is rate-limited by the removal of the hydroxide ion or by step(s) after the addition of indole.

As the mechanism of the lower row becomes dominant at alkaline pH, the overall velocity of the exchange reaction should fall to a value less than that of tryptophan synthesis. This condition, not observed in the present study from pH 6.6 to 8.6 at 20–25 °C was found for the exchange reaction with tritiated water at 37 °C and pH 6.8 (Turner *et al.*, 1985). Addition of indole to both rows of the mechanism is shown as an irreversible step since there was no evidence of the production of deuterated indole during tryptophan synthesis.

The pH-dependence of kinetic parameters can sometimes produce distorted values of molecular dissociation constants (pK_a) (Fersht, 1985; Tipton & Dixon, 1979). In the present cases, it seems the kinetic pK values may closely reflect molecular pK_a values of species A, B and C in the enzyme because the available evidence is that serine processing is slow so that binding steps approach equilibrium. This conclusion is strengthened by the obvious symmetry between the $pK_{A,B,C}$ of the free enzyme and $pK'_{A,B,C}$ of the enzyme-tryptophan complexes. In two experiments pK_a values corresponding to groups A and B were indistinguishable and a co-operative model was a satisfactory fit to the data. However, for the $k_{cat,B}/K_{m,B}$ data, which are easiest to fit, there was a preference for independent pK_a values. It seems likely that groups A and B have similar pK values and react in a similar way to perturbations at the active centre of the enzyme although they do not ionize co-operatively.

One of the groups must be B_1 (Lane & Kirschner, 1983b) required to be in the base form to convert the external aldimine to the quinoid form of enzyme-serine.

Early fluorescence studies implicated two ionizations in the binding of *E. coli* β_2 tryptophan synthase to serine (Goldberg *et al.*, 1968). One of these was contributed by a histidine residue of $pK_a 6.9$ (Miles & Kumagai, 1974; Miles *et al.*, 1989), and by sequence homology (Zalkin & Yanofsky, 1982) the remaining candidate residue in the yeast enzyme is His-379. It seems possible that the other member of the A+B pair could be a group on the pyridoxal phosphate coenzyme, possibly the phenolic OH which is required to stabilize aldimine and quinoid

complexes with L-serine. The third group C has a function which fits that of the proposed B_2 (Lane & Kirschner, 1983b); B_2H assists the removal of the OH group from the quinoid form of enzyme-serine and may be responsible for the high rate of the serine exchange reaction through EH forms. According to the formulation of Fig. 7, the acid form of C is also needed to release tryptophan from the enzyme, presumably by catalysis of proton transfer to the tryptophan quinoid. One of the few remaining possibilities at the active site is that this function is contributed by the pyridoxal-binding Lys-384, although the estimated pK_a values are low for a model lysine side chain.

Recent studies of the *E. coli* system by pre-steady-state kinetics (Lane & Kirschner, 1983a,b; Drewe & Dunn, 1985) disclose that serine binds to the enzyme in three significant rate processes. The fastest of these corresponds to the formation of a mixture containing external aldimine and quinoid and two slower steps reflect the formation of two species containing aminoacrylate. The suggested final mechanisms do not contain explicitly pH-dependent enzyme forms and do not admit the breakdown of eqn. (12). In order to account for differences in the reactions of serine in exchange and tryptophan synthesis, it seems plausible that indole does not react exclusively with enzyme aminoacrylates but can bind to earlier enzyme-serine species which have not lost the proton from the bound L-serine.

REFERENCES

- Bailey, C. J. & Turner, P. D. (1983) *Biochem. J.* **209**, 151–157
- Cleland, W. W. (1975) *Biochemistry* **14**, 3220–3224
- Drewe, W. F. & Dunn, M. F. (1985) *Biochemistry* **24**, 3977–3987
- Faeder, E. J. & Hammes, G. G. (1970) *Biochemistry* **9**, 4043–4049
- Fersht, A. R. (1985) *Enzyme Structure and Mechanism*, W. H. Freeman, New York
- Goldberg, M. E., York, S. & Stryer, L. (1968) *Biochemistry* **7**, 3662–3667
- Hyde, C. C., Ahmed, S. A., Padlan, E. A., Miles, E. W. & Davies, D. R. (1988) *J. Biol. Chem.* **263**, 17857–17871
- Keleti, T. (1984) in *Dynamics of Biochemical Systems* (Ricard, J. & Cornish-Bowden, A., eds.), pp. 103–113, Plenum Press, New York and London
- Kirschner, K. & Bisswanger, H. (1976) *Annu. Rev. Biochem.* **45**, 143–166
- Lane, A. N. & Kirschner, K. (1981) *Eur. J. Biochem.* **120**, 379–387
- Lane, A. N. & Kirschner, K. (1983a) *Eur. J. Biochem.* **129**, 561–570
- Lane, A. N. & Kirschner, K. (1983b) *Eur. J. Biochem.* **129**, 571–582
- Miles, E. W. (1979) *Adv. Enzymol. Relat. Areas Mol. Biol.* **49**, 127–186
- Miles, E. W. & Kumagai, H. (1974) *J. Biol. Chem.* **249**, 2843–2851
- Miles, E. W., Kawasaki, H., Ahmed, S. A., Morita, H., Morita, H. & Nagata, S. (1989) *J. Biol. Chem.* **264**, 6280–6288
- Mozarelli, A., Peracchi, A., Rossi, G. L., Ahmed, S. A. & Miles, E. W. (1989) *J. Biol. Chem.* **264**, 15774–15780
- Ogura, H., Arata, Y. & Fujiwara, S. (1967) *J. Mol. Spectrosc.* **23**, 76–85
- Ralston, M. (1981) in *BMDP Statistical Software* (Dixon, W. J., ed.), pp. 305–320, University of California Press, Berkeley
- Srere, P. A. (1987) *Annu. Rev. Biochem.* **56**, 89–124
- Tipton, K. F. & Dixon, H. B. F. (1979) *Methods Enzymol.* **63**, 183–234
- Tsai, M. D., Schleicher, E., Potts, R., Skye, G. E. & Floss, H. G. (1978) *J. Biol. Chem.* **253**, 5344–5349
- Turner, P. D., Loughrey, H. C. & Bailey, C. J. (1985) *Biochim. Biophys. Acta* **832**, 280–287
- Zalkin, H. & Yanofsky, C. (1982) *J. Biol. Chem.* **257**, 1491–1500