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Cooperative and Noncooperative Binding of Pyridoxal 5′-Phosphate to Tryptophan Synthase from *Escherichia coli*†

Peter Bartholmes,‡,§ Kasper Kirschner,* and Hans-Peter Gschwind

ABSTRACT: An improved purification procedure for the β_2 subunit of tryptophan synthase from *Escherichia coli* has led to an essentially pure and stable preparation with a specific enzymatic activity that is 30% higher than the previously reported maximum value. Sedimentation analysis shows that the apo-β_2 subunit is monodisperse and dimeric down to a concentration of 0.02 mg of protein/ml. The binding of pyridoxal 5′-phosphate (pyridoxal-P) to the apo-β_2 subunit and to the α_2–apo-β_2 complex was studied by equilibrium dialysis and spectroscopic titration. Both the β_2 subunit and the $\alpha_2\beta_2$ complex bind 2 mol of pyridoxal-P with no unspecific binding observable at higher concentrations of pyridoxal-P. The binding of pyridoxal-P to the apo-β_2 subunit is cooperative (Hill coefficient $n_H = 1.7$). The data have been fitted to the Adair equation, yielding the apparent microscopic dissociation constants for the complexes with one and two bound ligand molecules. They differ by a factor of 38, suggesting that the apo- and holo-β_2 subunits have distinct conformations. The binding of pyridoxal-P to the α_2–apo-β_2 complex is noncooperative with a value of the dissociation constant intermediate between the two values of the β_2 subunit. This finding suggests that the α subunit may stabilize a third conformational state of the β_2 subunit.

The dimeric β_2 subunit of tryptophan synthase from *Escherichia coli* (L-serine hydro-lyase (adding indole) (EC 4.2.1.20)) contains bound pyridoxal 5′-phosphate (pyridoxal-P[1]) which is the coenzyme participating in synthesis of L-tryptophan from L-serine and indole:

$$\text{indole} + \text{L-serine} \longrightarrow \text{L-tryptophan} + H_2O$$

This reaction is also catalyzed by the $\alpha_2\beta_2$ multienzyme complex with a 50-fold increase in turnover number over the isolated β_2 subunit. The literature on this enzyme has been reviewed recently by Yanofsky and Crawford (1972). The catalytic mechanism of tryptophan synthesis is qualitatively the same for the β_2 subunit and the complex (Faeder and Hammes, 1970, 1971). The heterologous interaction between α and β_2 subunits is thought to involve mutually induced conformational changes which are responsible for the observed simultaneous increases in catalytic efficiency and affinity for substrates (Faeder and Hammes, 1971; Kirschner et al., 1975a; Weischet and Kirschner, 1976). In this work, we investigate the equilibrium binding of pyridoxal-P to the β_2 subunit and the $\alpha_2\beta_2$ complex of tryptophan synthase as a means of probing the effects of heterologous protein–protein interaction on the active site.

Materials and Methods

Materials. Pyridoxal-P was purchased from Serva (Heidelberg) and purified by chromatography on Amberlite XE-64 (Peterson and Sober, 1954). Mutant strains of *E. coli* K12 *trp*A2/F′*trp*A2 and *trp*B8 were kindly donated by Drs. C. Yanofsky and I. P. Crawford. The cells were grown in 300- or 1500-l. fermenters in a minimal salts medium essentially as described by Creighton and Yanofsky (1970). The bacterial paste was stored frozen at −18 °C.

Buffers. Unless stated otherwise, all experiments were performed with buffer A: 0.1 M sodium pyrophosphate (pH 7.5) containing 10^{-4} M EDTA and 10^{-4} M 1,4-dithioerythritol. Other buffers can interfere with the binding of pyridoxal-P. For instance, phosphate is sometimes a competitive inhibitor for the binding of pyridoxal-P (Martinez-Carrion, 1975; Gianfreda et al., 1974), whereas Tris forms a Schiff base with the ligand (Simon and Kröger, 1974). Reaction of the carbonyl group with dithioerythritol is negligible (Lienhard and Jencks, 1966). Buffer B: 0.1 M imidazole-HCl (pH 7.0) containing 10^{-4} M pyridoxal-P, 2×10^{-4} M dithioerythritol, 2×10^{-3} M EDTA, and 10^{-4} M phenylmethylsulfonyl fluoride. Buffer C: 0.1 M potassium phosphate (pH 7.8) with 1×10^{-3} M dithioerythritol, 5×10^{-3} M EDTA, and 5×10^{-4} M phenylmethylsulfonyl fluoride. pH measurements of buffered ammonium sulfate solutions were performed at 22 °C after tenfold dilution with water.

Assays. Enzymatic activity and protein concentration were determined as described previously (Kirschner et al., 1975b).

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[1] Abbreviations used are: Pyridoxal-P, pyridoxal 5′-phosphate; DEAE, diethylaminoethyl; EDTA, (ethylenedinitrilo)tetraacetic acid; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol.

TABLE I: Purification of the β_2 Subunit of Tryptophan Synthase from 900g *E. coli trp*A2/F'*trp*A2.

	Total Protein (g)	10^{-6} × Total Act. (Units)	Specific[b] Act. (Units/mg)	Yield[b] (%)
Step 1: crude extract[a]	76.23	4.04	53	100
Step 2: DEAE-cellulose	8.15	3.31	406	82
Step 3: hydroxylapatite	2.53	2.77	1095	69
Step 4: ammonium sulfate (apoenzyme, 0–21% satn)	0.87	2.28	2628	56
Step 5: ammonium sulfate (holoenzyme, >19% satn)	0.44	1.80	4100	45

[a] Figures in this line are averages of 12 individual preparations. [b] Figures in these columns are averages of ten different preparations.

We observed that the activity of the β_2 subunit is decreased when plastic pipets are used instead of glass pipets. Moreover, the presence of bovine serum albumin (0.5 mg/ml), dithioerythritol (2×10^{-4} M), and EDTA (4×10^{-3} M) in the assay mixture increases the specific activity by approximately 20%. We therefore use only glass pipets for handling enzyme solutions and routinely use buffer C without phenylmethylsulfonyl fluoride but supplemented with 0.5 mg of bovine serum albumin/ml for diluting the β_2 subunit (cf. also Adachi and Miles, 1974). The concentration of pyridoxal-P was determined spectrophotometrically in 0.1 N NaOH at 388 nm (ϵ_{388nm} = 6.6×10^3 M^{-1} cm^{-1}, Peterson and Sober, 1954).

Enzymes. The α subunit of tryptophan synthase was prepared as described previously (Kirschner et al., 1975b). The β_2 subunit was purified from *E. coli trp*A2/F'*trp*A2 as follows: all operations were performed at 0–4 °C. The enzyme was found to be inactivated rapidly when exposed for more than several hours to dithioerythritol in the presence of air and ammonium sulfate. As a general precaution, solid dithioerythritol was added to buffers immediately before use and contact with ammonium sulfate was kept to a minimum. The preparation of cell-free extract (step 1) and the conditions for chromatography on DEAE-cellulose (step 2) were identical to steps 1 and 2 of the procedure used for the α subunit (Kirschner et al., 1975b). The β_2 subunit remained bound to the top of the column and was subsequently eluted with 10 l. of a linear gradient from 0 to 0.4 M NaCl in buffer B (without EDTA). The enzyme appearing at approximately 0.19 M NaCl is supplemented with $1/500$ volume of 1 M potassium phosphate buffer (pH 7.0).

Step 3: the protein solution was applied to a column (7 × 15 cm) of hydroxylapatite (Atkinson et al., 1973) equilibrated with buffer B containing 2×10^{-3} M phosphate but no EDTA. After washing the column with 500 ml of the same buffer, the enzyme was eluted with a linear gradient of 2×10^{-3} to 0.15 M phosphate in 6 l. of buffer B (no EDTA). The enzyme appearing at approximately 0.06 M phosphate was concentrated by adding 66.7 ml of a saturated solution of ammonium sulfate per 100 ml (40% saturation, Di Jeso, 1968) and finally adjusting the pH value to 7.0. The precipitate was dissolved in buffer C to give a final concentration of 70 mg of protein/ml. The pH of the solution was adjusted to 7.8 with 5 N KOH and it was subsequently clarified by centrifugation.

Step 4: the following method is adapted from the procedure devised by Adachi and Miles (1974). A column of Sephadex G-25 (2.5 × 40 cm) was equilibrated with buffer C. After applying 10 ml of 0.01 M hydroxylammonium chloride dissolved in buffer C, followed by 5 ml of buffer C, the protein solution was applied and then eluted with buffer C. The enzyme passes through the zone of hydroxylammonium chloride (where pyridoxal-P is removed as the oxime) and is eluted free of pyridoxal-P, hydroxylammonium chloride, and ammonium sulfate. The ammonium sulfate concentration of the solution of the apo-β_2 subunit (20 mg of protein/ml) was adjusted to 21% by dropwise addition of 2.66 ml of saturated ammonium sulfate solution per 10 ml of enzyme solution. The pH value was adjusted to 7.4 by addition of 5 N KOH. After stirring for 1 h, the precipitated enzyme was collected by centrifugation and dissolved in 10 ml of buffer C.

Step 5: the concentration of pyridoxal-P was adjusted to 2×10^{-3} M and the solution was dialyzed for 4 h against buffer C containing 2×10^{-4} M pyridoxal-P, 10^{-3} M dithioerythritol, 5×10^{-3} M EDTA, and ammonium sulfate at 19% saturation. The protein solution was clarified by centrifugation, supplemented with 20% glycerol, and stored at 4 °C. Table I summarizes the quantitative results. The preparation moves as a single band with two very faint bands of contaminating proteins in sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Laemmli, 1970; Studier, 1973). The specific activity of 4100 units/mg correlates well with the value calculated from the maximum specific activity of the α subunit (Kirschner et al., 1975b) and the molecular weights of the component polypeptide chains (6500 × 29 500/44 500 = 4300, Creighton and Yanofsky, 1970; Adachi et al., 1974).

Apo-β_2 subunit was prepared by resolution of the holoenzyme with 3×10^{-3} M hydroxylammonium chloride and extensive dialysis (De Moss, 1962). The complex was assembled by adding a twofold excess of α subunit to apo-β_2 subunit based on the known molecular weights. These were taken to be 29 500 for the α subunit (Henning et al., 1962; Li and Yanofsky, 1972) and 89 000 for the β_2 subunit (Hathaway and Crawford, 1970; Adachi et al., 1974).

Equilibrium dialysis was performed according to Kirschner et al. (1975b). The concentration of free and bound pyridoxal-P was determined fluorimetrically after appropriate dilution of samples with 0.1 N NaOH, using a Schoeffel RRS 1000 recording fluorimeter (excitation at 450 nm, emission at 516 nm) and a calibration curve. We assume that no pyridoxal-P is bound to the enzyme at pH 13. The enzyme activity after attainment of dialysis equilibrium (2–3 h) was at least 95% of the activity of the controls.

Spectroscopic titration of the apo-β_2 subunit was performed and evaluated as described previously (Kirschner et al., 1975b) with the modification described by Swaney (1971). A Cary Model 15 spectrophotometer was used for measuring difference spectra and a Schoeffel RRS 1000 fluorimeter for the fluorescence titrations. At least 15 min were allowed for attainment of equilibrium after each addition of pyridoxal-P, since the binding involves slow processes (P. Bartholmes and K. Kirschner, unpublished observations).

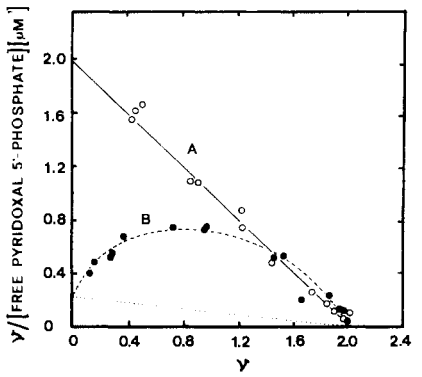

FIGURE 1: Binding of pyridoxal-P to tryptophan synthase: equilibrium dialysis in buffer A, 20 °C. (A, O) 9.25 μM apo-β_2 subunit plus 40 μM α subunit; (—) linear least-squares fit to $\nu/P = (n - \nu)/K$ yielding K = 1.0 μM and n = 2. (B, ●) 12.25 μM apo-β_2 subunit; (- - -) nonlinear least-squares fit to eq 1 yielding $\Psi_1 = 2.3 \times 10^5$ M^{-1} and $\Psi_2 = 5 \times 10^{11}$ M^{-2}; (···) slopes corresponding to $K_{d,1} = 8.7 \times 10^{-6}$ M and $K_{d,2} = 2.3 \times 10^{-7}$ M.

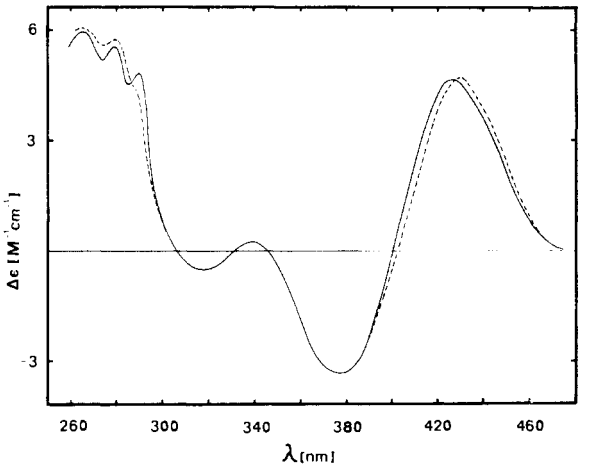

FIGURE 2: Difference spectra between pyridoxal-P bound to the β_2 subunit or the $\alpha_2\beta_2$ complex and free ligand. Buffer A, 20 °C. (- - -) β_2 subunit; (—) $\alpha_2\beta_2$ complex of tryptophan synthase. The enzymes were saturated with 4×10^{-5} M pyridoxal-P.

Results

The β_2 Subunit is a Stable Dimer. The apo-β_2 subunit sediments as a single boundary with $s^\circ_{20,w} = 4.9 \times 10^{-13}$ S, (published value $s_{20,W} = 5.0 \times 10^{-13}$ S, Wilson and Crawford, 1965). Equilibrium sedimentation experiments (Schachman, 1957; Yphantis, 1964) were performed with a Beckman Model E ultracentrifuge equipped with a scanner (λ = 280 nm) to check whether the apo-β_2 subunit dissociates at the concentrations used in the binding studies. A strictly linear dependence of log c vs. r^2 was observed for various loading concentrations ranging from 0.05 to 0.5 mg of protein/ml (data not shown). This is evidence for the homogeneity of the apo-β_2 subunit and the strong association of the monomers. The calculated weight-average molecular weight is $\bar{M}_w$ = 88 400 ± 2000, in agreement with the published values for the holoenzyme (Hathaway and Crawford, 1970; Adachi et al., 1974). Gel filtration studies on calibrated columns of Sephadex G-100 using the same buffer and temperature were performed according to Whitaker (1963) and led to a molecular weight of 86 000 ± 3000 (data not shown).

Equilibrium Dialysis. Figure 1 shows the results of binding studies conducted with pyridoxal-P, the apo-β_2 subunit, and the α_2-apo-β_2 complex. A true thermodynamic equilibrium is established. This is supported by the fact that the concentration of pyridoxal-P in the ligand chamber attains a finite, time-independent value after 2 h of dialysis under conditions for which intermediate degrees of saturation of the apo-β_2 subunit are obtained in the enzyme chamber. Control experiments failed to detect any binding of the ligand to the α subunit alone. The abscissa intercepts of the Scatchard plot show that 1 ± 0.05 molecule of coenzyme is bound per equivalent of β chain, both in the isolated subunit and in the complex. In earlier reports, this stoichiometry varied from 0.7 (Wilson and Crawford, 1965) to 1.6 (York, 1970).

The binding curve of the α_2-apo-β_2 complex of tryptophan synthase is hyperbolic (straight line in the Scatchard plot Figure 1A) with $K_d = (1.0 \pm 0.1)10^{-6}$ M. This indicates that the two binding sites for pyridoxal-P are identical and noninteracting. By steady-state kinetics, Wilson and Crawford (1965) have previously determined the K_M of the $\alpha_2\beta_2$ complex for pyridoxal-P. Its value is identical to the value of the thermodynamic dissociation constant directly determined here. In contrast, the Scatchard plot for the apo-β_2 subunit is convex (Figure 1B), which corresponds to a sigmoidal primary binding curve (i.e., positive cooperativity). The apo-β_2 binding data were fitted to the Adair (1925) equation

$$\nu = \frac{\Psi_1 P + 2\Psi_2 P^2}{1 + \Psi_1 P + \Psi_2 P^2} \quad (1)$$

where ν = mol of pyridoxal-P bound per β_2 subunit, P = concentration of free pyridoxal-P, and the Ψ_i are true thermodynamic parameters (Cornish-Bowden and Koshland, 1970; Buc et al., 1973). As shown in the Appendix, the ordinate intercept and the slopes to the Scatchard plot at $P \rightarrow 0$ and $P \rightarrow \infty$ can be used for obtaining initial estimates of Ψ_1 and Ψ_2. An iterative algorithm (Marquardt, 1963) was used to obtain a least-squares fit of the data to the Adair equation. The best fit was obtained with $\Psi_1 = 2.3 \times 10^5$ M^{-1} ± 18% and $\Psi_2 = 5.0 \times 10^{11}$ M^{-2} ± 18% and the theoretical curve shown in Figure 1B was calculated with these values. The degree of cooperativity is characterized by an Hill coefficient of n_H = 1.7 (which was calculated from the maximum of the Scatchard plot in Figure 1B; Dahlquist, 1974) and by a total free energy of interaction per mol of protomer of ΔF_0 = 2.1 kcal/mol (Whitehead, 1970).

Spectroscopic Titrations. The near-ultraviolet difference spectra between pyridoxal-P bound to the β_2 subunit or the $\alpha_2\beta_2$ complex of tryptophan synthase and free pyridoxal-P are shown in Figure 2. Figure 3 depicts the excitation and emission fluorescence spectra of free pyridoxal-P and the holo-β_2 subunit. The fluorescence excitation spectrum of the bound ligand is red-shifted, as expected from the absorption difference spectrum (Figure 2).

The spectroscopic effects shown in Figures 2 and 3 were used to titrate the apo-β_2 subunit with a concentrated stock solution of pyridoxal-P. The experimental saturation curves are sigmoidal but do not superimpose exactly on the equilibrium dialysis data. This is best shown by plotting the changes in absorbance at 280 nm or in fluorescence at 516 nm (each normalized with respect to the maximum change) against the degree of saturation predicted from the best-fit curve of Figure 1B. Figure 4 demonstrates that the spectroscopically determined binding curves are systematically displaced towards higher concentrations of total pyridoxal-P concentration.

Discussion

The improved purification procedure is a combination of the general strategy for purification of enzymes developed by

Atkinson (Atkinson, 1973; Bruton et al., 1975) and on the elegant procedure based on the large differences in solubility between the apo- and holo-β_2 subunits (Adachi and Miles, 1974). We have found that the latter method is more reproducible and efficient when used with partially purified preparations of the enzyme than with crude extracts.

The enzyme is well suited for physiochemical studies. It is 30% more active than material obtained by other procedures (Wilson and Crawford, 1965; Adachi and Miles, 1974), and does not exhibit "unspecific" binding (York, 1970) beyond 1 mol of pyridoxal-P per β polypeptide chain (Figure 1). The aggregates higher than the dimer described previously for the apo-β_2 subunit by Hathaway (1972) cannot be detected under the conditions studied here. Moreover, the apoenzyme does not dissociate at pH 7.5 even at concentrations as low as 0.02 mg of protein/ml. Under similar conditions, Hathaway et al. (1969) and Hathaway and Crawford (1970) found the apo-β_2 subunit to dissociate reversibly and almost completely to the monomer, and that the bound cofactor strongly stabilizes the dimer. We have, as yet, no explanation for the greater association constant of the apo-β_2 subunit prepared by our procedure.

The effect of binding to the enzyme on the absorption and fluorescence spectra of pyridoxal-P (Figures 2 and 3, respectively) are qualitatively similar to observations made with other pyridoxal-P-dependent enzymes (O'Leary and Malik, 1972; Jones and Cowgill, 1971). Above 310 nm, the spectral changes reflect the fact that a Schiff base is formed between pyridoxal-P and an ϵ-aminolysyl group of the enzyme (Fluri et al., 1971). The positive bands below 310 nm in Figure 2 probably arise from the perturbation of tryptophan and tyrosine side chains. Pyridoxal-P has an absorption minimum at 290 nm (Peterson and Sober, 1954). The differences between the difference spectra of the β_2 subunit and the $\alpha_2\beta_2$ complex are small but reproducible. Similar differences also show up when the apo- and the holo-β_2 subunit are titrated with α subunit (Kirschner et al., 1975a).

The cooperative binding of pyridoxal-P to the apo-β_2 subunit of tryptophan synthase (Figure 1B) has not been observed previously. A unique mechanism for cooperative binding of pyridoxal-P cannot be established from the equilibrium data at hand. However, one can exclude monomer–dimer equilibria from playing a significant role in generating cooperativity (Dunne and Wood, 1975), because both the apo- and the holoenzyme are very stable dimers under the experimental conditions used here. As found with other allosteric enzymes, the phenomenon is likely to arise from the coupling of conformational changes of the two subunits in response to the binding of pyridoxal-P (Kirschner, 1971; Janin, 1973; Hammes and Wu, 1974). The large difference in solubility observed for the apo- and holo-β_2 subunits in ammonium sulfate solutions (Adachi and Miles, 1974) is consistent with the notion of ligand-induced changes of the conformation.

The spectroscopic titrations are in qualitative agreement with the equilibrium binding curve measured by equilibrium dialysis. They show cooperative binding of pyridoxal-P but are systematically displaced toward higher ligand concentrations (Figure 4). Although the quantitative evaluation of spectroscopic titrations is very sensitive to the extrapolated plateau value (Halfman and Nishida, 1972), the relatively large deviations of the absorption data at low degrees of saturation appear to be outside of the experimental error. Cooperative binding curves obtained by spectroscopic titration may deviate from the behavior predicted by the Adair equation (eq 1) because more than one state exists for the bound ligand. This is

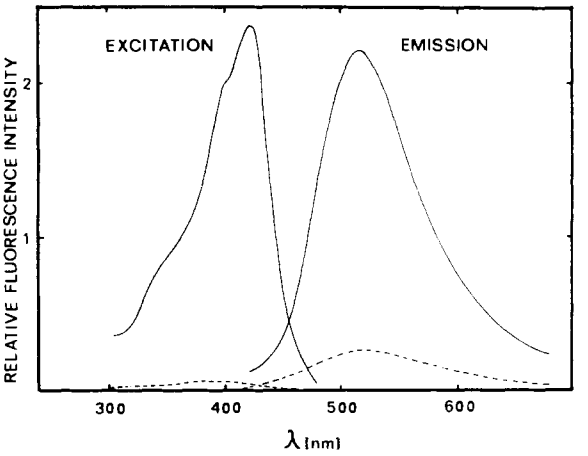

FIGURE 3: Effect of the apo-β_2 subunit on the fluorescence of pyridoxal-P. Buffer A, 20 °C, 1-cm path length. (—) 11.2 μM β_2 subunit; excitation measured at 516 nm, emission excited at 420 nm. (- - -) 50 μM pyridoxal-P; excitation measured at 516 nm, emission excited at 388 nm.

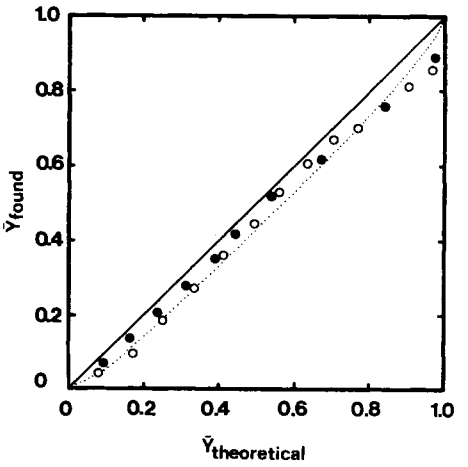

FIGURE 4: Comparison of spectroscopic titration data with results from equilibrium dialysis. Buffer A, T = 20 °C. (○) Normalized difference absorption: 35.5 μM apo-β_2 subunit was titrated with a 0.7 mM pyridoxal-P stock solution at 280 nm (cf. Figure 2) in tandem cuvettes with 4.4-mm path lengths for each compartment. The theoretical degree of saturation for each known total concentration of pyridoxal-P was calculated for 71 μM binding sites with eq 1 and $\Psi_1 = 2.3 \times 10^5$ M^{-1}, $\Psi_2 = 5 \times 10^{11}$ M^{-2}. (●) Normalized fluorescence enhancement: 23 μM apo-β_2 subunit was titrated as above in 2 × 10 mm fluorescence cuvettes; excitation at 450 nm, emission at 516 nm. The theoretical degree of saturation was calculated as above. (—) Predicted behavior for exact correspondence of spectroscopic change to amount of pyridoxal-P bound ($\varkappa$ = 1). (···) Calculated curve for the sequential mechanism with $\varkappa$ = 0.1 (eq 2).

true either for the sequential mechanism or for the nonexclusive concerted model (Koshland, 1970; Monod et al., 1965). Thus, the deviations between the binding curves obtained by the two methods (Figure 4) could be due to differences of extinction coefficients (or quantum yields) of the first and second molecule of bound pyridoxal-P. The latter assumption is supported by the fact that the difference spectrum (Figure 2) is generated almost entirely in slow first-order processes observed after mixing the apo-β_2 subunit with high concentrations of pyridoxal-P in a stopped-flow apparatus (P. Bartholmes and K. Kirschner, unpublished experiments). This means that the initial enzyme–pyridoxal-P complex (e.g., β_2P) has practically the same extinction coefficient as free pyridoxal-P (i.e., $\Delta\epsilon_1 \sim 0$). For illustrative purposes we have assumed that the sequential mechanism is valid and that the extinction coefficient

(or quantum yield) of β_2P ($\Delta\epsilon_1$) is less than that of β_2P_2 ($\Delta\epsilon_2$):

$$\beta_2 + P \rightleftharpoons \beta_2P;\ K_{d,1} = \frac{2[\beta_2][P]}{[\beta_2P]}$$

$$\beta_2P + P \rightleftharpoons \beta_2P_2;\quad K_{d,2} = \frac{[\beta_2P]\,[P]}{2[\beta_2P_2]}$$

where β_2 is the apo-β_2 subunit and P = pyridoxal-P. It can be shown easily that the spectroscopic saturation curve must obey the following equation

$$\frac{\Delta E}{\Delta E_{max}} = \frac{\kappa\Psi_1P_1 + 2\,\Psi_2P^2}{2\,(1 + \Psi_1P + \Psi_2P^2)} \qquad (2)$$

where $\kappa = \Delta\epsilon_1/\Delta\epsilon_2$, P is the concentration of free pyridoxal-P, $K_{d,1} = 2/\Psi_1 = 8.7 \times 10^{-6}$ M and $K_{d,2} = \Psi_1/2\Psi_2 = 2.3 \times 10^{-7}$ M (cf. Appendix). The diagonal in Figure 4 corresponds to $\kappa = 1$. Somewhat more involved expressions can be derived for the concerted mechanism but they predict the same qualitative deviation for $\kappa = \Delta\epsilon_T/\Delta\epsilon_R < 1$ as shown for the sequential mechanism. The theoretical curve for $\kappa = 0.1$ accounts reasonably well for the titration data obtained from absorbance measurements, supporting the qualitative notion that there is a correlation between the extinction coefficient and association constant of pyridoxal-P bound to the different (conformational) states of the β_2 subunit. The fluorescence data must be interpreted more cautiously.

The binding of the α subunit to the apo-β_2 subunit converts the curved Scatchard plot of the apo-β_2 subunit to a straight line (Figure 1). Moreover, the concentration of free pyridoxal-P required to half saturate the binding sites decreases from 1.3×10^{-6} to 1×10^{-6} M. The α subunit, therefore, is a positive effector for the β_2 subunit (Monod et al., 1965).

However, the apparent dissociation constant of the second coenzyme molecule ($K_{d,2} = 2.3 \times 10^{-7}$ M) of the β_2 subunit is fourfold smaller than the dissociation constant of the α_2-holo-β_2 complex ($K_d = 1 \times 10^{-6}$ M, cf. Figure 1). The interactions between the apo-β_2 subunit, on the one hand, and its ligands α subunit and pyridoxal-P, on the other, are summarized in the following scheme:

$$\begin{array}{ccc} \textcircled{1} & & \textcircled{2} \\ 2\alpha + 2P + \beta_2 & \rightleftharpoons & 2\alpha + \beta_2P_2 \\ \Downarrow & & \Downarrow \\ 2P + \alpha_2\beta_2 & \rightleftharpoons & \alpha_2\beta_2P_2 \\ \textcircled{3} & & \textcircled{4} \end{array}$$

where α = α subunit, P = pyridoxal-P, and β_2 = apo-β_2 subunit.

The binding of α subunit to the apo-β_2 subunit (transition 1 → 3) is weaker than its association with the holo-β_2 subunit (Goldberg et al., 1966; Adachi et al., 1974). This has been demonstrated indirectly by the fact that the apo-β_2 subunit is capable of activating the α subunit in the indoleglycerol phosphate → indole + glyceraldehyde 3-phosphate reaction (Wilson and Crawford, 1965; Creighton and Yanofsky, 1966). Because the enzyme concentrations used in our binding studies are at least 100 times greater than those employed in the standard enzyme assays, it appears reasonable to assume that transition 3 → 4 mainly involves the α_2-apo-β_2 complex of tryptophan synthase. Because the transition 1 → 2 is cooperative, the apo-β_2 and the holo-β_2 subunits (states 1 and 2, respectively) differ in their intrinsic affinities for pyridoxal-P. Since the binding of the coenzyme to the α_2-apo-β_2 complex is noncooperative (transition 3 → 4), the β_2 subunit in this case appears to be constrained in a third conformation of intermediate affinity for the ligand. The α_2-holo-β_2 complex (state 4) is also a 50-fold better catalyst than the holo-β_2 subunit (state 2, Yanofsky and Crawford, 1972). Moreover, the increase of catalytic efficiency in the transition 2 → 4 depends on a crucial isomerization step (Kirschner et al., 1975a). The scheme predicts that the equilibrium binding of the α subunit to apo-β_2 subunit (transition 1 → 3) must also be positively cooperative or that the transition 2 → 4 is negatively cooperative. These predictions are currently being tested.

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Appendix

The function describing convex Scatchard plots in terms of the Adair parameters Ψ_i is not explicitly known. However, it is possible to express ν and ν/P as functions of the denominator (D) of eq 1

$$D = 1 + \Psi_1 P + \Psi_2P^2 \ldots + \Psi_nP^n \qquad (3)$$

After differentiating D with respect to P ($dD/dP = \dot{D}$, $d^2D/dP^2 = \ddot{D}$)

$$\nu = P\dot{D}/D \qquad (4)$$

and

$$\nu/P = \dot{D}/D \qquad (5)$$

The slope at any point of the Scatchard plot is given by

$$\frac{d(\nu/P)}{d\nu} = \frac{d\,(\nu/P)}{dP}\Big/\frac{d\nu}{dP} \qquad (6)$$

After differentiating eq 3 and 4 with respect to P and inserting into eq 5

$$\frac{d(\nu/P)}{d\nu} = \frac{D\ddot{D} - \dot{D}^2}{P(D\ddot{D} - \dot{D}^2) + D\dot{D}} \qquad (7)$$

For the limiting conditions of $P \to 0$ and $P \to \infty$ we obtain for the slopes

$$\lim\left(\frac{d(\nu/P)}{d\nu}\right)_{P\to 0} = (2\Psi_2/\Psi_1) - \Psi_1 \qquad (8)$$

and

$$\lim\left(\frac{d(\nu/P)}{d\nu}\right)_{P\to\infty} = -n\Psi_n/\Psi_{n-1} = -K_{d,n} \qquad (9)$$

and for the intercepts

$$\lim\,(\nu/P)_{P\to 0} = \Psi_1 = n/K_{d,1} \qquad (10)$$

$$\lim\,(\nu)_{P\to\infty} = n \qquad (11)$$

These relationships have also been derived by a Taylor's series expansion of $\nu = f(P)$ by Schwarz (1976).

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