# Subunit Interactions of Tryptophan Synthase from *Escherichia coli* As Revealed by Binding Studies with Pyridoxal Phosphate Analogues†

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ABSTRACT: An improved purification procedure for the $\alpha_2\beta_2$ complex of tryptophan synthase from *Escherichia coli* has been developed. It consists of DEAE-Sephacel chromatography, followed by hydrophobic chromatography on Sepharose CL 4B, and leads to material with a higher specific activity than reported previously. Inhibition studies, equilibrium dialysis, and spectrophotometric titration were used to study the binding both of pyridoxal phosphate analogues and of bisubstrate analogues. Pyridoxine 5′-phosphate and *N*-phosphopyridoxyl-L-serine bind to the enzyme, but pyridoxamine 5′-phosphate and *N*-phosphopyridoxyl-L-alanine do not. *N*-Phosphopyridoxyl-L-tryptophan is bound only weakly, although L-tryptophan binds strongly to the $\alpha_2$holo$\beta_2$ complex. It is likely that either differences in protonation or in geometry are responsible for the low affinity of the bisubstrate analogues in comparison to that of the external aldimines of either L-serine or L-tryptophan with pyridoxal 5′-phosphate. As previously found with pyridoxal 5′-phosphate, pyridoxine 5′-phosphate, and *N*-phosphopyridoxyl-L-serine bind noncooperatively to two identical binding sites in the $\alpha_2$apo$\beta_2$ complex. The same ligands bind with positive cooperativity to two binding sites in the apo$\beta_2$ subunit. Because the analogues mimic the binding behavior of pyridoxal 5′-phosphate to both proteins, the internal aldimine of pyridoxal 5′-phosphate to the lysine amino group contributes only to the strength of that binding. The nicked apo$\beta_2$ subunit, which is produced by limited proteolysis with trypsin, binds pyridoxine 5′-phosphate noncooperatively to two identical sites. Therefore, the loop of polypeptide chain connecting the two autonomous domains of folding must be intact for enzyme activity, for the binding of the $\alpha$ subunit, and for cooperative binding of pyridoxine 5′-phosphate.

Tryptophan synthase from *Escherichia coli* is a bienzyme complex [L-serine hydro-lyase (adding indoleglycerol-phosphate), EC 4.2.1.20]. It contains bound pyridoxal 5′-phosphate (PLP)[1] which participates in the synthesis of L-tryptophan from L-serine and indole (Yanofsky & Crawford, 1970; Miles, 1979):

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

The complex has an $\alpha_2\beta_2$ structure and can be assembled from the $\alpha$ subunit and the tightly associated $\beta_2$ subunit, yielding a complex indistinguishable from the native enzyme. The general interest in this system arises from the way in which various properties of the subunits change when they associate with each other (heterologous subunit interactions). The mechanisms of catalysis of both the $\alpha$ and the $\beta_2$ subunit appear to be the same in the isolated and the associated forms. The catalytic efficiencies ($k_{cat}$) and the substrate affinities ($K_M$) are dramatically different, however. It is probable that these changes are caused by conformational changes that are mutually induced during the assembly (Faeder & Hammes, 1970, 1971; Weischet & Kirschner, 1976).

The reversible binding of specific ligands is also altered by heterologous subunit interactions (Goldberg & Baldwin, 1967; Faeder & Hammes, 1970, 1971; Kirschner et al., 1975). The binding curve of PLP is particularly interesting since it is sigmoidal (positive cooperativity, homologous subunit interactions) with the apo$\beta_2$ subunit and hyperbolic (noncooperativity) with the $\alpha_2$apo$\beta_2$ complex (Bartholmes et al., 1976). Thus, $\alpha$–$\beta$ interactions modulate $\beta$–$\beta$ interactions, and it is important to understand (a) by what mechanism cooperative binding occurs to the isolated apo$\beta_2$ subunit and (b) how the mechanism is changed in the $\alpha_2$apo$\beta_2$ complex.

Limited proteolysis of the holo$\beta_2$ subunit with trypsin generates autonomously folding structural domains ($F_1$ and $F_2$) that remain tightly associated as an $(F_1F_2)_2$ complex. This artificial multiprotein complex, having no detectable catalytic activity and altered ligand binding properties (Högberg-Raibaud & Goldberg, 1977; Decastel & Goldberg, 1978; Goldberg & Zetina, 1980), represents a third form of the $\beta_2$ subunit that is of potential interest for studying interdomain interactions in a native protein (Ptitsyn, 1978).

Ligand binding studies with equilibrium and kinetic methods are potentially useful methods for elucidating the underlying mechanisms. Because it forms an internal aldimine with a lysine amino group, PLP presents special problems as a probing ligand. By contrast, PNP and PMP are potentially useful PLP analogues, which cannot form an internal aldimine.

Various *N*-phosphopyridoxyl amino acids have been used as nonreactive bisubstrate analogues that specifically inhibit PLP binding to a number of PLP-dependent enzymes (Ikawa, 1967; Churchich et al., 1975; Borri-Voltattorni et al., 1975). Several catalytic intermediates have been detected by kinetic and spectroscopic studies, and tentative chemical structures have been proposed for these intermediates (Miles et al., 1968; Goldberg & Baldwin, 1967; Raibaud & Goldberg, 1976; Goldberg et al., 1968; York, 1972; Miles, 1979). The accumulated evidence indicates that PPS and PPT are analogues of the bound substrate L-serine and the bound product L-tryptophan. PPA is the bisubstrate analogue of the external aldimine of aminoacrylate with PLP presumably formed in the course of the indole → tryptophan reaction (Yanofsky &

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[1] Abbreviations used: PLP, pyridoxal 5′-phosphate; PNP, pyridoxine 5′-phosphate; PMP, pyridoxamine 5′-phosphate; PPA, *N*-phosphopyridoxyl-L-alanine; PPS, *N*-phosphopyridoxyl-L-serine; PPT, *N*-phosphopyridoxyl-L-tryptophan; DEAE, diethylaminoethyl; EDTA, (ethylenedinitrilo)tetraacetic acid; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol.

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Crawford, 1970; Miles, 1979).

These chromophoric ligands are used in this and the following paper (Tschopp & Kirschner, 1980) as tools for detecting qualitative and quantitative differences between the active sites of the nicked apo$\beta_2$ subunit, the intact apo$\beta_2$ subunit, and the $\alpha_2$apo$\beta_2$ complex of tryptophan synthase.

## Materials and Methods

*Materials.* PLP and phenylmethanesulfonyl fluoride were purchased from Serva (Heidelberg). PLP was used without further purification. DTE, PMP, ammonium sulfate, and ammonium hydrogen carbonate were purchased from Merck (Darmstadt). DEAE-Sephadex A-25, DEAE-Sephacel, and Sepharose CL 4B were products of Pharmacia (Uppsala). Tritium-labeled sodium borohydride was obtained from New England Nuclear. All other chemicals were of the highest degree of purity available from Merck (Darmstadt) and Fluka (Buchs). The mutant strains of *E. coli* W3110 *trp*R$^-$-$\Delta$*trp*LD102/F'$\Delta$*trp*LD102 were gifts from Drs. C. Yanofsky and I. P. Crawford. The cells were grown in a 100-L fermenter in a medium described by Adachi et al. (1974) supplemented with 30 $\mu$g of L-tryptophan/L. Bacteria were grown for 10–12 h at 37 °C to $2 \times 10^9$ cells/mL and disrupted with a Manton Gaulin Laboratory homogenizer.

*N-Phosphopyridoxyl Amino Acids.* The following method differs from that of Korytnyk & Ikawa (1970) in removing borate from the reaction mixture before purification of the products. Borate forms complexes with PLP and PNP (Stock et al., 1966). All operations were performed under yellow light to prevent photolysis. A total of 0.5 mmol each of PLP (free acid) and the corresponding L-amino acid were dissolved in 5 mL of water, and 1.5 mmol of 50% KOH was added. After 15 min of stirring at 25 °C, we added 2.5 mmol of solid $NaBH_4$ to the solution, which immediately turned colorless. After acidification with 12% HCl to pH 1 and removal of boric acid (Maire & Day, 1972), the mixture was adjusted to pH 8 with 5 N KOH and chromatographed on a column of DEAE-Sephadex A-25 (2.6 × 40 cm) equilibrated with 0.09 M $NH_4HCO_3$. The column was developed with 2 L of a gradient of increasing concentration of $NH_4HCO_3$ from 0.09 to 0.11 M. Fractions were analyzed by thin-layer chromatography on cellulose plates with 1-butanol–acetic acid–water (15:10:10 v/v) as the solvent (Ahrens & Korytnyk, 1970; McCoy et al., 1979). The pooled fractions were lyophilized, and the products were characterized by UV, NMR, and combustion analysis (Tschopp, 1978).

To synthesize PNP, we dissolved 0.5 mmol of PLP (free acid) in 5 mL of water and added 1 mmol of 50% KOH. The subsequent steps were identical with those used for the synthesis of phosphopyridoxyl amimo acids. Synthesis of tritium-labeled analogues were carried out by the same procedure, using $NaBT_4$.

*Assays.* Enzymic activity and protein concentration were determined as described by Miles & Moriguchi (1977). One unit of enzyme is defined as the amount catalyzing the synthesis of 0.1 $\mu$mol of tryptophan in 20 min at 37 °C (Creighton & Yanofsky, 1970). The concentrations of PLP and PMP were determined spectrophotometrically (Peterson & Sober, 1954). Molar concentrations of PNP and the phosphopyridoxyl amino acids required for calculating absorption coefficients were determined by phosphate analysis (King, 1932). Concentrations of analogues were determined spectrophotometrically in 0.1 M sodium phosphate, pH 7.5 (PNP, $\epsilon_{323} = 7.4 \times 10^3$ M$^{-1}$ cm$^{-1}$; PPS, $\epsilon_{325} = 8.4 \times 10^3$ M$^{-1}$ cm$^{-1}$; PPA, $\epsilon_{323} = 6.8 \times 10^3$ M$^{-1}$ cm$^{-1}$; PPT, $\epsilon_{331} = 7.6 \times 10^3$ M$^{-1}$ cm$^{-1}$).

*Buffers.* Buffer A is 0.1 M potassium phosphate (pH 7.5) containing $5 \times 10^{-3}$ M EDTA and $2 \times 10^{-4}$ M DTE. Buffer B is 0.05 M Tris-HCl (pH 7.5) with $5 \times 10^{-3}$ M EDTA, $2 \times 10^{-4}$ M DTE, $10^{-4}$ M PLP, $10^{-4}$ M phenylmethanesulfonyl fluoride, and $5 \times 10^{-3}$ M L-serine. Buffer C is 0.05 M sodium phosphate (pH 7.0) containing $5 \times 10^{-3}$ M EDTA, $2 \times 10^{-4}$ M DTE, $10^{-4}$ M PLP, and $5 \times 10^{-3}$ M L-serine.

*Enzyme Preparation.* All operations were performed at 0–4 °C. The preparation of cell-free extract was identical with step 1 of the procedure used for the $\alpha$ subunit (Kirschner et al., 1975). In step 2, 600 g wet weight of DEAE-Sephacel was equilibrated with 1 L of buffer B, washed with water, and sucked almost dry on a Buechner funnel. This material was added to the crude extract supplemented with $5 \times 10^{-3}$ M EDTA, $2 \times 10^{-4}$ M DTE, $10^{-4}$ M PLP, and $5 \times 10^{-3}$ M L-serine and was stirred for 20 min. The slurry was added to the top of a packed DEAE-Sephacel column (10 × 45 cm), equilibrated with buffer B. The column was washed with 1.5 L of buffer B, followed by 1.5 L of buffer B containing 0.07 M NaCl, and eluted with 10 L of a linear gradient of 0.07–0.35 M NaCl in buffer B. The excess $\alpha$ subunit was eluted at ~0.07 M NaCl, and the $\alpha_2\beta_2$ complex was eluted at ~0.19 M NaCl. The pooled $\alpha_2\beta_2$ complex fractions were adjusted to pH 7 with 5 N acetic acid and adjusted to 1.4 M with ammonium sulfate.

In step 3, the protein was applied to a column (7 × 60 cm) of Sepharose CL 4B equilibrated with buffer C containing 1.4 M ammonium sulfate. After the column was washed with 1.5 L of the same buffer, the enzyme was eluted with 4 L of a linear gradient of 1.4–0.7 M ammonium sulfate in buffer C. The enzyme, eluting at ~1.3 M ammonium sulfate, was concentrated with an Amicon PM-10 ultrafilter to 15–20 g of protein/L, clarified by centrifugation, and dialyzed against buffer A supplemented with $4 \times 10^{-5}$ M PLP, $10^{-3}$ M L-serine, and $10^{-3}$ M $NaN_3$. The enzyme can be stored under these conditions at 4 °C for several weeks with no loss of activity. It is less stable when precipitated with ammonium sulfate.

The excess free $\alpha$ subunit eluted at low salt concentration on DEAE-Sephacel was purified further by published procedures (Kirschner et al., 1975; Gschwind et al., 1979). Alternatively, it was obtained from the complex by precipitating the $\beta_2$ subunit at pH 4.5 (Henning et al., 1962). Free $\beta_2$ subunit was prepared from the complex shortly before use by eliminating the $\alpha$ subunit with a heat step (Högberg-Raibaud & Goldberg, 1977; Wilson & Crawford, 1965). Apo$\beta_2$ subunit was obtained from holo$\beta_2$ subunit by resolution with hydroxylamine (Bartholmes et al., 1976). The $\alpha_2$apo$\beta_2$ complex was assembled from the component subunits, adding a 20% molar excess of $\alpha$ subunit to counteract possible dissociation of the complex at low concentrations (Creighton & Yanofsky, 1966). The concentration of the pure subunits was determined as described by Kirschner et al. (1975). The extinction coefficient of the pure, stoichiometrically assembled $\alpha_2$apo$\beta_2$ complex was obtained by suitable weighting of the known extinction coefficients of the $\alpha$ and apo$\beta_2$ subunits: $E_{280}^{0.1\%} = 0.70$ cm$^2$ mg$^{-1}$ in 0.1 N NaOH; $E_{280}^{0.1\%} = 0.596$ cm$^2$ mg$^{-1}$ in 0.1 M Tris-HCl, pH 7.2 (Hathaway, 1972).

Nicked apo$\beta_2$ subunit was prepared by limited proteolysis with trypsin as described by Högberg-Raibaud & Goldberg (1977). Because the sedimentation coefficients of the nicked apoprotein ($s_{20,w} = 4.6 \pm 0.1$ S) is similar to that of the nicked holoprotein (4.65 S) and because the two forms of the protein are eluted from Sephadex G-100 at the same elution volume in 0.1 M potassium phosphate buffer, pH 7.5, and 4 °C, we conclude that removal of PLP does not lead to a dissociation

Table I: Purification of the $\alpha_2\beta_2$ Complex of Tryptophan Synthase[a]

| fraction | total protein (g) | $10^{-6}$ × total act.[b] (units) | sp act. (units/mg) | yield (%) |
|---|---|---|---|---|
| step 1: crude extract | 56.7 | 12.8 | 225 | 100 |
| step 2: DEAE-Sephacel chromatography | 13.0 | 8.8 | 671 | 69 |
| step 3: hydrophobic chromatography | 3.2 | 6.95 | 2175 | 54 |

[a] The crude extract was obtained from 550 g of fresh bacteria. [b] Activities were measured in the absence of either excess $\alpha$ or excess $\beta_2$ subunit.

of the $(F_1F_2)_2$ complex. The specific extinction coefficient ($E_{280}^{0.1\%}$ = 0.606 cm$^2$ mg$^{-1}$) of the nicked apo$\beta_2$ subunit in 0.1 M phosphate, pH 7, was obtained by suitable weighting of the extinction coefficients reported for the isolated fragments $F_1$ and $F_2$ (Högberg-Raibaud & Goldberg, 1977).

*Steady-state kinetics* were performed in buffer A (Wilson & Crawford, 1965). Since the binding of PLP involves slow processes (Bartholmes et al., 1980), PLP ($2 \times 10^{-7}$–$1 \times 10^{-4}$ M) and the competing analogue ($5 \times 10^{-5}$–$3 \times 10^{-4}$ M) were added and the mixtures were incubated at 4 °C in the dark for 6 h. The enzyme reaction was started at 20 °C with a mixture of L-serine and indole in buffer A and monitored at 290 nm with a Cary 118 CX spectrophotometer. The final concentration of L-serine was $2.5 \times 10^{-2}$ M ($K_M = 2 \times 10^{-4}$ M) and for indole it was $4 \times 10^{-4}$ M ($K_M = 1.8 \times 10^{-5}$ M).

*Spectroscopic titrations* were performed and evaluated as described previously (Kirschner et al., 1975; Bartholmes et al., 1976). A Cary Model 118 CX spectrophotometer was used for measuring difference spectra. At least 15 min were allowed for attainment of equilibrium after each addition of analogue since the binding involves slow processes (Tschopp & Kirschner, 1980).

*Equilibrium dialysis* was performed according to Kirschner et al. (1975) with $^3$H-labeled compounds. Control experiments showed that none of the ligands binds to the $\alpha$ subunit alone. In contrast to titration data, the abscissa intercepts of the Scatchard plots indicate less than 1 mol (0.70–0.95) of either PPS or PNP bound per equivalent of $\beta$ chain at saturation. This could be due either to a loss of enzyme activity or to enzyme dilution or to both during the dialysis run. Controls showed that the enzymes retained at least 90% of their activity. The dilution of protein was ~5–10%.

## Results

*Purification of the $\alpha_2\beta_2$ Complex of Tryptophan Synthase.* The previously reported method for purifying the $\alpha_2\beta_2$ complex of tryptophan synthase is time consuming and not easily reproducible (Adachi et al., 1974). Therefore, a more rapid and reproducible method was devised (Table I). Our current studies of different enzymes from the pathway of tryptophan biosynthesis (Bisswanger et al., 1979) require a method for purifying these enzymes from the same batch of bacteria and their separation in an early step of the purification procedure (Atkinson et al., 1973). Chromatography of the cell-free crude extract on DEAE-Sephacel separates phosphoribosyl-anthranilate isomerase:indoleglycerol-phosphate synthase from both the excess $\alpha$ subunit and the $\alpha_2\beta_2$ complex. The presence of 5 mM L-serine and 0.1 mM PLP in the gradient buffers is essential for preventing partial dissociation of the complex into free $\alpha$ subunit and the $\alpha\beta_2$ subcomplex (Creighton & Yanofsky, 1966; Adachi et al., 1974). When PLP and L-serine are omitted, the $\alpha\beta_2$ complex is eluted as a separate peak behind the $\alpha_2\beta_2$ complex (data not shown), probably because Tris forms an aldimine with PLP (Simon & Kröger, 1974).

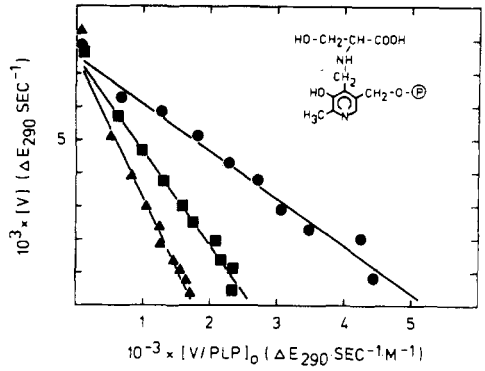



FIGURE 1: PPS is a competitive inhibitor of the PLP binding to the $\alpha_2$apo$\beta_2$ complex of tryptophan synthase. Initial velocity measurements were carried out as described under Material and Methods. PLP was varied in the absence or presence of inhibitor to obtain different initial velocities as shown in the figure. The buffer (A) was 0.1 M potassium phosphate, pH 7.5, containing 5 mM EDTA and 0.2 mM DTE. $T$ = 20 °C. The figure shows a plot of $v$ vs. $v$/[PLP] according to eq 1. (●) No inhibitor; (■) 0.147 mM PPS; (▲) 0.291 mM PPS. Concentration of enzyme: 2 $\mu$M $\beta$ site equiv; 2.4 $\mu$M $\alpha$ equiv. $K_d^{PLP}$ equals 1.4 $\mu$M. The value of $K_i$ was calculated from eq 1 and equals 0.15 mM.

The $\alpha_2\beta_2$ complex is obtained in essentially pure form by subsequent hydrophobic chromatography on Sepharose CL 4B in the presence of ammonium sulfate (von der Haar, 1976, 1979). Neither the $\alpha$ subunit nor the $\beta_2$ subunit can be adsorbed and eluted in this manner.

The isolated $\alpha$ and $\beta_2$ subunits are best prepared shortly before use from the purified complex, because it is better suited for prolonged storage than the subunits themselves. The described procedures lead to maximally active subunits and also remove traces of impurities contained in the preparation of the $\alpha_2\beta_2$ complex.

*Competitive Inhibition Studies.* Because specific binding of phosphopyridoxyl amino acids should lead to competitive inhibition with respect to PLP, we first used steady-state kinetics to screen the potential bisubstrate analogues for strong binding to the $\alpha_2$apo$\beta_2$ complex. When the concentrations of both indole and L-serine are much larger than the corresponding $K_M$ values, the velocity of tryptophan formation depends only on the concentration of PLP and on the concentration of the competitive inhibitor I (Orlacchio et al., 1979). It is convenient to plot the data according to Eadie-Hofstee, as given by

$$v = V - K_d^{PLP}(1 + [I]/K_i)(v/[PLP]) \quad (1)$$

where $v$ is the initial velocity, $V$ is the maximal velocity, $K_d^{PLP}$ is the dissociation constant of the enzyme–PLP complex, and $K_i$ is the dissociation constant of the enzyme–inhibitor complex. As seen by the linear plots in Figure 1, the binding of PLP to the $\alpha_2\beta_2$ complex of tryptophan synthase obeys the simple mass action law (Wilson & Crawford, 1965). As predicted by eq 1, $V$ is independent of PPS concentration, but the apparent equilibrium dissociation constant (i.e., the slope of the straight lines) increases with increasing PPS concentration. $K_d^{PLP}$ equals 1.4 $\mu$M, and $K_i$ equals 0.15 mM.

Similar studies revealed that also PNP is a competitive inhibitor for PLP binding ($K_i$ equals 0.11 mM). Surprisingly, PPT binds only weakly and influences both $K_d^{PLP}$ and V (mixed inhibition). No inhibition was detected either with PMP or with PPA.

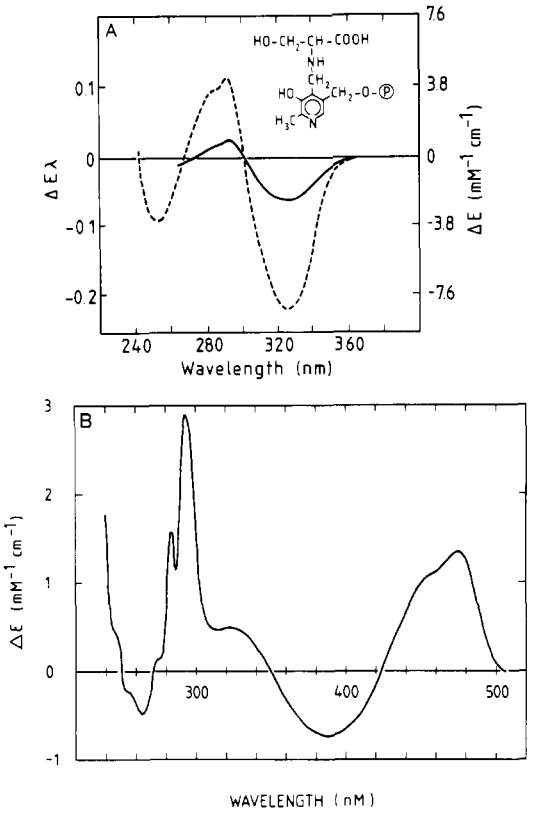



FIGURE 2: (A) Difference spectra between PPS bound either to the apo$\beta_2$ subunit or to the $\alpha_2$apo$\beta_2$ complex and the free ligand. Buffer A; $T$ = 20 °C: $d$ = 0.44 cm. (- - -) Concentration of apo$\beta_2$ subunit: 0.1 mM $\beta$ site equiv. Concentration of PPS: 0.393 mM. Calculated saturation: 60%. (—) Concentration of $\alpha_2$apo$\beta_2$: 0.1 mM $\alpha\beta$ site equiv. Concentration of PPS: 0.401 mM. Calculated saturation: 60%. (B) Difference spectrum between L-tryptophan bound to the $\alpha_2$holo$\beta_2$ complex and free L-tryptophan. Buffer A; $T$ = 20 °C. The spectra were measured with 0.1 mM site equiv and 0.52 mM L-tryptophan in 2-mm tandem cuvettes. The normalized difference spectrum was calculated with the known dissociation equilibrium constant [$K_d^{Trp}$ = 0.09 mM (cf. Figure 6)], giving a saturation of 80%.

*Binding Studies with the $\alpha_2$apo$\beta_2$ Complex.* The binding of PPS, PPT, and PNP was studied more directly by spectrophotometric titration and equilibrium dialysis. Although the fluorescence emission at 328 nm of the apo$\beta_2$ subunit (excitation at 278 nm) is quenched by bound PNP and PPS, and the fluorescence emission of the bound ligands at 398 nm is enhanced, probably by radiationless energy transfer (Stryer, 1978), these spectroscopic changes are not useful for the determination of dissociation constants of ~1 mM. In contrast, the pronounced absorbance difference spectrum of PPS shown in Figure 2A (drawn-out curve) is well suited for the purpose. The difference spectrum of bound PNP is similar (not shown here).

Only a weak and featureless difference spectrum is observable for bound PPT under the conditions of Figure 2A (data not shown). In contrast, the well-developed difference spectrum presented in Figure 2B shows that L-tryptophan binds to the $\alpha_2$holo$\beta_2$ complex under the same conditions, presumably as the external aldimine.

The binding data for both PPS and PNP as obtained by spectrophotometric titration and equilibrium dialysis are shown in Figures 3 and 4 (filled and empty circles) in the form of Scatchard plots. As previously found for PLP (Bartholmes et al., 1976), both PPS and PNP bind noncooperatively to two identical and noninteracting sites as given by

$$C + L \rightleftharpoons CL \qquad (2)$$

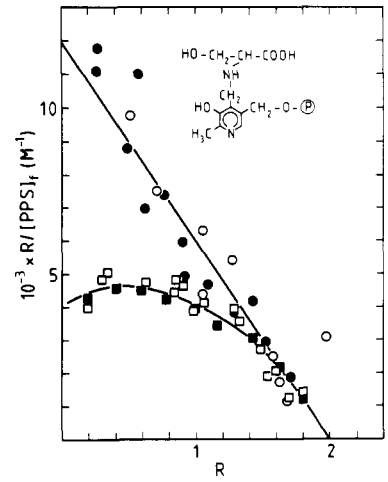



FIGURE 3: PPS binds noncooperatively to the $\alpha_2$apo$\beta_2$ complex and with positive cooperativity to the apo$\beta_2$ subunit of tryptophan synthase: Scatchard plots of spectrophotometric titration (● and ■) and equilibrium dialysis (○ and □) in buffer A, $T$ = 20 °C. (●) 0.10 mM site equiv of apo$\beta_2$ subunit and 0.12 mM $\alpha$ subunit; (○) 0.05 mM site equiv of apo$\beta_2$ subunit and 0.06 mM $\alpha$ subunit; (■) 0.10 mM site equiv of apo$\beta_2$ subunit; (□) 0.135 mM site equiv of apo$\beta_2$ subunit. Binding data from equilibrium dialysis are normalized to a stoichiometry of one molecule of PPS bound per equivalent of $\beta$ chain. Binding parameters are listed in Table II. The binding ratio $R$ = [PPS$_{bound}$]/[E$_0$] where [E$_0$] is the total concentration of binding sites. [PPS$_{free}$] is calculated from the expression [PPS$_{free}$] = [PPS$_{total}$] - ($\Delta E/\Delta E_{max}$)[E$_0$] where $\Delta E$ is the absorbance difference measured at a particular value and $\Delta E_{max}$ is the asymptotic value.

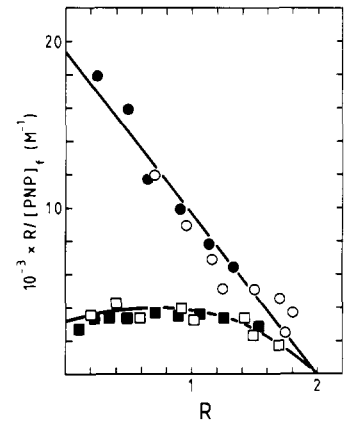



FIGURE 4: PNP binds noncooperatively to the $\alpha_2$apo$\beta_2$ complex and with positive cooperativity to the apo$\beta_2$ subunit of tryptophan synthase: Scatchard plots of spectrophotometric titration (● and ■) and equilibrium dialysis (○ and □). Buffer A; $T$ = 20 °C. (●) 0.063 mM site equiv of apo$\beta_2$ subunit and 0.076 mM $\alpha$ subunit; (○) 0.050 mM site equiv of apo$\beta_2$ subunit and 0.060 mM $\alpha$ subunit; (■) 0.069 mM site equiv of apo$\beta_2$ subunit; (□) 0.067 mM equiv of apo$\beta_2$ subunit. The binding data from equilibrium dialysis are normalized to a stoichiometry of one molecule of PNP bound per equivalent of $\beta$ chain. The binding parameters are listed in Table II. The definition of $R$ is as in Figure 3.

where C is the binding site on the tryptophan synthase complex and L is the ligand.

The data are described quantitatively by Scatchard's equation

$$R/[\bar{L}] = (n - R)/K_d \qquad (3)$$

where the binding ratio $R$ = [L$_{bound}$]/[E$_0$], [$\bar{L}$] is the concentration of free ligand, $n$ is the number of binding sites, and $K_d$ is the equilibrium dissociation constant. The $K_d$ values are very similar to the inhibition constants determined as described above: $K_i$ = 0.11 mM and $K_d$ = 0.12 mM for PNP and $K_i$ = 0.15 mM and $K_d$ = 0.17 mM for PPS. The agreement

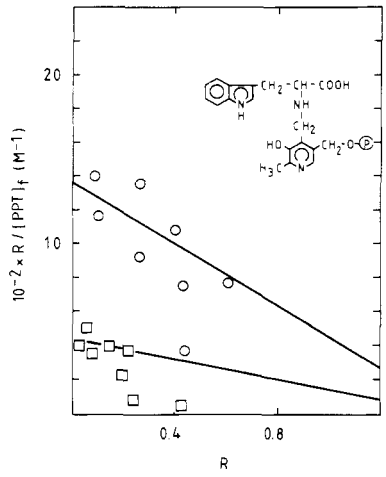



FIGURE 5: PPT binds noncooperatively and weakly to either the $\alpha_2$apo$\beta_2$ complex or the apo$\beta_2$ subunit of tryptophan synthase: equilibrium dialysis with [$^3$H]PPT in buffer A, $T$ = 20 °C. (○) 0.10 mM equiv of apo$\beta_2$ subunit and 0.12 mM $\alpha$ subunit; (□) 0.10 mM equiv of apo$\beta_2$ subunit. (—) Theoretical curves were calculated for the apparent stoichiometry of 0.75 molecule of PPT bound per equiv of $\beta$ chain due to enzyme inactivation and dilution (cf. Materials and Methods). Definitions and the method of evaluation are as in Figure 3.

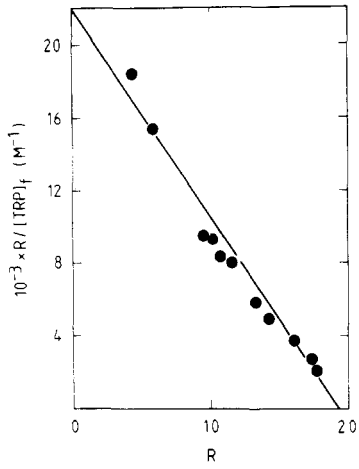



FIGURE 6: L-Tryptophan binds noncooperatively and strongly to the $\alpha_2$holo$\beta_2$ complex: spectrophotometric titration in buffer A, 20 °C. 0.1 mM site equiv of holo$\beta_2$ subunit and 0.12 mM equiv of $\alpha$ subunit were titrated with 20 mM L-tryptophan at 474 nm. Definitions and the method of evaluation are as in Figure 3.

indicates that no significant dissociation of the $\alpha_2$apo$\beta_2$ complex occurs (Bartholmes & Teuscher, 1979) in phosphate buffer at the low concentration (2 $\mu$M) of active sites used for the inhibition studies (Figure 1).

It is possible to detect weak binding of PPT to the $\alpha_2$apo$\beta_2$ complex by equilibrium dialysis (circles in Figure 5). Assuming two binding sites, the dissociation constant equals 1.1 mM, a value sixfold larger than the value obtained for PPS.

In contrast to PPT, L-tryptophan binds strongly to the $\alpha_2$holo$\beta_2$ complex (Figure 6), and $K_d$ equals 0.09 mM. This value is comparable to previous estimates (0.2–0.3 mM) obtained from inhibition studies under different conditions (Creighton, 1970).

*Binding Studies with the Apo$\beta_2$ Subunit.* The Scatchard plots of PPS and PNP binding to the apo$\beta_2$ subunit are concave toward the abscissa (squares in Figures 3 and 4). The shape of the binding curve corresponds to a sigmoidal binding curve when $R$ is plotted vs. [$\bar{L}$] and identifies positively cooperative interactions between the two binding sites. This behavior has also been observed for PLP binding (Bartholmes et al., 1976)

Table II: Quantitative Parameters of Ligand Binding to the Three Different States of the Apo$\beta_2$ Subunit[a]

| ligand | $n$ | nicked[b] apo$\beta_2$ subunit, $K_d$ (mM) | intact[c] apo$\beta_2$ subunit $K_{d,1}$ (mM) | intact[c] apo$\beta_2$ subunit $K_{d,2}$ (mM) | intact[c] apo$\beta_2$ subunit $n_H$ | $\alpha_2$apo$\beta_2$[d] complex, $K_d$ (mM) |
|---|---|---|---|---|---|---|
| PPS | 2 | | 0.5 | 0.13 | 1.3 | 0.16 |
| PNP | 2 | 1.0 | 0.6 | 0.13 | 1.5 | 0.12 |
| PPT | 2 | | 3.8[e] | 3.8[e] | 1.0 | 1.10[f] |

[a] $n$ is the number of binding sites, $n_H$ is the Hill coefficient, $K_d$ is the equilibrium dissociation constant for noncooperative binding (eq 3), and $K_{d,1}$ and $K_{d,2}$ are the apparent microscopic equilibrium dissociation constants for cooperative binding (eq 4). [b] Figure 7. [c] Squares in Figures 3 and 4. [d] Circles in Figures 3 and 4. [e] Squares in Figure 5. [f] Circles in Figure 5.

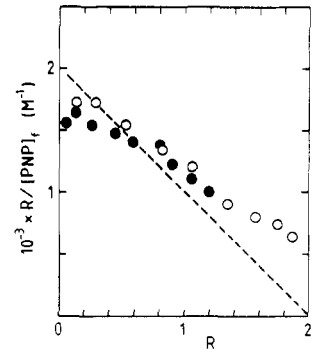



FIGURE 7: PNP binds noncooperatively to the nicked apo$\beta_2$ subunit of tryptophan synthase: (○ and ●) results from two independent spectrophotometric titrations in buffer A, 20 °C, and in 2-mm tandem cuvettes. 0.39 mM site equiv of nicked apo$\beta_2$ subunit. The theoretical curve (---) corresponds to $K_{d,1} = 1 \times 10^{-3}$ M and $\Delta\epsilon_{290} = 1.7$ mM$^{-1}$ cm$^{-1}$.

and contrasts sharply to the noncooperative manner in which these ligands are bound to the $\alpha_2$apo$\beta_2$ complex (circles in Figures 3 and 4). Cooperative binding may be described formally by a two-step overall binding reaction as given by

$$\beta_2 + 2L \rightleftharpoons \beta_2L + L \rightleftharpoons \beta_2L_2 \qquad (4)$$

where $\beta_2$ is the apo$\beta_2$ subunit and $K_{d,1}$ and $K_{d,2}$ are the apparent microscopic dissociation constants for the first and second binding reactions. $K_{d,1}$ and $K_{d,2}$ were calculated from a fit of the binding data to the Adair equation (Bartholmes et al., 1976):

$$R = \frac{2[[\bar{L}]/K_{d,1} + [\bar{L}]^2/(K_{d,1}K_{d,2})]}{1 + 2[\bar{L}]/K_{d,1} + [\bar{L}]^2/(K_{d,1}K_{d,2})} \qquad (5)$$

The values that best fit both the equilibrium data presented here and the kinetic data presented in the subsequent papers (Tschopp & Kirschner, 1980; Bartholmes et al., 1980) are collected in Table II together with the values of $n_H$, the Hill coefficient. The theoretical curves in Figures 3 and 4 are calculated with these values.

PPT binds even more weakly to the apo$\beta_2$ subunit than to the $\alpha_2$apo$\beta_2$ complex (squares in Figure 5), so that it is difficult to ascertain whether the binding is cooperative or not. The tentative value of $K_d$ obtained by assuming noncooperative binding ($n_H$ = 1) to two sites equals 3.8 mM (Table II).

*Binding to Nicked Apo$\beta_2$ Subunit.* We used spectrophotometric titration with PNP to characterize that state of the nicked $\beta_2$ subunit in relation to the intact protein. The binding difference spectrum is intermediate between those of either the $\alpha_2$apo$\beta_2$ complex or the apo$\beta_2$ subunit (not shown). As seen in Figure 7, removal of the peptide link between the $F_1$

and $F_2$ domains reduces cooperativity of PNP binding to an insignificant level and increases the value of $K_d^{PNP}$ to 1 mM (Table II).

## Discussion

The purification procedure described here is more rapid than the method of Adachi et al. (1974) and results in a higher specific activity of the enzyme. The method of hydrophobic chromatography on unsubstituted Sepharose CL 4B recently introduced by von der Haar (1976, 1979) is efficient and highly reproducible for the tryptophan synthase complex (Miles & Higgins, 1978). Because this method is based on a different principle than the DEAE chromatography used in the first step, purification of the tryptophan synthase complex can be achieved by a sequence of only two chromatographic separations. It is notable that neither the $\alpha$ subunit nor the $\beta_2$ subunit is bound to Sepharose CL 4B under comparable conditions. Thus, the association of the $\alpha$ and $\beta_2$ subunits appears to create the hydrophobic patches required for adsorption to the gel matrix (Shaltiel, 1974; Jennissen, 1978).

The $\alpha_2$apo$\beta_2$ complex binds not only PLP but also PNP and PPS to two identical and independent binding sites. The comparison of the dissociation constants of PNP (0.12 mM) and PLP (1.4 $\mu$M) gives a quantitative measure of the contribution of the internal aldimine formed between PLP and the lysine amino group to the overall free energy of binding of PLP.

It is likely that PMP is bound only weakly to the enzyme because, in contrast to PNP, it carries a positive charge on the $C_{4'}$ amino group at pH 7.5 (Jaworski & O'Leary, 1979). Adachi & Miles (1974) have reported that the holo$\beta_2$ subunit releases bound PLP in the presence of ammonium sulfate, presumably because it forms a positively charged imine. Electrostatic repulsion between similar charges both on the ligand and on an adjacent amino acid side chain would explain the low affinity of these ligands for tryptophan synthase.

It is surprising that, among the bisubstrate analogues tested, only PPS is bound significantly to the $\alpha_2$apo$\beta_2$ complex. In contrast, several different PLP-dependent enzymes have strong affinity for the conjugate *N*-phosphopyridoxyl amino acids: tyrosine aminotransferase [$K_d = 6.5 \times 10^{-9}$ M (Borri-Voltattorni et al., 1975)]; tyrosine decarboxylase [$K_d = 2.8 \times 10^{-7}$ M (Orlacchio et al., 1970)]; aspartate aminotransferase [$K_d < 10^{-9}$ M (Turano et al., 1970)]; tryptophanase (Morino & Snell, 1967; Raibaud & Goldberg, 1976).

Because the secondary amino group of PPS is also protonated at pH 7 (Khomutov et al., 1971; Jaworski & O'Leary, 1979), the comparison of $K_d^{PPS}$ (0.16 mM) with $K_d^{PMP}$ (estimated 10 mM) indicates that the L-serine side chain contributes strongly to the overall free energy of binding. Since PPA does not bind to tryptophan synthase under comparable conditions, the increase of affinity appears to be specifically contributed by the $\beta$-hydroxyl group of the L-serine side chain, possibly involving an hydrogen bond.

Because L-tryptophan is bound to the $\alpha_2$holo$\beta_2$ complex with approximately the same affinity as L-serine [$K_d^{Trp} = 0.09$ mM (cf. Figure 6); $K_d^{Ser} \sim 0.1$ mM (Faeder & Hammes, 1970)], the comparatively weak binding of PPT must be due to differences between the external aldimines of bound L-serine and L-tryptophan. This conclusion is supported by the 474-nm absorption band observed with bound L-tryptophan (Figure 2B) but not observed with bound L-serine (Miles, 1979). The 474-nm band is likely to be due to the quinonoid carbanion (Figure 8) that accumulates during the steady-state phase of tryptophan synthesis from indole and L-serine (Goldberg & Baldwin, 1967) and that must be involved in the exchange of the $C_\alpha$ proton of L-tryptophan with $D_2O$ reported by Tsai et al. (1978).

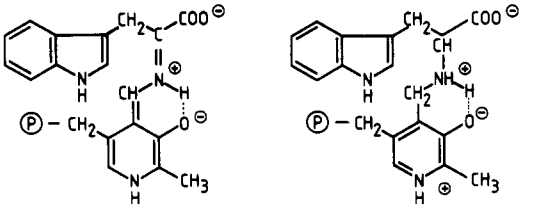
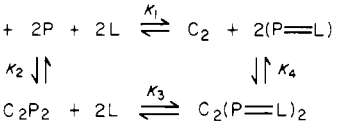


FIGURE 8: Structures of the quinonoid anion of the external tryptophan–PLP aldimine (left) and of *N*-phosphopyridoxyl-L-tryptophan (right).

Scheme I



Although several equilibria are involved in the binding of L-serine and L-tryptophan to tryptophan synthase (Goldberg & Baldwin, 1967; Goldberg et al., 1968; Miles & McPhie, 1974; Faeder & Hammes, 1970, 1971; York, 1972; Miles, 1979), it is feasible to calculate the overall dissociation constant $K_4$ (Scheme I) of the external aldimines of enzyme-bound PLP with both L-serine and L-tryptophan. In Scheme I, $C_2$ is the $\alpha_2$apo$\beta_2$ complex, P is PLP, L is either L-serine or L-tryptophan, (P=L) represents the external aldimine, and $C_2P_2$ represents the internal aldimine.

With $K_2 = 1$ $\mu$M (Bartholmes et al., 1976), $K_3 \sim 0.1$ mM for L-serine (Faeder & Hammes, 1970) and for tryptophan (Figure 6), and estimates of $K_1$ varying from 2 to 50 mM (Simon & Kröger, 1974; Metzler, 1957), the limits of $K_4 = K_2K_3/K_1$ are estimated to be 2 nM $< K_4 <$ 50 nM. $K_d^{PPS}$ (0.16 mM) is $10^4$-fold larger than $K_4$, and the discrepancy between $K_d^{PPT}$ and $K_4$ (1.1 mM) is even greater.

This comparison, and the similarity of $K_4$ with the dissociation constants of some enzyme–*N*-phosphopyridoxyl amino acid complexes, suggests that the binding site for external aldimines in tryptophan synthase is more selective than in the other PLP-dependent enzymes. As discussed above, the weak interaction of PPT with tryptophan synthase could therefore be due either to a different geometry of PPT compared to the quinonoid species or to a different charge distribution (Figure 8).

In contrast to the $\alpha_2$apo$\beta_2$ complex, the apo$\beta_2$ subunit binds PLP with positive cooperativity [sigmoidal binding curves (Bartholmes et al., 1976)]. Because both PNP and PPS also bind cooperatively to the apo$\beta_2$ subunit (Figures 3 and 4), formation of the internal aldimine between PLP and the lysine amino group is not important for eliciting the phenomenon. Therefore, the pyridine ring with its substituents must be the essential moiety for inducing the concerted changes of the enzyme described by Tschopp & Kirschner (1980) and by Bartholmes et al. (1980). Quantitative comparison shows, however, that the degree of cooperativity observed is larger with PLP as the ligand than with PNP or PPS as ligands. This can be seen by comparing the ratios $K_{d,1}/K_{d,2}$ ($\sim$30 for PLP) and $n_H$ [=1.7 for PLP (Bartholmes et al., 1976)] with the values given in Table II.

The difference spectra of PPS bound to either the $\alpha_2$apo$\beta_2$ complex or to the apo$\beta_2$ subunit (Figure 2A) indicate that the mode of binding of the pyridine ring cannot be exactly the same. The comparison between the extinction coefficients of free PPS ($\epsilon_{325} = 8.4 \times 10^3$ M$^{-1}$ cm$^{-1}$) and PPS bound to the apo$\beta_2$ subunit (dashed curve: $\Delta\epsilon_{325} = 8 \times 10^3$ M$^{-1}$ cm$^{-1}$ and

$\Delta\Delta\epsilon_{325} \sim 0.4 \times 10^3$ $M^{-1}$ $cm^{-1}$) demonstrates that the adsorption band at 325 nm is almost completely shifted to the "hidden band" at 290 nm in the case of the apo$\beta_2$ subunit. Similar shifts have been induced in reduced phosphorylase *b* by a change of pH from 5.3 to 7 (Feldmann et al., 1974) and in the reduced tryptophan synthase complex by the addition of indolepropanol phosphate (Kirschner et al., 1975). The species absorbing at 290 nm has been assigned to the tautomer of PMP in which the nitrogen in position 1 of the pyridine ring is unprotonated and the oxygen in position 3 is protonated. Thus, the binding sites for PPS in the complex and the $\beta_2$ subunit of tryptophan synthase differ with regard to the equilibrium between the neutral (290 nm) and zwitterionic (325 nm) species of bound PPS. These differences are probably due to differences in the detailed conformation at the binding sites involved.

Because the nicked apo$\beta_2$ subunit binds PNP weakly and noncooperatively (Figure 7), the loop of the polypeptide chain connecting the two autonomous domains of folding of the $\beta_2$ protomer (Goldberg & Zetina, 1980) must be intact not only for binding the $\alpha$ subunit, and for enzyme activity (Högberg-Raibaud & Goldberg, 1977), but also for the phenomenon of cooperative binding. It is likely that the removal of a short sequence of polypeptide chain by limited proteolysis (Crawford et al., 1978) restricts the oligomeric apoprotein to the low-affinity state. This notion is supported by the similarity between $K_d^{PNP}$ of the nicked protein (1 mM) and $K_{d,1}^{PNP}$ of the intact protein (0.6 mM; cf. Table II). The $\alpha$ subunit also binds cooperatively to the intact apo$\beta_2$ subunit (Bartholmes & Teuscher, 1979), but the binding sites for these two classes of allosteric ligands on the apo$\beta_2$ protomer must be quite different. Thus, tryptophan synthase represents another example of heterotropic interactions between a regulatory protein (the $\alpha$ subunit) and a specific ligand (PLP), reminiscent of the cyclic AMP dependent protein kinase system (Hoppe et al., 1978). Tryptophan synthase is particularly amenable to experimental study because pyridoxal phosphate and its analogues are naturally chromophoric ligands, and the regulatory $\alpha$ subunit possesses enzymic activity on its own account. Kinetic studies designed to unravel the elementary steps of ligand binding to either the apo$\beta_2$ subunit or the $\alpha_2$apo$\beta_2$ complex of tryptophan synthase are presented in the following papers (Tschopp & Kirschner, 1980; Bartholmes et al., 1980).

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## References

Adachi, O., & Miles, E. W. (1974) *J. Biol. Chem. 249*, 5430–5434.

Adachi, O., Kohn, L. D., & Miles, E. W. (1974) *J. Biol. Chem. 249*, 7756–7763.

Ahrens, H., & Korytnyk, W. (1970) *Methods Enzymol. 18A*, 489–494.

Atkinson, A., Bradford, P. A., & Selmes, I. P. (1973) *J. Appl. Chem. Biotechnol. 23*, 517–529.

Bartholmes, P., & Teuscher, B. (1979) *Eur. J. Biochem. 95*, 323–326.

Bartholmes, P., Kirschner, K., & Gschwind, H.-P. (1976) *Biochemistry 15*, 4712–4714.

Bartholmes, P., Balk, H., & Kirschner, K. (1980) *Biochemistry* (third paper of three in this issue).

Bisswanger, H., Kirschner, K., Cohn, W., Hansson, E., & Hager, V. (1979) *Biochemistry 18*, 5946–5953.

Borri-Voltattorni, C., Orlacchio, A., Giartosi, A., Conti, F., & Turano, C. (1975) *Eur. J. Biochem. 53*, 151–160.

Churchich, J. E., Beeler, T., & Oh, K. J. (1975) *J. Biol. Chem. 250*, 7722–7727.

Crawford, I. P., Decastel, M., & Goldberg, M. E. (1978) *Biochem. Biophys. Res. Commun. 85*, 309–311.

Creighton, T. E. (1970) *Eur. J. Biochem. 13*, 1–10.

Creighton, T. E., & Yanofsky, C. (1966) *J. Biol. Chem. 241*, 980–990.

Creighton, T. E., & Yanofsky, C. (1970) *Methods Enzymol. 18A*, 365–380.

Decastel, M., & Goldberg, M. E. (1978) *FEBS Lett. 89*, 64–68.

Faeder, E. J., & Hammes, G. G. (1970) *Biochemistry 9*, 4043–4049.

Faeder, E. J., & Hammes, G. G. (1971) *Biochemistry 10*, 1041–1045.

Feldmann, K., Gaugler, B. J. M., Winkler, H., & Helmreich, E. J. M. (1974) *Biochemistry 13*, 2222–2230.

Goldberg, M. E., & Baldwin R. L. (1967) *Biochemistry 6*, 2113–2119.

Goldberg, M. E., & Zetina, C. R. (1980) in *Protein Folding* (Jaenicke, R., Ed.) pp 469–483, Elsevier, Amsterdam.

Goldberg, M. E., York, S. S., & Stryer, L. (1968) *Biochemistry 7*, 3662–3667.

Gschwind, H. P., Gschwind, U., Paul, C., & Kirschner, K. (1979) *Eur. J. Biochem. 96*, 403–416.

Hathaway, G. M. (1972) *J. Biol. Chem. 247*, 1440–1444.

Henning, U., Helinski, R. D., Chao, F. C., & Yanofsky, C. (1962) *J. Biol. Chem. 237*, 1523–1530.

Högberg-Raibaud, A., & Goldberg, M. E. (1977) *Biochemistry 16*, 4014–4020.

Hoppe, J., Lawaczek, R., Rieke, E., & Wagner, K. G. (1978) *Eur. J. Biochem. 90*, 585–593.

Ikawa, M. (1967) *Arch. Biochem. Biophys. 118*, 497–500.

Jaworski, R. J., & O'Leary, M. H. (1979) *Methods Enzymol. 62D*, 436–454.

Jennissen, H. P. (1978) *J. Chromatogr. 159*, 71–83.

Khomutov, R. M., Dixon, H. B. F., Vdovina, L. V., Kirpchinikov, M. P., Morozow, Y. V., Severin, E. S., & Khurs, E. N. (1971) *Biochem. J. 124*, 99–106.

King, E. J. (1932) *Biochem. J. 26*, 292–306.

Kirschner, K., Wiskocil, R. L., Föhn, M., & Rezeau, L. (1975) *Eur. J. Biochem. 60*, 513–523.

Korytnyk, W., & Ikawa, M. (1970) *Methods Enzymol. 18A*, 524–566.

Maire, J. W., & Day, H. G. (1972) *Anal. Chem. 44*, 2015–2019.

McCoy, E. E., Colombini, C., & Strynadka, K. (1979) *Methods Enzymol. 62D*, 410–415.

Metzler, D. E. (1957) *J. Am. Chem. Soc. 79*, 485–492.

Miles, E. W. (1979) *Adv. Enzymol. Relat. Areas Mol. Biol. 49*, 127–186.

Miles, E. W., & McPhie, P. (1974) *J. Biol. Chem. 249*, 2852–2857.

Miles, E. W., & Moriguchi, M. (1977) *J. Biol. Chem. 252*, 6594–6599.

Miles, E. W., & Higgins, W. (1978) *J. Biol. Chem. 253*, 6266–6269.

Miles, E. W., Hatanaka, M., & Crawford, I. P. (1968) *Biochemistry 7*, 2742–2753.

Morino, Y., & Snell, E. E. (1967) *J. Biol. Chem. 242*, 5591–5601.
Orlacchio, A., Borri-Voltattorni, C., & Turano, C. (1979) *Biochem. J. 185*, 41–46.
Peterson, A. B., & Sober, H. A. (1954) *J. Am. Chem. Soc. 76*, 169–175.
Ptitsyn, O. B. (1978) *FEBS Lett. 93*, 1–4.
Raibaud, O., & Goldberg, M. E. (1976) *J. Biol. Chem. 251*, 2814–2819.
Shaltiel, S. (1974) *Methods Enzymol. 34*, 126–141.
Simon, D., & Kröger, H. (1974) *Biochim. Biophys. Acta 334*, 208–217.
Stock, A., Ortlanderl, F., & Pfleiderer, G. (1966) *Biochem. Z. 344*, 353–360.
Stryer, L. (1978) *Annu. Rev. Biochem. 47*, 819–846.
Tsai, M.-D., Schleicher, E., Potts, R., Skye, G. E., & Floss, H. G. (1978) *J. Biol. Chem. 253*, 5344–5349.
Tschopp, J. (1978) Ph.D. Thesis, Basel.
Tschopp, J., & Kirschner, K. (1980) *Biochemistry* (second paper of three in this issue).
Turano, C., Borri, C., Orlacchio, A., & Bossa, F. (1970) *Enzymes and Isoenzymes* (Shugar, D., Ed.) pp 123–131, Academic Press, London.
von der Haar, F. (1976) *Biochem. Biophys. Res. Commun. 70*, 1009–1013.
von der Haar, F. (1979) *J. Solid Phase Biochem. 4*, 207–220.
Weischet, W. O., & Kirschner, K. (1976) *Eur. J. Biochem. 64*, 313–320.
Wilson, D. A., & Crawford, I. P. (1965) *J. Biol. Chem. 240*, 4801–4808.
Yanofsky, C., & Crawford, I. P. (1970) *Enzymes, 3rd Ed. 7*, 1–31.
York, S. S. (1972) *Biochemistry 11*, 2733–2740.

# Kinetics of Cooperative Ligand Binding to the Apo$\beta_2$ Subunit of Tryptophan Synthase and Its Modulation by the $\alpha$ Subunit†

Jürg Tschopp‡ and Kasper Kirschner*

ABSTRACT: The different binding mechanisms of pyridoxine 5′-phosphate and *N*-phosphopyridoxyl-L-serine have been investigated by kinetic studies with rapid reaction techniques. Pyridoxine 5′-phosphate binds in a single rapid step to the $\alpha_2$apo$\beta_2$ complex and in a single slow step to the nicked apo$\beta_2$ subunit that is obtained by limited proteolysis with trypsin. Both pyridoxine 5′-phosphate and *N*-phosphopyridoxyl-L-serine bind to the apo$\beta_2$ subunit with a comparatively slow binding step, followed by an even slower isomerization reaction. These findings are consistent with the nonexclusive concerted mechanism of cooperative binding but cannot be explained by the simple sequential mechanism. A quantitative fit of the rate and equilibrium data to the concerted mechanism generally yielded the pertinent rate and equilibrium constants. In particular, the same value of $L_0 = [T_0]/[R_0] = 200 \pm 50$ simultaneously satisfies the data obtained with three different ligands. The comparison of the mechanisms of ligand binding to the three states of the apo$\beta_2$ subunit suggests that the $\alpha_2$apo$\beta_2$ complex is similar to the high-affinity R state and the nicked apo$\beta_2$ subunit is similar to the low-affinity T state of the apo$\beta_2$ subunit. The slow isomerization involved in the cooperative binding of the ligands to the intact apo$\beta_2$ subunit is discussed in terms of local and concerted conformational changes involving the two autonomously folding domains of the $\beta$ protomer.

Pyridoxal 5′-phosphate (PLP)[1] binds cooperatively to the apo$\beta_2$ subunit and noncooperatively to the $\alpha_2$apo$\beta_2$ complex of tryptophan synthase [L-serine hydro-lyase (adding indoleglycerol-phosphate), EC 4.2.1.20] from *Escherichia coli* (Bartholmes et al., 1976). The same binding patterns are found for both *N*-phosphopyridoxyl-L-serine (PPS), a bisubstrate analogue of pyridoxal 5′-phosphate and L-serine, and pyridoxine 5′-phosphate (PNP), a nonreactive analogue of PLP (Tschopp & Kirschner, 1980). The analogues are more convenient probes of subunit interactions than PLP because their binding to tryptophan synthase does not involve the formation of the internal aldimine. In this work we determine the different mechanisms of binding of PPS and PNP by kinetic studies, using rapid reaction techniques. The results shed light on the differences between the native apo$\beta_2$ subunit, the nicked apo$\beta_2$ subunit obtained by limited proteolysis with trypsin (Högberg-Raibaud & Goldberg, 1977a,b), and the $\alpha_2$apo$\beta_2$ multienzyme complex. Comparison of the mechanisms of binding of the PLP analogues to the three "states" of the $\beta_2$ subunit help us to understand the mechanism of cooperative binding to the native apo$\beta_2$ subunit.

## Materials and Methods

*Materials.* All chemicals were of the highest degree of purity available from Merck (Darmstadt) and Fluka (Buchs). The subunits and the complex of tryptophan synthase as well as PNP and PPS were prepared and assayed as described in the preceding paper (Tschopp & Kirschner, 1980).

*Buffer.* Unless stated otherwise, all experiments were performed with buffer A: 0.1 M potassium phosphate, pH 7.5, containing $2 \times 10^{-3}$ M EDTA and $2 \times 10^{-4}$ M DTE.

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[1] Abbreviations used: PNP, pyridoxine 5′-phosphate; PPS, *N*-phosphopyridoxyl-L-serine; EDTA, (ethylenedinitrilo)tetraacetic acid; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; DTE, 1,4-dithioerythritol.

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