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Mechanism of Reconstitution of the Apoβ_2 Subunit and the $\alpha_2$apoβ_2 Complex of Tryptophan Synthase with Pyridoxal 5′-Phosphate: Kinetic Studies†

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ABSTRACT: The mechanism of pyridoxal 5′-phosphate (PLP) binding to both the $\alpha_2$apoβ_2 complex and the apoβ_2 subunit of tryptophan synthase was investigated by rapid mixing experiments. Absorption and fluorescence changes were used to monitor the binding reaction directly. Reduction with sodium borohydride provided the rate of formation of the internal aldimine with the lysine amino group of the enzyme, and substrate turnover monitored the rate of formation of active enzyme. The $\alpha_2$apoβ_2 complex binds PLP in a sequence of three steps of decreasing rate: formation of a noncovalent complex, which isomerizes to an enzymically inactive internal aldimine, followed by formation of an active $\alpha_2$holoβ_2 complex. The two binding sites appear to bind PLP independently. The apoβ_2 subunit binds PLP cooperatively in a sequence of three steps of decreasing rate: formation of a noncovalent complex, which isomerizes to an enzymically inactive internal aldimine, followed by the formation of the enzymically active holoβ_2 subunit. Taken together with kinetic studies of pyridoxine phosphate binding [Tschopp, J., & Kirschner, K. (1980) *Biochemistry* (second paper of three in this issue)], the rate data of the apoβ_2 subunit are shown to be consistent with the concerted mechanism. The differences between the values of the isomerization rate constants of bound PLP and bound PNP appear to result from the covalent internal aldimine, which is formed with PLP but not with PNP.

The dimeric β_2 subunit of tryptophan synthase from *Escherichia coli* [L-serine hydro-lyase (adding indoleglycerol-phosphate), EC 4.2.1.20] requires pyridoxal 5′-phosphate (PLP)[1] as a coenzyme for catalyzing the synthesis of L-tryptophan from indole and L-serine [for reviews, see Yanofsky & Crawford (1972) and Miles (1979)]:

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

PLP and various PLP analogues bind cooperatively to the apoβ_2 subunit and noncooperatively to the $\alpha_2$apoβ_2 complex (Bartholmes et al., 1976; Tschopp & Kirschner, 1980a), but only PLP is an efficient coenzyme. In the resting state PLP forms an internal aldimine with a lysine amino group located at the active site (Miles, 1979; Rocha et al., 1979). Because it is bound by a covalent bond, the mechanism of PLP binding must differ from the mechanism of binding of PLP analogues such as pyridoxine phosphate (PNP) and *N*-phosphopyridoxyl-L-serine (PPS). These properties afford the opportunity of measuring several phases of the reconstitution process by such varied techniques as spectroscopy, chemical quenching, and recovery of enzyme activity. We here determine the different mechanisms of binding of PLP to the apoβ_2 subunit and the $\alpha_2$apoβ_2 complex of tryptophan synthase by kinetic studies. The results are interpreted on the basis of the binding mechanisms of the PLP analogues elucidated by Tschopp & Kirschner (1980b) and provide new information on the individual steps of holoenzyme formation.

Materials and Methods

Materials. PLP (A grade) was obtained from Serva (Heidelberg) and purified as described by Bartholmes et al. (1976). All other chemicals were of the highest degree of purity available either from Merck (Darmstadt) or from Fluka (Buchs). Doubly distilled water was used for making up solutions.

The mutant strains of *E. coli* K_{12} *trp*A2/F′*trp*A2, K_{12} *trp* B8, and W3110 *trp*R⁻Δ*trp*LD102/F′Δ*trp*LD102 were kindly donated by Drs. C. Yanofsky and I. P. Crawford.

Buffers. Unless stated otherwise, all experiments were performed with 0.1 M sodium pyrophosphate buffer, pH 7.5, containing 10^{-4} M EDTA, because previous equilibrium studies were performed with this buffer (Bartholmes et al., 1976). The use of Tris was ruled out because it forms an aldimine with PLP (Simon & Kröger, 1974). Enzyme solutions were equilibrated with buffer by chromatography on Sephadex G-25 just before use. All solutions containing PLP were prepared under yellow light to prevent photolysis of PLP (Reiber, 1972).

Enzyme Assays. Both the activity of the β_2 subunit in the indole to tryptophan reaction and the protein concentrations of the α and β_2 subunits and of the $\alpha_2\beta_2$ complex of tryptophan

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[1] Abbreviations used: PLP, pyridoxal 5′-phosphate; PNP, pyridoxine 5′-phosphate; PPS, *N*-(5′-phosphopyridoxyl)-L-serine; DTE, dithioerythritol; PMSF, phenylmethanesulfonyl fluoride.

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synthase were measured as described by Miles & Moriguchi (1977) and by Bartholmes et al. (1976). The concentration of PLP stock solutions was determined spectrophotometrically in 0.1 N NaOH at 388 nm [ϵ_{388} nm = 6.6×10^3 M^{-1} cm^{-1} (Peterson & Sober, 1954)].

Enzymes. The isolated α and β_2 subunits were prepared as described earlier (Kirschner et al., 1975; Bartholmes et al., 1976). The β_2 subunit was stored at −80 °C in 0.6 M potassium phosphate, pH 7.8, containing 2×10^{-4} M dithioerythritol, 2×10^{-4} M pyridoxal 5′-phosphate, 5×10^{-3} M EDTA, and 10^{-4} M PMSF. Apoβ_2 subunit was prepared by resolving the holoenzyme with 3×10^{-3} M hydroxylammonium chloride in pyrophosphate buffer and extensive dialysis against the hydroxylamine-free buffer (DeMoss, 1962). The $\alpha_2$apoβ_2 complex was assembled by adding a two- to threefold excess of the α subunit to the apoβ_2 subunit, based on the known molecular weights of the α subunit [M_r 29 500 (Li & Yanofsky, 1972)] and of the β_2 subunit [M_r 89 000 (Adachi & Miles, 1974)].

Stopped-flow experiments with detection of absorbance changes were performed either with a modified Gibson-Durrum stopped-flow spectrophotometer (Gibson & Milnes, 1964) or with the flow cuvette described by Strittmatter (1965) (d = 1.50 cm; volume = 0.106 mL) and a pneumatic drive for two 2-mL plastic drive syringes for enzyme and PLP solutions. The cuvette was thermostated with the cuvette holder of a Varian Techtron Model 634 double-beam spectrophotometer. The dead time was determined as described by Paul et al. (1980) and equals 0.4 s. The fully reacted equilibrium mixture was placed into the reference beam.

Monitoring of the progress curves by fluorescence was performed with the above-mentioned stopped-flow spectrophotometer equipped with a fluorescence cell by using a Corning cutoff filter, 3-72 ($\lambda_{50\%}$ = 455 nm). The solutions were briefly degassed shortly before being drawn into the drive syringes.

Temperature-jump experiments were carried out with the instrument described by Cohn et al. (1979). All rate data were collected and processed with a digital computer as described in the same paper.

Chemical quenching of the internal aldimine of PLP with the lysine amino group of the enzyme was performed as follows: PLP was rapidly added to apoenzyme contained in a thermostated tube and vigorously mixed by magnetic stirring. At different time intervals, 0.1-mL samples were withdrawn and rapidly mixed with 0.01 mL of 5 mM sodium borohydride in 0.05 N NaOH (Raibaud & Goldberg, 1973). As judged by bleaching of the yellow color of PLP, reduction was complete within 2 s after borohydride addition. After 60 min at 25 °C in the dark, the reduced samples were heated to 100 °C for 5 min and centrifuged. After two washes with 1 mL of water, the protein precipitate was dissolved in pyrophosphate buffer containing 6 M guanidine hydrochloride. The concentration of pyridoxamine phosphate bound covalently to the protein was determined fluorometrically by exciting at 330 nm and measuring the emitted fluorescence at 390 nm. A standard of reduced β_2 subunit of known concentration (Hathaway, 1972) was used to prepare a calibration curve.

Measurement of Formation of Active Enzyme. Starting at 10 s after manual mixing of apoenzyme with PLP, we withdrew 50-μL samples and rapidly added them to 1 mL of assay mixture containing no PLP. The final concentration of enzyme was 0.5 μM. The progress curves measured by absorbance changes at 291 nm were linear for at least 2 min. The rates of both PLP binding and PLP dissociation are so slow (cf. Results) that the degree of PLP saturation remains practically constant during the enzyme assay. Control experiments showed that the formation of active enzyme is accelerated approximately fivefold when 30 mM L-serine, the concentration used for the enzyme assay, is also included in the reconstitution solution. The observed acceleration could result from the binding of the aldimine formed between L-serine and PLP in solution (Simon & Kröger, 1974; Litwack & Cleland, 1968; Orlacchio & Borri-Voltattorni, 1979). The absolute rate is still much slower than that of the turnover, however.

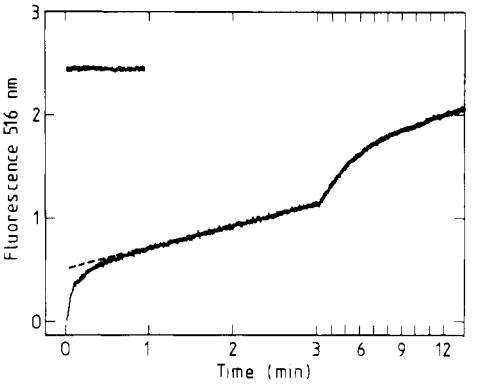

FIGURE 1: Two exponential reaction phases in the kinetics of binding of PLP to the $\alpha_2$apoβ_2 complex of tryptophan synthase. Enzyme and ligand were rapidly mixed, and the reaction was followed by fluorescence emission at 516 nm (excitation at 295 nm) as described under Materials and Methods. 0.1 M sodium pyrophosphate buffer, pH 7.5, and 0.1 mM EDTA were used. Temperature was 25 °C. The final concentrations were 22.4 μM site equiv of apoβ_2 subunit, 56 μM α subunit, and 300 μM PLP. Fluorescence increase is in arbitrary units. The horizontal trace on top is the base line after completion of the reaction. (---) Extrapolation of slow exponential to zero time.

Results

Kinetics of PLP Binding of the $\alpha_2$apoβ_2 Complex. The binding can be followed either by absorption or by fluorescence changes (Bartholmes et al., 1976). The most sensitive detection method is the measurement of sensitized PLP fluorescence emission obtained by exciting tryptophan at 295 nm (Isom & DeMoss, 1965).

PLP binds slowly to the enzyme complex, and the progress curve (Figure 1) obtained under these conditions can be decomposed into two well-separated exponentials according to $\Delta F_t = F_1^0 \exp(-k_{1,obsd}{}^c t) + F_2^0 \exp(-k_{2,obsd}{}^c t)$ where ΔF_T is the deviation of the observed fluorescence from the final equilibrium value. The analysis of the data in terms of superimposed exponentials is justified because only pseudo-first-order and first-order reactions will occur when PLP is present in large excess. Both with fluorescence excitation at either 410, 420, or 430 nm (excitation of bound PLP) and with absorption measurements at 436 nm [the peak of the difference spectrum (Bartholmes et al., 1976)], only the slower of the two processes is observable. The amplitude of the absorbance curve accounts completely for the difference absorbance measured at equilibrium (Bartholmes et al., 1976). No rapid relaxation processes were observable in temperature-jump experiments, neither with fluorescence nor with absorbance detection. As seen in Figure 2A, $k_{1,obsd}{}^c$ increases linearly with increasing PLP concentration as predicted for an elementary binding reaction to two identical and independent binding sites on the tryptophan synthase complex. In the reaction

$$C + L \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} CL \qquad (1)$$

Table I: Equilibrium and Rate Constants of PLP Binding to the $\alpha_2$apoβ_2 Complex of Tryptophan Synthase: Comparison with PNP Binding on the Basis of Equation 5 [C + L $\rightleftharpoons$ (CL) $\rightleftharpoons$ CL* $\rightleftharpoons$ CL**]

ligand	K_d[a] (M)	k_{12} (M^{-1} s^{-1})	k_{21} (s^{-1})	$K_{2,1}$ (M)	K_{obsd}[b] (M)	$k_{R,app}$[c] (M^{-1} s^{-1})	k_{23} (s^{-1})	k_{32} (s^{-1})	k_{34} (s^{-1})	k_{43} (s^{-1})
PLP	1×10^{-6}	n.d.[d]	n.d.	$\geq 10^{-3}$	5×10^{-5}	6.8×10^{2}	≥ 0.67	0.034	3.8×10^{-3}	7.6×10^{-5}
PNP[e]	1.2×10^{-4}	2.5×10^{6}	310	1.2×10^{-4}	1.2×10^{-4}	0	0	0	0	0

[a] The value for PLP is from Bartholmes et al. (1976); the value for PNP is from Tschopp & Kirschner (1980b). [b] $K_{obsd} = k_{32}K_{2,1}/k_{23}$. [c] $k_{R,app} = k_{23}/K_{2,1}$. [d] n.d., not determined. [e] Values for PNP are from Tschopp & Kirschner (1980b).

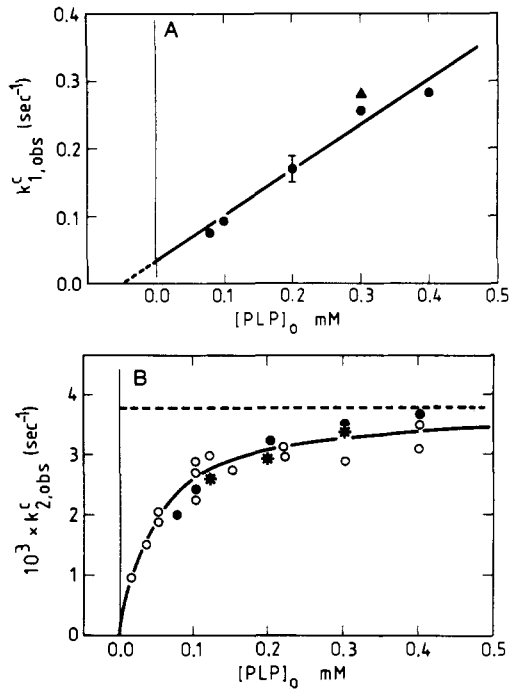

FIGURE 2: Binding of PLP to the $\alpha_2$apoβ_2 complex: concentration dependence of $k_{1,obsd}^c$ and $k_{2,obsd}^c$. Experimental conditions were as in Figure 1. (A) Increase of $k_{1,obsd}^c$ with increasing PLP concentration. (●) 22.4 μM site equiv of apoβ_2 subunit; 56 μM α subunit. (▲) Rate constant of internal aldimine formation measured by reduction with sodium borohydride (cf. Figure 3A). The bar indicates the average standard deviation. (B) Increase of $k_{2,obsd}^c$ with increasing concentration of PLP. (○) 11.2 μM site equiv of apoβ_2 subunit; 28 μM α subunit. (●) 22.4 μM site equiv of apoβ_2 subunit; 56 μM α subunit. (*) Rate constants of formation of active enzyme (cf. Figure 3B). (—) Calculated according to eq 6 with the values of k_{34}, k_{43}, and K_{obsd} from Table I. (---) Extrapolated maximum value of $k_{2,obsd}^c = k_{34} + k_{43}$.

C is the free binding site, CL is the occupied binding site, and L is the ligand (PLP). This mechanism predicts that $k_{1,obsd}^c$ depends on reactant concentrations as given by

$$k_{1,obsd}^c = k_{21} + k_{12}([\bar{L}] + [\bar{C}]) \sim k_{21} + k_{12}[L_0] \quad (2)$$

Because the experiments were performed with a large excess of PLP over enzyme concentration, $[\bar{L}] + [\bar{C}] \sim [\bar{L}] \sim [L_0]$, the total ligand concentration.

Because the value of k_{12} equals 6.8×10^2 M^{-1} s^{-1}, which is 6 orders of magnitude smaller than the diffusion-controlled limit (Hammes & Schimmel, 1970; Pecht & Lancet, 1977), the fastest observable process ($k_{1,obsd}^c$) probably represents an overall binding reaction

$$C + L \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} (CL) \underset{k_{32}}{\overset{k_{23}}{\rightleftharpoons}} CL^* \quad (3)$$

where a rapid initial binding step with a large equilibrium dissociation constant $K_{2,1} = k_{21}/k_{12}$ is followed by a slow isomerization of the primary protein–ligand complex CL to CL*. As indicated by the parentheses, the intermediate is present only at a low steady-state concentration.

Under these conditions only a single reaction phase is observable and $k_{1,obsd}^c$ depends on reactant concentrations according to eq 4 (Eigen & DeMaeyer, 1974):

$$k_{1,obsd}^c = k_{32} + k_{23}([\bar{L}] + [\bar{C}])/K_{2,1} \approx k_{32} + k_{23}[L_0]/K_{2,1} \quad (4)$$

The apparent second-order rate constant $k_{R,app}$ equals $k_{23}/K_{2,1}$ and can be determined from the slope of Figure 2A. Assuming a standard deviation of ±10% for the experimental values of $k_{1,obsd}^c$ at high PLP concentrations, the estimated lower limit of $K_{2,1} \geq 10^{-3}$ M. k_{32} is obtained from the ordinate intercept of Figure 2A. The lower limits of k_{23} are estimated from $k_{R,app}$ and $K_{2,1}$ (Table I). The kinetically determined equilibrium constant $K_{obsd} = [\bar{L}][\bar{C}]/[\overline{CL^*}] = k_{32}K_{2,1}/k_{23}$ can be determined from the intercept of the straight line in Figure 2A with the negative abscissa. It equals 50 μM.

The rate constant of the second rate process ($k_{2,obsd}^c$) increases with increasing PLP concentration asymptotically toward a plateau value (Figure 2B) as predicted for a slow isomerization CL* $\rightleftharpoons$ CL** following the binding reaction (Bernasconi, 1976; Lancet & Pecht, 1976; Cohn et al., 1979). The minimal formal scheme for the entire reconstitution reaction is summarized by

$$C + L \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} (CL) \underset{k_{32}}{\overset{k_{23}}{\rightleftharpoons}} CL^* \underset{k_{43}}{\overset{k_{34}}{\rightleftharpoons}} CL^{**} \quad (5)$$

According to this mechanism, $k_{2,obsd}^c$ depends on the excess PLP concentration as given by

$$k_{2,obsd}^c = k_{43} + k_{34}[[L_0]/([L_0] + K_{obsd})] \quad (6)$$

Since $k_{2,obsd}^c$ extrapolates to a very low value at zero PLP concentration, the data were analyzed by the direct linear plot (Eisenthal & Cornish-Bowden, 1974). The asymptotic value of the hyperbolic curve equals $k_{34} + k_{43} \approx k_{34} = 3.8 \times 10^{-3}$ s^{-1}. The median value of K_{obsd} was obtained independently from the half-maximal value of $k_{2,obsd}^c$ and equals 50 μM, in good agreement with the estimate from Figure 2A. By use of the independently determined overall equilibrium dissociation constant $K_d = k_{43}k_{32}k_{21}/(k_{12}k_{23}k_{34})$ (Bartholmes et al., 1976), the value of k_{43} was calculated and is collected together with the other equilibrium and rate constants in Table I.

The amplitudes of the two rate processes shown in Figure 1 are independent of PLP concentration (F_1^0 = 21%; F_2^0 = 79%). Unfortunately, the small signals prevented accurate measurement of progress curves below 50 μM PLP.

The kinetics of binding of PLP to the $\alpha_2$apoβ_2 complex differs markedly from the kinetics of binding of PNP and PPS (Tschopp & Kirschner, 1980b). Because the data for these two noncovalently bound PLP analogues are very similar, only the data for PNP are collected in Table I. The binding of PNP occurs rapidly, and only one process is observable. The rate of PLP binding is much slower, and at least two processes are observable.

It is likely that the differences between PLP and PNP are due in part to the formation of an internal aldimine between

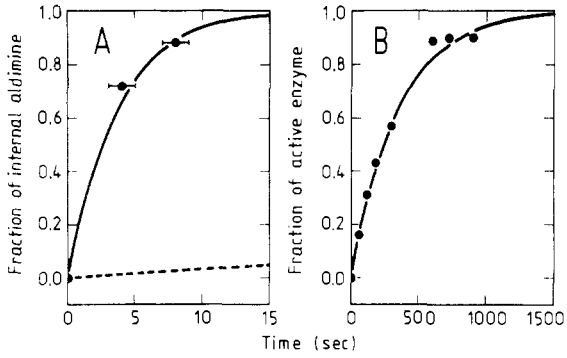

FIGURE 3: Different rates of formation of the internal aldimine and active enzyme during the binding of PLP to the $\alpha_2$apoβ_2 complex. Experimental conditions were as in Figure 1. (A) The internal aldimine is formed with $k_{1,obsd}{}^c$. The $\alpha_2$apoβ_2 complex was rapidly mixed with PLP, and the development of the internal aldimine was followed by reduction with sodium borohydride as described under Materials and Methods. Final concentrations: 22.4 μM site equiv of apoβ_2 subunit; 56 μM α subunit; 300 μM PLP. Fraction of internal aldimine $F = 1 - e^{-kt}$: (—) $k = 0.28\ s^{-1}$; (---) $k = 3.3 \times 10^{-3}\ s^{-1}$. The bars indicate the error limits of the sampling time. (B) Active tryptophan synthase is formed with $k_{2,obsd}{}^c$. The $\alpha_2$apoβ_2 complex was rapidly mixed with PLP, and the formation of active enzyme was followed as described under Materials and Methods. Final concentrations were as in (A) except the final concentration of PLP was 200 μM. (—) Fraction of active enzyme $A = 1 - e^{-kt}$ with $k = 2.9 \times 10^{-3}\ s^{-1}$.

PLP and the lysine amino group of the enzyme (Rocha et al., 1979). We therefore used a rapid sampling technique (reduction of the internal aldimine with sodium borohydride) to determine which of the two rate processes is responsible for the intramolecular internal aldimine formation. Moreover, it is of interest to determine in which of the two reaction phases the reconstituted holoenzyme attains its maximum catalytic efficiency.

A rapid sampling and assay technique was therefore devised to answer this question. Representative progress curves for the rate of internal aldimine formation and for the rate of active enyme formation are shown in parts A and B of Figure 3. The data points were fitted with single exponentials in each case. Although the limited time resolution of the chemical reduction assay permitted us to measure only the second half of the reaction (cf. Figure 3A), it is clear that the internal aldimine is formed with a rate constant indistinguishable from $k_{1,obsd}{}^c$. The value is presented in Figure 2A as a triangle. The dashed curve represents the exponential progress curve expected if the internal aldimine were formed during the slow reaction characterized by $k_{2,obsd}{}^c = 3.3 \times 10^{-3}\ s^{-1}$.

In contrast to the formation of the internal aldimine, active enzyme appears with a smaller rate constant, indistinguishable from $k_{2,obsd}{}^c$ (Figure 3B). The values of several experiments are included in Figure 2B as asterisks to demonstrate that formation of active enzyme coincides with the slower of the two transients observed in rapid mixing experiments. No further increase of enzyme activity is observed between 0.5 and 20 h.

Therefore, the two observable processes are both necessary and sufficient to account for generation of active enzyme. It is also clear that formation of the internal aldimine is by itself insufficient for generating the active $\alpha_2$holoβ_2 complex of tryptophan synthase.

Kinetics of PLP Binding to the Apoβ_2 Subunit. Similar to the studies with the $\alpha_2$apoβ_2 complex, the progress curve observed after rapid mixing of PLP with apoβ_2 subunit consists of two superimposed exponentials as given by $\Delta F_t = F_1{}^0 \exp(-k_{1,obsd}{}^B t) + F_2{}^0 \exp(-k_{2,obsd}{}^B t)$, where ΔF_t is the deviation of the observed amplitude from the final equilibrium value.

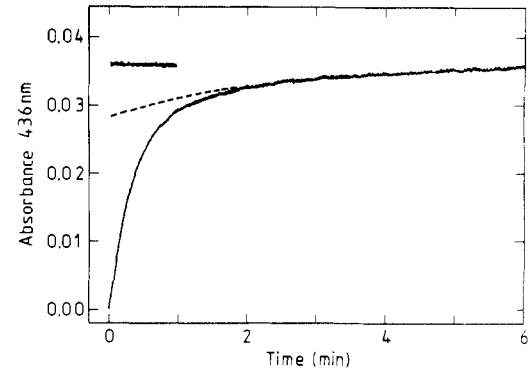

FIGURE 4: Two exponential reaction phases in the kinetics of binding of PLP to the apoβ_2 subunit of tryptophan synthase. Enzyme and ligand were rapidly mixed, and the absorbance at 436 nm was followed as described under Materials and Methods. Final concentrations: 22.4 μM site equiv of apoβ_2 subunit; 100 μM PLP. (---) Extrapolation of slow exponential to the instant of mixing.

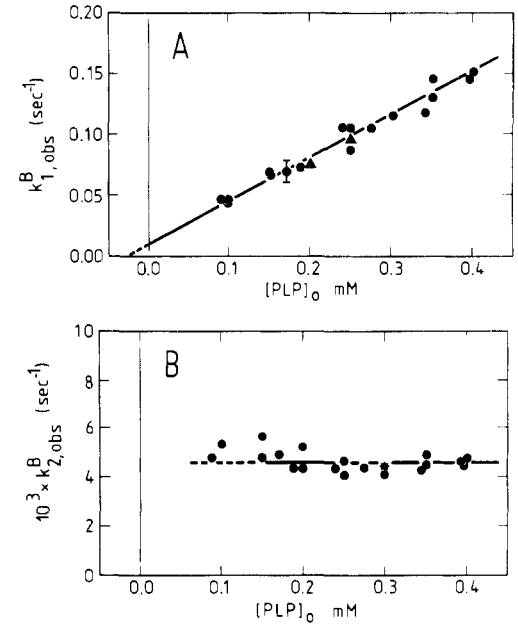

FIGURE 5: Concentration dependence of $k_{1,obsd}{}^B$ and $k_{2,obsd}{}^B$. Experimental conditions were as in Figure 4. (A) Increase of $k_{1,obsd}{}^B$ with increasing PLP concentration. Conditions of buffer and temperature were as in Figure 1. The final concentration of apoβ protomer equivalents was 22.4 μM. (●) Results of absorbance measurements (cf. Figure 4). (▲) Rate constant of internal aldimine formation measured with borohydride reduction (cf. Figure 6A). (B) $k_{2,obsd}{}^B$ is independent of PLP concentration. (●) Results of absorbance measurements (cf. Figure 4). (*) Rate constants of formation of active enzyme (cf. Figure 6B). The curves were calculated as described in the text with the rate constants collected in Table I.

These processes are observable with both fluorescence and absorbance measurements, and a representative progress curve is shown in Figure 4, where absorbance changes at 436 nm were used to follow the reaction. The sum of the amplitudes $F_1{}^0 + F_2{}^0$ accounts completely for the difference absorbance measured at equilibrium. The use of sensitized PLP fluorescence emission did not reveal any additional processes. Temperature-jump studies, however, did detect a more rapid relaxation process of small amplitude with $k = 58\ s^{-1}$, which was independent of PLP concentration between 5×10^{-5} and 3×10^{-3} M (data not shown). The relaxation with $k = 3 \times 10^3\ s^{-1}$ previously reported by Faeder & Hammes (1971) was not detectable under our conditions. In the following analysis we assume that the observed rapid process is due to an isomerization of bound PLP, possibly between tautomers, and that

Table II: Equilibrium and Rate Constants of PLP Binding to the Apoβ_2 Subunit of Tryptophan Synthase: Comparison with PNP Binding on the Basis of Equation 9 [B + L $\rightleftharpoons$ (BL) $\rightleftharpoons$ BL* $\rightleftharpoons$ BL**]

ligand	$K_{2,1}$[a] (M)	K_{obsd}[b] (M)	$k_{R,app}$[c] (M^{-1} s^{-1})	k_{23} (s^{-1})	k_{32} (s^{-1})	k_{34}[d] (s^{-1})	k_{43}[d] (s^{-1})
PLP	$\geq 10^{-3}$	2.5×10^{-5}	400	≥ 0.4	0.01	4.6×10^{-3}	6.4×10^{-6}
PNP[e]	$\geq 5 \times 10^{-3}$	7.0×10^{-4}	700	≥ 3.5	0.49	3.0×10^{-2}	4.0×10^{-3}

[a] Minimum values were estimated as described in the text. [b] $K_{obsd} = k_{32}K_{2,1}/k_{23}$. [c] $k_{R,app} = k_{23}/K_{2,1}$. [d] Calculated as described in the text. [e] Values for PNP are from Tschopp & Kirschner (1980b).

it is not directly related to the mechanism of binding.

As shown in Figure 5A, $k_{1,obsd}^B$ increases with increasing PLP concentration, whereas $k_{2,obsd}^B$ has a concentration-independent value of 4.6×10^{-3} s^{-1} (Figure 5B). Unfortunately, the small signal changes did not permit measurements below 90 μM PLP, so that it remains uncertain how $k_{2,obsd}^B$ varies at low PLP concentration. Moreover, the relative amplitudes F_1^0 (21%) and F_2^0 (79%) (cf. Figure 4) are independent of the concentration of PLP under the conditions of Figure 5 (data not shown).

Because $k_{1,obsd}^B$ (Figure 5A) is similar to $k_{1,obsd}^c$ (cf. Figure 2A), we assume that the first reaction phase of PLP binding to the apoβ_2 subunit also consists of a two-step mechanism analogous to eq 3:

$$B + L \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} (BL) \underset{k_{32}}{\overset{k_{23}}{\rightleftharpoons}} BL^* \qquad (7)$$

The data were evaluated according to (cf. eq 4)

$$k_{1,obsd}^B = k_{32} + k_{23}[L_0]/K_{2,1} \qquad (8)$$

and the rate and equilibrium constants are listed in Table II.

The concentration dependence of $k_{2,obsd}^B$ was evaluated according to the formal mechanism

$$B + L \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} (BL) \underset{k_{32}}{\overset{k_{23}}{\rightleftharpoons}} BL^* \underset{k_{43}}{\overset{k_{34}}{\rightleftharpoons}} BL^{**} \qquad (9)$$

where the transition from BL* to BL** represents a further, slow isomerization reaction. The values of k_{34} and k_{43} were estimated for the limit at high PLP concentration, where $k_{2,obsd}^B = k_{34} + k_{43}$ and the value of $L_2 = k_{43}/k_{34} = 1.4 \times 10^{-3}$ (Tschopp & Kirschner, 1980a). The data for PNP binding obtained in the first paper in this series (Tschopp & Kirschner, 1980a) are included in Table II for the comparison.

Similar to the studies with the $\alpha_2$apoβ_2 complex, we also determined the rate of formation of both the internal aldimine (Figure 6A) and the active enzyme (Figure 6B). The rate constants are included in Figure 5A as triangles and in Figure 5B as asterisks. It is clear that the formation of the internal aldimine occurs during the binding step characterized by $k_{1,obsd}^B$ (B + L $\rightarrow$ BL*) and that the formation of the active enzyme occurs with the final, slow isomerization characterized by $k_{2,obsd}^B$ (BL* $\rightarrow$ BL**; cf. eq 9).

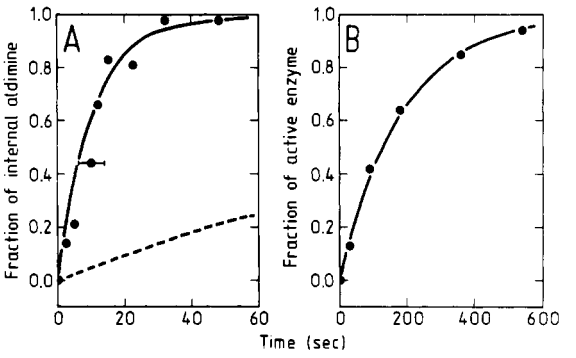

FIGURE 6: Different rates of formation of the internal aldimine and active enzyme during the binding of PLP to the apoβ_2 subunit. Experimental conditions were as in Figure 4. (A) The internal aldimine is formed with $k_{1,obsd}^B$. Apoβ_2 subunit was rapidly mixed with PLP, and the development of the internal aldimine was followed by reduction with sodium borohydride as described under Materials and Methods. Final concentration of PLP was 250 μM. The bar indicates the error limits of the sampling time. Fraction of internal aldimine $F = 1 - e^{-kt}$: (—) $k = 0.095$ s^{-1}; (---) $k = 0.0046$ s^{-1}. (B) Active holoβ_2 subunit is formed with $k_{2,obsd}^B$. The apoβ_2 subunit was rapidly mixed with PLP, and the formation of active enzyme was followed as described under Materials and Methods. Final concentration of PLP was 200 μM. (—) Fraction of active enzyme $A = 1 - e^{-kt}$ with $k = 5.4 \times 10^{-3}$ s^{-1}.

Discussion

Binding to the $\alpha_2$apoβ_2 Complex. The data on PLP binding rates are consistent with previous evidence, obtained from PLP binding studies at equilibrium (Bartholmes et al., 1976) and from studies with PNP and PPS (Tschopp & Kirschner, 1980a,b), that the two active sites of the $\alpha_2$apoβ_2 complex of tryptophan synthase are identical and do not interact. Thus, the $\alpha_2\beta_2$ complex is regarded in the following discussion as a dimer of two identical, functional $\alpha\beta$ protomers, each symbolized by C.

The comparison of the equilibrium and rate constants of the binding of both PLP and PNP, based on the mechanism of eq 5 (Table I), provides interesting clues for a physical interpretation of the three reaction steps observed with PLP. Because PNP cannot form an internal aldimine, it is likely that the initial step of PLP binding occurs by the same mechanism and with similar rate constants as observed for the single binding reaction of PNP (C + L $\rightleftharpoons$ CL). It is not clear why $K_{2,1}^{PLP}$ is at least 10-fold larger than $K_{2,1}^{PNP}$, however.

It is likely that the second step of PLP binding involves the formation of the enzymically still inactive internal aldimine (CL*) from the primary, noncovalent PLP–enzyme complex. The rate of formation of the aldimine of PLP with ϵ-aminocaproate occurs with an apparent second order rate constant of 1.5 M^{-1} s^{-1} under the same conditions (unpublished observations).

The final step of PLP binding generates the active holoenzyme with $k_{34} = 3.8 \times 10^{-3}$ s^{-1}. Because [CL**]/[CL*] = 50, only 2% of the holoenzyme exists in the catalytically inactive state CL* at equilibrium. Moreover, the low value of k_{43} (7.6×10^{-5} s^{-1}) predicts that the resolution of the holo$\alpha_2\beta_2$ complex by mere dialysis would be a very slow procedure (Miles & Moriguchi, 1977).

The rate of formation of the enzymically active holoenzyme could be determined by a slow conformational change. We suggest that PLP is deeply buried within the protein fold as found both for glycogen phosphorylase (Weber et al., 1978; Kavinsky et al., 1978) and for aspartate aminotransferase (Ford et al., 1980). Such a mode of binding would require a partial rearrangement of the tertiary structure to permit access of the covalently bound PLP to its final location and could explain the high activation energy of the isomerization reaction.

Comparison with Other PLP-Dependent Enzymes. The mechanism of PLP binding to other PLP-dependent enzymes has recently been studied with rapid mixing techniques. Fonda & Auerbach (1976) observed only one transient after rapidly

mixing PLP with the apoaspartate aminotransferase from pig heart cytosol. Both the internal aldimine and the active holoenzyme are simultaneously formed in a first-order reaction with $k = 0.02\ s^{-1}$. Several lines of evidence indicate that PLP binding is also accompanied by conformational changes at the active site (Arrio-Dupont, 1978).

The kinetics of binding of PLP to the monomeric D-serine apodehydratase from *E. coli* (Reed & Schnackerz, 1979) is characterized by three transients, and the proposed mechanism is identical with eq 5. The rapid preequilibrium $C + L \rightleftharpoons CL$ was detected by elegant stopped-flow temperature-jump experiments. The recombination rate constant k_{12} equals $2.6 \times 10^6\ M^{-1}\ s^{-1}$ and the dissociation rate constant k_{21} equals $5 \times 10^3\ s^{-1}$. The value of k_{21}/k_{12} (1.9×10^{-3} M) is similar to that observed for the binding of PLP to the $\alpha_2$apoβ_2 complex of tryptophan synthase (cf. Table I).

The second and third transients observed after mixing PLP with D-serine dehydratase depend on PLP concentration similar to the data on tryptophan synthase presented in parts A and B of Figure 2. Although neither chemical quenching with sodium borohydride nor active enzyme formation was used by Reed & Schnackerz (1979), the internal aldimine is thought to be formed in the $CL \rightleftharpoons CL^*$ reaction and the active enzyme is thought to be formed in the final isomerization step. This reaction is thought to be due to a conformational change that serves to lock the coenzyme into its active position. In conclusion, the mechanism of PLP binding to several enzymes that do not exhibit cooperative effects appears to be quite similar to the case of $\alpha_2\beta_2$ complex of tryptophan synthase, with regard to both the elementary steps and the equilibrium and rate constants.

In contrast to tryptophan synthase and the two enzymes mentioned above, tyrosine aminotransferase from rat liver (Borri-Voltattorni et al., 1975) and tyrosine decarboxylase from *Streptococcus faecalis* (Orlacchio & Borri-Voltattorni, 1979; Orlacchio et al., 1979) bind PLP comparatively rapidly and with high affinity ($K_d^{PLP} \sim 5 \times 10^{-9}$ M). Pyridoxamine–pyruvate transaminase from *Pseudomonas* also belongs in the class of enzymes that bind pyridoxal derivatives without slow isomerization steps (Gilmer & Kirsch, 1977). It remains to be seen whether these two different classes of PLP-dependent enzymes are characterized by two principally distinguishable microenvironments in the respective binding sites.

Binding to the Apoβ_2 Subunit. The cooperative binding of PLP, PNP, and PPS (Tschopp & Kirschner, 1980b) are described by the same formal mechanism (cf. eq 9). In the preceding paper we show that eq 9 is compatible with the concerted mechanism [eq 10 (Monod et al., 1965)] but not with the simple sequential mechanism [eq 11 (Koshland, 1970)]:

$$T_0 + 2L \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} (T_1) + L \underset{k_{32}}{\overset{k_{23}}{\rightleftharpoons}} T_1^* + L \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} (T_2) \underset{k_{32}}{\overset{k_{23}}{\rightleftharpoons}} T_2^* \underset{k_{43}}{\overset{k_{34}}{\rightleftharpoons}} R_2 \quad (10)$$

$$\beta_2 + 2L \underset{}{\overset{K_{d,1}}{\rightleftharpoons}} \beta_2 L + L \underset{}{\overset{K_{d,2}}{\rightleftharpoons}} \beta_2 L_2 \quad (11)$$

Equation 10 accounts only for the observable intermediates of the general scheme, expanded by including (T_1) and (T_2), intermediates present at steady-state concentrations. The rate constants correspond to those defined by the concerted mechanism [cf. Figure 6 in the preceding paper (Tschopp & Kirschner, 1980b)] as follows: $k_{23}/K_{2,1} = k_A'$, $k_{32} = k_D'$, $k_{34} = k_2'$, and k_{43} and k_2.

The fastest observable reaction ($k_{1,obsd}^B$, Figure 5A) involves the low-affinity state of the apoβ_2 subunit. There is no evidence for kinetic heterogeneity of the two binding sites. We have previously suggested that the isomerization step $(T_1) \rightarrow T_1^*$ and $(T_2) \rightarrow T_2^*$, which corresponds to $(BL) \rightleftharpoons BL^*$ (eq 9), involves some local change in the conformation of the binding site when the ligand is PNP (Tschopp & Kirschner, 1980b). It is likely, therefore, that the formation of the internal aldimine during both the $(T_1) \rightarrow T_1^*$ and the $(T_2) \rightarrow T_2^*$ transitions is accompanied by a similar conformation change when the ligand is PLP.

The rate of the second reaction phase observed after mixing PLP with the apoβ_2 subunit ($k_{2,obsd}^B$, Figure 5B) is independent of PLP concentration. In the analogous case of PNP binding, this reaction has been assigned to the concerted transition of the low-affinity T state to the high-affinity R state of the apoβ_2 dimer (Tschopp & Kirschner, 1980b).

The value of $k_{2,obsd}^B$ is equal to $k_2' + k_2 \approx k_2'$ ($= k_{34} = 5 \times 10^{-3}\ s^{-1}$) at high PLP concentration (Table II). The value of k_2 ($= k_{43} = 6 \times 10^{-6}\ s^{-1}$) is calculated from the values of k_2' and $[L_2] = k_2/k_2' = 1.4 \times 10^{-3}$ (Tschopp & Kirschner, 1980b). The values of k_0' ($\leq 10^{-4}\ s^{-1}$) and k_0 ($\leq 0.02\ s^{-1}$) have been estimated from the analysis of PNP and PPS binding rates (Tschopp & Kirschner, 1980b). Thus, the rate constants k_i' for the $T_i \rightarrow R_i$ transition change only moderately with increasing number of ligands bound, but the rate constants k_i for the $R_i \rightarrow T_i$ transition decrease dramatically. These relationships resemble closely those found for the cooperative binding of NAD^+ to yeast glyceraldehyde-3-phosphate dehydrogenase, which also obeys the concerted mechanism (Kirschner et al., 1971).

As seen from Table II, the rate constants k_{34} and k_{43} for the concerted $T_2 \rightarrow R_2$ transition are 10–100-fold smaller with PLP as the ligand than with PNP. A similar discrepancy has been noted for the rate constants k_{23} and k_{32} of the local conformational change ($T_1 \rightarrow T_1^*$; $T_2 \rightarrow T_2^*$; cf. eq 10) occurring during the binding reaction. The difference appears to result from the covalent bond between PLP and the lysine amino group. It is also interesting to note that the rate constants for the final generation of the enzymically active state of both the holoβ_2 subunit and the $\alpha_2$holoβ_2 complex are similar (cf. k_{34} and k_{43} in Tables I and II). This similarity suggests that the same kind of conformational change is involved, regardless whether the PLP binding sites interact (β_2 subunit) or not ($\alpha_2\beta_2$ complex). Our inability to detect the third reaction process predicted for the concerted mechanism with slow concerted isomerization (Tschopp & Kirschner, 1980b) is readily explained by the high concentrations of PLP used, relative to the magnitude of $K_T = K_{obsd} = 2.5 \times 10^{-5}$ M (cf. Table II). The small amplitudes allowed measurements to be done only above 1×10^{-4} M PLP (cf. parts A and B in Figure 5). Therefore, the conversion of the apoβ_2 subunit (99.5% T_0) to the holoβ_2 subunit (99.8% R_2) proceeds mainly via the pathway $T_0 \rightarrow T_2^* \rightarrow R_2$. A separate process related to $R_1 \rightarrow R_2$ apparently cannot be observed because T_1^* is populated only transiently, and the formation of R_1 from T_1^* is much slower than the formation of T_2^* from T_1^*.

In conclusion, all equilibrium and kinetic data of PLP binding cooperatively to the apoβ_2 subunit of tryptophan synthase are consistent both qualitatively and quantitatively with the nonexclusive binding concerted mechanism of Monod et al. (1965). The role of the bound α subunit in stabilizing a structure closely related to the R state of the β_2 subunit, which has been discussed in the preceding paper (Tschopp & Kirschner, 1980b), is also valid when the ligand is PLP.

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