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Kinetics of Cooperative Ligand Binding to the Apoβ_2 Subunit of Tryptophan Synthase and Its Modulation by the α Subunit†

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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 $\alpha_2$apoβ_2 complex and in a single slow step to the nicked apoβ_2 subunit that is obtained by limited proteolysis with trypsin. Both pyridoxine 5′-phosphate and *N*-phosphopyridoxyl-L-serine bind to the apoβ_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 apoβ_2 subunit suggests that the $\alpha_2$apoβ_2 complex is similar to the high-affinity R state and the nicked apoβ_2 subunit is similar to the low-affinity T state of the apoβ_2 subunit. The slow isomerization involved in the cooperative binding of the ligands to the intact apoβ_2 subunit is discussed in terms of local and concerted conformational changes involving the two autonomously folding domains of the β protomer.

Pyridoxal 5′-phosphate (PLP)[1] binds cooperatively to the apoβ_2 subunit and noncooperatively to the $\alpha_2$apoβ_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 apoβ_2 subunit, the nicked apoβ_2 subunit obtained by limited proteolysis with trypsin (Högberg-Raibaud & Goldberg, 1977a,b), and the $\alpha_2$apoβ_2 multienzyme complex. Comparison of the mechanisms of binding of the PLP analogues to the three "states" of the β_2 subunit help us to understand the mechanism of cooperative binding to the native apoβ_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 2×10^{-3} M EDTA and 2×10^{-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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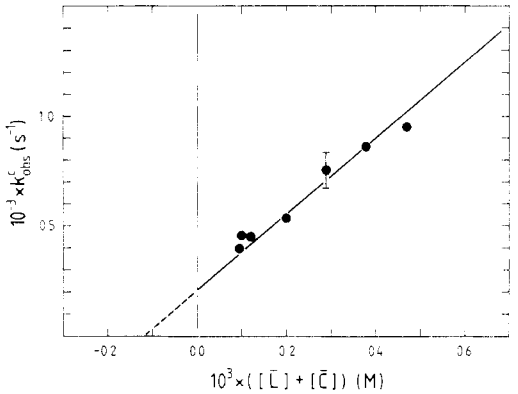

FIGURE 1: Kinetics of the binding of PNP to the $\alpha_2$apoβ_2 complex of tryptophan synthase: k_{obsd}^{c} increases with total PNP concentration. Temperature-jump measurements in buffer A, T = 20 °C, with observation of absorbance changes at 290 nm. Concentration of the enzyme was 0.08 mM site equiv of apoβ_2 subunit and 0.088 mM α subunit. The bar represents the average standard deviation. The theoretical curve was fitted to eq 2 as explained in the text, and the kinetic parameters are as listed in Table I.

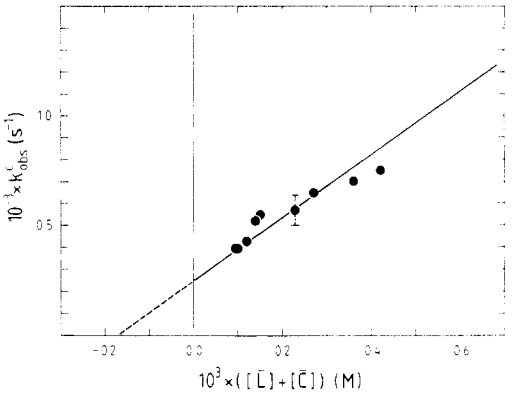

FIGURE 2: Kinetics of the binding of PPS to the $\alpha_2$apoβ_2 complex of tryptophan synthase: k_{obsd}^{c} increases with the total concentration of PPS. Concentration of the enzyme was 0.08 mM site equiv of apoβ_2 subunit and 0.088 mM α subunit. Other conditions were as under Figure 1.

Stopped-flow measurements were carried out with a Durrum-Gibson stopped-flow apparatus [dead time 2 ms; 1-cm path length (Paul et al., 1980)]. External clocking of the transient recorder allowed measurements of slow phases with half-times of up to 500 s. Enzyme and ligand solutions were Millipore filtered and partially degassed before mixing. About 10 measurements at each concentration were averaged, and the rate constants and amplitudes were calculated by least-squares fits of single- or double-exponential curves to averaged data and analyzed as described under Results.

Temperature-jump experiments were carried out with the apparatus described by Cohn et al. (1979). All solutions were clarified by Millipore filtration and briefly degassed in the cell. All progress curves were followed by absorbance measurements at 290 nm. The temperature was raised from 16 to ~24 °C in 10 μs by discharge of a 50-nF capacitor charged to 30 kV. About 20 measurements from each concentration were averaged before analysis with the same system as described for stopped-flow experiments. The absorption spectrum and the activity of the enzyme remained stable throughout the experiments.

Results

Binding to the $\alpha_2$apoβ_2 Subunit. Since the binding of PNP and PPS is rapid, the temperature-jump technique with absorbance measurements at 290 nm (Tschopp & Kirschner, 1980) was used to record the progress curves. Only a single-exponential increase of absorbance occurs, subsequent to an instantaneous increase during the temperature jump. The amplitude A^0 and the rate constant k_{obsd}^{c} obey the relationship $\Delta A_t = A + A^0 \exp(-k_{obsd}^{c} t)$, where ΔA_t is the time-dependent absorbance difference to the final equilibrium value. The total amplitude ($A + A_0$) agrees with static temperature difference spectra. No further relaxation process is observable with either temperature-jump or rapid mixing experiments. Neither enzyme solutions alone nor PNP nor PPS solutions alone give any measurable relaxation effects.

k_{obsd}^{c} increases when the total concentration of PNP is increased while the total concentration of enzyme is kept constant (Figure 1). The same concentration dependence is observed when the ligand is PPS (Figure 2), and it is consistent with an elementary binding reaction according to

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

Table I: Rate and Equilibrium Constants for PNP and PPS Binding to the $\alpha_2$apoβ_2 Complex of Tryptophan Synthase on the Basis of Equation 1 (C + L ⇌ CL)

ligand	K_d[a] (M)	k_{12} (M^{-1} s^{-1})	k_{21} (s^{-1})	$K_{2,1}$ (M)
PPS	1.7×10^{-4}	2.1×10^{6}	350	1.7×10^{-4}
PNP	1.2×10^{-4}	2.5×10^{6}	310	1.2×10^{-4}

[a] Data from Tschopp & Kirschner (1980).

where C is the free binding site, L is the free ligand, and CL is the complex.

The data were fitted to eq 2 (Eigen & DeMaeyer, 1974;

$$k_{obsd}^{c} = k_{21} + k_{12}([\bar{L}] + [\bar{C}]) \qquad (2)$$

Bernasconi, 1976), which expresses k_{obsd}^{c} as a function of the sum of the free reactant concentrations and the rate constants of eq 1.

The values of $[\bar{L}]$ and $[\bar{C}]$ were calculated from the known total concentrations of ligand and binding sites by an iterative procedure, assuming the know overall equilibrium dissociation constant.

As can be seen from Figures 1 and 2, the straight lines extrapolate to the negative abscissas at points corresponding to the observed equilibrium dissociation constants $K_{2,1} = k_{21}/k_{12}$ [0.12 mM for PNP and 0.17 mM for PPS (Tschopp & Kirschner, 1980)]. The values of the rate constants obtained from the ordinate intercepts (k_{21}) and slopes (k_{12}) are listed in Table I.

Binding to the Apoβ_2 Subunit. PNP and PPS are bound ~1000-fold more slowly to the apoβ_2 subunit of tryptophan synthase than to the $\alpha_2$apoβ_2 complex. Temperature-jump experiments did not reveal relaxation processes faster than τ < 10 ms, and therefore rapid mixing experiments were performed to detect slower rate processes. The total ligand concentration was always in large excess over the total enzyme concentration to ensure pseudo-first-order conditions.

After rapid mixing of enzyme with ligand, the absorbance at 290 increases in two distinct steps according to the expression

$$\Delta A_t = A_1^0 \exp(-k_{1,obsd}^{B} t) + A_2^0 \exp(-k_{2,obsd}^{B} t)$$

where ΔA_t is the time-dependent absorbance difference to the final equilibrium value.

The sum of the experimental amplitudes ($A_1^0 + A_2^0$) equals the absorption difference observable at equilibrium.

When an enzyme solution partially saturated with PNP was mixed rapidly with a large excess of the ligand, no additional

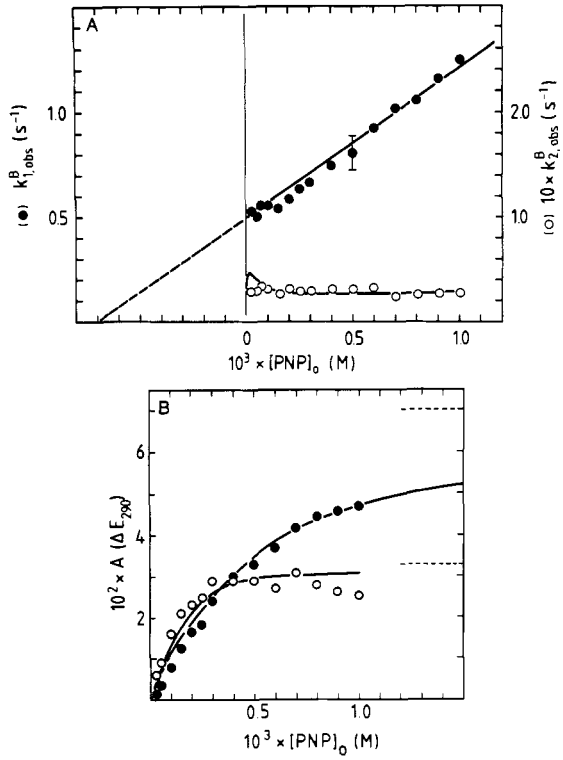

FIGURE 3: Kinetics of binding of PNP to the apoβ_2 subunit of tryptophan synthase: stopped-flow measurements in buffer A, T = 20 °C, with observation of absorbance changes at 290 nm. (A) Dependence of $k_{1,obsd}{}^B$ and $k_{2,obsd}{}^B$ on the total concentration of PNP. (●) Left-hand scale: $k_{1,obsd}{}^B$. (○) Right-hand scale: $k_{2,obsd}{}^B$. The concentration of apoβ_2 subunit was 0.022 mM site equiv. The kinetic parameters are listed in Tables II and IV, and the theoretical curves were calculated as described in the text. (B) Dependence of the reaction amplitudes on the total concentration of PNP. (●) Amplitude of the fast reaction phase. (○) Amplitude of the slow reaction phase. Theoretical curves were calculated according to the concerted mechanism with the equilibrium constants listed in Table III as described in the text.

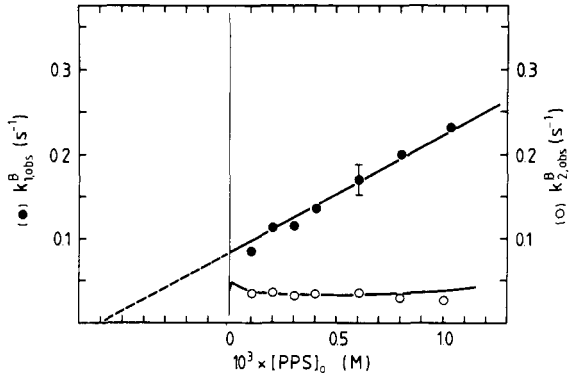

FIGURE 4: Kinetics of binding of PPS to the apoβ_2 subunit of tryptophan synthase: dependence of the rate constants of the fast (●) and slow (○) rate processes on the total concentration of PPS. Stopped-flow measurements in buffer A, T = 20 °C. Other conditions were as in Figure 3. Concentration of apoβ_2 subunit was 0.022 mM site equiv. The kinetic parameters are listed in Tables II and IV, and the theoretical curves were calculated as described in the text.

rate processes were discerned. Thus, the two rate processes observed in the previous experiments suffice to account for the overall reaction.

As seen in Figures 3A and 4, $k_{1,obsd}{}^B$ increases linearly as predicted for a binding reaction (cf. eq 1). The value of the apparent second-order rate constant $k_{R,app}$ equals 700 M^{-1} s^{-1}. This value is 6 orders of magnitude smaller than the diffusion-controlled limit (Hammes & Schimmel, 1970; Pecht & Lancet, 1977). Since no relaxation indicative of very rapid binding of ligands to the apoβ_2 subunit was observable in temperature-jump experiments, the first rate process ($k_{1,obsd}{}^B$) probably represents an overall binding reaction

Table II: Rate and Equilibrium Constants of Ligand Binding to the T State of the Native Apoβ_2 Subunit and to the Nicked Apoβ_2 Subunit on the Basis of Equation 3 [B + L ⇌ (BL) ⇌ BL*]

β_2 subunit	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})
intact	PPS	$\geq 5 \times 10^{-3}$	6×10^{-4}	1.5×10^2	≥ 0.75	0.09
	PNP	$\geq 5 \times 10^{-3}$	7×10^{-4}	7.0×10^2	≥ 3.5	0.49
nicked	PNP	$\geq 5 \times 10^{-3}$	1×10^{-3}	1.5×10^3	≥ 7.5	1.5

[a] Minimum values were estimated as described in the text. [b] $K_{obsd} = k_{32}K_{2,1}/k_{23} = K_T$. [c] $k_{R,app} = k_{23}/K_{2,1}$.

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

where B is the β protomer, (BL) and BL* are the enzyme–ligand complexes, and L is the ligand. As indicated by the parentheses, the intermediate is present only at a low, steady-state concentration.

Under these conditions only a single relaxation is observable and $k_{1,obsd}{}^B$ depends on reactant concentrations according to (Eigen & DeMaeyer, 1974; Bernasconi, 1976)

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

The apparent second-order rate constant $k_{R,app}$ equals $k_{23}/K_{2,1}$ and can be determined from the slopes of the straight lines in Figures 3A and 4 according to eq 4. Assuming a standard deviation of ±10% for the experimental values of $k_{1,obsd}{}^B$ at high concentrations of ligand, the estimated lower limit of $K_{2,1}$ equals $\geq 5 \times 10^{-3}$ M both for PNP and for PPS. k_{32} is obtained from the ordinate intercepts of Figures 3A and 4. The lower limits of k_{23} are estimated from $k_{R,app}$ and the values of $K_{2,1}$. The equilibium dissociation constant of the first binding reaction is $K_{obsd} = [\bar{L}][\bar{B}]/[\overline{BL}^*] = k_{32}/k_{R,app}$. It can be determined from the intercept of the straight lines in Figures 3 and 4 with the negative abscissa, and the values are collected in Table II.

The rate constant $k_{2,obsd}{}^B$ is practically independent of ligand concentration (cf. Figures 3A and 4). The simplest interpretation is that BL* (cf. eq 3) undergoes a further, slow isomerization (BL* ⇌ BL**). The overall mechanism of PNP and PPS binding is given in eq 5, an extension of eq 3:

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

As shown in Figure 3B, the amplitude $A_1{}^0$ of the fastest detectable binding process is half-maximal at 0.7 mM PNP concentration, in good agreement with the value of K_{obsd} (Table II). In contrast to $A_1{}^0$, the amplitude $A_2{}^0$ of the slow process is half-maximal at 0.1 mM PNP concentration. The sigmoidal equilibrium binding curve is half-maximal at the intermediate value of 0.27 mM PNP concentration (Tschopp & Kirschner, 1980).

These observations suggest that the mechanism of cooperative PNP binding involves (a) the comparatively rapid binding of ligand to a low-affinity state of the enzyme possessing two identical binding sites with $K_d = K_{obsd}$ = 0.7 mM and (b) a slow isomerization of the initially formed enzyme–ligand complex (BL*) to the thermodynamically stable state of high affinity (BL**).

Binding to the "Nicked" Apoβ_2 Subunit. PNP binds noncooperatively and weakly to the $(F_1F_2)_2$ complex (Tschopp &

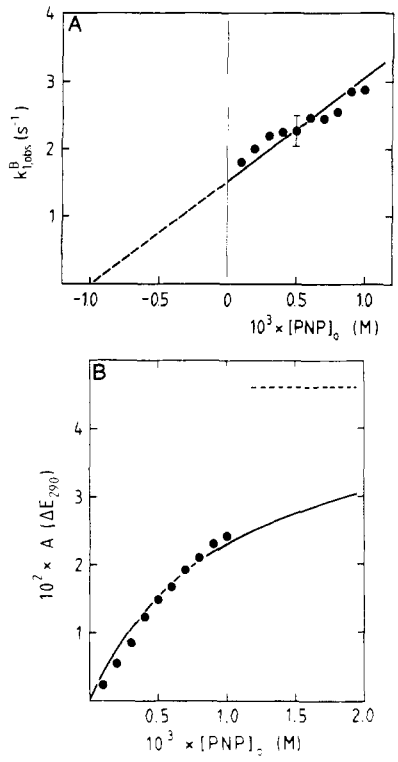

FIGURE 5: Kinetics of binding of PNP to the nicked apoβ_2 subunit of tryptophan synthase. (A) Dependence of $k_{1,obsd}{}^B$ on the total PNP concentration. Stopped-flow measurements in buffer A, T = 20 °C, with observation of absorbance changes at 290 nm. The concentration of nicked apoβ_2 was 0.024 mM site equiv. The kinetic parameters are listed in Table II, and the theoretical curve was calculated as described in the text. (B) Concentration dependence of the amplitude. The theoretical curve was calculated with K_T = 1.0 mM and $\Delta\epsilon$ = 1.92 mM^{-1} cm^{-1}.

Kirschner, 1980) that is obtained by limited proteolysis of the holoβ_2 subunit with trypsin (Högberg-Raibaud & Goldberg, 1977a,b; Crawford et al., 1978). Similar behavior has been found for the binding of PLP to the nicked apoβ_2 subunit (P. Bartholmes, personal communication). Thus, the nicked subunit seems to mimic the intact subunit constrained to the low-affinity state, and rapid mixing experiments were therefore performed to test this hypothesis.

Only a slow exponential reaction is observable after rapid mixing of the nickel apoβ_2 subunit with excess PNP [$\Delta A_t = A^0 \exp(-k_{1,obsd}{}^B t)$]. As seen in Figure 5A, $k_{1,obsd}{}^B$ increases linearly with the concentration of excess, total PNP. The rate constants evaluated according to eq 4 are listed in Table II. The amplitude of the binding process (A^0) increases hyperbolically to a plateau value (Figure 5B). The concentration dependences of both $k_{1,obsd}{}^B$ and A^0 are consistent with the equilibrium dissociation constant determined independently [$K_d = 10^{-3}$ M; $\Delta\epsilon_{290}$ = 1.7 mM^{-1} cm^{-1} (Tschopp & Kirschner, 1980)].

Discussion

Mechanism of Cooperative Binding to the Apoβ_2 Subunit. The observation of a slow isomerization reaction that is directly involved in generating a high-affinity state of the dimeric apoβ_2 subunit rules out the simple sequential mechanism (Koshland, 1970), because this model explicitly excludes isomers of enzyme–ligand complexes:

$$\beta_2 + 2L \overset{K_{d,1}}{\rightleftharpoons} \beta_2L + L \overset{K_{d,2}}{\rightleftharpoons} \beta_2L_2$$

The concerted mechanism with nonexclusive ligand binding

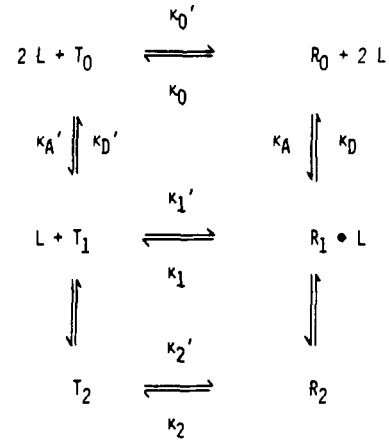

FIGURE 6: Concerted mechanism of cooperative binding to a dimeric protein. The scheme (Monod et al., 1965) is modified by including all-or-none transitions between the variously saturated species R_i and T_i (i = 0, 1, or 2). k_i and k_i' are the corresponding isomerization rate constants. k_A and k_A' and k_D and k_D' are the intrinsic rate constants of association and dissociation, respectively, of the vertical steps. The following relations between rate and equilibrium constants exist: $L_0 = [T_0]/[R_0] = k_0/k_0'$; $L_i = [T_i]/[R_i] = c^i L_0 = k_i/k_i'$, where $c = K_R/K_T$. $K_R = 2[R_0][L]/[R_1] = [R_1][L]/(2[R_2]) = k_D/k_A$. $K_T = 2[T_0][L]/[T_1] = [T_1][L]/(2[T_2]) = k_D'/k_A'$. $\alpha = [L]/K_R$; $c\alpha = [L]/K_T$.

[Figure 6 (Monod et al., 1965)] is consistent with the data, however, because a slow isomerization reaction is clearly observed (cf. $k_{2,obsd}{}^B$ in Figures 3 and 4). Under these conditions the concerted mechanism makes specific predictions for the number of observable rate processes. If the apoβ_2 subunit exists predominantly in the low-affinity T state, rapid mixing with excess ligand should result in only two exponential rate processes (Kirschner, 1971). The design of the experiment leads to the exclusive binding of ligand to two identical binding sites in the T state, while the T → R transition has not progressed significantly. The amplitude is expected to follow an hyperbolic saturation curve, and the values of K_{obsd} obtained from the concentration dependences of both $k_{1,obsd}{}^B$ and the half-maximal amplitude $A_1{}^0$ should be identical. As seen in parts A and B of Figure 3, this is the case. Rapid mixing experiments thus amount to an independent titration of the T state, and K_{obsd} is identical with K_T in the nomenclature of Monod et al. (1965).

In terms of the concerted mechanism, the slow isomerization with $k_{2,obsd}{}^B$ is due to the concerted transition of both protomers of the apoβ_2 dimer from a low-affinity T state to a high-affinity R state.

The relaxation spectrum of the concerted mechanism with slow isomerization consists of three time constants. The third relaxation process arises from the rapid binding of ligand to the R state of the apoβ_2 subunit (Eigen, 1967; Kirschner et al., 1971; Janin, 1973; Hammes & Wu, 1974).

Because the rate processes observed with the β_2 subunit are slow, the temperature-jump method cannot be used to perturb the equilibria. Cooling interferes after ~0.5 s. We therefore used perturbation by concentration jump as a method for detecting the predicted third rate process. When a solution of apoβ_2 subunit that was partially saturated with PNP was rapidly mixed with a large excess of PNP, only the two familiar exponential rate processes were observable, however. It is possible that both low concentrations and small relative extinction coefficients of the species R_0, R_1, and R_2 (Figure 6) prevent observation of the expected third rate process.

Alternative Mechanisms. We have considered more complicated models such as the general sequential mechanism

Table III: Equilibrium Constants of Cooperative Ligand Binding to the Apoβ_2 Subunit

ligand	parameters of Adair scheme[a]		parameters of concerted mechanism[b]					
	$K_{d,1}$ (M)	$K_{d,2}$ (M)	K_T (M)	K_R (M)	c	L_0	L_1	L_2
PLP	8.7×10^{-6}	2.3×10^{-7}	2.5×10^{-5}[c]	6.7×10^{-8}	2.7×10^{-3}	200	0.53	1.4×10^{-3}
PPS	5.0×10^{-4}	1.3×10^{-4}	6.0×10^{-4}	1.5×10^{-5}	2.5×10^{-2}	200	5	0.13
PNP	6.0×10^{-4}	1.2×10^{-4}	7.0×10^{-4}	2.1×10^{-5}	3.0×10^{-2}	200	6	0.18

[a] Calculated from the Adair parameters as given by Bartholmes et al. (1976) for PLP and by Tschopp & Kirschner (1980) for PNP and PPS. [b] Equilibrium constants as defined in Figure 6. [c] Bartholmes et al. (1980).

(Koshland, 1970) and mechanisms such as $B + 2L \rightleftharpoons BL + L \rightleftharpoons BL^* + L \rightleftharpoons BL_2^* \rightleftharpoons BL_2^{**}$ where the binding steps are in rapid equilibrium and the isomerization steps are slow. However, the assumption of more than two conformational states and more than three different types of elementary binding reaction is not warranted in view of only two observable rate processes.

The concerted mechanism with nonexclusive ligand binding [Figure 6 (Monod et al., 1965)] is the simplest consistent with the available equilibrium and rate data. In particular, the failure to observe additional rate processes when half-saturated β_2 subunit is mixed with excess ligand is most easily explained by the concerted mechanism. A definite assignment of mechanism requires a wealth of additional spectroscopic and structural information, however, and the following discussion merely emphasizes the consistency of the data with the concerted mechanism.

Data Fitting to the Concerted Mechanism. In order to fit the rate constants and amplitudes quantitatively to the concerted mechanism, the values of both the equilibrium dissociation constant of R_1 and R_2 (K_R) and the allosteric constant $[T_0]/[R_0] = L_0$ must be known. These constants were determined by a simultaneous fit of the binding curves of PLP (Bartholmes et al., 1976), of PNP, and of PPS (Tschopp & Kirschner, 1980) to the concerted mechanism. The value of K_{obsd} obtained from kinetic measurements [Figures 3A and 4 (Bartholmes et al., 1980)] is identical with K_T, the equilibrium dissociation constant of T_1 and T_2. Moreover, because the cooperative binding of all three ligands should be described by the same value of L_0, severe restrictions are imposed on the fit.

The best fit values of K_R and L_0 are found by using the relationships $K_{d,1} = K_R(1 + L_0)/(1 + cL_0)$ and $K_{d,2} = K_R(1 + cL_0)/(1 + c^2L_0)$, where $c = K_R/K_T$. $K_{d,1}$ and $K_{d,2}$ are directly related to the Adair parameters Ψ_1 and Ψ_2 (Bartholmes et al., 1976; Tschopp & Kirschner, 1980). By use of the known values of $K_{d,1}$ and $K_{d,2}$ and the values of K_T determined in this study, the best fit value of L_0 equals 200 ± 50. The other parameters are listed in Table III.

The rate constants for the binding of either PNP or PPS to the two identical binding sites in the T state of the apoβ_2 subunit ($T_0 + L \rightleftharpoons T_1$; $T_1 + L \rightleftharpoons T_2$; cf. Figure 6) are identical with $k_{23}/K_{2,1}$ and k_{32} (cf. eq 4). They are collected in Table II.

As seen from Table III, the ratio $[T_1]/[R_1] = L_1$ equals 5–6 for PNP and PPS as ligands. This distribution must lead to small amplitudes for the predicted third rate process involving the binding of ligands to the R state ($R_0 + L \rightleftharpoons R_1$; $R_1 + L \rightleftharpoons R_2$) unless the extinction coefficients are unusually large. The reaction amplitudes depicted in Figure 3B indicate that the extinction coefficients R_1 and R_2 are similar to those of T_1 and T_2, however. Thus, the concerted mechanism provides a consistent explanation for not observing a third rate process.

The concentration dependence of the rate constants of the slow concerted transitions is most easily described when the concentration of the free ligand does not change significantly during the isomerization (Eigen, 1967; Kirschner et al., 1971). This condition is fulfilled for the experiments of Figure 3A and 4, and the concentration dependence of $k_{2,obsd}^B$ is given by

$$k_{2,obsd}^B = \frac{k_0 + 2k_1\alpha + k_2\alpha^2}{(1 + \alpha)^2} + \frac{k_0' + 2k_1'c\alpha + k_2'(c\alpha)^2}{(1 + c\alpha)^2} \quad (6)$$

with the constants as defined in Figure 6.

Table IV: Rate Constants of the Concerted Transition $T_i \rightleftharpoons R_i$ in Ligand Binding to the Apoβ_2 Subunit[a]

ligand	rate constant (s^{-1})[b]					
	k_0	k_1	k_2	k_0'	k_1'	k_2'
PNP	≤0.02	0.06	0.005	$\leq 10^{-4}$	0.01	0.03
PPS	≤0.02	0.05	0.004	$\leq 10^{-4}$	0.01	0.03

[a] Fit of data from Figures 3A and 4 to eq 6. [b] Rate constants are as defined in Figure 6.

The individual values of the k_i and k_i' cannot be determined unequivocally. Nevertheless, a set of values was found, by trial and error, which gives a satisfactory fit to the data as shown by the theoretical curves in Figures 3A and 4. As seen from Table IV, the rate constants k_i' for the $T_i \rightarrow R_i$ transition increase with i, and the rate constants k_i for the $R_i \rightarrow T_i$ transition decrease with i. These relationships differ from the case of cooperative binding of NAD^+ to tetrameric yeast glyceraldehyde-3-phosphate dehydrogenase, where $k_0' = k_1' = k_2' = k_3' = k_4'$ and $k_0 > k_1 > k_2 > k_3 > k_4$ (Kirschner et al., 1971).

The concentration dependences of A_1^0 and A_2^0 (Figure 3B) are also consistent quantitatively with the predictions of the concerted mechanism. For rapid mixing of the apoβ_2 subunit, which is practically completely in the T state ($[T_0] = 200[R_0]$), with excess ligand, the expression for A_1^0 is given by

$$A_1^0 = \Delta\epsilon_T[B_0][[\bar{L}]/([\bar{L}] + K_T)] \quad (7)$$

where $[B_0]$ is the total concentration of β protomers and $[\bar{L}]$ may be replaced by $[L_0]$ to a good first approximation. $\Delta\epsilon_T$ equals 3.2 mM^{-1} cm^{-1} as calculated from a direct linear plot (Cornish-Bowden, 1976) of the amplitude data. $\Delta\epsilon_R$ was calculated from the sum of maximum values for A_1^0 and A_2^0 extrapolated to infinite ligand concentration. It equals 4.7 mM^{-1} cm^{-1} and agrees well with the difference spectrum observed at equilibrium (Tschopp & Kirschner, 1980). With the known difference extinction coefficients and equilibrium constants, the total amplitude A^0 is given by

$$A^0 = A_1^0 + A_2^0 = (\Delta\epsilon_R\bar{Y}_R + \Delta\epsilon_T\bar{Y}_T)[B_0] \quad (8)$$

where Y_R and $\bar{Y}_T$ obey the relationships $\bar{Y}_T = L_0c\alpha(1 + c\alpha)/[(1 + \alpha)^2 + L_0(1 + c\alpha)^2]$ and $\bar{Y}_R = \alpha(1 + \alpha)/[(1 + \alpha)^2 + L_0(1 + c\alpha)^2]$ with α, c, and L_0 as defined in Figure 6.

As seen in Figure 3B, the calculated values of A_2^0 agree well with the data.

The $\alpha_2$apoβ_2 Complex Resembles the R State of the Apoβ_2 Subunit. The effect of the α subunit on the cooperative binding of PLP to the apoβ_2 subunit follows the pattern predicted for allosteric activators (Monod et al., 1965). On one

hand, the α subunit itself binds cooperatively to the apoβ_2 subunit (Bartholmes & Teuscher, 1979) and noncooperatively to the holoβ_2 subunit (Creighton & Yanofsky, 1966). On the other hand, bound α subunit converts the binding of PLP and PLP analogues to the apoβ_2 subunit from cooperative to noncooperative.

Comparison of eq 1 with eq 5 shows that the change of equilibrium binding properties brought about by bound α subunit is based on a change of mechanism, chiefly by eliminating the two slow isomerization processes that are observed only with the apoβ_2 subunit. It appears that the binding site in the $\alpha_2$apoβ_2 complex exists in a rigid state that is optimally arranged for binding specific ligands with relatively high affinity ($K_{2,1} \sim 10^{-4}$ M) and rapidly ($k_{12} \sim 10^6$ M^{-1} s^{-1}) with a single step (eq 1). In contrast, the binding site in the T state of the apoβ_2 subunit has intrinsically very low affinity (B + L $\rightleftharpoons$ BL; $K_{2,1} \sim 10^{-2}$ M), and moderate overall affinity ($K_{obsd} \sim 10^{-3}$ M) is achieved only by the slow isomerization (BL $\rightleftharpoons$ BL*, eq 5).

The high-affinity state of the binding site stabilized in the $\alpha_2$apoβ_2 complex appears to be similar to, although not identical with, the R state of the apoβ_2 subunit. Differences are the low turnover number of the holoβ_2 subunit (the species R_2 in Figure 6) by comparison with the $\alpha_2$holoβ_2 complex (Miles, 1979) and the smaller value of K_R (Table III) by comparison with K_d (Table I).

In conclusion, it is likely that the mechanism of ligand binding to the $\alpha_2$apoβ_2 complex (eq 1) closely resembles the postulated mechanism of ligand binding to the R state of the apoβ_2 subunit ($R_0 + L \rightleftharpoons R_1$ and $R_1 + L \rightleftharpoons R_2$; compare eq 1 with Figure 6), which is not observable due to the relatively low concentrations of R_0 and R_1 under the experimental conditions used here.

The Nicked Apoβ_2 Subunit Resembles the T State of the Apoβ_2 Subunit. Removal of a short internal peptide converts the ligand binding properties of the apoβ_2 subunit from cooperative to noncooperative. The binding mechanism (eq 3) and the rate constants (Table II) are very similar to those observed for ligand binding to the T state of the apoβ_2 subunit.

Therefore, the nicked apoβ_2 subunit can be regarded as a stabilized form of the T state of the intact apoβ_2 subunit, unable to undergo the concerted conformational change to the R state in spite of saturation with PNP and PPS. This interpretation is supported by the low affinity of the α subunit for the nicked holoβ_2 subunit (Högberg-Raibaud & Goldberg, 1977a,b) and the low value of $K_{d,1}$ characterizing the cooperative binding of the α subunit to the native apoβ_2 subunit (Bartholmes & Teuscher, 1979).

Nature of the Isomerization Reactions. Ligand binding involves rate-limiting isomerization steps for the apoβ_2 subunit (eq 3). Since PNP and PPS do not form an internal aldimine, the isomerization is due to either a conformational change or a slow protonation step (Miles & McPhie, 1974). Whatever the exact structural interpretation may be, it is important to note that the process involves local changes that are not transmitted from one protomer to the other. Nicking of the polypeptide chain has no influence on the mechanism and rates of binding (Table II). Local conformational changes of this kind have been shown to occur during the cooperative binding of ligands to aspartate transcarbamylase (Howlett et al., 1977) and do not violate the basic assumptions of the concerted mechanism.

The concerted, slow transition of $T_i \rightleftharpoons R_i$ observable only with the intact apoβ_2 subunit (eq 5 and Figure 6) represents a gross conformational change. This notion is supported by the different solubilities of apo- and holoβ_2 subunits in ammonium sulfate solutions (Adachi & Miles, 1974). Complement fixation studies with antiserum directed against the holoβ_2 subunit (Zalkin et al., 1980) indicate that neither pyridoxal phosphate binding nor proteolytic nicking modifies the antigenic determinants of the apoβ_2 subunit significantly. The reactivity of cysteine-62 toward SH reagents is also unaltered when the holoβ_2 subunit is converted to the $(F_1F_2)_2$ holo complex (Higgins & Miles, 1978; Goldberg & Högberg-Raibaud, 1979). These results rule out major differences of tertiary structure between the apo- and holoβ_2 subunits. A more plausible explanation for the concerted isomerization is the change of relative orientation of the comparatively rigid F_1 and F_2 domains (Zakin et al., 1980) with respect to each other. It is an intriguing question as to how both the α subunit and PLP (or its analogues) bring about roughly the same conformational change, although they necessarily bind to different regions of the β protomer.

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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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