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Circular Dichroism Studies on the Interaction of Tryptophan Synthase with Pyridoxal 5′-Phosphate†

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ABSTRACT: The interaction between the β_2 subunit of tryptophan synthase and the coenzyme pyridoxal 5′-phosphate (PLP) is characterized by induced circular dichroism (CD) in the near-UV (260–285 nm) and in the visible region (320–480 nm, extrinsic Cotton effect). Because of its high mean residue ellipticity ($[\theta]$ = 56 deg cm^2 dmol^{-1} for the isolated holo-β_2 subunit and 102 deg cm^2 dmol^{-1} for the α_2-holo-β_2 complex, respectively) the latter has been used to define different conformational states of the β_2 dimer via CD titrations. Fitting the obtained binding parameters to the known data from equilibrium dialysis leads to the result that the low-affinity state of the isolated β_2 subunit shows a 3 times greater rotational strength than the holoenzyme in the high-affinity state. The generation of the final CD amplitude is characterized by a rate constant intermediate between the values for the formation of the internal aldimine and for the regain of enzymatic activity. Interaction of the α_2-apo-β_2 bienzyme complex with the cofactor leads to a hyperbolic binding curve which is apparently free of contributions caused by unspecific PLP binding outside the active center. The determined dissociation constant of 9×10^{-7} M is in good agreement with the value of 1×10^{-6} M as obtained by equilibrium dialysis. Binding kinetics reveal a very slow process with a rate constant of 2.6×10^{-4} s^{-1}, significantly smaller than that for the regain of catalytic activity during reconstitution of the enzyme.

The isolated β_2 subunit of tryptophan synthase [L-serine hydro-lyase (adding indole)] (EC 4.2.1.20) from *Escherichia coli* catalyzes the last step of L-tryptophan biosynthesis in the presence of pyridoxal 5′-phosphate (PLP):[1]

L-serine + indole → L-tryptophan + H_2O

PLP and its analogues pyridoxine 5′-phosphate (PNP)[1] and *N*-(5′-phosphopyridoxyl)-L-serine (PPS)[1] bind cooperatively to the apo-β_2 subunit and noncooperatively to the α_2-apo-β_2 bienzyme complex (Bartholmes et al., 1976, 1980; Tschopp & Kirschner, 1980a).

When bound to proteins, a number of optically inactive low molecular weight compounds become optically active (Blauer, 1974). Such induced Cotton effects result from the interaction of the chromophore with the asymmetric environment provided by the binding site on the native protein. For instance, the enzyme-bound PLP chromophore of L-aspartate aminotransferase has been shown to be optically inactive as long as the corresponding polypeptide chain is unfolded in the presence of strong denaturants (Fasella & Hammes, 1964a,b). However, interaction of PLP with the active center in its native conformation leads to a strong extrinsic Cotton effect (Pötsch, 1976; Miles & Moriguchi, 1977) in the $\pi \rightarrow \pi^*$ transition of the aldimine chromophore. In this work, we report CD–equilibrium binding measurements and kinetic experiments with tryptophan synthase and PLP in order to shed some light on the alteration of structural parameters during cofactor binding and the successive generation of the catalytically active holoenzyme.

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[1] Abbreviations used: PLP, pyridoxal 5′-phosphate; CD, circular dichroism; ^{3}H, tritium; PNP, pyridoxine 5′-phosphate; PPS, *N*-(5′-phosphopyridoxyl)-L-serine.

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Materials and Methods

Materials. Dithioerythritol and tris(hydroxymethyl)-aminomethane (Tris) were obtained from Roth (Karlsruhe). Bovine serum albumin (pure) and pyridoxal 5′-phosphate (PLP, A grade) were purchased from Serva (Heidelberg). All other chemicals were of the highest degree of purity available from Merck (Darmstadt). Quartz bidistilled water was used for making up solutions.

Buffer. Unless stated otherwise, all experiments were performed in 0.1 M sodium pyrophosphate (pH 7.5), N_2 saturated (Balk et al., 1981; Bartholmes et al., 1980). Solutions containing PLP were prepared under yellow light and stored in the dark to prevent photolysis of the cofactor (Reiber, 1972).

Enzymes. The α and β_2 subunits of tryptophan synthase were purified and stored as described by Kirschner et al. (1975) and by Bartholmes et al. (1976). The α_2-apo-β_2 complex was assembled by adding a 3-fold molar excess of α subunits to the apo-β_2 subunit (Bartholmes & Teuscher, 1979). Enzymatic activity of the bienzyme complex and its isolated subunits was tested by the standard methods of Smith & Yanofsky, (1963) and Faeder & Hammes (1970). Protein concentrations were determined as described by Miles & Moriguchi (1977) and by Bartholmes et al. (1976). The concentration of PLP was determined spectrophotometrically by using $\epsilon_{388} = 6.6 \times 10^3\ M^{-1}\ cm^{-1}$ in 0.1 M NaOH (Peterson & Sober, 1954).

CD Measurements. Measurements of circular dichroism were performed with a Roussel–Jouan dichrograph II. A thermostatable cell holder was used with cells of 5–10-mm thickness. The optical density was kept below 1.2 for all wavelengths. Values of mean residue ellipticity, $[\theta]$, were calculated by using the expression

$$[\theta] = 3300\Delta A m/(cl) \qquad (1)$$

where ΔA = CD signal, differential dichroic absorption; m = mean residue molecular weight; c = concentration in grams/liter; l = path length of the sample cell. The mean residue molecular weight for the β_2 subunit is 108, as taken from Goldberg et al. (1966). Titration experiments were carried out with separately prepared solutions for each ligand concentration. Complete spectra were recorded over extended periods of time.

As shown by control experiments, the induced positive Cotton effect in the ligand PLP is exclusively raised by the cooperative binding reaction in the active center; unspecific binding of excess PLP to lysine groups outside the active center does not lead to measurable ellipticity. The free coenzyme shows no CD, corrections for contributions of free PLP therefore being unnecessary.

In contrast to equilibrium dialysis, which allows the direct determination of the concentration of free ligand in order to construct corresponding Scatchard plots (Scatchard, 1949), analysis of spectroscopic titration data can only rest upon calculated values of this parameter according to

$$c_L = c_L^0 - \bar{Y} n c_E^0 \qquad (2)$$

where c_L = free ligand concentration, c_L^0 = total ligand concentration, c_E^0 = total concentration of β_2 dimers, $\bar{Y} = (\Delta[\theta]/\Delta[\theta]_{max})$ fractional saturation, and n = number of binding sites.

Thus, if the spectroscopically determined $\bar{Y}$ values for the given cooperative binding process significantly deviate from corresponding equilibrium data, different spectral contributions resulting from different states of the bound ligand have to be taken into consideration. For the sequential mechanism (Koshland, 1970), the respective deviations are formally described by the following modified Adair equation (Adair, 1925):

$$\bar{Y} = \frac{\Delta[\theta]}{\Delta[\theta]_{max}} = \frac{\varkappa_{Ad}\psi_1 P + 2\psi_2 P^2}{2(1 + \psi_1 P + \psi_2 P^2)} \qquad (3)$$

where $\varkappa_{Ad} = [\theta]_{(\beta_2PLP)}/[\theta]_{(\beta_2PLP_2)} > 1$, P = concentration of free PLP, $\psi_1 = 2/K_{d1}$, and $\psi_2 = 1/(K_{d1}K_{d2})$ [$K_{d1} = 8.7 \times 10^{-6}$ M and $K_{d2} = 2.3 \times 10^{-7}$ M are the corresponding microscopic dissociation constants as taken from Bartholmes et al. (1976)].

For the nonexclusive concerted mechanism, a somewhat more involved expression can be derived which, however, leads to a similar qualitative displacement for $\varkappa_{MWC} = [\theta]_T/[\theta]_R > 1$:

$$\bar{Y} = \frac{(1 + Lc^2)[\alpha(1 + \alpha) + \varkappa_{MWC}Lc\alpha(1 + c\alpha)]}{(1 + \varkappa_{MWC}Lc^2)[(1 + \alpha)^2 + L(1 + c\alpha)^2]} \qquad (4)$$

where T, R, L, c, and α are as defined by Monod et al. (1965).

Kinetic Experiments. Kinetic analysis made use of a 10-mm flow cuvette as described by Strittmatter (1964) and a pneumatic drive for 2-mL syringes. Since the time constant of the dichrograph equals 2-s, events occurring after 15 s were routinely observed. The solutions were carefully degassed before being drawn into the syringes.

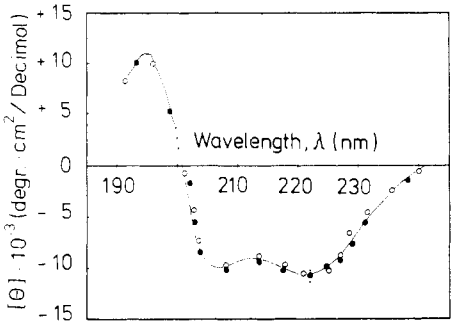

FIGURE 1: Far-UV CD spectrum of the β_2 subunit of tryptophan synthase. (○) Apo-β_2 subunit (4.7 mg/mL); (●) Holo-β_2 subunit (4.7 mg/mL); L = 0.01 cm; 20 °C.

Results

Protein CD Spectrum. Figure 1 shows the far-UV spectrum of the β_2 subunit of tryptophan synthase. According to the methods of Greenfield & Fasman (1969) and Chen et al. (1972), the helix content of the β chain can be estimated from the two extreme values of amide ellipticity at 208 and 222 nm. The resulting values of 21.5% and 27.7%, respectively, agree reasonably well with each other.

The spectra of both the apo- and holoenzyme are almost identical, thereby indicating that no major change in secondary structure occurs in the vicinity of the active center of the β_2 subunit upon PLP binding. Qualitatively the same holds true for the interaction of the $\alpha_2\beta_2$ complex with the coenzyme (data not shown).

That part of the CD spectrum which includes the near-UV and the visible region is shown in Figure 2. Thirteen phenylalanine residues, 12 tyrosine residues, and 1 tryptophan residue (Goldberg et al., 1966; Crawford et al., 1980) constitute the aromatic CD spectrum (250–300 nm), the characteristic fine structure of the phenylalanine residues being considerably less resolved than in the α subunit (Heyn & Weischet, 1975). The increase in the aromatic region of the dichroic spectrum occurring upon binding of PLP cannot unambiguously be interpreted since the internal aldimine itself

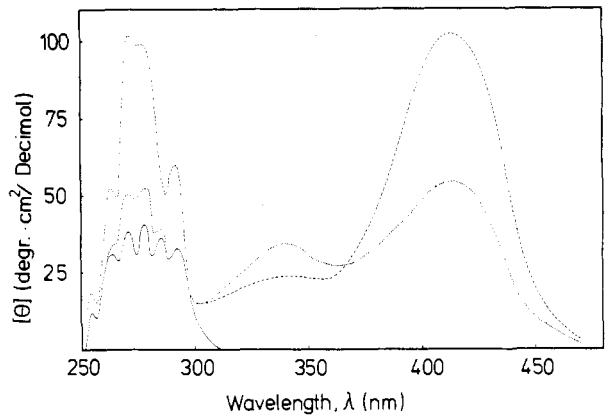

FIGURE 2: CD spectra of the apo-β_2 and holo-β_2 subunit and the native α_2-holo-β_2 bienzyme complex of tryptophan synthase in the near-UV and the visible regions. (—) Apo-β_2 subunit (2.5 mg/mL); (···) Holo-β_2 subunit (2.5 mg/mL); (---) α_2-holo-β_2 complex (2.0 mg/mL).

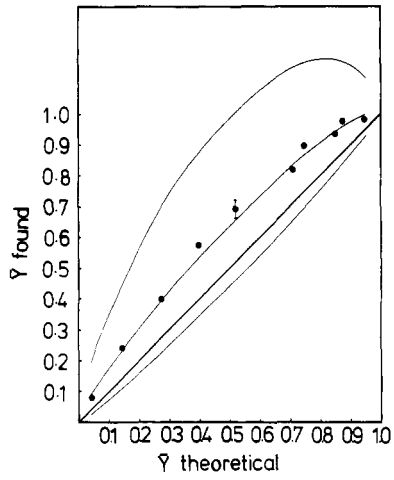

FIGURE 3: Titration of the apo-β_2 subunit of tryptophan synthase with PLP. Comparison with results from equilibrium dialysis. (●) Normalized dichroic absorption at 415 nm. Data points are averaged from ten titration experiments. Enzyme concentrations: 0.8 mg/mL $\leq c \leq$ 2.1 mg/mL; 20 °C. Solid curves from bottom to top: calculated for $\varkappa$ = 0.1, 3.0, and 7.0. Diagonal line: calculated for $\bar{\nu}$ = 1.0 with data from equilibrium dialysis.

absorbs in this wavelength range (Metzler et al., 1980).

In the visible region (320–500 nm), both the holo-β_2 subunit and the α_2-holo-β_2 complex show a significant positive, extrinsic Cotton effect which is characteristic for most PLP-dependent enzymes (Shimomura & Fukui, 1978; Pötsch, 1976; Dunathan, 1971; Martinez-Carrion & Jenkins, 1965). The observed ellipticity values are in accordance with those published by Miles & Moriguchi (1977). At the given wavelength, both apo species reveal no measurable dichroic absorption. Moreover, only the specific interaction of the cofactor with the lysine residue in the active center induces the abovementioned cofactor ellipticity (Balk et al., 1981). Unspecific binding at high concentrations of cofactor (40-fold molar excess) neither influences the specific activity of the enzyme nor gives rise to additional CD effects. Together, these findings provide an excellent means for titrating the apoenzyme with PLP.

CD Titrations. Binding of the cofactor to the apo-β_2 subunit and the $\alpha_2\beta_2$ complex was followed at 415 nm, which is the maximum value of the observed extrinsic Cotton effect, and analyzed as described under Materials and Methods. As shown in Figure 3, the data points, which are averaged from ten titration experiments with apo-β_2 subunit, by no means superimpose on the data obtained from equilibrium dialysis (Bartholmes et al., 1976; H. Balk and P. Bartholmes, unpublished experiments with [^{3}H]PLP). Rather, the titration curve is displaced systematically toward lower values of PLP concentration, leading for low fractional saturation to the calculation (eq 2, Scatchard evaluation) of physically meaningless negative concentrations of free cofactor. The only explanation for this fact is that the induced CD for the initial enzyme–coenzyme complex (β_2·PLP) is significantly higher than that of β_2·PLP$_2$. The $\varkappa$ factor derived from the general formalism in eq 3 is operative also for the nonexclusive concerted binding model of Monod et al. (1965), as proposed recently for the cooperative interaction of the β_2 subunit with PLP and its analogues (Tschopp & Kirschner, 1980b; Bartholmes et al., 1980). The theoretical curve calculated for $\varkappa_{MWC} = [\theta]_T/[\theta]_R = 3.5$ is in good accordance with the primary CD titration data. On the other hand, the binding data collected following long term incubation (specific activity after ≥24 h at 20 °C was about 96%) for the interaction of the completely assembled $\alpha_2\beta_2$ bienzyme complex with PLP are in excellent agreement with those from earlier equilibrium dialysis experiments (Bartholmes et al., 1976). The binding curve is strictly hyperbolic, with $K_d = (0.9 \pm 0.2) \times 10^{-6}$ M and $n = 2$, as estimated from a corresponding Scatchard plot. This had to be expected since the two binding sites for PLP have been shown to be identical and independent (Bartholmes et al., 1976).

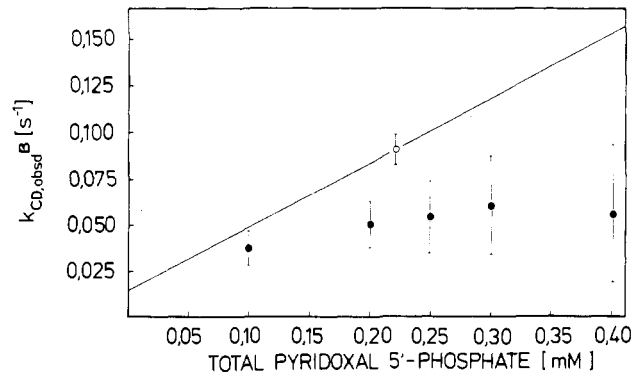

FIGURE 4: Kinetics of PLP binding to the apo-β_2 subunit (●) of tryptophan synthase. Concentration dependence of the observed reaction. Experimental conditions: 1 mg/mL apo-β_2 subunit, 20 °C. (○) Concentration dependence of the formation of the internal aldimine as taken from Bartholmes et al. (1980).

CD Kinetics of PLP Binding to the Apo-β_2 Subunit and to the α_2-Apo-β_2 Complex. The progress curve observed 15 s after the apo-β_2 subunit was rapidly mixed with excess PLP generates ~50% of the amplitude of the measured equilibrium cofactor ellipticity. It consists of a single exponential according to $\Delta[\theta] = \Delta[\theta]_0 \exp(-k_{CD,obsd}^{B} t)$, where $\Delta[\theta]$ is the deviation of the observed ellipticity from the final equilibrium value. $k_{CD,obsd}^{B}$ depends on cofactor concentration, as shown in Figure 4. Control experiments with tandem cuvettes proved that no additional processes with measurable CD amplitude precede the observed reaction which at low PLP concentrations apparently parallels the formation of the internal aldimine as shown recently (Bartholmes et al., 1980). Unfortunately, the low time resolution of the instrument prevented accurate measurements of reaction rates at concentrations higher than 2×10^{-4} M PLP. This finding indicates that the recently postulated concerted isomerization reaction (Bartholmes et al., 1980, eq 9 therein) has to occur at an early stage during the overall binding of PLP to the apo-β_2 subunit.

Cofactor binding to the α_2-apo-β_2 bienzyme complex is characterized by two exponential processes. The first one resembles the fast reaction observed for the interaction of PLP with the isolated apo-β_2 subunit. The second process, which

is 20 times slower than the formation of the active holoenzyme, is described by a concentration-independent rate constant $k_{CD,2,obsd}^{C} = 2.6 \times 10^{-4}$ s^{-1}. Again it has been ascertained by activity measurements that this extremely slow progress curve is not an artifact which, e.g., might be brought about by slow photodecomposition of the holoenzyme. Since the sum of the amplitudes of the two events equals the observed equilibrium value, no further slow reactions with measurable ellipticity are expected to occur.

Discussion

From the two negative extreme values of amide ellipticity in the far-UV region, a rough estimate of the secondary structure of a protein can be given. The far-UV CD spectra of both the apo- and the holo-β_2 subunit are identical within the limits of error. This is in good agreement with the observations of Zakin et al. (1980), who found that both the apo- and the holo-β_2 subunits are immunologically indistinguishable. On the other hand, several lines of evidence [e.g., altered binding properties with respect to the α subunit (Bartholmes & Teuscher, 1979; K. Kirschner and C. Paul, unpublished experiments), different thermal inactivation (Zetina & Goldberg, 1980), and altered neutral salt precipitation (Adachi & Miles, 1974)] exist for changes in the protein conformation upon binding of PLP. Our results are not necessarily in conflict with these findings since the sensitivity of CD in the amide region with respect to small conformational changes is limited (Woody & Tinoco, 1967; Bayley, 1973). Therefore, local alterations in the spatial arrangement of side chains or of elements of secondary structure may not be detectable. Similar observations have been put forward for the interaction of other PLP-dependent enzymes with their cofactor (Wilson & Meister, 1966; Martinez-Carrion et al., 1970). Even the interaction of the holo-β_2 subunit with the corresponding α chain which, e.g., increases the respective turnover numbers by factors of 50–100 via mutually induced conformational changes does not produce a measurable difference spectral effect in the amide ellipticity either.

The near-UV CD spectrum of the β_2 subunit is significantly influenced upon PLP binding. However, since both the aromatic side chains of the enzyme and the internal aldimine absorb in this region, part or all of the spectral changes could be due to induced dichroism in the internal aldimine (Metzler et al., 1980). The fully assembled α_2-holo-β_2 bienzyme complex of course shows a near-UV CD spectrum in which the individual contributions of intrinsic aromatic side-chain ellipticity can by no means be resolved.

In the visible region, holotryptophan synthase exhibits two maxima of dichroic absorption at 335 and 415 nm where the amplitude of the latter is considerably greater than that of the former. The species giving rise to the CD at 415 nm is well-defined as to the internal aldimine between PLP and ϵ-Lys-86 (Crawford et al., 1980) in an aqueous environment in the active center. In addition, the weak Cotton effect around 335 nm may be explained by a different mode of cofactor binding, mainly involving the interaction with hydrophobic side chains. As judged from model reactions (Gani et al., 1978), the probability for the existence of an asymmetric 4′-carbinolamine type of interaction, i.e., an inherent asymmetry of the chromophore, is extremely low. We are thus led to assume that the observed ellipticity at 415 nm is due to an induced extrinsic Cotton effect in the aldimine chromophore. Moreover, several additional points of attachment (e.g., the 5′-phosphate group, the 3-hydroxyl group, and possibly the ring nitrogen) would allow for a rigid anchoring of the ligand to the protein. Such a complex seems to be required to explain a large induced effect (Breusov et al., 1964; Chignell, 1972). The cofactor itself when in solution or unspecifically bound to other proteins such as bovine serum albumin (Johnson & Graves, 1966) does not show ellipticity in the visible region. Excess unspecific binding of PLP to ϵ-lysyl groups other than the essential one leads to a similar effect (Balk et al., 1981). Comparable observations have been made for denatured PLP-dependent holoenzymes (Fasella & Hammes, 1964a,b).

Binding of the α subunit to the holo-β_2 protein increases the induced CD at 415 nm by a factor of 2. This effect, which is even more pronounced than that found by Miles & Moriguchi (1977), indicates a comparatively higher dissymmetry factor $g = \Delta\epsilon/\epsilon$ (Kuhn, 1958) in the bienzyme complex ($g = 6.8 \times 10^{-3}$) than in the isolated β_2 enzyme ($g = 3.7 \times 10^{-3}$). The corresponding molar absorbance ϵ of the internal aldimine is not significantly changed upon formation of the native complex. The observed dissymmetry factors are in accordance with those obtained for other PLP-dependent enzymes (Shimomura & Fukui, 1978; Martinez-Carrion et al., 1970).

It has been shown previously that the cofactor binds cooperatively to the isolated apo-β_2 subunit. CD titrations at 415 nm, which are apparently free of spectroscopic contributions as brought about from unspecific binding to nonessential lysine side chains (see above), lead to the observation that the rotational strength of the aldimine chromophore at low fractional saturation is appreciably higher than in the fully saturated state. This can only be explained when at least two different conformational states of the protein are assumed. So far, this may be taken as evidence favoring the recently proposed minimum scheme for cofactor binding. The generation of the maximum ellipticity at 415 nm is characterized by a rate constant $k_{obsd,CD}^{B}$ of $\sim 5 \times 10^{-2}$ s^{-1}. Consequently, no "overshoot" kinetics are observable in the time range during which the reconstitution of enzymatic activity had been tentatively proposed to be paralleled by a concerted T $\rightleftarrows$ R transition ($k_{obsd} = 4.6 \times 10^{-3}$ s^{-1}; Bartholmes et al., 1980). Thus we are led to suggest that the conversion from the low-affinity T state to the high-affinity R state has to occur at an earlier step of the respective binding mechanism.

The fact that upon binding of cofactor to the α_2-apo-β_2 complex the final CD amplitude is generated in a very slow reaction, which significantly runs after the final regain of enzymatic activity of the holoenzyme complex, can only be explained when a slow reorientation reaction of the enzyme with its cofactor in the resting state is assumed. This effect, however, cannot play an important role in the course of enzymatic turnover. On the other hand, such extremely slow processes during reconstitution of apoenzymes with PLP have been reported previously for tryptophanase (Högberg-Raibaud et al., 1975) and for glutamate decarboxylase (O'Leary & Malik, 1972) as well.

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