Interactions between Tryptophan Synthase from *Escherichia coli* and Derivatives of the Coenzyme Pyridoxal 5′-Phosphate

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Z. Naturforsch. **36 c**, 778–783 (1981); received March 27, 1981

Coenzyme Derivatives, Pyridoxal 5′-Phosphate, Tryptophan Synthase

The interaction of the coenzyme analogues pyridoxal (**A**), pyridoxine 5′-phosphate (**B**), pyridoxic acid 5′-phosphate (**C**) and N-phosphopyridoxyl-L-serine (**D**) with both the isolated apo β_2 subunit and the native α_2 apo β_2 bienzyme complex of tryptophan synthase from *Escherichia coli* has been investigated using enzyme kinetics and CD spectroscopy. A 500-fold molar excess of (**A**) yields a maximum activation of the α_2 apo β_2 complex of 12% compared to the native holo bienzyme complex. The corresponding Michaelis constant K_M equals 0.16 mM. Compounds (**B**–**D**) which lack the reactive carbonyl group in the 4-position cannot act as cofactor during enzymic turnover. However, they are competitive inhibitors with respect to the natural coenzyme pyridoxal 5′-phosphate. The corresponding inhibition constants K_I are for (**B**): 0.10 mM, (**C**): 0.03 mM and (**D**): 0.16 mM.

The CD spectra of the aromatic side chains of both protein species ($[\Theta]_{278nm}$ for the β_2 subunit = 26 degr cm² decimol^{-1}, for the bienzyme complex = 40 degr cm² decimol^{-1}) remain unchanged and no measurable dichroic absorption is induced in the visible region at 415 nm upon addition of (**A**), (at this wavelength productive binding of pyridoxal 5′-phosphate induces a significant extrinsic Cotton effect in the internal aldimine). Reaction with (**B**) leads to an enhancement of the dichroic amplitude at 278 nm of the isolated β_2 subunit ($\Delta[\Theta]$ = 6 degr cm² decimol^{-1}) and of the $\alpha_2\beta_2$ complex ($\Delta[\Theta]$ = 17 degr cm² decimol^{-1}) respectively. Compound (**C**) shows no effect in the aromatic region of the β_2 subunit, but a decrease in the $\alpha_2\beta_2$ complex ($\Delta[\Theta]$ = 5 degr cm² decimol^{-1}). At 315 nm, however, a remarkable extrinsic Cotton effect of + 20 degr cm² decimol^{-1} is induced in (**C**). Ligand (**D**) causes a similar increase at 278 nm of $\Delta[\Theta]$ = 14 degr cm² decimol^{-1} in both protein species. The given data are discussed on the basis of the mode of binding of the natural coenzyme.

Introduction

The isolated, dimeric β_2 subunit of tryptophan synthase from *Escherichia coli* (L-serine hydrolase (adding indole) EC 4.2.1.20) holds bound pyridoxal 5′-phosphate which is the coenzyme participating in the essentially irreversible β-replacement reaction:

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

Work on this enzyme has been reviewed recently by Miles [1]. As shown previously in our laboratory pyridoxal 5′-phosphate binds cooperatively to the β_2 dimer and non-cooperatively to the fully assembled, native $\alpha_2\beta_2$ bienzyme complex suggesting that the β_2 subunit exists in at least three distinct conformations: apo β_2-, holo β_2-subunits and α_2 holo β_2 complex [2, 3]. Employing equilibrium and rapid mixing experiments with β_2 hybrid molecules containing one native protomer and one borohydride reduced protomer [4] and with the coenzyme analogue pyridoxine 5′-phosphate which lacks the reactive carbonyl group in the 4-position of the pyridine ring [5] the mechanism of cofactor binding has been demonstrated to be compatible with the nonexclusive concerted model of Monod *et al.* [6].

The interaction between pyridoxal 5′-phosphate as coenzyme and both the native $\alpha_2\beta_2$ bienzyme complex and the isolated β_2 subunit of tryptophan synthase leads to significant changes in the dichroic absorption of enzyme and ligand. In the near-UV (260–285 nm) a positive intrinsic Cotton effect is induced in the wavelength range of aromatic absorption of the protein; at the same time a large positive extrinsic Cotton effect is observed in the absorption range of the coenzyme (320–480 nm). The latter does not show dichroic absorption in its unbound state [7, 8].

This yields a means of assigning different conformational states of enzyme-ligand complexes to distinct steps of the previously suggested cooperative binding mechanism [9]. In this work, we present binding data for the reaction of pyridoxal 5′-phosphate analogues with tryptophan synthase from *Escherichia coli* in order to characterize the contri-

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0341-0382/81/0900-0778 $ 01.00/0

butions to enzyme-coenzyme interaction brought about by the essential side groups in the 4- and 5′-position of the cofactor.

Materials and Methods

Bovine serum albumin (pure) and pyridoxal 5′-phosphate (A grade) were obtained from Serva (Heidelberg). Tris (hydroxymethyl) aminomethane and dithioerythritol were purchased from Roth (Karlsruhe). All other chemicals were of the highest degree of purity available from Merck (Darmstadt). Quarz bidistilled water was used throughout.

Buffer

Unless stated otherwise all experiments were performed in 0.1 M sodium pyrophosphate (pH 7.5), saturated with N_2 [9, 10]. Solutions containing pyridoxal 5′-phosphate and its various analogues were prepared under yellow light and stored in the dark to prevent photolysis [11].

Coenzyme analogues

Pyridoxal (A grade) was purchased from Serva (Heidelberg) and purified by chromatography on Dowex 1 × 2, 200–400 mesh, acetate [12, 13]. Pyridoxine 5′-phosphate was prepared according to [15] by borohydride reduction of pyridoxal 5′-phosphate. Pyridoxic acid 5′-phosphate was prepared by photooxidation of alkaline pyridoxal 5′-phosphate using a 150 W xenon lamp with a cut-off filter (transparent at ≧ 300 nm). The crude product was recristallyzed 3 times from water [14]. N-phosphopyridoxyl-L-serine was prepared by borohydride reduction of the aldimine between cofactor and L-serine according to [15–18]. Boric acid was removed as described by [19].

Enzymes

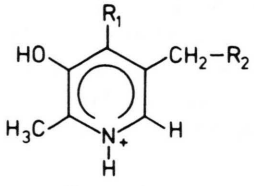
The α and β_2 subunits of tryptophan synthase from *Escherichia coli* (EC 4.2.1.20) were purified and stored as described earlier [2, 20]. The α_2 apo β_2 bienzyme complex was assembled by adding a three-fold molar excess of α subunits to the apo β_2 subunit [21]. Enzymatic activity of the bienzyme complex and its isolated subunits was tested by standard methods according to [22, 23]. Protein concentrations were determined as described [2, 8]. The concentration of pyridoxal 5′-phosphate was determined according to [12].

CD Measurements

Measurements of circular dichroism were performed with a Roussel-Jouan dichrographe II. A thermostated 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 using the expression:

$$[\Theta] = 3300 \cdot \frac{\Delta A \cdot m}{c \cdot l}$$

with $\Delta A = CD$ signal, differential dichroic absorption; m = mean residue molecular weight = 108 [24]; c = concentration in grams/litre; l = pathlength of the sample cell. Complete spectra were recorded over extended periods of time: ~ 1–10 nm/min.



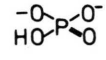
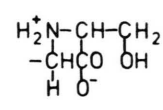
Compound	R_1	R_2
(A) Pyridoxal	–CHO	–OH
Pyridoxal 5′-phosphate	–CHO	
(B) Pyridoxine 5′-phosphate	$-CH_2OH$	–"–
(C) Pyridoxic acid 5′-phosphate	$-COO^-$	–"–
(D) N-phospho-pyridoxyl-L-serine		–"–

Fig. 1. Structres of the derivatives of the coenzyme pyridoxal 5′-phosphate as used in this investigation.

Results and Discussion

As shown in Fig. 1 the coenzyme analogues under investigation differ from each other with respect to the 4- and the 5′-position.

It is generally accepted that the productive interaction between the coenzyme pyridoxal 5′-phosphate and the respective pyridoxal 5′-phosphate dependent enzyme [25–29] includes (a) the generation of an internal aldimine between the 4-carbonyl group of the coenzyme and an ε-amino-lysyl side chain (b) ionic interactions between the mono- or dianionic form of the 5′-phosphate group and positively charged side chains of the protein and (c) hydrophobic interactions between the pyridine ring and apolar parts of the active center.

The transaldimination from the internal aldimine (a) to the external aldimine between L-serine and pyridoxal 5′-phosphate is the first step in the catalysis of the abovementioned β-replacement reaction. Moreover, pyridoxal (**A**) contains the residual structure which is sufficient to achieve the required function as a relay of charges in order to destabilize the respective bond in the substrate L-serine. Hence, derivative (**A**) should be able to substitute the coenzyme as a catalyst. Fig. 2 shows that with high concentrations of pyridoxal (molar excess: 500 fold) in fact a considerable activation of the α_2 apo β_2 bienzyme complex is observed yielding the Michaelis parameters $K_M = 0.16 \times 10^{-3}$ M and $V_{max} = 18$ IU/mg. This indicates, that pyridoxal binds specifically to the enzyme. The interaction however, is by a factor of

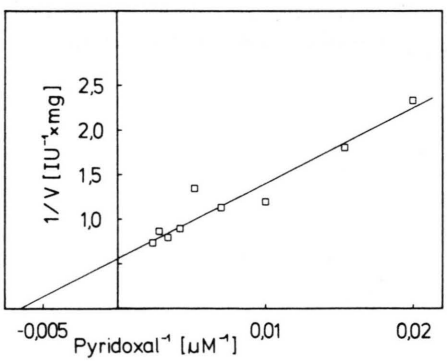


Fig. 2. Pyridoxal (**A**) can substitute the native coenzyme as a catalyst. Lineweaver-Burk plot of initial velocity experiments with the α_2 apo β_2 bienzyme complex of tryptophan synthase. Excess pyridoxal (50–500 µM) was preincubated with the enzyme (1 µM β sites) at 25 °C for 24 h in the dark. The buffer was 0.1 M sodium pyrophosphate, N_2 saturated.

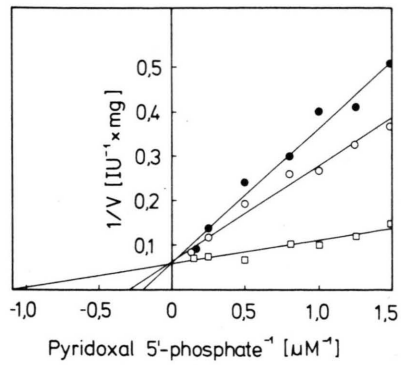


Fig. 3. Pyridoxic acid 5′-phosphate is a competitive inhibitor of cofactor binding to the α_2 apo β_2 bienzyme complex of tryptophan synthase. For initial velocity experiments pyridoxal 5′-phosphate was varied in the absence or presence of inhibitor. Data are plotted according to Lineweaver-Burk. Concentration of enzyme: 1 µM β sites, $T = 25$ °C. (□) No inhibitor, (○) 50 µM (**A**), (●) 100 µM (**C**).

ten weaker than with the natural coenzyme which leads to a maximum specific activity of about 17.5 IU/mg. Since the enzymatic activity of the isolated β_2 subunit for L-tryptophan synthesis shows only 1–2% of the value obtained for the native bienzyme complex no accurate measurement of pyridoxal-activation could be performed with the apo β_2 dimer. Therefore it remains open whether binding of (**A**) to apo β_2 occurs in a cooperative way, as described recently for pyridoxal 5′-phosphate [2] as well as for (**B**) and (**D**) [15].

Binding of compounds (**B–D**) cannot be detected via activation of the apo enzyme. However, if they interact with specific sites in the active center of the enzyme, these derivatives should act as competitive inhibitors with respect to the function of pyridoxal 5′-phosphate during the catalytic process. Therefore we investigated the inhibition pattern of (**B–D**) for the activity in L-tryptophan synthesis of the bienzyme complex in detail. Plotting the obtained data according to Lineweaver and Burk leads to the following inhibition constants K_I: 1.0×10^{-4} M for (**B**), 3.0×10^{-5} M for (**C**) and 1.6×10^{-4} M for (**D**). Fig. 3 shows that the corresponding inhibition pattern for pyridoxic acid 5′-phosphate (**C**) clearly indicates a competitive mode of interaction. This holds also for compounds (**B**) and (**D**) confirming recently published results [15].

The given results suggest that (apart from the catalytically active 4-carbonyl group) the 5′-phos-

phate group is of crucial importance for the correct position of the native coenzyme molecule.

Considering the individual contributions of both the 4- and the 5′-ring substituents to the enzyme-coenzyme interaction, the two groups clearly do not yield the total binding energy: The overall equilibrium constant taken from the corresponding binding constants of the respective derivatives (**A**) and (**B**) would be 2.6×10^{-8} M, which is one order of magnitude below the actual value for the coenzyme, $K_d = 1 \times 10^{-6}$ M [2]. This indicates clearly that additional interactions between coenzyme and the active center of the enzyme according to (c) are of importance.

Since at pH 7.5 the ε-amino group of the reactive lys 86 [24] is expected to be in its protonated form the electrostatic attraction of residues in the 4-position carrying negative charges may provide additional contributions to the overall free energy of interaction. This view is supported comparing the respective inhibition constants of compounds (**B**–**D**): pyridoxic acid 5′-phosphate (**C**) with its strongly electronegative 4-carboxyl group reveals a fife times lower inhibition constant indicating this analogue to be more tightly bound compared to pyridoxine 5′-phosphate (**B**) with a hydroxyl group in the same position.

Considering the fact, that several other pyridoxal 5′-phosphate dependent enzymes have strong affinity for the conjugate N-phosphopyridoxyl amino acids (tyrosine aminotransferase, $K_d = 6.5 \times 10^{-9}$ M, [30]; aspartate aminotransferase, $K_d \leqq 10^{-9}$ M, [31]) it is surprising that compound (**D**) interacts only weakly with tryptophan synthase. Obviously this enzyme has a binding site for external aldimines which is more selective than in other pyridoxal 5′-phosphate dependent enzymes.

In Fig. 4A the CD spectra in the near-UV and in the visible region (250–480 nm) induced by the interaction of the isolated β_2 subunit with pyridoxal 5′-phosphate and derivatives (**A**–**D**) are compared. The near-UV spectrum of the native apo β_2 subunit consists of positive, intrinsic Cotton effects contributed by 1 tryptophan-, 12 tyrosine- and 13 phenylalanine residues. In addition to a significant enhancement of the dichroic absorption in the aromatic region ($\Delta[\Theta] = 12$ degr. cm^2 decimol^{-1}) the holo β_2 subunit shows a large positive, extrinsic Cotton effect in the visible region at 415 nm ($\Delta[\Theta] = 36$ degr. cm^2 decimol^{-1}). This is caused by the induced asymmetry of the aldimine chromophore, and seems to be a characteristic feature of most pyridoxal 5′-phosphate dependent enzymes [25, 32]. A second lower maximum at 335 nm may be explained by a different mode of cofactor binding *via* hydrophobic interactions with the protein.

Compound (**B**) induces a significant increase of $\Delta[\Theta] = 6$ degr. cm^2 decimol^{-1} in the aromatic region. On the other hand, compound (**D**) with its bulky L-serine substituent revealing weaker binding than (**B**) [15], causes a much larger effect in the same wavelength region: $\Delta[\Theta] = 14$ degr. cm^2 decimol^{-1}. This may indicate an altered orientation of the phosphopyridoxyl ring in the active center. No

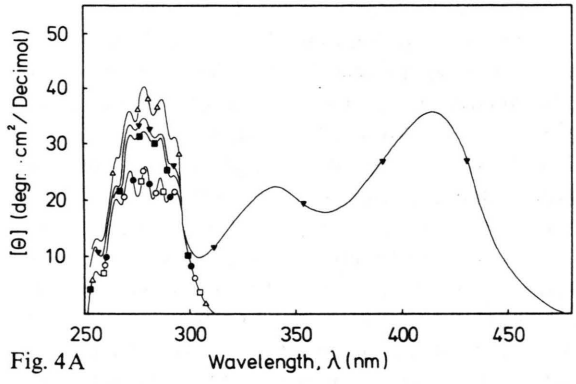


Fig. 4A

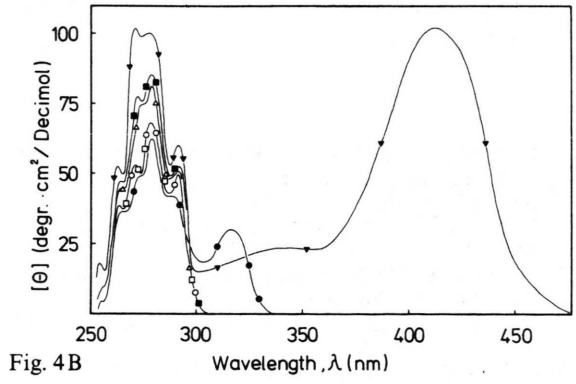


Fig. 4B

Fig. 4. Changes in the circular dichroism spectra of tryptophan synthase in the near-UV and in the visible region as observed upon binding of the cofactor and derivatives (**A**, **B** + **D**) respectively. 4A: (○) 46 μM isolated apo β_2 subunit; same enzyme concentration + (□) 500 μM (**A**), (■) 200 μM (**B**), (●) 350 μM (**C**), (△) 250 μM (**D**), (▼) holoenzyme. 4B: (○) 46 μM β sites/apo complex same enzyme concentration + (□) 500 μM (**A**), (■) 200 μM (**B**), (●) 350 μM (**C**)? (△) 250 μM (**D**), (▼) holo bienzyme complex. The enzymes were preincubated in the presence of the respective analogues for 24 h at 25 °C in the dark.

interaction of derivatives (**A**) and (**C**) with the apo β_2 subunit was detected *via* CD spectroscopy.

The mode of binding of compounds (**A**–**D**) differs drastically for both enzyme species. As shown in Fig. 4B the interaction of the coenzyme analogue (**B**) with the α_2 apo β_2 complex clearly exhibits the maximum change of the near-UV dichroic absorption: ($\Delta[\Theta] = 17$ degr. cm^2 decimol^{-1}).

Compound (**D**) induces a similar enhancement as observed for the isolated β_2 subunit whereas binding of pyridoxic acid 5′-phosphate (**C**) causes a small decrease in the aromatic dichroic absorption. However, a remarkable extrinsic Cotton effect of + 20 degr. cm^2 decimol^{-1} is induced at 315 nm which corresponds to the absorption maximum of (**C**). It should be noted that the binding of pyridoxal (**A**), as established by activation of the apo bienzyme complex, is not accompanied by significant changes in the wavelength region from 250–480 nm. Since rigid anchoring of pyridoxal 5′-phosphate to the protein is essential for a significant effect on dichroic absorption of the ligand [33], one may assume that the 5′-phosphate group (which is absent in (**A**)) plays an important role in generating the observed asymmetry of the productive aldimine chromophore between lys-86 [24] and the cofactor. The fact, that unspecific binding of additional cofactor molecules to other lysine side chains outside the active center does not induce asymmetry in the resulting unproductive aldimines points into the same direction [10].

Conclusions

The given results prove clearly that derivatives of the coenzyme pyridoxal 5′-phosphate carrying altered substituents in the 4-position or lacking the 5′-phosphate group still can bind specifically to the active center of tryptophan synthase. However, the respective interactions are much weaker indicating that the 5′-phosphate group is of crucial importance in attaining the correct orientation of the cofactor molecule in the active center. As demonstrated by dichroic absorption measurements, only productive binding of the cofactor molecule to the active site of the enzyme leads to prominent Cotton effects. Obviously the simultaneous and rigid anchoring of the coenzyme *via* (i) the internal aldimine, (ii) the electrostatic attraction of the 5′-phosphate group by a specific phosphate binding site and (iii) the binding of the pyridine ring with its residual substituents is required for the observed spectral effects.

Acknowledgements

Work was supported by grants of the Deutsche Forschungsgemeinschaft and the Fonds der Chemie. We are grateful for the expert technical assistance of Mrs. B. Teuscher. We thank Dr. K. Kieslich and Mr. W. Wania, GBF Stöckheim for generous aid in growing bacterial strains on a large scale.

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