# THE CHARACTERISTICS OF ALKYL SUBSTITUTED ANALOGUES OF PYRIDOXAL PHOSPHATE AS COENZYMES FOR ASPARTATE TRANSAMINASE

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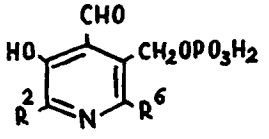
To obtain information concerning the role of different parts of the pyrodoxal phosphate molecule (PLP) in binding to the apoenzyme and realization of the enzymatic reaction, the interaction between L-aspartate: 2-oxoglutarate apo-aminotransferase of pig heart (E.C.2.6.1.1) and a series of alkyl derivatives of PLP has been studied:




| | I | II | III | IV | V | VI | VII |
|---|---|---|---|---|---|---|---|
| $R^2$: | $CH_3$ | $C_2H_5$ | n-$C_4H_9$ | iso-$C_3H_7$ | $CH_3$ | H | H |
| $R^6$: | H | H | H | H | $CH_3$ | H | $CH_3$ |

A commercial sample of PLP was purified according to Peterson and Sober (1954). Compound II was a kind gift from Prof. Snell, the other analogues were synthesized by us (Drobinskaya et al., 1968) they were homogeneous chromatographically and electrophoretically. The apoenzyme was prepared according to Wada and Snell (1962); its residual enzymatic activity was about 5%. There was no absorption due to PLP in visible range of spectra of the apoenzymes samples. A "Jouan" Dichrograph with sensitivity about $10^{-4}$ O.D. units was employed for the recording of circular dichroism (CD). The data at $\lambda$ values $< 300$ m$\mu$ were obtained, using a 0.5 cm cell and with

protein concentrations of about 2 mg/ml. The enzymatic activity of the reconstituted holoenzymes was measured by determination of oxaloacetate formation (Cammarata et al., 1951). The CD spectra were recorded eith an excess of coenzyme or its analogue after attainment of constant values of CD in the absorption band of protein-bound artificial coenzymes. For recording of the absorption spectra the excess of coenzyme analogue was removed by dialysis.

Results and Discussion. PLP is known to be present in aspartate transaminase (AT) as a Schiff base with the $\varepsilon$- $NH_2$ - group of a lysyl residue (Huges et al.,1962). Addition to the apoenzyme of anyone of the artificial coenzymes (I-VII) results in appearance of a positive Cotton effect in the absorption band of the respective Schiff bases (Fig.1 and Table 1). At the same time a negative dichroic band arises at 295-300 mμ which is presumably due, as suggested previously (Ivanov et al., 1967), to induced optical activity of a tyrosyl anion, that may take part in coenzyme binding. It is remarkable that in the case of the 2-butyl (but not of the 2-ethyl) analogue the magnitude of this effect is greatly increased. It is tempting to interpret this fact as a result of steric interference of the larger substituent upon the tyrosyl group.

CD titration with coenzyme indicated saturation of CD peaks at a coenzyme: apoenzyme ratio of 1:1, assuming a molecular weight of 44000 for the enzyme subunit. We obtained the same stoichiometry upon resolution of the dialysed artificial holoenzymes into protein and coenzyme in alkaline medium. Since the quantity of titrable apoenzyne molecules cannot exceed the total protein concentration, the value obtained is the upper bound of molecular weight.

All holoenzymes reconstituted with compounds I-VII are

TABLE I. PROPERTIES OF RECONSTITUTED TRANSAMINASES

| | I | II | III | IV | V | VI | VII |
|---|---|---|---|---|---|---|---|
| $\lambda_{max}$, pH 5.2 | 430 | 435 | 440 | 440 | 455 | 425 | 455 |
| $\lambda_{max}$, pH 8.1 | 360 | 365 | 370 | 370 | 370 | 360 | 370 |
| $pK_a$ | 6.25 | 6.5 | 6.4 | 6.6 | 6.3 | 5.8 | - |
| $\Delta D/Dx10^4$, 420-450m | 28 | - | 25 | 27 | 25 | 29 | - |
| $\Delta D/Dx10^4$ at 360 m | 19 | - | 15 | 10 | 15 | 21 | - |
| $k_2$, $M^{-1}$ $min^{-1}$ | 1500 | 750 | 150 | - | 10000 | 450 | - |
| $V_{max}$, relative | 1 | 0.32* | 0.5 | - | 0.56 | 1.2 | 0.47 |
| $K_M$ for α-oxoglutarate | 0.1 | 0.05* | 0.08 | - | 0.14 | 0.24 | - |
| $K_M$ for L-aspartate | 2.0 | 0.5* | 0.6 | - | 1.0 | 1.8 | - |

Bimolecular constants ($k_2$) were measured in 0.1 M phosphate buffer, pH 7.4, by continuous recording of CD at $\lambda$ = 365 mμ immediately upon addition of the coenzyme analogue to the apo-AT, t = 22°C. Apoenzyme concentration was varied from 1.4 to 5.0 mg/ml; concentrations of coenzyme or analogues were varied from 1.5 to 3.0 - fold excess over the apoenzyme.

pH-indicators. Their $pK_a$ values were calculated on the basis of pH-dependence of the CD patterns. The $pK_a$ values and some other optical parameters of the reconstructed holoenzymes are listed in Table 1. A noteworthy feature is the pronounced shift of pK in the case of compound VI, which has no substituent in position 2**

Holoenzymes reconstituted with the amino analogues exhibited positive CD in the absorption band of these analogues, as demonstrated earlier for pyridoxamine phosphate. This constitutes

*The data from study Morino and Snell (1967).

**When this work was in progress, Prof. E.E.Snell informed us about his studies, in collaboration with Y.Morino, on the coenzymatic activity of compounds II and VI (now published, Morino and Snell, 1967). Our data on the spectra of aspartate holotransaminases reconstituted with II and VI and their pK values are in full quantitative accordance, and the data on enzymatic activity are in qualitive accordance with the results of these authors.

evidence for specific binding of the aminoanalogues to the active site of the apoenzyme.

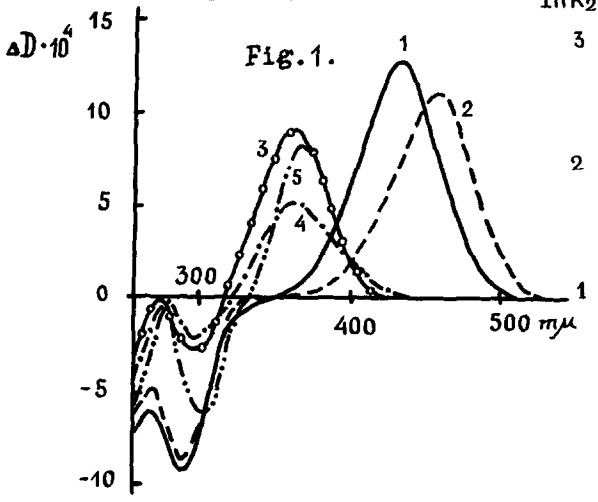



Fig.1.

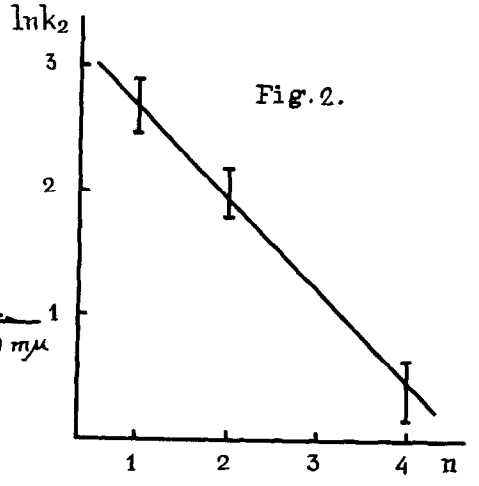



Fig.2.

Fig.1. CD spetra of AT reconstituted with PLP and its analogues. Here and in the other figures the protein concentration is 3.8 mg/ml. ΔD is the difference between optical densities in left and right circulary polarized light. 1, with PLP; 2, with V } pH 5.2 + succinate (1M); 3, with PLP; 4, with V; 5, with III } pH 8.1

Fig.2. The dependence of log bimolecular rate constant for coenzyme association with apoenzyme plotted versus length of 2-alkyl substituent.

Having ascertained the fact of specific binding of compounds I-VII in the active site of apo-AT, we studied the timecourse of this binding. In reaction: apo-AT + coenzyme $\xrightarrow{k_2}$ intermediates $\xrightarrow{k_1}$ active holo-AT (Banks et al., 1963) the increase in concentration of end product will follow a bimolecular rate equation if the rate-determining step is the one with overall constant $k_2$. In the absence of extremely large excess of coenzyme this proved to be the case. It is seen from Table 1 that the artificial coenzymes markedly differ in their specific rates of reaction with apo-AT. Amongst the compounds I-VII only V reacts more rapidly than PLP. This may possibly be due to increased reactivity of the carbonyl group in compound V. For the series I, II, III, IV the electrondonating effects of substituents should

be similar. Hence, it is likely that the decrease in association rates with increasing of length of the 2-substituent is primarily determined by the entropy factor.

Fig.2 demonstrates that the plot of $\ln k_2(n)$ values is actually a straight line, the slope of which corresponds to 1.4 entropy units per one $CH_2$ group. Thus, there is present in the vicinity of the 2-methyl group of PLP a region of the apoenzyme which markedly restricts the conformational mobility of the 2-substituents in compounds II-IV. This is in line with our hypothesis (Karpeisky and Ivanov, 1966) concerning interaction of the 2-methyl group with protein moiety of AT.

Next, we studied the kinetics of enzymatic transamination catalysed by the aformentioned forms of AT. All artificial holoenzymes had fairly high enzymatic activity; the respective Michaelis parameters are listed in Table 1. The reaction proceeds through the same intermediates as in the case of PLP (Ivanov et al., 1967); the intermediates with $\lambda_{max}$ at 430-450 mμ are optically inactive, as with the natural enzyme.

We may summarize the above results as follows: (1) the 2-methyl group of PLP is likely to be in close contact with the protein moiety of AT; (2) the alkyl substituents examined modulate the kinetic parameters both of the enzymatic reaction and of the reaction of cofactor association with the protein; (3) the experimental data provide evidence in support of the occurrence of an ionized tyrosyl residue in the active site of AT; (4) although natural coenzyme is not the "best coenzyme" in terms of certain kinetic parameters, it may be considered as an optimal coenzyme if the totality of parameters is taken into consideration.

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