APPLICATION OF SPIN-LABELED VITAMIN B_6 ANALOGUES FOR THE STUDY OF ASPARTATE AMINOTRANSFERASE

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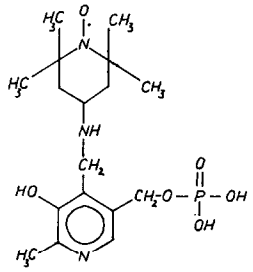
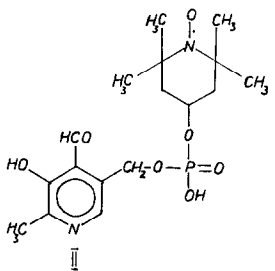
1. Introduction

For elucidation of the chemical nature and functional role of amino acid residues in an enzyme's active site, valuable information can be obtained by introducing a coenzyme analogue which carries either a reactive or 'reporter' group sensitive to its immediate surroundings. This role can be played by a spin label, e.g., an iminoxyl radical. Use of spin-labeled inhibitors and coenzyme analogues has proved an adequate approach to investigations of the topography of active sites of some enzymes [1]. In this work, the following vitamin B_6 analogues were used (see scheme I).



I



II

It is shown that compounds I and II are highly specific and interact with the apotransaminase, being bound in the positions corresponding to pyridoxamine-5'-phosphate (PMP) and pyridoxal-5'-phosphate (PLP), respectively. In either case, the iminoxyl group of the radical oxidizes one thiol group near the enzyme's active site. It is found that this group belongs to Cys-390. The distance from this group to the centre of coenzyme's pyridine ring does not exceed 8 Å.

2. Materials and methods

Mixture of α- and β-forms of the aspartate aminotransferase (EC 2.6.1.1, Asp-transaminase) from cytosol of pig heart muscle was used in the experiments; apoenzyme was obtained by the procedure in [2]. Transaminase activity was determined by the direct spectrophotometric method [3].

Synthesis and properties of the spin labels I and II used in this work have been described in ref. [4]; I and II are analogues of PMP and PLP, respectively. Absorbance spectra of I and II, within a wide pH range ($1 \leqslant pH \leqslant 14$), are similar in regard to the positions of the maxima to those of PMP and PLP, respectively. This indicates that the ionic forms of these compounds in aqueous solutions are identical to those of PMP and PLP. Thiol groups were determined as described previously [5].

3. Results and discussion

The spin labels I and II have high affinities for apotransaminase. Upon titration of apoenzyme with spin labels I and II (fig. 1), specific binding is complete at a label–apoenzyme ratio of about 1:1 (per enzyme subunit, mol.wt. 46 300). Titration was followed by recording the appearance of typical maxima in CD spectra.

Upon interaction of apotransaminase with I and II a positive maximum appears in the CD spectrum of the enzyme in the absorption band of bound coenzyme (as happens with the natural coenzyme forms). This strongly suggests that the analogues bind

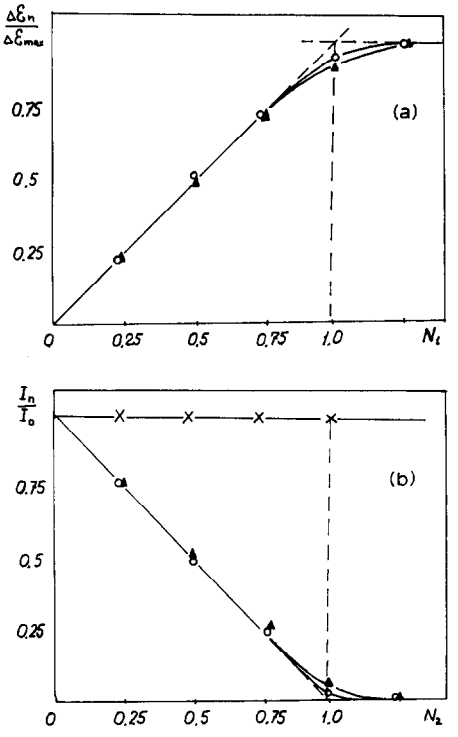


Fig. 1. Interaction of I and II with apotransaminase: (a) Titration of the apoenzyme with spin labels (I or II). Dependence of the relative intensity of the characteristic maxima in the CD spectra ($\Delta\epsilon_n/\Delta\epsilon_{max}$) on the ratio [spin label]/[apotransaminase] = n_1. ▲–▲–▲, spin label I. ○–○–○, spin label II; (b) Titration of the spin label with CM-apotransaminase. Dependence of the relative intensity of the central component of the EPR signal inherent to the spin label (I_n/I_0) on the ratio [CM-transaminase]/[spin label] = n_2. ▲–▲–▲, spin label I. ○–○–○, spin label II. X–X–X, 2,2,6,6-tetramethyl-4-hydroxypiperidine-1-oxyl.

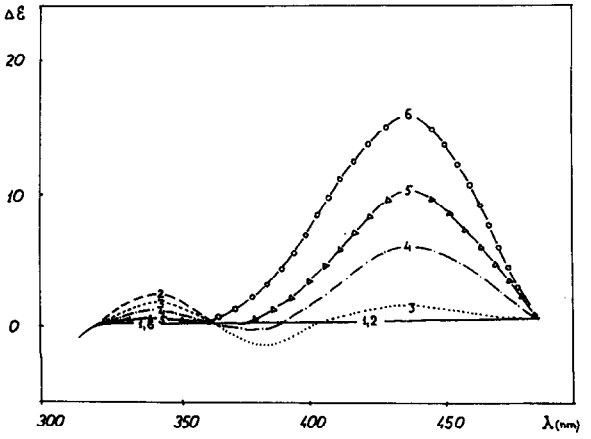


Fig. 2. Interaction of PLP with the apotransaminase–I complex at pH 5.2 (CD spectra). 1 – apotransaminase; 2 – apotransaminase–I complex; 3–6 – CD spectra of the complex at following concentrations of PLP; 3 – $0.25 \cdot 10^{-4}$ M; 4 – $0.5 \cdot 10^{-4}$ M; 5 – $1.0 \cdot 10^{-4}$ M; 6 – $1{,}5 \cdot 10^{-4}$ M. Protein concentration $1.0 \cdot 10^{-4}$ M, concentration of I $3.0 \cdot 10^{-4}$ M. Spectra were read 30 min after addition of PLP into each sample.

specifically at the active site [6–8].

The apotransaminase–II complex is characterized by absorption and CD spectra at pH 8.3 coinciding in position of the maximum (360 nm) with those of the Asp-transaminase aldimine form. Appearance of a maximum at 360 nm in the CD spectrum, i.e. within a region not interfering with absorption peaks of both aldehyde and hydrate forms which suggests the formation of an aldimine bond between spin label II and the ε-amino group of a lysine residue located in the active site. The CD maximum at 360 nm persists at lower pH values up to 5.2. The positive maximum in the CD spectrum at 430 nm, which is typical of the holoenzyme's aldimine form at this pH value, is absent in the spectrum of apotransaminase-II complex.

Complexes of I and II with apotransaminase are catalytically inactive; intensity of the maxima in CD spectra of these complexes is lower than that of amino and aldimine forms of the native holoenzyme. However, there is no doubt that binding of I and II proceeds specifically at the active site. The specificity of binding of I and II was verified in experiments on the interaction of PLP with the apotransaminase–I complex (fig. 2). At a low PLP concentration, the coenzyme is being bound to the enzyme non-specifically (378 nm maximum in the CD spectrum) because the active site is occupied by the spin-labeled analogue. Increase of PLP concentration results in gradual displacement of I from the active site. The CD spectrum acquires a form typical of the native aldimine form. The family of curves displays an isosbestic point (fig. 2), and equilibrium is reached rather rapidly (at relatively small excess of PLP). This proves once again the specificity of the interaction between the spin-labeled coenzyme derivatives and the active site of Asp-transaminase.

Quite unexpected was the irreversible disappearance of a signal in EPR spectra observed upon binding of

I and II with apotransaminase. Measurement of the intensity of EPR signals for I and II in the course of titration with the protein showed that the relative decrease of free radical concentration in solution is directly proportional to the relative concentration of the apotransaminase complexes with I and II. The same phenomenon was observed in experiments where the thiol groups of two 'external' cysteine residues [9] of the enzyme (Cys-45 and Cys-82) had been carboxymethylated before addition of the labeling reagent. Titration of thiol groups in the complexes formed between the labels and native apoenzyme or the carboxymethylated one revealed loss of a single thiol group per transaminase subunit. It follows from this that the iminoxyl fragment upon location in the active site reacts with one of the 'internal' thiol groups.

Further experiments have shown that oxidation of this thiol group by the iminoxyl radical spin labels I and II does not considerably affect the potential catalytic properties of the enzyme protein: reactivation in the presence of PLP resulted in 75% restitution of the initial activity. It is known that upon so-called 'syncatalytic' modification one of the thiol groups is being modified, viz. Cys-390 located in the region of the Asp-transaminase active site [10, 11]. The degree of inactivation depends on the size of the modifying agent; e.g., with thiocyanocysteine formation it is rather slight. To check the possibility of oxidation of the SH group of Cys-390, we performed syncatalytic alkylation of transaminase modified with I or II and reconstituted with PLP (transaminase^{rec}). For this *N*-ethylmaleimide was used as an alkylating agent in the procedure described by Christen [10]. Under similar conditions, the same treatment was applied to the initial native holoenzyme. In a number of experiments it was observed that upon syncatalytic alkylation the transaminase^{rec} is inactivated extremely slowly; residual activity was found to be one order of magnitude higher than that of the alkylated holoenzyme. Results of these experiments are shown in table 1. It seems likely that the partial inactivation of transaminase^{rec} in the course of syncatalytic modification was due to a decrease in enzyme stability resulting from the numerous treatments and modifications. The results obtained indicate that the iminoxyl moiety of spin labels I and II attacks Cys-390 which is being oxidized whereas the label undergoes reduction leading to formation of di-*N*-substituted hydroxylamine. However the possibility cannot be excluded that some other cysteine residue is modified in the region of the active site; although this seems rather unlikely.

The use of compounds I and II for the study of the Asp-transaminase active site allows one, in principle, to evaluate the distance between the functional thiol group of the active site and certain regions of the coenzyme molecule. However, the conformation assumed by the molecules of I and II upon their binding at the active site of Asp-transaminase is not known. Therefore, we can evaluate only the

Table 1
Modifications of aspartate transaminase

Preparation	Activity	PLP reactivation	Number of SH-groups
Asp-transaminase	(100%)	–	4.1 ± 0.2*
CM-Asp-transaminase	110	–	2.1 ± 0.2
CM-apotransaminase	–	100–105	2.1 ± 0.2
CM-apotransaminase-I	0	–	1.0 ± 0.2
The same, after removal of I	0	65–75	0.9 ± 0.2
Syncatalytic modification NEM			
CM-Asp-transaminase	5**	–	1.0 ± 0.2
Transaminase^{rec}	50**	–	Not determined

* Four of the five thiol groups are titratable in these conditions.
** The activity was compared with that of the initial preparations: CM-Asp-transaminase and transaminase^{rec}, respectively.

maximum distance between the pyridine nucleus and the thiol group of Cys-390, taking into account that the protein thiol group and the iminoxyl radical of the label can react only when the distance between them is close to the length of the Van-der-Waals radii. Evaluation made with use of a space model of the spin label I shows that this distance should not exceed 8 Å. Upon binding to the enzyme-active site the iminoxyl moiety of spin label II is very probably located in the same way as that of spin label I in respect to the pyridoxal nucleus. This is proven by the complete identity of the apoenzyme preparation modified with two labels. In other words, such a bulky substituent as the 2,2,6,6-tetramethyl-1-oxyl-piperidine-4 can be located in the enzyme active site at a unique, strictly determined position in proximity to the cysteine residue. This results in an inevitable oxidation of the thiol group of this residue.

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