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REORIENTATIONS OF COENZYME IN ASPARTATE TRANSAMINASE STUDIED ON SINGLE CRYSTALS OF THE ENZYME BY POLARIZED-LIGHT SPECTROPHOTOMETRY *

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Summary

Investigation of polarized-light absorption spectra of single crystals of cytosolic aspartate transaminase (L-aspartate 2-oxoglutarate aminotransferase, EC 2 6 1 1) from chicken heart has revealed that the coenzyme's absorption bands at 430 and 360 nm are polarized in opposite directions, both in crystals of the free enzyme and in its complex with a quasi-substrate, 2-methylaspartate. The opposite signs of polarization of the 430 and 360 nm bands of the free enzyme indicate different orientation of the pyridine ring of pyridoxal 5'-phosphate in the protonated and non-protonated forms of the 'internal' coenzyme-lysine aldimine These data suggest that reorientation of the coenzyme ring occurs mainly in the first step of the catalytic reaction, associated with proton transfer from the NH_3^+ group of amino acid substrate to the coenzyme-lysine aldimine.

Absorption bands at 333 and 430 nm are seen in the spectra upon soaking the crystals in solutions containing aspartate, glutamate or cysteinesulfinate Both bands are polarized in the same direction as is the 430 nm absorption band of the protonated internal aldimine. Soaking the crystals in solutions containing 2-oxoglutarate, glutarate or maleate reverses the sign of polarization of the 430 nm band. High concentrations of acetate induce the same effect. Thus, binding of dicarboxylate or acetate anions in the active site of aspartate transaminase appears to result in partial or complete return of the coenzyme ring to a position similar to that of the non-protonated internal aldimine

* A preliminary short communication has been published [9]

Introduction

Rotatory changes in position of the coenzyme, pyridoxal 5′-phosphate, associated with the catalytic cycle of aspartate transaminase, were first suggested in 1966 by Braunstein et al [1] and Karpeisky and Ivanov [2] on the basis of general typochemical considerations and of studies concerning changes in optical activity of enzyme-bound pyridoxal 5′-phosphate at sequential stages of the transamination reaction [3,4] Support for this idea came from investigations of the stereochemistry of tritium addition to the C-4′ atom of pyridoxal 5′-phosphate on reduction with $NaB[^3H]H_4$ of the internal and the substrate aldimines in the active site of the transaminase [5] Recent X-ray findings confirmed that movement of the pyridine ring of pyridoxal 5′-phosphate does take place in the course of the catalytic reaction [6] However, the actual mode of the change in coenzyme position is still uncertain, and it is not known at which intermediate steps it occurs To clarify these questions, polarized-light absorption spectroscopy of single crystals of the enzyme can be used. Changes of polarization ratios of absorption bands may result either from a change in direction of the transition dipole moment within the coenzyme ring or from reorientation of the ring. This approach was first applied by Metzler et al. [7] in their studies of crystals of cytosolic aspartate transaminase from pig heart. Reported below are the results of our studies on polarized-light absorption spectra of crystals of chicken heart cytosolic aspartate transaminase and its complexes with substrates and substrate analogues. Crystals of the chicken enzyme differ from those of the homonymous pig isozyme in shape and the packing arrangement of protein molecules in the unit cell [8]. Our findings provide additional insight into the nature of reorientations of pyridoxal 5′-phosphate in the active site of the transaminase.

Materials and Methods

Chemicals L-Aspartic, 2-methyl-DL-aspartic, L-glutamic, L-cysteinesulfinic, 2-oxoglutaric, glutaric and maleic acids were purchased from Sigma Chemical Co. Poly(ethylene glycol) was obtained from LOBA-Chemie (Vienna) All other chemicals were standard reagent grade.

Preparation and crystallization of the enzyme Cytosolic aspartate transaminase was isolated from chicken hearts by using the procedure of Kochkina et al. [10] Crystals were grown essentially as described earlier [8] in test-tubes by overlaying of a combined CsCl and $(NH_4)_2SO_4$ solution and protein solution containing 0 1 M 2-methyl-DL-aspartate. The following modifications were introduced in the crystallization procedure (a) EDTA was omitted from solutions, (b) 0.1 M potassium phosphate buffer, pH 7 5, was included in both saline and protein solutions. In a series of crystallization experiments, 2-methyl-DL-aspartate was replaced by 5 mM 2-oxoglutarate Within a week, crystals reached dimensions of up to 0.15 × 0.5 × 1 0 mm. Crystals of smaller size were obtained in the absence of 2-methyl-DL-aspartate or 2-oxoglutarate Crystals were stored in the following stabilizing solution. 60% saturated $(NH_4)_2SO_4$ in 0.3 M potassium phosphate, pH 7.5. The crystals are of prismatic shape, they belong to space group $P2_12_12_1$ and have four dimeric enzyme molecules in the unit cell [8]

Spectral studies Polarized-light absorption spectra were measured in a Cary 118 spectrophotometer equipped with a polarizer and a special microcell wherein single crystals were mounted. Spectra were recorded with two orientations of the polarizer. In one orientation, the plane of polarization of the incident light beam was parallel to the crystallographic *c*-axis, in the other it was perpendicular to that axis, but not coincident with the *a*- or *b*-axis * [9].

The orthorhombic crystals described above are not suitable for circular dichroic (CD) measurement, since they are optically anisotropic. To record CD spectra, we used tetragonal crystals of aspartate transaminase grown in 40% saturated $(NH_4)_2SO_4$ solution [11]. These plate-shaped crystals with dimensions of 0.3 × 0 5 × 0.5 mm consist of ordered layers of molecules irregularly spaced in the direction of the 4-fold axis (the hkO precession X-ray photograph presented a regular pattern of reflections, whereas on the hkL graph the distribution of reflections was irregular). CD spectra were measured with a Jobin Yvon dichrograph mark III. Crystals were oriented in such a way that the axis of 4-fold symmetry coincided with the direction of the light beam.

Results

Spectra of crystals of the free enzyme

Absorption spectra of a linearly polarized light transmitted through a single crystal of aspartate transaminase are shown in Fig. 1a. The positions of absorption maxima differ from those of the enzyme in solution. In the latter case, absorption maxima are situated at 430 and 360 nm, the two bands correspond to the protonated and non-protonated forms of the internal pyridoxal 5′-phosphate-lysine aldimine, respectively [12]. In the spectrum of a transaminase crystal, the 430 nm band proved to be shifted to 405 nm, the position of the 360 nm band is markedly changed in one of the two polarizations. The 405 nm band displays a pH dependence which is characteristic of pyridoxal 5′-phosphate-aldimines, i.e , its intensity diminishes in alkaline media and increases when crystals are soaked in acidic buffer solutions. It seemed plausible that the shift of the absorption peak from 430 to 405 nm might be caused by formation of an aldimine between enzyme-bound pyridoxal 5′-phosphate and NH_3 **. Actually, we found that transfer of the crystals from $(NH_4)_2SO_4$ solution into 35% (w/v) buffered poly(ethylene glycol) (M_r 6000) or 2.1 M potassium phosphate solutions led to 'normalization' of the spectrum, i.e., to reappearance of the usual bands at 430 and 360 nm *** (Figs. 1b and 3). These bands proved to

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* Since the plane of one of the polarizations does not coincide with any crystallographic axis, some distortion in the absorption spectrum of this polarization might occur However, such distortion appears to be insignificant, judging from the fact that very similar polarization ratios were obtained with crystals differing in thickness

** The reaction between the internal pyridoxal 5′-phosphate-lysine aldimine and NH_4^+ in the enzyme's activity site may proceed as follows An NH_4^+ in proximity to the imine double bond donates one of its protons to the imino N atom (as does the NH_3^+ group of substrate amino acid), thereupon the neutral NH_3 molecule attacks the C-4′ atom of the coenzyme to form an aldimine with pyridoxal 5′-phosphate The dissociation constant of the pyridoxal-ammonia aldimine was found to be very large in solution [13] But in our case, the position of equilibrium is evidently displaced in favour of formation of the aldimine, due to the high concentration of NH_4^+ in the medium (2 5 M)

*** The distribution of reflections and their intensities on precession X-ray photographs were not altered upon transfer of crystals into poly(ethylene glycol) or 2 1 M phosphate solutions, i e , the crystals remain isomorphous

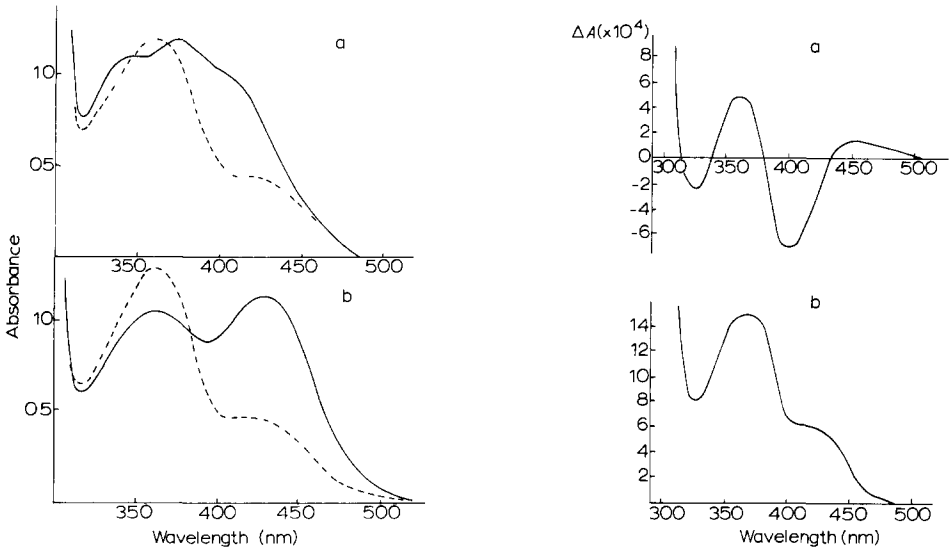


Fig 1 Polarized-light spectra of single orthorhombic crystals of aspartate transaminase (———) $A_{\parallel}$, absorbance of light polarized in the plane parallel to the crystallographic c-axis, (— — —) $A_{\perp}$, absorbance of light polarized in the plane perpendicular to the c-axis (a) A crystal in 60% saturated $(NH_4)_2SO_4$ solution containing 0 3 M potassium phosphate (pH 7 5) (b) A crystal in 35% (w/v) poly(ethylene glycol) solution buffered with 0 1 M potassium phosphate (pH 7 5)

Fig 2 Circular dichroic spectra of single tetragonal crystals of aspartate transaminase (a) A crystal in 45% saturated $(NH_4)_2SO_4$ solution containing 0 2 M potassium phosphate (pH 7 5) (b) A crystal in 2 0 M potassium phosphate (pH 7 0)

be polarized in opposite directions, both in crystals grown in the absence of 2-methyl-DL-aspartate and in crystals first grown in the presence of 2-methyl-DL-aspartate and then freed from it by thorough washing

To verify further the formation of 'external' pyridoxal 5'-phosphate-ammonia aldimine in the crystalline enzyme, CD spectra were measured of tetragonal transaminase crystals grown in $(NH_4)_2SO_4$ solution Breaking of the imine bond between pyridoxal 5'-phosphate and a lysine residue and formation of external aldimines in the active site of transaminase are known to be associated either with disappearance of CD in the coenzyme's absorption bands or with inversion of its sign from positive (in the free holoenzyme) to negative [3,4,12] Actually, we found that the crystalline enzyme displays negative CD in its 405 nm band (Fig 2a). Transfer of the tetragonal crystals from $(NH_4)_2SO_4$ solution to 2 M phosphate buffer led to the disappearance of the negative CD band and appearance of a positive one at 430 nm, as is displayed by the free holoenzyme in solution (Fig 2b)

Spectra of crystalline complexes of the enzyme with substrate and with NH_2OH

Soaking of the orthorhombic crystals of aspartate transaminase in stabilizing $(NH_4)_2SO_4$ solution containing a quasi-substrate (2-methyl-DL-aspartate) leads to normalization of the spectrum, i.e., to the appearance of clear-cut peaks at 430 and 360 nm [9] Similar absorption maxima are seen in the spectrum of

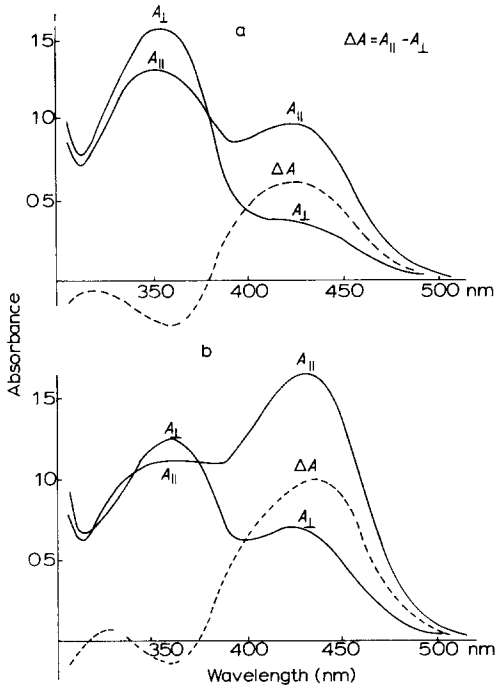


Fig 3 Effect of 20methylaspartate on polarized-light spectra of single orthorhombic crystals of aspartate transaminase $A_{\parallel}$ and $A_{\perp}$ as in the legend to Fig 1 (a) A crystal in 2 1 M potassium phosphate buffer, pH 7 0 (b) A crystal in 2 1 M potassium phosphate buffer (pH 7 0) containing 0 1 M 2-methyl-DL-aspartate

the 2-methyl-DL-aspartate-enzyme complex in solution [14]. Evidently, 2-methyl-DL-aspartate displaces NH_3 from its bond with pyridoxal 5′-phosphate, yielding the quasi-substrate aldimine. The effect of 2-methyl-DL-aspatate on the spectrum normalized by prior transfer of a crystal into 2.1 M phosphate buffer is shown on Fig. 3 It can be seen that on addition of 2-methyl-DL-aspartate, absorbance is diminished at 360 nm and increased at 430 nm.

TABLE I

POLARIZATION RATIOS OF ABSORPTION MAXIMA OF ASPARTATE TRANSAMINASE AND ITS COMPLEXES WITH SUBSTRATES AND QUASI-SUBSTRATES

$A_{\parallel}$ and $A_{\perp}$ as in the legend to Fig 1

	$\frac{A_{\parallel} - A_{\perp}}{A_{\parallel} + A_{\perp}}$			
	430 nm maximum	360 nm maximum	333 nm maximum	492 nm maximum
Free enzyme	0 43	—0 10		
Complex with L-aspartate	0 51		0 30	
Complex with 2-methyl-DL-aspartate	0 54	—0 10		
Complex with erythro-3-hydroxy-aspartate *			0 16	0 08
Complex with 2-oxoglutarate	—0 02			

* Data from paper Ref 9

Similar results are obtained with crystals transferred into poly(ethylene glycol) solution. The 430 and 360 nm bands of the 2-methyl-DL-aspartate-enzyme complex are polarized in opposite directions Judging from absorption and CD spectra recorded in aqueous solution of transaminase [12,15], the 360 and 430 nm bands of the 2-methyl-DL-aspartate-complex can be assigned to the non-protonated internal and to the protonated external (substrate) aldimine, respectively In accordance with this assumption, 2-methyl-DL-aspartate does not change the polarization ratio of the 360 nm band and slightly increases that ratio in the 430 nm band (Table I).

Soaking of crystals in solutions containing L-aspartate or L-glutamate leads to the appearance of absorption bands at 333 and 430 nm (Fig. 4) The former band belongs to the pyridoxamine form, and the latter to the protonated substrate aldimine These two forms of transaminase are in equilibrium, just as in solution The position of the equilibrium was displaced in favor of the pyridoxamine form when cysteinesulfinate was diffused into the crystals. Both the 333 and 430 nm bands observed in the presence of aspartate or glutamate have the same sign of polarization, identical with that of the 430 nm bands displayed by the free enzyme and its complex with 2-methyl-DL-aspartate.

The effect of NH_2OH on the polarized-light spectrum of crystals is of interest This carbonyl reagent breaks the imine bond between pyridoxal 5′-phosphate and a lysine residue to form an oxime of pyridoxal 5′-phosphate in the enzyme's active site The spectrum of a crystal into which NH_2OH was diffused is shown in Fig 5. A strong absorption band at 380 nm is seen which is polarized in the same direction as the 430 nm band displayed by the protonated pyridoxal 5′-phosphate lysine aldimine

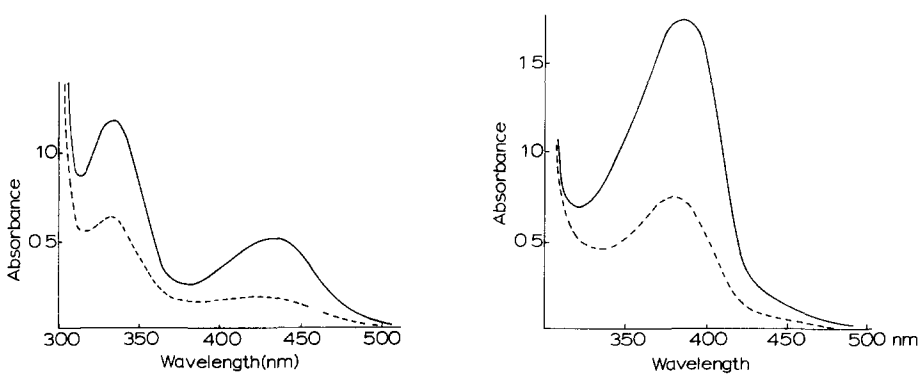


Fig 4 Polarized-light spectra of a single orthorhombic transaminase crystal soaked in 35% (w/v) poly-(ethylene glycol)/0 1 M potassium phosphate (pH 7 5), containing 0 1 M L-aspartate $A_{\parallel}$ (———) and $A_{\perp}$ (— — —) as in the legend to Fig 1

Fig 5 Polarized-light spectra of a single orthorhombic transaminase crystal soaked in 2 1 M potassium phosphate buffer (pH 7 0) containing 0 05 M NH_2OH $A_{\parallel}$ (———) and $A_{\perp}$ (— — —) as in the legend to Fig 1 Similar spectra were obtained in 35% poly(ethylene glycol)/0 05 M NH_2OH solution

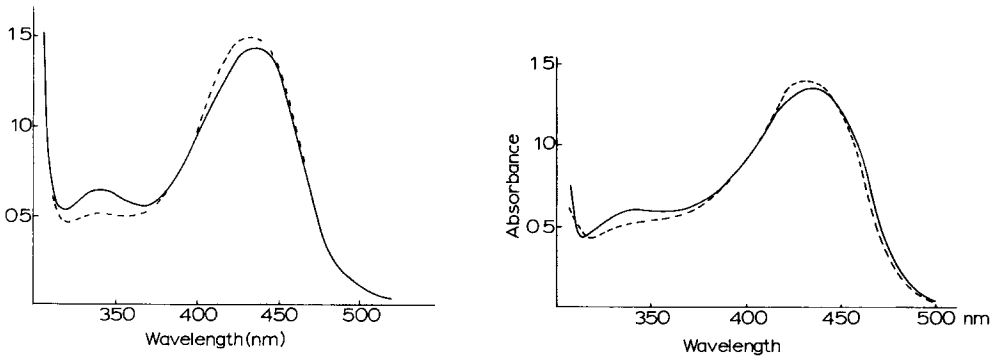


Fig 6 Polarized-light spectra of a single orthorhombic crystal of the complex of aspartate transaminase with 2-oxoglutarate (0 1 M) in 35% (w/v) poly(ethylene glycol) solution buffered with 0 1 M potassium phosphate (pH 7 5) $A_{\parallel}$ (———) and $A_{\perp}$ (— — —) as in the legend to Fig 1

Fig 7 Polarized-light spectra of a single orthorhombic crystal of aspartate transaminase in 60% saturated $(NH_4)_2SO_4$ solution containing Tris/0 5 M acetate (pH 7 0) $A_{\parallel}$ (———) and $A_{\perp}$ (— — —) as in the legend to Fig 1

The effect of dicarboxylate and acetate anions on polarized-light spectra of the crystalline enzyme

The spectrum of a crystal into which 2-oxoglutarate was diffused is shown in Fig. 6. Comparison of Figs. 6 and 1b shows that 2-oxoglutarate not only increases the pK value of the coenzyme's chromophore (cf. Ref 12), but moreover, inverts the sign of polarization of the 430 nm band, i.e , induces polarization in the direction characteristic for the 360 nm band, although lesser in degree Similar effects are induced by glutarate and maleate. The different polarization ratios of the 430 nm bands displayed by complexes of aspartate transaminase with inhibitory dicarboxylate anions, on the one hand, and by the enzyme complex with 2-methyl-DL-aspartate, on the other, point to differences in the nature of these complexes.

Strong effects upon polarized-light spectra of the enzyme crystals are produced by the acetate anion Addition of 0.15—0 25 M acetate buffer to the stabilizing $(NH_4)_2SO_4$ solution leads to normalization of the spectrum, i.e , to replacement of the 405 nm band by the usual 430 nm band [9]. In higher concentrations (at least 0.5 M), acetate increases the pK value of the internal aldimine and induces depolarization or even inversion of polarization of the 430 nm band (Fig. 7), i e , it produces an effect similar to that of dicarboxylate anions This effect was observed in both $(NH_4)_2SO_4$ and poly(ethylene glycol) solutions

Discussion

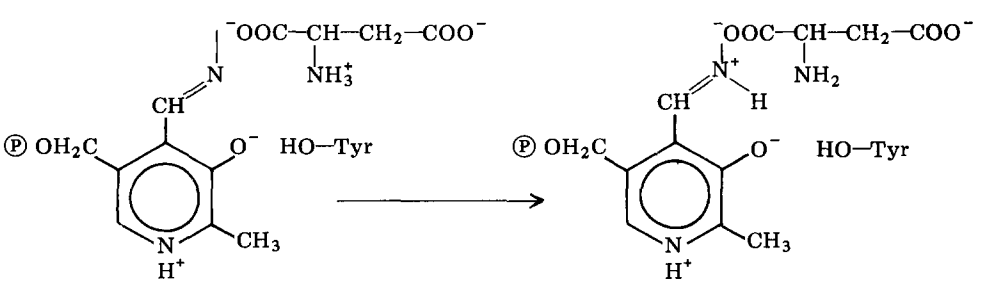
Opposite polarization of the 430 and 360 nm absorption bands of crystals of free (non-liganded) aspartate transaminase is of considerable interest. This fact can be explained either by different orientation of the coenzyme ring in the protonated and the non-protonated forms of the internal aldimine or by

differences in direction of the transition dipole moment within the coenzyme ring The latter assumption seems unlikely, since quantum-mechanical calculations indicate that protonation of the aldimine N does not noticeably change the direction of the transition dipole moment (Savin, F A , personal communication)

Another possibility is that a *trans*-oid to *cys*-oid reorientation of the imine double bond (relative to the 3-OH group of pyridoxal 5′-phosphate) may occur in the protonation step Quantum-mechanical calculations indicate that such reorientation would be accompanied by a 10° shift in direction of the transition dipole moment [15]. However, recent X-ray findings seem to rule out such reorientation of the imine bond in the first steps of the catalytic reaction [6]

Finally, one should take into account that the crystals under study contain four dimeric enzyme molecules in the unit cell [8] All these molecules have identical projections along the crystallographic axes, however, projections of the two subunits in one dimeric molecule may be non-coincident Since the immediate surroundings of subunits in crystals differ, this might cause a difference in pK values of the coenzyme Thus, one may imagine that one subunit contains mainly the protonated internal aldimine and the other its non-protonated form at the same pH If this were the case, opposite polarization of the 430 and 360 nm bands might be due to differences in position of the two subunits in relation to crystallographic axes rather than to differences in orientation of the coenzyme ring in one subunit This suggestion can, however, be rejected for two reasons. First, the pH dependence of the absorption spectrum of the crystalline enzyme within the pH range from 5 to 8 proved similar to that of the enzyme in solution. Second, opposite polarization of the 430 and 360 nm bands has also been observed in linear dichroism spectra of aspartate transaminase artificially oriented in polyacrylamide gel [16].

Summarizing the facts discussed above, we can infer that protonation of the internal pyridoxal 5′-phosphate-lysine aldimine does induce reorientation of the pyridine ring in the active site of aspartate transaminase Judging from the change in polarization ratio, the angle of rotation of the ring may amount to approx 25° Such rotation is apparently associated with the first step of the catalytic reaction, namely with proton transfer from the NH_3^+ group of substrate amino acid to the imine N Protonation of this atom should lead to formation of a chelate hydrogen bond with the 3′-OH group of the coenzyme and consequently to breaking (or much weakening) of a hydrogen bond between this group and a nucleophilic group of the enzyme protein (such a bond with the residue Tyr-225 was recently postulated for the chicken mitochondrial aspartate transaminase on the basis of X-ray investigations [6]):



Breaking of the latter bond makes possible rotation of the pyridine ring As a result, the C-4′ atom of the coenzyme moves towards the nucleophilic neutral amino group of the substrate Thus, protonation of the internal aldimine provides not only electronic, but also necessary geometric prerequisites for realization of the next step of the enzymic reaction, namely transamination Our findings do not exclude the possibility that a further minor rotation of the pyridoxal 5′-phosphate ring occurs in this step, wherein the substrate's amino group displaces the N^{ϵ} atom of a lysine residue from its imine bond with pyridoxal 5′-phosphate to form the substrate aldimine.

The scheme outlined above is consistent with our observation that soaking of crystals of the free enzyme in solutions containing dicarboxylates or a high concentration of acetate reverses the sign of polarization of the 430 nm band Many spectral and kinetic data strongly indicate that the imine N atom serves as binding site for certain mono- and dicarboxylate anions [17—19] Hence, it may be inferred that the anion bound in the active site of transaminase forms a hydrogen bond with the imine N As a consequence, a hydrogen bond between this atom and the coenzyme's 3′-OH group is broken and the hydrogen bond between the latter and a nucleophilic protein group (such as the OH group of Tyr-225) is reconstituted This may result in partial or complete return of the pyridoxal 5′-phosphate ring into a position similar to that of the enzyme-bound non-protonated coenzyme

It is of interest to compare the spectra described in this paper with those of crystals of pig heart cytosolic aspartate transaminase reported by Metzler et al [7] In their paper, the polarization directions for the crystalline 2-methyl-DL-aspartate-complex were incorrectly assigned (Metzler, D E , personal communication) Metzler revised those data and found that the 360 nm bands of the free enzyme and of the 2-methyl-DL-aspartate-complex are similarly polarized whereas their 430 nm bands are polarized in opposite directions In contrast to our data, no essential difference was observed in polarization of the 360 and 430 nm bands of the free crystalline enzyme from pig heart This discrepancy may be due to different orientation of crystallographic axes relative to the light beam, and to different packing arrangement of enzyme molecules in the unit cell of crystals of the pig and chicken transaminases It follows from our data that a major change in orientation of the pyridoxal 5′-phosphate ring accompanies the protonation step According to Metzler's revised data, this change occurs in the transaminase step We may suppose that the coenzyme moves in a complex manner and various aspects of its movement are seen with crystals of the pig and chicken cytosolic transaminases.

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