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THE BINDING OF PYRIDOXAMINE 5-PHOSPHATE TO ALANINE AMINOTRANSFERASE AS MEASURED BY FLUORESCENCE SPECTROSCOPY

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SUMMARY

The interaction between pyridoxamine 5-phosphate and alanine aminotransferase (L-alanine:2-oxoglutarate aminotransferase, EC 2.6.1.2) was investigated by fluorescence spectroscopy. The cofactor bound to the catalytic site of the enzyme is subject to environmental perturbations which cause a blue shift of 17 mμ in the band position of the emission spectrum, a 10-fold decrease in the fluorescence yield and a uniform increase in the polarization of fluorescence values. An analysis of the results of polarization of fluorescence according to Perrin's equation indicates that pyridoxamine 5-phosphate has rotational flexibility when bound to the catalytic site of the enzyme alanine aminotransferase. Since the fluorescence properties of the cofactor are sensitive to conformational changes of the enzyme, it was possible to investigate the mechanism of inhibition by *p*-mercuribenzoate and obtain further information on the functional role of the reactive sulfhydryl groups of the enzyme alanine aminotransferase.

INTRODUCTION

The enzyme alanine aminotransferase (L-alanine:2-oxoglutarate aminotransferase, EC 2.6.1.2) from pig heart has been investigated in several laboratories[1–4] and considerable effort has been devoted to the elucidation of its physical and chemical properties[4]. These investigations have revealed that the enzyme contains approximately 1 mole of pyridoxal 5-phosphate per mole of enzyme (mol. wt., 100 000) and that the cofactor is bound through a Schiff's base linkage to an ε-amino group of a lysine residue[5]. The work of GREIN AND PFLEIDERER[2] has shown that the purified enzyme is easily inactivated by mercurial compounds which are known to react specifically with sulfhydryl groups. However, the functional role played by these highly reactive sulfhydryl groups of the enzyme alanine aminotransferase remains to be elucidated. It is the purpose of this work to investigate, by means of fluorescence spectroscopy, the nature of the environment surrounding the cofactor pyridoxamine 5-phosphate when it is firmly bound to the catalytic site of the enzyme alanine aminotransferase. It is shown that the fluorescence properties of the cofactor (fluorescence

Abbreviation: PCMB, *p*-chloromercuribenzoate.

yield, polarization of fluorescence and emission spectra) are sensitive to changes in the original conformation of the enzyme. This is illustrated by the interaction of *p*-chloromercuribenzoate (PCMB) with the enzyme, where the decrease in the affinity of pyridoxamine 5-phosphate for the apoenzyme is easily detected by measurements of fluorescence and polarization of fluorescence.

EXPERIMENTAL PROCEDURE

Preparation of the enzyme

The enzyme alanine aminotransferase from pig heart was purified according to the method of TURANO AND RIVA[3], except that in the last step of the purification, the enzyme was passed through a Sephadex G-100 column (1.5 cm × 11 cm) equilibrated with 0.1 M phosphate buffer (pH 6.8) containing 1 mM EDTA and 1 mM mercaptoethanol. This preparation had a specific activity of 130 units/mg of enzyme and exhibited an absorption maximum at 425 mμ (pH 5.5). The pyridoxal 5-phosphate content of the enzyme (1 mole of pyridoxal 5-phosphate per 10 000 g of protein) was determined by the phenylhydrazine method of WADA AND SNELL[6].

The enzymatic activity was determined by the spectrophotometric method of GREIN AND PFLEIDERER[2]. All reagents were prepared in 0.1 M Tris–acetate buffer (pH 7.6). The reaction was carried out in a volume of 3 ml at 25° in the presence of 300 μmoles of Tris buffer (pH 7.6), 100 μmoles of L-alanine, 20 μmoles of α-ketoglutarate, 0.30 μmole of NADH and an excess of lactate dehydrogenase. The reaction was initiated by addition of 0.1 ml of the enzyme solution and the decrease in absorbance at 340 mμ was measured for 1 min in the Cary model 15 spectrophotometer. Protein concentrations were determined by the procedure of LOWRY *et al.*[7].

A unit of enzyme activity is defined as the amount which produces 1 μmole of pyruvate per min under the conditions of this enzymatic assay. Specific activity is defined as units/mg of protein.

Preparation of the phosphopyridoxamine form of the enzyme

The aminic form of the enzyme alanine aminotransferase was prepared by dialyzing the enzyme solution against 2-l volumes of 0.1 M phosphate buffer containing 0.1 M L-alanine followed by dialysis against 2-l volumes of 0.1 M phosphate buffer (pH 6.8) at 4°. This preparation was used throughout the studies reported in the paper. The enzyme aspartate aminotransferase from pig heart was purified according to the method of MARTINEZ CARRION *et al.*[8]. The phosphopyridoxamine form of the enzyme was prepared by the procedure described by SCARDI *et al.*[9]. The Sephadex resins were purchased from Pharmacia. Lactic dehydrogenase NADH, L-alanine, α-ketoglutarate, EDTA, Tris, pyridoxamine, pyridoxamine 5-phosphate, pyridoxal 5-phosphate, from Sigma. Guanidine · HCl (ultrapure) from Mann Research Laboratories. Other materials, commercially obtained, were of the highest purity available.

METHODS

Fluorescence spectra

Fluorescence spectra were obtained with the use of a spectrofluorimeter de-

signed in our laboratory[10]. Radiation from a 150-W xenon lamp (Hanovia) was passed through a 500-mm Bausch and Lomb monochromator (blazed at 300 mμ/mm) and focused onto the cell compartment. The fluorescence was observed at right angles to the exciting beam with a 500-mm Bausch and Lomb monochromator (blazed at 300 mμ dispersion 3.3 mμ/mm) and detected by a photomultiplier tube (EMI 6256 S). The signal was amplified and fed to the Y axis input of a Moseley recorder (Model 135 AM). The X axis of the recorder was coupled to the wavelength drive of the analyzing monochromator. Calibration of the exciting source and detector system of the spectrofluorimeter was carried out as described in a previous publication[10]. In all fluorescence measurements the exciting monochromator was operated with slit widths of 1 mm (band width 3.3 mμ). Under the experimental conditions chosen for excitation, the rate of fluorescence decrease due to irreversible chemical changes of pyridoxamine 5-phosphate was negligible. Fluorescence spectra recorded with a minimum time of illumination (1 min) are reproducible. A decrease of 15% in the intensity of fluorescence emitted at 390 mμ was observed when the samples of pyridoxamine 5-phosphate were irradiated for 6 min at 25°.

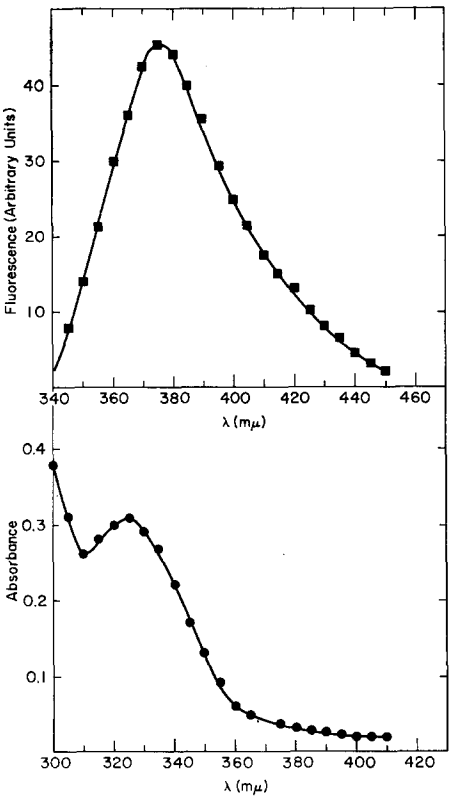
Polarization of fluorescence

Polarization of fluorescence measurements were performed in an apparatus built in our laboratory. This instrument is similar to the double beam photometer designed by WEBER[11]. Illumination was provided by a xenon lamp (Hanovia 150 W) with wavelengths selected by a quartz prism monochromator (Schoeffel, QPM, 30 S). The fluorescence emitted by the sample in the cell compartment was isolated by Corning glass filters (CS-0-51) which transmit light at wavelengths longer than 350 mμ. The band width for excitation was 5 mμ in the spectral region 310–350 mμ. This device is capable of measuring polarization of fluorescence values to an accuracy of 1% for polarization values greater than 0.1. Absorbance measurements were performed in a Beckman DU and in Cary 15 spectrophotometers.

RESULTS

Fluorescence of the cofactor

The phosphopyridoxamine form of the enzyme alanine aminotransferase shows an absorption maximum at 325 mμ, which is attributed to the cofactor bound to the active site of the enzyme[4,5]. While the absorption properties of free and bound pyridoxamine 5-phosphate appear to be similar, the fluorescence characteristics of the cofactor bound to the protein are markedly different from those of free pyridoxamine 5-phosphate in aqueous solution. Thus, the specialized environment surrounding the cofactor at the catalytic site causes two fluorescence changes (a) a blue shift of 17 mμ in the band position of the emission spectrum and (b) a substantial decrease in the fluorescence yield. Both effects are illustrated in Table I and Fig. 1, where it may be seen that the emission spectrum of bound pyridoxamine 5-phosphate (maximum 375 mμ) is shifted towards shorter wavelengths when compared to pyridoxamine 5-phosphate (maximum 392 mμ) under similar experimental conditions. In addition, the interaction of pyridoxamine 5-phosphate with the catalytic site of the enzyme leads to a 10-fold decrease in the fluorescence emitted over the spectral range 340–450 mμ.



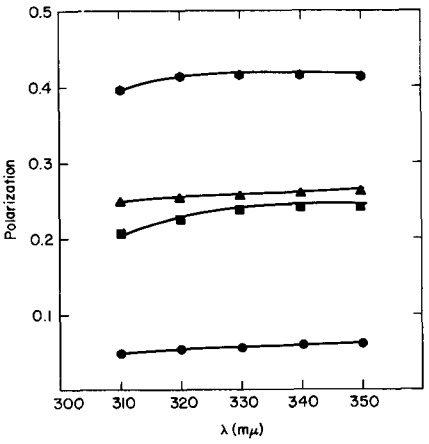


Fig. 1. Absorption spectrum of the phosphopyridoxamine form of alanine aminotransferase in 0.1 M phosphate buffer (pH 6.8). Protein concn., 3 mg/ml. Emission spectrum of the phosphopyridoxamine form of alanine aminotransferase excited at 327 mμ.

Fig. 2. Polarization of fluorescence spectra of pyridoxamine 5-phosphate (●) and alanine aminotransferase (phosphopyridoxamine form) (■) in 0.1 M phosphate buffer (pH 6.8) at 10°. Polarization of fluorescence spectra of pyridoxamine 5-phosphate in 60% sucrose (▲) and pyridoxamine 5-phosphate in 98% glycerol (◆) at 10°.

TABLE I

FLUORESCENCE PROPERTIES OF ALANINE AMINOTRANSFERASE (PHOSPHOPYRIDOXAMINE FORM)

Sample	λ_A (mμ)	λ_F (mμ)	$\frac{F}{F_0}$ *	p**
Pyridoxamine 5-phosphate (pH 7)	327	392	1	0.05 ± 0.01
Alanine aminotransferase (pH 6.8)	326	375	0.10	0.22
Alanine aminotransferase (pH 5)	326	375	0.10	0.22
Alanine aminotransferase (pH 8.5)	326	375	0.10	0.22
Alanine aminotransferase (pH 6.8) in 5 M guanidine·HCl	327	393	1	0.05 ± 0.01
Alanine aminotransferase treated with PCMB	326	392	0.9	0.05 ± 0.01

* Relative fluorescence yield. F_0 is the fluorescence yield of pyridoxamine 5-phosphate in water at pH 7.

** Polarization of fluorescence excited with plane polarized light at 330 mμ.

Polarization spectra

The polarization spectra of the pyridoxamine form of the enzyme alanine aminotransferase, which is the set of polarization of fluorescence values obtained upon excitation with light of varying wavelengths, was measured in 0.1 M phosphate buffer (pH 6.8) at 10°. The results of these measurements together with the polarization of fluorescence values of free pyridoxamine 5-phosphate in solvents of varying viscosity are shown in Fig. 2.

The effect of viscosity on the polarization of fluorescence values of free pyridoxamine 5-phosphate is easily explained in terms of the rotational motion of the chromophore during the interval τ_M (fluorescence lifetime), since the parameters quantum yield (Q) and τ_M are not affected by changing the viscosity of the medium[12]. Accordingly, the fluorescence is largely depolarized ($p = 0.05$) in solvents of low viscosity (water, 10°) because the molecules of pyridoxamine 5-phosphate assume a nearly random orientation during the brief lifetime of the excited state. Conversely, the polarization is substantially increased in viscous solvents (sucrose 60% and glycerol 98%) because the molecules of pyridoxamine 5-phosphate exhibit limited motion during the interval τ_M.

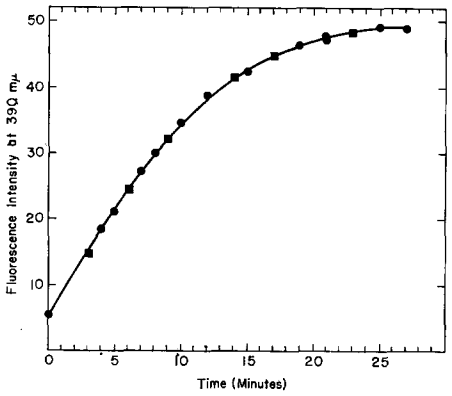
As shown in Fig. 2, the binding of pyridoxamine 5-phosphate to the enzyme leads to a uniform increase in polarization values over the entire range of wavelengths examined (310–350 mμ). This change in the polarization of fluorescence values of bound pyridoxamine 5-phosphate, as compared to the free chromophore in aqueous solution, is due to several effects which are not easily distinguished experimentally. Two factors to consider which may account for the uniform increase in the polarization values of bound pyridoxamine 5-phosphate are: (1) a decrease in the fluorescence yield (Q) of the cofactor which influences the lifetime of the excited state (τ_M), since $\tau_M = \tau_{FM} Q$, and (2) a change in the rate of tumbling of pyridoxamine 5-phosphate as a result of the interaction with the enzyme. It was clearly shown by PERRIN[13] and WEBER[14] that either a decrease in the fluorescence lifetime (τ_M) or an increase in the rotational relaxation time of the hydrodynamic unit in solution (ϱ_h) would cause an increase in the observed polarization values (p).

Interaction of the enzyme with p-mercuribenzoate

As shown in Table I, the fluorescence properties of pyridoxamine 5-phosphate in alanine aminotransferase remain virtually unchanged from about pH 5 to pH 8, showing one maximum of emission at 375 mμ which is independent of the wavelength of excitation. Since these fluorescence properties are drastically altered by the addition of denaturing agents (guanidine · HCl), it was thought of interest to investigate whether the emission spectra and polarization of fluorescence of the cofactor are sensitive to the conformational changes leading to distortion of the catalytic site.

To this end the interaction of the phosphopyridoxamine form of the enzyme with PCMB was investigated by means of fluorescence and polarization of fluorescence spectroscopy.

In order to investigate the effect of PCMB on the affinity of pyridoxamine 5-phosphate for the enzyme, fluorescence measurements were conducted on samples of enzyme treated with the mercurial compound. In a typical experiment, the enzyme, 0.5 mg/ml, was incubated with an 8-fold excess of PCMB, and the course of the reaction was followed by fluorescence measurements at 390 mμ (excitation 330 mμ).



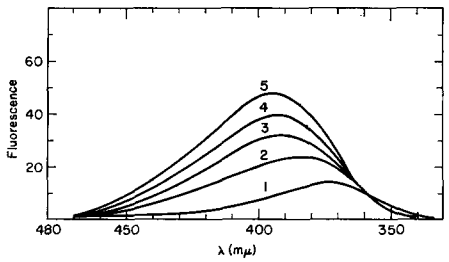


Fig. 3. Increase in fluorescence intensity at 390 mμ (excitation wavelength, 330 mμ) that follows the release of pyridoxamine 5-phosphate from the enzyme alanine aminotransferase after addition of PCMB. The enzyme (1 mg) was incubated with 0.08 μmole of PCMB in 2 ml of 0.1 M phosphate buffer (pH 6.8) at 25°. Results obtained in the presence (●) and absence (■) of 0.1 M L-alanine.

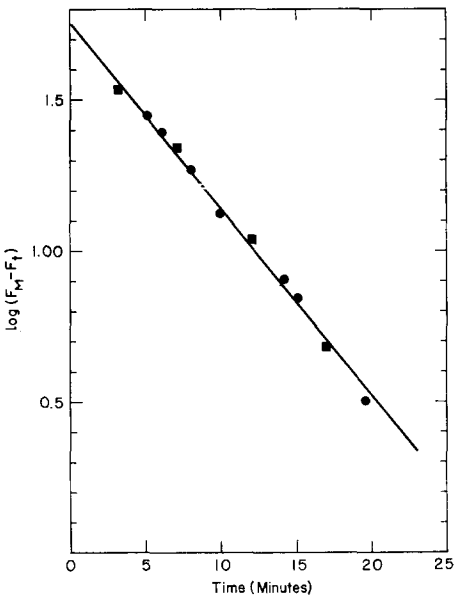
Fig. 4. Changes in emission spectra that follow the addition of PCMB to the enzyme alanine aminotransferase. The reaction mixture (2 ml) contained 1 mg of enzyme and 0.08 μmole of PCMB in 0.1 M phosphate buffer (pH 6.8). The incubation times are (1) 0, (2) 5, (3) 8, (4) 15, and (5) 22 min.

For the fluorescence measurements, a blank cuvette containing the same concentration of pyridoxamine 5-phosphate as the enzyme solution was used as standard. At pH 6.8, the reaction of PCMB with the sulfhydryl groups of the enzyme was followed by a substantial increase in the fluorescence emitted at 390 mμ as illustrated in Fig. 3. The increase in fluorescence intensity was accompanied by a progressive shift in the emission spectra towards longer wavelengths (Fig. 4). The process was completed within 20 min and the recorded emission spectra showed the characteristic features of free pyridoxamine 5-phosphate in solution. After completion of the fluorescence measurements, the sample of inactivated enzyme was dialyzed against 0.1 M phosphate buffer (pH 6.8) and examined by fluorescence spectroscopy. It was found that the fluorescence emitted by the dialyzed sample was negligible over the entire range of wavelengths examined (340–450 mμ). This observation is compatible with the hypothesis that the fluorescence enhancement that follows the inhibition of the phosphopyridoxamine form of the enzyme alanine aminotransferase is related to the release of the cofactor from the catalytic site. Since the change in fluorescence intensity as a function of the time of incubation with PCMB can be adequately described by Eqn. 1

$$\ln (F_M - F_t) = K_{obs} \cdot t + c \qquad (1)$$

where F_M is the maximum intensity of fluorescence at completion of the reaction, F_t the fluorescence intensity at time t, it was possible to determine the apparent rate constant for removal of pyridoxamine 5-phosphate ($K_{obs} = 0.14$ min^{-1}) (Fig. 5).

In order to evaluate the amount of pyridoxamine 5-phosphate released into solution during the reaction of the enzyme with the mercurial compound, the fluorescence emitted by the inactive species was compared with those of pyridoxamine



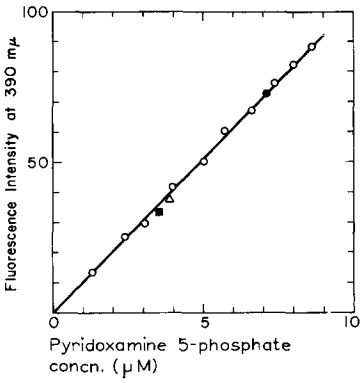
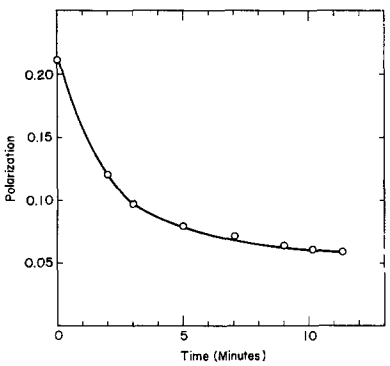


Fig. 5. Plot of log ($F_m - F_t$) (fluorescence increase at 390 mμ) *vs*, time for the enzyme alanine aminotransferase treated with PCMB in the presence (●) and absence (■) of 0.1 M L-alanine.

Fig. 6. Fluorescence increase at 390 mμ (excitation wavelength, 330 mμ) as a function of pyridoxamine 5-phosphate concentration in 0.1 M phosphate buffer (pH 6.8) at 25° (○). ●, results obtained with aspartate aminotransferase at a concentration of 0.35 mg/ml; △, with alanine aminotransferase at a concentration of 0.4 mg/ml in the presence of 5 M guanidine·HCl. ■, results obtained with alanine aminotransferase (0.4 mg/ml) inactivated by PCMB in the absence of 5 M guanidine·HCl. Experiments conducted at 25°.

5-phosphate solutions of known concentration. Samples of aspartate and alanine aminotransferase in the phosphopyridoxamine form were dissolved in 5 M guanidine·HCl and used as standards. The denaturing agent guanidine·HCl was used in these experiments to insure the complete dissociation of the enzymes into apoenzyme and pyridoxamine 5-phosphate. As shown in Fig. 6, the fluorescence emitted at 390 mμ is strictly proportional to the concentration of pyridoxamine 5-phosphate over the concentration range $1 \cdot 10^{-6}$–$9 \cdot 10^{-6}$ M. The samples of enzyme treated with guanidine·HCl yield a pyridoxamine 5-phosphate content which agrees with values published in the literature[4,8]. Thus aspartate aminotransferase contains 2.0 moles of pyridoxamine 5-phosphate per 100 000 mol. wt., while alanine aminotransferase yields 1 mole per 100 000 mol. wt. The sample of alanine aminotransferase inactivated by PCMB gives 0.9 mole of pyridoxamine 5-phosphate per mole of protein when examined in the absence of guanidine·HCl (Fig. 6). Since this value is very close to that obtained in the presence of guanidine·HCl, it seems reasonable to conclude that almost 90% of the pyridoxamine 5-phosphate content of the enzyme alanine aminotransferase is released into solution after addition of the mercurial compound. It is interesting to note that the decrease in the affinity of pyridoxamine 5-phosphate for alanine aminotransferase is also shown by polarization of fluorescence measurements conducted on samples of enzyme incubated with an excess of PCMB.



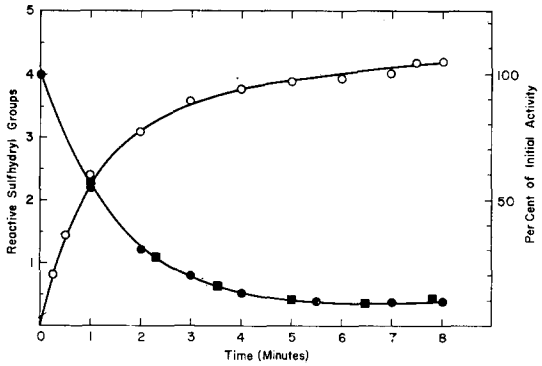


Fig. 7. Changes in the polarization of fluorescence of the phosphopyridoxamine form of the enzyme alanine aminotransferase after addition of PCMB at 25°. The wavelength of excitation for polarized light was 330 mμ. The enzyme (2 mg) was incubated with 0.16 μmole of PCMB in 2 ml of 0.1 M phosphate buffer (pH 6.8).

Fig. 8. Comparative rates of binding of PCMB (left ordinate) and loss in enzymatic activity in the presence (●) and absence (■) of 0.1 M L-alanine (right ordinate). The reaction mixture contained per ml: 0.70 mg of enzyme and 0.06 μmole of PCMB in 0.1 M phosphate buffer (pH 6.8). Incubation temperature was 25°. Small aliquots were removed from the incubation mixture at various times for measurements of their enzymatic activity.

Fig. 7 shows how the polarization of fluorescence of the cofactor is affected by the subsequent addition of an 8-fold excess of PCMB to a sample of alanine aminotransferase. In the absence of PCMB, the polarization of fluorescence ($p = 0.22$) measures the rotational motion of the cofactor bound to the enzyme. As soon as the affinity of the cofactor for the enzyme is altered by the addition of PCMB, the polarization of fluorescence decreases in the manner depicted in Fig. 7 and approaches the value of free pyridoxamine 5-phosphate in solution ($p = 0.05$). The polarization of fluorescence values obtained at intermediate stages of the reaction reflect the relative contribution of both free and bound pyridoxamine 5-phosphate to the fluorescence of the system.

Reaction of PCMB with sulfhydryl groups

The reaction between PCMB and the sulfhydryl groups of the enzyme alanine aminotransferase was studied by spectrophotometric methods under experimental conditions similar to those used in the fluorimetric studies. For spectrophotometric measurements, the enzyme at a concentration of 0.7 mg/ml in 0.1 M phosphate buffer (pH 6.8) was allowed to react with an 8-fold excess of PCMB, and the course of the reaction was followed by absorbance measurements at 250 mμ as described by BOYER[15]. Under these experimental conditions, no turbidity was formed, and an immediate increase in absorbance at 250 mμ was observed. From the results included in Fig. 8, it is immediately apparent that the reaction of 4 sulfhydryl residues per mole of enzyme occurred within 5 min of incubation at 25°. In order to correlate the rate of mercaptide formation with that of disappearance of enzymatic activity, the aminotransferase at a concentration of 0.6 mg/ml in 0.1 M phosphate buffer (pH 6.8) was incubated with an 8-fold excess of PCMB at 25°. Aliquots were withdrawn from the reaction mixture, diluted with 0.1 M phosphate buffer (pH 6.8) and assayed for enzymic activity. As illustrated in Fig. 8, the reaction of the ligand with sulfhydryl

groups measured by the increase in absorbance at 250 mμ, proceeds at a rate closely comparable to the rate of loss of enzymatic activity. The loss of approx. 90% of the original activity corresponds to the reaction of about 4 sulfhydryl residues within 5 min of incubation at 25°. This finding strongly suggests that four sulfhydryl residues per mole of enzyme (mol. wt., 100 000) are essential for enzymatic activity. Therefore, if the assumption is made that the four sulfhydryl residues are equally reactive with PCMB, then it is possible to calculate the apparent rate constant for inactivation of alanine aminotransferase ($K_{obs} = 0.54$ min^{-1}) when the data of Fig. 8 are analyzed according to Eqn. 2

$$A = A_0 \cdot e^{-K_{obs} \cdot t} \tag{2}$$

where A is the amount of active enzyme at time t and A_0 is the initial concentration of active enzyme at time $t = 0$. It is worthy of note that the rate of inhibition by PCMB remains essentially the same when L-alanine at a concentration of 0.1 M is present in the reaction mixture (Fig. 8). This observation is taken as an indication that the presence of substrate in the reaction mixture does not hinder the approach of PCMB to the reactive residues of the enzyme alanine aminotransferase.

Finally, it should be emphasized that the inhibition of alanine aminotransferase, treated with an 8-fold excess of PCMB at 25° for 20 min, is partially reversed by addition of cysteine (0.1 M), followed by dialysis at 4° for 20 h against 0.1 M phosphate buffer (pH 6.8) containing mercaptoethanol (10^{-3} M) and pyridoxal 5-phosphate (10^{-4} M). These experimental conditions restore 65% of the original activity.

DISCUSSION

The fluorescence studies presented in this paper indicate that pyridoxamine 5-phosphate bound to the enzyme alanine aminotransferase is subject to environmental perturbations which cause a blue shift in its emission spectrum and a decrease in the fluorescence yield. The origin of these fluorescence changes must be sought in specific short-range interactions, perhaps complex formation between pyridoxamine 5-phosphate and amino acid residues of the catalytic site.

In an effort to gain further information on the "specialized environment" surrounding the molecule of pyridoxamine 5-phosphate bound to the enzyme, the polarization spectra of the phosphopyridoxamine form of the enzyme was compared to the free chromophore dissolved in viscous solvents. Although the binding of pyridoxamine 5-phosphate to the enzyme results in a uniform increase of the polarization values over the entire spectral range, it was found that the p values of the cofactor are not comparable in magnitude to those observed when free pyridoxamine 5-phosphate is immersed in a medium of large viscosity values (98% glycerol). In evaluating these results it is important to consider the relative contribution of several parameters which are known to affect the measured polarization of fluorescence. Thus a change in the relative strength of a particular transition in the absorption spectra may be expected to produce a change in the shape of the polarization spectrum[16,17]. Furthermore, it is well established that changes in the fluorescence lifetime τ_M would be reflected in the observed polarization values (p)[13]. The relative contribution of intrinsic effects, such as changes in the strength of absorption transitions, were not

considered in the analysis of the polarization results because of the similarity between the absorption properties of free and bound pyridoxamine 5-phosphate.

However, an analysis of the polarization of fluorescence results according to Perrin's equation (Eqn. 3)

$$\frac{1}{p} - \frac{1}{3} = \left(\frac{1}{p_0} - \frac{1}{3}\right)\left(1 + \frac{3\tau}{\varrho_h}\right) \tag{3}$$

shows that the rotational relaxation time (ϱ_h) of the rotating unit in solution must be smaller than the value expected for a macromolecule of 100 000 mol. wt. According to Eqn. 3, it may be anticipated that for $\tau_M = 1.5 \cdot 10^{-9}$ sec (fluorescence lifetime of pyridoxamine 5-phosphate)[18] and $\varrho_h = 150 \cdot 10^{-9}$ sec (rotational relaxation time of the macromolecule) (J. E. CHURCHICH, unpublished results), the polarization of fluorescence of bound pyridoxamine 5-phosphate should approach the limiting value ($p = 0.41$) observed in glycerol at 10°. Since the p values of the bound cofactor ($p = 0.22$) are actually smaller than the limiting polarization of fluorescence, it follows that the parameter ϱ_h does not correspond to the rotation of a macromolecule of 100 000 mol. wt. Similar conclusions are obtained by assuming a decrease in the fluorescence lifetime. Thus for $\tau_M = 1.5 \cdot 10^{-10}$ sec a rotational relaxation time of $4.5 \cdot 10^{-10}$ sec is obtained for a polarization value of 0.22. This rotational relaxation time is of the same order of magnitude as the value observed for free pyridoxamine 5-phosphate[12], but 300 times shorter than the rotational relaxation time of the whole macromolecule.

A behavior of this kind is explained by proposing that the rotational motion of the emission oscillator is affected by either the Brownian motion of the protein as a whole or by rotations at the level of the bound chromophore. Since the lifetime of the excited state of bound pyridoxamine 5-phosphate is presumably shorter than $1.5 \cdot 10^{-9}$ sec, it is very unlikely that the Brownian motion of the macromolecule *per se* would promote considerable changes in the observed polarization values. This contention is strengthened by the polarization of fluorescence studies reported by several laboratories on the binding of small molecules to macromolecules in solution[19–22]. In view of these considerations, it seems reasonable to conclude that the polarized fluorescence emitted by the cofactor bound to the catalytic site of the enzyme alanine aminotransferase is strongly influenced by its local freedom of rotation.

Another interesting feature of these studies is that the fluorescence properties of the cofactor bound to the enzyme are sensitive to variations in the structure of the catalytic site; therefore, the cofactor itself acts as an indicator of events occurring at the level of the catalytic site. This was demonstrated in the inactivation of the enzyme by PCMB; where the reaction of the mercurial compound with sulfhydryl residues of the protein gives rise to significant changes in the fluorescence parameters. In this connection it is interesting to note that a comparison between the observed rate constants ($K = 0.54$ and $K = 0.14$ min^{-1}) corresponding to enzyme inactivation and fluorescence enhancement, respectively, reveals that the first process proceeds faster than the fluorescence increase at 390 mμ, which is related to the dissociation of the enzyme into apoenzyme and free pyridoxamine 5-phosphate. This lack of parallelism between the two processes suggests that the binding of PCMB to the enzyme results in immediate loss of activity, and that the increase in fluorescence at

390 mμ occurs only after a change in the structure of the catalytic site. These inhibition studies, however, do not permit a clear decision concerning whether the functional role of the reactive sulfhydryl groups lies in binding the substrate or in maintaining the structural integrity of the enzyme. Although further studies are required to clarify this problem, it is important to note that the presence of substrate failed to modify the rate of inactivation by PCMB, or the release of the cofactor as measured by fluorescence spectroscopy (Fig. 3). These results tend to support the concept that the sulfhydryl residues are indeed involved in the maintenance of a particular conformation of the active species in solution.

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