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The study of the catalytic sites of enzymes using fluorescent compounds

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The use of fluorescent componds can be a valuable tool to probe the active site of enzymes. Several examples of this approach are discussed, particularly the use of pyridoxal phosphate analogs. The study of protein-protein interactions by means of florescent-labeled proteins is also analyzed.

Key terms: *fluorescent probes; macromolecular aggregates; pyridoxal phosphate fluorescent analogs.*

INTRODUCTION

Fluorescence spectroscopy has proven to be a valuable technique in the study of ligand binding to enzymes. The method can provide information concerning the active site of enzymes provided a fluorescent chromophore has reacted with specific amino acid residues located at the active center. The fluorescent adducts bound to the protein should have absorption and fluorescent properties distinct from that of natural chromophores of the protein; i.e., tyrosyl and tryptophanyl residues.

This review consists of two parts. The first section is devoted to the reaction of pyridoxal-5'-P analogs with amino acid residues of the protein; the second section deals with the use of chemically modified enzymes as probes of macromolecular aggregates.

Analogs of pyridoxal-5'-P

The catalytic domain of vitamin B_6 requiring enzymes has been investigated by using analogs of the coenzyme pyridoxal-5'-P which interacts specifically with amino acid residues located near the Schiff base linkage formed between ε-lysine and the carbonyl group of the cofactor (Snell, 1952). For example, the analogs depicted in Figure 1 have been synthesized (Pfeuffer *et al*, 1972; Choi *et al*, 1985) and tested as cofactors of 4-aminobutyrate aminotransferase (Choi *et al*, 1985).

Aminotransferase catalyzes the reversible transamination of the neurotransmitter 4-aminobutyrate (Baxter & Roberts, 1961) with the active site pyridoxal-5'-P to yield succinic semialdehyde and pyridoxamine-5'-P. Pyridoxal-5'-P is reformed by transamination with α-ketoglutarate to yield glutamate and enzyme bound pyridoxal-5'-P.

When the apoprotein of the enzyme 4-aminobutyrate aminotransferase is incubated with equimolar amounts of either PLP-O-methyl ester or PLP(S), the absorption spectra resemble that of pyridoxal-5'-P covalently linked to a specific lysyl residue of the protein (Choi *et al*, 1985). Two bands centered at 330 and 415 nm are readily detected in the absorption spectra.

However, PLP-O-methyl ester bound to the apoprotein does not restore catalytic activity, whereas the binding of PLP(S) (phosphorothioate-PLP) results in a good recovery of the original catalytic activity. Judging from these observations, it appears that a decrease in the negative charges of the phosphate groups due to the formation of a methyl ester prevents the formation of species endowed with catalytic activity. On the other hand, modification of the phosphate side chain of the coenzyme by introducing a sulfur atom covalently bound to phosphorous renders a phosphorothioate analog which serves as cofactor during the transamination reaction.

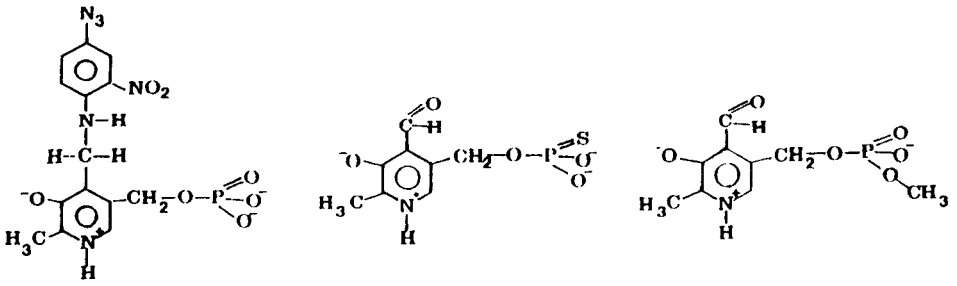

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Fig 1. Structure of pyridoxal-5'-P analogs. (1) Arylazido derivative of pyridoxamine-5'-P. (2) Pyridoxal-5'-phosphorothidate. (3) Pyridoxal-5'-P methyl ester.

Apparently, preservation of the negative charges of the phosphate group is needed for correct alignment of the cofactor at the binding site of the aminotransferase. Consistent with this interpretation of the enzymatic results is the finding that ^{31}PNMR measurements conducted on pyridoxal-5'-P covalently bound to the aminotransferase have shown that the chemical shift of the bound coenzyme is pH independent within the range 6.05-7.5 (Churchich *et al*, 1983). According to the results derived from NMR measurements, the cofactor pyridoxal-5'-P is bound in its dianionic form via a rigid salt bridge to positively charged amino acid residues.

The observation that the phosphorothioate analog reacts with amino acids located at the catalytic domain of the enzyme opens new avenues in the study of the binding sites of vitamin B_6 dependent enzymes by means of ^{31}P NMR spectroscopy.

The arylazido derivatives of pyridoxamine and pyridoxamine-5'-P have been synthesized and used as photoaffinity reagents of enzymes capable of binding pyridoxamine and pyridoxamine-5'-P (Scholz *et al*, 1990). 4-4-azido-2-nitrophenyl pyridoxamine (N_3N_pPxn), the arylazido derivative of pyridoxamine, shows two intense absorption bands centered at 340 and 475 nm when examined by absorption spectroscopy. A band at 2130 cm^{-1} characteristic of N_3 stretching vibrations, is detected by infrared spectroscopy.

This photoaffinity reagent was selected as affinity label of the enzyme pyridoxal kinase for the following reasons (a) N_3N_pPxn acts as a competitive inhibitor with respect to pyridoxal (K_i = 2μM) under dark conditions (b) Upon illumination of the arylazido derivative, a singlet nitrene is generated, which reacts with amino acids of the protein and (c) the binding of the affinity label to the kinase is easily detected by absorption spectroscopy or by radioactivity measurements using N_3N_p[^{3}H]Pxn. When a mixture containing pyridoxal kinase and 5-fold molar excess of N_3N_pPxn was irradiated at pH 6.5, it was found that the catalytic activity of the enzyme decreases in the manner depicted in Figure 2. After dialysis against phosphate buffer (pH 6.5), the modified enzyme displayed strong absorption bands in the range 360 to 450 nm due to the incorporation of nitrophenyl residues.

Apparently, the bonds connecting the two chromophores, i.e. pyridoxamine and 3-nitrophenyl-azide, do not undergo a splitting reaction during photolysis because the modified enzyme reduced with sodium dithionite shows a fluorescent band centered at 400 nm which can be attributed to pyridoxamine (Fig 2). For the correlation of inactivation of pyridoxal kinase with incorporation of radioactive N_3N_p[^{3}H]Pxn, the irradiated protein was denatured and digested with chymotrypsin (Scholz *et al*, 1990). Complete chymotryptic digestion of the labeled kinase yielded at least two radioactive peptides upon separation of the protein fragments by reverse-phase HPLC. Although the amino acid sequences of the radioactive peptides have not been determined, it appears that the photolabeling reagent reacts with more than one amino acid residue implicated in the binding of the substrate pyridoxamine.

One of the problems arising from the reaction of affinity label reagents with proteins

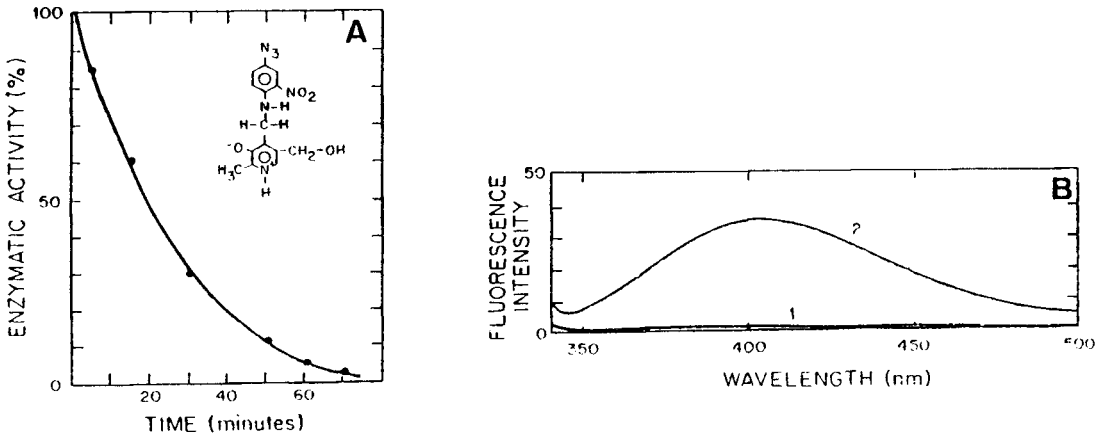

Fig 2. Inactivation of pyridoxal kinase by N_3N_pPxn. (A) Change in the catalytic activity of pyridoxal kinase (12.5 μM) incubated with N_3N_pPxn (63 μM) and irradiated with 340-nm light (intensity 8 mJ . cm^{-2} . s^{-1}). Aliquots withdrawn from the incubation mixture at the indicated times were assayed for enzymatic activity. (B) Emission spectrum of modified pyridoxal kinase reacted with N_3N_pPxn (1). The same sample was reacted with sodium dithionite (1 mM) and the emission spectrum recorded after excitation with 330-nm light (2).

is the precise identification of the modified amino acid residues. The singlet nitrene generated by photoirradiation is so reactive that modification of more than one amino acid residue is generally observed. Even in the presence of substrates, the modification of amino acids not implicated in the recognition of the substrate has been reported.

Adenosine polyphosphate pyridoxal

Adenosine diphosphate pyridoxal and uridine diphosphate pyridoxal have been synthesized (Tagaya *et al*, 1985; Tamura *et al*, 1986) and used as catalytic site probes of the enzymes pyruvate kinase and glycogen synthetase, respectively.

AP_4-PL (adenosine tetraphosphate pyridoxal) (Fig 3) has several attractive features for its application in chemical studies of the catalytic site of pyridoxal kinase (Dominici *et al*, 1988). First, the adenosine polyphosphate derivative of pyridoxal contains structural features of the substrates pyridoxal and ATP which are assumed to bind to different domains of the kinase; second, the introduction of an active site-directing moiety to pyridoxal-5'-P makes the resultant reagent an effective probe for the pyridoxal binding site of the kinase.

The effect of addition of AP_4-PL on the catalytic function of pyridoxal kinase (see Table I) was investigated by preincubating the enzyme with increasing concentrations of the affinity labeling reagent at 25°C in phosphate buffer (pH 7). Inactivation of the kinase was attained by preincubating the enzyme with 5-fold molar excess of AP_4-PL as shown in Figure 4. The inactivation reaction is fast, and it was virtually impossible to determine the apparent rate constant at various concentrations of inhibitor.

Substantial protection against inhibition was observed by preincubating the enzyme with ATP prior to addition of AP_4-PL. Only slight protection was observed by the

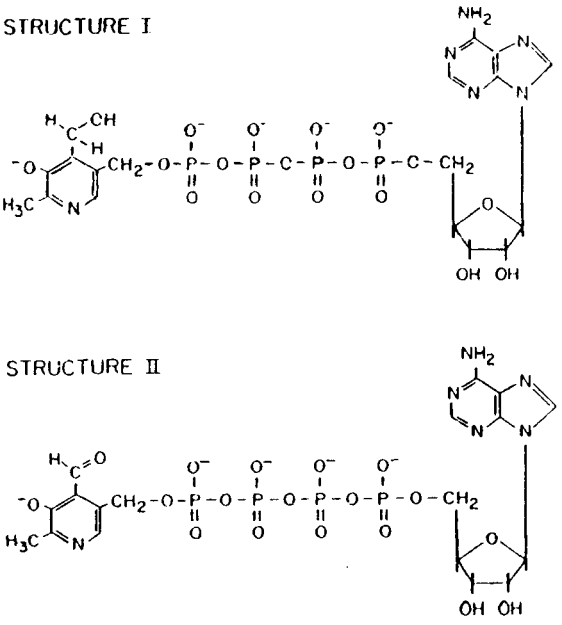

Fig 3. Structure of AP_4 pyridoxine (structure I) and AP_4 pyridoxal (structure II).

Table I

Catalytic parameters of pyridoxal kinase

Substrate or inhibitor	K_{cat}	K_m or K_i
	s^{-1}	μM
ATP	1.9	70
2'-Deoxy-ATP	1.9	90
GTP	0.87	200
AP_4		6000
AP_4-PN		6
PLP[a]		
PL	1.9	30

[a] PLP showed 20% inhibition of kinase activity when incubated with the enzyme at a concentration of 8 mM.

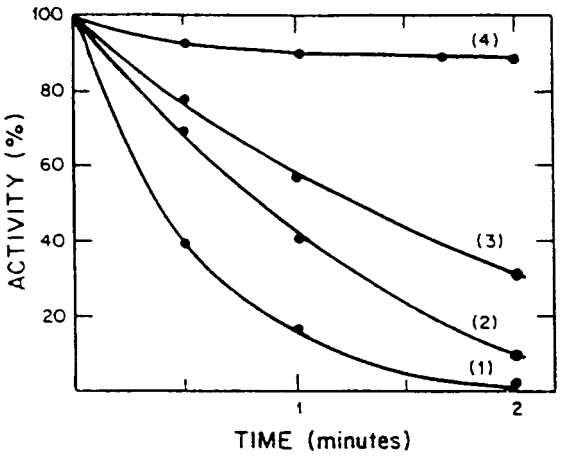

Fig 4. Time course of inactivation of pyridoxal kinase by AP_4-PL. Results obtained when a sample of pyridoxal kinase (20 μM) was preincubated with AP_4-PL (200 μM) (1) and AP_4-PL (100 μM) (2). The results of enzymatic activity measurements of samples of enzyme (20 μM), preincubated with pyridoxal (1 mM) (3), and ATP (1 mM) (4), prior to the addition of AP_4-PL (100 μM) are included in the figure. Experiments conducted in 0.1 M sodium phosphate (pH 7) at 25°C. Aliquots withdrawn at the indicated time intervals were assayed for catalytic activity. The inactivation reaction is rather fast, and a pseudo-first order rate constant K_{obs} = 0.04 s^{-1} is obtained for the sample incubated with 10-fold molar excess of AP_4-PL.

substrate pyridoxal at concentrations of 1 mM. To ascertain whether the loss of catalytic activity produced by reaction with AP_4-PL could be related to the modification of lysyl residues, absorption spectra were taken for samples of enzyme reacted with AP_4-PL. Adenosine polyphosphopyridoxal exhibits two intense absorption bands at 260 and 388 nm, due to the adenine and pyridoxal-5'-P moieties, respectively. An increase in the intensity of the absorption band covering the range 390-480 nm was observed upon reaction of AP_4-PL with the enzyme. The spectral changes are indicative of the formation of a Schiff base linkage between the carbonyl group of pyridoxal and ε-amino groups of lysyl residues.

Upon reduction with $NaBH_4$, the enzyme treated with AP_4-PL displayed an intense absorption band at 325 nm characteristic of pyridoxyl chromophores. Using an extinction coefficient of 9,000 $M^{-1}cm^{-1}$ for the pyridoxal chromophores, it was possible to determine a degree of labeling of AP_4-pyridoxyl molecules bound per enzyme dimer. In order to determine the amino acid sequence of the labeled peptide, the enzyme treated with five-fold molar excess of AP_4-PL was reduced with tritiated $NaBH_4$, denatured with guanidinium-HCl, dialyzed against ammonium bicarbonate, and digested with chymotrypsin. Upon separation by HPLC chromatography using a reverse-phase column, only one radioactive peptide was isolated (Fig 5). After a second chromatography, one radioactive peptide absorbing at 325 nm was detected. The radioactive fractions were pooled, lyophilized and characterized by automated Edman degradation. The amino acid sequence val-asp-ala-val-val-gly-ala-gly-asp-leu-ala-ala-met-leu-leu-ala-thr-*x*-his is consistent with the amino acid composition determined by amino acid analysis. The position indicated by *x* corresponds to a lysine derivatized with AP_4-PL for the following reasons: (a) After Edman degradation, all the radioactivity was detected in cycle number 18; and (b) pyridoxal ε-lysine, arising from the hydrolysis of the radioactive peptide was detected in the amino acid analysis.

Several lines of experimental evidence indicate that AP_4-PL functions as a specific affinity label of pyridoxal kinase. Thus, competition for binding between AP_4-PL and the natural substrate ATP was demonstrated by the ability of the latter to exert a protective effect against inactivation of the enzyme. On the other hand, AP_4-PL binds strongly to the enzyme whereas AP_4 and pyridoxal-5'-P do not inhibit the enzyme at concentrations of 1 mM.

An intriguing question raised by the reported results is related to the position of

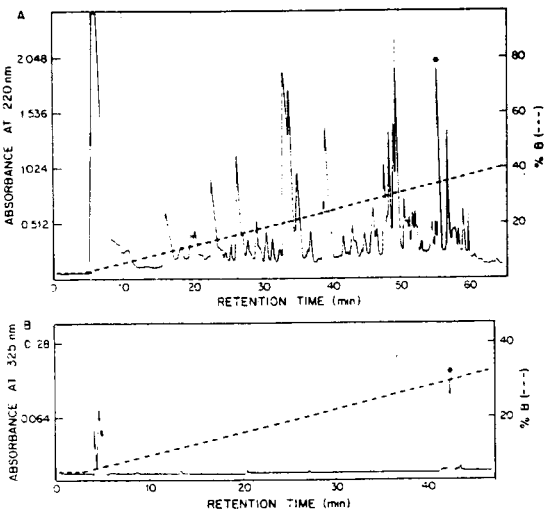

Fig 5. Panel A, a separation of chymotryptic peptides from modified pyridoxal kinase by reverse-phase HPLC. The separation was performed with a linear gradient from 5 to 60% B in 90 min at a flow rate of 0.5 ml/min (A = 0.1% TFA (trifluoroacetic acid); B = 0.1% TFA in acetonitrile/ H_2O 80:20). Elution was monitored at 220 nm. Fractions of 0.5 ml were collected, and 10 µl of each fraction were withdrawn for counting radioactivity in a Beckman LS 7500 counter. Panel B, the fractions showing radioactivity (*) were pooled, lyophilized, and purified by reverse-phase HPLC using a linear gradient of acetonitrile (5-40%) containing 0.1% TFA for 60 min at a flow rate of 0.5 ml/ min. The elution profile was monitored at 325 nm. The labeled peptide, showing absorbance at 325 nm, was used for amino acid analysis and sequenced by Edman degradation.

the modified lysyl residue in the sequenced peptide with respect to the adenosine binding site of ATP. Without a knowledge of the complete tertiary structure of pyridoxal kinase it is rather difficult to provide an answer to that challenging question.

Interaction of proteins

Proteins tagged with fluorescent probes have been used to study their interaction with other macromolecules in solution. Two methods based on fluorescence intensity and emission anisotropy measurements are suitable to monitor association of proteins in solution.

When the tagged protein is the only component of the system, the emission anisotropy A_0 is determined by the Brownian rotation of the labeled protein. Upon addition of increasing concentrations of a second unlabeled protein, the observed emission anisotropy A should increase when heterologous aggregates are formed:

$$\text{Lab - Prot}_1 + \text{Prot}_2 \rightarrow \text{Lab - Prot}_1 \text{ - Prot}_2$$

In the presence of an excess concentration of the unlabeled protein ($Prot_2$), the equilibrium is shifted in the direction of formation of the protein-protein complex until the emission anisotropy approaches a maximum value (A_M). The fraction of Lab-Pro_1 (α) bound to $Prot_2$ is a function of the emission anisotropy values, A_0, A and A_M and of ß, the fluorescence yield of free/bound Lab-$Prot_1$ (Equation 1).

$$\alpha = \frac{A - A_0}{(A_m - A)ß + (A - A_0)} \quad (1)$$

The apparent equilibrium constant (K_D) is determined by plotting $1/\alpha$ against 1/[L], where [L] is the concentration of free Lab-$Prot_1$.

$$\frac{1}{\alpha} = 1 + \frac{K_D}{[L]} \quad (2)$$

This experimental approach was applied to investigate the interaction between pyridoxal kinase and the apoprotein of aspartate aminotransferase (Kim *et al*, 1988). For these experiments the kinase was labeled with fluoresceine isothiocyanate (FIT) and emission anisotropy measurements were performed upon excitation at 480 nm, a wavelength coinciding with the absorption of the fluorescent probe.

A typical titration curve obtained at a fixed concentration of FIT-pyridoxal kinase and varying concentrations of aspartate aminotransferase is given in Figure 6, where it can be seen that a progressive increase in the emission anisotropy takes place as a result of association between the proteins.

The change in emission anisotropy elicited by addition of the unlabeled protein can be attributed to variations in the rotational correlation of Lab-$Prot_1$ trapped by the unlabeled protein ($Prot_2$). If this is the case, then rotational correlation time measurements in the presence and absence of $Prot_2$

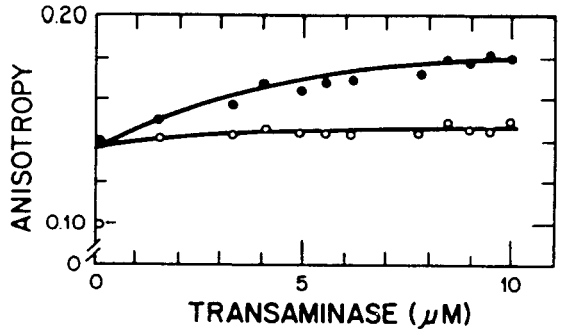

Fig 6. Changes in the emission anisotropy of FIT-kinase following the addition of increasing concentrations of apotransaminase at pH 6.8 in 0.05 M phosphate buffer (pH 6.8) containing 150 mM KCl (•). Effect of addition 400 mM KCl on the emission anisotropy of the samples (•). Experiments conducted at a fixed concentration of FIT-kinase (4 μM) at 25° C. Excitation wavelength 460 nm. Emission was selected by glass filters (Corning C-S-3-69).

would provide some insight into the stability of the Lab-Prot$_1$-Prot$_2$ complex in the nanosecond time scale.

For precise determination of rotational correlation times, the protein is labeled with a reagent characterized by a fluorescent lifetime at least 25% shorter than the rotational correlation time of the macromolecule.

In order to ensure complete binding of Lab-Prot$_1$ to Prot$_2$, time-dependent emission anisotropy measurements are conducted at protein concentrations at least 10-fold greater than the determined dissociation constant (K_D).

Fluorescence and emission anisotropy measurements are made using the monophotonic technique (O'Connor & Phillips, 1984) in a nanosecond spectrometer. A free running flash lamp pulsed at 10 kHz is used as exciting source.

Rotational correlation times are determined by recording the fluorescence decay curves of the polarized components F//(t) and F⊥ (t), parallel and perpendicular, respectively, to the plane of the incident polarized light.

The functions F//(t) and F⊥(t) are deconvoluted (Kim *et al*, 1988) and used to calculate the emission anisotropy A(t):

$$A(t) = \frac{F//(t) - F\perp(t)}{F//(t) + 2\perp(t)} \qquad (3)$$

The emission anisotropy function A(t) is fitted to mono or biexponential decays using nonlinear least square analysis.

For a protein characterized by a rotational correlation time (∅R) and one fluorescent lifetime (τF), the following equation is valid

$$F//(t) - F\perp(t) = 3A_0 e^{-t/\varnothing R}\ e^{-1/\tau F} \qquad (4)$$

Figure 7 shows the results of time dependent emission anisotropy measurements of EDANS-kinase in the absence and presence of aspartate aminotransferase. The anisotropy decay function of the kinase labeled with the fluorescent probe N-iodoacetylaminoethyl-5-naphtylamine-1-sulfonic acid (I-EDANS) yields a single rotational correlation time compatible with the rotation of a rigid spherical body, whereas binding of the transaminase influences the emission anisotropy decay function of EDANS-kinase. The decay function of EDANS-kinase in the presence of

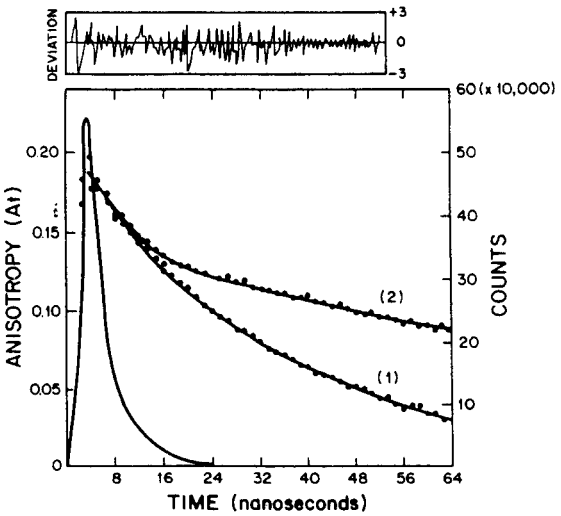

Fig 7. Anisotropy decay functions of EDANS-kinase in the absence (1) and presence (2) of apotransaminase. The following concentrations were used: EDANS-kinase 10μM and apotransaminase 50 μM in phosphate buffer (pH 6.8) containing 150 mM KCl at 25°C. The best fit parameters for EDANS-kinase are A_0 = 0.21, ϕ = 36 ns. The best fit parameters for the double exponential decay of EDANS-kinase in the presence of apotransaminase are A_1 = 0.67, ϕ = 36 ns, A_2 = 0.33, ϕ_2 = 62 ns. It should be noted that the extrapolated value of A(t), A = 0.21 is smaller than the theoretical value expected for a chromophore rigidly bound to the protein A_0 = 0.32. Included is the deviation function corresponding to the deconvoluted data fitted to a double exponential function. Excitation wavelength 340 nm. The emission was selected by glass filters (Corning C-S-3-72).

the transaminase is no longer mono-exponential; indeed the results are fitted by a two exponential function (Kim *et al*, 1988). The longest rotational correlation time, $\varnothing_2$ = 62 ns, could be assigned to EDANS-kinase complexed to the aminotransferase, whereas the shortest rotational correlation time, $\varnothing 1$ = 36 ns, corresponds to free EDANS-kinase.

The binary complex EDANS-kinase-transaminase is stable at moderate ionic strength (150 mM KCl), but it dissociates upon increasing the ionic strength of the medium (Kim *et al*, 1988).

What is the physiological role of protein aggregates (clusters)? The main function of protein compartmentation is to ensure direct transfer of pyridoxal-5'-P to the apotransaminase without leakage to the surrounding environment.

It is well established that pyridoxal-5'-P is bound to vitamin B_6-dependent enzymes in eukaryotic cells and that the concentration of free pyridoxal-5'-P is very low, ranging from 0.1 to 1 μM (Li & Lumeng, 1985). The reason for the existence of low concentrations of free pyridoxal-5'-P in mammalian tissues is clear: it is due to the high catalytic activity of phosphatases that cleave the phosphoryl group of free pyridoxal-5'-P. Hence, the main function of multienzyme clusters consists in preventing the hydrolysis of pyridoxal-5'-P by the phosphatases. Pyridoxal-5'-P generated by the kinase binds rapidly to the catalytic site of the amino-transferase, and it is no longer available to the phosphatases.

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