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Modification of Carboxyl Residues of Proteins with Pyridoxamine as a Fluorophore

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Abstract: A general procedure to quantitate the reaction of carbodiimides with carboxy groups of proteins is described. Pyridoxamine reacts with the *o*-acylisourea intermediate generated during the reaction of carboxyl residues with carbodiimides. The extent of the reaction is determined by measuring the spectroscopic properties, absorption and emission, of pyridoxyl residues covalently attached to the proteins. Resolved pig brain aspartate aminotransferase (apoenzyme), inactivated by 1-ethyl-3-(3-dimethylamino propyl) carbodiimide, reacts with [^{3}H]pyridoxamine. After trypsin digestion, one peptide labeled with radioactive pyridoxyl was separated by reverse phase HPLC.

Key words: aspartate aminotransferase, carbodiimide, pyridoxamine, spectroscopic properties.

The carboxyl groups of proteins such as aspartic and glutamic acid have been found to be implicated in specific carbodiimide protein interactions (Solioz, 1984). The *o*-acylisourea intermediate formed during the reaction of carboxyl residues with carbodiimide can react with a nearby amino group of an amino acid chain to yield inter and intramolecular crosslinked protein species (Herz and Packer, 1981; Pennington and Fisher, 1981). This zero-length cross linker has been used extensively to localize the binding site or the catalytic site of proteins (Mornet *et al.*, 1981; Toner-Webb *et al.*, 1987; Andreeva *et al.*, 1993). Moreover, a rearrangement reaction of the *o*-acylisourea intermediate can lead to the formation of a stable N-acylurea adduct (Borders *et al.*, 1989). The reaction of the *o*-acylisourea intermediate with radiolabeled nucleophiles ([^{14}C] glycine ethyl ester) has been used to quantitate the extent of the chemical modification of carboxyl residues (Matsuo *et al.*, 1980). If an appropriate nucleophilic reagent is used after the selective modification of carboxy groups in the proteins by carbodiimide derivatives, the nucleophilic reagent can attack the activated carboxy group stoichiometrically to yield stable products (Hoare and Koshland, 1966). This carbodiimide nucleophile approach has been successfully used for the quantitative determination of carboxy groups in enzyme activity studies (Carraway and Koshland, 1972; Pho *et al.*, 1977; Ramachandran and Colman, 1977).

The aim of the present work is to examine the spectroscopic properties of pyridoxamine (PM) covalently attached to proteins and to investigate the possibility of using this fluorophore as a probe of the rotational and diffusional dynamics of proteins in solution. PM reacts with the *o*-acylisourea intermediate formed during the reaction of carboxyl residues of the protein with carbodiimide. Using aspartate aminotransferase as a model system, the modified peptide has been isolated. The spectral changes detected when the ligand is bound to apoproteins of the pyridoxal-5′-phosphate (PLP) dependent enzyme can be used to investigate the micro environment of the cofactor binding sites.

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Materials and Methods

Materials

NaB[^{3}H]$_4$ (340 mCi/mmol) was purchased from Du Pont New England Nuclear, and pyridoxamine, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), dicyclohexyl carbodiimide (DCCD) from Sigma (St. Louis, USA). The proteins bovine serum albumin, trypsin, lysozyme and malate dehydrogenase were purchased from Boehringer Mannheim (Indianapolis, USA). Resins used in chromatography for enzyme preparation were purchased from Biorad (Richmond, USA) and Pharmacia (Uppsala, Sweden).

Labeling of proteins

Reactions were carried out in 25 mM *o*-morpholino ethanesulfonic acid (MES), pH 6, containing 5 mg/ml of protein, 10 mM EDC and 1 mM pyridoxamine-5′-P (PMP), or 10 mM pyridoxamine. The reaction mix-

ture was incubated at 25℃ for 3 h. At the end of the reaction, the modified proteins were passed through a Sephadex-G-25 column, precipitated with 70% acetone (v/v) and dialysed against 0.1 M phosphate buffer (pH 7).

The reaction of resolved aspartate aminotransferase with the carbodiimide reagent was carried out as described above with the exception that tritiated pyridoxamine (10 mCi/mmol) was used in the incubation reaction.

The degree of pyridoxamine incorporation in the modified proteins was determined by measuring the absorbance at 325 nm (ε_{325}=9,000 $M^{-1}cm^{-1}$) or by diluting the protein solution and determining the fluorescence at 395 nm (excitation at 325 nm) using P-pyridoxyl-acetyl as a standard of known quantum yield (q=0.08). P-pyridoxyl-acetyl was prepared as described in reference (Higgins and Wilson Miles, 1978).

Preparation of aspartate aminotransferase

The method used in the purification of aspartate aminotransferase from pig brain is based on methods (Jenkins and Sizer, 1960; Martinez-Carrion *et al.*, 1967) previously used in the purification of the enzyme from pig heart. Protein concentration was determined by the colorimetric method of Lowry *et al.* (1951) and by spectophotometric measurement using a molar absorption coefficient of the dimer $\varepsilon_{280}=7.0\times10^4$ M^{-1} cm^{-1}.

The method used for resolution of the enzyme is based on the conversion of the pyridoxal phosphate form of the enzyme to the pyridoxamine phosphate form by addition of the substrate aspartate (20 mM) at pH 7.8 in the presence of 0.1 mM 2-mercaptoethanol. Then, the dissociation of the cofactor pyridoxamine phosphate from the enzyme is achieved by the addition of 0.5 M H_2KPO_4. The sample is incubated at room temperature (25℃) for 1 h and then dialyzed against 0.1 mM potassium phosphate (pH 7.5) containing 0.1 mM 2-mercaptoethanol.

Inactivation of aspartate aminotransferase

It was studied at pH 6 and pH 6.5, using a concentration of resolved enzyme of 0.5 mg/ml, an EDC concentration of 10 mM, and a pyridoxamine concentration of 1mM in 25 mM MES. Aliquots withdrawn from the incubation mixture at several time intervals were mixed with pyridoxal 5′-phosphate (1 mM) incubated for 25 min at 25℃ prior to enzymatic assays. A control of holotransaminase (pyridoxal 5′-P form) was treated with EDC and PM under identical experimental conditions and assayed for catalytic activity.

The enzyme activity was measured in a coupled assay with malate dehydrogenase and 20 mM aspartate plus 20 mM 2-oxoglutarate as substrates (Birchmeier *et al.*, 1973).

Synthesis of [^{3}H] Pyridoxamine-5′-P

[4′,4′,^{3}H] pyridoxamine was synthesized by tritium labeled sodium borohydride (340 mCi/mmol) reduction at room temperature of the imine formed by pyridoxal in NH_4OH. The compound was purified through chromatography on an amberlite-CG-50 column (H^+ form) (1×30 cm) eluted with water and 0.01 M HCl.

Identification of a modified peptide

The modified enzyme was treated with guanidine hydrochloride (6 M), dialysed against 50 mM ammonium bicarbonate (pH 8.6), and digested with trypsin (mixing molar ratio, substrate : trypsin, 50 : 1) at 37℃ for 5 h. The digested sample was lyophilized dissolved in 0.1% TFA and applied to a vydac C_{18} column (25×0.46 cm) for reverse-phase HPLC. The peptides were eluted using a linear gradient from 5 to 50% of eluant B in 90 min. Eluant A: 0.1% TFA; eluant B: 0.1% TFA in 80 : 20 acetonitrile/H_2O. The fractions were lyophilized, dissolved in the scintillation mixture and counted in a scintillation counter (Beckman, LS 7500).

The sequence of the isolated peptide was determined by Edman degradation using an Applied Biosystems model 411A pulsed liquid-phase sequencer equipped with a phenyl-thiohydantoin analyzer.

Spectroscopy

Fluorescence measurements were conducted in a precision fluorimeter equipped with two Bausch and Lomb monochromators and a light source (Xenon lamp, 150 watt). Polarization measurements (steady state) were conducted in a SLM double beam polarization apparatus equipped with a RACAL-DANAN digital multimeter. Illumination provided by a Xenon lamp (150 watts) was passed through a grating monochromator (Bausch and Lomb). Emission light was filtered through glass filters (Corning glass C.S. 3-72). Emission polarization values were measured with a precision of +/−0.002.

Fluorescence decay measurements were made using the monophotonic technique with an Ortec Nanosecond spectrometer. Excitation was set at 330 nm and the emission filtered through a glass filter (Corning, CS-3-72). The excitation source was a free-running flash lamp operating in air at 1×10^5 Pa. The fluorescence decay function was deconvoluted and fitted to either a mono or a biexponential decay using non-linear least square analysis (O'Connor and Phillips, 1984).

Spectroscopic measurements were carried out in a

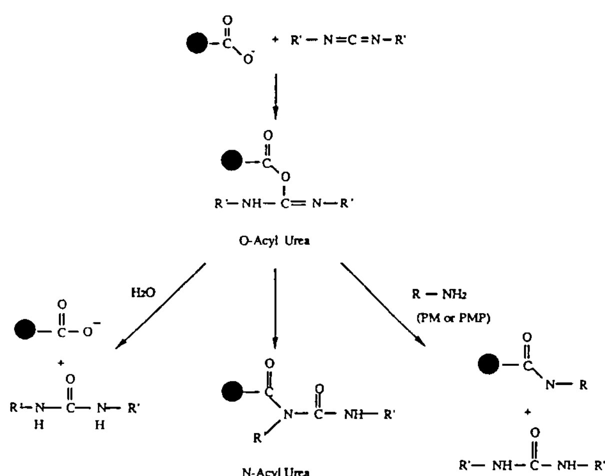

Fig. 1. Possible reaction mechanism for the interaction of PM or PMP with a carboxy group of protein activated by EDC. The protein molecule is indicated by the solid black circle.

Shimadzu, UV-160 spectrophotometer.

Results

Modification of carboxyl residue of proteins

The results of the labeling process indicate that the reaction of carbodiimides with carboxy groups of proteins yields the *o*-acylisourea intermediate that reacts with additional nucleophiles such as pyridoxamine or pyridoxamine-5′-P. A possible reaction mechanism for the modification is shown in Fig. 1.

Derivatization of protein carboxy groups by carbodiimides is efficient at acid pH in the region 5.0~6.5, but still proceeds at a reasonable rate at neutral pH. Incubation of bovine serum albumin and lysozyme with carbodiimide and pyridoxamine at pH 6 resulted in the incorporation of pyridoxyl residues.

A potential side reaction during modification of the carboxy groups of proteins by carbodiimides is covalent cross-linking between adjacent subunits. To establish whether such intersubunit crosslinking had occurred, resolved aspartate aminotransferase modified by EDC was subjected to SDS-polyacrylamide gel electrophoresis. The modified protein migrated as a single band of 45 kDa that was indistinguishable from the control, untreated enzyme. Therefore, there was no evidence that intersubunit cross linking had occurred during inactivation of the aminotransferase.

Another potential side reaction taking place during modification of carboxy groups by carbodiimide is the formation of stable N-acylisourea adducts. To ascertain whether the formation of stable N-acylisourea adducts had taken place, the resolved aminotransferase was treated with 5 mM dicyclohexyl [^{14}C] carbodiimide, and tested for incorporation of radiolabel. Although the resolved enzyme was inactivated by DCCD, there was little incorporation of radiolabeled ligand (0.2 mol/dimer) in the modified enzyme after dialysis against 25 mM MES (pH 6).

As indicated in Fig. 1, if the nucleophile is water instead of internal or external nucleophile, the protein remains unmodified, although the inhibition process observed should be reversible in this case. The modification of the resolved aminotransferase by EDC was irreversible, since extensive dialysis against 4 L of 25 mM MES (pH 6.0) for 24 h at 4℃ did not restore catalytic activity.

Thus, the results mentioned above provided strong evidence in support of the hypothesis that most of the *o*-acylisourea intermediate generated by reaction with carbodiimides is trapped by the nucleophilic group of pyridoxamine. The irreversible covalent modification was clearly confirmed by identification of modified tryptic peptide.

Table 1. Spectroscopic properties of pyridoxamine bound to proteins

Sample	λ_f (nm)	τ_f (ns)	P	Degree of Labeling (mol/mol)[a]	Activity (%)
BSA	395	3.0	0.3	2.0	–
Lysozyme	395	3.2	0.18	1.9	60
Apo GOT	395	3.0	0.3	1.8	10

Excitation wavelength at 325 nm. λ_f is the maximum wavelength of fluorescence emission, τ_f is fluorescence lifetime, P is polarization of fluorescence. Apo GOT represents apo aspartate aminotransferase.
[a] mol/dimer for Apotransaminases.

Spectroscopic properties of modified protein

Table 1 lists the properties of proteins tagged with pyridoxamine. The incorporation of approximately 2 pyridoxyl residues/mol of lysozyme resulted in 40% loss of catalytic activity. Pyridoxyl bound to the proteins displays an emission band centered at 395 nm (excitation at 325 nm) as shown in Fig. 2.

The fluorescence emitted by the pyridoxyl residues of aspartate aminotransferase decays in a multiexponential manner with an average lifetime of approximately 3 nanoseconds (Fig. 3). The polarization of fluorescence values obtained by excitation at 330 nm are influenced by the size of the macromolecule to which the fluorescent residues are covalently attached. Aspartate aminotransferase and bovine serum albumin of molecular masses ranging between 94 and 68 kDa exhibit polarization of 0.3, whereas lysozyme (16 kDa) is characterized by a polarization value of 0.18.

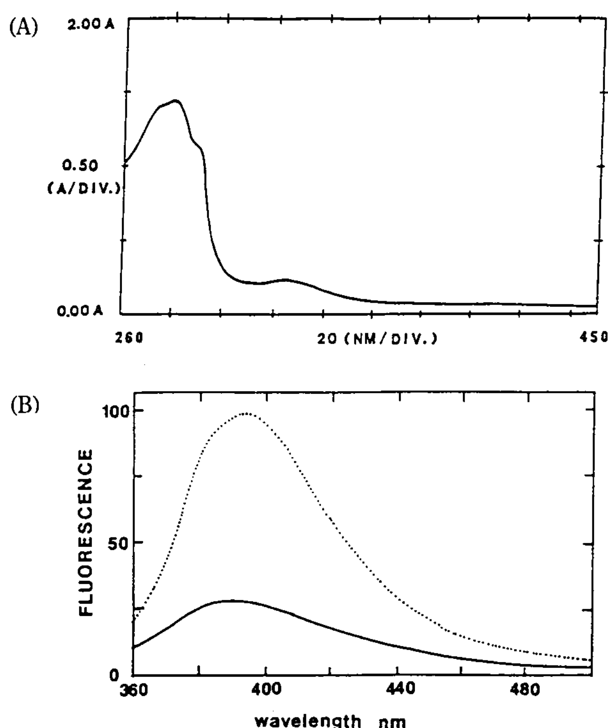

Fig. 2. (A) Absorption spectrum of lysozyme modified with pyridoxamine. A degree of labeling of 1.8 mol PM/mol enzyme was detected by absorption spectroscopy. The enzyme is partially inactivated (40%). (B) Fluorescence spectra of pyridoxamine free (····) and bound to lysozyme(—) at pH 6.0. The excitation wavelength was 325 nm.

Covalent binding of pyridoxamine to the proteins examined causes an increase in the polarization of fluorescence. It is important to note that the observed polarization is dependent on the rotational dynamics of the macromolecules bearing the chromophore. If the fluorescence lifetime is constant and if the extrinsic chromophore rotates in common with the macromolecule, then one should observe a linear correlation between the reciprocal of the anisotropy (A=2P/3-P) and the reciprocal of the molecular weight of the macromolecule, as predicted by Perrin's equation for spherical bodies undergoing brownian motion. For globular proteins used in this study, the observed polarization of the probe is mainly influenced by the mass of the macromolecule undergoing rotational diffusion.

Inactivation of resolved aminotransferase

Inactivation of resolved aminotransferase by EDC takes place at pH 6.5 and 6 as shown in Fig. 4. At the lower pH value, the half life for inactivation is 50 min. The inhibitory effect exerted by chemical modification of the protein is completely prevented when pyridoxal −5′-P is bound to the aminotransferase, indicating that carbodiimide reacts with aminoacid residues located in the catalytic site.

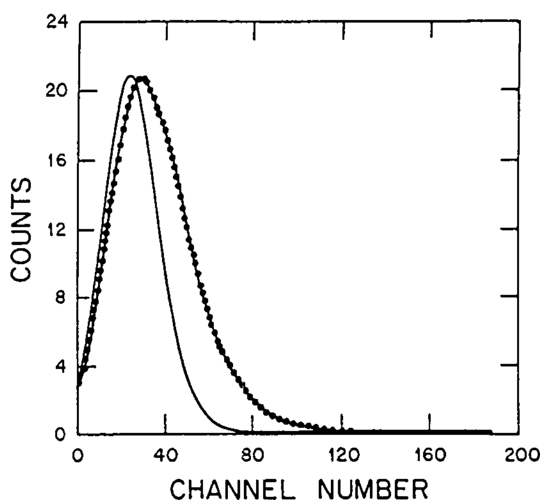

Fig. 3. Fluorescence decay of pyridoxyl residues bound to aspartate aminotransferase (●) and lamp profile (—). The deconvoluted decay with fitted to a biexponential curve. $\alpha_1=0.99$, $\tau_1=3$ ns, $\alpha_2=0.01$, $\tau_2=18$ ns.

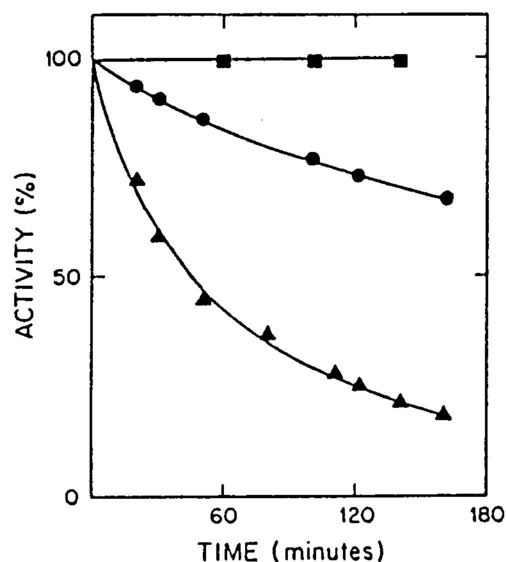

Fig. 4. Inactivation of resolved aspartate aminotransferase by EDC and pyridoxamine at pH 6.5 (●) and pH 6 (▲) in 25 MES buffer. 0.5 mg/ml of resolved enzyme was incubated in the presence of EDC (10 mM) and pyridoxamine (1 mM) at 25℃. Aliquots withdrawn at the indicated times were mixed with pyridoxal-5′-P (1 mM), incubated for 15 min and assayed for catalytic activity. Results obtained with the holoenzyme (PLP form) incubated with EDC and pyridoxamine under similar experimental conditions are represented with closed squares.

Identification of modified peptides

To ascertain whether the loss of catalytic activity produced by the reaction of EDC could be related to the modification of a few carboxyl residues of the aminotransferase, resolved enzyme (5 mg) reacted with EDC and tritiated pyridoxamine (10 mCi/mmol) was digested with trypsin, and the peptides separated by reverse-phase HPLC. Upon separation by reverse phase chro-

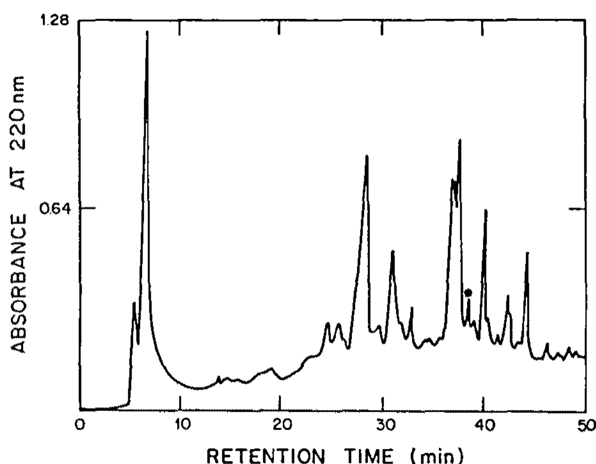

Fig. 5. Separation of tryptic peptides from modified aspartate aminotransferase by reverse-phase HPLC. The separation was performed with a linear gradient from 5 to 60% B eluant in 90 min at a flow rate of 0.8 ml/min (eluant A=0.1% TFA, eluant B=0.1% TFA in 80 : 20 acetonitrile/H_2O). Elution was monitored at 220 nm. Fractions of 0.5 ml were collected and 10 μl of each fraction were withdrawn for counting radioactivity. The fraction indicated in the figure (*) contains 3,000 cpm. Amino acid sequence for the radioactive peptide: FLFPFFXSAYQGFASGN-LEK. X indicates the position of the radiolabeled amino acid.

matography, only one radioactive peptide was detected. A typical elution profile obtained with the digested aminotransferase is shown in Fig. 5.

Automated Edman degradation was performed to identify the modified peptide. The following amino acid sequence was obtained for the radioactive peptide:

FLFPFFXSAYQGFASGNLEK

X indicates the position of the radiolabeled amino acid. This sequence overlaps a segment of 20 amino acids, positions 216~235 of the entire primary sequence of cytosolic aspartate aminotransferase from pig brain (Christen *et al.*, 1985). Accordingly, the modified amino acid (X) corresponds to aspartic acid 222. In this respect, it should be noted that aspartic acid 222 is involved in either binding pyridoxal-5'-P or in assisting pyridoxal catalysis (Jansonius *et al.*, 1985).

Discussion

The purpose of this study was to develop a chemical modification method of carboxyl residues of proteins with a fluorescent probe pyridoxamine, and to investigate the possibility of using this chromophore as a probe to study the catalytic binding site of PLP dependent enzymes.

Water soluble carbodiimide EDC theoretically could react with several residues of protein such as Asp, Glu, Cys, and Tyr (Carraway and Koshland, 1972). However, only carboxyl residues under mild acidic conditions have been found to be implicated in specific carbodiimide-protein interactions (Solioz, 1984). If the modification reaction is carried out at alkaline pH, the carbodiimide can be considered as a specific reagent for modification of the phenolic hydroxy group of tyrosine residues in proteins (Grouselle and Pudles, 1977). The inactivation of aspartate aminotransferase proceeded more rapidly at acid pH (pH 6). The increased rate of inactivation occurring at low pH is due probably to increased concentration of the protonated carbodiimide, since this is the species of carbodiimide that reacts preferentially with a nucleophilic group (Smith *et al.*, 1958). Also, as the pH is lowered from neutral to 6, it is clear that the best available choice of a nucleophile is a carboxyl group. Under these experimental conditions, therefore, it is most likely that the reactive group being modified in the protein is a carboxylic acid.

It is also raises the interesting question of why only a few of the carboxy groups are modified by carbodiimide and pyridoxamine under the chosen experimental condition. It should be noted that carbodiimides have been extensively used, under similar conditions and pH, to inactivate other proteins such as carboxypeptidase A (Nau and Riordan, 1975), hexokinase (Pho *et al.*, 1977) and isocitrate dehydrogenase (Ramachandran and Colman, 1977). Results from these studies indicate that only one or two essential carboxylic acid residues per subunit of each enzyme are modified. A simple explanation for this is that the carboxylic groups modified may have relatively higher pKa values. The unusual pKa may arise from a low dielectric constant in the local environment such as catalytic site due to a multiple hydrophobic interaction of other nearby side chains. Therefore, those groups are largely protonated at pH 6, whereas other carboxy groups that are not involved in the protein substrate recognition or binding are ionized. It is known that protonated carboxylic groups react with the carbodiimide reagent (Kurzer and Douraghi-Zaceh, 1967).

Moreover, if the enzyme to be modified is a PLP dependent, the cofactor binding site should be more suitable for this modification. In the present work, the aspartate aminotransferase was successfully used as a model system. The spectroscopic properties of the cofactor bound to the catalytic site of the aminotransferase provide important information about the microenvironment of the catalytic site.

The emission anisotropy of pyridoxamine labeled aspartate aminotransferase is A=0.22. The maximum emission anisotropy expected for a pyridoxamine rigidly immobilized is A_o=0.32. If one applies Perrin's equation: $A_o/A = 1 + \tau/\phi$, one obtains a value of rotational

correlation time ϕ=6.7 ns for a decay time τ=3 ns. The observed rotational correlation time is considerably shorter than the value predicted for the rigid sphere of MW 90,000 (45 ns). This may be due to segmental flexibility. If the fluorophore covalently bound to the protein exhibits some degree of internal rotation, then it might be anticipated that the apparent rotational relaxation time should be smaller than the value expected for the rotation of the entire macromolecule in solution.

In view of these considerations it seems reasonable to suggest that pyridoxamine is a suitable probe to detect fast rotational motions of macromolecules. Dynamic processes such as solvent relaxation and energy transfer, which often occur in time scale of 0.1~10 ns, can also be detected by measuring the fluorescence properties; i.e., quantum yield, band position and lifetime of the excited singlet state of pyridoxamine covalently bound to proteins.

Therefore, the method described in this paper can be directly applied to investigate the microenvironment of the cofactor binding sites of proteins, particularly pyridoxal phosphate dependent enzymes. Since the pyridoxamine is smaller than most of the extrinsic probes commonly used in labeling macromolecules, it is anticipated that they would produce minor conformational changes when reacted with nucleophilic groups of enzymes. In this aspect, it should be noted that using another vitamin B_6 derivative pyridoxic acid, a similar approach has recently been developed by the author (Kwon *et al.*, 1994). The method has been applied to modify the amino residue of proteins and to demonstrate the possibility of using the probe to investigate structural features of macromolecules.

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