Journal of Biochemistry and Molecular Biology, Vol. 33, No. 1, January 2000, pp. 43-48

JOURNAL OF
Biochemistry and Molecular Biology

Structural Arrangement for Functional Requirements of Brain Recombinant 4-Aminobutyrate Aminotransferase

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Received 5 August 1999, Accepted 16 September 1999

4-Aminobutyrate aminotransferase is a key enzyme of the 4-aminobutyric acid shunt. It converts the neurotransmitter 4-aminobutyric acid to succinic semialdehyde. In order to study the structural and functional aspects of catalytically active Cys residues of pig brain 4-aminobutyrate aminotransferase, we purified the active form in *E. coli* by coproduction of thioredoxin. The structural arrangement for functional requirements of a dimeric protein using a bifunctional sulfhydryl reagent was then characterized, and the spatial proximity between the essential SH groups and a cofactor (pyridoxal-5'-phosphate) binding site was determined. The bifunctional sulfhydryl reagent DMDS reacted with the enzyme at the ratio of one molecule per enzyme dimer. This resulted in an approximately 50 % loss of enzymatic activity. The spatial proximity of the distance between the essential SH groups and the cofactor-binding site was determined by the energy transfer measurement technique. The result (approximate 20 Å) suggested that cross-linking of two sulfhydryl groups with DMDS is not near a PLP binding site.

Keywords: 4-Aminobutyrate aminotransferase, Dimeric structure, Energy transfer, Fluorescence, Stability.

Introduction

4-Aminobutyric acid is the major inhibitory neurotransmitter in mammalian brain tissue . The holoenzyme 4-aminobutyrate aminotransferase (4-aminobutyrate: 2-oxoglutarate aminotransferase, EC 2.6.1.19) contains 1 mole of pyridoxal 5'-phosphate (PLP) and appears to be dimeric, composed of identical subunits, with a molecular weight of 100 kDa. This enzyme is important in the 4-aminobutyric acid shunt and catalyzes the reversible transamination of 4-aminobutyrate to yield succinic semialdehyde and pyridoxamine-5-phosphate (Churchich and Moses, 1981). The peptide bearing the cofactor PLP, a vitamin B6 derivative, covalently attached to a lysine residue has been sequenced (Kim and Churchich, 1989). Also, several cysteine residues critical for enzyme function have been identified by chemical modification (Kim and Churchich, 1991). The cDNA encoding pig brain 4-aminobutyrate aminotransferase has been isolated and characterized (Kwon *et al.*, 1992; Park *et al.*, 1993; Kim *et al.*, 1997). When recombinant 4-aminobutyrate aminotransferase was overexpressed in an *E. coli* expression system, the protein formed insoluble inclusion bodies (Lee *et al.* 1996; Kim *et al.* 1997). The precursor protein 4-aminobutyrate aminotransferase also exhibits catalytic activity comparable to that of the mature enzyme (Kim *et al.* 1997). In a recent study, recombinant 4-aminobutyrate aminotransferase was overexpressed as a catalytically active form in *E. coli* by coproduction of thioredoxin (Sung *et al.*, 1999). The lysyl residue at the 330 position of the amino acid sequence has been verified as serving as the catalytic site of the enzyme by site-specific mutagenesis (Kim *et al.*, 1997). In this study we characterized the structural arrangement of dimeric 4-aminobutyrate aminotransferase for functional requirements using a bifunctional sulfhydryl reagent, and determined the spatial proximity between the essential SH groups and a cofactor (PLP) binding site.

Materials and Methods

Materials DMDS (4,4'-dimaleimide distilbene 2,2'-disulfonate) was obtained from Molecular Probes Inc. (Eugene, USA). *E. coli* strains BL21(DE3) and HB101 have been described by Kim and Richardson (1993; 1994). Restriction enzymes were purchased from New England Biolabs Inc. (Cambridge, USA) and Phamacia-Amersham (Cleveland, USA). Hydroxylamine and 4-aminobutyric acid were obtained from Eastman Kodak Inc. (Boston, USA). Gabase, succinic semialdehyde, 2-oxoglutarate, pyridoxal-5'-P, and other chemicals were purchased from Sigma Chemical Co. (St. Louis, USA).

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Purification of recombinant 4-aminobutyrate aminotransferase Cloning of premature and mature forms of 4-aminobutyrate aminotransferase has been described by Kim *et al.*, 1997 and Sung *et al.*, 1999. Plasmids used to produce recombinant 4-aminobutyrate aminotransferases were constructed using the appropriate pT expression vector containing the T7 promoter (Kim and Richardson, 1993 and 1994; Kim *et al.*, 1992a, b; 1995), and functionally expressed using T7 RNA polymerase (Tabor and Richardson, 1985) and thioredoxin (Yasukawa *et al.*, 1995). Purification of the expressed protein was performed as described (Kim *et al.*, 1997, Sung *et al.*, 1999).

Enzyme assays A coupled assay system consisting of 4-aminobutyrate aminotransferase and succinic semialdehyde dehydrogenase was used to study the catalytic conversion of 4-aminobutyrate to succinic semialdehyde (Beeler and Churchich, 1978).

Reaction with the Cross-linking reagent DMDS DMDS (4,4'-dimaleimide distilbene 2,2'-disulfonate) reacts with specific SH residues in proteins resulting in formation of a disulfide linkage. A sample (10 μM) of purified 4-aminobutyrate aminotransferase was preincubated with varying concentrations of DMDS. Experiments were conducted in 0.1 M phosphate buffer (pH 7.0). The reaction was stopped after 30 min incubation at 25°C. Aliquots withdrawn from the incubation mixtures were tested for enzymatic activity.

4-aminobutyrate aminotransferase (10 μM) in 0.1 M phosphate buffer (pH 7.0) was preincubated with DMDS (20 μM). The reaction was stopped after 30 min incubation at 25°C and unreacted reagent was removed by chromatography at 4°C on a Sephadex G-25 column (1×20 cm) that had been equilibrated with 0.1 M potassium phosphate (pH 7.0). The number of DMDS molecules bound to the enzyme was determined by measuring the absorbance at 340 nm using an extinction coefficient of 5×10^4 $M^{-1}cm^{-1}$.

Determination of free SH residues A sample (10 μM) of purified 4-aminobutyrate aminotransferase was preincubated with sulfhydryl reagent (20 μM) in 0.1 M phosphate buffer for 30 min. Excess reagent was removed by chromatography at 4 °C on a Sephadex G-25 column (1×20 cm) that had been equilibrated with 0.1 M potassium phosphate (pH 7). The column eluate was monitored at 280 nm and fractions with the greatest protein concentrations were combined. Aliquots of the combined fractions were mixed with guanidinium HCl to a final concentration of 5 M and titrated with DTNB (5,5'-dithiobis (nitrobenzoic acid)). The concentration of free sulfhydryl groups was determined spectrophotometrically at 412 nm using an extinction coefficient of 13,600 M^{-1} cm^{-1}.

Proteolysis of the labeled enzyme with trypsin Aminotransferase (2 mg/ml) preincubated with DMDS (10 μM) was incubated with trypsin (0.1 mg/ml) in 0.1 M Na-phosphate (pH 7.0) at 25°C for 3 hours. Aliquots withdrawn from the incubation mixture were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Spectroscopy Absorption spectra were measured with a Kontron UV-932 spectrophotometer (Cho *et al.*, 1997). Fluorescence spectra were measured with a Perkin Elmer LS 50B luminescence spectrophotometer. The slits of the monochromators were set to give a bandwidth of 3 nm.

Other methods Protein concentrations were determined by the method of Bradford (1976). 4-aminobutyrate aminotransferase was resolved according to the modified method of Beeler and Churchich (1978). SDS-PAGE, performed on 12 % polyacrylamide slab gel by the method described by Laemmli (1970), was used to determine the molecular mass of the monomeric unit of the enzyme as well as the molecular mass of subunits cross-linked with DMDS.

Results

4-Aminobutyrate aminotransferase was purified as a functionally active form by coproduction of *E. coli* thioredoxin. The enzyme, produced in soluble form by coproduction of thioredoxin, appeared to have a native protein conformation (Sung *et al.*, 1999).

Crosslinking of the subunits Aminotransferase behaves as a dimeric protein over a wide pH range (5-8). The monomeric form is not catalytically competent. Thus, we investigated whether the catalytic function of both subunits is influenced by insertion of intermolecular cross-linking agents. We also tested the catalytic activity of 4-aminobutyrate aminotransferase with titration of DMDS by the chemical modification method. A time course of inactivation of 4-aminobutyrate aminotransferases by DMDS was performed. When a sample of aminotransferase was treated with varying concentrations of DMDS the catalytic activity decayed in the manner shown in Fig. 1. A 50 % loss of the original catalytic activity was detected after preincubating premature aminotransferase with an equal molar concentration of DMDS. However, loss of catalytic activity reached a similar plateau at increased concentrations of DMDS, indicating that the catalytic action of DMDS binding interfered with the specific Cys residues responsible for enzyme catalysis. DMDS reacts specifically and irreversibly with SH residues in protein to yield adducts characterized by an emission band centered at approx 420 nm (Fig. 2). Therefore, the emission observed upon reaction of DMDS with SH groups was used to monitor the time course of the reaction. When the number of DMDS molecules bound to the enzyme was determined by measuring the absorbance at 340 nm ($\varepsilon = 5\times10^4$ $M^{-1}cm^{-1}$), it was found that one molecule of DMDS is bound per enzyme dimer, assuming that one molecule of DMDS reacts with two SH residues. If intersubunit crosslinking occurs in the modified enzyme the molecular mass of the oligomeric species would be higher than 50 kDa and they would be detected by SDS-PAGE. The electrophoresis results clearly show the presence of oligomeric species (50 %) which can be assigned to dimers (molecular mass of 100 kDa) of 4-aminobutyrate aminotransferase (Fig. 3). It should be noted,

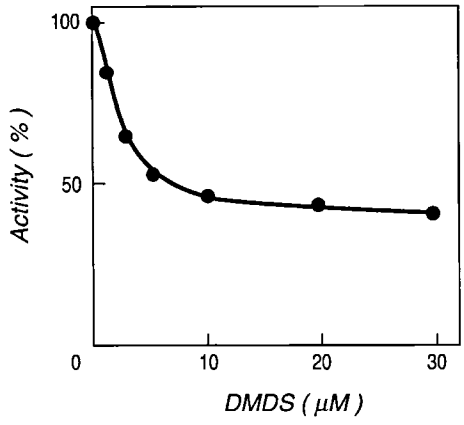

Fig. 1. Time course of inactivation of 4-aminobutyrate aminotransferase reacted with DMDS. A sample of 4-aminobutyrate aminotransferase (10 μM) was preincubated with varying concentrations of the bifunctional cross-linking reagent DMDS for 30 min at room temperature in 0.1 M phosphate buffer (pH 7.0). Aliquots withdrawn from the incubation mixtures were tested for enzymatic activity.

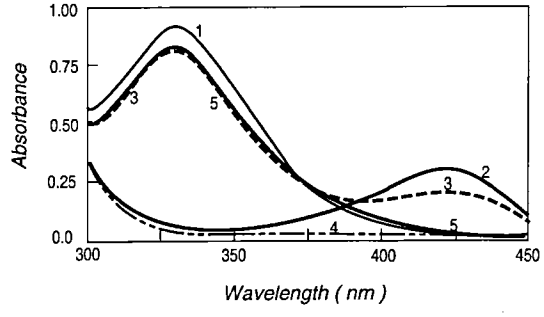

Fig. 2. Absorption spectra of native and modified enzymes. Absorption spectra represented with free DMDS (1.6 μM) 280 nm (1), native aminotransferase (holoenzyme) only (1.6 μM) (2), DMDS labeled aminotransferase (mole ratio 1:1) (3), resolved aminotransferase (apoenzyme) only (1.6 μM) (4), and resolved aminotransferase labeled with DMDS at an equal molar ratio (5).

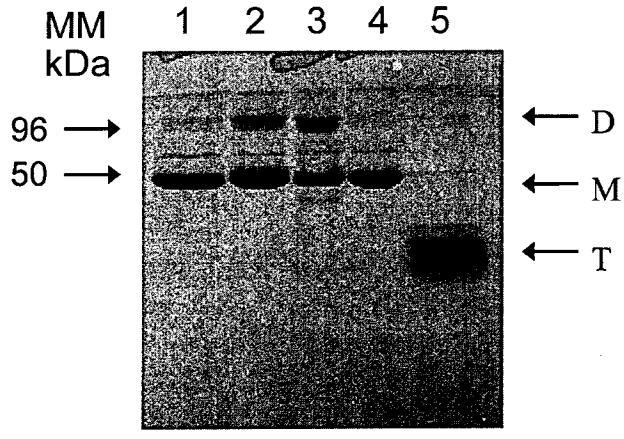

Fig. 3. SDS-polyacrylamide gel electrophoresis of aminotransferase before and after treatment with DMDS. Equal molar concentrations of the enzyme and DMDS were mixed in 0.1 M potassium phosphate at pH 7 at room temperature for 30 min. After extensive dialysis against 0.1 M potassium phosphate at pH 7 the samples were subjected to 10% SDS-PAGE. Lane 1, native aminotransferase (50 μg), lane 2, aminotransferase (50 μg)+DMDS, lane 3, aminotransferase (50 μg)+DMDS+2-mercaptoethanol during sample treatment before SDS-PAGE, lane 4, aminotransferase (50 μg) reacted with iodosobenzoate+DMDS, and lane 5, aminotransferase (50 μg) reacted with DMDS was digested by trypsin.

Table 1. Reaction of 4-aminobutyrate aminotransferase with DMDS and determination of free sulfhydryl groups by titration with DTNB in 5 M guanidinium HCl.

Reaction mixture	Activity(%)	SH group(dimer)	Disulfide bond(dimer)
WT-enzyme	100	5.9	0
premature + DMDS	46	3.8	1
mature + DMDS	48	4.0	1

however, that despite the uncertainties in the absolute values of intrasubunit crosslinkers, the observation that intersubunit cross-linking has taken place is significant since the enzyme preserves some catalytic function. As shown in Fig. 3, when aminotransferase treated with 1 mM 2-mercaptoethanol after preincubation with DMDS was run on SDS-PAGE, the enzyme was not disassociated into monomeric species. This observation suggests that the intrasubunit cross-linking reagent DMDS reacts with SH residues in protein in an irreversible reaction. To determine the SH groups of the protein involved in the reaction with DMDS, enzyme samples (2 mg/ml) were allowed to react with a 5 molar excess of DMDS for 30 min at 25°C. They were then passed through a Sephadex G-25 gel filtration column. The protein was mixed in 5 M guanidinium HCl and titrated with DTNB. The results, as included in Table 1, showed that one pair of SH residues per enzyme dimer reacted with DMDS. Thus, the enzyme forms the dimeric structure of the protein aligned along the subunit interface.

Essential sulfhydryl groups as a probe of conformational change Purified 4-aminobutyrate aminotransferase exhibits a molecular mass of 100 kDa when examined in native PAGE. Drastic denaturing brings about irreversible dissociation of the aminotransferase into two subunits of identical molecular mass of 50 kDa. Iodosobenzoate, which reacts with vicinal sulfhydryl (SH) groups in proteins to form disulfide bonds, blocks the SH groups of 4-aminobutyrate aminotransferase and reversibly inhibits catalytic activity (Choi and Churchich, 1985; Kim and Churchich, 1989). To investigate whether vicinal SH groups of the protein are involved in the reaction with DMDS, enzyme samples (2 mg/ml) were allowed to react with a 5 molar excess of iodosobenzoate for 30 min at

25°C, then applied to a Sephadex G-25 gel filtration column. The protein was mixed with a 5 molar excess of DMDS for 30 min at 25°C and analyzed by SDS-PAGE. The results, as shown in Fig. 3 (lane 4), showed that one of the vicinal SH residues involved in the reaction with iodosobenzoate had been labeled with DMDS. The effect of cross-linking on the stability of the dimeric structure was investigated by proteolysis with trypsin. As shown in Fig. 3 (lane 5), the subunits cross-liked by DMDS were also susceptible to proteolytic digestion. The enzyme reacted with DMDS was digested by trypsin, assuming that the effect of intersubunit cross-linking is to change the stability of the enzyme.

Energy transfer of DMDS-labeled SH residues to a PLP binding site After attachment of an intersubunit cross-linking chromophore to the enzyme, the critical distance of the essential SH residues to a PLP binding site can be measured using fluorescence energy transfer techniques. The cofactor pyridoxal 5'-phosphate is covalently bound through a Schiff base linkage to the e-amino group of lysyl residues in most of the pyridoxal enzymes thus far investigated (Churchich, 1976; 1982; Kwok *et al.*, 1987). A similar covalent linkage is formed when pyridoxal-5'-phosphate (PLP) is allowed to react with several proteins at a neutral or alkaline pH. The results of the fluorescence measurements are shown in Fig. 4. It can be seen that the fluorescence yield of DMDS-labeled apoenzyme is significantly reduced in the presence of the fluorescence labeled-holoenzyme bound to a PLP. If the donor fluorescence (Q_D) is quenched in the presence of an acceptor fluorophore to give fluorescence (Q_A), the efficiency (E) of radiation-less energy transfer can be determined from equation (1). The diminished fluorescence yield of DMDS-bound holoenzyme may be ascribed to the presence of a PLP acting as an energy acceptor in a process of radiationless energy transfer based on the theory of Föster's mechanism (Lakowicz, 1983). If this type of quenching mechanism is operative the efficiency of energy transfer is dependent on the sixth power of the distance (R) separating the donor-acceptor pair, and on the critical distance of transfer (Ro) for which the efficiency of energy transfer is 50 % (Equation 1).

$$E = \frac{Q_D - Q_A}{Q_D} = \frac{1}{1+(R / R_o)^6} \quad (1)$$

The critical distance of transfer (R_o) was evaluated with the aid of Equation (2).

$$Ro = (9.70 \times 10^{-25} \cdot X^2 \cdot Q_D \cdot J(\nu) n^{-4})^{1/6} \quad (2)$$

The spectral overlap of donor emission and acceptor absorption was determined by the equation: $J(\nu) = \Sigma F_D(\lambda)\, \varepsilon_A(\lambda)\, \lambda^4 d\lambda / \Sigma F_D(\lambda) d\lambda$, where is the wavelength in centimeters, $F_D(\lambda)$ is the fluorescence at of an unquenched donor, and $\varepsilon_A(\lambda)$ is the acceptor absorption coefficient in $mmole^{-1}cm^{-1}$. For the donor-acceptor pair DMDS-PLP, the recorded spectrum shown in Fig. 5 gives an overlap integral of $J(\nu) = 4.2 \times 10^{-14}$ cm^6mmole^{-1} using a value of 0.475 for the orientation factor (X^2), a value of 0.015 for the quantum yield of the donor (Q_D), and a refractive index of 1.33 (n). The critical distance of transfer = 20 Å is evaluated with the aid of Equation (2). Since the efficiency of energy transfer for the DMDS-PLP pair bound to the holoenzyme, evaluated from the recorded spectrum shown in Fig. 5 approaches a value of 0.60, it follows that the actual distance of transfer is R = 20 Å when determined with the aid of Equation (1). Assuming that aminotransferase is represented by a hydrated-spherical model of 100 kDa, then it is easy to show that this spherical structure is characterized by a radius of 38 Å.

Therefore, it seems reasonable to conclude that the SH residues carrying the DMDS fluorescent probe span two subunits and reach the PLP binding site near the center of the spherical protein. The simplest model, compatible with the fluorescent measurements, is outlined as shown in (Fig. 6). The results of these experiments suggest that there is no physical overlap between the catalytic sites of the enzyme. The chromophores are located on different subunits since they are separated by a distance greater than 20 Å.

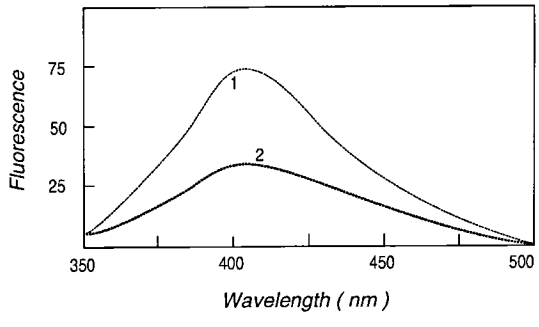

Fig. 4. Fluorescence spectra of DMDS-modified apo- and holo-aminotransferases. Fluorescence spectra of DMDS-labeled apoenzyme (1) and DMDS-labeled holoenzyme (2) obtained by excitation at 330 nm in aqueous solution (pH 7.4). The protein samples have an absorbance of 0.1 at 280 nm.

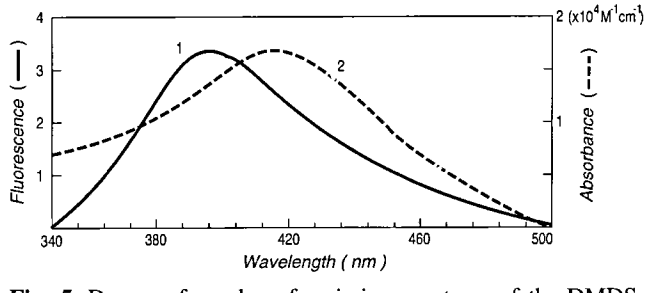

Fig. 5. Degree of overlap of emission spectrum of the DMDS-labeled enzyme and the absorption spectrum of PLP bound to the enzyme. Experiments were conducted in 0.1 M potassium phosphate at pH 7. The spectral overlap integral was calculated from the recorded spectral overlap $J(\nu) = 4.2 \times 10^{-14}$ cm^6mmole^{-1}.

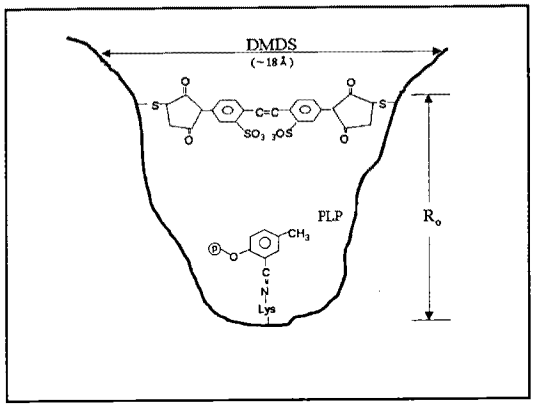

Fig. 6. Schematic model for spatial proximity of the distance between the catalytic sites of aminotransferase.

Discussion

We overproduced cloned 4-aminobutyrate aminotransferase in a catalyticallyactive form by coexpressing *E. coli* thioredoxin (Sung *et al.*, 1999). This allows preparation of a large amount of active protein suitable for structural studies. The enzyme contains the catalytically important SH groups (Kim and Churchich, 1989). Vicinal sulfhydryl (SH) groups in 4-aminobutyrate aminotransferase formed by disulfide bonds, blocked SH groups of 4-aminobutyrate aminotransferase and reversibly inhibited catalytic activity (Choi and Churchich, 1985). This study identified the SH residues involved in the formation of intersubunit disulfide bonds. The model chosen to explain the effect of intersubunit cross-linking on the catalytic function is based on the assumption that the PLP binding site, one per dimer, and sulfhydryl groups susceptible to oxidation, are situated in a cleft at the subunit interface. Although direct evidence for the location of the PLP binding site at the subunit interface is lacking due to our limited knowledge of the structure of this enzyme, it should be noted that intersubunit cross-linking via disulfide bonds has an effect on the orientation and degree of mobility of the cofactor PLP. Crosslinking of the two subunits of 4-aminobutyrate aminotransferase is brought about by oxidation of either pair of sulfhydryl groups. If disulfide bonds are not formed in the native enzyme in the cell, the ease with which they are generated upon oxidation with artificial reagents yields information regarding the structure and dynamics of 4-aminobutyrate aminotransferase. The cysteinyl residues participating in the formation of disulfide bonds are situated in a cleft at the subunit interface, indicated by their close proximity within 20 Å of each other. A necessary condition for this mechanism to be operative is extensive overlap between the emission band of the donor (DMDS) and the absorption band of the acceptor (PLP). This requirement is fulfilled by the DMDS-phosphopyridoxyl pair, for which the overlap integral has been determined in several proteins. According to Förster's theory, the strength of the interaction is conveniently expressed in terms of the critical distance (R_o) of separation between the donor-acceptor pair, for which the probability of dipole-dipole energy exchange is 50%. The critical distance of transfer is related to the overlap integral J (ν). Consequently, the critical distance of transfer may vary from $R_o = 0$ to $R_o = 50$ for most of the proteins carrying phosphopyridoxyl residues. For random directional distribution of the donor-acceptor pairs ($X^2 = 2/3$), the critical distance of transfer for several phosphopyridoxyl-protein conjugates is compatible with the dimension of the macromolecule. However, two chromophores were firmly fixed to the enzyme in our experiment because the polarization excitement of DMDS was $P = 0.38$, which indicates very rigid binding to the protein. PLP binding to the enzyme was also very rigid. Therefore, the orientation factor gives 0.475 for rigid binding. The efficiency of energy transfer (E) can be used to calculate the actual distance of separation between the donor-acceptor pair using Equation (1). The method of radiation-less energy transfer has been used to deduce proximity relationships between the catalytic sites of proteins made up of two subunits. In order to determine the actual distance of separation between the essential SH residues and the PLP binding site of aminotransferase, DMDS-labeled enzyme was prepared in one subunit and the natural chromophore PLP in the other subunit. After measuring the efficiency of energy transfer from the donor DMDS to the acceptor PLP it was possible to estimate an actual distance of transfer of 20Å for the chromophores located in different binding sites. In most applications of radiation-less energy transfer designed to determine the distance of separation of chromophores located on different subunits of oligomeric proteins, it has been assumed that the orientation factor has a value of $X^2 = 0.66$, which corresponds to rapid Brownian rotation of both donor and acceptor. Another interesting aspect of our work is the finding that cross-linking of SH residues which are located on opposite sides of the dimer does not completely impair the catalytic function of the enzyme. This indicates that the two subunits function independently during catalysis, even when constrains are imposed at the subunit interfaces by the insertion of covalent bridges. The crosslinking reagent DMDS, which is specific for SH groups, is non-fluorescent until the maleimide groups have been conjugated. Stilbene disulfonate derivatives have been used as a cross-linking agent of amine residues in proteins (Pickover *et al.*, 1979). Therefore, this experimental approach may be useful in studying the structural aspects of subunit interactions in other oligomeric enzymes.

Acknowledgments This study was supported by the Academic Research Fund of the Ministry of Education, Republic of Korea (BSRI-98-3434).

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