NANOSECOND EMISSION ANISOTROPY OF INTERACTING ENZYMES

ASPARTATE AMINOTRANSFERASE GLUTAMATE DEHYDROGENASE

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SUMMARY

Nanosecond fluorescence studies were performed on mitochondrial aspartate aminotransferase from beef liver to determine whether the dimeric enzyme displays any modes of flexibility in the nanosecond range. The most informative quantities calculated from nanosecond fluorescence measurements S(t) and D(t) decay in a monoexponential manner with decay times τ_S=13 and τ_D=10 nanoseconds respectively. The observed rotational correlation time θ=43 M-seconds yields a volume for the dimeric enzyme of 1.97 x 10^5 $A^{\circ 3}$. The rotational correlation time of aspartate aminotransferase is influenced by the presence of the enzyme glutamate dehydrogenase.

INTRODUCTION

The interaction between beef liver mitochondrial aspartate aminotransferase and glutamate dehydrogenase has been studied by Fahien et al. (1,2). They found that glutamate dehydrogenase has transaminase dehydrogenase activity; i.e., in the presence of NADH, NH_4^+ and aspartate aminotransferase, the enzyme glutamate dehydrogenase catalyzes the conversion of the transaminase in the P-Pyridoxal form to the P-Pyridoxamine form. For several reasons, Fahien, et al. have suggested that a complex is formed between the two enzymes; and, in this complex, the enzyme glutamate dehydrogenase reacts with aspartate aminotransferase.

It is the purpose of this communication to report the results obtained when the interaction between the two enzymes is monitored by nanosecond emission techniques. It is well established that nanosecond emissions spectroscopy is one of the most sensitive techniques available for studying the dynamic interaction of macromolecules (3,4,5). Direct support for the concept that the two enzymes form a stable complex in solution is obtained from steady

and nanosecond fluorescence measurements conducted on samples of aspartate aminotransferase tagged with the fluorescent dye DNS.

MATERIALS AND METHODS

Mitochondrial aspartate aminotransferase from beef liver was prepared according to the method of Lee and Churchich (6). The purified enzyme gives one cationic band at pH 7.8 and pH 8.6 in polyacrylamide gel electrophoresis. The enzyme glutamate dehydrogenase was purchased from Boehringer Mannheim Corporation (Lot No. 7244165). The enzyme was dissolved in 0.1M phosphate pH 7.4 and passed through a column of sephadex G-50 prior to the experiments.

The aspartate aminotransferase was labeled with 1-dimethylaminonaptalene-5-sulphonyl chloride (DNS-chloride) following the procedure developed by Weber (7). The average extent of labeling (1.1-1.4 DNS molecules/molecule of enzyme) was determined by measuring the fluorescence and absorption of the enzyme-dye conjugate. The activity of the dansylated aminotransferase was found to be 95% of the native enzyme when assayed according to the method of Sizer and Jenkins (8). Steady polarization of fluorescence measurements were performed in a souble beam polarization instrument. Illumination was provided by a Xenon Lamp (Hanovia, 150W) with wavelengths selected by a Quartz Prism Monochromator. The degree of fluorescence polarization was measured with a precision of ±0.005.

Fluorescence decay measurements were performed in an Ortec model 9200 nanosecond spectrometer. Time-base calibration of the multichannel analyzer was performed both directly, using a standard delay line, and indirectly, using a solution of quinine sulphate, whose lifetime is 19.5 ns. The excitation was set at 340 nm, and the emission was filtered through a glass filter C-S-3-72 (Corning). Fluorescence decay times were corrected for the finite duration of the exciting light pulse using the least square method developed by Ware et al. (9).

The emission anisotropy was determined from the fluorescence decay curves of the polarized components $F_{11}(t)$ and $F_{\perp}(t)$, parallel and perpendicular, respectively, to the plane of the incident polarized light. Polaroid HNB sheet polarizers were used for excitation and emission. The exciting wavelength was set at 340 mm and the emission was passed through a glass filter C-S-3-72 (Corning). Transmission was observed at wavelengths longer than 420 nm.

RESULTS

The fluorescence properties of the dansylated aminotransferase containing an average of 1.4 moles of DNS per mole of enzyme were examined at pH 7.4 in 0.1M sodium phosphate. The dansyl residues exhibit an emission band centered at around 520 mm when excited at 340 mm, and the fluorescence decays in a monoexponential manner with a fluorescence lifetime of 13 n seconds (Table I). According to the theory developed by Jablonski (10), the

TABLE I

FLUORESCENCE PROPERTIES OF DNS-AMINOTRANSFERASE

SAMPLE	FLUORESCENCE (nm)	DELAY TIME (n seconds)	ROTATIONAL CORRELATION TIME (n seconds)
DNS-Aminotransferase	520	13 ± 0.2	43 ± 4
DNS-Aminotransferase + Glutamate Dehydrogenase	520	13 ± 0.2	43 and 175

emission anisotropy A(t) is an explicit function of time (Equation 1).

$$A(t) = \frac{F_{11}(t) - F_{\perp}(t)}{F_{11}(t) + 2F_{\perp}(t)} = \frac{D(t)}{S(t)} \qquad (1)$$

For a rigid spherical macromolecule, the anisotropy A(t) is described by a single exponential function of time where Θ is the rotational correlation time.

$$A(t) = A_o \; e^{\frac{-t}{\Theta}} \qquad (2)$$

If D(t) and S(t) are single exponential functions characterized by the decay times τ_D and τ_S respectively, then the rotational correlation time is given by equation (3).

$$\Theta = \frac{\tau_S \times \tau_D}{\tau_S \quad \tau_D} \qquad (3)$$

Figure 1 shows the results obtained with the dansylated aminotransferase. The most informative quantities calculated from nanosecond measurements, D(t) and S(t) decay in a monoexponential manner, with decay times of τ_D=10 and τ_S=13 nanoseconds respectively. The observed rotational correlation time (Θ=43 n seconds) agrees well with the value expected for a dimeric protein of 90,000g. Assuming a spherical shape for the dimeric enzyme, the observed

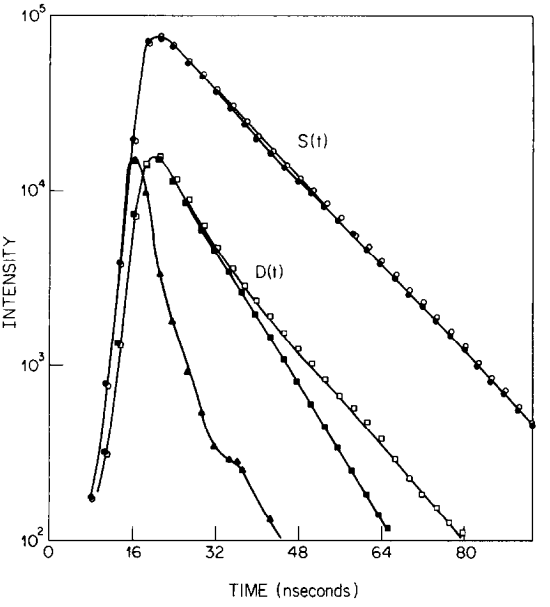

FIG. 1 Decay of the sum $F_{\perp 11}(t) + 2F_{\perp}(t) = S(t)$ (●) and difference $F_{11}(t) - F_{\perp}(t) = D(t)$ (■) fluorescence for DNS-aminotransferase (3μM) in 0.1M phosphate (pH 7.4) at 25°C. Decay of S(t) (○) and D(t) (□) fluorescence for the mixture DNS-aminotransferase (3μM) + glutamate dehydrogenase (8μM) in phosphate (pH 7.4) at 25°C. Ordinate, intensity in arbitrary units on a log scale; abscissa time in nanoseconds. The intensity profile of the lamp flash is also shown (▲).

rotational correlation time yields a volume of 1.97×10^5 A°^{3}. Thus the volume calculated from the observed rotational correlation time is larger than the volume calculated for the unhydrated sphere ($V_o = 1.24 \times 10^5$ A°^{3}). This difference can be accounted for, at least in part, by the hydration of the enzyme.

Since the rotational correlation time is related to the volume of the rotating macromolecule (Equation 4)

$$\Theta = \frac{\eta V}{KT} \qquad (4)$$

it is evident that a decrease by one half in the rotational correlation time would be expected in a dimer to monomer conversion if there were no significant changes in hydration or shape within the limits of detection of the method. The finding that the observed rotational correlation time of dansylated aminotransferase is compatible with the size of a dimer of 90,000g, strongly suggests that no dissociation of the dimer into monomers could be detected

in the nanosecond range. This finding is not surprising since it is well established that dissociation of the dimer into monomers is only attained under drastic denaturing conditions.

Furthermore, the absence of rotational correlation times shorter than 43 nanoseconds indicates that strong interactions within the dimeric structure prevents independent rotation of the monomeric units. In order to detect the formation of a stable complex in solution between the two interacting enzymes, the fluorescence polarization emitted by the dansylated transaminase was measured after the addition of increasing concentrations of glutamate dehydrogenase until no further changes in the polarization of fluorescence could be detected.

As illustrated in Fig. 2, the polarization of fluorescence values increase from 0.22 to 0.25 after the addition of approximately 2.5 moles of glutamate dehydrogenase per mole of dansylated transaminase. This change in the degree of fluorescence polarization was not accompanied by any change in the fluorescence lifetime of the dansyl chromophore as shown in Table 1. These results suggest that the increase in the polarization of fluorescence is mainly due to a restriction in the rotational diffusion of the danylated

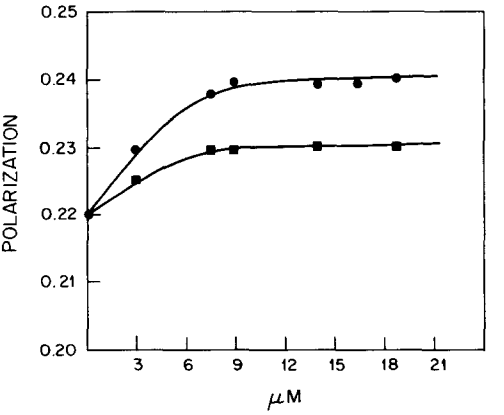

FIG. 2 Changes in polarization of fluorescence of DNS-aminotransferase (3μM) on titration with increasing concentration of glutamate dehydrogenase (●) at pH 7.4 in 0.1M phosphate. Results obtained when the titration experiments were conducted in the presence of GTP (1mm) (■) at pH 7.4. Excitation wavelength 340 nm. Temperature 15°C.

aminotransferase as a result of interaction with the dehydrogenase. It should be noted that this increase in the degree of polarization of fluorescence observed at protein concentrations of 8 μM is influenced by the presence of GTP in the reaction mixture as shown in Fig. 2. In order to evaluate the macromolecular size of the complex resulting from the association of dansylated aminotransferase with glutamate dehydrogenase, we decided to perform emission anisotropy decay measurements at constant temperatures (25°C). The results of these experiments are included in Fig. 1, where it may be seen that the plots of log S(t) versus time for DNS transaminase in the absence and presence of glutamate dehydrogenase are virtually superimposable. In contrast to the S(t) plots, the D(t) curves are influenced by the presence of glutamate dehydrogenase in the reaction mixture. Thus, the plot of log D(t) vs time for dansylated aminotransferase is virtually linear (τ_D=10 NS), whereas the same plot for dansylated aminotransferase in the presence of glutamate dehydrogenase exhibits multiexponential decay kinetics (Fig. 1).

A good fit of the experimental curve D(t) for the mixture containing both enzymes was obtained with the decay time constants τ_D=10.1 and τ_{D_2}=12.2 nanoseconds. This finding implies that the emission anisotropy decay function contains more than one exponential term. Thus, it appears that the rotational correlation time of the dansylated aminotransferase in the presence of glutamate dehydrogenase varies between 45 and 175 nanoseconds depending upon the decay values chosen for the function D(t). The longest rotational correlation time, Θ=175 n seconds is obtained with τ_{D_2}=12.1 nanoseconds. It is important to note that the decay time of dansylated aminotransferase as well as the emission anisotropy decay function. A(t) are not influenced by the presence of proteins such as Bovine serum albumin, ovoalbumin and γ-globulin at concentrations larger than 5 mg/ml. This finding is interpreted to mean that we are dealing with a specific effect due to the interaction of glutamate dehydrogenase with aspartate aminotransferase.

DISCUSSION

An interesting and important result of the nanosecond fluorescence measurements is the observation that the rotational correlation time of the enzyme aspartate aminotransferase is influenced by the presence of the enzyme glutamate dehydrogenase. Thus, the emission anisotropy decay measurements indicate the presence of macromolecular species characterized by a rotational correlation time of ϕ=175 nanoseconds, which is longer than the rotational correlation time of free aspartate aminotransferase (ϕ=43 ns). Although these results lend support to the concept that the two enzymes tend to form a macromolecular aggregate in neutral solutions, it should be noted that the enzyme aspartate aminotransferase is not rigidly trapped by the subunits of glutamate dehydrogenase when the concentration of the latter enzyme is of the order of 2 mg per ml. Indeed, the longest rotational correlation time determined when the dansylated aminotransferase is allowed to interact with glutamate dehydrogenase (ϕ=175) is definitely shorter than the value expected for macromolecular species of molecular weight larger than 300,000g (ϕ=300 nanoseconds or longer).

These results can be interpreted in terms of a model in which the dimeric aminotransferase interacts with the catalytic subunit of glutamate dehydrogenase (300,000 ms), but this interaction does not prevent the rotation of the aminotransferase. It is well established that glutamate dehydrogenase tends to polymerize in neutral solutions when the protein concentration is larger than 0.3 mg per ml (11). In this connection, it should be noted that at the concentration at which glutamate dehydrogenase exerts its maximal effect on the rotational correlation time of aspartate aminotransferase, it exists mainly in a polymeric form, suggesting that only this form of the enzyme is bound to the aminotransferase. The steady polarization of fluorescence measurements conducted on the mixture of the two enzymes also indicate that dissociation of the glutamate dehydrogenase by either addition of GTP or dilution of the incubation mixture brings about

a decrease in the measured polarization of fluorescence. These lines of experimental evidence are consistent with the idea that aspartate aminotransferase binds to the polymeric form of glutamate dehydrogenase.

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