Rotational dynamics of 4-aminobutyrate aminotransferase

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Received 21 June 1983

The fluorescence dye 1-anilinonaphthalene-8-sulfonate (ANS) was used as a probe of non-polar binding sites in 4-aminobutyrate aminotransferase. ANS binds to a single binding site of the dimeric protein with a K_d of 6 μM. Nanosecond emission anisotropy measurements were performed on the ANS-enzyme in an effort to detect independent rotation of the subunits in the native enzyme. The observed rotational correlation time (ϕ = 65 ns) corresponds to the rotation of a rather rigid dimeric structure. The microenvironment surrounding the natural probe pyridoxal-5-P covalently bound to the dimeric structure was explored using ^{31}P-NMR at 72.86 MHz. In the native enzyme, the pyridoxal-5-P ^{31}P-chemical shift is pH-independent, indicating that the phosphate group is well protected from the solvent. The correlation time determined from the ^{31}P-spectrum of the aminotransferase exceeds the value calculated for the hydrated spherical model (ϕ = 40 ns). It is concluded that the phosphate of the pyridoxal-5-P molecule is rigidly bound to the active site of 4-aminobutyrate aminotransferase.

1. INTRODUCTION

4-Aminobutyrate aminotransferase (EC 2.6.1.19) is a dimeric protein made up of two subunits of M_r 50000 [1].

The inhibition of 4-aminobutyrate aminotransferase by compounds bearing a strong resemblance to the substrate have received considerable attention in recent years, because the transaminase is a key enzyme in central nervous system neurotransmitter metabolism [2–4].

We have shown that the holoenzyme possesses two non-equivalent catalytic binding sites differing in their affinities for the cofactor and P-pyridoxal analogues [5,6]. The behavior of the aminotransferase is very unusual in the sense that the k_{cat} and K_m-values of the enzyme containing 1 mol pyridoxal-5-P/dimer are identical with those of the enzyme containing 2 mol pyridoxal-5-P/dimer [5]. This unusual behavior might be due to subunits which interact strongly in a negatively cooperative manner during the catalytic cycle.

However, there is insufficient information on the quaternary structure of the aminotransferase to establish any correlation between protomers' interaction and catalytic function.

Here, we investigate certain aspects of the quaternary structure of 4-aminobutyrate aminotransferase using nanosecond fluorescence and ^{31}P-NMR spectroscopy.

Nanosecond emission anisotropy is used to detect any modes of flexibility due to independent rotation of the subunits in the nanosecond time range, whereas ^{31}P-NMR spectroscopy is used to explore the microenvironment surrounding the molecule of the cofactor pyridoxal-5-P covalently attached to the dimeric structure.

2. MATERIALS AND METHODS

2.1. *Purification of the enzyme*

4-Aminobutyrate aminotransferase was purified as in [5]. Using 40 kg pig brain as starting material, we obtained 100 mg homogeneous enzyme. The purification steps were conducted at 4°C.

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00145793/83/$3.00

The pyridoxal-5-P content of the purified aminotransferase (1 mol pyridoxal-5-P/100000 M_r), was determined by the colorimetric method in [7]. Protein concentration was determined as in [8]. Succinic semialdehyde dehydrogenase was purified from pig brain as in [9].

2.2. *Enzymatic assays*

A coupled assay system consisting of two purified enzymes (i.e., 4-aminobutyrate aminotransferase and succinic semialdehyde dehydrogenase) was used to monitor the catalytic conversion of 4-aminobutyrate into succinic semialdehyde [5].

2.3. *Polyacrylamide gel electrophoresis*

The enzyme 4-aminobutyrate aminotransferase was examined by polyacrylamide gel electrophoresis using slab gels containing 7.5% polyacrylamide. Protein bands were detected by staining with Coomassie blue dye.

2.4. *Fluorescence spectroscopy*

Fluorescence decay measurements were made using the monophoton technique with an Ortec model 8200 ns spectrometer. A free-running flash lamp operating in air at 1 atmosphere was used as exciting source. The lamp was pulsed at 10 kHz. Excitation was set at 365 nm and the emission was filtered through a corning glass filter (CS 3-72). Rotational correlation times were determined by recording the fluorescence decay curves of the polarized components ($F_\parallel(t)$ 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 functions:

$$D(t) = F_\parallel(t) - F_\perp(t)$$

and

$$S(t) = F_\parallel(t) + 2\,F_\perp(t)$$

were deconvoluted, and the deconvoluted decay functions were each accurately fitted to either a mono- or bi-exponential decay using non-linear least squares analysis [10].

The values of the anisotropy decay parameters were appreciated by examining the shape of the deviation function (DV_k) and computation of the mean weighted residue (R) [11]. Steady state fluorescence spectra were recorded in a precision spectrofluorimeter equipped with two Bausch and Lomb monochromators. Absorption spectra were recorded in a Cary model 15 spectrophotometer.

2.5. *Binding of 1-anilinonaphthalene-8-sulfonate (ANS)*

The fluorescence enhancement at 475 nm (excitation at 360 nm) that follows the addition of increasing concentrations of ANS to a fixed concentration of enzyme (8 μM) in 100 mM triethanolamine–HCl buffer (pH 7) was used to determine the stoichiometry of binding. The results of the fluorometric measurements were analyzed as in [12] using eq.(1):

$$\bar{\nu}/L = K(n - \bar{\nu}) \qquad (1)$$

An extinction coefficient of $4.9 \times 10^3\ M^{-1}.cm^{-1}$ was used for free and bound ANS.

2.6. *NMR-spectra*

Fourier transform ^{31}P-NMR spectra were recorded at 72.86 MHz on a Bruker WH-180 spectrometer. A concentric 5-mm NMR tube containing ^2H_2O was employed as a field/frequency lock.

All spectra of 4-aminobutyrate aminotransferase were proton-decoupled. Normally a 60° pulse angle was applied, and the recycle time for the experiment was 1 s. Sample volumes of 11.12 ml in 20-mm diameter tubes were used. Continuous air flow through the spectrometer probe head kept the temperature at 10°C. For further experimental details see [13].

Prior to the ^{31}P-NMR measurements, the enzyme at 6.7 mg/ml was dialyzed against 100 mM triethanolamine HCl (pH 7) containing 1 mM dithioerythreitol and 2 mM EDTA.

Dialysis for 24 h against 4 changes of buffer was required to remove phosphate ions. The pH of each enzyme sample was determined before and after the NMR measurements with a Radiometer model 26 pH meter.

3. RESULTS AND DISCUSSION

The fluorescence probe ANS is ideally suited for emission anisotropy measurements because of its water solubility and the magnitude of the changes in fluorescence yield associated with binding to the enzyme.

When the probe was bound to the aminotransferase, the overall fluorescence quantum yield increased 80-fold when compared to the free dye at pH 7. The maximum emission of bound ANS is centered at 475 nm upon excitation with a wavelength of 360 nm. Its fluorescence decay is monoexponential and reaches a value of 16.7 ns.

The fluorescence enhancement that follows the addition of increasing concentrations of ANS to a fixed concentration of enzyme was used to determine the stoichiometry of binding.

Fig.1 shows the results of the titration of the enzyme with increasing concentrations of ANS at pH 7. It is evident that one binding site per dimer has the ability to interact with the fluorescent probe with a K_d of 6 μM.

It should be noted that upon formation of the protein–ANS complex, the catalytic activity of the aminotransferase remains essentially invariant. Furthermore, the overall quantum yield of bound ANS is not influenced by addition of the substrate 4-aminobutyrate up to 0.01 M.

These lines of experimental evidence are consistent with the concept that the probe binds to the enzyme at some site other than the catalytic binding site. Emission anisotropy measurements were performed at 25 μM protein and 10 μM ANS.

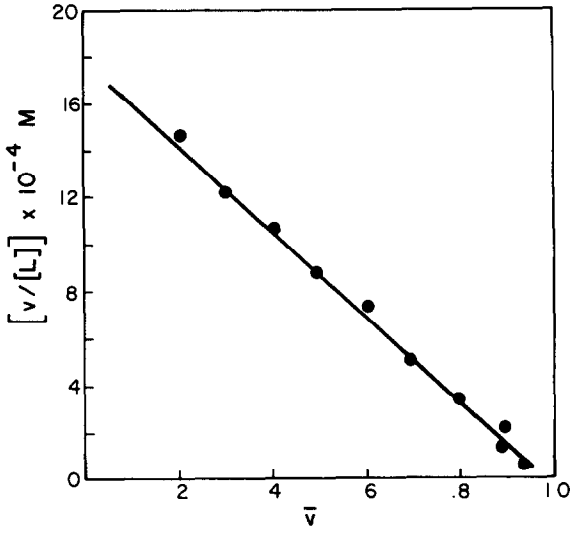

Fig.1. Analysis of the titration results of the enzyme (8 μM) with varying concentrations of ANS. Plot of $\bar{v}/L$ *vs* $\bar{V}$. A dissociation constant $K_d = 6$ μM was determined for one binding site per dimer.

Under this set of experimental conditions, the fluorescence emitted at 475 nm is entirely due to bound ANS. A representative set of experimental data for the decay functions $S(t)$ and $D(t)$ are given in fig.2.

The $S(t)$ function gives one fluorescence lifetime τ_S = 16.7 ns, whereas the $D(t)$ function yields a decay time τ_D = 12.4 ns.

Fig.3 shows that a plot of the emission anisotropy function $A(t)$ *vs* time is linear up to about 70 ns. From the linear plot, a rotational correlation time of 65 ns is obtained for ANS bound to the enzyme.

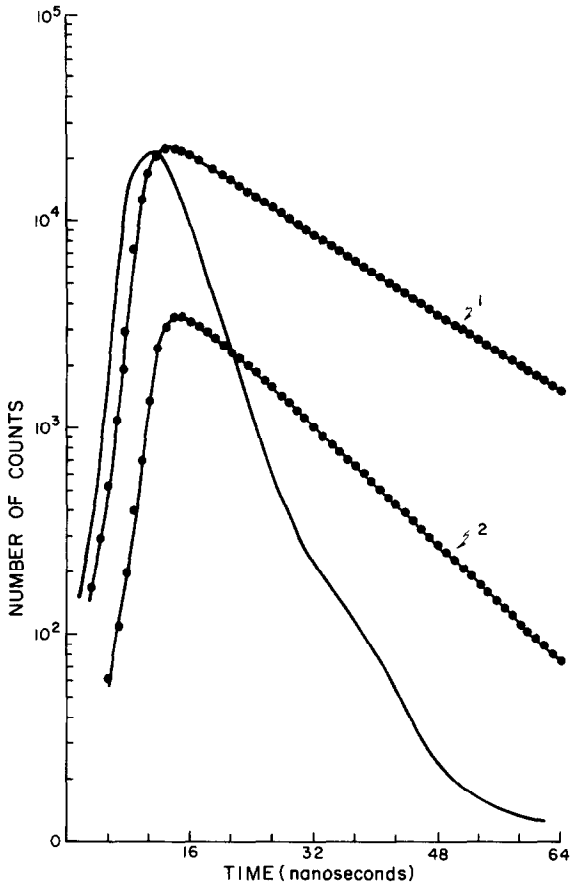

Fig.2. Fluorescence decay curves of ANS (10 μM) in the presence of 4-aminobutyrate aminotransferase (25 μM) at pH 7 in 0.1 M triethanolamine–HCl. The experimental decay functions $S(t)$ (1) and $D(t)$ (2) are given together with the lamp profile. The points are the experimental values and the solid lines are calculated convolution products. The function $S(t)$ yields a decay time τ_S = 16.7 ns, $D(t)$ yields a decay time τ_D = 12.4 ns.

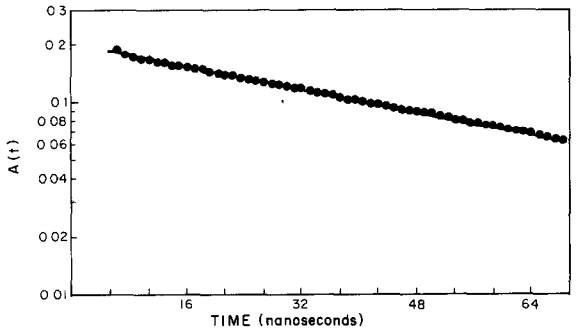

Fig.3. Plot of emission anisotropy $S(t)$ *vs* t for ANS bound to 4-aminobutyrate aminotransferase. A rotational correlation time $\phi = 65$ ns was obtained using the equation $A = A_o\, e^{-t/\phi}$ for $A_o = 0.2$.

The overall correlation time of a spherical macromolecule can be calculated using eq.(2):

$$\phi_o = 4\,\pi\eta R^3/3\,kT \qquad (2)$$

Assuming a radius R of 33 Å for the dimeric protein of M_r 100000, one obtains a rotational correlation time of 40 ns for the hydrated spherical model.

Thus, the time emission anisotropy measurements indicate that the motion of the probe is rather restricted in the nanosecond time scale. Since the preceding emission anisotropy measurements do not provide any information about the degree of mobility of a molecule interacting with the active site, it was desirable to investigate the spectroscopic properties of the cofactor pyridoxal-5-P which is known to interact with amino acid residues at the catalytic binding site of the aminotransferase.

Emission anisotropy measurements of the cofactor are not feasible, because upon interaction with the protein, pyridoxal-5-P exhibits a weak fluorescence quantum yield ($q = 0.01$) and an estimated fluorescence decay of 0.1 ns.

Therefore, it was necessary to resort to ^{31}P-NMR spectroscopy for correlation time measurements.

At pH 7, the ^{31}P-NMR spectra of 4-aminobutyrate aminotransferase (1 mol of pyridoxal-5-P/dimer) exhibits a linewidth of 36 Hz at 72.86 MHz (fig.4). As shown in fig.5, the chemical shift relative to external 85% phosphoric acid (−4.43 ppm) is pH-independent in the range 7.5 to 6.05. This is in contrast to the behavior of

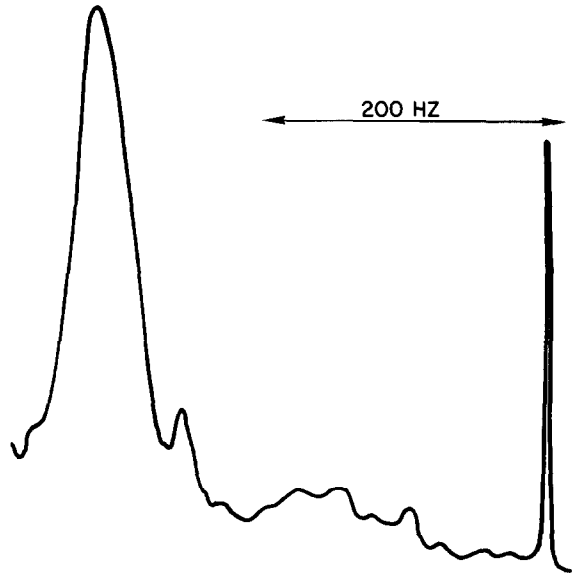

Fig.4. ^{31}P-NMR spectra at 72.86 MHz of 4-aminobutyrate aminotransferase. The concentration of enzyme is 6.7 mg/ml at pH 7; 20000 scans.

free pyridoxal-5-P which displays a chemical shift upon conversion of the monoanionic to the dianionic form of the phosphoric ester (fig.5) [14].

Thus, the cofactor of the aminotransferase is likely to be bound in its dianionic form via a rigid salt bridge to a positively charged region of the active site, leading to a considerable lowering of the pK-value for the dianion–monoanion transition. At a frequency of 72.86 MHz a linewidth of 36 Hz was determined for the ^{31}P-spectrum of 4-aminobutyrate aminotransferase. The predominant relaxation mechanisms governing the ^{31}P-linewidth are expected to be dipole–dipole interactions and contributions due to chemical shift anisotropy (CSA) [15].

For globular proteins undergoing isotropic tumbling, the ^{31}P-linewidth is proportional to the overall correlation time (τ_c):

$$\pi\Delta\nu = \frac{1}{5}\left(\frac{\gamma_p^2.\gamma_H^2.h^2}{R^6}\right)\cdot\tau_c \qquad (3)$$

where γ_p and γ_H are the gyromagnetic ratios and R is the distance P–H ($R = 1.7$ Å) [15].

For a $\Delta V = 36$ Hz, equation 3 yields a correlation time of 80 ns, which exceeds the overall cor-

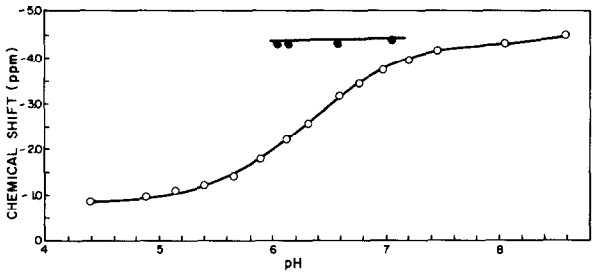

Fig.5. The effect of pH on the ^{31}P-NMR chemical shift of 4-aminobutyrate aminotransferase (●). The effect of pH on the chemical shift of free pyridoxal-5-P (○) is included in the figure. A pK = 6.34 was determined for pyridoxal-5-P.

relation time (ϕ_o = 40 ns) calculated for the hydrated sphere of M_r 100000. Hence, restricted rotational freedom of the phosphate group around the C–O bond linking the phosphate ester to the pyridine moiety of the cofactor could be asserted from linewidth data.

The only other enzyme in which the cofactor phosphate group behaves similarly to that of 4-aminobutyrate aminotransferase is glycogen phosphorylase [16]. The linewidth obtained for glycogen phosphorylase (M_r 200000) is 55 Hz [16].

The most important conclusion derived from time emission anisotropy measurements is that the mobility of the probe ANS, bound to a site on the enzyme distinct from the catalytic binding site, is rather restricted in the nanosecond time range.

This implies that the probe does not sense a change in the configuration of the dimeric enzyme due to independent rotation of the subunits during the brief interval of the excited state.

Rapid structural fluctuations on the nanosecond time scale have been detected in several proteins [17,18], and changes in enzyme catalysis induced by alterations of the environmental viscosity have been related to protein fluctuations [19]. Moreover, theoretical calculations based on statistical thermodynamics have predicted that thermal energy fluctuations in proteins of M_r 100000 reach a value of 30 kcal/mol on a molar accounting basis at the temperature of 37°C [20]. This energy is large enough to induce substantial unfolding of the native structure of a dimeric protein.

The finding that the rotational correlation time of 4-aminobutyrate aminotransferase is greater than the value calculated for the hydrated spherical model, is interpreted to mean that there is no disruption of the subunit interfaces within the dimeric structure. This finding strongly suggests that 4-aminobutyrate aminotransferase is a rather rigid structure which preserves communication between protomers even in the nanosecond time scale.

ACKNOWLEDGEMENTS

This work was supported by a NIH grant, GM 27639-03 and by a NATO Research Grant (no.05582).

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