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# NANOSECOND SPECTROSCOPY OF A DIMERIC ENZYME: PLASMA AMINE OXIDASE *

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The fluorescence dye 1-anilino-naphthalene-8-sulphonic acid (ANS) was used as a probe of non-polar binding sites in the enzyme plasma amine oxidase. Steady fluorescence measurements indicate that ANS binds to a single binding site of the dimeric enzyme with a dissociation constant of 5 μm. This binding site is different from the catalytic binding site. 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 ($\phi$ = 105 ns) corresponds to the rotation of a rigid dimeric macromolecule. A rotational correlation time of 120 ns was obtained with the enzyme labelled with pyrenebutyric acid. It is concluded that the dimeric enzyme does not exhibit any modes of flexibility due to independent rotation of the subunits in the nanosecond range.

## 1. Introduction

The enzyme plasma amine oxidase has been studied in several laboratories [1,2,3]. The presence of copper has been firmly established and extensive progress has been made in elucidating the role of the cupric atoms in the catalytic mechanism [3,4]. Despite extensive kinetic studies, the role played by pyridoxal-P remains uncertain and there is insufficient information about the quaternary structure of this enzyme. It is the aim of the present studies to investigate certain aspects of the quaternary structure of the enzyme by using the technique of Nanosecond Fluorescence Spectroscopy. The dye ANS is used to probe the presence of non-polar binding sites and to study the interaction between the subunits in the dimeric macromolecule.

## 2. Experimental procedure

### *2.1. Fluorescence spectroscopy*

Fluorescence emission spectra were obtained with the use of a spectrofluorometer designed in our laboratory [5]. Polarization of fluorescence measurements were performed in an apparatus similar to that described by Weber [6]. Illumination was provided by a xenon lamp with wavelengths selected by a quartz prism monochromator. Fluorescence polarized light was passed through glass filters. The detector system consisted of an EMI 9502 B photomultiplier and a digital voltmeter. The degree of polarization of fluorescence was measured with a precision of ±0.02.

Fluorescence decay measurements were made using the monophotonic technique with an Ortec Model 9200 nanosecond spectrometer. Time base calibration of the multi-channel analyzer was performed both directly using a standard decay line and indirectly, using a solution of quinine sulfate, whose lifetime is 19.5 ns. A free running flash lamp operating in air at 1 atmosphere was used as exciting source. The lamp is pulsed at 10 kHz. The excitation was set at 360 nm and the emission was filtered through a glass filter, Corning, C-S-3-72.

Rotational relaxation 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 $D(t)$=

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$F_{\parallel}(t) - F_{\perp}(t)$, $S(t) = F_{\parallel}(t) + 2F_{\perp}(t)$, and $I(t)$ [decay for unpolarized emission] were deconvoluted and analyzed for a decay function of one, two, or three exponentials. The deconvoluted decay functions $D(t)$, $S(t)$, and $I(t)$ were each accurately fitted to a monoexponential decay using nonlinear least squares analysis.

Deconvolution of the data was performed with a computer program based on the least square method developed by Ware et al. [7]. This method reduces the problem of solving an integral equation to one of solving a set of simultaneous equations. For criterion of "goodness of fit" for this deconvolution method, the deconvoluted data was checked by three methods [8]:

(a) A visual comparison of experimental $F(t)$ and computed convolution curves $F_c(t)$.

(b) A drawing of the deviation function defined by equation

$$D_{vi} = [F_{ci}(t) - F_i(t)]/\sqrt{F_i(t)}. \quad (1)$$

(c) A computation of the mean weighted residue $R$:

$$R = \frac{1}{n}\sum_{i=1}^{n} [F_{ci}(t) - F_i(t)]^2/F_i(t). \quad (2)$$

### 2.2. Titration with 1-8 ANS

Titration of the enzyme plasma amine oxidase with ANS was followed by the increase in the fluorescence emitted at 470 nm upon excitation at 350 nm. The analysis of the titration results were performed by the method of Scatchard [9]. The fluorescence intensity of free ($F_0$) and bound ANS ($F_{max}$) as well as the fluorescence ($F$) observed when both free and bound are in equilibrium were used to calculate the fraction of ligand bound (eq. (3)).

$$\alpha = (F - F_0)/(F_{max} - F_0), \quad (3)$$

where $F_{max}$ is the actual observed fluorescence when all the ANS has been adsorbed. The average number of ligand molecules bound per mole of protein ($\bar{v}$) were calculated for points along the titration curve by means of eq. (4).

$$\bar{v} = \alpha[L_0]/[P_0], \quad (4)$$

where $[L_0]$ is the total ligand concentration and $[P_0]$ total protein concentration. The results were analyzed by eq. (5).

$$\bar{v}/[L] = Ka(n - \bar{v}). \quad (5)$$

### 2.3. Purification of the enzyme

The enzyme plasma amine oxidase from pig blood was purified by the procedure of Buffoni and Blaschko [1] as modified by Lindstrom and Petterson [2]. The enzyme was shown to be homogeneous by polyacrylamide gel electrophoresis and isoelectric focusing. Enzymatic activity was assayed spectrophotometrically by the method of Tabor et al. [10]. Protein concentrations were determined by using a Millimolar extinction coefficient of 250 $mM^{-1}$ $cm^{-1}$ at 280 nm [2].

The specific activity of plasma amine oxidase is 0.14 unit/mg (international unit: μmoles/min) when assayed with benzylamine as a substrate.

### 2.4. Sodium dodecyl sulfate polyacrylamide gel electrophoresis

The method described by Weber and Osborn [11] was used to determine the molecular weight of the monomeric unit of plasma amine oxidase. Electrophoresis was performed on 5% polyacrylamide gels containing 0.1% SDS in 0.1M sodium phosphate (pH 7.2).

### 2.5. Reaction with pyrenebutyric

The reaction of the pyrenebutyric anhydride reagent (PB) with plasma amine oxidase was performed according to the method of Knopp and Weber [12]. The number of pyrene chromophores bound to the protein was determined by measuring the absorption at 346 nm assuming a molar extinction coefficient of $4 \times 10^4$ [12].

## 3. Results and discussion

### 3.1. Binding of ANS

The enzyme preparations used throughout this work were homogeneous as determined by the criteria of polyacrylamide gel electrophoresis and isoelectric

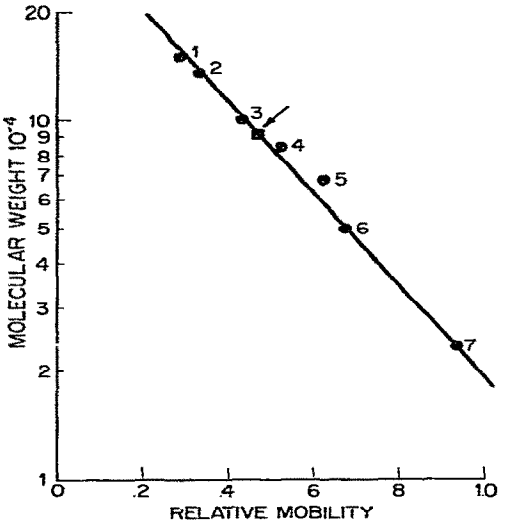


Fig. 1. Determination of the molecular weight of plasma amine oxidase by SDS polyacrylamide gel electrophoresis. The following proteins were used as standards: (1) γ-globulin, (2) bovine serum albumin (dimer), (3) phosphorylase A, (4) conalbumin, (5) bovine serum albumin, (6) reduced γ-globulin, H, chain, (7) reduced γ-globulin, L, chain. The relative mobility of plasma amine oxidase corresponds to a molecular weight of 92,000.

focusing. Only one band was detected by polyacrylamide gel electrophoresis after straining with reagents which are specific for the detection of protein, carbohydrates, and enzymatic activity. The finding that plasma amine oxidase is a glycoprotein confirms the reports from other laboratories [1,4]. The enzyme has a molecular weight of approximately 180 000 [1]. Under drastic denaturing conditions, i.e., treatment with sodium dodecyl sulphate (0.1%) and β-mercaptoethanol, the enzyme is irreversibly dissociated into two subunits of identical molecular weight (92 000) (fig. 1). Since the enzyme plasma amino oxidase is made up of two subunits of identical molecular weight, it was thought of interest to study the interaction between the subunits in the native state by nanosecond fluorescence spectroscopy using fluorescence probes of long fluorescence lifetime. Among the probes examined, it was found that 1–8 ANS is suitable for these studies because its binding to the enzyme does not exert any effect on the catalytic activity.

The binding of the fluorescence probe ANS to the enzyme benzylamine oxidase was examined over a wide range of protein concentrations at pH 7.0 in 0.05 M phosphate buffer. At a protein concentration of 10 μM, and dye concentration of 5 μM, the fluorescence of the ligand is one hundred fold enhanced with a concomitant shift in the wavelength of the maximum of emission from 510 to 475 nm (table 1). The fluorescence enhancement that follows the addition of increasing concentrations of ANS to a fixed concentration of enzyme (13 μM) was used to determine the affinity constant of the ligand for the protein. Fig. 2 shows how the fluorescence intensity changed with increasing concentrations of 1–8-ANS while keeping the protein concentration constant. The maximum number of binding sites ($n$) was estimated by analysis of the data at constant protein

Table 1
Spectroscopic properties of ANS-plasma amine oxidase

| Sample | Solvent | Absorption ($cm^{-1}$) | Emission ($cm^{-1}$) | Fluorescence lifetime (ns) | Polarization |
|---|---|---|---|---|---|
| 1–8 ANS (12 μM) + Enzyme (25 μM) | Buffer pH 7 | 27,777 | 21,052 | 10.2 | 0.33 |
| 1–8 ANS (12 μM) + Enzyme (25 μM) + Benzylamine (0.1 mM) | Buffer pH 7 | 27,777 | 21,052 | 10.2 | 0.33 |

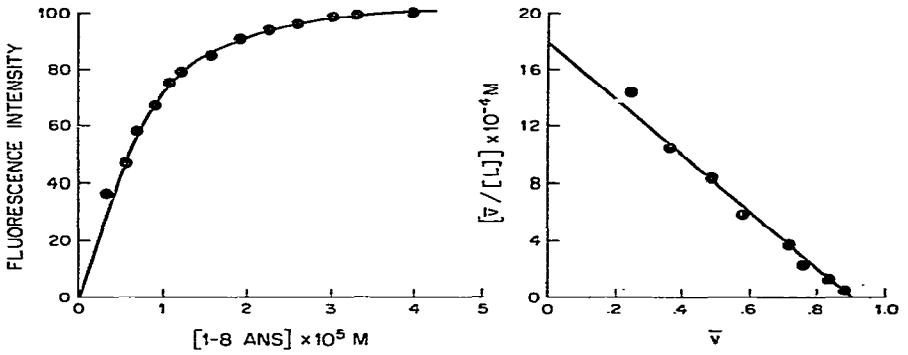


Fig. 2. *Left.* Changes in fluorescence intensity at 470 nm (excitation 350 nm) upon addition of varying concentrations of 1–8-ANS to a fixed concentration of enzyme (13 μM) in 0.05 M phosphate (pH 7) at 25°C. *Right* Plot $V/[L]$ versus $\bar{\nu}$. A dissociation constant of 5 μM was obtained for 0.9 binding sites per dimer.

concentrations (13 μM), yielding values ranging from 0.9 to 1.2 ANS binding sites per mole of enzyme and a dissociation constant of 5 μM (fig. 2). This unusual stoichiometry of binding, 1 mole of dye per mole of enzyme, is particularly significant since the enzyme is a dimer composed of monomers of identical molecular weight. It is interesting to note that addition of the substrate benzylamine to the ANS-enzyme complex has no effect on the fluorescence yield, emission spectra and affinity of the dye for the enzyme (table 1). In addition, 1–8-ANS has no effect on the catalytic activity. These lines of experimental evidence are consistent with the idea that 1–8-ANS binds to the enzyme at some site other than the catalytic binding site.

### *3.2. Rotational correlation time*

Steady polarization of fluorescence measurements give a polarization of fluorescence value of 0.33 for the dye bound to the enzyme, indicating that the dye is rigidly bound to a non-polar site of the enzyme. Therefore, it seems possible to perform nanosecond fluorescence measurements designed to probe the strength of the interaction between the two subunits in the dimeric macromolecule. If the dimeric enzyme displays any mode of flexibility in the nanosecond range due to independent rotation of the subunits, then one should be able to detect the presence of rotational correlation times shorter than the rotational correlation time of a compact macromolecule of 180 000 molecular weight. If, on the other hand, the two subunits are rigidly bound in the dimeric macromolecule, then one should obtain rotational correlation times corresponding to a compact rotating body of 180 000 daltons.

Emission anisotropy measurements were performed at a protein concentration of 25 μM to ensure binding of the dye to the protein. Under this set of experimental conditions, there is a minimum interference from unbound dye and the emission anisotropy measurements yielded reproducible rotational correlation times for bound 1–8-ANS.

The data collected for the deconvoluted decay functions $I(t)$, $D(t)$, and $S(t)$ could be fitted to single exponential decays with very low root mean square deviations (RMS). Thus the RMS values for the monoexponential functions $I(t)$, $D(t)$, and $S(t)$ were $3.2 \times 10^{-3}$, $5 \times 10^{-3}$, and $3.9 \times 10^{-3}$, respectively.

A representative set of experiment data for the decay of $S(t)$, $D(t)$, and $I(t)$ are given in fig. 3. The $S(t)$ function gives one fluorescence decay time $\tau_S$ = 10.2 ns, indicating the homogeneity of the ANS enzyme complex. The $D(t)$ function yields a decay value $\tau_D$ = 9.3 ns, and the $I(t)$ function yields the decay value $\tau$ = 10.2 ns for unpolarized emission (table 1).

The time dependent anisotropy is depicted in fig.

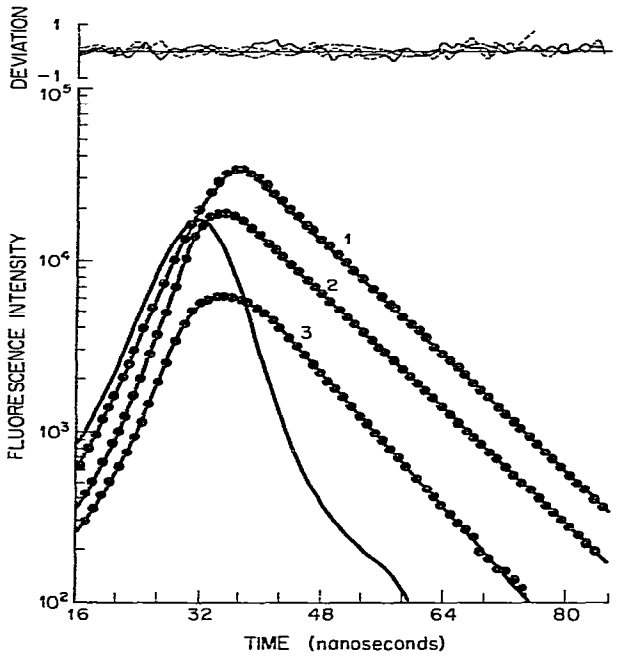


Fig. 3. Fluorescence decay curves of 1–8 ANS (12 μM) + plasma amine oxidase (25 μM) in 0.05 phosphate (pH 7). The experimental decay functions $D(t)(3)$, $S(t)(1)$, and $I(t)(2)$ are given together with the lamp profile. The points are the experimental values and the solid lines are calculated convolution products. The insert is a representation of the deviation function between convolution products and experimental values (see section 2).

4. It can be seen that there is little scatter in the anisotropy data points up to a time of about 90 ns. Although the rotational correlation time of a rigid macromolecule of 180 000 daltons is too long relative to the excited lifetime of the chromophore to permit the computation of an accurate value for the rotational correlation time, it is possible to estimate an approximate lower limit by $\phi$ by resorting to eq. (6)

$$A = A_0 \exp(-t/\phi) \qquad (6)$$

The logarithmic plot of the anisotropy ($A$) versus time is linear within experimental uncertainty and yields a rotational correlation time of 105 ns.

The preceding emission anisotropy results have been obtained using a fluorescent probe which decays with a lifetime of 10 ns. Under this set of experimental conditions, only the initial part of the emission anisotropy decay curve can be measured with accuracy. In order to avoid some ambiguity about the value of the rotational correlation time of plasma amine oxidase, we decided to study the emission anisotropy of the enzyme labelled with pyrenebutyric acid (PB). This chromophore decays with a fluorescent lifetime of 100 ns in air saturated solutions. It has been used to determine the rotational correlation time components of several immunoglobulins [12,13].

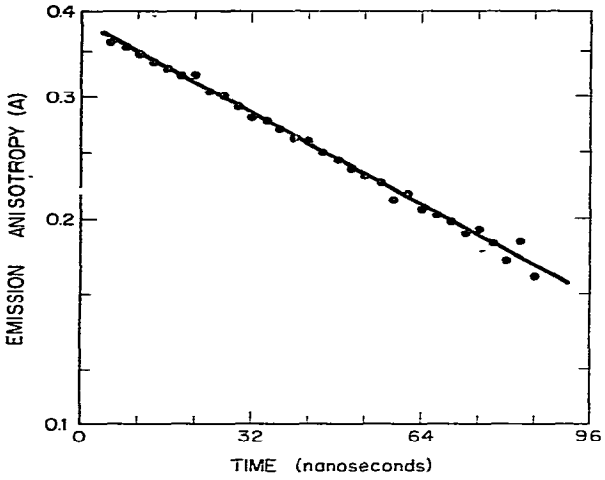


Fig. 4. Plot of emission anisotropy ($A$) versus time for ANS bound to plasma amine oxidase. A rotational correlation time of 105 ns was obtained by using eq. (6).

The time dependent anisotropy of plasma amine oxidase carrying PB is given in fig. 5. It is immediately apparent from these results that a fast initial decay of the emission anisotropy is followed by a slower decay up to a time of about 500 ns. The initial decay is too fast (shorter than 5 ns) to be measured by our instrument, but it can be attributed to a rapid motion of the probe itself. The longest anisotropy decay component, on the other hand, could be unambiguously assigned to the rotation of the entire macromolecule since it has a rotational correlation time of 120 ns.

An unhydrated rigid sphere of the volume of plasma amine oxidase would have a rotational correlation time of 55 ns when the partial specific volume is 0.73 ml/g. If the degree of hydration of the dimeric protein ($h$) is 0.2 g water/1 g of protein, the rotational

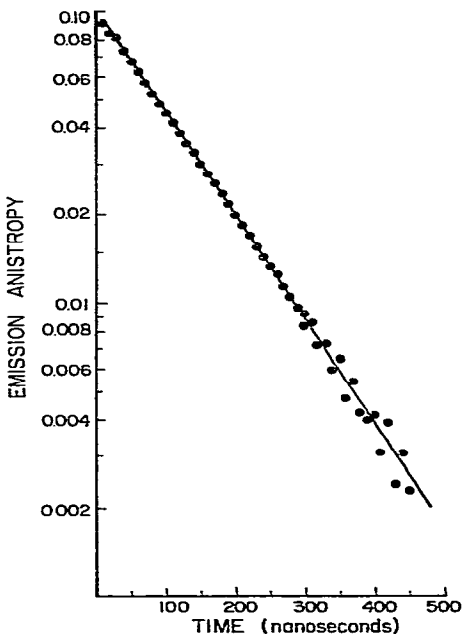


Fig. 5. Time dependent emission anisotropy of PB plasma amine oxidase. The solid line is the least-squared best fit to the observed data. The rotational correlation time is 120 ns. The sample contains approximately 2 moles of pyrene-butyric per 180 000 g of enzyme.

correlation time would be 77 ns. All these calculations were performed using eqs. (7) and (8)

$$\Theta = v\eta/kt, \tag{7}$$

$$V = (\bar{v} + h)M/N. \tag{8}$$

It is evident that the observed rotational correlation times, 105 and 120 ns, obtained with ANS-plasma amine oxidase and PB-plasma amine oxidase, respectively, are greater than the rotational correlation time calculated for the hydrated dimeric spherical macromolecule. This observation is consistent with the finding that plasma amine oxidase is a globular protein characterized by a $f/f_0$ ratio of 1.1 [14]. The most important conclusion to be drawn from these measurements is that the dimeric protein does not exhibit any degree of flexibility due to independent rotation of the subunits in the nanosecond range.

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