The Application of Nanosecond Fluorometry to an Allosteric Protein

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Summary

The fluorescent probe 1-anilino-8-naphthalene sulfonate, when bound by the inactive form of phosphorylase b, behaves as if distributed between two different binding sites, whose bound dye emits fluorescence with decay times of 19 and 8 nanosecs. In 0.1 M glycylglycine, pH 7.0, the addition of the allosteric activator adenosine-5'-phosphate or the substrate glucose-1-phosphate results in the progressive loss of the component of longer decay time. At intermediate levels of activator the decay curves could be represented as the sum of the weighted contributions of two species, corresponding to those prevailing in the absence of activator and in excess activator.

The activity of glycogen phosphorylase b, which is one of the most extensively studied allosteric enzymes, is controlled by the allosteric activator AMP (1, 2). Binding studies have shown that the binding of AMP in the absence of substrate is strongly cooperative, with a value of the Hill coefficient of 1.7 (2). Binding and kinetic studies have indicated that strong heterotropic cooperativity exists between the combination of the enzyme with AMP and either of the substrates glucose-1-phosphate and inorganic phosphate (1, 2, 3, 4).

Phosphorylase b (molecular weight 185,000) consists of two equivalent subunits of molecular weight 92,500. Each subunit contains a single strong binding site for AMP(1,2,3).

The AMP-induced activation of phosphorylase b has usually been attributed to a conformational transition, AMP being preferentially bound by the active form (1). The high degree of cooperativity of AMP binding has been attributed to the simultaneous conversion of both subunits to the active conformation upon the binding of a molecule of AMP by one subunit (1, 2).

The existing kinetic studies have been interpreted as indicating that the binding of substrate likewise favors the transition to the active form, although differences of opinion exist as to the number and equivalence of the active states (1, 2, 5). However, a basic weakness of the model described above is the paucity of direct physical evidence for a conformational change.

In recent years a rapid evolution of technique has made it possible to monitor the time decay of fluorescence directly (6). The technique of nanosecond fluorometry has been applied to a number of systems with the objective of analyzing complex decay curves or detecting excited state reactions (7).

With the development of programs for analyzing multicomponent decay curves in terms of the lifetimes and amplitudes of the individual emitting species (8) it has become possible to apply nanosecond fluorometry to the problem of monitoring conformational transitions of proteins, and in particular, to the activator - or substrate - induced transformations of allosteric proteins.

In general, when emission occurs from a collection of sources with different decay times, we have for the fluorescence intensity i (t) as a function of time, t:

$$(1) \qquad i(t) = \sum_j \alpha_j e^{-t/\tau_j}$$

where α_j and τ_j are the amplitude and decay time, respectively, corresponding to the jth component. If the number of components is small, the experimental curves of i(t) versus t may, in principle, be analyzed by the method of moments to yield the set of values of α_j and τ_j.

In this report, we shall describe the results of the application of nanosecond fluorometry to the phosphorylase b system, using the fluorescent probe 1-anilino-8-naphthalene sulfonate (ANS). This probe, which is virtually non-

fluorescent in aqueous solution, acquires an intense fluorescence when bound by a non-polar site on a protein (9, 10).

In an earlier report, Seery and Anderson have found that the fluorescence intensity of ANS bound to phosphorylase b is sharply reduced in the presence of AMP or substrates. From parallel equilibrium dialysis studies, it was concluded that the greater part of the effect arose from the dissociation of ANS, although it was not possible to demonstrate a quantitative correlation (11). For this reason, it was not possible to make any statement as to the quantum yield or excited lifetime of the residual bound dye.

Experimental

Phosphorylase b (EC 2.4.1.1) was extracted from rabbit skeletal muscle and purified according to the procedure of Fischer and Krebs (12). Each preparation was recrystallized three times and stored at 3°C. The enzyme was assayed by the method of Cori, et al (13). The preparations used in this study had specific activities of 60-70 units per mg. protein.

Enzyme solutions in buffer were prepared and freed from AMP by passage through a Sephadex G-25 column, followed by passage through a short, activated charcoal-G-25 column. Complete removal of AMP was indicated by a ratio of absorbances, A_{260}:A_{280} of 0.48-0.49. The protein concentration was determined from the absorbance at 280 nm, assuming a value of A_{280} for a 1 mg/ml solution equal to 1.32 (2). Molar concentrations were computed using a molecular weight of 92,500 for the phosphorylase monomer.

The ammonium salt of 1-anilino-8-naphthalene sulfonate (ANS) was purchased from the Sigma Chemical Co. and recrystallized twice from water. AMP was purchased from the Sigma Chemical Co. and used without further treatment.

Measurements of the intensity and spectral distribution of fluorescence were made using an Aminco spectrofluorometer. Measurements of the time decay of fluorescence were made using an Ortec 9200 single photon counting nanosecond fluorometer. A Corning 5540 filter was used in the excitation beam.

A Shoeffel monochromator intercepted the emission beam. The selected emission wavelength was normally 500 nm for these studies.

The emission intensity, which is observed as a function of time after excitation, includes components stemming from scattered light, the intrinsic fluorescence arising from bound pyridoxal-5′-phosphate, and the fluorescence of free ANS. The appropriate controls were therefore examined and the corresponding contributions subtracted from the experimental curves. The residual emission should arise solely from bound ANS. For the conditions employed here, the above corrections were significant only for short (<5 nanosecs) intervals after excitation.

The analysis of decay curves in terms of the amplitudes and lifetimes of fluorescent components was made using the program developed by Dyson and Isenberg, which is based upon the method of moments (8). We are indebted to Dr. Dyson for supplying us with a copy of the Flortran program.

Results and Discussion

Figure 1 shows the variation of total emission intensity as a function of AMP level for phosphorylase b plus ANS in 0.1 M glycylglycine, pH 7.0. In agreement with Seery and Anderson (11), a major decrease in intensity with increasing concentrations of AMP is observed (11). The degree of quenching approaches a limiting value above 10^{-3} M AMP.

If the corrected time decay curves for different AMP levels are examined, it is seen that a major change in shape occurs (Figures 1 and 2). With increasing AMP concentrations, the intensity decreases much more rapidly with time, corresponding to a reduced average excited lifetime. As Figure 1 shows, the decay curve for a intermediate level of AMP may be represented as the sum of the weighted contributions of two limiting curves, corresponding to zero AMP and to a saturating (10^{-2} M) concentration of AMP.

Thus, if i(t) is the intensity as a function of time for an intermediate level of AMP, we have

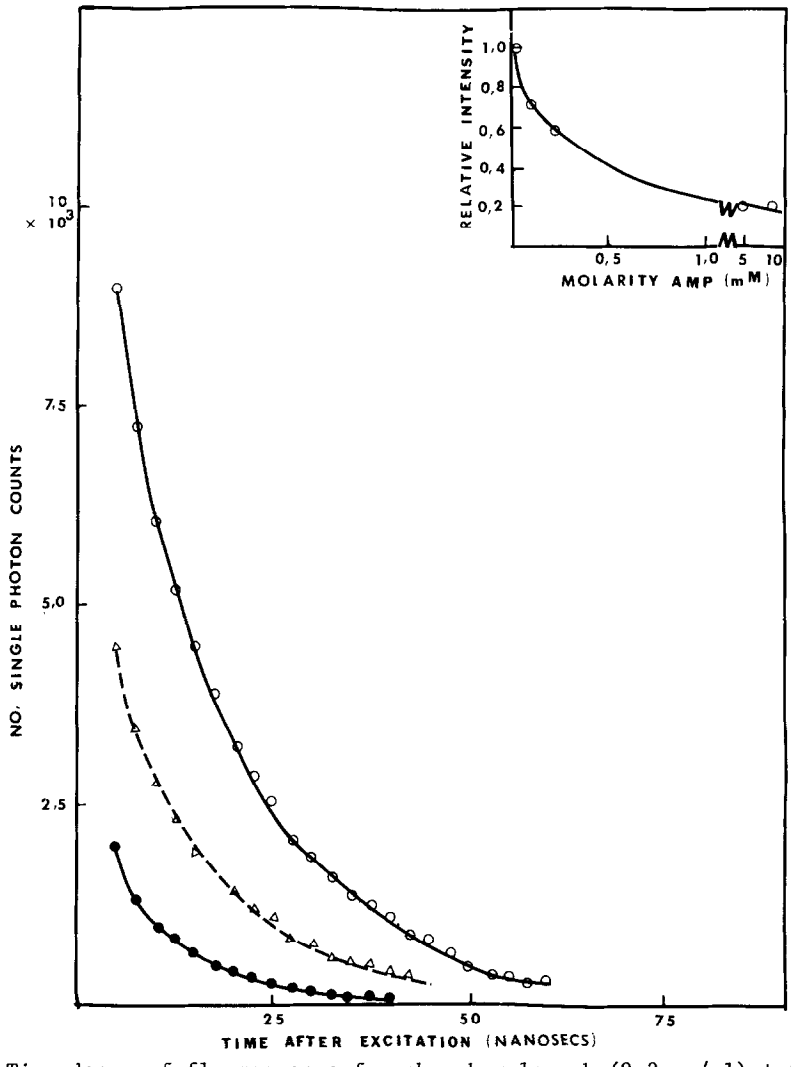

Fig. 1 Time decay of fluorescence for phosphorylase b (9.2 mg/ml) + ANS (7.6×10^{-5} M) in 0.1 M glygly, pH 7.0, for different concentrations of AMP. The ordinate is the corrected number of single photon counts per channel; the abscissa is the time in nanosecs since the excitation maximum. Every fifth channel is plotted.

⊙ no AMP

△ 2.1×10^{-4} M AMP

● 9.1×10^{-3} M AMP

The dashed line (- - -) corresponds to the curve for 2.1×10^{-4} M AMP which is computed from equation 2, for x = 0.35.

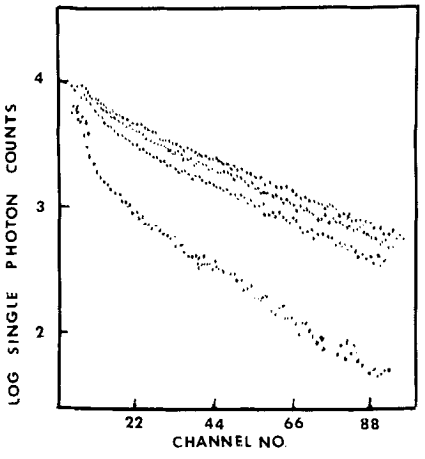

Fig. 2 Logarithmic plot of single photon counts versus channel number for phosphorylase b plus ANS under the above conditions. The levels of AMP are, from top to bottom: 0, 8.7×10^{-5} M, 2.1×10^{-4} M, and 9.1×10^{-3} M.

(2) $$i(t) = x\, i_1(t) + (1-x)\, i_2(t)$$

and $$x = (i-i_2)/(i_1-i_2)$$

where i_1 and i_2 are the intensities corresponding to the molecular states prevailing in the absence of AMP and at a saturating level of AMP, respectively, and x is the mole fraction of fluorochromes in state 1.

Thus the time decay curves as a function of AMP concentration can be accounted for in terms of an AMP-induced transition between two molecular states, which interact differently with ANS to yield distinct and characteristic decay curves. In view of the results of Seery and Anderson (11), state 2 appears to have a reduced affinity for ANS as well as an altered decay curve.

Fig. 1 (inset) Dependence of emitted intensity at 500 nm upon AMP level for phosphorylase b plus ANS under the above conditions. The excitation wavelength is 350 nm.

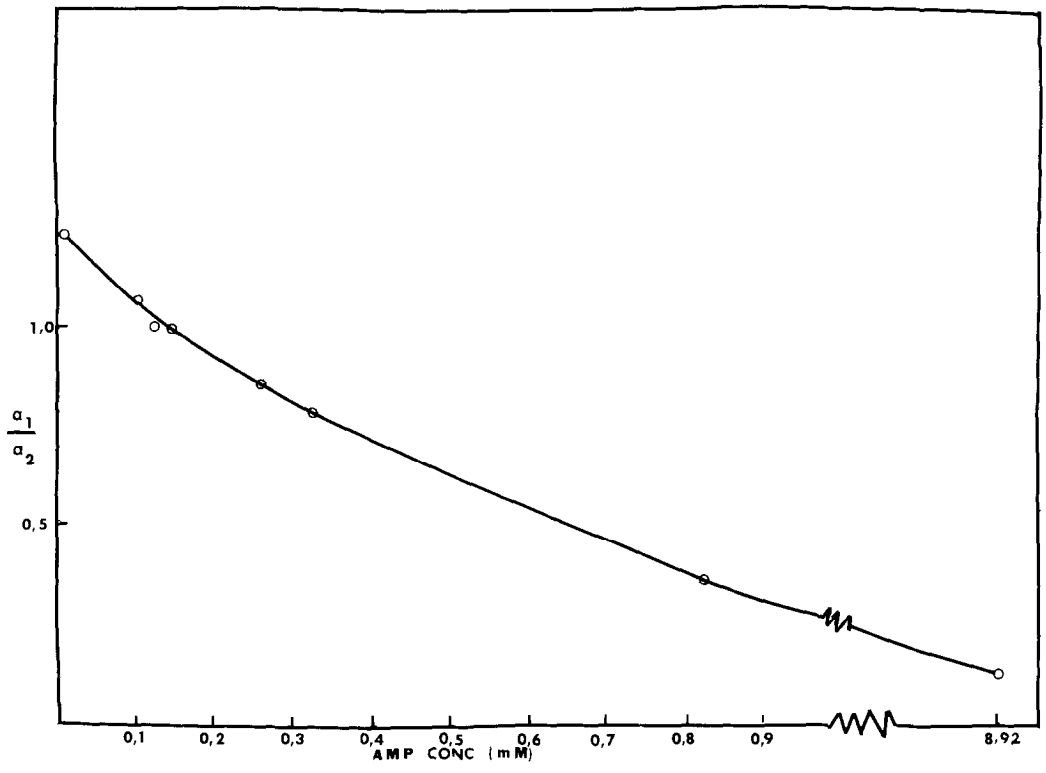

Fig. 3 Dependence upon AMP level of the ratio of the amplitudes of the 19 and 8 nanosec components (α_1/α_2) for phosphorylase b plus ANS in 0.1 M glycylglycine, pH 7.0. The conditions are the same as for Table 1.

A more explicit analysis is provided by application of the method of moments to the time decay curves (8). The results are shown in Table 1. The component incrementation test shows that for each AMP level, the same two lifetimes found in the two-component analysis recur in the three-component analysis, plus a third component of negligibly small amplitude or decay time. This is a strong indication that the decay curves can be represented as the summed contributions of two species of different lifetimes.

Moreover, at all AMP levels lifetimes close to 19 and 8 nanosecs

Table 1

Effect of AMP upon the Time Decay of Fluorescence for Phosphorylase b plus ANS*

Molarity of AMP	one component analysis		two component analysis		three component analysis	
	α	τ	α	τ	α	τ
0 **	0.0279	15.6	0.0172 0.01395	19.4 7.6	0.0172 0.0140 (3×10^{-8})	19.4 7.6 (-75)
1.0×10^{-4} ***	0.0432	15.5	0.0195 0.0271	20.7 10.1	0.0249 0.0232 (7.9×10^{-3})	19.5 8.3 (-2.9)
1.23×10^{-4} ***	0.0426	15.2	0.0219 0.246	19.8 8.9	0.0235 0.02345 (4×10^{-5})	19.4 8.4 (-6)
1.45×10^{-4} ***	0.0418	15.1	0.0231 0.232	19.4 8.2	0.02345 0.02265 (1.7×10^{-5})	19.2 7.8 (-7.8)
2.52×10^{-4} ***	0.03435	14.2	0.0129 0.0245	20.3 9.5	0.0182 0.0216 (2×10^{-3})	18.6 7.3 (-1.82)
3.22×10^{-4} **	0.0161	14.1	0.00791 0.0102	18.9 7.7	0.00809 0.0100 (8×10^{-6})	18.9 7.5 (-10)
8.92×10^{-3} **	0.00635	10.2	0.000769 0.00676	20.0 7.3	0.00089 0.0068 (3.4×10^{-5})	19.4 7.0 (-5.9)

* Analyzed by the method of moments, using the Flortran program of Dyson and Isenberg (8), assuming 1,2, or 3 components. Because of differences in lamp intensity and counting times, the absolute values of α for different AMP levels are not comparable.

** The concentrations of phosphorylase b and ANS are 5.0 mg/ml and 4.9×10^{-5} M, respectively.

*** The concentrations of phosphorylase b and of ANS are 11.1 mg/ml and 1.2×10^{-4} M, respectively. The buffer is 0.1 M glycylglycine, pH 7.0.

appear. The effect of increasing AMP concentration is to reduce the relative amplitude of the component corresponding to the 19 nanosec lifetime, so that the decay curve comes to be dominated by the component of shorter lifetime (Figure 2 and Table 2).

Table 2

Effect of Glucose -1- phosphate upon the Time Decay of Fluorescence for Phosphorylase b plus ANS*

Molarity Glucose -1-phosphate	one component analysis		two component analysis		three component analysis		α/α_2
	α	τ	α	τ	α	τ	
0	0.0279	15.6	0.0172 0.0139	19.4 7.6	0.0172 0.0140 (3×10^{-8})	19.4 7.6 (-75)	1.23
0.714×10^{-2}	0.028	14.7	0.0172 0.0149	18.8 6.4	0.0172 0.0149 (1.5×10^{-9})	18.8 6.4 (-50)	1.15
1.786×10^{-2}	0.0483	14.3	0.0266 0.0278	18.8 7.2	0.0270 0.0276 (0.5×10^{-5})	18.7 7.0 (-9.7)	0.98
3.2×10^{-2}	0.0463	13.9	0.0199 0.0311	19.6 8.4	0.0221 0.0295 (0.68×10^{-4})	19.0 7.8 (-5.6)	0.75

* The concentrations of phosphorylase b and ANS are 5.7 mg/ml and 1.54×10^{-4} M, respectively. The other conditions are the same as for Table 1.

The two decay times clearly reflect the presence of two different microenvironments. Thus, at least two different binding sites (or classes of binding site) must be present on each phosphorylase b protomer. The binding of AMP results in the loss of one of these. As Figure 3 indicates, the fraction of the emitted intensity arising from the site corresponding to the 19 nanosec component falls progressively with increasing AMP level. However, the amplitude of the second component also decreases, indicating that the binding affinity of the second site is also reduced in the presence of AMP.

The addition of the substrate glucose-1-phosphate has consequences

similar to those produced by AMP (Table 2). In particular, the relative magnitude of the 19 nanosec component is progressively reduced. The implication is that the molecular events produced by substrate and activator have qualitatively parallel effects upon the bound fluorochrome.

These results are consistent with a model of the symmetrical type (14) which postulates the simultaneous transition of both protomers to the conformation characteristic of the active state upon binding a single molecule of AMP. If an intermediate conformation is present, it is not detected by the bound fluorochrome.

It should be stressed that phosphorylase b is notoriously sensitive to a variety of non-physiological ions and that these include many of the common buffers (3,4). Thus, the buffer glycerol phosphate, as compared with glycylglycine, appears to function as an allosteric activator (3,4). The conclusions cited here should therefore be regarded as applying explicitly to the cited conditions of buffer, pH and temperature.

From the preceding it is clear that nanosecond fluorometry can provide an index of the molecular events accompanying the interaction of an allosteric protein with its modifier and that it may prove to be among the more powerful techniques which are applicable to this problem.

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