A FLUORESCENT PROBE FOR THE DIMER TO TETRAMER CONVERSION OF GLYCOGEN PHOSPHORYLASES *a* AND *b*

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Received 17 October 1970

1. Introduction

Environmentally sensitive fluorescent probes have been used to follow conformational changes in glutamate dehydrogenase [1] and the conversion of chymotrypsinogen A to α-chymotrypsin [2].

We now describe the use of 2-methylanilino-naphthalene-6-sulphonate (MNS) as a probe for the formation of phosphorylase *b* tetramer and for the enzymic conversion of the inactive phosphorylase *b* to the active phosphorylase *a*.

2. Materials and methods

MNS was synthesized by the method of Cory et al. [3] and recrystallised from dilute NaOH.

Phosphorylase *b* was prepared by the method of Fischer and Krebs [4] and phosphorylase kinase as described by Krebs and Fischer [5]. Phosphorylase activity was measured on a Technicon Autoanalyser essentially following the method of Cori et al. [6] except that inorganic phosphate was measured continuously to obtain initial rates.

Fluorescence measurements were made as described previously [7]. Fluorescence emission spectra were recorded using a Hitachi-Perkin-Elmer MPF-2A recording spectrofluorimeter with band widths of 2 nm on the two monochromators. Fluorescence lifetimes were measured using a TRW model 32A decay time fluorimeter.

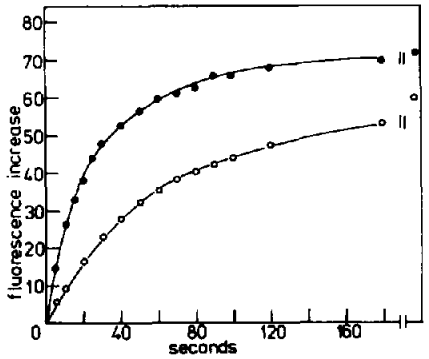

Fig. 1. The dimer to tetramer conversion of phosphorylase *b* as followed by the increase in fluorescence of MNS (20 μM). At zero time AMP and $MnCl_2$ were added to concentrations of 2 mM and 5 mM respectively. The concentrations of phosphorylase *b* were 2.6 mg/ml (●——●) and 0.7 mg/ml (○——○). Excitation was at 365 nm and emission at 450 nm. The buffer was 0.05 M triethanolamine containing 0.1 M KCl and 1 mM β-mercaptoethanol at pH 8.5 and 25°.

3. Results

MNS binds to phosphorylase *b* with an 80 fold enhancement of the fluorescence at 430 nm and a shift in the emission maximum from 520 nm in buffer to 430 nm on the protein. Addition of AMP (1 mM) and a divalent metal ion (Mg^{2+}, Mn^{2+}, or Ca^{2+} at 10–20 mM) causes a further 2–3 fold fluorescence enhancement. This further enhancement is relatively slow (fig. 1) and the rate is second order in phosphorylase *b* concentration (rate constant = 3.1×10^5 M^{-1} min^{-1} at 25°). This ligand induced fluorescence increase can always be correlated with an increase in

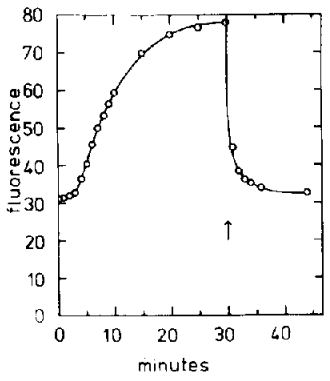

Fig. 2. Change in MNS fluorescence on the conversion of phosphorylase *b* to *a* and the effect of glucose. The reaction mixture contained 2 mg/ml phosphorylase *b*, 3 mM ATP, 10 mM magnesium acetate, 1 μM cyclic 3′, 5′-AMP. The buffer was 0.05 M triethanolamine containing 1 mM EDTA, 1 mM β-mercapthoethanol, and 0.1 M KCl at pH 7.0 and 25°. At the point indicated by the arrow glucose was added to a concentration of 0.1 M.

$S_{20,w}$ from 8.8. to about 13.6. MNS at a concentration of 20 μM has no effect on the phosphorylase *b* activity.

The conversion of phosphorylase *b* dimer to phosphorylase *a* tetramer is accompanied by a fluorescence increase of the same magnitude which is reversed by the addition of glucose (fig. 2). The appearance of phosphorylase *a* activity (in the absence of AMP) roughly parallels the fluorescence increase except that there is an initial lag period in the latter (fig. 3). The rate of fluorescence increase after the lag period is proportional to the amount of phosphorylase kinase added over a

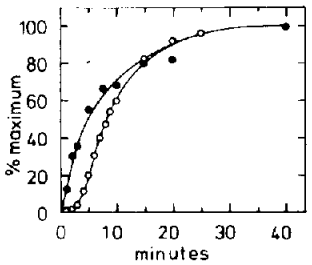

Fig. 3. The phosphorylase kinase catalyzed conversion of phosphorylase *b* to *a* as followed by MNS fluorescence (○——○), and by phosphorylase activity assayed in the absence of AMP (●——●). The reaction conditions were the same as in fig. 2. MNS at a concentration of 20 μM has no effect on the phosphorylase *a* activity.

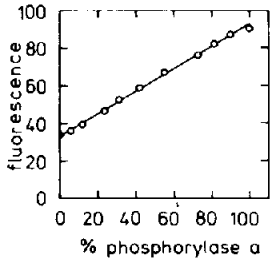

Fig. 4. Fluorescence of MNS (20 μM) in mixtures of phosphorylase *a* and *b*. The total protein concentration was kept constant at 2 mg ml^{-1} while the proportions of the *a* and *b* forms were varied. The buffer was 0.05 M triethanolamine containing 0.1 M KCl, 1 mM EDTA, and 1 mM 2-mercaptoethanol, at pH 7.0 and 25°.

four fold concentration range. At higher kinase concentrations the rate tends to level off. The fluorescence of MNS in mixtures containing varying proportions of phosphorylases *a* and *b* is proportional to the amount of the tetrameric *a* form present (fig. 4).

The fluorescence lifetime of MNS bound to phosphorylase *b* is 8.4 nsec and this is increased to 11.4 nsec on conversion of the *b* to the *a* form.

4. Discussion

MNS can be used to detect the formation of both phosphorylase *b* and phosphorylase *a* tetramers. The possibility in the case of the *b* form that part of the fluorescence increase is due to a ligand induced conformational change can be excluded on the grounds that the rate of fluorescence change can be described by a single second order rate constant. This probe, therefore, provides a simpler and more sensitive measure of tetramer formation than does ultracentrifugation. In the case of phosphorylase *a* formation a lag phase is observed by fluorescence which is not present when the appearance of activity is used to measure phosphorylase *a* formation. The rate of aggregation of dimer *a* to tetramer *a* will depend on the concentration of dimer *a* and may thus be rate limiting in the initial stages of the kinase reaction. The lag phase can be reduced, but not abolished, by including in the reaction mixture 10% of the *a* form so that this cannot be the only explanation. Another possibility is that, initially, dimers of the form a_1 b_1 are produced which do not aggregate. The binding of MNS to phosphor

ylases *a* and *b* is rapid, being completed within the mixing time of about 5 sec.

After the lag phase a linear increase in fluorescence is observed, the rate of which is proportional to the amount of kinase added so that, in this part of the reaction, the phosphorylation of phosphorylase *b* is rate limiting. This proportionality breaks down at high kinase concentrations probably because, under these conditions, agregation again becomes rate limiting.

Glucose, which dissociates phosphorylase *a* tetramer to the dimer [8], causes a decrease in the fluorescence to approximately the same value as that observed with the dimer *b*. These considerations suggest that MNS detects formation of the tetrameric form while the fluorescence of MNS bound to phosphorylase *a* dimer is the same as that of MNS bound to phosphorylase *b* dimer.

The extent of the fluorescence decrease induced by glucose depends on the concentration of the glucose added and can thus be used as a measure of the binding of this ligand. At pH 8.5, formation of tetramer *b* only occurs in the presence of both AMP and metal ion. Thus, in the presence of saturating concentrations of one, the MNS fluorescence can be used to measure the binding of the other. Using this method, the apparent K_D for AMP at 25 mM Mn^{2+} is 4×10^{-5} M and the K_D for Mn^{2+} in the presence of 1 mM AMP is 6 mM. This represents binding of the metal to sites involved in tetramer formation, and these are not the same as those sites observed using NMR proton relaxation enhancement at lower Mn^{2+}concentrations ($K_D = 1.7 \times 10^{-4}$ M) [9].

The increase in fluorescence lifetime of MNS shows that the quantum yield of fluorescence is higher when the dye is bound to the tetramer than when bound to the dimer. This change does not fully explain the observed enhancement so that there is also probably an increase in the amount of dye bound. The similarity in the behaviour of this dye to that of bromthymol blue described by Ullman et al. [10] suggests that the increase in binding of the latter dye may have been due to tetramer formation and not directly to an AMP induced conformational change.

Acknowledgements

We wish to thank the Science Research Council for financial support.

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