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The binding of substrates and inhibitors to the metal center of myoinositol monophosphatase

Francis Kwok[a,*], Jorge E. Churchich[b]

[a]*Department of Applied Biology and Chemistry Technology, Hong Kong Polytechnic, Hong Kong, Hong Kong*
[b]*Department of Biochemistry, University of Tennessee, Knoxville, TN 37996-0840, USA*

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Abstract

The synthetic substrate anthraniloyl-β-glycerol-P binds to myoinositol monophosphatase with a $K_d = 5$ μM at pH 7.5. The anthraniloyl chromophore, excited at 330 nm, sensitizes the long lived luminescence of bound Tb(III) at 490, 545, 585 and 620 nm. Assuming a mechanism of radiationless energy transfer, the actual distance of separation between the donor-acceptor pair was calculated to be $R = 10$ Å. Tb(III) binds to the monophosphatase with a $K_d = 2$ μM, whereas Ca-(II) displaces the lanthanide at concentrations above 0.1 mM. The binding studies support the notion that Tb(III), Ca(II) and Mg(II) interact with a common binding site on the protein. Phosphate ion, a strong competitive inhibitor, perturbs the luminescence of bound Tb(III), whereas the substrate β-glycero-P has no effect on the luminescence yield and long-lived emission of bound Tb(III). It is suggested that the phosphate group of the substrate is not in direct contact with the metal ion coordinated to several amino acid residues of the enzyme.

Key words: Inositol; Monophosphatase; Terbium; Inhibitor; Energy transfer; Regulation

1. Introduction

Inositol monophosphatase catalyzes dephosphorylation of myoinositol-I-phosphate to myoinositol, which is needed for resynthesis of phosphatidyl inositol [1]. The complete cDNA and deduced amino acid sequence of the human, bovine and rat brain enzymes has been reported [2,3].

The structure of human myoinositol monophosphatase has been determined to 2.1 Å resolution by X-ray crystallography [4]. A kinked structure, formed by amino acid residues 90–95, surrounds the lanthanide Gd(III) which is also coordinated to water molecules and one sulphate ion [4].

It has been suggested that the sites occupied by the lanthanide Gd(III) and sulphate in the crystal structure are likely to be occupied by Mg(II) and the phosphoate of the substrate when the enzyme is engaged in catalysis. The metal ion Mg(II) is essential for catalytic activity [5], and it is bound to the site occupied by the lanthanide Tb(III) complexed to the protein [6]. Although lanthanides have been used as calcium analogues to probe the metal binding environment of calcium binding proteins [7], there is no information available on the effect of Ca(II) and substrates on the luminescence properties of Tb(III) coordinated to the monophosphatase. Here, we have investigated certain aspects of the binding of the substrate aminobenzoyl-β-glycerol-P and the inhibitor Tb(III) to the monophosphatase by means of luminescence techniques.

Fluorescence spectroscopy is used to monitor the binding of the substrate anthraniloyl-β-glycerol-P to the monophosphatase, whereas phosphorescence spectroscopy is used to detect the effects of inhibitors and substrates on the long-lived luminescence of Tb(III) coordinated to the protein.

2. Experimental

2.1. Purification of the enzyme

The procedure developed by Meek et al. [8] for the purification of bovine brain myoinositol monophosphatase was used in the isolation of the enzyme from pig brain. Starting with 6.4 kg of fresh pig brain, 16 mg of pure myoinositol monophosphatase were obtained. Purity of the enzyme was tested on SDS-PAGE using 12.5% polyacrylamide gels. The specific activity of the purified enzyme was 8 μmol/min/mg with DL-β-inositol-I-P and 4 μmol/min/mg with DL-β-glycero-P at pH 7.5, 25°C. Protein concentration was determined by the method of Bradford [9]. The formation of inorganic phosphate was assayed with Malachite green as described by Lanzetta et al. [10]. Enzymatic assays were performed at pH 7.5 in 10 mM Tris-HCl containing DL-β-glycero-P (2 mM), $MgCl_2$ (2 mM) and NaCl (0.1 M).

2.2. Binding of anthraniloyl-β-glycerol-P (Ant-β-Gly-P)

Anthraniloyl-β-glycerol-P was synthesized as described in reference [11]. The purity of the compound was tested by thin-layer chromatography and NMR spectroscopy [11]. The NMR spectrum was recorded using a 250 MHz Brücker spectrometer. The emission spectra of Ant-β-Gly-P were measured in the presence and absence of myoinositol monophosphatase at pH 7.5 in 20 mM Tris-HCl buffer. The fraction of ligand bound $\alpha = F - F_o/F_M - F_o$ was determined by adding increasing concentrations of Ant-β-Gly-P to a fixed concentration of enzyme upon excitation at 330 nm. F_M, the maximum increase in fluorescence, was determined directly by adding increasing concentrations of enzyme to a fixed concentration of Ant-β-Gly-P (5 μM). The average number

*Corresponding author. Fax: (852) 605 6259.

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of ligand molecules bound per mol of monomer (ν) was determined for points along the titration curve by means of Eqn. 1:

$$\nu = \alpha \times [(L_o/P_o)] \quad (1)$$

where L_0 and P_0 are the total ligand and protein concentrations, respectively. Plots of ν/[L] vs. ν were used in the analysis of binding results.

2.3. Fluorescence spectroscopy

A Perkin-Elmer model LS 50B spectrofluorimeter was used in the fluorescence and phosphorescence measurements. For the study of Tb(III) phosphorescence, the sample was excited with a pulsed Xenon flash lamp with pulse width at half peak height of 10 μs. The delay and gate time were 1 μs and excitation and emission slits set at 5 nm. Phosphorescence decay curves were fit by computer to single or double exponential functions using Statgraphics. Absorption spectra were recorded in a Shimadzu spectrophotometer.

2.4. Materials

All the resins used in the purification of the enzyme were purchased from Pharmacia (Hong Kong). The substrates DL-β-inositol-I-P, DL-β-glycero-P and 2'-AMP were purchased from Sigma. $TbCl_3$ was purchased from Aldrich. Pig brains were obtained from a local slaughterhouse.

3. Results and discussion

3.1. Binding of anthraniloyl-β-glycerol-P

Insertion of an aminobenzoyl group in the structure of β-Glycero-P renders a compound that is a stubstrate of myoinositol monophosphatase in the presence of $MgCl_2$ [11]. In order to prevent any hydrolysis of the fluorescent substrate, the fluorometric titrations were conducted in the presence of $CaCl_2$ (5 mM) which is known to be a competitive inhibitor of Mg(II) [5].

Addition of one equivalent of Ant-β-Gly-P to the enzyme (13 μM, monomer) brings about a 21% enhancement of the fluorescence emitted by the ligand when excited at 330 nm. The enhancement of substrate fluorescence was used to determine the stoichiometry of binding. The results of fluorometric titrations obtained when

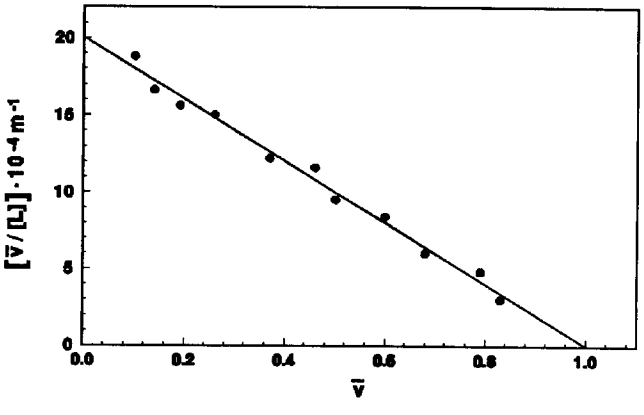

Fig. 1. Plot of ν/[L] vs. ν. Analysis of the titration results of myoinositol monophosphatase (6 μM) with varying concentrations of anthraniloyl-β-glycerol-P in 20 mM Tris-HCl buffer (pH 7.5) containing $CaCl_2$ (5 mM). A dissociation constant K_d = 5 μM was determined for one binding site/monomer.

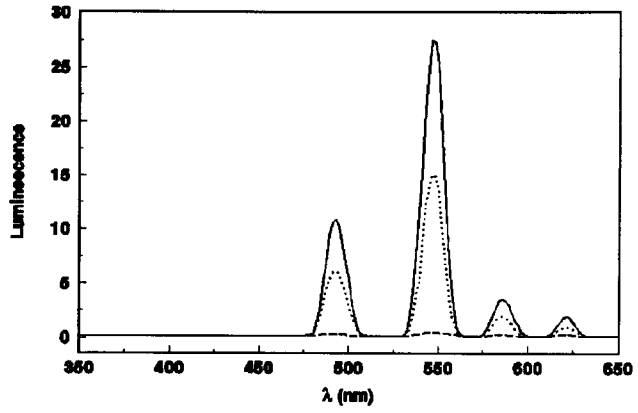

Fig. 2. Luminescence spectra of Tb(III)-myoinositol monophosphatase (20 μM). Spectra were recorded in the absence (—) and presence of anthraniloyl-β-glycerol-P (20 μM) (· · · ·) upon excitation at 290 and 330 nm, respectively, with a pulsed Xenon flash lamp. The luminescence spectrum of Tb(III)-myoinositol monophosphatase (20 μM) excited at 330 nm (- - - -) is included in the figure.

the enzyme (6 μM, monomer) was allowed to interact with increasing concentrations of Ant-β-Gly-P are given in Fig. 1. A dissociation constant K_d = 5 μM was obtained for the binding of 1 mol of Ant-β-Gly-P per monomer of enzyme.

3.2. Sensitized Tb(III) luminescence

Tb(III) exhibits strong luminescence bands when bound to myoinositol monophosphatase at pH 7.5. Two major bands centered at 490 and 545 nm and two minor bands at 585 and 620 nm are detected upon excitation at 290 nm (Fig. 2). The sensitization of Tb(III) luminescence is predominantly due to radiationless energy transfer from tryptophanyl residues as revealed by the excitation spectrum.

When Ant-β-Gly-P (20 μM) was allowed to mix with Tb(III)-monophosphatase (20 μM) and the luminescence of bound Tb(III) recorded upon excitation at 330 nm, it was found that the luminescence of the lanthanide is substantially increased when compared to Tb(III)-monophosphatase excited at 330 nm (Fig. 2).

If the observed sensitized luminescence is due to radiationless energy transfer from the anthraniloyl donor to bound Tb(III), then the actual distance of separation can be determined by resorting to Forster's theory of resonance transfer [12]. Accordingly, the critical distance of transfer R_o = 5 Å was obtained from Eqn. 2:

$$R_o = (8.78 \times 10^{-25}) x^2 \times Q_D \times J \times n^{-4} \quad (2)$$

when the orientation factor x^2 = 2/3, the refractive index n = 1.33, the quantum yield Q_D = 0.12 and the overlap integral $J = 1.4 \times 10^{-19}$ cm^6 · mol^{-1} were used in the calculations. The overlap integral was determined from the recorded spectra included in Fig. 3. The efficiency of energy transfer E = 0.0128 was obtained with the aid of Eqn. 3:

$$E = \frac{A(\lambda_A}{A(\lambda_D} \times \left[\frac{IAD}{IA} - 1\right] \quad (3)$$

where $A(\lambda_A)$ and $A(\lambda_D)$ are the absorbances of donor and acceptor at the excitation wavelength of 330 nm and *IAD/IA* is the ratio of the areas of the emission spectra of the acceptor (Tb(III)) in the presence and absence of donor. An actual distance of transfer of R_o = 10 Å was calculated with the aid of Eqn. 4:

$$R = R_o\left[\frac{1}{E} - 1\right]^{\frac{1}{6}} \quad (4)$$

Since the amino acids residues coordinated to Gd(III) in the X-ray crystallographic structure of myoinositol monophosphatase are within 2.5 Å of the metal center [4]. It is evident that the anthraniloyl chromophore is not in direct contact with the metal ion Tb(III) coordinated to myoinositol monophosphatase.

3.3. *Effect of inhibitors and substrates on Tb(III) luminescence*

The effect of Ca(II) and phosphate ions on the luminescence properties of Tb(III) bound to the enzyme was examined by phosphorescence spectroscopy. The luminescence spectra recorded in Fig. 4 shows the concentrations of Ca(II) and phosphate required to displace the lanthanide from its binding site. The decrease in luminescence yield induced by $CaCl_2$ suggests that the site of coordination of Tb(III) can be occupied by Ca(II) ions. On the other hand, inorganic phosphate a strong competitive inhibitor with respect to the substrate β-Glycero-P (K_i = 0.1 mM) displaces Tb(III) from its coordination site. This dramatic reduction in the sensitized luminescence of Tb(III) is reversed by addition of increasing concentrations of the lanthanide. When the concentration of Tb(III) approaches 0.2 mM, the original luminescence of Tb(III) enzyme complex is restored.

In marked contrast to inorganic phosphate, the substrates of the enzyme DL-β-Glycero-P, and inositol-I-P do not perturb the sensitized luminescence of bound Tb(III). Indeed, the luminescence yield and long-lived

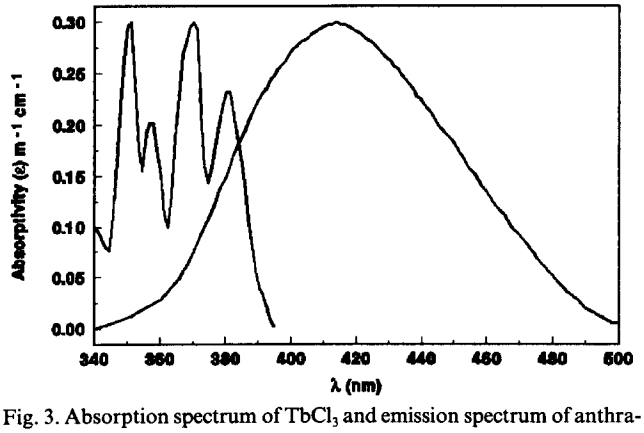

Fig. 3. Absorption spectrum of $TbCl_3$ and emission spectrum of anthraniloyl-β-glycerol-P used in the calculation of the overlap integral (*J*).

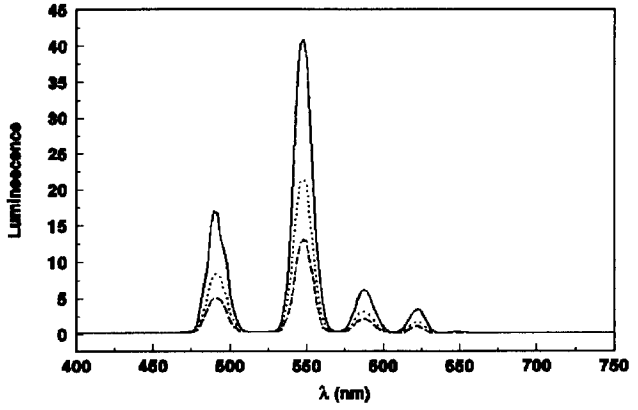

Fig. 4. Luminescence spectra of Tb(III)-myoinositol monophosphatase (20 μM). Spectra were recorded in the absence (—) and presence of $CaCl_2$ (1 mM) (· · ·) and in the presence of phosphate ions (0.1 mM) (- - - -) in 20 mM Tris-HCl (pH 7.5). Excitation with a pulsed Xenon flash lamp at 290 nm; excitation and emission slits, 5 nm.

luminescence of Tb(III) (τp = 1.1 ms) remains essentially invariant in the presence of 1 mM concentrations of inositol-I-P and DL-β-Glycero-P. Moreover, the presence of the substrates do not prevent the reduction of luminescence yield caused by inorganic phosphate.

Hence, the luminescence studies reported in this work do not support the hypothesis that the phosphate group of the substrate is coordinated to Tb(III) bound to amino acid residues of the enzyme. Moreover, the energy transfer measurements indicate that an anthraniloyl moiety covalently bound to β-Glycero-P is 10 Å away from the metal center.

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