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THE INCREASE IN YEAST ENOLASE FLUORESCENCE PRODUCED BY SUBSTRATES AND COMPETITIVE INHIBITORS IN THE PRESENCE OF EXCESS Mg^{2+}

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SUMMARY

1. Substrates or competitive inhibitors produce an increase in the fluorescence of yeast enolase (2-phospho-D-glycerate hydro-lyase, EC 4.2.1.11) in the presence of a high concentration (0.01 M) of Mg^{2+} or other activating metal ions.

2. The increase in fluorescence is ascribed to a decrease in quenching of one or more tryptophans in the enzyme upon binding metal ion and substrate.

3. Fluorometric titrations of the enzyme with substrate in the presence of excess Mg^{2+} have given substrate binding constants in agreement with substrate Michaelis constants.

4. Titrations with Mg^{2+} in the presence of excess substrate show two binding sites. The site with the smallest dissociation constant is identical with the site associated with structural changes in the enzyme when the metal ion is bound. The second site appears only in the presence of substrate and is not connected with enzymic activity, but perhaps with actual inhibition.

INTRODUCTION

Mg^{2+} causes a structural change in yeast enolase (2-phospho-D-glycerate hydro-lyase, EC 4.2.1.11)[1] at concentrations insufficient for maximal activity. Substrate (an equilibrium mixture of about 20% 2-phosphoglycerate and 80% phosphoenolpyruvate) in the presence of higher concentrations (0.01 M) of Mg^{2+} also produces changes in the fluorescence emission spectrum of the enzyme[1]. This paper presents an analysis of these changes in terms of the structure of protein, and examines the relationship between metal ion and substrate binding and enzymic activity[2].

MATERIALS AND METHODS

Yeast enolase was prepared by the method of WESTHEAD AND MCLAIN[3]. Phosphoserine, 3-phosphoglyceric acid and 2-phosphoglyceric acid were purchased from Sigma; glycerol phosphate came from Eastman Kodak. 2-Phosphoglycerate was

converted from the barium salt to the tricyclohexylammonium salt before use[1]. Glycolic acid phosphate, β-chlorolactic acid phosphate and lactic acid phosphate were generous gifts of Drs. F. Hartman and F. Wold.

The methods and equipment used in these studies were described previously[1]. "Metal-free" enolase was prepared by adsorbing the Mg^{2+}-contaminated enolase on phosphocellulose at pH 6.0 and washing the adsorbed enzyme six times with 10^{-3} M sodium acetate, pH 6.0 (ref. 3). The preparations had 0.5–1.5 moles of Mg^{2+} left per mole of enzyme, estimated from the fluorescence changes resulting from the addition of excess EDTA and Mg^{2+} (ref. 1). After correcting for this remaining metal ion, the preparations gave results which were reproducible and consistent with results obtained using enzyme which was treated with an excess of EDTA and chromatographed on Sephadex G-25 (ref. 1).

RESULTS AND DISCUSSION

Addition of substrate (an equilibrium mixture of 20% 2-phosphoglycerate and 80% phosphoenolpyruvate) to a solution of yeast enolase which is 0.01 M in Mg^{2+} causes an increase in the fluorescence emission of the enzyme[1] (Fig. 1). The reagents are together and separately optically transparent at the exciting wavelength (275–300 nm) and non-fluorescent under any known conditions. The effect of substrate and

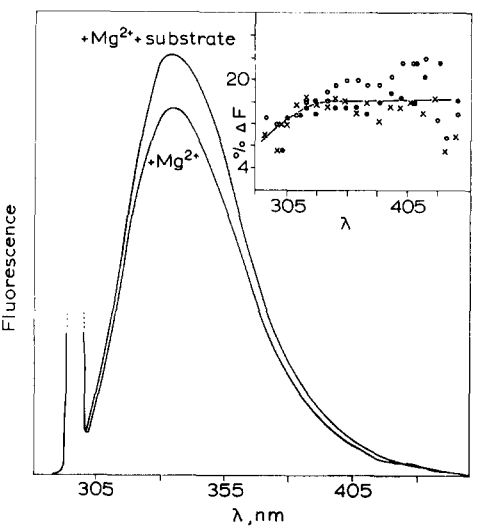

Fig. 1. Effect of substrate and excess Mg^{2+} on the fluorescence emission spectrum of yeast enolase. The fluorescence emission spectrum of a solution containing 0.06 M Tris–HCl, pH 7.8, 0.01 M $MgCl_2$ and $7.5 \cdot 10^{-6}$ M yeast enolase in a total volume of 2.0 ml was scanned after the solution had been allowed to come to 25° in the dark. Then 10 μl of 0.2 M 2-phosphoglyceric acid (as the tricyclohexylammonium salt) was added, mixed into the solution, and the fluorescence emission scanned again. The excitation wavelength was 295 nm. The excitation and emission band widths were both 2.2 nm. Front face ilumination was used[1]. The spectra are not corrected for monochrometer transmission efficiency or photomultiplier spectral response, as these corrections are small[1]. Inset: Typical changes in emission spectrum of the enzyme in 0.01 M Mg^{2+} produced by addition of substrate to 10^{-3} M, expressed as the percentage of change in fluorescence ($\%\Delta F$) as a function of emission wavelength. ×, excitation at 275 nm; ○, 285 nm; ●, 295 nm. Excitation at different wavelengths is done in an attempt to separate tyrosine and tryptophan contributions to the emission and changes in emission.

TABLE I

EFFECT OF VARIOUS METAL IONS AND PHOSPHATE ESTERS ON THE FLUORESCENCE AND ACTIVITY OF YEAST ENOLASE

The conditions of measurement were similar to those described in the legend to Fig. 1, except that the excitation wavelength was 280 nm and the emission wavelength was 335 nm. The data are not corrected for changes in the absorption of the enzyme (see text), as these are small.

Effector	*Maximum % change in fluorescence*	*activity with enolase*[4]
Mg^{2+}	+16	++
Mn^{2+}	+12	+
Zn^{2+}	+14	+
Ni^{2+}	+ 2	Weak
Ca^{2+}	+ 2	o
Co^{2+}	− 2	Weak
Substrate	+16	Substrate
β-Chlorolactic acid phosphate	+13	Weak substrate
Glycolic acid phosphate	+20	Competitive inhibitor
Lactic acid phosphate	+12	Competitive inhibitor
3-Phosphoglyceric acid	+ 4	Competitive inhibitor
Glycerol phosphate	− 1	No effect
Phosphoserine	− 1	No effect

excess Mg^{2+} occurs over a pH range of 5.5 to 8.8, where the enzyme is active. It is independent of buffer.

This effect should not be confused with the magnesium effect described in reference 1. The latter requires only Mg^{2+} and is saturated at Mg^{2+} concentrations above 10^{-4} M. The former requires considerably higher concentrations of metal ion and substrate or competitive inhibitor; neither alone is sufficient.

A greater increase in the fluorescence of the enzyme is produced by glycolic acid phosphate, a competitive inhibitor of the enzyme (Table I). Other competitive inhibitors also produce increases in fluorescence, the magnitude of the increase varying with the inhibitor; compounds which are not inhibitors produce no effect. There is no correlation between the binding constant of the inhibitor to the enzyme and the size of the fluorescence change.

Other metal ions besides Mg^{2+} also produce an increase in enzyme fluorescence in the presence of substrate (Table I). It is evident that, except for Co^{2+} and Ni^{2+} (which produce only very low levels of activity)[4], only those metal ions which activate the enzyme allow the increase in fluorescence. The ion-specificity data should be interpreted with caution, however, since some metal ions at high concentrations have effects on the fluorescence of the protein which are not connected with the metal–substrate effect described here. The percent increases in Table I are the maximum increases found during a titration with the indicated metal ion, covering a concentration range between 10^{-6} and 10^{-2} M.

These data suggest that these increases in fluorescence are produced by binding at the active site of the enzyme.

An analysis of the change in fluorescence produced by metal and substrate is also presented in Fig. 1. The percentage increase in fluorescence is plotted as a function

of emission wavelength. A similar method of analysis has been used in determining the effects of magnesium and chloride and acetate salts on the conformation of yeast enolase[1,5].

In the case of the ion–substrate effect, a relatively constant percentage increase is found at emission wavelengths above 320 nm, where only tryptophan emits. This pattern suggests that tyrosine fluorescence is not affected by metal ion and substrate binding, and that the increase in tryptophan emission is due to a decrease in quenching of one or more tryptophans in the enzyme. Both protonated amine and unionized carboxyl groups can quench tryptophan[6]. Mg^{2+} and substrate have no effect on the fluorescence of free tryptophan, so a direct interaction is not likely to be involved.

The binding of substrate in the presence of high concentrations of Mg^{2+} is also accompanied by a change in the absorption spectrum of the enzyme (not shown). The absorption difference spectrum exhibits two nearly equal peaks of decreased absorption at 291 and 284 nm. The molar difference extinction coefficients, using a mol. wt. of 90 000 (ref. 7) is 1200 at 291 nm and 1000 at 284 nm. This is considerably larger than the value expected for "electrostatic" perturbations (such as pH) of tryptophan, which are about 200 (ref. 8). However, more than one tryptophan may be involved.

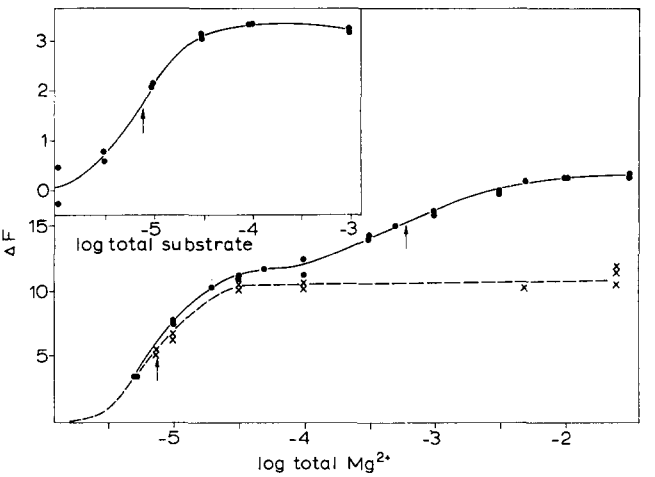

Fig. 2. Fluorometric titrations of yeast enolase with Mg^{2+} in the presence of excess substrate. 2.0 ml of a solution containing 0.035 M Tris–HCl pH 7.8, $7.5 \cdot 10^{-6}$ M yeast enolase and 0 or $3.3 \cdot 10^{-4}$ M 2-phospho-D-glyceric acid, (potassium salt) was allowed to come to 25° in the dark. Then the solution was excited with light at 280 nm and the fluorescence emission was observed at 318 nm. The slit band-widths for excitation and emission were 1.7 nm and 3.3 nm, respectively. Small amounts of concentrated Mg^{2+} solutions were added with microliter pipettes and the change in fluorescence emission intensity (ΔF) was recorded after mixing. No correction was made for dilution, as this was less than 0.5%. ΔF is the change in fluorescence, in arbitrary units. The experiment was done in duplicate. The estimate of Mg^{2+} initially present in the enzyme was made from the fluorescence changes occurring on addition of excess EDTA and Mg^{2+} to a duplicate sample[1]. The arrows indicate the inflection points in the titration curves. ×, results obtained in the absence of substrate; ●, results in the presence of $3.3 \cdot 10^{-4}$ M substrate. Inset: Typical titration of yeast enolase with substrate in the presence of 0.01 M Mg^{2+}. The conditions and procedures of the titration were the same as those described above, except that 0.1 M imidazole–acetate, pH 6.6, was used as the buffer, and that no substrate was initially present. The data are from an experiment done at a different time, and the fluorescence units are not comparable to those of the above experiment.

There is no significant effect of ion–substrate binding on the sedimentation constant or rotational relaxation time of the enzyme (not shown).

The fluorescence increase can be used to investigate the binding of both metal ion and substrate. Fig. 2 shows an example of a fluorescence titration at low ionic strength of the enzyme with Mg^{2+} in the presence of substrate. There are two binding sites for Mg^{2+}, the stronger one having a dissociation constant of about $3 \cdot 10^{-6}$ M and the weaker site having a dissociation constant of $5 \cdot 10^{-4}$ M. The data also show that the weaker site appears only in the presence of substrate.

A number of similar titrations were made, using competitive inhibitors instead of substrate, and titrating with substrate in the presence of varying concentrations

TABLE II

DISSOCIATION CONSTANTS FOR COMPLEXES OF YEAST ENOLASE WITH Mg^{2+}, SUBSTRATE AND COMPETITIVE INHIBITORS

Corrections of observed binding constants for protein concentration and for nonspecific interactions between K^+ or Mg^{2+} and the phosphate esters were made using the association constants of WOLD AND BALLOU[9] and as described in ref. 1. An average association constant of 200 was assumed for the Mg^{2+}–"substrate" complex, and 280 was taken as the association constant for Mg^{2+} and glycolic acid phosphate. Small changes in these values would not greatly affect the calculated dissociation constants. The figures in brackets are those of HANLON AND WESTHEAD[10,11]. Where two values are given separated by a semicolon, these are from separate experiments. Most individual experiments were done in duplicate PEP = phosphoenolpyruvate; 2-PGA = 2-phosphoglyceric acid.

(A) Mg^{2+}-enolase dissociation and Michaelis constants:

Other additions	*K_d (M)*	*K_m (Mg^{2+}, M)*
–	$3.5 \cdot 10^{-6}$	
0.5 M KCl	$1.2 \cdot 10^{-5}$ [(1) $1 \cdot 10^{-5}$] [(2) $5 \cdot 10^{-4}$]	
0.5 M potassium acetate	$8 \cdot 10^{-6}$	
$3 \cdot 10^{-4}$ M substrate	(1) $3.2 \cdot 10^{-6}$ (2) $5 \cdot 10^{-4}$	$2.4 \cdot 10^{-5}$ (conc. enzyme) $6 \cdot 10^{-5}$ (dil. enzyme)
$3 \cdot 10^{-4}$ M substrate + 0.5 M KCl	(1) $8 \cdot 10^{-6}$ (2) $1.5 \cdot 10^{-3}$–$1.7 \cdot 10^{-3}$; $1.8 \cdot 10^{-3}$–$2.1 \cdot 10^{-3}$	$5.4 \cdot 10^{-4}$ (dil. enzyme) [$4 \cdot 10^{-4}$ (2-PGA); $5.5 \cdot 10^{-4}$ (PEP)]
10^{-3} M substrate + 0.5 M KCl	(2) $8.1 \cdot 10^{-4}$; $1.0 \cdot 10^{-3}$–$1.1 \cdot 10^{-3}$ [(1) $1 \cdot 10^{-5}$; (2) $1.7 \cdot 10^{-4}$; (3) $1.3 \cdot 10^{-3}$]	
10^{-3} M glycolic acid phosphate + 0.5 M KCl	$2.7 \cdot 10^{-3}$; $3.2 \cdot 10^{-3}$–$3.5 \cdot 10^{-3}$	
$3 \cdot 10^{-3}$ M glycolic acid phosphate + 0.5 M KCl	$1.6 \cdot 10^{-3}$–$8 \cdot 10^{-3}$	

(B) Phosphate ester–enolase dissociation and Michaelis constants:

Other additions	*K_d (M)*	*K_m (substrate, M)*
$3 \cdot 10^{-3}$ M $MgCl_2$	$5.0 \cdot 10^{-5}$	
$3 \cdot 10^{-2}$ M $MgCl_2$	$9.0 \cdot 10^{-6}$	
$3 \cdot 10^{-3}$ M $MgCl_2$ + 0.5 M KCl	$2.0 \cdot 10^{-4}$	[$1.85 \cdot 10^{-4}$ at $1 \cdot 10^{-3}$ M Mg^{2+} (2-PGA)] [$2.2 \cdot 10^{-4}$ at $2 \cdot 10^{-3}$ M Mg^{2+} (PEP)]
$1 \cdot 10^{-2}$ M $MgCl_2$ + 0.5 M KCl	$8 \cdot 10^{-5}$	
$3 \cdot 10^{-2}$ M $MgCl_2$ + 0.5 M KCl	$3.9 \cdot 10^{-5}$	

of Mg^{2+}, and in the presence or absence of 0.5 M KCl. A summary of the binding constants, corrected for protein concentration and nonspecific binding between metal ion (Mg^{2+} and K^+) and substrate[9] is presented in Table II.

The stronger binding site corresponds to the one shown by BREWER AND WEBER to involve a conformational change in the enzyme. A recent study of ion binding to enolase by equilibrium dialysis and an investigation of the kinetics of the enolase reaction have been made by HANLON AND WESTHEAD[10,11]. These authors found that 2 moles of Mg^{2+} are bound in the absence of substrate, and that the most firmly bound Mg^{2+} is bound with a dissociation constant in 0.5 M KCl of $1 \cdot 10^{-5}$ M, which agrees with our data (Table II). The second mole of Mg^{2+} is bound with a dissociation constant in 0.5 M KCl of $5 \cdot 10^{-4}$ M, but our fluorescence titrations give no hint of this occurring (see below).

In the presence of substrate, HANLON AND WESTHEAD[10] found that two more moles of Mg^{2+} were bound, the two new sites having dissociation constants of about $1.3 \cdot 10^{-3}$ M (Table II). The precision of the equilibrium dialysis technique is low in this concentration range, however.

Our data confirm the appearance of this additional site(s), though we cannot measure the stoichiometry of Mg^{2+} binding by fluorescence titrations. If 2 moles of Mg^{2+} are bound, they are bound independently of each other, for the slope of our titration curves is that expected for a single binding site, or several independent sites. The binding constant we find for the additional site(s) is in fair agreement with that calculated by HANLON AND WESTHEAD[10], though we believe our data are more reliable in this concentration region.

Titrations of the enzyme with substrate in the presence of excess Mg^{2+} show a single dissociation constant and either a single site or several independent sites. Yeast enolase is known from titration studies to bind 2 moles of a competitive inhibitor[12].

The dissociation constant for the additional Mg^{2+}-site(s) and the dissociation constant for substrate or competitive inhibitors appears to be affected by the concentration of the other ligand (Table II). This is reasonable, if the additional Mg^{2+}-binding site(s) consists in part of the substrate or competitive inhibitor.

Table II gives a comparison of the binding constants obtained from this study and that of HANLON AND WESTHEAD[10] and the Michaelis constants obtained from measurements by us and by HANLON AND WESTHEAD[11] of the kinetics of the enolase reaction. There is close agreement between the substrate binding and Michaelis constants.

However, the additional Mg^{2+}-binding site(s) has a dissociation constant that is clearly greater than the Michaelis constant for Mg^{2+}. The "silent" Mg^{2+}-binding site, with a dissociation constant in 0.5 M KCl of $5 \cdot 10^{-4}$ M, appears to correspond most closely to the Michaelis constant. We conclude that the second, "silent" Mg^{2+}-binding site is the one that is kinetically relevant, and that the additional site(s) appearing in the presence of substrate have nothing to do with the activity of the enzyme. Indeed, they may represent the Mg^{2+}-binding site(s) which are associated with the inhibition of the enolase reaction found at high Mg^{2+} concentrations[11]. The data in this paper confirm and extend Hanlon and Westhead's findings.

Yeast enolase is now known to consist of two identical subunits[13] and there are probably two substrate binding sites[12]. The central question in the activation of

enolase by Mg^{2+} now concerns the very different binding affinities of the two strongest Mg^{2+}-binding sites and the very different effects of the binding of these first two Mg^{2+} on the enzyme. Either the two sites are chemically different (that is, different amino acids are involved in the binding) or they are originally identical and the binding of the first mole of Mg^{2+} produces a change in the enzyme that lowers the affinity of the second site for Mg^{2+}.

Related to this question is what relation the Mg^{2+}-binding sites bear to the substrate binding sites. There appears to be only one detectable type of substrate binding site, whether the affinity constant is measured enzymically or fluorometrically[11]. It is possible that the 2 active sites are each controlled by one of the Mg^{2+}, but one has a low turnover number[14], or that both sites are controlled by a single Mg^{2+}. Further work must be done to decide these questions.

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