DIRECT MEASUREMENT OF PROTON RELEASE BY YEAST ENOLASE UPON BINDING MAGNESIUM IONS

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1. Introduction

We recently reported that the binding of divalent metal ions to yeast enolase (2-phospho-D-glycerate hydrolyase, EC 4.2.1.11) is coupled to proton equilibria [1]. This conclusion was based on flow microcalorimetric measurements of the heat of metal binding in three buffers with different heats of ionization. The thermal data were shown to be consistent with the release of two protons when the two, specific cation binding sites on the apoenzyme [2] are filled with Mg^{2+} ions at pH 7.5 and 0.05 M ionic strength. The calorimetric experiments could not distinguish between the release of one proton as each Mg^{2+} ion binds and the release of both protons in response to the conformational change triggered by the binding of the first Mg^{2+} ion [3,4]. Neither could calorimetric measurements at a single pH establish the number or the identity of the titratable groups involved. We report direct measurements of proton release by yeast enolase upon binding Mg^{2+} ions undertaken to clarify these points.

The proton release–pH profile is an unsymmetrical bell-shaped curve. The maximum number of protons is released at pH 6.5 and exceeds two, implicating more than two titratable groups in metal binding. The shape of the curve above pH 6.5 is consistent with the complexation of metal by histidyl residues. Above pH 7 two Mg^{2+} ions bind with dissociation constants $\leqslant$ 5 μM, and proton release coincides with metal binding. The affinity of enolase for Mg^{2+} ions decreases below pH 7. The sharp decrease in the number of protons released below pH 6.5 may result from a change in the conformation of the protein.

2. Materials and methods

Enolase was purified from baker's yeast and made metal-free as previously described [1]. The specific activity at 240 nm [5] of the enzyme used in the experiments reported was 300 ± 30 units. An optical factor of 0.89 cm^{-1} mg^{-1} ml [6] and a molecular weight of 88 000 [7] were used to convert absorbance readings at 280 nm to the concentration of dimeric enzyme. Throughout the manuscript an equivalent is based on the dimer molecular weight. Analyzed reagent grade $Mg(C_2H_3O_2)_2 \cdot 4\,H_2O$ and ultra pure KCl were purchased from J. T. Baker Chemical Company. Standardized 0.1 M NaOH was obtained from the Hartman-Leddon Company.

The release of protons was monitored with a Radiometer PHM26/T TT11/SBR2/SBU1/TTA31 pH-stat system thermostatted at 25°C. In a typical experiment 1 ml of 0.1 mM metal-free enolase was adjusted to the desired pH, and the pH was kept constant by the addition of 1 mM NaOH, as microliter aliquots of 1 mM Mg $(C_2H_3O_2)_2$ were added. The ionic strength of the enzyme and titrant solutions was maintained at 0.05 M with KCl. Care was taken to purge all solutions of CO_2, and the titrations were performed under a water-saturated nitrogen atmosphere. The base strength was determined volumetrically using potassium hydrogen phthalate as a primary standard and either phenolphthalein or brom thymol blue as an indicator. Metal ion concentrations were measured with a Perkin-Elmer Model 303 atomic absorption spectrophotometer.

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3. Results and discussion

Typical complexometric titration curves at pH 6 and at pH 7 are shown in fig. 1. At the latter pH the release of protons is directly proportional to the number of equivalents of magnesium added per mole of dimeric enzyme for the first 85% of the reaction. Since this corresponds to the addition of 1.7 equivalents of metal, the data confirm our earlier report [1] that two Mg^{2+} ions bind immeasurably tightly to the apoenzyme above pH 7 and at low ionic strength. The solid line through the experimental points is the theoretical curve calculated for the binding of magnesium to two independent sites with dissociation constants of 0.02 and 1 μM. Two earlier studies [1,2] have demonstrated that the first Mg^{2+} ion binds approximately fifty times more tightly than the second. A conservative estimate of the maximum dissociation constant for the weaker bound Mg^{2+} ion, based upon all of our complexometric titrations above pH 7, is 5 μM.

Although two Mg^{2+} ions bind to the apoenzyme, only the first is required to cause ultraviolet absorption and fluorescence emission changes that have been interpreted as a change to the catalytically active conformation of enolase [3,4]. The possibility existed, therefore, that proton release coincided with the conformational change triggered by the binding of a single Mg^{2+} ion. The direct proportionality at pH 7 between proton release and the binding of nearly two equivalents of metal clearly demonstrates that protons are released as each of the two metal binding sites is filled.

It is apparent from a comparison of the titration curves at pH 6 and at pH 7 that Mg^{2+} ions bind less tightly at pH values below neutrality. It has previously been reported that the binding of Zn^{2+} ions to enolase is impaired below pH 7 [8]. The experiments reported here do not permit a decision on whether the total number of Mg^{2+} ions that bind is reduced at low pH values. The dashed line drawn through the pH 6 data in fig. 1 was calculated for the binding of two metal ions with dissociation constants of 1 and 50 μM. The dotted line was calculated by assuming that only one Mg^{2+} ion binds at pH 6 with a dissociation constant of 35 μM.

Fig. 2 shows the number of equivalents of protons released, following the addition of approximately 10 equivalents of metal, as a function of pH. Each plotted point is the average of several determinations. The

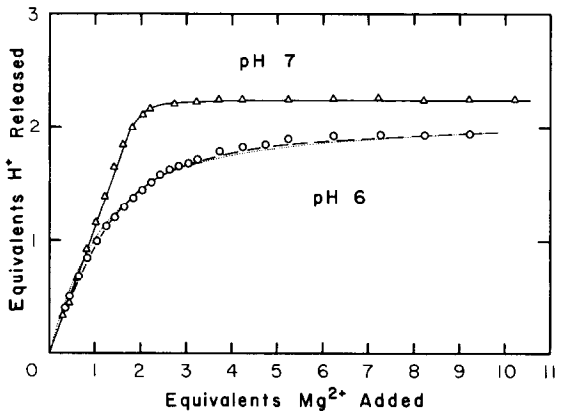

Fig. 1. The number of protons released per mole of enolase dimer as a function of the number of equivalents of Mg^{2+} ions added. Ionic strength 0.05 M and 25°C. (△) pH 7. (○) pH 6. Calculated curves: (——) two independent metal binding sites with dissociation constants K_1 = 0.02 μM and K_2 = 1 μM, (- - - -) two independent metal binding sites with dissociation constants K_1 = 1 μM and K_2 = 50 μM, (. . . . .) one metal binding site with dissociation constant K = 35 μM.

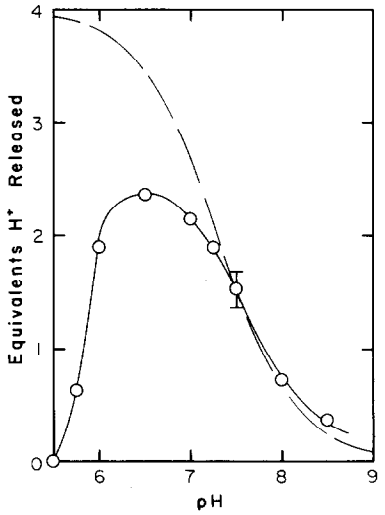

Fig. 2. The average number of protons released per mole of enzyme dimer when Mg^{2+} ions bind to yeast enolase as a function of pH. Ionic strength 0.05 M and 25°C. The vertical bar indicates the standard deviation in the measurements at pH 7.5. The dashed line is the proton release–pH profile expected for the binding of two metal ions to four titratable groups with pK_a = 7.3.

principal sources of uncertainty in the number of protons released are the amount of endogenous metal present in the enzyme sample and the strength of the dilute base used to maintain the pH constant. The vertical bar in fig. 2 represents the standard deviation in seven measurements at pH 7.5 made with different enzyme preparations and different titrant solutions. The average number of protons released at pH 7.5 is in satisfactory agreement with the value of two previously deduced from calorimetric measurements [1], especially considering the sharp dependence of the number of protons released on pH in this region.

The maximum number of equivalents of protons is released at about pH 6.5 and exceeds two. Consequently, it appears that more than two titratable groups are involved in metal binding to enolase. The experimental results reported here do not permit a choice between three and four titratable groups, although the former possibility is less attractive because of the stoichiometry of metal binding and the apparent identity of the enzyme's subunits [9].

Histidine has been implicated in the active site of enolase by metal binding [8], by kinetic [10,11], and by chemical modification [12] experiments. The observed decrease in the number of protons released as the pH is raised above 6.5 is reasonably consistent with the titration of the imidazole side chain of histidine. The dashed line in fig. 2 is the proton release–pH profile that would be expected if two metal ions bound throughout the pH range studied to four identical groups with a pK_a of 7.3. The pK_a of the 4-methyl imidazolium ion is 7.52, and histidyl side chains in proteins normally titrate in the pH range 6.4–7.4 [13]. Either nonidentity, or interaction of the titrating groups would broaden the expected titration curve.

Histidine was first implicated in the binding of metal ions by enolase to explain an apparent decrease in the number of Zn^{2+} ions bound as the pH was lowered from 7.8 to 5.1 [8]. The decrease in the number of protons released that we observe below pH 6.5 appears to be too sharp to attribute to a decrease in the number of metal ions bound as a result of either a first, or a second-order protonation process. An alternative possibility is that the enzyme undergoes a conformational change to a form unable to bind metal ions. Two laboratories have reported an abrupt change in the conformation of yeast enolase with a midpoint at about pH 5 [14,15].

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Note added in proof

Complexometric titrations in the absence of added salt confirm that more than two titratable groups are involved in metal binding to yeast enolase and permit a choice between three and four chelating groups. Isoionic protein was adjusted to the desired pH, and the number of protons released upon addition of an excess of magnesium was measured. An average of 3.0 moles of protons are released per mole of dimeric enzyme at pH 7.5, and an average of 3.4 equivalents of protons are released at pH 6.5. These results strongly suggest that each of the two Mg^{2+} ions that bind to the apoenzyme complexes with two titratable groups.

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