THE METAL SPECIFICITY OF MAMMALIAN α-AMYLASE AS REVEALED BY ENZYME ACTIVITY AND STRUCTURAL PROBES

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1. Introduction

The α-amylases which have been studied to date all contain one firmly bound atom of calcium per enzyme molecule [1]. Previous investigators [2, 3] have suggested that the Ca^{2+} acts to stabilize a tertiary structure of the enzyme which yields a catalytically active molecule and which renders the enzyme resistant to proteolytic degradation. α-Amylase binds calcium very tightly with a binding constant of 2×10^{11} [4]. Previous work has been complicated by this high affinity of α-amylase towards calcium. Thus, studies performed using glass equipment are of questionable validity. In addition, and because of the almost ever-present calcium contamination, previous workers [5] have studied metal specificity in the presence of EDTA. EDTA binds most divalent metal ions more strongly than Ca^{2+}; therefore Ca–EDTA may act as a Ca^{2+} donor when divalent metal ions are added to an assay system containing both Ca^{2+} and EDTA. Indeed, lanthanides were found to activate α-amylase when studied in the presence of EDTA [5]. Later, this effect was demonstrated to be the result of Ca^{2+} contamination and, in the absence of EDTA, no lanthanide activation of α-amylase was noted [6]. In addition, as mentioned in this communication, starch, which is usually used as the substrate for α-amylase contains tightly bound calcium which is resistant to chelation and removal by extensive treatment with EDTA.

The present report describes the metal specificity of α-amylase using an assay system which does not include glass equipment, EDTA, or starch. In addition, the role of calcium in the maintenance of the active conformation of α-amylase is explored using structural probes.

2. Materials and methods

Amylase was prepared from undiluted porcine pancreatin (Miles Marschall Division, Elkhart, Indiana) according to the method of Loyter and Schramm [7]. Chelex-100 was purchased from Biorad Co. DTNB was purchased from Calbiochem. PNPM was synthetized according to the method of Matsubara [8]. Dialysis tubing, 20 mm, was purchased from Union Carbide. $[^{203}Hg](NO_3)_2$ was purchased from the Radiochemical Center. The Mn(II), Cd(II), Co(II), Ni(II) and Mg(II) ions were used as their sulfate salts and were purchased from Johnson Matthey Chemicals. The other metals used, $BaCl_2$, $SrCl_2$, $CaCl_2$ and Nd_2O_3, as well as all other reagents were of the highest purity available. Calcium contamination in all metal solutions, reagents, etc., was measured using a Perkin-Elmer 290 atomic absorption spectrophotometer.

2.1. *Removal of Ca^{2+} from buffers and solutions*

Buffers, water and PNPM were passed through a Chelex-100 column (5.5 × 8 cm) equilibrated to pH 7.0. They were stored in polypropylene tubes and bottles boiled in 0.01 M EDTA pH 7.0 and washed with Chelex treated double distilled water. All assays were

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Abbreviations:
DTNB: 5,5′-dithio-bis(2-nitrobenzoic acid); DFP: diisopropylfluorophosphate; HEPES: *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; PIPES: piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); PNPM, *p*-nitrophenylmaltoside.

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performed in similarly prepared test tubes. Eppendorf micropipette tips and polypropylene pipettes were used.

2.2. *Preparation of calcium free enzyme*

Purified porcine pancreatic α-amylase was placed in a continuous flow dialysis cell (Colowick cell [9]) using 20 mm dialysis tubing as the membrane and dialysed at 4° for 10 days against a continuous flow of 0.01 M EDTA pH 7.0, containing 2 μg/ml DFP. The enzyme was then dialysed at 4° against Chelex treated 0.02 M HEPES or PIPES buffer pH 7.0 containing 2 μg/ml DFP for an additional 5 days to remove residual EDTA and Ca–EDTA.

2.3. *Assay for metal specificity*

Two types of assays were employed. The first, using PNPM as a substrate measures directly the extent of enzyme activation. The second technique measures the ability of the added metal ion to mask SH groups from reacting with DTNB.

2.4. *PNPM assay*

This assay uses p-nitrophenyl maltoside. The k_{cat} of α-amylase towards PNPM is 24,000 times less than the k_{cat} towards starch [10]. Because calcium may be easily removed from PNPM by passage through a Chelex-100 column, this assay is more reliable than a starch assay. 2.5×10^{-5} M enzyme was incubated with 0.02 M Chelex treated PNPM in 0.02 M PIPES pH 7.0 for 30 min at room temp. The reaction was stopped and the pH raised to 9.0 by addition of 0.5 M glycine-NaOH buffer pH 9.0 and activity calculated from the absorption by liberated p-nitrophenol at 400 nm. Using native α-amylase under similar conditions, this reaction was found to be linear during the first 45 min. The maximal k_{cat} of α-amylase for PNPM is 5.0×10^{-2} sec [10]. Metals were added to 2.5×10^{-4} M concentrations.

2.5. *DTNB assay*

The SH groups of α-amylase react with DTNB only after prior removal of Ca^{2+} [4, 11]. In the native enzyme, these SH groups are masked. Replenishment of calcium in the calcium free apoenzyme will remask the SH groups and prevent reaction with DTNB. Calcium free α-amylase (4×10^{-5} M) and apo-amylase with the various metals added was incubated at room temp. for 45 min with DTNB (8×10^{-4} M) in 0.02 M Chelex treated HEPES pH 8.0. The various metals added were in final conc. of 1×10^{-4} M. Absorption at 412 nm was measured spectrophotometrically. Using the molar extinction coefficient of TNB^- = 13,600 [12], the number of reacting SH groups per enzyme was calculated. The DTNB solutions used were calcium free.

2.6. *Preparation of calcium free mercury-enzyme derivatives*

As previously reported [4], with 2 SH groups of porcine α-amylase, these groups lie close enough to each other so that the 2 derivatives $(-S-Hg)_2$ and (–S–Hg–S–) may be prepared. These derivatives are fully active in the presence of calcium. Therefore, the Ca^{2+}-free Hg(II) derivatives were prepared to determine if, even in the absence of Ca^{2+}, the (–S–Hg–S–) bridge would maintain the enzymatic activity.

Amylase was incubated at 4° with $[^{203}Hg](NO_3)_2$ in equimolar and 3:1 molar ratios of mercury to enzyme. Completion of this reaction was demonstrated by reacting aliquots of the Hg-enzyme derivatives with DTNB in the presence of EDTA pH 8.0. After Hg(II) reaction with the SH groups, no reaction occurs with DTNB [4]. The Hg-enzyme derivatives were then dialyzed at 4° against 0.01 M EDTA pH 7.0 containing 2 μl/ml DFP using 20 mm dialysis tubing. Aliquots were then counted in a Packard Tricarb scintillation counter and activity determined by using starch as the substrate in the presence of 10^{-2} M EDTA. Calcium contamination of the Hg–enzyme derivatives was determined using a Perkin-Elmer 290 atomic absorption spectrophotometer.

3. Results

3.1. *Preparation of calcium free HEPES, PIPES, water and PNPM*

After passage of these solutions through the Chelex column, residual calcium could not be detected by atomic absorption spectrophotometry.

3.2. *Calcium contamination of metals studied*

Atomic absorption spectrophotometric analysis of the metals used revealed that 0.1 M solutions of the metal ions used contain less than 10^{-5} M Ca^{2+}.

Table 1
Metal specificity of α-amylase.

Metal	Activation using PNPM[a] (%)	SH groups per enzyme reacting with DTNB[b]
None	< 5	1.7
Ca^{2+}	100 ± 5	0.4
Sr^{2+}	100 ± 5	0.4
Ba^{2+}	88 ± 5	0.9
Mn^{2+}	75 ± 5	1.4
Cd^{2+}	28 ± 5	1.5
Nd^{3+}	5 ± 5	1.8
Co^{2+}	5 ± 5	1.5

[a] Activity with Ca^{2+} was taken as 100% (see text); enzyme present in the assay = 2.5×10^{-5} M; metals added to a final conc. of 2.5×10^{-4} M. Other experimental details are given in the text.

[b] Binding of appropriate metal masks SH groups (see text); enzyme present in the assay = 4×10^{-5} M; metals added to 1×10^{-4} M final conc. Other details are given in the text.

3.3. *Preparation of calcium free α-amylase*

Using the described procedure, amylase was found by atomic absorption spectrophotometry to contain less than 0.05 moles calcium atoms per mole enzyme (50,000 molecular weight). The specific activity of this apoenzyme after replenishment of calcium and assay using starch varied between 1200 and 1300 units per mg, which represents about 85% of the usual native enzyme activity. The apoenzyme was found to be stable when stored with DFP at 4° in the absence of calcium for over 2 weeks. Maximal calcium effect was obtained with *equimolar* calcium and enzyme concentrations using either the PNPM or DTNB techniques eliminating the concern over residual EDTA in the system (see below).

Table 2
The activity of amylase $\langle \overset{S}{S} \rangle$Hg(II) and amylase $\langle \overset{SHg^+}{SHg^+}$.

Derivatives as a function of Ca^{2+} content:		
Hg(II) content (atoms per enzyme)	Ca^{2+} content (atoms per enzyme)	Activity (%)
1	1	100
2	1	100
0.85	0.19	18
1.20[a]	0.19	24

[a] This represents probably a mixture of molecules containing one mercury atom per enzyme and two mercury atoms per enzyme.

3.4. *Assay of metal specificity of calcium free apoenzyme using PNPM*

The Chelex treated PNPM was found to be free of residual calcium and gave basal activity for the calcium free apoenzyme of less than 5% in the *absence* of EDTA (table 1). The metal specificity results are summarized in table 1. The enzyme is fully activated by Ca^{2+} and Sr^{2+}; Ba^{2+}, Mn^{2+} and Cd^{2+} give sub-maximal activation while the activity with Co^{2+} and Nd^{3+} are below the limits of significance. Similarly the ions Mg^{2+}, La^{3+}, Ni^{2+} were found to be incapable of activating apo-α-amylase.

3.5. *Assay of metal specificity of calcium free apoenzyme using DTNB*

The results of the DTNB assay of metal specificity are also summarized in table 1. In the presence of calcium, 0.4 SH groups per enzyme react with DTNB while 1.7 SH groups react in the calcium free apoenzyme.

Ca^{2+} and Sr^{2+} which activate apo-α-amylase fully are also capable of masking the 2 SH groups. Ba^{2+}, which activates the apoenzyme, masks the SH groups only partially. Other metal ions which either activate apo-α-amylase partially (Mn^{2+}, Cd^{2+}) or not at all (Nd^{3+}, Co^{2+}) fail to mask the SH groups against DTNB.

3.6. *Preparation and assay of activity of calcium free Hg^{2+} enzyme derivative*

After extensive dialysis against 0.01 M EDTA with DFP, the Hg^{2+}-enzyme derived from reacting equimolar concentrations of enzyme and $[^{203}Hg](NO_3)_2$ was found to contain 0.85 Hg^{2+} atoms per enzyme molecule while that derived from reacting α-amylase with a 3 fold molar excess of $[^{203}Hg](NO_3)_2$ contained 1.2 atoms of Hg^{2+} per enzyme molecule. The activity of these Hg^{2+} enzyme derivatives was assayed using starch as the substrate in the presence of 10^{-2} M EDTA. The assay was repeated in the presence of 10^{-2} M Ca^{2+} to determine maximal activity. Using this technique, the Hg^{2+} enzyme containing 0.85 atoms of Hg^{2+} per enzyme molecule had 17% activity without added Ca^{2+} while the enzyme containing 1.2 Hg^{2+} per enzyme molecule had 24% activity (table 2).

Atomic absorption spectrophotometric study of the Hg-enzyme derivatives revealed that they contained 19% residual calcium contamination (0.19 atoms per enzyme molecule, table 2). Therefore, in the absence of all calcium, the Hg-enzyme would be devoid of catalytic activity.

4. Discussion

4.1. *Calcium content of starch*

We were unable to remove *all* the calcium from starch even after prolonged treatments with EDTA according to published procedures [3, 13]. The starch polymer probably contains firmly bound and non-exchangeable calcium. Whenever the calcium free enzyme was assayed in the absence of added EDTA 20–30% activity was obtained. This is probably the reason why previous workers [5, 13] included EDTA in the assay. This problem was solved when PNPM was prepared and was easily cleaned of Ca^{2+} contamination.

4.2. *Metal specificity*

Upon removal of Ca^{2+} from the enzyme, 2 SH groups become exposed and the enzyme loses its amylolytic activity. Upon binding Ca^{2+} to apo-α-amylase, full activity is regained and the two SH groups are masked. The ability of metal ions to activate apo-α-amylase and to mask the 2 SH groups was investigated. Ca^{2+} and Sr^{2+} cause 100% reactivation and mask completely the 2 SH groups (table 1). Ba^{2+} activates amylase to 88% activity but masks only 1 SH group. Mn^{2+} and Cd^{2+} which cause 75% and 28% activity, respectively, fail to mask the two SH groups even partially. The ions Co^{2+}, Ni^{2+}, Mg^{2+}, Y^{3+}, La^{3+}, Nd^{3+}, UO_2^{2+}, VO^{2+} fail to activate the apoenzyme or to mask the two SH groups.

In conclusion, all metal ions which are capable of masking the two SH groups against DTNB even partially, activate amylase. However, some of the metal ions which fail to mask the SH groups can still activate the enzyme.

4.3. *The role of Ca^{2+}*

The apoamylase in which the two SH groups have been bridged by Hg(II) was found to be inactive in the absence of Ca^{2+}. This indicates that keeping the two SH groups close to each other is not enough to maintain the active architecture of the enzyme active site. One should note, however, that the mercury derivatives of α-amylase are fully active in the presence of calcium (table 2).

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