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Abortive Complexes of α-Amylases with Lanthanides†

Alexander Levitzki* and Jacques Reuben

ABSTRACT: Apoamylases from both hog pancreas and *Bacillus subtilis* were prepared by removing the tightly bound Ca^{2+}. In both cases lanthanide ions fail to activate the enzyme. Furthermore, in both cases the rare earth ions do not compete for the essential Ca^{2+} binding site and therefore do not inhibit the Ca^{2+} activation process. *B. subtilis* α-amylase possesses two Ca^{2+} binding sites, one of which can bind Gd^{3+}, with an additional site for the lanthanide ion.

The lanthanide series constitutes a group of 14 elements in which the electronic f shell is progressively filled providing them with diverse spectral and magnetic properties. Because of the close similarity in ionic radii of the lanthanides with the calcium ion it has been suggested (Birnbaum *et al.*, 1970; Darnall and Birnbaum, 1970; Williams, 1970) that these ions can be used as replacements for calcium and used as probes for the calcium binding sites in biological systems. This possibility seems to be highly attractive since the calcium ion has filled electron shells and thus is devoid of spectral and magnetic properties which can be used to probe its mode of interaction with its binding site. The lanthanides seem to open a new dimension in the study of numerous biological systems which require calcium for their function and stability. Indeed it has been recently reported that some lanthanide ions can replace Ca^{2+} in a few systems such as the enzyme α-amylase from *Bacillus subtilis* (Smolka *et al.*, 1971), thermolysin from *B. thermoproteolyticus* (Colman *et al.*, 1972), and as substitutes for Ca^{2+} in the activation of trypsinogen to trypsin (Darnall and Birnbaum, 1970).

α-Amylases are known to be calcium metalloenzymes in which the metal ion is absolutely required for functional integrity, yet the specific role of the metal has not been delineated. We wish to report in this article our attempts to substitute the calcium ion by lanthanide ions in α-amylases from hog pancreas and *B. subtilis*.

Materials and Methods

Soluble starch Analar was obtained from British Drug House. Charcoal (Darco G60) was obtained from the British Drug House and treated according to Whistler and Durso (1950). Solutions of lanthanide were prepared by dissolving the metal sesquioxides (Alfa Inorganics) in HCl. All other chemicals used were of the highest purity available.

B. subtilis α-amylase was purchased from Calbiochem. The enzyme was dialyzed against Hepes[1] (0.02 M, pH 6.9) in the cold and centrifuged (12,000*g*) to remove insoluble material. The enzyme was found to be over 95% homogeneous using gel electrofocusing (Wrigley, 1968).

Hog pancreatic α-amylase was prepared according to Loyter and Schramm (1962) except for the charcoal–calcite stage. The α-limit dextrins were removed from the enzyme by charcoal treatment in batches at pH 8.5 using washed Darco G60 charcoal activated by heating at 100° overnight, before use. The activity of α-amylases was measured at pH 6.9 as described previously (Loyter and Schramm, 1962). The specific activity of the enzyme was found to be 1550 units/mg using the modified Bernfeld assay (Loyter and Schramm, 1962).

Protein concentration was measured using the extinction coefficients $A^{280}_{1\%} = 24.3$ for pancreatic α-amylase and $A^{280}_{1\%} = 25.3$ for the *B. subtilis* α-amylase (Stein *et al.*, 1964).

The preparation of apo-α-amylases was performed by continuous dialysis against 0.01 M EDTA (pH 7.0 at 4°) using a

† From the Departments of Biophysics (A. L.) and Isotope Research (J. R.), The Weizmann Institute of Science, Rehovot, Israel. *Received August 8, 1972.*

[1] Abbreviations used are: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Mops, 2-(*N*-morpholino)propanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; DFP, diisopropyl phosphorofluoridate.

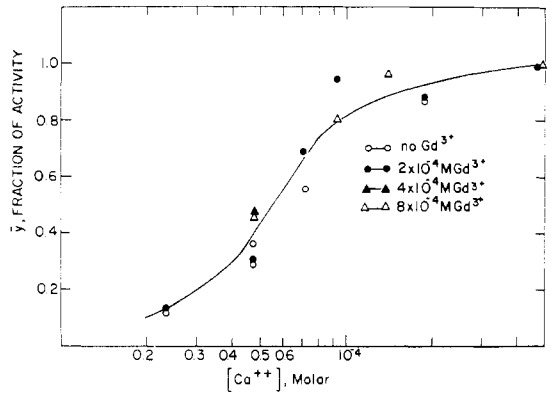

FIGURE 1: The activation of *B. subtilis* α-amylase by Ca^{2+}. Enzyme activity was measured at pH 4.6 maintained by 0.02 M sodium acetate buffer in the presence of increasing concentrations of $CaCl_2$. Different $GdCl_3$ concentrations were incorporated in the assay mixture as is indicated in the figure.

Colowick cell (Colowick and Womack, 1969). The enzymes were treated with DFP prior to the removal of Ca^{2+}. The calcium content was monitored by a Perkin-Elmer Model 303 atomic absorption spectrophotometer.

Ca^{2+}-free buffers were prepared by the removal of metal ions by the passage through Chelex-100 (Dowex A-1) columns. The removal of Ca^{2+} from soluble starch was achieved by prolonged (10 days) dialysis against 0.01 M EDTA (pH 7.0 at 4°) or by the passage of the starch through a long (2 × 50 cm) Chelex-100 column at pH 7.0.

All Ca^{2+}-free solutions were stored in polypropylene containers which were thoroughly washed with EDTA and double distilled water. It was found that the treatment of glass by the method suggested by Smolka *et al.* (1971) was insufficient to eliminate the "leakage" of Ca^{2+} from the glass. When the Bernfeld assay of apo-α-amylase (hog) was performed in a treated glass, 80–90% activity was detected although no external calcium was added to reactivate α-amylase. When, however, the assay was conducted in polypropylene or polycarbonate test tubes, not more than 7% activity was found in the absence of added Ca^{2+}. This residual activity probably reflects residual Ca^{2+} contamination in the assay system.

Pmr Measurements. Longitudinal proton relaxation rates (T_1) were measured at 24.3 MHz on a pulsed NMR Instrument (Bruker Inc., Germany), by the 180–90° null method (Reuben 1971). The paramagnetic contribution by Gd(III) to the water relaxation rate was obtained as described earlier (Reuben, 1971).

Results

The Inability of Rare Earth Ions to Activate α-Amylases. Apo-α-amylases were prepared from pancreatic α-amylase and *B. subtilis* α-amylase as described under Materials and Methods. At pH 6.9, no activation by lanthanide ions could be detected for either enzyme as is shown in Tables I and II whereas activation by Ca^{2+} took place readily. It was also found that the addition of 10^{-2} M Ca^{2+} to the apo-α-amylases after dilution in the presence of 6.65×10^{-4} M lanthanides did not restore the activity. This phenomenon may be due to the irreversible denaturation of the apoprotein by the hydroxo complexes of lanthanide ions. Such complexes are known to be formed at pH values close to neutrality.

The residual activity detected in pancreatic α-amylase in the presence of lanthanide ions (Table I) probably reflects the Ca^{2+} contamination in the system since doubling the amount of apoenzyme did not cause an increase in the absolute enzymatic activity measured and thus resulted in a decrease in the relative activity (Table I). The extreme affinity of α-amylases from various sources toward Ca^{2+} is well documented (Fischer and Stein, 1960; Stein *et al.*, 1969). Because of the hydrolysis of lanthanide ions and the high affinity of α-amylase toward calcium at neutral pH, measurements were made at pH 4.6. Under these conditions the affinity of the *B. subtilis* α-amylase toward calcium is reduced to values easily measured, and no hydroxo complexes of lanthanide ions are formed. Furthermore the *B. subtilis* α-amylase is stable at pH 4.6.

TABLE I: Activity of Pancreatic Apo-α-amylase in the Presence of Rare Earth Ions.

Ion	% Activity: 0.1-ml Apoenzyme Sample	% Activity: 0.2-ml Apoenzyme Sample
Y^{3+}	19	10.0
La^{3+}	19.5	10.5
Nd^{3+}	12.5	7.0
Sm^{3+}	16.0	7.5
Eu^{3+}	13.0	----
Gd^{3+}	16.0	9.0
Dy^{3+}	14.0	10.0
Ho^{3+}	17.0	9.0
Er^{3+}	17.0	8.0
Ca^{2+}	100[a]	100[a]
None	7.0	4.0
None[b]	88	

[a] The specific activity of the Ca^{2+} reactivated enzyme is 990, namely 76% of the native activity (specific activity = 1300 U/mg). [b] Assay conducted in a Pyrex test tube treated according to Smolka *et al.* (1971). Apo-α-amylase (7.35 mg/ml, 1.47×10^{-4} M) was diluted 800-fold into 0.02 M Hepes buffer (pH 6.9) containing 0.01 M KCl in the presence of 6.65×10^{-4} M lanthanide ion. The amylocytic assay was conducted in polypropylene test tubes and all the aliquots were taken using Eppendorf disposable tips.

The Effect of Gd^{3+} and Ca^{2+} on B. subtilis α-Amylase Activity at pH 4.6. At low pH it has been found that the dissociation constant of the Ca^{2+}–enzyme complex is increased to 5×10^{-5} M (Figure 1) and thus the sensitivity to Ca^{2+} impurities is decreased. Also, at low pH, lanthanide ions do not undergo hydrolysis as they do at neutral pH. Therefore the effect of Ca^{2+} and Gd^{3+} on the activation of apoamylase was checked at pH 4.6 (Figure 1). It can be seen that the activation of apoamylase by Ca^{2+} is not affected by Gd^{3+}. Also, Gd^{3+} alone, up to concentrations of 1×10^{-3} M, did not activate apoamylase. The two facts taken together mean that Gd^{3+} does not bind to the tight Ca(II) binding site of α-amylase. The presence of lanthanides at concentrations higher than 1×10^{-3} M in the assay system (pH 4.6) causes 20–30% inhibition. The addition of Ca^{2+} (0.01 M) restores the activity to normal.

Binding of Gd^{3+} to Native Pancreatic α-Amylases. When a solution of 5.5×10^{-5} M $GdCl_3$ at pH 6.3 (0.002 M Mops

TABLE II: Activity of *B. subtilis* Apo-α-amylase in the Presence of Rare Earth Ions.[a]

Ion	Concn in Assay (M)	% Activity
Gd^{3+}	1.06×10^{-3}	0.7
Dy^{3+}	1.24×10^{-3}	0.3
Er^{3+}	1.22×10^{-3}	1.5
Yb^{3+}	1.00×10^{-3}	1.8
Ca^{2+}	1.88×10^{-3}	100[b]

[a] Apo-α-amylase from *B. subtilis* was assayed in the presence of various metal ions in 0.02 M Hepes buffer (pH 6.9). [b] The specific activity of the reactivated apoenzyme was 1180 U/mg at pH 6.9.

buffer) was titrated with 6.1×10^{-6} M native α-amylase no change in the water relaxation rate (T_1) was observed. This indicated that no binding of Gd^{3+} to native α-amylase or any appreciable exchange of Ca^{2+} by Gd^{3+} occurred. This did not change within a period of 24 hr, indicating the absence of a slow exchange of the tightly bound Ca(II).

Binding of Gd^{3+} to B. subtilis α-Amylase. When a Gd^{3+} solution is titrated with *B. subtilis* α-amylase at pH 4.6 an enhancement (ϵ^*) of the water proton relaxation was observed (Figure 2). The enhancement of bound Gd^{3+} is $\epsilon_b = 3.4$. The number of binding sites on the amylase protein and the dissociation constant for the amylase–Gd(III) complex were calculated from the Scatchard plots (Reuben, 1971). The results are shown in Figure 3. It is readily seen that the *B. subtilis* α-amylase possesses two Gd(III) binding sites. The Gd^{3+}–amylase dissociation constant was found to be $K = 2.6 \times 10^{-5}$ M. Furthermore, Ca(II) effectively competes for one of those sites. It appears, therefore, that *B. subtilis* possesses two calcium binding sites, one being the Ca(II) binding site essential for the functional integrity of the enzyme.

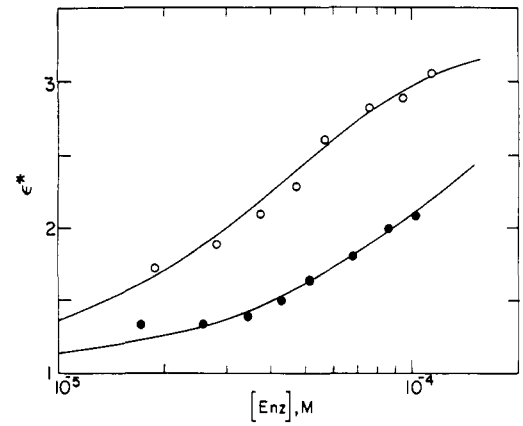

FIGURE 2: The enhancement (ϵ^*) of the paramagnetic contribution by Gd(III) to water proton relaxation rate as a function of the concentration of *B. subtilis* apo-α-amylase. The Gd(III) concentration was 1×10^{-4} M. The solutions were 0.05 M in sodium acetate buffer (pH 4.6). Curves are calculated with $\epsilon_b = 3.4$ and equilibrium constants obtained from Scatchard plots: open symbols, no Ca^{2+} added; closed symbols, 8.55×10^{-4} M Ca^{2+} added.

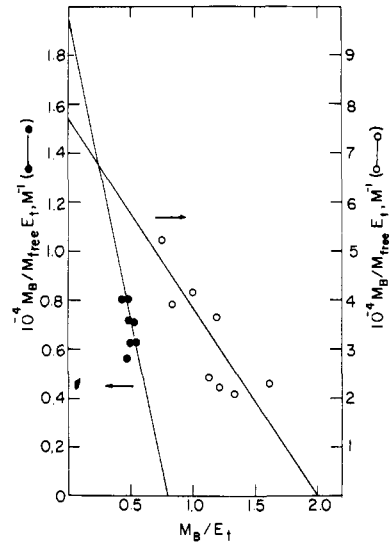

FIGURE 3: Scatchard plots for Gd(III) binding to apo-α-amylase. The plots in the presence of Ca^{2+} were calculated from the data in Figure 2. A similar titration was carried out in the presence of 8.55×10^{-4} M Ca^{2+}; open symbols, no Ca^{2+} present; closed symbols, titration in the presence of 8.55×10^{-4} M Ca^{2+}.

Discussion

Our results with both pancreatic α-amylase and *B. subtilis* α-amylase indicate that the lanthanide ions are incapable of binding to the essential Ca^{2+} site. It should be noted that at neutral pH values the affinity for the essential Ca^{2+} is so high that minute Ca^{2+} impurities will activate the apoamylase (Table I). Thus, in our hands the treatment of glass as recommended by Smolka *et al.* (1971) was insufficient. Enough Ca^{2+} leaks from the glass to activate the apoamylase (Table I).

Pancreatic α-amylase, both in the native state and in the apoenzyme form, does not possess any binding sites for Gd^{3+}. *B. subtilis* apo-α-amylase, on the other hand, possesses two Ca^{2+} binding sites, one of which can accommodate Gd^{3+} with an additional site for Gd^{3+} (Figure 2). The Ca^{2+} binding site essential for enzyme function *cannot* bind the Gd^{3+} ion, whereas the nonessential Ca^{2+} binding site can accommodate Gd^{3+} (Figure 1, Table II). In conclusion, lanthanide ions will in some cases bind to Ca^{2+} binding sites; however, this is not universal, since both in the case of α-amylases and concanavalin A (M. Shoham, I. Pecht, and A .J. Kalb,[2]) rare earth elements fail to replace Ca^{2+} and form biologically active complexes. It is interesting to note that both α-amylases and concanavalin A exhibit extreme specificity toward Ca^{2+} and in both cases lanthanide ions fail as replacements. On the other hand, in proteins in which the metal ion is not essential for the biological activity but rather provides structural stability, the specificity is less restrictive. Indeed, the activation by lanthanide ions of the trypsinogen to trypsin conversion and the successful replacement of Ca^{2+} by Sm^{3+} in thermolysin have been observed (Colman *et al.*, 1972).

[2] Manuscript in preparation.

Added in Proof

Recently we were able to demonstrate (M. Steer and A. Levitzki, manuscript in preparation) that the ability to activate hog pancreatic apo-α-amylase is paralled by the ability to mask the two SH groups which become exposed upon Ca^{2+} removal from the enzyme. Using this tool it can be demon-

strated that Y^{3+}, lanthanides, as well as many other divalent cations, fail to activate the apoenzyme.

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Peptide Chloromethyl Ketones as Irreversible Inhibitors of Elastase†

Robert C. Thompson‡ and Elkan R. Blout*

ABSTRACT: Three peptide chloromethyl ketones have been prepared which irreversibly inhibit elastase. Amino acid analyses of the inactivated enzyme show that inhibition results from the alkylation of a histidine residue of the enzyme. The three peptide chloromethyl ketones differ markedly in their ability to inactivate the enzyme with the longer peptides reacting more rapidly. A parallel dependence of the ease of substrate hydrolysis on peptide chain length has been observed previously with amide substrates of elastase.

Elastase (EC 3.4.4.7) is a serine proteinase secreted by the pancreas in the form of a zymogen. Its catalytic mechanism, amino acid sequence, and tertiary structure show that it is a member of the same family of enzymes as chymotrypsin and trypsin. Nonetheless, certain properties distinguish elastase from the other enzymes of this family. One of the more puzzling features has been the failure, despite considerable effort (Kaplan *et al.*, 1970; Visser *et al.*, 1971, and unpublished results), to develop reagents which alkylate the histidine residue known to be at the active site of the enzyme (Shotton and Watson, 1970). Such alkylating reagents are available for both chymotrypsin and trypsin and have played an important role in implicating the active-site histidine residue in their catalytic mechanisms (Shaw, 1970).

Recently, it has become apparent that the catalytic efficiency of elastase is considerably enhanced by interaction with amino acid residues of the substrate N terminal to the scissile bond (Atlas *et al.*, 1970; Thompson and Blout, 1970, 1973b,c). The major increase in catalytic efficiency arises from the increased ability of the enzyme to transfer an acyl group of the substrate to the hydroxy group of serine-188. It appeared possible that the factors contributing to this increased ability to acylate serine-188 would also facilitate the alkylation of histidine-45 by the proper reagent. Other investigators have previously reported that peptide chloromethyl ketones can react more rapidly than tosylamino acid chloromethyl ketones with other, related, enzymes (Morihara and Oka, 1970; Powers and Wilcox, 1970). Consequently, we have prepared three peptide chloromethyl ketones and report here that these reagents inactivate elastase rapidly and irreversibly. Evidence is presented that the inactivation is the result of alkylation of an histidine residue.

Materials and Methods

Porcine pancreatic elastase (>99.8% pure) was purchased from Whatman Biochemicals, England.

Carbobenzoxyalanine and acetylproline were purchased from the Fox Chemical Co., Los Angeles.

N-Carbobenzoxyalanine chloromethyl ketone was prepared according to the method used by Shaw (1967) to prepare the phenylalanine analog: yield based on Z-Ala-OH, 38%; mp 87–88°, $[\alpha]_D^{25}$ −43.6° (*c* 2.8, MeOH). *Anal.* Calcd for $C_{12}H_{14}ClNO_3$: C, 56.4; H, 5.49; Cl, 13.9; N, 5.5. Found: C, 56.4; H, 5.5; Cl, 13.3; N, 5.6.

Alanine Chloromethyl Ketone Hydrobromide. Z-Ala-CMK[1] (1.0 g; 3.9 mmol) was dissolved in a saturated solution of hydrogen bromide in glacial acetic acid (2 ml). After 20 min, anhydrous ether (200 ml) was added and the mixture was shaken vigorously. The ether was decanted when clear, and

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‡ Present address: Bacterial Physiology Unit, Harvard Medical School.

[1] The abbreviation Ala-CMK is used to denote the chloromethyl ketone derived from alanine, *viz.*, 1-chloro-3-aminobutan-2-one.