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Lanthanide Ions Activate α-Amylase†

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ABSTRACT: *Bacillus subtilis* α-amylase, which has had its calcium removed, can be reactivated under carefully controlled conditions by lutetium(III) as well as by a variety of other lanthanides. The reactivation process is extremely sensitive to concentrations of the lanthanide ions and buffer system. Thus at pH 6.9 in Hepes (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer, concentrations of Lu^{3+} above 10^{-4} M inhibit the enzyme, whereas in maleate buffer at pH 6.9, apoamylase is activated by concentrations up to 10^{-3} M Lu^{3+}.

The lanthanide ions have recently been suggested as calcium ion substitutes for calcium ion binding sites in proteins and enzymes. (Darnall and Birnbaum, 1970; Birnbaum *et al.*, 1970; Williams, 1970). In contrast to the calcium ion, however, the varied magnetic and spectral properties of the rare earth metal ions should make excellent spectroscopic probes of the metal ion binding sites in proteins. It has been shown recently that the lanthanide ions isomorphously replace the calcium ion in α-amylase (Smolka *et al.*, 1971), in thermolysin (Colman *et al.*, 1972), and in the activation of trypsinogen to trypsin (Darnall and Birnbaum, 1970). In addition it has been shown that lanthanide ions replace Mg^{2+} ion in leucine tRNA synthetase (Kayne and Cohn, 1972) and pyruvate kinase (Valentine and Cottam, 1973). Lanthanide ions also replace the calcium ion and the transition metal ion in concanavalin A (Sherry and Cottam, 1973).

α-Amylase from *Bacillus subtilis* contains two identical subunits each of which appears to bind two calcium ions (Stein *et al.*, 1964; Imanishi, 1966). Removal of calcium from the enzyme results in loss of activity whereas addition of calcium back to the apoenzyme results in the recovery of full activity. Smolka *et al.* (1971) showed that the rare earth metal ions are capable of substituting for the calcium ion in *B. subtilis* α-amylase and produce active enzymes. Levitzki and Reuben (1973) utilizing somewhat different experimental conditions were unable to obtain activation of α-amylase with lanthanide ions and as a result have challenged our assertion that lanthanide ions can activate α-amylase. They reported that each subunit of α-amylase binds two gadolinium ions which do not activate the enzyme and hence lanthanides form only "abortive complexes" with α-amylase.

We have repeated and extended our original experiments and herein resolve the apparent discrepancies between the two earlier reports.

Materials and Methods

Crystalline *B. subtilis* α-amylase (lot 108B-0590, Sigma) was used without further purification. The enzyme sedimented as a single symmetrical boundary in the ultracentrifuge and gave a single band upon polyacrylamide gel electrophoresis.

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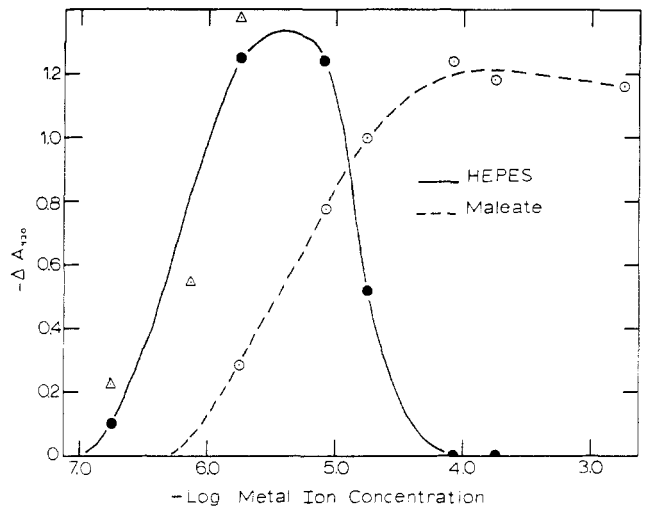

FIGURE 1: The activation of *B. subtilis* α-amylase by lutetium. Enzyme activity was measured at pH 6.9 in either 0.02 M Hepes buffer or 0.02 M maleate buffer. Atomic absorption analysis showed less than 0.2 M calcium ion per subunit in the apoenzyme. The assay mixtures each contained 0.63 μg of apoamylase and were incubated for 6 min. Absorbances of apoamylase assays (with no metal ion added) were always substracted from absorbances in the presence of metal ions in order to correct for trace calcium impurities (see text). A decrease of one absorbance unit corresponds to the production of 357 μg of maltose. The specific activity of the lutetium-activated α-amylase at maximum activation was 244 U/mg: (●) lutetium in Hepes; (⊙) lutetium in maleate; (△) calcium in Hepes.

Apoamylase and calcium-free starch were prepared exclusively by electrodialysis. The native enzyme was dissolved in water, treated with phenylmethylsulfonyl fluoride for several hours as described previously (Smolka *et al.*, 1971). Calcium-free water, buffers, starch, and glassware were prepared as described previously (Smolka *et al.*, 1971). All solutions were analyzed for calcium impurities using the Varian Techtron AA-5 atomic absorption spectrometer.

Amylase activity was determined by a modification of the alkaline ferricyanide method used by Robyt *et al.* (1972) rather than by the traditional Bernfeld (1955) method since Robyt and Whelan (1972) have shown that molar reducing values obtained with 3,5-dinitrosalicylate are dependent upon the chain length of maltodextrins produced from starch. In addition metal ions such as calcium and therefore, presumably, lanthanide ions affect the dinitrosalicylate reducing values. The assay consisted of 0.5 ml of 1.0% electrodialyzed starch, 1.0 ml of buffer, and 0.1 ml of metal ion at appropriate concentrations. The reaction was initiated by the addition of 0.10 ml of electrodialyzed enzyme and allowed to proceed at 25° for 4–15 min, depending upon the experiment. Then 3.0 ml of a freshly prepared solution containing 3.08×10^{-3} M $K_3Fe(CN)_6$, 0.10 M Na_2CO_3, and 8.0×10^{-3} M NaCN were added. The reaction mixture was then immediately immersed in boiling water for 5 min, cooled for 1 min in ice–water, and absorbances were measured at 420 nm.

The reverse process of incubating the apoenzyme with the lanthanide ions and initiating the reaction by addition of the lanthanide–enzyme complex to the starch–buffer solution led to variable results depending upon how long the metal ion was in contact with the enzyme and what concentrations of metal ion were present. At high concentrations of lanthanide ion inhibition was observed, which was not reversed upon dilution of the enzyme–lanthanide solution into the assay mixture.

Results and Discussion

Earlier we had reported (Smolka *et al.*, 1971) that *B. subtilis* apo-α-amylase was activated by various lanthanides (10^{-4} M) in 0.02 M maleate buffer at pH 6.0, whereas Levitzki and Reuben (1973) observed no activation of the apoenzyme by various lanthanides (10^{-3} M) in 0.02 M Hepes[1] buffers at pH 6.9. Thus they asserted that lanthanide ions will not activate *B. subtilis* α-amylase. Figure 1 shows the activation of apoamylase as a function of Lu^{3+} concentration in maleate buffer and Hepes buffer at pH 6.9. We have used Lu^{3+} since earlier it was shown that lutetium ion activates apoamylase better than the other lanthanides (Smolka *et al.*, 1971). It is clear that there is a rather narrow lutetium ion concentration region where α-amylase is activated in the Hepes system. At concentrations above 10^{-4} M Lu^{3+}, the enzyme is inhibited. In maleate buffer, on the other hand, not only must more lutetium ion be present to effect activation, but the enzyme is not inhibited nearly as much at higher concentrations of the metal ion. We feel that the different results obtained in the two buffer systems is a reflection of the difference in metal ion binding capacities of the two buffers. Hepes has a very low affinity for metal ions (Good *et al.*, 1966) whereas a dicarboxylic acid such as maleate would be expected to have a higher affinity for the metal ions (K_1 for the Nd^{3+} complex is 4610, Roulet *et al.* (1970)). In maleate buffer there is less free metal ion present and hence the effective metal ion concentration is much lower than in Hepes buffer. The fact that in Hepes Lu^{3+} concentrations above 10^{-4} M inhibit the enzyme suggests that the metal ion is binding at other low-affinity sites on the enzyme which causes the inhibition. These lower affinity sites are not as able to effectively compete with maleate for the metal ion as do the strong binding sites on the enzyme and hence less inhibition is observed in maleate. At pH 6.0, where the interaction of maleate and Lu^{3+} would be expected to decrease, we do observe slight inhibition of the activation of amylase with high concentrations of Lu^{3+} (10^{-3} M).

Since Hepes does not bind metal ions, the apoenzyme in this buffer is particularly sensitive to calcium ion contaminants. Thus Levitzski and Reuben (1973) state that enzyme assays carried out in glassware treated according to Smolka *et al.* (1971) were insufficient to prevent leakage of calcium ion from the glass and thus activate the apoenzyme. We have also observed this in the Hepes buffer system, and it is necessary to perform the assays in polypropylene tubes where less calcium ion contaminants are found. However, when assays are conducted in maleate buffer at pH 6.0 or 6.9, assays in glass tubes (Kimax) treated according to Smolka *et al.* (1971) are possible; not more than 5–10% residual activity is found in the absence of added calcium or lanthanide ion.

Because of the high affinity of apoamylase for calcium ion and because any trace calcium impurities will activate the enzyme in Hepes at pH 6.9, it is necessary to show that calcium contaminants in the lutetium solutions are not causing the reactivation. Atomic absorption analysis showed that a 0.1 M solution of $LuCl_3$ contained 6.3×10^{-5} M Ca. Figure 1 shows that the maximum reactivation of the enzyme occurred between 1.76×10^{-6} and 8.8×10^{-6} M Lu^{3+}. Therefore the calcium ion concentration due to the contaminants in the lutecium solutions would be between 10^{-9} and 10^{-8} M. Atomic absorption analysis of buffers, water and starch solutions showed that in all cases the calcium concentration was below

[1] Abbreviation used is: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

10^{-6} M. In addition, the absorbance readings of the apoenzyme assays (with no added calcium ion or lanthanide ion) were always subtracted from those obtained in the metal ion activation assays so that any activation due to trace impurities of calcium in the buffers and starch were taken into account. The only other source of calcium ion contaminant is the Lu^{3+} solutions which, in the concentrations used, clearly do not have enough calcium ion to produce the reactivation observed.

The effectiveness of Lu^{3+} as an activator of apoamylase as compared to Ca^{2+} varies depending upon the metal ion concentrations compared. Thus at pH 6.9 in Hepes, Lu^{3+} and Ca^{2+} at low concentration are equally effective as activators of α-amylase (Figure 1). If however the maximum activation due to lutetium at optimum concentrations (1.76–8.8 × 10^{-6} M) is compared to the maximum activation due to calcium, the lutetium-activated amylase is only approximately 40% that of the calcium-activated enzyme.

At pH 4.6 Levitzki and Reuben (1973) found that the dissociation constant of the Ca^{2+}–enzyme complex was decreased and thus the sensitivity to calcium ion contaminants is decreased. Figure 2 shows the dependence of Lu^{3+} concentration on the activation of apoamylase in 0.02 M acetate (pH 4.6). As at the higher pH in Hepes buffer, high concentrations of Lu^{3+} inhibit the enzyme whereas lower concentrations activate. The degree of activation is somewhat less at this pH than at the higher pH. The activity observed at the optimum concentration of Lu^{3+} (8.8 × 10^{-4} M) is only 23% of that observed by 1.8 × 10^{-4} M calcium ion in the assay mixture.

In addition to Lu^{3+}, we have tested Dy^{3+}, Sm^{3+}, Pr^{3+}, Gd^{3+}, and Nd^{3+} as activators of α-amylase in 0.02 M maleate buffer at pH 6.9. At 10^{-4} M all the lanthanides activate amylase. Amounts of reactivation by the lanthanides as compared to reactivation of the enzyme by 1.76 × 10^{-4} M calcium ion were: Lu^{3+}, 54%; Dy^{3+}, 39%; Sm^{3+}, 21%; Pr^{3+}, 13%; and Gd^{3+}, 11%. A plot of crystal-ionic radius of the lanthanides as a function of activity show the same straight-line relationship as observed earlier (Smolka *et al.*, 1971), with the possible exception of Gd^{3+} which has slightly lower activity.

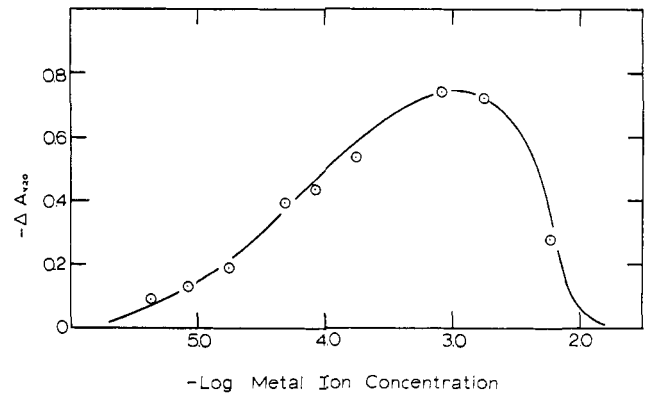

FIGURE 2: The activation of *B. subtilis* α-amylase by lutetium at pH 4.6. Enzyme activity was measured in 0.02 M acetate buffer. Each assay contained 10.5 μg of apoamylase and was incubated 15 min. The maximum activity of the lutetium-activated amylase corresponded to 23% of the activity of an assay containing 1.76 × 10^{-4} M calcium ion.

Conclusion

It is clear from the data presented above that the lanthanide ions are activators of *B. subtitlis* α-amylase. The activity of the lanthanide–amylase complexes are, however, very sensitive to the free metal ion concentration present in the assay system. The free metal ion concentration, in turn, is strongly dependent upon the buffer composition and thus a variety of metal ion concentrations must be tested before conclusions concerning the activity of the enzyme can be made. This also holds true for the lanthanide acceleration of the activation of trypsinogen to trypsin where high concentrations of the lanthanides inhibit the reaction (J. E. Gomez, E. R. Birnbaum, and D. W. Darnall, unpublished observations).

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Added in Proof

Steer and Levitzki (1973) were recently unable to activate porcine pancreatic α-amylase with 2.5 × 10^{-4} M Nd^{3+}. If the pancreatic and bacterial amylases are analogous, then the lack of activity in the pancreatic enzyme may be due to the high concentration of Nd^{3+} ion as can be seen from Figure 1. Alternatively the pancreatic enzyme may be sufficiently different from the bacterial enzyme, at least as regards ion binding, to make it impossible to draw effective parallels.

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