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Rare Earth Metal Ions as Substitutes for the Calcium Ion in *Bacillus subtilis* α-Amylase*

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ABSTRACT: Rare earth metal ions have been used to replace the calcium ion in *Bacillus subtilis* α-amylase. The lanthanide α-amylases were found to be enzymatically active. The effectiveness of various lanthanide ions to activate apoamylase was found to follow the order: $Ca^{2+} \sim Lu^{3+} > Yb^{3+} > Er^{3+} \sim Y^{3+} \sim Dy^{3+} \sim Tb^{3+} > Sm^{3+} > Nd^{3+} > Pr^{3+} > La^{3+}$. The smallest rare earth ions were most effective as calcium ion substitutes whereas the larger lanthanides were less effective. The circular dichroic spectra of the native calcium-containing α-amylase, apoamylase, and Nd^{3+}–amylase are identical in the spectral region from 200 to 250 nm. This suggests there are no large structural changes in the enzyme upon removal of the calcium ion from native amylase.

Research on the nature of the interaction of the calcium ion with proteins has lagged far behind studies of the nature of transition metal ion interactions with proteins. This situation has come about simply because it is difficult to obtain any spectroscopic or magnetic information about the calcium ion which can be used to reveal the nature of its interaction with proteins. We have recently proposed that the rare earth metal ions should make good isomorphous replacement substitutes for the calcium ion (Darnall and Birnbaum, 1970; Birnbaum *et al.*, 1970). In contrast to the calcium ion, however, the lanthanide ions can be scrutinized by a variety of magnetic and spectral methods and hence should make good probes for the calcium ion binding sites in proteins. Williams (1970) has recently come to similar conclusions.

We have shown previously that the neodymium ion binds to trypsin and trypsinogen with concomitant changes in the visible absorption spectrum of the neodymium ion (Darnall and Birnbaum, 1970). Neodymium ion was shown to function in a manner similar to the calcium ion acceleration of the activation of trypsinogen to trypsin.

If information concerning the lanthanide ion binding sites in proteins is to be extrapolated to the calcium ion binding sites in proteins, it must be shown that the lanthanide ions are isomorphic replacements for the calcium ion. Strong evidence that this obtains in the α-amylase system is presented herein.

The α-amylases of various origins so far investigated have been found to contain at least one atom of calcium firmly and specifically bound to the enzyme (Stein *et al.*, 1964, and references therein). *Bacillus subtilis* α-amylase, which contains two apparently identical subunits, has been shown to bind 4–5 calcium ions/mole of enzyme of mol wt 48,000 (Stein *et al.*, 1964; Imanishi, 1966; Connellan and Shaw, 1970). It has been shown by Hsui *et al.* (1964) and Imanishi (1966) that when the calcium is removed from *B. subtilis* α-amylase, enzymatic activity is lost. Upon addition of calcium back to the apoamylase full enzymatic activity is recovered.

We have prepared calcium-free α-amylase and tested the lanthanide ions as possible activators of the apoenzyme. We have been able to observe reactivation of *B. subtilis* α-amylase with many of the rare earth metal ions, and indeed we have observed a correlation of enzyme activation with the crystal ionic radius of the rare earth metal ion tested.

Experimental Section

Enzyme. 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 component upon polyacrylamide electrophoresis. Before calcium was removed from the enzyme, it was always treated with 10^{-3} M phenylmethylsulfonyl fluoride for 12–20 hr at 4° to inactivate traces of proteolytic enzymes (Hsui *et al.*, 1964). Unless these contaminants were thoroughly inactivated, α-amylase was irreversibly inactivated by proteolysis upon removal of the calcium. After treatment with phenylmethylsulfonyl fluoride, the enzyme was dialyzed against the desired solution and centrifuged at 15,000 rpm, and the protein content determined by absorption at 280 nm using $E^{1\%}_{1\,cm} = 25.3$ (Hsui *et al.*, 1964). If the solution was to serve directly for enzymic studies it was diluted in 0.02 M maleic acid–Tris buffer (pH 6.0) to approximately 2 μg/ml. If the enzyme was to be electrodialyzed or dialyzed against EDTA for calcium removal, it was not diluted with buffer, but immediately put in the appropriate vessel for electrodialysis.

Calcium Analysis. Calcium concentrations were analyzed by means of a Perkin-Elmer 303 atomic absorption spectrometer. The procedure followed was that outlined by the manufacturer, except that the purity of our water eliminated the need for a 1% lanthanum chloride solution in the analysis. Calcium ion concentration was determined at 422 nm from

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a standard curve prepared from Baker Analyzed calcium carbonate dissolved in 0.1 M HCl and diluted to concentration with a micropipet and calcium-free water.

Water Purification. Deionized water was doubly distilled in a Corning all-glass distillation apparatus. This doubly distilled water was then passed through a column of Dowex 50 ion-exchange resin which was in the hydrogen ion form. Analysis showed that calcium content in the purified water was lower than 0.001 μg/ml. The purified water was then used to make all buffer and metal solutions.

Calcium-Free Glassware. All glassware, polyethylene bottles, and the electrodialysis chamber were treated with aqua regia for 1–2 hr, washed with tap water, soaked for 2–4 hr in 0.05 M EDTA (adjusted at pH 9.0 with Mann Ultra Pure Tris), rinsed several times with calcium-free water, allowed to soak in such water overnight, and given a final rinse. Unless this procedure was strictly followed it was found that Ca^{2+} was leached from the walls of containers at a high enough rate to activate apoamylase. Even with the EDTA–Tris treatment it was found that the calcium ion concentration in solutions increased to unacceptable levels within a few weeks.

Metal Solutions. Samples of lanthanide oxides with a purity greater than 99.9% were provided by Molybdenum Corp. of America and the American Potash Chemical Corp. Stock solutions of the lanthanide chlorides were prepared by digesting a slight stoichiometric excess of the oxide with HCl, filtering, and diluting to volume with calcium-free water. The calcium contamination of these and all other metal solutions was then analyzed by the atomic absorption method. Solutions of Mn^{2+}, Mg^{2+}, Sr^{2+}, and Fe^{2+} were prepared from Baker Analyzed $MnCl_2$, $MgCl_2 \cdot 6H_2O$, $Sr(NO_3)_2$, and $FeSO_4 \cdot 7H_2O$.

Removal of Calcium from α-Amylase. ELECTRODIALYSIS. Electrodialysis at 4° was carried out in a Gelman electrodialysis cell, under constant stirring at a maximum voltage of 500 V. To minimize current flow through the solution, the dialysis was started using various voltages depending on the relative salt concentration of the substance being electrodialyzed. For heavily contaminated materials voltages on the order of 50 V were initially applied and gradually increased to the maximum voltage as the resistance of the solution increased. For less heavily contaminated substances higher initial voltages were used. Amperage was kept below 10 mA by changing the low Ca^{2+} water whenever the amperage reached close to this value. After about 2–6-hr dialysis, the amperage reached a steady value of less than 1 mA and the maximum voltage was continued for 14–20 hr if the enzyme was being electrodialyzed, and from 4 to 8 hr if the starch substrate was being electrodialyzed. Electrodialysis was discontinued for the enzyme when it showed only residual amylolytic activity.

EDTA DIALYSIS. Simple dialysis was performed against 0.05 M EDTA. Dialysis of the enzyme was carried out in a Tris–EDTA system at pH 6.0, and dialysis of the substrate was carried out in Tris–EDTA at pH 9.0. All dialyses of this type were carried out at 4° for at least 1 week with the appropriate EDTA solution being changed at 2-day intervals after an initial three changes on the first day. The EDTA was then removed from the enzyme and substrate by dialysis against low Ca^{2+} water for 2 more days in the case of the enzyme, and for another week in the case of the substrate. As with electrodialysis, dialysis was suspended when the enzyme showed only residual activity.

Substrate Preparation. Substrates were prepared from Baker's soluble starch by various techniques: (1) for the Nd^{3+} inhibition studies, the commercial product was dissolved in 0.02 M Tris–Maleate buffer at pH 6.0 by boiling and diluted with the same buffer to the proper concentrations. Substrates were usually boiled at regular intervals to effect solution, since they tended to precipitate on standing at 4°. (2) Ca^{2+}-free substrates were prepared by dialyzing a 50-mg/ml solution of the commercial product against 0.05 M EDTA for 3 weeks where the EDTA solution was changed after 24 hr, 48 hr, and then once a week. It was then dialyzed against double-distilled, deionized water for another week. The product was lyophilized in containers which had been carefully treated to remove surface Ca^{2+} contaminants, and oven dried at 60° for 4 days. The dry starch was then dissolved (10 mg/ml) in Ca^{2+}-free buffer (pH 6.0) and stored in Ca^{2+}-free volumetric flasks. (3) Lower Ca^{2+} level substrates were effected by dissolving the EDTA-dialyzed product in water (10 mg/ml) by boiling and then electrodialyzing until the current was less than 1.0 mA at 500 V at which point the electrodialysis was continued for 4 more hr. The substrate was then used directly for reactivation studies. Analysis for Ca^{2+} showed that a 10% starch solution contained approximately 10^{-4} M Ca^{2+}. After electrodialysis this value generally dropped to $1–5 \times 10^{-6}$ M Ca^{2+}.

Tris buffer (Ultra Pure, Mann) was shown to be very low in Ca^{2+} and was used directly for buffer preparations. Maleic acid (Baker Analyzed) was calcium contaminated and was purified by passing a solution through a column of Dowex 50 ion-exchange resin in the H^+ form. Atomic absorption analysis showed the final Tris–maleate buffers to be lower than 10^{-7} M in calcium ion.

Amylase Activity Assay. Amylase activity was determined by a modification of Hoffman's (1937) ferricyanide technique. An incubation mixture consisting of 0.50 ml of the appropriate concentration of starch dispersion was diluted with 1.00 ml of low Ca^{2+} water to which 10.0 μl of enzyme (0.2 μg) was then added with vigorous mixing. The mixture was allowed to incubate at 25° in a constant-temperature water bath for 5 min and 50 sec. During the last 10 sec of the incubation time, 2.00 ml of a solution of 2.5×10^{-3} M $K_3Fe(CN)_6$, 0.14 M Na_2CO_3, and 0.01 M NaCN was added quickly with vigorous stirring. The reaction mixture was then immersed in boiling water for 5 min and cooled for 1 min in cool water. Absorbance was measured at 420 nm. The ferricyanide and cyanide were freshly mixed just prior to the beginning of any series of tests and blanks consisting of substrate, water, and reagents were tested at intervals not exceeding 3 hr. The values for these blanks were then used for any calculations involving the appropriate unknowns. Standard reducing maltose samples were made from D(+)-maltose hydrate obtained from Nutritional Biochemicals. All standards contained 1.00 ml of sugar solution instead of water plus the 0.50 ml of 10 mg/ml starch dispersion, and were treated with 2 ml of oxidizing reagents. A standard curve of maltose concentration *vs.* absorbance at 420 nm showed the analysis was linear from 10 to 400 μg of maltose per sample.

The "maltose equivalent" production by α-amylase was found to be linear between 3 and 8 min at an assay starch concentration of 3.3 mg/ml and hence 6 min was chosen as the incubation time for all studies in this report.

Reactivation Studies. These were of two types. The first, labeled "instant reactivation," consisted of adding enough of a given metal salt solution to a calcium-free substrate mixture to make the assay mixture a given concentration in test metal. Since the electrodialyzed starch solution did not contain any buffers, 1.00 ml of Ca^{2+}-free buffer solution was substituted for the water used in the standard assay system. An aliquot of apoenzyme (0.5–2.5-μg/ml final incubation concentration)

TABLE I: Calcium Contaminant Concentrations in the Metal Solutions Used for Reactivation of the *B. subtilis* α-Amylase Apoenzyme.

Metal[a]	Ca Concn × 10^6 (M)	Soln Tested (M)
Nd	1.25	0.001
La	6.50	0.07
Er	3.20	0.001
Sm	<0.1	0.001
Yb	17.8	0.1
Lu	31.0	0.1
Dy	10.0	0.1
Gd	2.50	0.1
Y	3.75	0.1
Mg	5.50	0.1
Fe	1.00	0.1
Mn	1.00	0.1
Sr	1.25	0.1

[a] All lanthanides were in the III oxidation state as was Y. Other metals were in the II state.

was added to the mixture and allowed to incubate only for the 6 min allotted for the activity assay.

The second type of reactivation experiments consisted of incubating a 100-μl sample of approximately 10^{-4} M apoenzyme with various metals at 10^{-2}, 10^{-3}, and 10^{-4} M concentrations. Activities were checked at 15, 30, 60, and 90 min by taking 50-μl aliquots, diluting these with 0.95 ml of calcium-free water, and immediately using 10-μl aliquots of the diluted enzyme for Ca^{2+}-free activity assays. Concomitant controls were carried out without any metal addition and the reactivation in excess of that exhibited by the controls was considered the degree of reactivation. Incubations were conducted with and without buffers.

For both types of assays great care had to be exercised to avoid calcium ion contamination from various sources. The most trivial carelessness resulted in increasing the calcium level to 10^{-5} M, thereby invalidating a reactivation experiment.

In all the above reactivation studies, activity controls for the apoenzyme were determined at the same time. These consisted of carrying out a calcium-free assay without adding any metals to the mixture.

TABLE II: Inhibition of Native α-Amylase Activity by the Neodymium Ion.[a]

Nd^{3+} Concn (M)	Substrate Concn Used in Assay (mg/ml)	Act. (mg of Maltose/ml per min)
0	10	138
10^{-4}	10	98
10^{-3}	10	42
0	5	108
10^{-4}	5	83
10^{-3}	5	31

[a] Assays were performed as described in text.

TABLE III: Electrodialysis of α-Amylase.

Enzyme Prepn Code	Final Protein Concn after Electrodialysis (Moles/l. × 10^5)	Moles of Ca^{2+}/ Mole of Protein after Electrodialysis
ED-9	3.78	0.825
ED-10	10.1	0.755
ED-11	6.50	0.377
ED-13	5.76	0.104
ED-14	3.18	0.204

Circular Dichroism. Circular dichroism measurements were performed on a Cary 60 recording spectropolarimeter equipped with a Model 6002 circular dichroism attachment and programmed to a bandwidth of 15 Å.

Results and Discussion

Calcium Anaylsis. Since *B. subtilis* α-amylase binds calcium so strongly (Stein *et al.*, 1964), it was necessary to show that any reactivation of the apoenzyme with other metal ions was not due to trace contamination of calcium ion. For this reason we analyzed all of our metal ion solutions for Ca^{2+} by means of atomic absorption analysis. These results are seen in Table I and will be discussed later in relation to reactivation experiments.

Periodic analysis for Ca^{2+} levels in substrate, buffers, apoenzyme preparations, and 0.5–0.005 μg/ml of calcium standards showed that there was a significant amount of leaching of calcium from the walls of the containers regardless of their nature. The least amount came from decalcified volumetric glassware. Treatment with the Tris–EDTA maintained the Ca^{2+} contamination at acceptable levels for a longer period of time but could not eliminate eventual contamination. Average values for a 2-month period were a 6- to 10-fold increase in Ca^{2+} contamination, with buffer systems most easily and quickly contaminated.

Neodymium Inhibition of Native α-Amylase. Studies were undertaken to determine the effect of neodymium ion on the native, calcium-containing enzyme. Results of incubating neodymium ion for 24 hr with the native enzyme are seen in Table II. It is clear that under these conditions Nd^{3+} is inhibiting the enzyme reaction. This could be due to the fact that Nd^{3+} is specifically competing for the Ca^{2+} binding sites but is a poor activity substitute, or it could be due to nonspecific binding of Nd^{3+} to the enzyme. To distinguish between these possibilities all further studies were done with calcium-free enzyme.

Electrodialysis. Table III shows the results of calcium analysis of several apoamylase preparations as prepared by electrodialysis. As can be seen from Table III it was difficult to remove the last traces of calcium ion. These apoamylase preparations always showed a small amount of amylolytic activity which was determined and subtracted from any activity produced by various test metal ions.

Instant Reactivation Experiments. The amount of reactivation of apoamylase with various metal ions was determined by comparing the activity of the test metal ion to the activity obtained upon incubation of apoenzyme (0.2–2.0 μg/ml) with 10^{-4} M Ca^{2+} in the assay mixture. The degree of

TABLE IV: Sensitivity of Instant Reactivation of Apoamylase to Calcium Ion.

Metal Ion (M)	% Reactivation
Ca^{2+} (10^{-4})	100[a]
Ca^{2+} (10^{-5})	29
Ca^{2+} (10^{-6})	0
Nd^{3+} (10^{-4})	39
Nd^{3+} (10^{-4}) + Ca^{2+} (10^{-6})	41

[a] The samples with 10^{-4} M Ca^{2+} were arbitrarily set at 100% reactivation for comparison purposes.

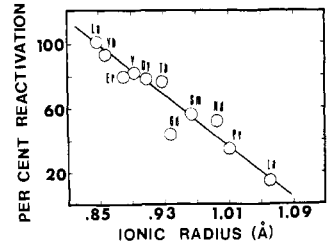

FIGURE 1: Reactivation of apo-α-amylase by lanthanide ions plotted as a function of crystal ionic radius of the metal ions. The per cent reactivation by the lanthanide ions was determined by comparison to the reactivation produced by calcium ion. See text for experimental details.

reactivation achieved with 10^{-4} M Ca^{2+} was arbitrarily set at 100%, and the test metal ion activation was compared to this calcium control.

Early in these studies it became apparent that trace amounts of Ca^{2+} in chemicals would reactivate apoamylase. Therefore, it was imperative to determine the minimum amount of Ca^{2+} necessary to show reactivation of the apoenzyme under the conditions of "instant reactivation." Table IV shows that under our conditions at least 10^{-5} M Ca^{2+} must be present in order to significantly reactivate the enzyme. The enzyme was not reactivated by 10^{-6} M Ca^{2+}. Table IV also shows that 10^{-4} M Nd^{3+} significantly reactivated the apoenzyme but to a lesser extent than 10^{-4} M Ca^{2+}. The effect of 10^{-6} M Ca^{2+} on the amount of reactivation with Nd^{3+} was negligible within experimental error. Since the concentration of all other metal ions tested for reactivation was 10^{-4} M in the assay system, it can be readily seen from the data in Table I that the calcium contamination of the various metal ion solutions is far too small to activate the enzyme under the conditions of instant reactivation. We therefore feel that the reactivation obtained with Nd^{3+} and the other metal ions cannot be due to the presence of calcium ion.

Table V shows results of various metal ions tested as activators of apoamylase. It is clear that many of the rare earth metal ions are good activators of apoamylase. It is also evident that there is a linear relationship between the size of the rare earth metal ion and the degree of activation (Figure 1). Table V also shows that Sr^{2+}, Mn^{2+}, and Fe^{2+} are nearly as good as calcium in the reactivation process, whereas Mg^{2+} is a very poor reactivator.

In experiments with relatively poor activators such as Nd^{3+} and La^{3+}, poor reproducibility of the amount of reactivation was obtained. Table VI shows some selected data for Nd^{3+}. The puzzling results obtained for Nd^{3+} with respect to reproducibility may indicate that some as yet unknown parameter is influencing the degree of activation. This effect is much more pronounced for poor replacements of calcium ion than it is for the good replacements such as Lu^{3+} or even Ca^{2+} itself. There seems to be no good correlation between this effect and apoenzyme residual activity, assay protein concentration, or different substrate preparations. Despite this drawback, any simultaneous activation tests with a series of lanthanides always gave the same relative order of activation shown in Figure 1 and Table V.

Incubation Reactivation. When 10^{-4} M apoamylase was incubated with 10^{-2} or 10^{-3} M Ca^{2+} in the absence of buffer full activity was restored to the enzyme within 15 min. Neodymium ion at these same concentrations in the absence of buffer did not restore any activity in 30 min. Identical studies

TABLE V: Reactivation of Apoamylase with Various Metal Ions.

Metal Ion Tested	Crystal Ionic Radius (Å)[a]	Av % Reactivation
La	1.061	14
Sr	1.034	89
Pr	1.013	33
Nd	0.995	43
Ca[b]	0.979	100
Sm	0.964	56
Gd	0.938	43
Tb	0.928	76
Dy	0.908	77
Y	0.893	80
Er	0.881	78
Yb	0.858	93
Lu	0.848	101
Mn	0.80[c]	80
Fe	0.76[c]	81
Mg	0.65[c]	10

[a] Data from Templeton and Dauben (1954). [b] Calcium was picked as the arbitrary standard with which to compare the other metal ions. [c] Data from Weast (1967).

TABLE VI: Reactivation Effected by Nd in Different Experiments on Various Apoenzyme Preparations.

Enzyme Code	M Assay Concn × 10^8	Residual Apoenzyme Act. (%)[a]	% Reactivation
ED-9	3.78	14.6	37.2
ED-10	5.05	35.0	32.0
ED-12	4.62	31.0	22.6
ED-12	4.62	10.0	52.0

[a] Apoenzyme activities were calculated relative to the blank and compared to the amount of reactivation produced by 10^{-4} M Ca^{2+}.

with ytterbium showed the same results. However, when 10^{-3} M Yb^{3+} was incubated with 10^{-4} M apoamylase with 2×10^{-3} M maleate–Tris buffer (pH 6.0), 24% reactivation (relative to 10^{-3} M Ca^{2+}) was noted after 30 *min* incubation. Longer incubation times produced corresponding higher activities. The metal ion:enzyme ratio in these incubation studies is 10–100 whereas in the instant reactivation studies this ratio is ~10,000. It would therefore appear it is more difficult, kinetically speaking, for Yb^{3+} to bind the enzyme than Ca^{2+}. That Yb^{3+} was found to be nearly as good as calcium as an activator in the instant reactivation experiment may be explained by the large excess of Yb^{3+} over enzyme.

Circular Dichroism. It has been previously suggested that the calcium ion may serve a structural role in α-amylase rather than by participating in amylolysis at the active site of the enzyme (Hsui *et al.*, 1964). It was suggested that the calcium ion substitutes for missing disulfide linkages in the enzyme. It appeared likely that if the calcium ion were indeed behaving in this manner, removal of Ca^{2+} from the enzyme should result in structural changes in the protein. These changes should be observable in the circular dichroic (CD) spectrum of the enzyme such as is seen for ribonuclease or lysozyme when the disulfide bands are broken (Jirgensons, 1970; Pflumm and Beychok, 1969). The circular dichroic spectra of native α-amylase, apo-α-amylase, and apo-α-amylase activated with 10^{-4} M Nd^{3+} were determined in the region between 250 and 200 nm. Spectra of all three samples were identical within experimental error and corresponded quite closely with the previously published circular dichroic spectrum of native α-amylase (Jirgensons, 1970). The spectrum is typical of a protein with a significant amount of α helix and exhibits negative Cotton effects at 222 and 210 nm. Using a molecular weight of 48,700 and 110 as the average residual weight we find $[\theta]_{222} = -8850$ (deg cm²) dmole^{-1} whereas Jirgensons (1970) obtained a value of −8300. Since the CD spectra of native amylase and apoamylase are identical, we therefore feel it is unlikely that the calcium ion is behaving in a structural manner as suggested by Hsui *et al.* (1964).

Discussion

It is apparent from the data in Tables IV and V and Figure 1 that the rare earth metal ions are activators of α-amylase at low concentrations. The smaller rare earth metal ions are good activators of apoamylase; in fact Lu^{3+} is fully as effective as calcium in the reactivation process. On the other hand, the larger lanthanides are very poor activators of α-amylase with La^{3+}, the largest producing very little activity. Initially we felt the neodymium ion (ionic radius 0.995 Å) should make the best replacement for the calcium ion (ionic radius 0.990 Å). It is obvious, however, that Nd^{3+} is one of the poorer substitutes of the calcium ion. The crystal ionic radii of the lanthanides listed in Table V were determined from X-ray analysis of the sesquioxides.

Several different values have been reported for ionic radii of the lanthanide ions. Pauling (1960) has reported radii ranging from 1.15 Å for La^{3+} to 0.93 Å for Lu^{3+}, Rich (1965) has reported 1.14 Å for La^{3+} to 0.85 Å for Lu^{3+}, while Templeton and Dauben (1954) have reported 1.06 Å for La^{3+} to 0.85 Å for Lu^{3+}. Our data in Figure 1 were plotted according to Templeton and Dauben's ionic radii. The correlation between size and activity within the rare earth series is not significantly affected by the choice of radii made, but when comparing ionic radii obtained from different sources complications occur. This is partly a result of the experimental method used to obtain ionic radii. Ideally ionic radii should only be compared for ions which crystallize in the same crystal lattice. A change in the lattice parameters will result in a different "size" for the same ion. Since the radii for all lanthanides can be obtained from a series of isomorphous salts, the relative radii are quite good. When comparing them to other cations, however, such as Ca^{2+} in a different crystal structure, the radii can no longer be regarded as accurate measures of ion size. These difficulties are magnified when trying to compare ions in the first transition series with the alkali or alkaline earth metal ions. Thus plots of activity *vs.* ionic radii for several different types of metal ions may not be smooth curves as is the plot observed in Figure 1, simply because the radii are obtained from different crystal lattices. It is clear from the plot made here, however, that size is of great importance in determining the ability of an ion to activate α-amylase.

A second factor of importance in the activation process is the charge of the metal ion. The attractive force between metal ion and protein is a function of both charge and the inverse of the size of the ion. Our results indicate that the protein can discriminate on a basis of both size and charge. The particular balance of size and charge for a tripositive ion interacting with α-amylase occurs with Lu^{3+}, at an ionic radius of 0.85 Å.

The activity with divalent ions falls off with ionic size less than that of Ca^{2+} and an identical phenomenon may occur with a tripositive charge. Thus as the ionic radius continues to decrease below 0.85 Å, activity may again decrease for ions such as Sc^{3+} and Al^{3+}. The effect of the increase in ionic charge is to shift the optimum ionic radius needed for maximum activity from 0.99 Å (+2) to 0.85 Å (+3).

The increase of activity with decreasing size of the metal ion might be explained by the following analysis. Consider α-amylase with a negatively charged metal binding site in contact with an aqueous solution. The metal ion preferred by the protein binding site will be that cation which experiences the greatest decrease in free energy when its nearest neighbor becomes the protein site rather than water. In general the relative affinities of the site for two different metal ions x and y will be governed by the free energy difference: $\Delta G_{x,\text{protein}} - \Delta G_{y,\text{protein}} - \Delta G_{x,\text{water}} + \Delta G_{y,\text{water}}$, where $\Delta G_{x,\text{protein}}$ and $\Delta G_{y,\text{protein}}$ are the free energies of interaction between the metal ion and the protein and $\Delta G_{x,H_2O}$ and $\Delta G_{y,H_2O}$ are the free energies of hydration. If the protein binding site has a high electric field strength (high negative charges) then the metal ion–protein attractive forces (ΔG's) and their differences will be much higher than the hydration energies and their differences, respectively. The affinities will then be controlled by $\Delta G_{x,\text{protein}} - \Delta G_{y,\text{protein}}$ (Diamond and Wright, 1969). The smallest metal ion will have its center of charge nearest the protein binding site and will experience the largest attractive force, which for a strong site outweighs its also having the highest free energy of hydration, so affinity will increase with decreasing ionic radius. This analysis assumes that increasing affinity of the protein for a metal ion is seen directly in the activity of the enzyme. It is, of course, possible that this is not true.

An additional inherent assumption made in this analysis is that only a single charged site is involved in the binding of each metal ion. Under these conditions the smaller the ion, the closer it can get to the binding site. However, if two or more negative functional groups are involved, a metal ion which can interpose itself between the two (or more) negative

charges and most effectively neutralize the repulsive forces generated will bind most strongly. This effect should place a lower limit on the possible size of the metal ion, since if the ion is too small it will not effectively shield the negative binding sites from each other. In view of the extreme difficulty in removing Ca^{2+} from α-amylase, multidentate binding would be expected, and this could explain the inability of small ions such as Mg^{2+} to activate α-amylase.

Since all of the lanthanide reactivations of α-amylase were carried out in maleate buffers (which certainly will bind the lanthanides), it is possible that the observed effects of different lanthanides on α-amylase could be just an effect of different affinities of the lanthanides for the buffer anion. We feel that this is not the case, since in general, the binding of lanthanides to most ligands increases as the size of the lanthanide ions decreases (Moeller *et al.*, 1968). This means that lutetium should bind the buffer anion most strongly leaving the smallest amount of free lanthanide ion in solution available for activation. Since we observe the greatest degree of reactivation with lutetium, this would seem to indicate that buffer anion binding does not affect the *relative* reactivation capacities of the lanthanides.

It was somewhat surprising that Mn^{2+} and Fe^{2+} were fairly good activators of apoamylase, since in general the transition metal ions form different types of complexes than do the alkaline earth metal ions or the lanthanides. Mn^{2+} and Fe^{2+} do not fit on the line in Figure 1 and do not show the same correlation of size and activity as do the lanthanides. This is probably explained by the fact that, in general, there is no correlation between metal ion size and strength of complex formation for d-transition metals, since covalent overlap of d orbitals becomes an important consideration.

Our circular dichroism data support Imanishi's (1966) findings that α-amylase does not change its conformation when calcium was removed, and we were unable to note any change in the CD spectrum when Nd^{3+} was bound to the enzyme. This casts some doubt on the contention that calcium functions primarily as a conformational stabilizer in this amylase (Hsui *et al.*, 1964).

The fact that the rare earth metal ions are activators of α-amylase is strong evidence that these metal ions are binding at the same site in the enzyme as calcium. It should now be possible to take advantage of the many and varied magnetic and spectral properties of the lanthanide ions (Darnall and Birnbaum, 1970) to further probe the calcium ion binding site in this enzyme.

The lanthanide ions have been shown to substitute for calcium in trypsinogen (Darnall and Birnbaum, 1970), *Staphylococcus* nuclease (Williams, 1971), and now α-amylase. Morallee *et al.* (1970) have also used Eu^{3+} to probe the active site of lysozyme. It thus appears that the rare earth metal ions will be generally useful to probe calcium binding sites in other proteins and enzymes.

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