Further, as shown in Table I, not all of the hydrogen peroxide in the steady-state system is utilized to oxidize ferrocyanide at pH 7.8. In addition it has been observed that a large excess of hydrogen peroxide added to cytochrome *c* peroxidase at pH 8 causes rapid destruction of the heme group.

The above results suggest that at high pH, ferrocyanide is such a poor substrate for I and II that hydrogen peroxide can effectively compete with ferrocyanide in reactions with these enzyme intermediates, producing a number of side reactions including oxidation of the heme and amino acid residues of the protein. During the side reactions, an intermediate is most likely produced which is a better oxidant of ferrocyanide than II. This could account for the faster oxidation of ferrocyanide in the steady state compared to the single turnover transient state studies where only I and II are produced.

pH Dependence. The pH dependence of the steady-state parameters is difficult to interpret due to the specific ion effects, the abnormal ionic strength dependence of V_m^{app}/e at pH 6, and the side reactions between enzyme and hydrogen peroxide at high pH. Certainly the pH dependence of V_m^{app}/eK_m^{app}, after correction for the side reactions, is consistent with the interpretation of k_2^{app} and k_3^{app} in the transient state (Jordi and Erman, 1974). The pH dependence is due primarily to the variation in electrostatic interaction between ferrocyanide and the enzyme intermediates as the net charge on the enzyme changes with pH.

V_m^{app}/e, essentially a unimolecular rate constant, should be relatively insensitive to electrostatic effects although the ionic strength dependence of V_m^{app}/e at pH 6 casts some doubt on this expectation. Nevertheless, if V_m^{app}/e is insensitive to the charge on the enzyme, then the strong pH dependence of V_m^{app}/e suggests that a proton or a protonated form of the complex may be involved in the intramolecular electron transfer reaction. This is an interesting area for further investigation.

K_m^{app} is relatively independent of pH, varying by only a factor of 10 over the pH range 4–8. This is due to the cancellation of the pH variation of the intramolecular electron-transfer rate constants in the numerator and the pH dependence of the bimolecular association rate constants in the denominator of the expression for K_m^{app}, eq 7.

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The Metal Ion Acceleration of the Conversion of Trypsinogen to Trypsin. Lanthanide Ions as Calcium Ion Substitutes†

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ABSTRACT: The lanthanide ions are shown to be effective calcium ion substitutes in accelerating the conversion of trypsinogen to trypsin. The rate of activation of the zymogen in the presence of lanthanides is much greater than that of the calcium ion. In addition this increased activation takes place at nearly 100-fold lower concentrations of the lanthanide ions than with calcium ion. The effect of the lanthanide ions in accelerating the activation of the zymogen, like the calcium effect, is reflected in a decrease in the K_m of the trypsin–trypsinogen interaction. Whereas a calcium concentration of 50 mM reduces the K_m by a factor of 3 (over 4 mM Ca^{3+}), 0.5 mM Nd^{3+} reduces the K_m by a factor of 14. Inhibition of the activation occurs with concentrations of lanthanide ions ranging from 5×10^{-4} to 10^{-3} M, depending on the particular lanthanide ion.

Within the last 10 years there has been substantial attention focused on specific interactions of proteins with metal ions. It has become apparent that the functions of metal ion–protein complexes are biologically important not only in a catalytic capacity, but also in a structural capacity. A majority of the work in this area has been conducted on systems which contain transition metal ions which are amenable to spectroscopic and magnetic investigations. Little has been accomplished on systems containing calcium ion since its rare-gas electronic configuration makes it difficult to probe by conventional spectroscopic techniques.

A solution to this inherent difficulty can be achieved by using lanthanide metal ions as substitutes for calcium (Birnbaum *et al.* 1970; Darnall and Birnbaum, 1970; Williams, 1970). In contrast to the calcium ion, the varied magnetic and spectral properties of the rare earth metal ions should make excellent spectroscopic probes of the metal ion binding sites in

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proteins. Moreover since the sizes of the lanthanide ions decrease across the series (with very little change in chemical properties) an assessment of the importance of size of the metal ion in the biochemical system can be made.

Since these initial proposals were made, it has been shown that lanthanide ions isomorphously replace the calcium ion in α-amylase (Smolka *et al.*, 1971; Darnall and Birnbaum, 1973), in thermolysin (Colman *et al.*, 1972), and in the conversion of trypsinogen to trypsin (Darnall and Birnbaum, 1970). In addition, it has been shown that lanthanide ions replace Mg^{2+} in leucine tRNA synthetase (Kayne and Cohn, 1972) and in pyruvate kinase (Valentine and Cottam, 1973). Lanthanide ions also replace both Ca^{2+} and transition metal ions in conconavalin A (Sherry and Cottam, 1973). Other investigators have taken advantage of the spectroscopic properties of lanthanide ions to probe their binding sites in various other enzymes and proteins (Dwek *et al.*, 1971; Luk, 1971; Reuben, 1971a,b).

Trypsinogen is known to bind calcium ions at two sites, one being associated with the N-terminal hexapeptide, and the other being located somewhere in the body of the molecule, with the latter being the stronger of the two binding sites (Delaage and Lazdunski, 1967; Abita *et al.*, 1969; Radhakrishnan *et al.*, 1969). Trypsin possesses only one binding site for calcium and it appears to be the same as the strong site on trypsinogen (Delaage and Lazdunski, 1967), but little is known about its position in the protein or the coordination geometry surrounding the metal ion (Stroud *et al.*, 1972).

The calcium ion accelerated conversion of trypsinogen to trypsin has been known for some time (McDonald and Kunitz, 1941). The activation of trypsinogen involves the hydrolysis of the Lys^6–Ile^7 bond resulting in the cleavage of the hexapeptide Val-$(Asp)_4$-Lys from the amino terminus of the molecule (Davie and Neurath, 1955; Desnuelle and Fabre, 1955). Radhakrishnan *et al.*, (1969) showed that the Ca^{2+} accelerated tryptic hydrolysis of the Lys^6–Ile^7 bond of the NH_2-terminal hexapeptide of trypsinogen was eliminated upon chemical modification of the aspartyl carboxyl groups of the peptide. The rate of conversion of the modified trypsinogen to trypsin was nevertheless faster than the conversion of native trypsinogen to trypsin in the absence of Ca^{2+}. Abita *et al.* (1969) showed that the ligands involved in the weak calcium binding site in trypsinogen are the β-carboxyl groups of the two aspartyl residues which are the nearest neighbors of the important Lys-Ile bond. The saturation of this site by Ca^{2+} favors the formation of the trypsinogen–trypsin complex, but calcium ion has no effect on the decomposition of this complex into products.

We reported earlier that neodymium ion accelerates the trypsinogen–trypsin conversion to a greater degree than does calcium ion (Darnall and Birnbaum, 1970). In this paper we extend these studies and assess the importance of the size of the metal ion in the conversion.

Experimental Section

Materials. The trypsin used (lot 22C-8121, Sigma Chemical Co.) was a twice crystallized, lyophilized, essentially salt-free preparation. The trypsinogen (lot 117B-1980, Sigma Chemical Co.) was once crystallized and contained 28% $MgSO_4$. Tris (Ultra Pure) was purchased from Mann Research Laboratories. The lanthanide metals were obtained as the oxide (99.99% pure) from the Molybdenum Corporation of America and the Kerr-McGee Corp. *p*-Toluenesulfonyl-L-arginine methyl ester hydrochloride (Tos-ArgOMe)[1] was purchased from Sigma (lot 81C-0550) and used without further purification. The remainder of the chemicals used in the study were reagent grade from Baker Chemical Co. Distilled, deionized water from a Corning all-glass distillation apparatus was used throughout.

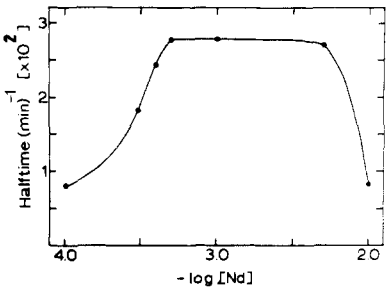

FIGURE 1: Effect of neodymium concentration on the rate of activation of trypsinogen. The reciprocal of the half-time for complete conversion of trypsinogen to trypsin is plotted as a function of the metal ion concentration, at 0°, ionic strength 0.3 and pH 8. Trypsin concentrations were determined by assay at 25° in 10^{-3} M Tos-ArgOMe.

Methods. A stock trypsinogen solution was prepared by dissolving the commercial preparation in cold 10^{-3} M HCl so that a solution of approximately 14–20 mg/ml was obtained. The cloudy suspension that resulted was centrifuged at 30,000*g* for 5 min to remove the insoluble or denatured material. The clear supernatant solution was filtered into a dialysis bag and dialyzed against 10^{-3} M HCl for 16 hr at 2° using 4–5 changes of dialysate. A stock solution of trypsin was prepared in much the same manner except that the dialysis was carried out for only two changes of dialysate since the preparation was essentially salt free initially. After dialysis, the protein concentrations were determined spectrophotometrically at 278 nm using $E_{1\,cm}(1\%)$ 15.4 for trypsin and 13.7 for trypsinogen. The trypsinogen solution was then divided into 2–3-ml aliquots in screw-top test tubes and frozen until needed. The trypsin was diluted to a concentration of 2 mg/ml with 10^{-3} M HCl and stored at 8° until needed. Fresh enzyme was prepared weekly or sooner if the specific activity decreased, and the trypsinogen was rarely stored more than 30 days.

Metal Ion Solutions. The lanthanide ion solutions were prepared by dissolving the oxide in a slight stoichiometric excess of HCl. These were then diluted to approximately 0.15 M with water and the actual concentrations were determined by titration with standard EDTA at pH 6 using Xylenol Orange as the indicator. The metal ion solutions were then adjusted to precisely 0.10 M with water (final pH 2–3) and all subsequent dilutions were made from these adjusted stock solutions. The calcium stock solution (0.50 M) was prepared by dissolving the anhydrous chloride in water (final pH 6).

Assay Procedure. Trypsin activity was determined by a slight modification of the method of Hummel (1959). Tos-ArgOMe was dissolved to a concentration of 10^{-3} M in 0.05 M Tris-Cl buffer at pH 8.0 at 25°. The assay mixture also contained 0.05 M calcium chloride and the ionic strength was adjusted to 0.3 with sodium chloride. Trypsin activity was monitored by following the increase in OD at 247 nm using a Gilford 222A spectrophotometer. All activity measurements were conducted at 25°.

Activation of Trypsinogen. The metal ion was mixed with trypsinogen and, at time zero, a catalytic amount of trypsin was added to initiate the conversion of trypsinogen to trypsin. All incubation experiments were conducted at 0° and a pH of 8.0 (measured at 0°). The ionic strength was maintained at 0.3 with NaCl. The final trypsinogen concentration used in all of the experiments was in the range 0.28–1.25 mg/ml, while the

[1] Abbreviation used is: Tos-ArgOMe, *p*-tosylarginine methyl ester.

TABLE I: Lanthanide Ion Concentrations for Maximal Zymogen Conversion.[a]

Metal Ion	Concn × 10^4 (M)
La^{3+}	5.0
Pr^{3+}	4.5
Nd^{3+}	4.5
Sm^{3+}	3.5
Gd^{3+}	3.0
Tb^{3+}	2.0
Ho^{3+}	2.0
Lu^{3+}	0.8

[a] The initial trypsinogen concentration was 0.48 mg/ml. The reaction was initiated by addition of trypsin (final concentration, 0.05 mg/ml) at pH 8 and 0°. $\mu = 0.3$.

final trypsin concentration used to initiate the reaction was 0.05 mg/ml. A typical experiment involved diluting the stock trypsinogen to 1.10 mg/ml with water and adding to this an equal volume of the proper Tris buffer. Aliquots of this solution were then pipetted into test tubes according to the following scheme: 1.75 ml of trypsinogen–Tris; 0.20 ml of metal ion solution; 0.05 ml of 2 mg/ml trypsin (at time zero). The incubation mixtures were maintained at 0° and were stirred gently with micro Teflon-covered stirring bars. At various time intervals, 0.05-ml aliquots were removed and assayed for trypsin activity.

Dependence of Activation Rate on Lanthanide Ion Concentration. All of these experiments were conducted at a final trypsinogen concentration of 0.48 mg/ml. A series of lanthanide ion solutions were prepared that spanned the range of 10^{-4}–10^{-1} M. Each of these solutions was used in a separate activation experiment and the rate of conversion of trypsinogen to trypsin was followed. A typical experiment consisted of eight incubation vials, seven of which contained different concentrations of a particular lanthanide metal ion; the eighth vial was identical except that in place of the lanthanide, a calcium solution was added to achieve optimal conversion (final calcium ion concentration of 0.05 M). This procedure allowed the rates obtained to be compared with the optimal rate obtained with calcium.

Results and Discussion

Lanthanide Ion Effects on Trypsin and Tos-ArgOMe. It was shown earlier that Nd^{3+} stimulated the apparent conversion of trypsinogen to trypsin (Darnall and Birnbaum, 1970). This stimulation could have three possible explanations: (1) the conversion rate is accelerated, (2) the lanthanides increase the activity of any trypsin formed in the conversion, and/or (3) since the amount of trypsin converted was monitored by activity measurements, the lanthanides themselves catalyze the hydrolysis of Tos-ArgOMe. Possibility (3) was ruled out since the addition of Nd^{3+} to the assay system in the absence of enzyme produced no hydrolysis of Tos-ArgOMe. Possibility (2) was ruled out when it was shown that, up to certain concentrations, none of the lanthanide ions had any effect on trypsin activity. At high concentrations, the lanthanide ions did inhibit trypsin activity (see below). Therefore it appeared that Nd^{3+} was indeed accelerating the rate of conversion of zymogen to enzyme.

Dependence of the Activation Rate on Metal Ion Concentration. Figure 1 shows the dependence of the activation rate of trypsinogen on the concentration of neodymium(III). Under these conditions, concentrations of Nd^{3+} between 4.5×10^{-4} and 3×10^{-3} M give maximal conversion rates. At higher concentrations of the metal ion the conversion is inhibited. The metal concentration dependence of the activation of trypsinogen was examined for several lanthanide ions, and the lowest metal ion concentration for maximum conversion rate is tabulated in Table I.

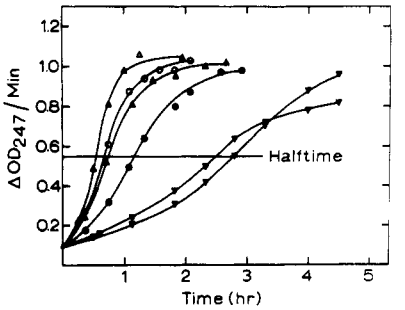

FIGURE 2. Conversion rates of trypsinogen to trypsin in the presence of various metal ions. Conditions are those for Figure 1. Initial trypsinogen concentrations were 0.42 mg/ml. (△) La, Pr, Nd; (○) Gd; (▲) Sm; (●) Ho; (▽) 0.05 M Ca; (■) Ln. Metal ion concentrations were those listed in Table I.

Dependence of the Activation Rate on Various Lanthanides. Since the data in Table I show that the maximum rate of activation of trypsinogen by trypsin occurs at slightly different concentrations of metal ion, a comparison of the effect of several lanthanides on the conversion rate was made at that metal ion concentration (Table I) which produced the maximal rate. Figure 2 shows the full conversion rate profiles for a series of lanthanide ions. The lanthanide ions were all capable of accelerating the conversion of zymogen and furthermore essentially 100% conversion of zymogen to enzyme was obtained when either a calcium ion or a lanthanide ion was present. Figure 2 shows that all of the lanthanide ions accelerate the conversion of trypsinogen more rapidly and at much lower concentrations than does the calcium ion.

A plot of the half-time for the complete conversion against the crystal ionic radius of the lanthanides is shown in Figure 3. From this graph it is clearly seen that, under the conditions employed, the larger lanthanides accelerate the activation of trypsinogen much better than the smaller ones. Since the two aspartate residues closest to the Lys–Ile bond on trypsinogen have already been implicated in the binding of the calcium ion, it seems reasonable to suppose that the larger lanthanides are

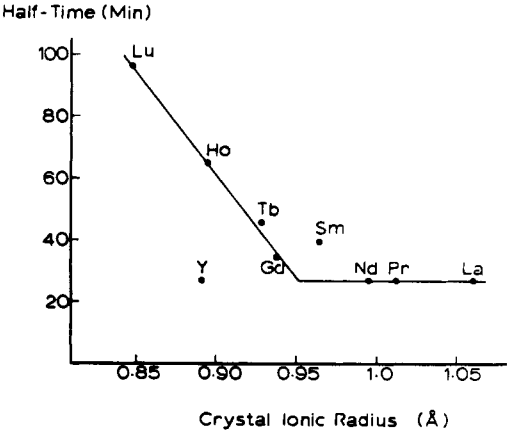

FIGURE 3: Plot of half-time of conversion of trypsinogen to trypsin as a function of the metal ion crystal ionic radius. Half-times are those from Figure 2. Crystal ionic radii taken from Templeton and Dauben (1954).

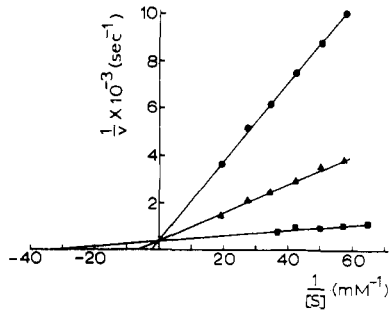

FIGURE 4: Lineweaver-Burk plots for the activation of trypsinogen by trypsin: (●) 4 mM Ca^{2+}, K_m = 0.445 mM; (▲) 50 mM Ca^{2+}, K_m = 0.159 mM; (■) 0.5 mM Nd^{3+}, K_m = 0.033 mM. Data for 0.1 mM Lu^{3+} were nearly identical with 50 mM Ca^{2+}.

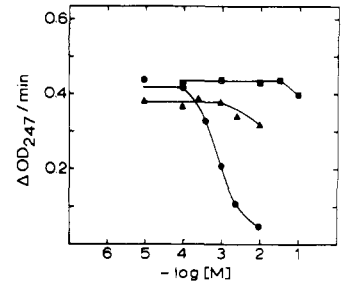

FIGURE 6: Effect of metal ion concentration on trypsin activity. Conditions were arranged to follow as closely as possible to those during the activation of trypsinogen. Trypsin concentrations were 0.017 mg/ml; assays were run 3 min after mixing trypsin and metal ions. All assays run at 0°, ionic strength 0.3 at pH 8. (■) Ca^{2+}; (▲) La^{3+}; (●) Lu^{3+}.

capable of bridging the gap between the two aspartate carboxyls, whereas the smaller ions are less efficient at bridging this gap, and hence at neutralizing the region of anionic charge on the hexapeptide. This is in agreement with the fact that magnesium ion (ionic radius 0.65 Å) has no effect on the conversion rate, whereas calcium ion (ionic radius 0.99 Å) accelerates the rate. The fact that the lanthanides, such as Nd^{3+} and Pr^{3+}, accelerate the conversion markedly better than calcium ion yet have nearly the same ionic radius, can be ascribed to the higher charge of the lanthanides, which more effectively neutralizes the highly anionic region near the bond being cleaved.

Effect of Lanthanides on K_m and V_{max}. Typical Lineweaver-Burk plots for the conversion of trypsinogen to trypsin in the presence of calcium and neodymium ions are seen in Figure 4. The effect of calcium ion is the same as that noted by Abita *et al.*; *i.e.*, calcium bound at the hexapeptide site decreases the K_m of the reaction by a factor of 3 while the V_{max} is unaffected. Neodymium ion, on the other hand, at 5×10^{-4} M causes a decrease in the K_m by a factor of 14 while V_{max} remains unchanged. Thus the effect of the lanthanide ion is to increase the affinity of trypsinogen for trypsin much more than does the calcium ion.

The idea that larger metal ions are able to bridge the two aspartate residues more effectively than the smaller ones is attractive. If the larger lanthanides do bridge the binding site better, then we would expect that the trypsinogen-lanthanide binding constants would reflect this. However, one piece of data was inconsistent with this prediction. A study of the lanthanide ion concentration dependence of the activation showed that all the lanthanide ions produced bell-shaped curves typical of an acceleration of the conversion at low lanthanide concentrations followed by inhibition at high lanthanide concentrations (see Figure 1). As shown in Figure 5, lutetium ion, the smallest lanthanide, accelerated the conversion at a lower concentration than did praseodymium ion, one of the largest lanthanides. This suggests that the lutetium ion has a higher binding constant than does praseodymium ion, which is clearly not consistent with the concept of the larger lanthanide bridging the two aspartate carboxyl groups better. Figure 5 also shows that the conversion of trypsinogen is inhibited at a lower concentration of lutetium than praseodymium. This inhibition could be due to inhibition of trypsin activity itself or to inhibition of zymogen activation by combination of the metal ion with trypsinogen. To distinguish between these possibilities the effect of varying lanthanide concentrations on trypsin itself was tested. Figure 6 shows the effects of various concentrations of lanthanide ions on trypsin activity using Tos-ArgOMe as the substrate. At low concentrations of lanthanide or calcium ions there is no effect on trypsin activity. However, it is clear that at higher lanthanide concentrations there is inhibition of trypsin and furthermore Figure 6 shows that lutetium inhibits at lower concentrations than does the larger lanthanum. This means that the concentrations of lanthanides that produce the maximum observed acceleration of the trypsinogen conversion (shown in Table I) are in reality a balance between the concentrations that produce maximum acceleration and those which inhibit trypsin activity.

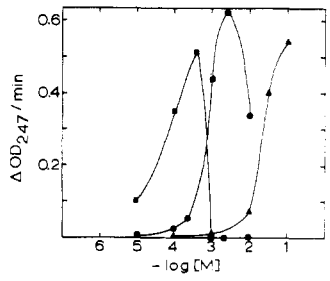

FIGURE 5: Effect of metal ion concentration on the activation of trypsinogen to trypsin. Initial trypsinogen concentrations were 1.5 mg/ml. The reaction was initiated by adding trypsin (0.05 mg/ml final concentration) at zero time; 18 min later samples were withdrawn and assayed for trypsin activity at 25°. All samples incubated at 0°, ionic strength 0.3 and pH 8. (▲) Ca^{2+}; (■) Lu^{3+}; (●) Pr^{3+}. The activities of the Ca^{2+} and Lu^{3+} were multiplied by 2 and 5, respectively, to fit on the same scale as Pr^{3+}.

In an attempt to rationalize the kinetic data across the lanthanide series with the metal ion concentration dependence data, the activation profiles of trypsinogen in the presence of all the lanthanide ions were reexamined at lanthanide concentrations low enough (10^{-4} M) so that there was no inhibition of trypsin activity. A completely different plot of the half-time of conversion as a function of lanthanide ionic radius was obtained under these conditions as is seen in Figure 7 (compare to Figure 3). This plot reveals no simple relationship between the effectiveness of the ion in accelerating the conversion and the size, charge to ionic radius ratio, or any other known parameter which reflects the size of the cation. The plot does bear a similarity to thermodynamic data across the lanthanide series for some simple carboxylate ligands (Moeller *et al.*, 1965) and also for sulfate ligands (Fay, 1969). Fluctuations of this type in stability constant data have generally been attributed to variations in the coordination number of the hydrated ion and to variations in the amount of bound water on the complex species as one crosses the lanthanide series. Since the stability constants for weak complexes of the lanthanides have a large contribution from the entropy term of complexation, this type of variation is particularly important (Moeller *et al.*, 1968). If we can extrapolate from the simple ligands to the protein, and if the

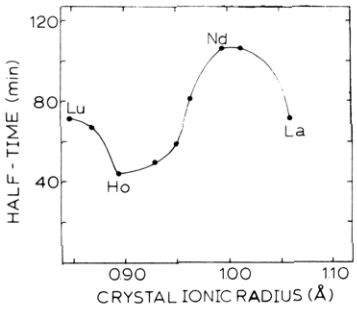

FIGURE 7: Plot of half-time of conversion of trypsinogen to trypsin as a function of the metal ionic radius. Conditions were identical with those in Figure 2 except all metal ions were at 10^{-4} M.

observed kinetics of the conversion reflects the variation in the binding constant at the hexapeptide of trypsinogen, then this variation in the conversion rate may be due to the different degrees of hydration of the lanthanide ion when it is bound to the protein. Thus although the size of the metal ion may be very important in determining metal ion specificity, an additional factor may be the extent of dehydration of the metal ion which occurs upon binding. If complete dehydration of the cation occurs upon binding, then a straightforward dependence on size would be expected.

Conclusions

All of the evidence so far accumulated indicates that metal ions accelerate the activation of trypsinogen to trypsin by binding to at least two aspartate carboxyl groups on the N-terminal hexapeptide of trypsinogen. The binding of the metal ion, be it a lanthanide ion or a calcium ion, increases the affinity of trypsinogen for trypsin without affecting the maximum velocity of the reaction. Others have suggested that the metal ion functions in this manner by decreasing the high anionic charge on the hexapeptide (Abita *et al.*, 1969; Radhakrishnan *et al.*, 1969). It is reasonable to ask, therefore, how a decrease of high negative charge on the N-terminal hexapeptide would decrease the K_m (*i.e.*, increase the affinity of trypsinogen for the enzyme). We have depicted one possible explanation in Figure 8. Figure 8A shows a fully extended space-filling model of the N-terminal hexapeptide, Val-Asp-Asp-Asp-Asp-Lys. It is known that the specificity site, which is the site on trypsin responsible for recognizing lysine or arginine side chains, is composed of a pocket which has a negatively charged carboxyl group at the end of it (Stroud *et al.*, 1972). The lysine or arginine side chains near the bond to be cleaved by trypsin must be free in order for binding of the substrate (in this case trypsinogen) to take place. Figure 8B shows one possible conformation of the hexapeptide in the absence of metal ions. Since there is such a high density of negative charge near the lysine side chain, it seems reasonable that there should be an electrostatic attraction between the lysyl side chain and the carboxyl groups, resulting in a situation depicted by the model shown in Figure 8B, where the protonated amino group is bound to the negative carboxylate groups. In this conformation the lysine side chain could not be as free for binding in the specificity pocket on trypsin as would the side chain shown in Figure 8A. The rate of the conversion of trypsinogen to trypsin would then be dependent on the extent to which the conformations shown in Figure 8A and B existed in solution. This in turn would depend on the affinity of the protonated amine for the deprotonated carboxyl groups. Anything which shifts the equilibrium between the two conformations toward the extended side chain would accelerate the conversion of zymogen to enzyme. Presumably then the binding of the metal ion is able to effect this shift in the conformational equilibrium.

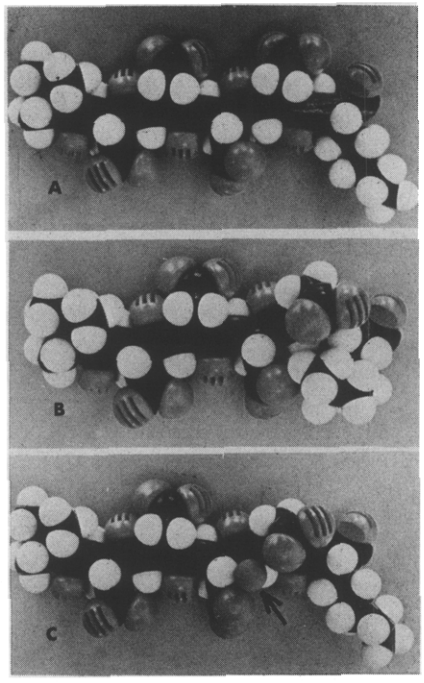

FIGURE 8: (A) Fully extended space-filling model of the N-terminal hexapeptide of trypsinogen. (B) Space-filling model of the N-terminal hexapeptide of trypsinogen with the ϵ-amino group of lysine bound to the two aspartate carboxyls nearest the lysine residue. (C) Space-filling model of the N-terminal hexapeptide of trypsinogen with a metal ion chelated between the two aspartate carboxyls nearest the lysine residue. The arrow points to the position of the metal ion.

Figure 8C shows a probable conformation of the hexapeptide in the presence of the bound calcium ion or a lanthanide ion. The metal ion can be effectively coordinated by the chelate consisting of the two carboxyl groups on the aspartate residues nearest the lysyl residue and this significantly reduces the interaction of the lysyl side chain with the carboxyl groups. The greater the binding constant of the metal ion at this site on trypsinogen, the greater the shift in the equilibrium towards the extended side chain conformation and the more rapid the acceleration of the conversion.

Another possible explanation of the effect of metal ion binding is that, instead of changing the conformation of the substrate as shown in Figure 8, direct interactions between the aspartate carboxyls and the trypsin molecule are altered so that the enzyme–substrate affinity is altered.

The differences between the shapes of the curves shown in Figures 3 and 7 point out the care which must be exercised in using lanthanides as calcium ion substitutes. All the lanthanides activate the conversion of trypsinogen to trypsin, but they also inhibit trypsin activity at higher concentrations. Furthermore the metal ion concentrations at which the activation and inhibition occur are different for each of the lanthanide ions. This same phenomenon, *i.e.*, activation at low concentrations and inhibition at higher concentrations of lanthanide ion, has recently been observed for α-amylase (Darnall and Birnbaum, 1973). It may be that this is a general phenomenon; thus, studies involving lanthanide ion binding to other proteins and enzymes should be carried out under a variety of lanthanide ion concentrations.

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Equilibrium Kinetic Study of Bovine Liver Glutamate Dehydrogenase at High pH†

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ABSTRACT: The kinetics at equilibrium of bovine liver glutamate dehydrogenase have been studied by isotopic exchange of substrate and coenzyme at 25° with glutamate and alanine systems in 126 and 136 mM Veronal buffer at pH 8.8 and in 138 mM Veronal buffer at pH 9.5. At pH 8.8 increase in substrate concentration (alanine:pyruvate or glutamate:α-ketoglutarate) at equilibrium from below K_m to more than tenfold greater resulted in marked and progressive suppression of the $NAD^+ \leftrightarrow$ NADH equilibrium reaction rate (measured with tracer [^{14}C]NADH) while the alanine $\leftrightarrow$ pyruvate (measured with [^{14}C]pyruvate) and glutamate $\leftrightarrow$ α-ketoglutarate (measured with [^{14}C]-α-ketoglutarate) rates simultaneously rose progressively toward maximum values. In similar experiments at pH 9.5 only a slight suppression in the $NAD^+ \leftrightarrow$ NADH equilibrium rates was observed with increasingly saturated substrate concentration while alanine $\leftrightarrow$ pyruvate and glutamate $\leftrightarrow$ α-ketoglutarate rates, respectively, rose toward maximum values. Increase in NAD^+:NADH concentration from highly unsaturating to near saturating resulted in a rise in alanine $\leftrightarrow$ pyruvate and glutamate $\leftrightarrow$ α-ketoglutarate rates to plateau values without any inhibition. These results are compatible with a compulsory binding order mechanism for bovine liver glutamate dehydrogenase with both glutamate and alanine substrate systems at pH 8.8, and an alternative order mechanism (partially compulsory) at pH 9.5, in contrast to an alternative order (random) mechanism previously demonstrated at pH 8.0 (Silverstein and Sulebele, 1973, 1974). Minimum estimates for some dissociation constants were obtained from the kinetic data. These findings further confirm the general similarity in the mechanism of bovine liver glutamate dehydrogenase with respect to glutamate and alanine substrate systems despite marked differences between them with respect to rate, pH optima, and modulation of activity by allosteric effectors, and suggest that similar major alterations in kinetic mechanism by changes in pH and perhaps other factors may obtain for other enzyme systems as well.

Bovine liver glutamate dehydrogenase (L-glutamate: NAD(P) oxidoreductase (deaminating), EC 1.4.1.3) is a six subunit allosteric enzyme important in amino acid and carbohydrate metabolism which catalyzes the oxidative deamination of glutamate, alanine, and other amino acids to the corresponding α-keto acids and ammonium ion (Frieden, 1963; Eisenberg, 1970). While the catalysis with glutamate and alanine differs markedly with respect to rate, pH optima, and modulation of activity by allosteric effectors (Struck, Jr., and Sizer, 1960; Frieden, 1959, 1963, 1964, 1971; Tomkins *et al.*, 1961), we have shown in equilibrium kinetic studies of the catalytic and allosteric mechanisms of regulatory enzymes that the enzyme

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