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Fluorescence Energy-Transfer Measurements between the Calcium Binding Site and the Specificity Pocket of Bovine Trypsin Using Lanthanide Probes†

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ABSTRACT: Using fluorescence energy-transfer experiments we have measured the distance between the specificity pocket and the calcium ion binding site of bovine pancreatic trypsin. Proflavin and thionine were used to block the specificity site, whereas various lanthanide ions were substituted for the calcium. It was then possible to choose various donor–acceptor pairs which exhibited suitable energy transfer. We have calculated the distance between proflavin and Nd(III), Pr(III), and Ho(III) to be 10.9, 10.3, and 10.3 Å, respectively. This agrees very well with the value of approximately 10 Å we obtained between the methyl protons of *p*-toluamidine (a competitive inhibitor) and Gd(III) using nuclear magnetic resonance techniques (Abbott, F., Gomez, J. E., Birnbaum, E. R., and Darnall, D. W. (1975), *Biochemistry 14,* 4935). This is strong evidence that, in solution, the calcium binding site is composed of the side chains of Ser-190 and Asp-194.

It has been established for some time that bovine pancreatic trypsin binds one calcium ion (Delaage and Lazdunski, 1967). Although the presence of calcium has no apparent effect upon the activity of trypsin, the metal ion does retard denaturation and degradation of the protein by autolysis (Green and Neurath, 1953; Delaage and Lazdunski, 1967; Gabel and Kasche, 1973; Lazdunski and Delaage, 1965). The exact nature of the single trypsin calcium ion binding site has been the subject of several investigations. Chemical modification and titration of carbonyl side chains on trypsin indicate that at least one carboxyl group is involved in calcium ion coordination to the enzyme (Abita and Lazdunski, 1969; Delaage and Lazdunski, 1967; Duke et al., 1952). It was earlier proposed that the cal-

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cium ion binding site might be composed of one or more of the carboxyl groups of Asp-71,[1] Glu-77, or Asp-153 (Abita and Lazdunski, 1969; Stroud et al., 1971). This site lies approximately 25 Å from the specificity site of the enzyme Asp-189. Results from nuclear magnetic resonance (Darnall et al., 1975; Abbott et al., 1975a,b) and electron spin resonance (Cohen-Addad and Leyssieux, 1973) indicate, however, that the single metal site on the enzyme is only 10–13 Å away from the specificity pocket. Based upon this distance requirement and the requirement that at least one carboxyl group is involved in the binding site, we proposed that the most likely calcium ion binding site would consist of the side chains of Asp-194 and Ser-190 (Abbott et al., 1975a,b). An explanation of how the metal ion stabilized the enzyme toward autolysis was suggested by this metal ion binding site.

Recently Bode and Schwager (1975a,b) completed an x-ray structure determination of crystalline bovine trypsin containing a single calcium ion bound between Glu-70 and Glu-80. This particular site is approximately 25 Å from the specificity site and does not offer any obvious explanations for the stabilizing effects of calcium ion.

In view of the large discrepancy between the distance measurements we obtained in solution using magnetic resonance methods compared with the results obtained by crystallographic methods we have employed fluorescence energy transfer techniques to measure distances from the active site of trypsin to the metal binding center.

Isomorphous replacement of bound calcium in various proteins by lanthanide ions has been widely demonstrated (Darnall and Birnbaum, 1973; Matthews and Weaver, 1974; Shimomura and Johnson, 1973). Lanthanide ions have been shown to influence the stability of trypsin in the same manner as does calcium (Gomez et al., 1974) and to compete with calcium for the single metal ion binding site in solution (Abbott et al., 1975b). We have taken advantage of replacing the calcium ion in trypsin with various lanthanides and have observed energy transfer from proflavin, a competitive inhibitor, to the lanthanides. To our knowledge this is the first demonstration that lanthanide ions can be suitable energy-transfer acceptors in biological systems.

Theory

Förster proposed that the transfer of electronic excitation energy between two chromophores, one of which is fluorescent, can occur by a dipole–dipole mechanism under certain conditions (Förster, 1948). Verification of this theory for the measurement of molecular distances has been found for organic chromophores (Bennett, 1964; Bennett et al., 1964; Kellogg, 1964) and recently for inorganic chromophores where terbium ion was the fluorescent donor and cobalt the energy acceptor (Berner et al., 1975; Horrocks et al., 1975).

The efficiency of such energy transfer from the fluorescent donor to an acceptor is given by

$$E = \frac{1}{1 + \left(\frac{r}{R_0}\right)^6} \tag{1}$$

where r is the distance between donor and acceptor, and R_0 is the "critical distance" at which energy transfer between chromophores is 50% efficient. R_0 in Å may be calculated by the following relationship (Förster, 1966):

$$R_0^6 = \frac{9000 \ln 10 K^2 \Phi_0}{128 \pi^5 n^4 N} J_\nu \tag{2}$$

where K^2 is the orientation factor for the transition dipole moments of the donor and acceptor, Φ_0 is the fluorescence quantum yield of the donor in the absence of acceptor, n is the refractive index of the medium, and N is Avogadro's number. J_ν is the spectral overlap integral of acceptor absorbance and donor fluorescence, normalized to unity:

$$J_\nu = \frac{\int_0^\infty \epsilon_A(\lambda) F_D(\lambda) \lambda^4 d\lambda}{\int_0^\infty F_D(\lambda) d\lambda} \tag{3}$$

$\epsilon_A(\lambda)$ is the molar extinction coefficient of the acceptor as a function of wavelength λ, and $F_D(\lambda)$ is the relative fluorescence intensity of the donor at wavelength λ.

Experimental Section

Twice-crystallized, dialyzed, and lyophilized bovine pancreatic trypsin (Sigma Chemical Co., type III) was dialyzed against three changes of 10^{-3} M hydrochloric acid at 4 °C and lyophilized. Concentrations of stock trypsin solutions were determined by their absorption at 278 nm using an extinction coefficient of 1.67 cm^{-1} mg^{-1} ml (Abbott et al., 1975b). Activity of the enzyme was monitored as described previously (Gomez et al., 1974).

Chloride salts of praseodymium(III), neodymium(III), terbium(III), and holmium(III) were prepared by adding 0.1 N HCl to an excess of the sesquioxide (99.9+% from Kerr-McGee Corp. and Molybdenum Corp. of America), filtering the solution, and evaporating the filtrate to dryness. Nitrate salts of lanthanum(III) and gadolinium(III) were prepared in the same manner, substituting 0.1 N HNO_3 for HCl. Stock lanthanide solutions were analyzed by complexometric titration in pH 6 acetate buffer with an EDTA[2] solution standardized against fired La_2O_3. Xylenol orange (0.5% in 50:50 ethanol–water) was used as the indicator (Lyle and Rahman, 1963).

Acetamidine hydrochloride (Aldrich Chemical Co.), proflavin (3,5-diaminoacridine, Aldrich Chemical Co.), thionine (J. T. Baker Chemical Co.), and Mes buffer (2-(*N*-morpholino)ethanesulfonic acid, Sigma Chemical Co.) were used as received. *p*-Toluamidinium nitrate was prepared by passing the hydrochloride salt (Columbia Organic Chemicals Co.) through an anion-exchange column containing Ag 1-X8 (20–50 mesh, Bio-Rad Laboratories) in the nitrate form. Concentrations of stock solutions of proflavin and thionine were determined spectrophotometrically. For proflavin a molar extinction coefficient, ϵ_m, of 3.34×10^4 at 444 nm was used (Bernhard et al., 1966), and $\epsilon_m = 6.0 \times 10^4$ at 595 nm for thionine (Havemann et al., 1962) was used.

Chromatographically purified porcine pancreas elastase was purchased from Signia Chemical Co. Congo red elastin (Sigma) was used to measure elastase activity.

Trypsin–lanthanide ion and elastase–lanthanide ion binding constants were determined by equilibrium dialysis as described earlier (Abbott et al., 1975b) with the exception that the inhibitor was either *p*-toluamidine or *p*-aminobenzamidine.

Fluorescence spectra were obtained with an Aminco-Bow-

[1] Throughout this paper we shall use the amino acid sequence numbering system of chymotrypsinogen A.

[2] Abbreviations used: EDTA, ethylenediaminetetraacetic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; NMR, nuclear magnetic resonance.

TABLE I: Transfer Efficiencies and Distances between Chromophores in Bovine Trypsin.[a]

Donor	Acceptor	Overlap Integral J_ν ($cm^3\ M^{-1}$)	Critical Distance, R_0 (Å)	Efficiency of Transfer, E (%)	R (Å)
Terbium(III)[b]	Thionine	1.58×10^{-13}	44.1	>99	<21
Proflavin[c]	Praseodymium(III)	6.22×10^{-18}	8.19	20.2	10.3
Proflavin[c]	Neodymium(III)	7.49×10^{-18}	8.45	17.6	10.9
Proflavin[c]	Holmium(III)	5.98×10^{-18}	8.14	19.7	10.3

[a] At pH 6 with 0.05 M Mes buffer, $\mu = 0.3$ adjusted with NaCl. [b] Excitation at 280 nm. [c] Excitation at 442 nm.

man Ratio spectrophotofluorimeter using an R818 photomultiplier tube and 3-mm cuvettes. Using the R818 phototube and quinine sulfate as a standard, it was shown that no correction was needed to obtain "true" emission spectra between 450 and 600 nm. Lanthanide ion concentrations varied from 5×10^{-4} to 5×10^{-3} M, while concentrations of trypsin and inhibitor (either thionine or proflavin) were in the range 10^{-6} to 8×10^{-5} and 9×10^{-7} to 2×10^{-6} M, respectively. Optical density at the excitation wavelength was less than 0.1 (for 1-cm cell) to ensure linearity in fluorescence response.

Proton relaxation times were determined with a JEOL PS-100, high-resolution, nuclear magnetic resonance spectrometer as described previously (Abbott et al., 1975b). Absorption spectra were recorded with a Cary 14 recording spectrophotometer.

Results

A prerequisite for use of the Förster relationship for energy transfer is that there must be appreciable overlap between the donor fluorescence and acceptor absorption for the two chromophores (Förster, 1959). Thionine and proflavin are strong reversible inhibitors of tryptic activity and possess suitable fluorescent and absorption characteristics for such investigations. Although only europium(III) and terbium(III) ions can act as fluorescent donors, most of the lanthanides have absorption bands in the visible–ultraviolet region and can therefore act as acceptors.

The fluorescence bands of terbium(III) and the absorption of thionine were found to have suitable overlap in the 500–625-nm range. The overlap integral between these two chromophores (Table I) was evaluated using the absorption spectrum of trypsin-bound thionine since the thionine absorption band at 597 nm undergoes a red shift of approximately 20 nm upon binding with trypsin (Glazer, 1967). Although the fluorescence of terbium(III) is greatly enhanced upon binding to proteins (Epstein et al., 1974; Sherry and Cottam, 1973; Berner et al., 1975), no shifts of the fluorescent bands were observed.

In calculating the "critical distance", R_0, the refractive index, n, was taken to be that of the solvent, 1.33. K^2, the orientation factor, was assumed to be ⅔ (possible deviations from this value will be discussed later) and a quantum yield, Φ_D, of 0.25 was used for terbium(III). Although quantum yields for terbium(III) vary from 0.15 to 0.40 depending upon the nature of the chelating agent (Berner et al., 1975), the resultant change in R_0 for this system is less than ±10% using either extreme value for Φ_D.

An excitation wavelength of 280 nm was used for recording the fluorescence spectra for solutions containing trypsin, terbium(III), and thionine. Amounts of ternary complex were calculated using a $K_d = 2.3 \times 10^{-5}$ M for the trypsin–thionine complex (Glazer, 1967) and $K_a = 3.85 \times 10^3\ M^{-1}$ for terbium(III) binding to trypsin. Monitoring either the quenching of terbium(III) fluorescence or the enhancement of the thionine fluorescence, the efficiency of energy transfer was found to be greater than 99%. Since the energy transfer is essentially 100% efficient, one can only calculate an upper limit for the value of the distance between these two chromophores. Using eq 1 we calculate that the distance between thionine and the terbium ion must be *less* than 21 Å (Table I).

In order to obtain a more discriminating value of the distance between the active site and the metal ion binding site, other suitable donor–acceptor chromophore pairs were sought. In order to be able to measure distances smaller than 21 Å, the critical transfer distance, R_0, must be reduced from the value of 44 Å in the Tb–thionine donor–acceptor pair. Of the terms in eq 2, only the expression for the overlap integral, J, is susceptible to a large variation. This may be accomplished by changing the absorbing chromophore from one which has a high extinction coefficient, such as thionine ($\epsilon \sim 60\,000$), to one with a low extinction coefficient, such as a lanthanide ion ($\epsilon \sim 10$). As can be seen from eq 3, a change in ϵ of 10^3 will make a proportionate change in J, and a change of roughly a factor of ⅓ in the R_0, assuming an r^6 exponential dependence for a dipole–dipole mechanism. Proflavin was found to be a suitable fluorescent donor chromophore for such investigations since it has an excitation maximum at 442 nm and a broad fluorescent band at 520 nm, a region where several lanthanide ions have absorption bands with molar extinction coefficients on the order of 2.0–10.0. Praseodymium(III), neodymium(III), and holmium(III) were chosen as acceptors chromophores since they exhibit appreciable spectral overlap with proflavin fluorescence. Figure 1 shows the lanthanide absorption curves and the proflavin fluorescence spectrum from which overlap integrals were calculated. The overlap integrals for these three ions with proflavin are given in Table I. Due to their low absorption coefficients, the actual spectra of these ions bound to trypsin were impossible to obtain. Comparisons between the absorption spectra for the free ions and those complexed with EDTA showed no significant changes in the spectral range of concern and, therefore, the absorption spectra would not be expected to undergo significant changes upon binding with trypsin. None of the lanthanide ions used is known to have a hypersensitive absorption band (Jorgensen and Judd, 1964) in the range of spectral overlap with the proflavin fluorescence. As a result, the absorption spectra of the chloride salts of the lanthanides in water was used in our calculations of the overlap integral.

A quantum yield of 0.27 has been measured for proflavin at pH 4 in 0.05 M acetate buffer (Melhuish, 1964). Using such a solution as a reference, the quantum yield for proflavin was determined to be 0.26 at pH 6 (0.05 M Mes buffer) and $\mu = 0.3$. This value was not affected by the presence of low concentrations of trypsin or lanthanide ions but was slightly in-

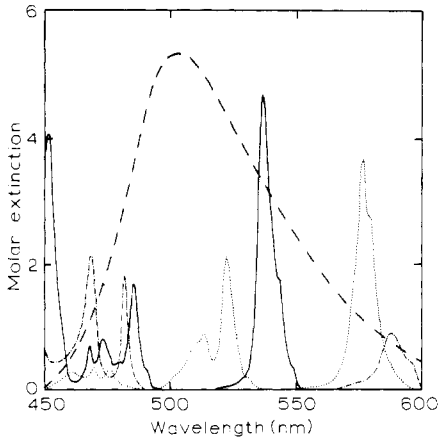

FIGURE 1: Spectral overlap between proflavin fluorescence and Nd(III), Pr(III), and Ho(III) adsorption. Spectra were taken at pH 6.0, 0.05 M Mes buffer at 25 °C. The ionic strength was adjusted to 0.3 with sodium chloride. (···) Nd(III) absorbance; (—) Ho(III) absorbance; (- - -) Pr(III) absorbance; (- - -) proflavin fluorescence. The Pr(III) and Nd(III) extinction coefficients have been multiplied by 0.5 for clarity in representation.

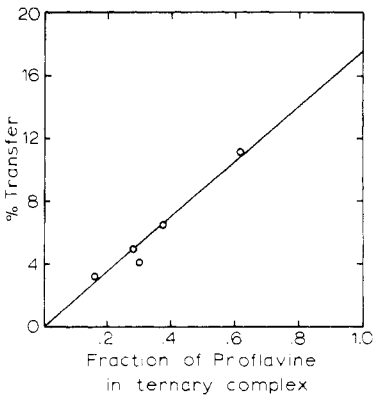

FIGURE 2: Energy transfer from proflavin to Nd(III) as a function of the fraction of proflavin in the trypsin-Nd(III)-proflavin complex. Conditions were those of Figure 1.

fluenced by changes in ionic strength.

Fluorescence emission spectra for proflavin from 450 to 600 nm were obtained using an excitation wavelength of 442 nm. Figure 2 shows the amount of quenching of proflavin fluorescence by neodymium(III) bound to trypsin. The relative amount of proflavin in the ternary complex was calculated using a dissociation constant of 4.2×10^{-5} M which we determined from measurements of the competitive inhibition of the hydrolysis of tosylarginine methyl ester under the same conditions as for the fluorescence experiments. This value compares very favorably with the value of 4.0×10^{-5} obtained under slightly different conditions (Bernhard and Gutfreund, 1965). The trypsin metal ion association constants were obtained by equilibrium dialysis as described earlier (Abbott et al., 1975a,b). Data showed a single metal ion was bound to trypsin with the following binding constants: Nd(III), 1.7×10^3 M^{-1}; Ho(III), 2.6×10^3 M^{-1}; and Pr, 1.1×10^3 M^{-1}. All association constants were determined at pH 6.0, 25 °C, 0.1 M Mes buffer, and the ionic strength was adjusted to 0.3 with sodium chloride.

Control experiments showed that the addition of trypsin to a solution containing only proflavin resulted in no quenching of proflavin fluorescence. Likewise the addition of Pr(III), Ho(III), or Nd(III) alone produced no quenching of proflavin fluorescence. In addition no quenching of proflavin was observed for the ternary proflavin–metal–trypsin complex when a nonabsorbing metal ion (i.e., La(III) or Ca(II)) was used. From these experiments we can conclude that the sole quenching mechanism for the proflavin–trypsin–Ho^{3+} (Nd^{3+} or Pr^{3+}) complexes is that of energy transfer between chromophores in the ternary complex. Addition of benzamidine to these solutions resulted in a decrease in the amount of quenching due to displacement of proflavin from the specificity pocket of trypsin. By the same token the amount of quenching was decreased when Ca(II) was added, as one would expect if the calcium ion and lanthanide ion are competing for the same site.

Table I shows a compilation of the results obtained by monitoring the quenching of proflavin by praseodymium(III), noedymium(III), and holmium(III) in the presence of trypsin. The metal ion–inhibitor distances obtained by the fluorescence techniques for the proflavin–lanthanide–trypsin complexes are all on the order of 10–11 Å. This is in excellent agreement with the NMR results (Abbott et al., 1975a,b), where a distance of 10 Å was obtained between the methyl protons of bound *p*-toluamidine and the metal ion.

Discussion

The largest uncertainty in using the energy-transfer approach for making distance measurements between two chromophores has been the evaluation of K^2 in eq 2. K^2, which takes into account the orientation of the donor and acceptor transition dipole moments, can range from 0 to 4, although it is usually set equal to $\frac{2}{3}$ which is the value obtained for the random orientation of the donor and acceptor dipoles with complete rotational freedom for both dipoles (Förster, 1959). In looking at energy transfer between Tb(III) and Co(II) in thermolysin, we (Berner et al., 1975) and later Horrocks et al. (1975) set the orientation factor equal to $\frac{2}{3}$ on the basis that the energy levels of both the donor and acceptor ions are degenerate or near degenerate. In this study we have an organic chromophore whose transition moment may be fixed when it is bound to the protein. The lanthanide ion acceptor (or donor in the case of the thionine experiments) can be assumed to have a random orientation due to the degeneracies existing in the ground and excited state energy levels. Under conditions such as these (one chromophore transition moment fixed and the other random), Dale and Eisinger (1975) have calculated that K^2 can only vary between $\frac{1}{3}$ and $\frac{4}{3}$. Because R_0 depends on the sixth root of K^2, a variation in K^2 from $\frac{1}{3}$ to $\frac{4}{3}$ only makes for a ±12% difference in the value of R_0. Hence unknown deviations of K^2 from $\frac{2}{3}$ (still fulfilling $\frac{1}{3} \leq K^2 \leq \frac{4}{3}$) will not change our calculations significantly. We have therefore used the value for K^2 of $\frac{2}{3}$ in our distance calculations.

The fact that the same distance (10–11 Å) from the specificity site to the metal binding site is obtained by two completely independent experimental techniques is comforting. On the one hand we have observed the effect of paramagnetic gadolinium(III) on the nuclear spin relaxation of protons of *p*-toluamidine (Abbott et al., 1975a,b), while on the other hand we have examined the quenching of the fluorescence of proflavin by three different lanthanides. Both techniques give the same answer. This in itself would indicate that energy transfer between an organic chromophore and a lanthanide ion takes place by a dipole–dipole (r^6 dependence) rather than by a dipole–quadrupole (r^8 dependence) or by a quadrupole–quadrupole (r^{10} dependence) as has been suggested for energy transfer between two lanthanide ions (Kleinerman, 1969; Dexter, 1953; Nakazawa and Shionoya, 1967; Reisfeld, 1973).

It should be pointed out in this regard that, if a dipole-quadrupole mechanism were responsible for the energy transfer, the resulting R_0 and R would be reduced by roughly a factor of 2, placing the metal ion even closer to the active site of the enzyme (Nakazawa and Shionoya, 1967), close enough, in fact, that the metal ion would be expected to interfere with the binding of substrate and inhibitors to the enzyme, which is not observed. A dipole-dipole mechanism for energy transfer has previously been demonstrated for energy transfer between a terbium ion donor and a cobalt ion acceptor in thermolysin (Berner et al., 1975; Horrocks et al., 1975).

The value of R_0 for energy transfer between Tb(III) and Co(II) in thermolysin was calculated to be 16-17 Å (Berner et al., 1975), whereas here we find the R_0 for the Tb(III)-thionine couple to be 44 Å and for the proflavin-Nd(III) (Ho(III) or Pr(III)) couple to be on the order of 8 Å. This means that one can measure distances between lanthanides and other chromophores ranging from 5 to 65 Å apart depending upon the donor-acceptor pair chosen. The R_0 of 44 Å for the Tb(III) to thionine couple is one of the highest ever found for a protein system (Steinberg, 1971). The factor to which the magnitude of R_0 is most sensitive is the extinction coefficient of the acceptor. Thus using thionine as the acceptor with an extinction coefficient of nearly 60 000, one obtains an R_0 of 44 Å; while using neodymium ion as an acceptor with extinction coefficients of from 2 to 8, one obtains an R_0 of 8 Å (Table I). Since several of the lanthanides fluoresce in the visible region (Tb(III) and Eu(III)) and many absorb in the visible region, it should be possible to choose an organic chromophore (as either the donor or acceptor) which will be suitable for distance measurements of nearly any range. Thus it would appear that the lanthanide ions will become much more generally useful for energy-transfer experiments than we first envisioned (Darnall and Birnbaum, 1970).

Although we have obtained the same distance (~10 Å) from the specificity site of trypsin to the metal binding site by two independent techniques, the fact that the crystallographers measure a distance of nearly 25 Å from the specificity site to a bound calcium ion requires some explanation. We have therefore searched for possible errors in our experiments which could cause the discrepancy. The NMR experiments were conducted under the following conditions (Abbott et al., 1975b): 0.2 M *p*-toluamidine, 10^{-4} to 10^{-3} M Gd(III) and 10^{-4} to 10^{-3} M trypsin. With such high concentrations of inhibitor, it was possible that multiple inhibitor molecules were binding the protein. This was discounted by varying the concentration of *p*-toluamidine and by competition experiments with acetamidine (Abbott et al., 1975b). Multiple metal binding sites were also discounted through displacement experiments of Gd(III) by Ca(II). In addition we obtained the same answers whether the inhibitor was titrated with protein (at constant Gd(III)) or with Gd(III) (at constant protein). The fluorescence experiments described herein were done under the following conditions: proflavin 10^{-7} to 10^{-6} M, trypsin 10^{-6} M, and Nd(III) (Ho(III) or Pr(III)) 10^{-4} to 10^{-3} M. This means that, under the NMR conditions, we had 10^2 to 10^3 M excess inhibitor over protein and Gd(III), while under the fluorescence conditions we had 10^3 excess metal ion over the protein and inhibitor. Obtaining the same distance measurement under these very diverse experimental conditions is strong evidence that we are looking at single binding sites for both the inhibitor and the metal ion.

Bode and Schwager (1975a,b) observed a heavy metal atom bound in a roughly octahedral environment in bovine trypsin between the two side chains carboxyls of Glu-70 and Glu-80 and between the carbonyl oxygens of Asn-72 and Val-75. The other ligands of the metal ion are water molecules positioned trans to one another.

The trypsins from various species as well as chymotrypsin bind a single calcium ion. Since the conformation of these proteins are similar, it seems likely that the calcium ion binding site is the same in all of them and indeed it appears that the calcium ion is bound at the same site in bovine α-chymotrypsin and trypsin (Birnbaum et al., 1976, unpublished). We have therefore examined the amino acid sequences of the various trypsins and chymotrypsins in order to compare the conservation of the metal binding site proposed by Bode and Schwager (1975a,b) and us (Abbott et al., 1975b). Of the residues involved in the crystallographers' site, only the residues Glu-70 and Glu-80 need be conserved since the carbonyl groups of residues 72 and 75 should be able to bind the metal ion regardless of which particular amino acid is substituted. The binding site we have proposed is composed of the side chains of Ser-190 and Asp-194. These residues should likewise be conserved to maintain the binding site. Table II shows the amino acid sequence between residues 69 and 81 and between residues 190 and 194 for several serine proteolytic enzymes. It is clear that Glu-70 and Glu-80 are conserved in dogfish, porcine, and bovine trypsin, whereas the binding site would be lost for chymotrypsin and Streptomyces trypsin both of which are known to bind calcium ion. The binding site we have proposed between Ser-190 and Asp-194 is conserved in all cases with the exception of Streptomyces trypsin where Ser-190 is replaced with a threonine, still maintaining the hydroxyl group.

We have shown that a single calcium ion is bound to chymotrypsin with the same affinity as that to trypsin, and we have also shown they have very probably the same calcium binding sites (Birnbaum et al., 1976, unpublished). The sequence data shown in Table II would certainly mediate against the calcium ion being found between the side chains of residues 70 and 80 in chymotrypsin and trypsin, but would fit well with binding between the side chains of residues 190 and 194.

Table II shows that Glu-70 and Glu-80 are conserved in elastase, while Ser-190 is replaced by a glycine. Since the conformation of elastase, trypsin, and chymotrypsin are nearly the same (Shotton et al., 1971), one would expect elastase to bind calcium ion in the same manner as trypsin if the metal ion is bound between Glu-70 and Glu-80, whereas the binding should be considerably weaker (or nonexistent) if the metal site is between residues 190 and 194. Previous work indicates that there is no agreement among investigators as to whether elastase binds a calcium ion or not. Shotton (1970) has indicated that calcium ion has no effect on elastase. Likewise Lamy et al. (1961) and Lewis et al. (1956) asserted calcium ion was without effect on elastase. In contrast Hormann and Fujii (1962) found Ca(II) stabilized elastase and Marshall et al. (1969) observed that Ca(II) changed the solubility properties of elastase. In view of these discrepancies, we have compared the calcium binding properties of elastase with the calcium binding properties of trypsin under the same conditions. At pH 8.0, $\mu = 0.1$ and 5 °C, the binding constant, as determined by equilibrium dialysis, for the calcium-trypsin interaction is 10^3, whereas that for the calcium-elastase interaction is 10^2. Since the binding constant for the calcium-elastase is so low, it is difficult to tell if more than one metal ion is bound under these conditions. The essential point remains, however: *calcium is bound to trypsin with an affinity of at least tenfold greater than it is bound to elastase.* These data by themselves would argue against the binding site at Glu-70 and Glu-80 (since this

TABLE II: Comparison of Serine Protease Amino Acid Sequences at Proposed Calcium Ion Binding Sites.

Protein	Sequence between Residues 69 and 81												Sequence between Residues 190 and 194	
	70									80			190	194
Elastase[a]	Gly-	Glu-	His-	Asn-	Leu-	Asn-	Gln-	Asn-	Asn-	Gly-	Thr-	Glu-	Gln-	Gly-Cys-Gln-Gly-Asp
Chymotrypsin A[b]	Gly-	Glu-	Phe-	Asp-	Gln-	Gly-	Ser-	Ser-	Ser-	Glu-	Lys-	Ile-	Gln-	Ser-Cys-Met-Gly-Asp
Bovine trypsin[c]	Gly-	Glu-	Asp-	Asn-	Ile-	Asn-	Val-	Val-	Glu-	Gly-	Asp-	Glu-	Gln-	Ser-Cys-Glu-Gly-Asp
Porcine trypsin[d]	Gly-	Glu-	His-	Asn-	Ile-	Asp-	Val-	Leu-	Glu-	Gly-	Asn-	Glu-	Gln-	Ser-Cys-Glu-Gly-Asp
Streptomyces trypsin[e]	Ile-	Thr-	Ala-	Thr-	Gly-	Gly-	Val-	Val-	Asp-	Leu-	Gln-	Ser-	Ala-	Thr-Cys-Glu-Gly-Asp
Dogfish trypsin[e]	Gly-	Glu-	His-	Asp-	Ile-	Ser-	Ala-	Asn-	Glu-	Gly-	Asp-	Glu-	Thr-	Ser-Cys-Gln-Gly-Asp

[a] Shotton and Hartley (1970). [b] Smillie et al. (1968). [c] Titani et al. (1975). [d] Hermodson et al. (1973). [e] Olafson et al. (1975).

site is conserved in both elastase and trypsin) but is consistent with the loss of a hydroxyl group in the binding site.

All of our data point to the calcium ion binding site in trypsin as being the side chains of Asp-194 and Ser-190. This site was proposed first by Abbott et al. (1975a,b) on the NMR distance measurements, on the basis that at least one carboxyl group was involved in the binding and on the fact that it explained the stabilization of trypsin against autolysis. There are, however, objections to this site as pointed out by Abbott et al. (1975a,b). Whether or not Ser-190 and Asp-194 form the calcium binding site, we do measure a distance of 10 Å from the metal ion to the specificity pocket of trypsin by two completely independent techniques as compared with the 25-Å distance seen by the crystallographers. The question then remains, what accounts for the difference in results? In the solid state, the metal ion is clearly seen at a point which would be impossible according to our solution measurements. Since x-ray studies have agreed well with chemical studies on trypsin and chymotrypsin, one hesitates to invoke differences in the structure of the solution and crystalline enzyme as possible explanations of the data. Nevertheless this may be the answer. Certainly calcium ion must be much more strongly bound in the crystalline state than in solution. At pH 8.0 and 25 °C we have determined the binding constant of the calcium–trypsin complex to be 3700 M^{-1} (Abbott et al., 1975b). The enzyme was crystallized from 2.4 M ammonium sulfate (Bode and Schwager, 1975a,b). Taking into account the solubility of $CaSO_4$ and using our Ca–trypsin binding constant, one can calculate that the enzyme should not nearly be fully bound with calcium ion. Yet 100% occupancy of the crystal was determined by analysis. The binding site suggested from crystallography is a logical one. The concurrence of two carboxyl groups ought to bind a calcium ion with an equilibrium constant on the order of 10^3. If this is the case, we should see two calcium ions binding trypsin in order to explain our solution results, i.e., one site 10 Å away from the active site and a second site 25 Å away. Since we see no evidence of such a second site, the implication would be that in solution the site available to bind calcium is 10 Å from the active site, while in the crystal the available site is 25 Å from the active site.

This leaves us with three possibilities: (1) the NMR and fluorescence experiments are not measuring the correct distance; (2) the x-ray determination of the calcium site is incorrect; (3) the difference in ionic strength between the two types of experiments, solution and crystal, causes a conformational change such that there are *two* different binding sites present dependent upon the ionic strength. None of these possibilities is very palatable, although the third choice at least allows us to continue to place faith in the physical techniques. In order to test the third choice we have undertaken fluorescence experiments at an ionic strength approaching that where the enzyme would begin to crystallize. These experiments have proved to be inconclusive because of precipitation problems. Thus for the time being the question is still unresolved.

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