Binding of Lanthanides and of Divalent Metal Ions to Porcine Trypsin†

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ABSTRACT: The single binding site for Ca^{2+} on the porcine trypsin molecule was found to be capable of binding trivalent lanthanide ions as well as Y^{3+} and the divalent ions Mn^{2+} and Cd^{2+}. The affinity of the lanthanide ions to trypsin depends strongly on their ionic radius; the smaller the lanthanide ion, the higher the binding constant. The affinity of metal ions to trypsin does not depend on their chemical similarity to Ca^{2+}. The fluorescence of Tb^{3+} is enhanced 10^5-fold when bound to porcine trypsin. The pH dependence of the Tb^{3+} fluorescence indicates that the metal binding site of the enzyme consists of one or more carboxyl side chains. Measurements on the fluorescence excitation spectrum of the trypsin–Tb^{3+} complex reveal energy transfer from one or more tryptophans in close proximity to the bound metal ion.

The autocatalytic conversion of trypsinogen to trypsin is markedly activated by calcium ions. Trypsinogen possesses two binding sites for Ca^{2+} (Lazdunski *et al.*, 1970). One of them is on the hexapeptide cleaved upon the conversion (of the bovine enzyme) and the other is on the trypsin moiety. While there is no connection between the enzymatic activity of trypsin and the presence of calcium ions (Sipos and Merkel, 1970), it has been observed that the binding of Ca^{2+} to the enzyme inhibits its autodigestion (Buck *et al.*, 1962). Thus, calcium binding to trypsin has an important biological function. The three-dimensional structure of a diisopropylphosphoryl derivative of bovine trypsin has been elucidated by Stroud *et al.* (1971). However, attempts to obtain a heavy atom derivative at the calcium site by diffusing neodymium ions into the crystal have failed. The authors speculate that Ca^{2+} binds at a site where three carboxylic side chains come together and form a cluster of negative charges.

The divalent calcium ions, as well as other metal ions with closed electronic shells, are devoid of useful spectroscopic characteristics which could serve as a handle in probing the nature of their binding sites. Recently there has been great interest in the potential use of the spectroscopic and magnetic properties of the trivalent lanthanides (Williams, 1970; Birnbaum *et al.*, 1970; Morallee *et al.*, 1970; Reuben, 1971a,b; Luk, 1971; Sherry and Cottam, 1973; Gafni and Steinberg, 1974). Particularly attractive is the possibility that lanthanides may bind specifically to the calcium sites of proteins (Colman *et al.*, 1972). In this regard it should be noted that the activation of the trypsinogen to trypsin conversion by neodymium has been found to be much more efficient than that effected by calcium (Darnall and Birnbaum, 1970).

The intensity of the fluorescence spectrum of terbium is tremendously enhanced upon the binding of Tb^{3+} to the ion-carrying protein transferrin (Luk, 1971). We have observed similar effects with trypsin. The fluorescence enhancement can be used to monitor the binding of terbium to the enzyme and, by competition, of other cations as well. Confirmation of this approach was sought from equilibrium dialysis experiments using radioactive ytterbium, from electron spin resonance (esr) measurements of free manganese in solutions containing the enzyme, and from water proton relaxation rate enhancements in solutions of manganese and of gadolinium. In this communication we report in detail the results of our study of metal ion binding to porcine trypsin.

Experimental Section

Fluorescence spectra were recorded on a Turner Spectro Model 210 spectrofluorimeter. This instrument has the advantage of correcting intensities throughout the whole spectral range. Single-wavelength fluorescence operations such as lanthanide titrations of trypsin and pH dependence of the fluorescence intensity were performed on an Aminco-Keirs spectrophosphorimeter. Esr measurements were done on a Varian Associates E-12 esr spectrometer operating at the X-band. The water proton relaxation times, T_1, were measured at 24.3 MHz on a Bruker pulsed spectrometer and enhancements evaluated as previously described (Reuben, 1971, and references therein). Radioactivity of ^{169}Yb was monitored on a Packard Auto-Gamma spectrometer. Trypsin activity was assayed spectrophotometrically on a Gilford 2400 spectrophotometer.

Porcine trypsin was the product of Novo Industri A/S, Copenhagen, Denmark. Tos-LysCH_2Cl,[1] and Pipes were purchased from Sigma Chemical Co., St. Louis, Mo. The lanthanides used were either the sesquioxides or the chlorides obtained from Alfa Inorganics, Beverly, Mass. The radioactive $^{169}Yb^{3+}$ isotope was obtained from the Radiochemical Centre Ltd., Amersham, England. Arsenazo indicator was from Fluka AG, Buchs, Switzerland. NphOGdnBz was purchased from Nutritional Biochemicals Corp., Cleveland, Ohio. *N*-Acetyltryptophanamide and BzArgOEt were synthesized and purified in our laboratory. Calcium, magnesium, strontium, manganese, and cadmium chlorides were all of analytical grade.

Unless otherwise stated, all the experiments were carried out in solutions buffered at pH 6.3 with Pipes.

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[1] Abbreviations used are: Tos-LysCH_2Cl, tosyllysyl chloromethyl ketone; BzArgOEt, benzoylarginyl ethyl ester; Pipes, piperazine-*N*,*N*′-bis(ethanesulfonate); NphOGdnBz, *p*-nitrophenyl-*p*-guanidinobenzoate.

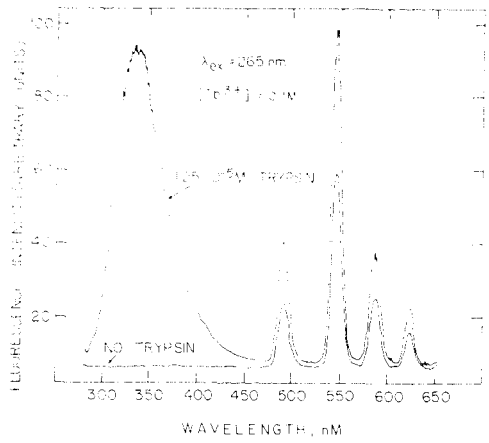

FIGURE 1: Fluorescence enhancement of Tb^{3+} by porcine trypsin. Emission spectra of 0.1 M $TbCl_3$ with and without 1.25×10^{-5} M trypsin in 0.01 M Pipes (pH 6.3). Excitation wavelength, 265 nm.

Stock solutions of trivalent lanthanides were prepared in the 2 M concentration range mainly from the sesquioxides. The oxide was dissolved in slightly less than 6 equiv of hydrochloric acid. The solution was mixed under constant moderate heating until the pH was 4–5. Residual insoluble material was filtered off through a Millipore filter (0.45 μ).

Lanthanide concentrations were determined by EDTA titration using Arsenazo I as the end point indicator (Woyski and Harris, 1963). This procedure was selected from a number of determination procedures and found to be both accurate and convenient. The EDTA was standardized using La_2O_3.

Trypsin activity was assayed spectrophotometrically at 253 nm (Schwert and Takenaka, 1955), using BzArgOEt as a substrate.

Tosyllysyl trypsin was routinely used in order to avoid continuous autodigestion during experiments. The reaction of trypsin with Tos-LysCH_2Cl was carried out (Shaw and Glover, 1970) in a ~20 mg/ml of enzyme solution with approximately a threefold molar excess of Tos-LysCH_2Cl. After 1 hr, the activity dropped down to 2% of its original value and after 2 hr the reaction was practically complete with no detectable activity left. The reacted protein was then dialyzed against dilute HCl solutions, pH 2.5, to eliminate excessive reagent, and was subsequently lyophilized. This pH gives maximum stability to the native enzyme.

The extinction coefficient of the protein at 280 nm was determined by measuring the absorbance of known concentrations of trypsin. The concentration of the enzyme was determined by the micro-Kjeldahl method using the nitrogen content of the protein as calculated from its amino acid composition (Walker and Keil, 1973). The value of the extinction coefficient was found to be 1.50 cm^{-1} mg^{-1} ml.

The concentration of active trypsin was determined by active site titration using NphOGdnBz (Chase and Shaw, 1967). Enzyme preparations used in this study contained 78.5% active material. It yielded one major band in disc electrophoresis (Davis, 1964), with one faint additional band.

The concentration of free Mn^{2+} was monitored by esr following the procedure of Reuben and Cohn (1970).

For the equilibrium dialysis experiments 5 Ci/l. of $^{169}Yb^{3+}$ was diluted with nonradioactive Yb^{3+} to give a solution of 4.95×10^{-3} M $YbCl_3$ and a specific radioactivity of 800,000 cpm/μmol of $YbCl_3$. Eleven dilutions were prepared which served both for dialysis against trypsin and for calibration counting. Equilibrium dialyais was performed in duplicates in 0.3-ml dialysis cells. The solutions were 0.2 M in Pipes (pH 6.3).

TABLE I: The Magnitude of Fluorescence Enhancement of Tb^{3+} Induced by Binding to Trypsin.

λ_{ex} (nm)	λ_{em} (nm)	Enhancement Factor
265	490	5,900
265	545	9,400
280	490	34,000
280	545	53,000
295	490	50,000
295	545	90,500

From each compartment duplicate samples of 100 μl were drawn for radioactivity counting. The optical density of each compartment without protein was found to be practically zero, which eliminates the possibility of protein leakage. Protein concentration in all the protein-containing compartments was found to be constant within ±3%.

Results

Enhancement of Tb^{3+} Fluorescence upon Binding to Trypsin. When trivalent terbium binds to trypsin, its fluorescence is markedly enhanced. Figure 1 shows corrected fluorescence spectra of Tb^{3+} and of the Tb^{3+}–trypsin complex. The spectra reveal the protein fluorescence peak at 335 nm and the characteristic Tb^{3+} fluorescence quartet between 480 and 630 nm. Table I shows the fluorescence enhancement of the Tb^{3+} ion brought about by trypsin when excited at different wavelengths. The enhancement was calculated from spectra taken in the 10^{-6}–10^{-5} M concentration range of protein. Under these conditions, the optical density is sufficiently small to avoid any nonlinearity in the concentration dependence of the fluorescence. The aqueous Tb^{3+} solution has its excitation peak at 262 nm. A gradual growth of an excitation peak at 280 nm is observed upon titrating the Tb^{3+} with trypsin.

Binding Constants for Lanthanides and Divalent Cations to Porcine Trypsin. The binding behavior of Tb^{3+} was studied. The association constant of trivalent terbium to tosyllysyltrypsin was determined by fluorescence titration of the protein with Tb^{3+}. The enzyme (2.5×10^{-5} M) was titrated with a solution containing the same concentration of enzyme containing 0.1 M $TbCl_3$. Therefore, the optical density of the titration mixture remained constant, thereby excluding the possibility of perturbations in the fluorescence measurements. The fluorescence of free Tb^{3+} was subtracted from the observed total fluorescence. The fluorescence of free Tb^{3+} was found from a titration in which *N*-acetyltryptophanamide replaced the protein. The optical density of the *N*-acetyltryptophanamide solution was adjusted to be identical with that of the trypsin solution and both titrations were carried out simultaneously.

The normalized Scatchard plot (Scatchard, 1949) was constructed according to

$$F/[Tb^{3+}]_{free} = (F_\infty/K) - (F/K) \quad (1)$$

where F is the measured fluorescence intensity (in arbitrary units), F_∞ the fluorescence at infinite Tb^{3+} concentration (extrapolated value), and K the dissociation constant of the trypsin–Tb^{3+} complex. This is a normalized Scatchard plot since F/F_∞ replaces the usual term (bound ligand)/(total protein) and as a result the plot of $F/[Tb^{3+}]$ *vs.* F will intercept the abscissa always near unity even though more than one binding site may be involved. Throughout the titration

TABLE II: Binding Constants of Various Metal Ions to Porcine Trypsin.

Metal Ion	Ionic[a] Radius (Å)	$\bar{K}_a$ (M^{-1})
La^{3+}	1.061	180
Pr^{3+}	1.013	370
Nd^{3+}	0.995	540
Sm^{3+}	0.964	670
Eu^{3+}	0.950	800
Gd^{3+}	0.938	340
Tb^{3+}	0.923	880
Dy^{3+}	0.908	930
Er^{3+}	0.881	1160
Tm^{3+}	0.869	1630
Yb^{3+}	0.858	1810
		4000[c]
Lu^{3+}	0.848	2210
Ca^{2+}	0.990[b]	6200
Mn^{2+}	0.800[b]	5500
		6500[d]
Cd^{2+}	0.970[b]	4120
Y^{3+}	0.893[b]	820
Mg^{2+}	0.660	Negligible
Sr^{2+}	1.120[b]	Negligible

[a] Templeton and Dauben (1954). [b] Handbook of Physics and Chemistry (1971). [c] By using equilibrium dialysis at 4°, pH 6.3 (see Figure 3). [d] By using esr (see Figure 6).

$[Tb^{3+}]_{free}$ is close to $[Tb^{3+}]_{total}$ and in fact $F/[Tb^{3+}]_{total}$ was plotted against F. A straight line was obtained in the Scatchard plot describing the binding of Tb^{3+} to porcine trypsin (Figure 2). This behavior is typical for equivalent and independent binding sites. The intrinsic binding constant for Tb^{3+} was found to be 880 M^{-1} (Table II).

The stoichiometry of lanthanide binding was determined by equilibrium dialysis against $^{169}Yb^{3+}$ solutions (Figure 3). This experiment demonstrates directly that trypsin has *one* binding site for lanthanides. The binding constant to trypsin at 4° and pH 6.3 found by this experiment is 4000 M^{-1}.

Calcium was shown to compete for the lanthanide binding site with a binding constant of 6200 M^{-1}. It displaces Tb^{3+} from porcine trypsin completely as evidenced by the diminution

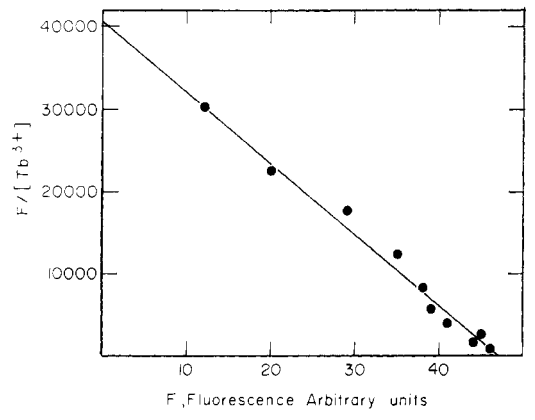

FIGURE 2: Scatchard plot for Tb^{3+} binding to trypsin. Trypsin concentration was kept constant at 2.5 × 10^{-5} M in 0.2 M Pipes (pH 6.3) at 25°. $TbCl_3$ concentration was raised up to 0.05 M. In order to obtain the fluorescence of the free Tb^{3+}, the same titration was performed with *N*-acetyltryptophanamide instead of trypsin to produce the same absorbance.

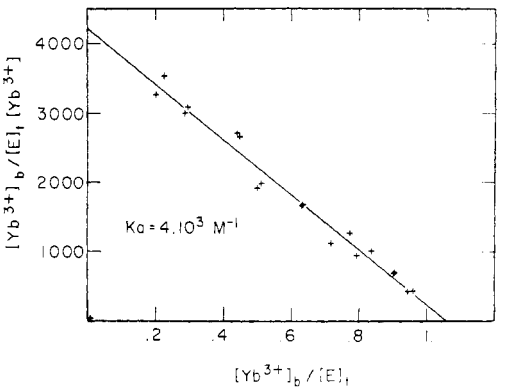

FIGURE 3: Scatchard plot for $^{169}Yb^{3+}$ binding to trypsin. Eleven dilutions of a stock solution of 4.95 × 10^{-3} M $YbCl_3$ with specific radioactivity of 800,000 γcpm/μmol were dialyzed against 4.2 × 10^{-4} M porcine trypsin in 0.2 M Pipes (pH 6.3) at 4°.

of the fluorescence down to that of free Tb^{3+}. The binding of other lanthanides as well as of Y^{3+} and a number of bivalent cations was studied by competition with Tb^{3+} in a similar way. A solution of *ca.* 2.5 × 10^{-5} M trypsin and 0.005 M $TbCl_3$ was titrated in the fluorescence cell with a solution containing exactly the same concentrations of trypsin and Tb^{3+} as well as about 0.1 M of the competing cation, thus keeping the concentration of protein and Tb^{3+} constant. The results were analyzed in terms of a normalized Scatchard equation for two competing ligands:

$$\bar{Y}_L/[L] = (1/K_L) - (1/K_L)(\bar{Y}_L + \bar{Y}_T) \quad (2)$$

(see Appendix for the derivation). $\bar{Y}_L$ is the fractional saturation with respect to the metal L, $\bar{Y}_T$ is the fractional saturation for Tb, and K_L the dissociation constant for the lanthanide L. The Scatchard plot for Ca^{2+} is shown in Figure 4 and the results for all the cations studied are summarized in Table II.

The dependence of the lanthanide–trypsin complex formation constant on their radius is presented in Figure 5.

Binding of Mn^{2+} Using Esr. For the Mn^{2+} ion an independent method of measuring the binding of trypsin was used. This was done by measuring the intensities of the esr peaks of the free manganese. The measurement was based on the fact that the very broad "smeared out" esr signal of the bound manganese has a negligible contribution to the signal composed of the characteristic sextet (Cohn and Townsend, 1954). The method has the advantage of measuring directly the

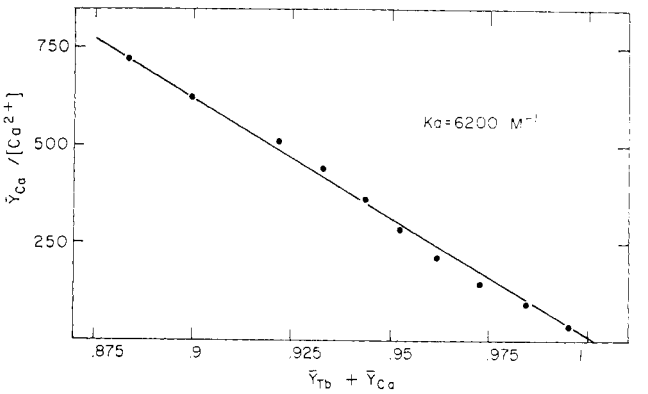

FIGURE 4: Scatchard plot for Ca^{2+} binding to trypsin. A trypsin solution of 2.5 × 10^{-5} M containing 0.005 M $TbCl_3$ in 0.02 M Pipes, pH 6.3, was titrated at 25° with an identical solution containing 0.1 M $CaCl_2$.

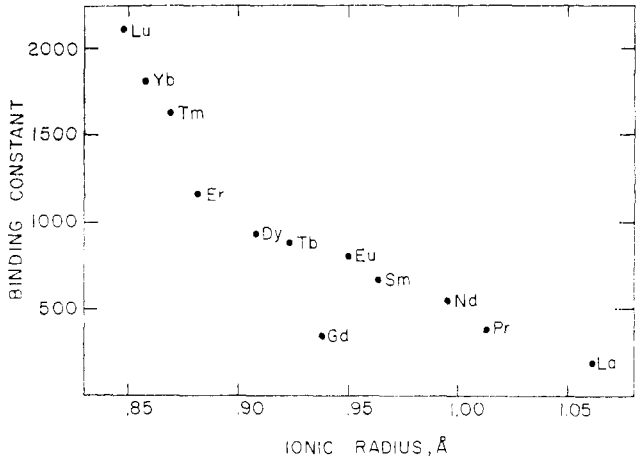

FIGURE 5: The dependence of the lanthanide–trypsin complex formation constants on the ionic radius.

free metal ion concentration, thus yielding both the binding constants and the number of binding sites. A constant porcine trypsin concentration was titrated with Mn^{2+} ion in the concentration range 10^{-5}–10^{-3} M. The amounts of free Mn^{2+} present were calculated from a calibration curve. The results in the form of a Scatchard plot are shown in Figure 6 and agree with those obtained from the competition with Tb^{3+} as described in the previous section.

The Stability of the Trypsin–Tb^{3+} Complex as a Function of pH. The pH dependence of the Tb^{3+} trypsin complex formation was measured. Stock solutions of tosyllysyl-trypsin and $TbCl_3$ were added together and adjusted close to the final volume with an unbuffered aqueous solution at pH 6.3. The pH of each solution was then adjusted with dilute hydrochloric acid to the desired value and to the final value and the fluorescence intensity of the Tb^{3+} band was subsequently measured. Each solution contained 2.7×10^{-5} M trypsin and 9.3×10^{-3} M $TbCl_3$. Under these conditions at pH 6.3 the enzyme is 90% saturated with Tb^{3+}. The results (Figure 7) show a sigmoidal titration curve with an apparent pK of 5.2.

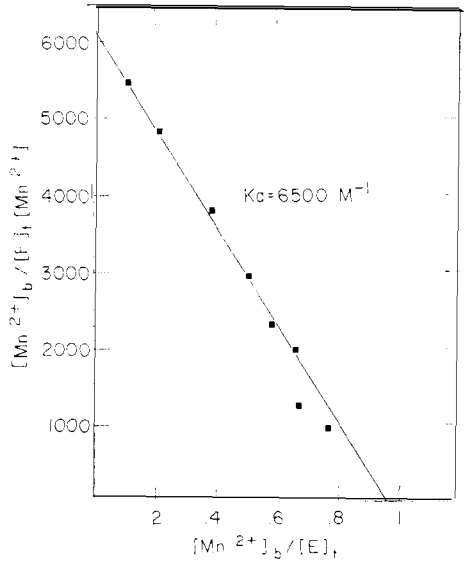

FIGURE 6: Scatchard plot for Mn^{2+} binding to trypsin. A constant trypsin concentration of 3×10^{-4} M was titrated with $MnCl_2$ in the concentration range of 10^{-5}–10^{-3} M, and the intensity of the esr signal of free Mn^{2+} was measured. The solutions were 0.2 M in Pipes and the temperature was 22°.

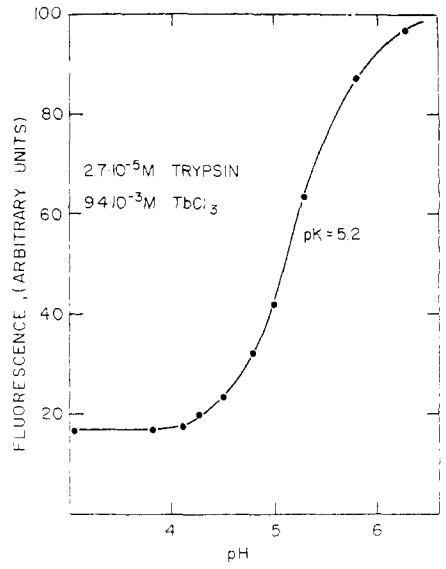

FIGURE 7: The stability of the trypsin–Tb^{3+} complex, as a function of pH. Unbuffered solutions of 2.7×10^{-5} M trypsin containing 9.4×10^{-3} M $TbCl_3$ were brought to the desired pH using dilute HCl, and the Tb^{3+} fluorescence was measured.

No proton release from porcine trypsin was found to occur in the pH range between 4 and 6.3 upon Ca^{2+} or Tb^{3+} binding.

Protein Fluorescence Quenching. Unlike the terbium ion, calcium does not quench the protein fluorescence. With the protein almost fully saturated with Tb^{3+}, its fluorescence (λ_{ex} 280 nm; λ_{em} 340 nm) is quenched by 21 ± 2.5%. The same effect is observed in both ways: quenching of the excitation peak with fixed emission wavelength and of the emission peak with fixed excitation wavelength.

Metal Ion Effects on Enzymatic Activity. It was found that neither Ca^{2+} nor trivalent lanthanide ions affect the enzymatic activity of porcine trypsin under the conditions used for the physicochemical measurements. Both K_m and k_{cat} remained essentially constant. (At pH 6.3 the activity is 50% of the maximal activity at pH 8.)

Nuclear Magnetic Resonance (Nmr). Porcine trypsin causes an enhancement of the water proton longitudinal relaxation rate, $1/T_1$, in solutions of Mn^{2+} and of Gd^{3+}. The observed enhancements are small, 1.6 for Mn^{2+} and 1.4 for Gd^{3+} at 24.3 MHz, and, therefore, cannot be used with a sufficient degree of accuracy for analytical purposes.

Discussion

The fluorescence of trivalent terbium is tremendously enhanced upon binding to trypsin. The magnitude of the enhancement can be used to monitor the binding equilibrium of Tb^{3+} and by competition of other cations as well. Stoichiometry and binding constants independently determined by equilibrium dialysis using radioactive ytterbium and by monitoring free manganese from the esr spectral intensities show that the terbium displacement titration is a valid one and that the binding constants obtained in this way are correct.

Trivalent lanthanides compete with calcium for its binding site on porcine trypsin. Their binding, however, is somewhat disfavored by the higher charge. The divalent manganese and cadmium bind almost as strongly as calcium. On the other hand, Sr^{2+} and Mg^{2+} bind to trypsin with negligible affinity (Table II).

The pH dependence of Tb^{3+} binding to trypsin indicates

that the metal ion is bound to carboxylate side chains of the protein. The apparent pK of the equilibrium

$$Tb^{3+} + \text{protein-(COOH)}_n \rightleftharpoons \text{protein-(COO}^-)_n Tb^{3+} + nH^+ \quad (3)$$

is 5.2 (Figure 7). This equilibrium is the sum of two equilibria.

$$\text{protein-(COOH)}_n \rightleftharpoons \text{protein-(COO}^-)_n + nH^+ \quad (4)$$

$$\text{protein-(COO}^-)_n + Tb^{3+} \rightleftharpoons \text{protein-(COO}^-)_n Tb^{3+} \quad (5)$$

With a binding constant of 880 M^{-1} (pK = 2.94), Tb^{3+} cannot perturb to any measurable extent a group with a pK value of 5.2 and in fact no proton liberation was observed upon binding. Thus, the overall pK of 5.2 actually described the pK of ionization of these side chains which function as the metal ligands. From the value of this pK (5.2) one can safely conclude that the metal ligands are carboxyl side chains of aspartic and/or glutamic acid residues.

There is a possibility for multiple ligands at the binding site similar to that suggested by Stroud *et al.* (1971) for the bovine enzyme. This should result in extensive dehydration of the cation upon binding, leading to relatively low values of the enhancements of the water proton relaxation rates. Indeed, the enhancements observed for both Mn^{2+} and Gd^{3+} are very low; however, their detailed interpretation must await further investigation of the temperature and frequency dependence.

The enhancement of the Tb^{3+} fluorescence in its trypsin complex depends strongly on the excitation wavelength (Table I). The fact that maximal Tb^{3+} fluorescence is obtained when the complex is excited at 295 nm demonstrates that energy transfer occurs from one or more tryptophans to the bound Tb^{3+}. The extent of energy transfer from tyrosines to Tb^{3+} is probably extremely small.

Since the absorption of Tb^{3+} and Tb^{3+}–trypsin in the 320–400-nm range is negligible, the mechanism of energy transfer from the protein to the Tb^{3+} is probably not due to dipole interaction (Förster mechanism; Förster (1948)). We therefore conclude that tryptophan residue(s) must be within a very short distance from the Ca^{2+} site in porcine trypsin.

The binding of Tb^{3+} to trypsin is accompanied by quenching of the protein fluorescence to the extent of 21%. Since a similar effect does not occur upon Ca^{2+} binding, it may be concluded that the observed quenching is due to the energy transfer from tryptophans to the bound Tb^{3+}.

The results of this study together with the amino acid sequence of porcine trypsin (Hermodson *et al.*, 1973) should be of value in the identification of the metal ion binding site.

Appendix

The binding constants of metal ion (L) to trypsin were calculated from the terbium-displacement titration by a two-step procedure. In the first step a double reciprocal plot of $1/(F_0 - F)$ *vs.* $1/[L]_{total}$ is constructed and the data are fitted by linear regression, where F is the measured fluorescence in arbitrary units, F_0 is the initial fluorescence without the competing ion present, and $[L]_{total}$ is the total molar concentration of the competing ion. The approximaion $[L]_{total} = [L]_{free}$ was found to be valid over the entire titration range since throughout the titration $[L] \gg [E]$, where E denotes enzyme. The intercept of the extrapolation of this plot on the ordinate yields $1/(F_0 - F_\infty)$, F_∞ being the lowest fluorescence value at infinite [L].

The next step is to calculate the Scatchard plot for competition of two species for the same site on a macromolecule. The two equilibria are described by eq 6 and 7 where K_T is the

$$K_T = [E][Tb]/[ETb] \quad (6)$$

$$K_L = [E][L]/[EL] \quad (7)$$

enzyme–Tb^{3+} dissociation constant and K_L the enzyme–metal (L) dissociation constant. The fraction of enzyme with bound Tb^{3+} and the fraction of enzyme with bound L are $\bar{Y}_T$ and $\bar{Y}_L$, respectively:

$$\bar{Y}_T = [ETb]/[E]_{total} \quad (8)$$

$$\bar{Y}_L = [EL]/[E]_{total} \quad (9)$$

If only one binding site is involved, the Scatchard characteristic term: (bound metal)/(total enzyme) equals $\bar{Y}_L$. The Scatchard ordinate would then be

$$\frac{\bar{Y}_L}{[L]} = \frac{\bar{Y}_L[E]}{K_L[EL]} = \frac{\bar{Y}_L[E]_{total}}{K_L[EL]} - \frac{\bar{Y}_L[EL]}{K_L[EL]} - \frac{\bar{Y}_L[ETb]}{K_L[EL]}$$

which yields

$$\frac{\bar{Y}}{[L]} = \frac{1}{K_L} - \frac{1}{K_L}(\bar{Y}_L + \bar{Y}_T) \quad (10)$$

This is the Scatchard equation applied in our study (eq 2 in the text). To calculate it, the left-hand side becomes

$$\frac{\bar{Y}_L}{[L]} = \frac{\bar{Y}_L}{([L]_{total} - [E]_{total}\bar{Y}_L)} \quad (11)$$

The right-hand side contains terms for $\bar{Y}_T$ and $\bar{Y}_L$. To evaluate $\bar{Y}_T$, one has to start with $Y_T{}^0$, which is the fraction of enzyme with bound Tb^{3+} in the absence of L. From the relations

$$[E][Tb]/[ETb] = K_T$$

$$[ETb] + [Tb] = [Tb]_{total}$$

$$[ETb] + [E] = [E]_{total}$$

one can obtain an expression for [ETb] and from it, using (8), the expression for $\bar{Y}_T{}^0$ becomes

$$\bar{Y}_T{}^0 = \frac{Z - (Z^2 - 4[E]_{total}[Tb]_{total})}{2[E]_{total}} \quad (12)$$

where

$$Z = [Tb]_{total} + K_T + [E]_{total}$$

$\bar{Y}_T$ is calculated from $\bar{Y}_T{}^0$ by

$$\bar{Y}_T = \frac{\bar{Y}_T{}^0(F - F_\infty)}{(F_0 - F_\infty)} \quad (13)$$

To evaluate $\bar{Y}_L$ an expression for [EL] is required:

$$[EL] = [E]_{total} - [E] - [ETb] = [E]_{total} - ([ETb]K_T/[Tb]) - [ETb] \quad (14)$$

Substituting (14) in (9), one obtains

$$\bar{Y}_L = 1 - \frac{[ETb]K_T}{[E]_{total}[Tb]} - \frac{[ETb]}{[E]_{total}} \quad (15)$$

and using (8)

$$\bar{Y}_L = 1 - \bar{Y}_T[1 + (K_T/[Tb])] \quad (16)$$

and hence

$$\bar{Y}_L = 1 - \bar{Y}_T\left(1 + \frac{K_T}{[Tb]_{total} - [E]_{total}\bar{Y}_T}\right) \quad (17)$$

A plot of eq 10 (eq 2 in the text), when linear, yields the association constant for the metal L. Linearity of this plot proves the existence of equivalent and independent binding sites.

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