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Calcium Binding Site of Trypsin as Probed by Lanthanides†

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ABSTRACT: A number of physicochemical techniques have been applied to identify, locate, and characterize the Ca^{2+}-binding site in porcine and bovine trypsin. The fluorescent lanthanide Tb^{3+} and the paramagnetic lanthanide Gd^{3+} were used as probes for the Ca^{2+}-binding site in the trypsin molecule. The fluorescent lanthanide Tb^{3+} was found to bind to the specific Ca^{2+}-binding site on the trypsin molecule concomitant with a large increase in its fluorescence. The pH dependence of the Tb^{3+}-binding process to the trypsin molecule and studies on proton release upon Tb^{3+} binding to the protein reveal the involvement of two carboxyl residues in metal binding. Proton relaxation rate measurements on the Gd^{3+}-trypsin complex reveal that upon metal binding six of the eight water molecules coordinating the aquo-Gd^{3+} ion are released where two water molecules remain bound to the protein-bound Gd^{3+} ion. Model building of the trypsin molecule identifies the two carboxylate residues at the metal-binding site as Glu-70 and Glu-80, as recently revealed by x-ray crystallographic studies. Fluorescence excitation studies on the trypsin-Tb^{3+} complex reveal energy transfer from a tryptophan residue to the bound Tb^{3+} ion. This residue is identified as Trp-141. Trypsin was also found to possess a low-affinity site for lanthanide ions which is incapable of binding Ca^{2+}. The existence of a secondary lanthanide-binding site is responsible for the variation of the circular polarized luminescence spectrum of the Tb^{3+}-trypsin complex with the Tb^{3+} to protein ratio. Some differences are found between the spectroscopic properties of the lanthanide complexes of bovine trypsin and porcine trypsin. These differences stem from the structural differences of the Ca^{2+}-binding sites of the two types of trypsins.

Calcium binding to trypsin is known to inhibit the enzymatic autodigestion of the protein (Buck et al., 1962). This effect seems to underlie the biological function of calcium in this system and there has been considerable interest in locating the calcium site on the enzyme (Stroud et al., 1971, 1974; Abbott et al., 1975a,b; Bode and Schwager, 1975a,b). In a previous communication, we have shown that the trivalent lanthanide ions as well as Mn^{2+} and Cd^{2+} are capable of binding at the single Ca^{2+}-binding site of porcine trypsin (Epstein et al., 1974). Using the fluorescence enhancement of Tb^{3+}, the electron spin resonance spectrum of Mn^{2+}, and the radioactive $^{169}Yb^{3+}$ isotope, the stoichiometry and association constants of the cation-trypsin complexes were determined. These studies are now extended to include measurements of water proton relaxation rates, proton release, and circularly polarized luminescence with porcine trypsin as well as some measurements with bovine trypsin. The present work involves the determination of the number of carboxylate ligands at the binding site, the determination of the number of water molecules coordinated to the bound Gd^{3+} ion, and the identification of the chromophore involved in the energy transfer that leads to fluorescence enhancement of bound Tb^{3+}. Our results, viewed in conjunction with the molecular model of trypsin,

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permit a positive identification of the metal ion binding site.

Most of the trivalent lanthanides exhibit strong fluorescence either in their crystals or in solution. Weissman (1942) has shown that lanthanide complexes when excited with light absorbed by the ligand give rise to enhanced fluorescence at frequencies similar to those of the aquo-ions. Typical spectra of the Tb^{3+} aquo ion and its complexes with porcine trypsin have been presented in a previous communication (Epstein et al., 1974).

Although the J levels of the lanthanides are split by ligand fields of low symmetry, splittings are generally not discernible in the fluorescence spectra. However, they are observed in the circularly polarized luminescence (CPL).[1] Thus, CPL spectra reflect the asymmetry of the ligand field in the excited electronic state of the ion and may provide detailed information on its binding site. The CPL of Tb^{3+} bound to transferrin and to conalbumin has been studied by Gafni and Steinberg (1974). They found that both the emission and CPL spectra for the two complexes were very similar and concluded that the structure and conformation of the metal ion binding site of the two proteins must be similar. A detailed treatment of the CPL of Eu^{3+} and Tb^{3+} is given by Luk and Richardson (1975) in their study of carboxylic and amino acid complexes. It seems at present that a CPL spectrum by itself can provide only qualitative information. However, spectral changes or similarities can form the basis for more quantitative and detailed conclusions. The fluorescence enhancement can be used to quantitate the binding of Tb^{3+} to macromolecules. Displacement of bound Tb^{3+} by other cations competing for the same site will result in an apparent quenching of the fluorescence, the magnitude of which can be used to monitor the binding of the competing ions (Epstein et al., 1974).

A large enhancement of the fluorescence intensity results from the binding of Tb^{3+} to the protein. The maximum enhancement is usually observed for the $^5D_4 \rightarrow {}^7F_5$ transition. It is also found that the enhancement, E^*, strongly depends upon the excitation wavelength. From the value of λ_{ex} leading to maximum enhancement (E^*), the residue involved in energy transfer can often be identified.

Because energy transfer occurs from the protein to the lanthanide ion, the fluorescence of the protein itself is quenched upon binding of Tb^{3+}. Thus, for example, the fluorescence of porcine trypsin is quenched to the extent of 21% by Tb^{3+}, whereas Ca^{2+} produces no quenching (Epstein et al., 1974). Such effects can be useful in the assignment of residues involved in energy transfer. It is noteworthy that several of the lanthanides can produce quenching effects. Ricci and Kilichowski (1974) report the following order of efficiency in the quenching of indole fluorescence: $Eu^{3+} > Yb^{3+} > Sm^{3+} > Tb^{3+} > Gd^{3+} > Ho^{3+} \approx Dy^{3+}$.

Paramagnetic Properties and Lanthanides. Paramagnetic ions by virtue of the large magnetic moment associated with their electronic spin greatly enhance the relaxation rates of nuclei in their vicinity through the electron–nuclear hyperfine interaction. Outstanding in this respect is Gd^{3+} which exerts its influence almost solely through the electron–nuclear *dipolar* interaction. The longitudinal relaxation rate of a nucleus due to this interaction may be expressed as

$$1/T_{1M} = Df(\omega,\tau_c)/r^6 \quad (1)$$

where D is a constant containing the product of the squares of the interacting magnetic moments, r is the distance between the latter, and $f(\omega,\tau_c)$ is a function of the resonance frequency, ω, and of the correlation time, τ_c, characteristic of the process responsible for interruption of the interaction. The book by Dwek (1973) should be consulted for details not given here.

In aqueous solution, owing to rapid chemical exchange between the coordinated and free water molecules, an average effect is observed in the form of an increment in the relaxation rate. The increment is given by

$$1/T_{1p} = P_M/T_{1M} \quad (2)$$

where P_M is the fractional population of the given nuclei in the vicinity of the ion. Thus, for water proton relaxation rates (PRR) $P_M = q[M]/55.5$, [M] being the molar concentration of metal ion of hydration number q and 55.5 is the molarity of water. Equation 4 assumes rapid chemical exchange with respect to the relaxation T_{1M}, which is the case for aquo-Gd^{3+} (Reuben, 1975). Upon binding of Gd^{3+} to proteins, part of its water of hydration is substituted by protein ligands.

The water PRR can be used an an analytical method for monitoring the binding of Gd^{3+} to macromolecules. From its detailed interpretation, the number of water molecules remaining in the coordination sphere of the bound Gd^{3+} ion may also be obtained. This is an important quantity, since it reflects on the number of ligands contributed by the macromolecule and may serve in the identification of the binding site.

Potentiometric Measurements. If the binding site is composed of carboxylates or other groups that ionize by proton dissociation, the protons themselves will compete with the lanthanide ion. In this case, the binding will depend upon the pH and the overall protonation constant, K_H, as well as on the lanthanide association constant, K_L. It can be shown (see Appendix) that:

$$\log \{(1/\bar{Y} - 1)K_L[L] - 1\} = n \log [H^+] + \log K_H \quad (3)$$

where n is the number of proton dissociating groups and $\bar{Y}$ is the lanthanide saturating function defined as

$$\bar{Y} = [EL]/[E]_t \quad (4)$$

the square brackets denoting concentrations and the subscript standing for total. A plot of the left-hand side of eq 1 against log $[H^+]$ should be linear with a slope of n and an intercept on the ordinate of log K_H. The value of n can be further checked by measuring the concentration of protons released upon cation binding.

Materials and Methods

Bovine and porcine trypsins were products of Novo Industri A/S, Copenhagen, Denmark. These preparations were found to be superior to several other commercial preparations with respect to their active-site content, their specific activity, and the high percentage of the β form of the enzyme. Benzoylarginyl ethyl ester and N-acetyltryptophanamide were synthesized and purified by Mr. I. Jacobson from the Department of Biophysics. The lanthanides were used as the trichloride salts and were prepared from their corresponding sesquioxides or obtained as such. Both types of lanthanide compounds were obtained from Alfa Inorganics, Beverly, Mass. All other organic and inorganic chemicals used in this study were of the highest analytical grade available commercially.

Fluorescence measurements were performed on a Hitachi-Perkin-Elmer MPF 3L spectrofluorimeter equipped with a corrected spectrum accessory. Single-wavelength fluorescence measurements which included the determination of metal-

[1] Abbreviations used are: CPL, circular polarized luminescence; PRR, proton relaxation rate; TLCK, tosyllysyl chloromethyl ketone; NTA, nitrilotriacetic acid; EDTA, ethylenediaminetetraacetic acid; Pipes, 1,4-piperazinediethanesulfonic acid; BSA, bovine serum albumin.

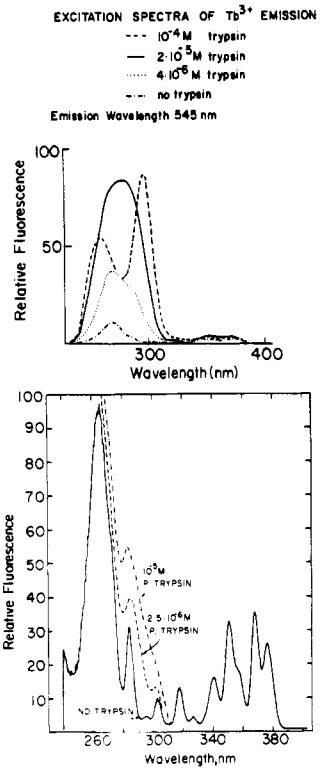

FIGURE 1: Enhancement of Tb^{3+} fluorescence by porcine trypsin. Corrected excitation spectra of 0.1 M $TbCl_3$ (A, top) and 0.01 M $TbCl_3$ (B, bottom) in the presence and absence of porcine trypsin at the concentrations shown. In B, the sensitivity is enhanced compared to A, and at wavelengths higher than 310 nm the spectra overlap. t = 25 °C, pH ~6.3 in 0.2 M Pipes.

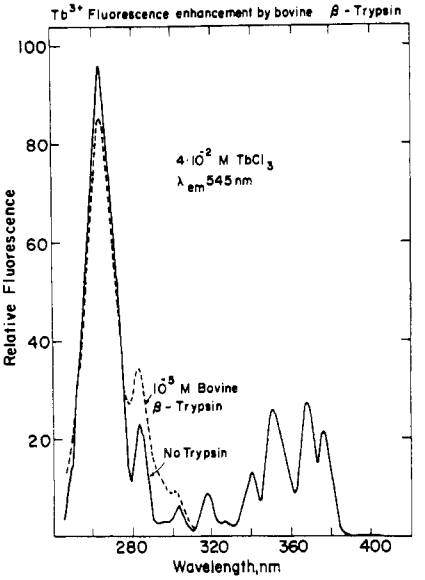

FIGURE 2: Enhancement of Tb^{3+} fluorescence by bovine trypsin. Corrected excitation spectra of 0.01 M $TbCl_3$ in the presence and absence of trypsin. At wavelengths higher than 310 nm the spectra overlap. t = 25 °C, pH 6.3 in 0.2 M Pipes.

binding constants and the pH dependence of the Tb^{3+} fluorescence were performed either with an Aminco-Keirs spectrophosphorimeter or with the Hitachi-Perkin-Elmer MPF 3L.

Circular polarized luminescence (CPL) measurements were performed as described by Steinberg and Gafni (1972).

Nuclear magnetic longitudinal relaxation times, T_1, were measured with a system consisting of a 12-in. iron-core electromagnet manufactured by Varian Associates, Palo Alto, Calif. with a variable power supply producing magnetic fields of 1-14 kG which correspond to proton resonance frequencies of 4-56 MHz and a programmed pulse spectrometer with a variable frequency synthesizer manufactured by Bruker Physik AG, Germany. The spectrophotometric assay of trypsin was performed according to Schwert and Takenaka (1955). Inactivation by TLCK was performed according to Shaw and Glover (1970). The chromatographic separation of pure β-tosyllysyltrypsin was performed according to Schroeder and Shaw (1968). The concentration of bovine and porcine trypsin was determined routinely at 280 nm. The exact extinction coefficients for both bovine and porcine trypsin were determined and found to be $\epsilon_{280\ nm}^{1\%} = 16.0$ and $\epsilon_{280\ nm}^{1\%} = 15.0$, respectively.

Lanthanide ion concentrations were determined by titration with EDTA using Arsenazo 1 as the end-point indicator (Woyski and Harris, 1963).

The three-dimensional model of bovine trypsin was built from coordinates obtained by Huber et al. (1974).

Results

Enhancement of Tb^{3+} Fluorescence upon Binding of Trypsin. When trivalent terbium binds to trypsin, its fluorescence is markedly enhanced. The corrected fluorescence spectra of Tb^{3+} and of the Tb^{3+}-porcine trypsin complex have already been published (Epstein et al., 1974). The spectra reveal the protein fluorescence peak at 335 nm and the characteristic Tb^{3+} fluorescence quartet between 490 and 625 nm. Figure 1 shows the corrected *excitation* spectra of the Tb^{3+} emission at 543 nm at different complexation degrees of the metal by porcine trypsin. Figure 1A shows the fluorescence enhancement caused by relatively high concentrations of porcine trypsin. Figure 1B shows the excitation spectrum of aqueous $TbCl_3$ at a higher sensitivity so that the fine structure becomes evident. In this figure, the fluorescence enhancement caused by low porcine trypsin concentrations is shown. It can be seen from Figure 2 that the complexation of porcine trypsin with Tb^{3+} causes a large fluorescence enhancement when the complex is excited in the range of 280-310 nm. At 10^{-4} M porcine trypsin when the fluorescence of the bound Tb^{3+} finally dominates the observed fluorescence, an excitation peak at 295 nm becomes evident. The largest enhancement occurs at 295 nm also with lower protein concentrations (Figure 1).

The Tb^{3+} fluorescence enhancement due to bovine trypsin is shown in Figure 2. It is lower than the enhancement caused by porcine trypsin.

Table I demonstrates that the structure of the emission spectrum of the bound Tb^{3+} is different in the two Tb^{3+}-trypsin complexes, porcine and bovine. Both enzymes enhance to a different extent the three main Tb^{3+} emission bands, following the order: $\epsilon_{545}^* > \epsilon_{490}^* > \epsilon_{585}^*$. The selective enhancement changes the relative intensities of the Tb^{3+} emission lines (compared to the relative emission intensities of free Tb^{3+}). However, as can be seen from Table I, this change in the structure of the Tb^{3+} emission spectrum is larger upon Tb^{3+} complexation to porcine trypsin than upon Tb^{3+} complexation to bovine trypsin. The enhancement ratio is obtained by dividing the emission intensity ratio of the bound Tb^{3+} by the same ratio of the free Tb^{3+}, namely: $(F_{545}/F_{490})_{bound}/(F_{545}/F_{490})_{free} = [(F_{545})_{bound}/(F_{545})_{free}]/[(F_{490})_{bound}/(F_{490})_{free}] = \epsilon_{545}^*/\epsilon_{490}^*$.

Quenching of Protein Fluorescence by Cations. The excitation data of Figure 1, which show maximum enhancement

TABLE I: Differences between the Tb^{3+} Emission Patterns of the Free Ion and its Porcine and its Bovine Trypsin Complex.[a]

Type of ratio[b]	Free Tb^{3+}	Tb^{3+}-porcine tosyllysyltrypsin	Tb^{3+}-bovine β-tosyllysyltrypsin
F_{545}/F_{490}	2.80	3.90	3.25
$\epsilon_{545}^*/\epsilon_{490}^*$	1.00	1.39	1.16
F_{545}/F_{585}	2.30	4.45	2.85
$\epsilon_{545}^*/\epsilon_{585}^*$	1.00	1.94	1.24
F_{490}/F_{585}	0.82	1.17	0.88
$\epsilon_{490}^*/\epsilon_{585}^*$	1.00	1.43	1.07

[a] Taken at 285-nm excitation wavelength. [b] The subscript denotes the Tb^{3+} emission line identified by the peak wavelength in nm.

TABLE II: Quenching of Protein Fluorescence by Cations.[a]

Metal Ion	% Quenching of Porcine Trypsin	% Quenching of Bovine Trypsin
Ca^{2+}	3.5	8
Tb^{3+}	26	7
Yb^{3+}	34	10

[a] The emission band of 3×10^{-6} M trypsin. Both types were recorded at two excitation wavelengths: 280 and 295 nm. Cations were added and the emission was recorded upon each addition until no further change in the protein emission spectrum was observed. The curves were integrated by weighing the paper surrounded by the curve and the baseline.

of Tb^{3+} fluorescence at 295 nm, imply the existence of an energy transfer, radiative or nonradiative, from aromatic chromophores of the protein to the Tb^{3+} ion. The alternative possibility is that by binding to the protein a forbidden spectral transition of the aquo-ion becomes allowed by a perturbation of the ligand field symmetry. In order to facilitate the differentiation between these possibilities, a few additional measurements were performed.

Energy transfer from the protein to Tb^{3+} is expected to be accompanied by a certain loss of the protein's own fluorescence at 335 nm. This actually was found to be the case with porcine trypsin and to a smaller extent with bovine trypsin, as is seen in Table II. Yet, this quenching is not necessarily related to the secondary emission, characteristic of Tb^{3+}. This can be seen from the fact that Yb^{3+} quenches the protein fluorescence of both enzymes 1.3–1.4 times more than Tb^{3+}. Also, Ca^{2+} quenches the bovine trypsin fluorescence better than Tb^{3+} (Table II).

On the other hand, possible new f–f transitions caused by the removal of degeneracy of Tb^{3+} energy levels due to symmetry perturbation mean a shortening of the fluorescence lifetime by the same factor as the enhancement observed. Lifetime measurements of Tb^{3+}-trypsin, performed in collaboration with Dr. A. Grinvald (of the Chemical Physics Department), clearly show that the lifetime of the excited state of Tb^{3+} remains very long upon binding to trypsin (in the order of a few milliseconds).

The fact that the excitation peak of the enhancement occurs at 295 nm where there is initially no trace of an excitation peak of the aqueous Tb^{3+} and the fact that the lifetime of the emission is characteristic of a forbidden spectral transition are both in favor of an energy-transfer mechanism from a tryptophan to the Tb^{3+} ion.

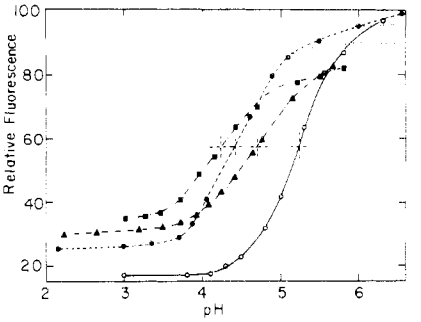

FIGURE 3: The stability of the Tb^{3+}-trypsin complexes as a function of pH. Unbuffered solutions of 2.5×10^{-5} M trypsin containing $TbCl_3$ were brought to the desired pH using dilute HCl, and the Tb^{3+} fluorescence was measured. (○) Porcine trypsin with 10^{-2} M $TbCl_3$; (▲) bovine trypsin with 3.6×10^{-2} M $TbCl_3$; (●) bovine trypsin with 10^{-2} M $TbCl_3$; (■) bovine trypsin with 4×10^{-3} M $TbCl_3$.

The identity of the chromophore as either a tyrosine or a tryptophan is usually implied by changes introduced in the shape of the protein emission line (335 nm) upon its quenching by Tb^{3+}. In both trypsins, quenching by Tb^{3+} introduced no changes in the protein emission line shape. This result was obtained at two different excitation wavelengths: 280 and 295 nm. It seems, therefore, that this approach of differentiation between the two types of chromophores is inapplicable to trypsin.

pH Dependence of Tb^{3+} Fluorescence. In order to obtain information concerning the identity of the amino acid side chains to which Tb^{3+} binds, the pH dependence of Tb^{3+} binding to the two trypsins was examined. Figure 3 shows such a dependence with porcine trypsin and at three Tb^{3+} concentrations with bovine trypsin.

The vertical scale of Figure 3 is drawn as experimentally measured, which means that all the curves are at approximately the same conditions yet not exactly the same. The zero of this scale has been shifted in order to bring the calculated midpoints together on the ordinate.

In all four experiments, the protein concentration was approximately 2.2×10^{-5} M (see legend to Figure 3). In the case of porcine trypsin, this was the concentration of active trypsin present, already known to equal the concentration of metal-binding trypsin. The unbuffered solutions were brought to pH 6.3 and the Tb^{3+} was added. At pH 6.3 with the specified concentrations, porcine trypsin was 90% saturated with Tb^{3+}, and bovine trypsin was 94 to 99.3% saturated. The Tb^{3+} fluorescence intensity was measured at 545 nm with excitation at 295 nm. By adding minute volumes of dilute hydrochloric acid, the pH was lowered and accurately measured, and the fluorescence was recorded. Between pH measurements, the electrode was rinsed and immersed in 0.1 M HCl to prevent formation of protein- or lanthanide-containing films.

None of the observed titrations is complete at the high pH range due to the formation of Tb^{3+}–OH complexes at high pH values. With porcine trypsin, the titration is almost complete and the curve is fairly symmetric. With bovine trypsin, an additional process effecting the Tb^{3+} fluorescence occurs at a higher pH, generating a "tail" above pH 5.0. When the tail effect is subtracted, a symmetric titration curve is obtained, as is shown by the dashed line of Figure 3. This subtraction seems to be legitimate, since all the points fall on the titration curve except for those which constitute the tail. The corrected titration curve is either the upper or the lower dashed line or in-between. The two curves differ in their midpoint by 0.08 pH unit (pK = 4.42 for the lower dashed line and 4.50 for the upper dashed line).

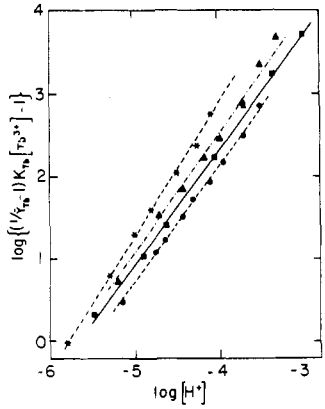

FIGURE 4: A double logarithmic plot of the data presented in Figure 3. (*) Porcine trypsin with 10^{-2} M $TbCl_3$; (▲) bovine trypsin with 3.6×10^{-2} M $TbCl_3$; (×) bovine trypsin with 10^{-2} M $TbCl_3$; (●) bovine trypsin with 4×10^{-3} M $TbCl_3$.

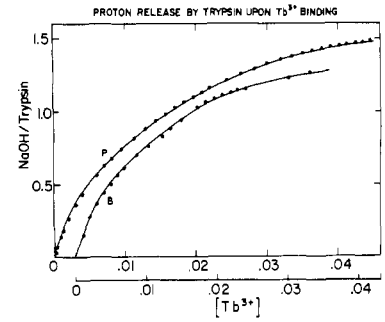

FIGURE 5: Proton release by porcine and bovine trypsin upon Tb^{3+} binding. Small Tb^{3+} aliquots were added to 2 mL of trypsin at the same pH. The resulting pH changes were back-titrated with dilute standardized NaOH. (P) Porcine trypsin at pH 4.5; (B) bovine trypsin at pH 4 for which 10% initial deprotonation was assumed.

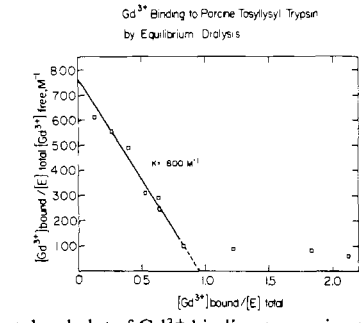

FIGURE 6: Scatchard plot of Gd^{3+} binding to porcine trypsin measured by equilibrium dialysis. 2×10^{-5}–3×10^{-2} M Gd^{3+} and 1.8×10^{-3} M trypsin were measured after dialysis at 24 °C. 100-μL aliquots were titrated with EDTA and Arsenazo I as indicator. The solution contained 0.16 M Pipes, pH 6.3, and 0.4 M NaCl. Dialysis was performed in 300-μL dialysis cells.

Regardless of the precision of the midpoint determination in the bovine trypsin titration, it can be seen that the midpoint of the porcine trypsin titration is 5.24, 0.8 pH unit higher than that for bovine trypsin with the same Tb^{3+} concentration.

The question could arise whether the change in pH blocks the enhancement of Tb^{3+} fluorescence or causes protons to displace Tb^{3+} from its binding site. To answer this question, the pH-dependence measurement was performed at three Tb^{3+} concentrations. Increasing the Tb^{3+} concentration in the titration mixture shifts the curve toward lower pH values, indicating a competition between the protons and Tb^{3+} for the binding site. It was possible to show the same phenomenon for porcine trypsin.

From the midpoints of the pH titration curves, apparent pK values for the reaction protein + $Tb^{3+} \rightleftarrows$ protein-$Tb^{3+} + nH^+$ can be obtained. These pK values are for bovine trypsin: pK 4.24 at 3.6×10^{-2} M Tb^{3+}, pK 4.5 at 10^{-2} M Tb^{3+}, and pK 4.7 at 4×10^{-3} M Tb^{3+}. For porcine trypsin, the pK is 5.24 at 10^{-2} M Tb^{3+}. The observed pK values for both trypsins are strongly indicative of the participation of carboxylic side chains in the metal binding.

The same results, somewhat differently analyzed, will provide information concerning the number of ionizable groups (from now on regarded as carboxyl groups) which participate in the binding of the metal ion. For this purpose, the linear relationship of eq 3 will be applied. The binding constant measured independently at pH 6.3 will be taken as K_L.

The result of this analysis, shown in Figure 4, shows a slope of 1.4 for the bovine trypsin and 1.6 for the porcine trypsin. This means that more than one proton is released upon Tb^{3+} binding for both trypsins.

Proton Release Experiments. In an attempt to reconfirm the number of protons involved in Tb^{3+} binding, the proton liberation upon Tb^{3+} binding was measured. In this experiment, Tb^{3+} at a certain pH is added in small quantities to an enzyme solution of known concentration at precisely the same pH. The small changes in the pH resulting from the binding are titrated back with dilute, standardized NaOH and the amounts of the base are recorded. The total amount of NaOH needed to maintain the same pH corresponds to the amount of protons liberated. The results are shown in Figure 5. For porcine trypsin, a minimum release of 1.5 protons is indicated. For bovine trypsin, a minimum release of 1.3 protons is indicated.

To summarize this part, the identification of the dissociable binding groups of Tb^{3+} as carboxylates was more straightforward than determining their number which is most probably two.

Binding of Gd^{3+}. The Tb^{3+} fluorescence was used to measure the binding constants of a series of metal ions to trypsin via their competition with Tb^{3+} (Epstein et al., 1974). With such measurements, one must make sure that the competing ions have no spectral response at the wavelengths at which the binding experiment is performed, namely, 295 and 545 nm. However, upon preparation of the Gd^{3+}–Tb^{3+}–trypsin mixture and the Tb^{3+}–trypsin solution for the Gd^{3+}-binding experiment, an absorption difference at 280 and 295 nm was repeatedly observed. This spectral difference was not due to a difference spectrum between a Gd^{3+}–trypsin mixture and free Gd^{3+} plus trypsin in two different half-cells as reference. If it was due to Gd^{3+} binding, this spectral difference would not interfere with the determination of the Gd^{3+}-binding constant of trypsin. The phenomenon could well alter the Tb^{3+} fluorescence, but, in a case like this, where both the Tb^{3+} fluorescence and the absorption difference are due to the same chemical process, namely, the Gd^{3+} binding, the observed binding constant should not change. Even though the difference was not due to Gd^{3+} binding, the result of the Gd^{3+}-binding experiment by monitoring the Tb^{3+} fluorescence (K = 390 M^{-1} at 25 °C) remained questionable. Therefore, the binding of Gd^{3+} was investigated by two other independent methods: equilibrium dialysis and the Gd^{3+}-induced enhancement of proton relaxation rate. The binding of Gd^{3+} using equilibrium dialysis is shown in Figure 6.

Due to the higher protein concentrations used with Gd^{3+}, binding sites with low affinity can also be detected, as can be seen in Figure 6. The primary binding site was found to possess

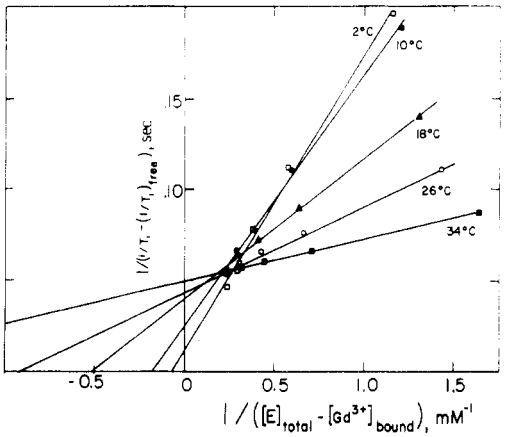

FIGURE 7: A double-reciprocal plot of Gd^{3+} titration by porcine trypsin, monitoring the water PRR. The τ_{null} was measured at 100-μL samples containing 0.5 mM Gd^{3+} and porcine trypsin at a varying concentration up to 4.5 mM. The aqueous solutions were buffered at pH 6.3 by 2 M Pipes. For the details of the calculation see text.

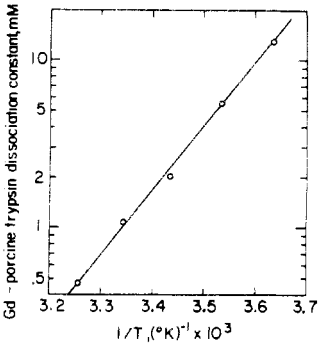

FIGURE 8: The dissociation constant of the Gd^{3+}–porcine trypsin complex as a function of the reciprocal absolute temperature. The dissociation constants are obtained by the double-reciprocal plotting in Figure 7.

an affinity constant of 800 M^{-1}. This value was confirmed by independent measurements of magnetic relaxation enhancement.

The relaxation behaviors of the aquo-Gd^{3+} and its porcine trypsin complex were established by titrating 0.5 mM Gd^{3+} with the enzyme and monitoring the change in the PRR.

A quantity of 90 mg of enzyme was added to 0.5 mL of 0.2 M Pipes, pH 6.35. The pH which was lowered by the acidified protein was readjusted to 6.3 and any precipitate was centrifuged away. Measurement of the OD_{280} of a 500-fold dilution gave a result of 6.4 mM which means 5.0 mM active enzyme concentration, 78.1% active material as was also shown previously (Epstein et al., 1974). 5.0 mM Gd^{3+} was prepared in the same buffer, adjusted to pH 6.3.

The τ_{null} of water protons was measured in the presence of 0.5 mM Gd^{3+} and five concentrations of porcine trypsin: 0.9, 1.8, 2.7, 3.6, and 4.5 mM. The measurements were done at 44 MHz proton resonance frequency in samples with a total volume of 100 μL at temperatures of 2, 10, 18, 26, and 34 °C. The τ_{null} of the same enzyme concentrations without Gd^{3+} was also measured.

The results were treated in the following way: (1) ln $2/\tau_{null}$ equals the observed relaxation rate. (2) Subtraction of the diamagnetic contribution to the relaxation rate yields the relaxation rate due to Gd^{3+} in the particular solution. The diamagnetic contribution is the relaxation rate measured for the buffer and the various enzyme concentrations without Gd^{3+}. (3) A direct complexation curve of $1/T_{1p}$ vs. $[E]_{total}$ is plotted and $(1/T_{1p})_{bound}$ is estimated from the fact that this is the asymptotic value to which $1/T_{1p}$ tends at a high $[E]_{total}$. (4) The concentration of the bound Gd^{3+} at each titration point is calculated by:

$$[Gd^{3+}]_{bound} = [Gd^{3+}]_{total}\left(\frac{1/T_{1p} - (1/T_{1p})_{free}}{(1/T_{1p})_{bound} - (1/T_{1p})_{free}}\right)$$

(5) With $[Gd^{3+}]_{bound}$ known, the results were fitted by least squares according to the linear equation:

$$\frac{1}{1/T_{1p} - (1/T_{1p})_{free}} = \left(\frac{1}{(1/T_{1p})_{bound} - (1/T_{1p})_{free}}\right) \times \left(1 + \frac{1}{K_L(n[E]_t - [Gd^{3+}]_{bound})}\right)$$

(6) If the resulting $(1/T_{1p})_{bound}$ does not coincide with the estimated value at 3, the procedures of 4 and 5 are repeated with the newly obtained value. This successive correction had never to be repeated more than twice.

Figure 7 shows the final cycle of the successive correction in which the $(1/T_{1p})_{bound}$ used to estimate $[Gd^{3+}]_{bound}$ is the same as $(1/T_{1p})_{bound}$ yielded by the linear fit. The resulting binding constants of Gd^{3+} to porcine trypsin are shown in Figure 8 as a function of the inverse absolute temperature. The interpolated result for the binding constant at 24 °C, 830 M^{-1}, coincides within the experimental error with the result obtained by equilibrium dialysis.

The Hydration Number of Bound Gd^{3+}. With the value of $(1/T_{1p})_{bound}$ determined in the preceding paragraph one can proceed with the calculation of the number of water molecules coordinated to the bound Gd^{3+} ion using eq 2. For this, an independent estimate of $1/T_{1M}$ is needed. The latter involves the calculation of the function $f(\omega,\tau_c)$, which requires the knowledge of the correlation time, τ_c, and the knowledge of the Gd^{3+}–water proton distance, r. Unpublished observations by Epstein and Koenig on the frequency dependence of PRR in Gd^{3+} solutions containing porcine trypsin have shown that the correlation time is frequency dependent and is therefore governed to a large extent by the electron-spin relaxation time. The electron-spin resonance spectrum of a solution containing 0.5 mM Gd^{3+} and 2.7 mM porcine trypsin recorded at 9.5 GHz consists of a single line with a peak to peak line width of 250 G, corresponding to $1/T_{2e} = 3.8 \times 10^9$ s^{-1}. The electron-spin resonance frequency corresponding to the magnetic field at which the PRR measurements were done is 29 GHz. Using the formulas previously given by Koenig and Epstein (1975) and a correlation time for electron-spin relaxation of 7.5×10^{-12} s (Reuben, 1971a), one obtains $1/T_{1e} = 7.4 \times 10^8$ and 2.25×10^9 s^{-1}. The correlation time for the isotropic rotational motion of the trypsin molecule is 1.3×10^{-8} s, as determined from fluorescence polarization measurements (Yguerabide et al., 1970). In order to estimate the value of r, the crystallographic data on the $La(EDTA)^-$ chelate was used. There the lanthanide–water oxygen distance is 2.58 Å (Hoard et al., 1965). Assuming axial symmetry around the lanthanide–oxygen bond and using 0.95 Å for the O–H distance and 106° for the H–O–H angle (Chidambaram, 1962), one obtains r = 3.22 Å. Thus, $T_{1M} = 6.6 \times 10^{-7}$ s is calculated by eq 1. With $[M] = 5 \times 10^{-4}$ and $(1/T_{1p})_{bound} = 30.2$ s^{-1}, one obtains from eq 2 $q = 2.2$. This result indicates that approximately two water molecules are coordinated to Gd^{3+} in its trypsin complex.

CPL of Tb^{3+} and Low-Affinity Sites. The CPL spectra of Tb^{3+}–porcine trypsin exhibit unique features. Figure 9 shows that the main component of the Tb^{3+} emission spectrum, the 545-nm line, is actually composed of five spectral transitions

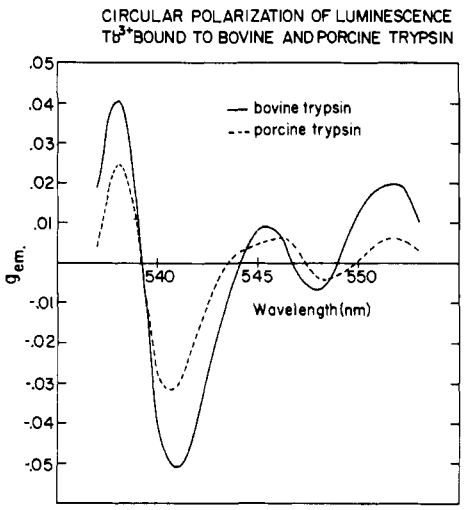

FIGURE 9: CPL spectrum of Tb^{3+} emission of the Tb^{3+}-porcine trypsin and Tb^{3+}-bovine trypsin complex. The Tb^{3+}-trypsin solutions, buffered at pH 6.3 by 0.2 M Pipes, were measured as described by Steinberg and Gafni (1972). At each wavelength, the AC and DC were measured separately and their respective ratio, multiplied by a calibration factor, yielded g_{em}.

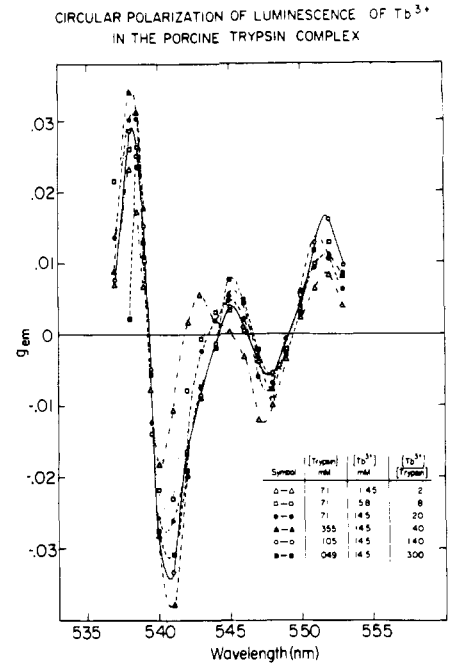

FIGURE 10: The dependence of the CPL spectrum on Tb^{3+} to trypsin ratio. (△) 0.71 mM trypsin, 1.45 mM Tb^{3+}; (□) 0.71 mM trypsin, 5.8 mM Tb^{3+}; (●) 0.71 mM trypsin, 14.5 mM Tb^{3+}; (▲) 0.355 mM trypsin, 14.5 mM Tb^{3+}; (○) 0.105 mM trypsin, 14.5 mM Tb^{3+}; (■) 0.049 mM trypsin, 14.5 mM Tb^{3+}.

(at least) as seen in the CPL. The plus and the minus signs of g_{em} denote a dominance of a right- or left-handed circular component in the emitted light, respectively. Therefore, a change in sign differentiates between spectral transitions. The other two emission lines at 490 and 585 nm reveal three transitions each.

Figure 9 also shows that in the Tb^{3+}-porcine trypsin system the g_{em} spectrum depends on both the Tb^{3+} and the trypsin concentrations.

This result strongly indicates that the population of the Tb^{3+}-trypsin complex is inhomogeneous. Measurements carried out with purified porcine and bovine β-tosyllysyltrypsins show the same phenomenon, not reduced even slightly by using the purified preparations. This leads to the conclusion that the trypsin molecule possesses more than one binding site for Tb^{3+} with different affinities and different anisotropy factors.

For a more detailed examination of the concentration dependences of g_{em}, attention will be focused on the intense CPL band at 540.5 nm (Figure 10). All the other transitions are subject to relatively large experimental error, either due to the low g_{em} or due to the very low total intensity (e.g., the transition at 538 nm).

The observed absolute increase of g_{em} at 540.5 nm with increasing protein concentration is easily explained. 14.5 mM Tb^{3+} has a significant fluorescence intensity which is completely unpolarized. Upon addition of protein, the emission intensity increases but the circular polarization increases proportionally more (starting at zero) so that an increase in g_{em} is observed.

If we assume that the low-affinity binding sites have on the average a larger g_{em} value at 540.5 nm, an increase of the g_{em} value as a function of the Tb^{3+} concentration is expected (Figure 10). Thus, for example, an increase of the Tb^{3+} concentration from 1.45 to 14.5 mM results in an increase of the occupancy of the high-affinity site ($K = 800\ M^{-1}$) from 47 to 92%, whereas the increase in occupancy of the low-affinity sites ($K = 100\ M^{-1}$, Figure 6) will be from 9 to 59%.

It can be observed that the increase of g_{em} at 540.5 nm with increasing protein concentration does not last at higher protein concentrations. Rather, a decrease is observed at 7.1×10^{-4} M trypsin. The same assumption, that the low-affinity binding sites have a larger contribution to the CPL, explains this phenomenon as well. When the molar protein concentration has reached the level where it is only a few percent of the Tb^{3+} concentration, the fluorescence of free Tb^{3+} becomes totally negligible and a further increase of the protein concentration will result in a parallel increase of the fluorescence emission and the fraction which is circularly polarized. The relative degree of saturation of the high- and low-affinity binding sites is different, such that, as the concentration of free Tb^{3+} decreases by a finite amount due to binding, preferential binding to the high affinity site (with the lower g_{em}) occurs, yielding a decrease in the observed g_{em}.

The CPL of Tb^{3+} could also be used to show that the symmetry properties of the metal-binding site of bovine and porcine trypsin are very similar. Figure 9 shows that the Tb^{3+} CPL spectrum is similar in both enzymes. The intensity differences have to be ignored in view of the results shown in Figure 10.

Discussion

The Ligands Involved in Metal Binding. The results of the proton release measurements and the pK values of the groups involved in metal-ion binding in trypsin indicate that two carboxylates are present at the binding site. Thus, the chromophore responsible for the enhancement of Tb^{3+} fluorescence need not be a ligand of the metal ion. Spatial proximity and proper orientation could render an aromatic ring of the protein a suitable energy-transfer source for Tb^{3+} without direct association between the two. We attribute the observed fluorescence enhancement effects to energy transfer from a tryptophan residue. In support of this assignment are the findings that Yb^{3+} and Tb^{3+} quench the protein fluorescence in the same sequence as the quenching of indole fluorescence (Ricci and Kilichowski, 1974) and that the quenching effect is not correlated to any secondary emission. The fact that the excitation spectrum of the Tb^{3+} emission has a peak at 295 nm does not necessarily imply the involvement of a tyrosine residue as

TABLE III: Thermodynamic Parameters for Gd^{3+} Complexation with Proteins and Chelating Agents.

Ligand	$\Delta H°$ (kcal/mol)	$\Delta S°$ (eu)	$G°_{25}$ (kcal/mol)
BSA[a]	5.1	35	−5.33
Trypsin	16.9	70	−3.96
NTA[b]	1.02	56.2	−15.73
EDTA[c]	−1.73	71.2	−22.95

[a] From Reuben (1971a). [b] From Moeller and Ferrus (1962). [c] From Mackey et al. (1962).

some authors seem to advocate (Mikkelsen and Wallach, 1974; Nieboer, 1975). Luk (1971) has found that in transferrin Tb^{3+} binds to a phenolate characteristically absorbing at 295 nm; however, his experiments were carried out at pH 8.5.

The dissociation constant of the Gd^{3+}–trypsin complex decreases with increasing temperature, indicating that complex formation is an endothermic process. Endothermic binding of Gd^{3+} has also been observed with bovine serum albumin (BSA) (Reuben, 1971b) and with lysozyme (Jones et al., 1974), as well as for Ce^{3+}, Pm^{3+}, and Eu^{3+} with glycine (Tanner and Choppin, 1968). Analysis of the results for BSA and trypsin according to eq 5 shows that the binding process is entropy driven.

$$\ln K_L = -\Delta H°/RT + \Delta S°/R \tag{5}$$

The thermodynamic parameters are summarized in Table III along with data for the NTA and EDTA chelates. The main difference between the small chelates and the macromolecular complexes is in the large positive enthalpies for the latter. Entropy changes are often attributed to the extensive dehydration accompanying complex formation (Choppin, 1971). The entropy of hydration of Gd^{3+} is 98 eu (Bertha and Choppin, 1969). Assuming that the entropies of complexation with proteins are due to dehydration only, one concludes from the results in Table III that the Gd^{3+} ion has lost more than 35% of its water of hydration upon complexation with BSA and more than 70% upon complexation with trypsin. With a hydration number of 8 for the aquo-ion, this means that not more than 5.2 and 2.4 water molecules remain in the coordination sphere of a Gd^{3+} ion bound to BSA and trypsin, respectively. The analysis of water PRR data in terms of the number of water molecules yielded $q = 2.2$. Using the same value of r with the BSA data of Reuben (1971b), one obtains $2.8 < q < 5.5$. Despite the oversimplifications involved, the agreement between the estimates of q obtained from entropy considerations and from the analysis of PRR data is remarkable. From the above discussions, it may be concluded that lanthanide complexation with proteins is an entropy-driven process similar to the chelation by smaller ligands. The differences in dissociation constants observed with different lanthanides (Epstein et al., 1974) probably arise from differences both in the entropies and in the enthalpies of complexation.

Inspection of a molecular model constructed according to the crystallographic data of Huber et al. (1974) suggests that the metal-ion binding site is composed of the carboxylates of Glu-70 and Glu-80 and the peptide carbonyl oxygens of Asn-72 and Val-75, with the carboxylates acting as bidentate ligands. There is no other clustering of carboxyls in the trypsin molecule. Facing this site at a distance of about 7 Å is Trp-141, which is probably the residue involved in energy transfer. Recent x-ray difference maps of the bovine enzyme and its Ca^{2+} complex have identified this same site as the calcium-binding site (Bode and Schwager, 1975a,b).

The Secondary Lanthanide-Binding Site. A different conclusion however has been reached by Abbott et al. (1975a,b). These authors measured Gd^{3+}-induced PRR of *p*-toluamidine, an inhibitor of the enzyme, and using independently determined dissociation constants calculated a distance of 10 Å between the Gd^{3+} and the methyl group of the inhibitor. With the known position of the inhibitor, this places the Gd^{3+} ion at a site composed of the carboxyl side chain of Asp-194 and the hydroxyl of Ser-190. This site is approximately 15 Å away from the one mentioned above. The more recent study of Epstein and Reuben (1977) has shown however that the Gd^{3+}–trypsin–inhibitor system is heterogeneous, as revealed by the dependence of the water and inhibitor proton NMR line widths upon both the Gd^{3+} and the enzyme concentrations and in the effects of added Ca^{2+} on the line widths. These findings imply that in the previous work the distance from a weak rather than from a strong metal-ion binding site of trypsin may have been determined.

The CPL results reported here also show the presence of low-affinity sites. Gomez et al. (1974) have studied the acceleration by lanthanide of the trypsinogen conversion by trypsin. They found that the reaction is inhibited at high lanthanide concentrations (ca. 10^{-2} M) due to the inhibition of trypsin activity. It seems now that this inhibitory effect could result from the weak lanthanide binding at the carboxyl of Asp-194 and the hydroxyl of Ser-190, the latter being essential for enzyme specificity.

Acknowledgment

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Appendix

The Displacement of Protons by Metal Ions. It is assumed that a number of chelating residues are involved in metal binding and that only the deprotonated species bind the metal ion according to the equilibrium:

$$K_L = \frac{[EL]}{[E][L]} \tag{1a}$$

It is also assumed that the species EH, EH_2 . . . EH_{n-1} do not have a significant affinity for the metal ion. Thus:

$$[E]_{total} = [EL] + [E] + [EH_n] \tag{2a}$$

n denotes the number of protons ionizing from the metal-binding site. The free ligand concentration in solution remains unchanged during the titration and therefore:

$$[L]_{free} = [L]_{total} \tag{3a}$$

When the ligand is in large excess one can therefore write:

$$K_H = \frac{[EH_n]}{[E][H^+]^n} \tag{4a}$$

From eq 3a and 4a one obtains:

$$[EH_n] = [EL]\left(\frac{K_H[H^+]^n}{K_L[L]}\right) \tag{5a}$$

Equation 2 therefore becomes:

$$[E]_{total} = [EL]\left(1 + \frac{1}{K_L[L]} + \frac{K_H[H^+]}{K_L[L]}\right) \tag{6a}$$

If one defines the saturation function $\bar{Y}$:

$$\bar{Y} = \frac{[EL]}{[E]_{total}} \quad (7a)$$

one obtains from eq 6a and 7a:

$$\frac{1}{\bar{Y}} = \frac{[E]_{total}}{[EL]} = 1 + \left(\frac{1}{K_L[L]} + \frac{K_H}{K_L[L]}\right)[H^+]^n \quad (8a)$$

namely:

$$\frac{1}{\bar{Y}} - 1 = \left(\frac{1}{K_L[L]}\right)(1 + K_H[H^+]^n) \quad (9a)$$

or

$$\left(\frac{1}{\bar{Y}} - 1\right)(K_L[L] - 1) = K_H[H^+]^n \quad (10a)$$

From eq 10a one obtains eq 3 of the text:

$$\log\left\{\left(\frac{1}{\bar{Y}} - 1\right)K_L[L] - 1\right\} = n \log [H^+] + \log K_H \quad (11a)$$

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