Lanthanide Probes in Biological Systems: The Calcium Binding Site of Pancreatic Elastase As Studied by Terbium Luminescence†

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ABSTRACT: In the presence of porcine pancreatic elastase, Tb^{3+} ions can be excited by energy transfer from an aromatic side chain of the protein. The enhancement of Tb^{3+} luminescence (λ_{ex} = 280 nm; λ_{em} = 543 nm) due to binding of Tb^{3+} to elastase is $\sim 2 \times 10^4$. The luminescence excitation spectrum indicates that energy transfer is from a tryptophan side chain to the bound Tb^{3+}. Analysis of the luminescence intensity data shows that Tb^{3+} ions bind to elastase, forming a 1:1 complex, with an association constant of 3×10^5 M^{-1} (pH 6.58, 25 °C). Binding constants for Ca^{2+}, Mg^{2+}, and Zn^{2+} to elastase were also determined by the luminescence method (displacement of Tb^{3+}), showing that under physiological conditions elastase exists as Ca^{2+}·elastase. Addition of the competitive inhibitor acetyltrialanine to Tb^{3+}·elastase causes a twofold enhancement of luminescence intensity. By measuring the effect of acetyltrialanine concentration on the intensity, it was shown that this effect is due to binding of the inhibitor to the Tb^{3+}·enzyme, and a dissociation constant K_d was determined. K_d was found to be a factor of 4 smaller than K_i for acetyltrialanine as a competitive inhibitor of the hydrolysis of succinyltrialanine *p*-nitroanilide, determined under the same conditions. This and other evidence indicates that acetyltrialanine can bind to two distinct sites in Tb^{3+}·elastase. This finding requires reassessment of other work using lanthanide probes of calcium binding sites in elastase and other enzymes.

Calcium ions are involved in a wide variety of important biological processes, commonly exerting their effects by binding to a protein, thereby causing a change in its biological activity. The study of calcium proteins has been hampered by the inapplicability of the spectral techniques so useful in the study of other metalloproteins. Lanthanide ions have a number of properties which make them excellent spectroscopic probes for Ca^{2+} (Nieboer, 1975; Martin & Richardson, 1979). Tb^{3+} ions have been used quite widely in luminescence experiments. When Tb^{3+} is bound to a protein, luminescence may be excited by energy transfer from nearby aromatic side-chain chromophores which are excited directly by ultraviolet radiation (Brittain et al., 1976). The green luminescence of Tb^{3+} (540–550 nm) may thus be strongly enhanced compared with aqueous Tb^{3+}, and analysis of the system can give information on the Tb^{3+} (and Ca^{2+}) binding site(s). The general aim of this work has been to investigate further the qualitative and quantitative information which can be gained about calcium binding proteins by this technique.

Pancreatic elastase (EC 3.4.21.11) was used in these experiments because of the results obtained in a survey of terbium binding proteins by Brittain et al. (1976). They showed that Tb^{3+} bound to elastase (probably in a 1:1 complex) showed strongly enhanced luminescence. Further, the intensity of the luminescence was reported to change on the addition of competitive inhibitors, raising the possibility that reactions at the active site could thus be monitored. In 1976, Darnall et al. reported that pancreatic elastase, in contrast to trypsin, has a very low affinity for Ca^{2+}. However, Dimicoli & Bieth (1977) showed by equilibrium dialysis experiments that Ca^{2+} and Gd^{3+} form stable complexes with elastase. They found that the presence of bound Ca^{2+} or Gd^{3+} had no effect on k_{cat} and K_m for the hydrolysis of the specific substrate succinyltrialanine *p*-nitroanilide nor on K_i for the competitive inhibitor (trifluoroacetyl)trialanine. Relaxation measurements on the fluorine nuclei in the elastase–inhibitor–Gd^{3+} complex were used to calculate a distance of 20 Å between the fluorine nuclei and the Gd^{3+} ion.

In the present paper, we describe the use of the terbium luminescence technique (1) to characterize the metal ion binding site of elastase and (2) to measure directly the binding of inhibitors to Tb^{3+}·elastase.

Experimental Section

Materials. The following materials were obtained from the Sigma Chemical Co., St. Louis, MO: elastase from porcine pancreas, Type III, lot 48C-8095; succinyltrialanine *p*-nitroanilide; trialanine; acetyltrialanine; acetyltetraalanine; acetyltrialanine methyl ester; phenylmethanesulfonyl fluoride; diethyl *p*-nitrophenyl phosphate (paraoxon).[1] $TbCl_3 \cdot 6H_2O$ was from Alfa-Ventron, Danvers, MA, and elastinal was a gift from Dr. H. Umezawa, Institute of Microbial Chemistry, Tokyo. Buffer components were analytical grade reagents or the best commercially available. pH measurements were made on a Radiometer PHM 64 pH meter at 25 °C and are accurate to ±0.01 pH unit.

Methods. Fluorometric experiments were done at 25 °C on an SLM 8000 spectrofluorometer combined with an SLM PR-8000 spectrum processing system supplied by SLM Instruments, Urbana–Champaign, IL. In all experiments, the photon-counting mode was used, usually with an integration time of 10 s. The ratiometric method with rhodamine 6-G in the reference beam was used to correct for variations in lamp intensity with wavelength and with time. Semimicrofluorometer cells (1-mL capacity) were used throughout to minimize the amount of enzyme required and to decrease effects due to absorption of the exciting light by the protein. Titrations of elastase solutions with Tb^{3+} were performed by repeated

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[1] Abbreviations used: paraoxon, diethyl *p*-nitrophenyl phosphate; PMS, phenylmethanesulfonyl; Mops, 3-(*N*-morpholino)propanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid.

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addition of small volumes (5 or 10 μL) of stock aqueous $TbCl_3$ to 1 mL of enzyme in 0.05 M Mops buffer, μ = 0.1 with KCl, pH 6.58, in the thermostated cell holder. After each addition, the intensity was recorded for a period of several minutes ($\geq$15 readings) to ensure stability, and the readings were averaged. Intensities were checked before and after recording of spectra to ensure that no intensity changes occurred over the periods required (up to 90 min). Mops buffer at pH 6.58 was used in preference to piperazine buffer, as used in the earlier experiments (Brittain et al., 1976). Representative experiments performed in both Mops and piperazine buffers gave similar results, arguing against any significant interaction between Mops and Tb^{3+} or Mops and enzyme.

In all binding experiments, λ_{ex} = 280 nm and λ_{em} = 543 nm. Association constants for the binding of Tb^{3+} to elastase were obtained from the luminescence data as follows. In every case, it was possible to estimate the maximum intensity (I_{max}) accurately, and therefore α could be evaluated for each intensity reading ($\alpha = I/I_{max} = [Tb^{3+}\cdot E]/[E]_0$). By the assumption of a simple equilibrium with association constant K_T ($K_T = [Tb^{3+}\cdot E]/[E][Tb^{3+}]$), a value of K_T was calculated for each reading. The binding of other metal ions to elastase was estimated from their ability to compete with Tb^{3+} for a single binding site on the protein. Tb^{3+} was added to elastase, the intensity measured, and α calculated from K_T and the initial concentrations. Aliquots of the other metal ion were then added, causing a stepwise decrease in intensity. For each intensity reading, it was possible to estimate an association constant for the second metal ion (Ca^{2+}, Mg^{2+}, or Zn^{2+}). The constancy of this association constant over a series of additions supports the simple scheme in which Tb^{3+} and the other metal ions compete for a single binding site.

The binding of peptide inhibitors to Tb^{3+}·elastase could be measured directly, since binding produced an increase in the luminescence intensity of the bound Tb^{3+}. Concentrated aqueous solutions ($\sim$50 mM) of the inhibitors (acetyltrialanine, acetyltetraalanine, and trialanine) were prepared, with NaOH being added where necessary to titrate the carboxylic acid groups. Small aliquots of these solutions were then added to elastase in the presence of sufficient Tb^{3+} to give $\sim$95% saturation of enzyme with Tb^{3+}, with the intensity I being read after each addition. The simple binding equation is $Tb^{3+}\cdot E + X \rightleftharpoons Tb^{3+}\cdot E\cdot X$, where X is the inhibitor, and the dissociation constant $K_d = [Tb^{3+}\cdot E][X]/[Tb^{3+}\cdot E\cdot X]$. If I_0 is the intensity in the absence of X and I_{max} is the maximum intensity at saturating [X], it can be shown that $(I - I_0)/(I_{max} - I_0) = [Tb^{3+}\cdot E\cdot X]/[E]_0 = [X]_0/(K_d + [X]_0)$, assuming that $[X]_0 \gg [E]_0$. Hence, a plot of $[X]_0/(I - I_0)$ against $[X]_0$ should be linear, with intercept $K_d/(I_{max} - I_0)$ and slope $1/(I_{max} - I_0)$. In all experiments, linearity of such plots was observed and constants were evaluated by linear regression analysis.

Elastase dissolves readily in Mops buffer (0.05 M, μ = 0.1, pH 6.58) to give a clear stock solution. Protein concentration (usually 5–10 μM) was determined from the ultraviolet absorption spectrum (determined on a Cary 14 spectrophotometer) by using $A_{1cm}^{1\%}$ = 20.2 and a molecular weight of 25900 (Hartley & Shotton, 1971). Elastase activity was determined by using the specific elastase substrate succinyltrialanine *p*-nitroanilide (Bieth et al., 1974). Release of *p*-nitroaniline was followed at 410 nm on a Cary 14 spectrophotometer at 25 °C (ϵ_{410} = 8800). Stock solutions of the substrate in water ($\sim$8 mM) were prepared by neutralizing a weighed amount with an equimolar amount of NaOH (to pH $\sim$5.6); a small amount of insoluble material was removed by filtration. The concentration of *p*-nitroanilide was determined by measuring the

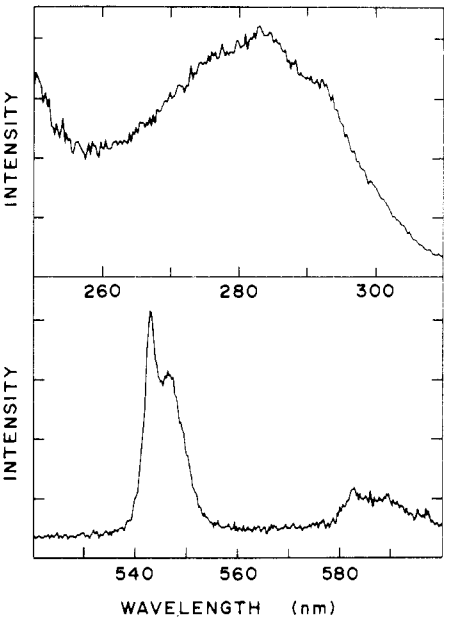

FIGURE 1: Excitation (upper) and emission (lower) spectra of Tb^{3+}·elastase in 0.05 M Mops buffer, μ = 0.1, pH 6.58, $[elastase]_0$ = 1.53 μM; $[Tb^{3+}]_0$ = 33.5 μM; integration time 20 s; 0.25-nm increments; resolution 2 (excitation) and 1 nm (emission).

p-nitroaniline produced on complete hydrolysis of an aliquot by elastase. This substrate solution was stable for at least 1 week at 4 °C. Elastase assays were carried out in the same pH 6.58 Mops buffer as used in the luminescence measurements. Elastase solutions were normally used on the day of preparation, but no loss of activity was observed in 1 week at 4 °C. For some experiments, elastase was dialyzed at 4 °C against either pH 6.58 Mops buffer or Mops buffer containing 5 mM EGTA. In the latter case, subsequent dialysis against Mops buffer (μ = 0.5 with KCl) followed by pH 6.58 Mops buffer (μ = 0.1) was required to remove all traces of EGTA which would interfere with metal ion binding experiments. The concentration of active sites in elastase solutions (undialyzed) was determined by titration with paraoxon (Bender et al., 1966), using 0.05 M phosphate buffer at pH 7.5 and a paraoxon concentration of 2.41 mM. The enzyme concentration determined by titration was 84% of the protein concentration determined from the ultraviolet spectrum, showing the probable presence of some inactive protein in the elastase solutions. The active-site concentration was used in the enzyme kinetic experiments to calculate k_{cat}, while the protein concentration was used in the binding experiments.

For the determination of k_{cat} and K_m for the elastase-catalyzed hydrolysis of succinyltrialanine *p*-nitroanilide and K_i for acetyltrialanine, 100 μL of concentrated Mops buffer (0.55 M, μ = 1.0) was added to an aqueous solution (1 mL) of the substrate (plus other components), to give a final buffer of 0.05 M Mops, μ = 0.1, pH 6.58. After equilibration at 25 °C, the reaction was initiated by adding an aliquot of enzyme. Kinetic constants were evaluated by linear regression analysis of plots of $[S]/v$ vs. [S]. PMS-elastase was prepared by treatment of elastase in 0.05 M Mops buffer, pH 6.58 (78 μM; 1 mL) with phenylmethanesulfonyl fluoride in acetonitrile (20 mM; 25 mL) for 2 h at 25 °C, followed by chromatography on Sephadex G-25. The pooled protein fractions (15.7 μM) had a specific activity 3.8% of that of native elastase.

Results

Excitation and Emission Spectra of Tb^{3+}·Elastase. Figure 1 shows typical excitation and emission spectra of Tb^{3+}·elastase

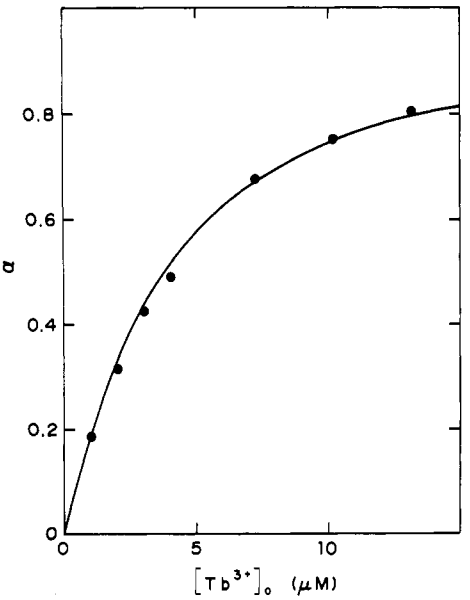

FIGURE 2: Luminescence titration of elastase with Tb^{3+} in 0.05 M Mops buffer, $\mu = 0.1$, pH 6.58, at 25 °C; $[E]_0 = 1.55$ μM. The solid line is calculated for $K_I = 3.2 \times 10^5$ M^{-1}.

at pH 6.58. Spectra were also run at higher elastase concentrations (up to 6.9 μM) to ensure that the observed excitation spectrum ($\lambda_{max} = 283$ nm; shoulder at 292 nm) was not affected by the absorbance of the elastase ($\lambda_{max} = 280$ nm; A_{280} of a 1.53 μM solution = 0.08). Very similar excitation spectra were obtained. The emission spectrum shown in Figure 1 shows the principal emission band in the 540–550-nm region ($\lambda_{max} = 543$ nm with a second peak at 547 nm), due to the $^5D_4 \rightarrow {}^7F_5$ transition, together with a weaker band in the 580–600-nm region. Control experiments showed that under these conditions, Tb^{3+} alone and elastase alone gave no significant signal in either excitation or emission spectra. An identical emission spectrum was obtained in piperazine hydrochloride buffer (0.05 M, $\mu = 0.1$, pH 6.47).

For determination of the enhancement of the Tb^{3+} luminescence due to energy transfer from a protein side chain, emission spectra of the Tb^{3+}·enzyme complexes were determined in the wavelength range 530–560 nm ($\lambda_{ex} = 283$ nm), with $[E]_0 = 4.3$ μM and $[Tb^{3+}]_0 = 113$ μM, 1.03 mM, and 9.86 mM in Mops buffer, pH 6.58. Similar spectra were run in the absence of protein with $[Tb^{3+}] = 9.9$ mM. The emission intensities were estimated by measuring the areas under the peak centered at 545 nm. From these values, an enhancement of $(1.9 \pm 0.1) \times 10^4$ was calculated at each of the three $[Tb^{3+}]_0/[E]_0$ ratios. (Enhancement = ratio of emission intensities, on a molar basis, of protein bound vs. free Tb^{3+}.)

Binding of Metal Ions to Elastase. Determination of Binding Constants. Figure 2 shows a plot of data obtained in a typical titration of elastase with Tb^{3+}, where $\alpha = I/I_{max}$, and the solid line is calculated for $K_T = 3.2 \times 10^5$ M^{-1}. Similar experiments were performed on several different samples of elastase at various protein concentrations. The data are summarized in Table I. Included in Table I are binding constants for Ca^{2+}, Mg^{2+}, and Zn^{2+} estimated by the terbium displacement method. At enzyme concentrations above ~7 μM, Tb^{3+} binding data were not consistent with a simple equilibrium. Qualitatively, these data might be rationalized in terms of the formation of significant amounts of associated enzyme such as E·Tb^{3+}·E. Attempts to analyze this effect quantitatively were defeated by Tb^{3+}-induced precipitation of the enzyme at the relatively high enzyme concentrations required (>50 μM).

Table I: Association Constants for the Binding of Metal Ions to Elastase at 25 °C[a]

metal ion	enzyme sample	$[E]_0$ (μM)	$10^{-5}K$ (M^{-1})
Tb^{3+}	untreated	0.315	3.8 ± 0.3
		1.55	3.2 ± 0.4[b]
		1.55[c]	3.8 ± 0.4
		5.26	3.2 ± 0.2
Tb^{3+}	EGTA-treated, dialyzed	0.43	3.1 ± 0.3[d]
		3.97	2.3 ± 0.1
Tb^{3+}	dialyzed	0.46	2.3 ± 0.2
		0.92	2.4 ± 0.3[d]
		4.26	2.3 ± 0.2
Tb^{3+}	PMS-enzyme	0.38	2.3 ± 0.3
Ca^{2+}	dialyzed	0.92	2.1 ± 0.5
Zn^{2+}	dialyzed	0.92	0.07 ± 0.02
Mg^{2+}	dialyzed	0.92	0.03 ± 0.01

[a] In 0.05 M Mops buffer, $\mu = 0.1$, pH 6.58, unless otherwise specified. [b] Average of three determinations. [c] In 0.05 M piperazine hydrochloride buffer, $\mu = 0.1$, pH 6.47. [d] Average of two determinations.

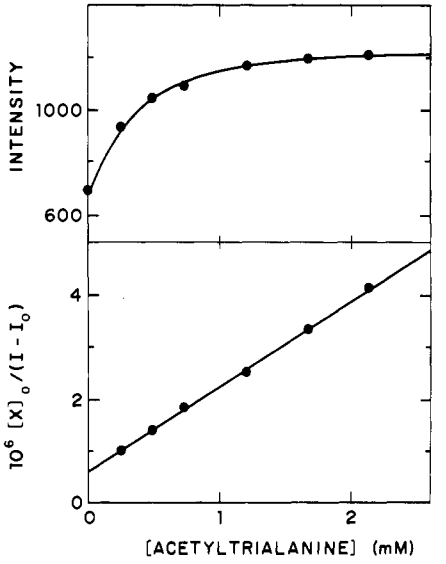

FIGURE 3: Effect of acetyltrialanine on the luminescence intensity (arbitrary units) of Tb^{3+}·elastase in 0.05 M Mops buffer, $\mu = 0.1$, pH 6.58, at 25 °C: $[E]_0 = 3.9$ μM; $[Tb^{3+}]_0 = 34$ μM. In the lower graph, the solid line is the least-squares line: $K_d = (3.7 \pm 0.3) \times 10^{-4}$ M; $I_{max} - I_0 = 608 \pm 10$.

Binding of Peptide Inhibitors to Tb^{3+}·Elastase. Figure 3 shows the effect of acetyltrialanine on the luminescence intensity of Tb^{3+}·Elastase, under conditions where Tb^{3+} is in a saturating concentration (>90%) at all concentrations of the peptide. As described under Experimental Section, these data may be evaluated by plotting $[X]_0/(I - I_0)$ against $[X]_0$, as in Figure 3, yielding K_d, the dissociation constant of inhibitor X from Tb^{3+}·E·X, and $I_{max} - I_0$. The ratio I_{max}/I_0 shows the effect of binding of the peptide on the intensity of Tb^{3+} luminescence (Table II). K_d increases as the protein concentration increases. For 13 elastase concentrations in the range 0.38–22 μM, the points conform approximately to a straight line described by $K_d = (4.0 \times 10^{-4} + 1.2 \times 10^2[E]_0)$ M with some suggestion of leveling off at the highest enzyme concentrations. The emission spectrum of Tb^{3+}·elastase is unchanged from that shown in Figure 1 in the presence of a saturating concentration of acetyltrialanine.

Elastinal, a potent competitive inhibitor of elastase (Umezawa et al., 1973), produces a small decrease in the luminescence intensity of Tb^{3+}·elastase. A maximal decrease of 25% was caused by 10.5 μM elastinal ($[E]_0 = 1.18$ μM; $[Tb^{3+}]_0 = 34$ μM). The magnitude of this decrease was not

Table II: Binding of Peptide Inhibitors to Tb^{3+}·Elastase and Tb^{3+}·PMS-Elastase at 25 °C[a]

inhibitor	$[E]_0$ (μM)	$[Tb^{3+}]$ (μM)	K_d (mM)	I_{max}/I_0	K_i (mM)
acetyltrialanine	0.38	34	0.37 ± 0.03	1.9	1.4,[b] 1.1[c]
	1.53	34	0.60 ± 0.01	2.3	2.7[d]
acetyltrialanine + elastinal (17 μM)	1.16	33	0.70 ± 0.04	2.5	
acetyltrialanine + elastinal (49 μM)	5.42	31	0.89 ± 0.02	1.9	
acetyltrialanine	0.37[e]	33	0.38 ± 0.02	2.4	
	1.40[e]	31	0.59 ± 0.02	3.0	
	15.0[e]	67	1.23 ± 0.03	3.4	
acetyltetraalanine	1.49	34	0.61 ± 0.04	2.6	
trialanine	1.49	34	1.02 ± 0.04	2.1	7[c]

[a] In 0.05 M Mops buffer, μ = 0.1, pH 6.58. [b] This work (Table III). [c] At pH 8; Dimicoli et al. (1979). [d] At pH 8; Shaw & Whitaker (1973). [e] PMS-elastase.

affected by increasing $[Tb^{3+}]$. Elastinal inhibition of the elastase-catalyzed hydrolysis of succinyltrialanine *p*-nitroanilide was measured under similar conditions: 6.4 μM elastinal gave ~99% inhibition with $[S]_0$ = 1.55 mM (K_m = 2.14 mM). The addition of acetyltrialanine to Tb^{3+}·elastase in the presence of elastinal caused a similar increase in intensity to that observed in the absence of elastinal. Conversely, elastinal even at 39 μM was unable to reverse the increase in intensity due to 1.19 mM acetyltrialanine. It was possible to measure K_d for the binding of acetyltrialanine to Tb^{3+}·elastase in the presence of elastinal. Results of these experiments are included in Table II. Acetyltrialanine also increases the luminescence intensity of Tb^{3+}·PMS-elastase, allowing K_d and I_{max}/I_0 to be measured (Table II).

In view of the effects of acetyltrialanine, several related compounds were examined. Trialanine and acetyltetraalanine, compounds known as competitive inhibitors of elastase, also cause increases in the luminescence intensity of Tb^{3+}·elastase similar to those produced by acetyltrialanine (Table II). Acetyltrialanine methyl ester is a good substrate for elastase (Feinstein et al., 1973). Its addition to Tb^{3+}·elastase ($[S]_0$ = 1.08 mM; $[E]_0$ = 0.34 μM; $[Tb^{3+}]_0$ = 30.6 μM) resulted in a rapid increase in intensity of about twofold, due to the acetyltrialanine released on hydrolysis of the methyl ester. Extrapolation of the intensity back to the time of addition showed that the ester, in contrast to the product acid anion, has no effect on the emission intensity of Tb^{3+}·elastase. When acetyltrialanine methyl ester was added to Tb^{3+}·PMS-elastase ($[S]_0$ = 2.0 mM; $[E]_0$ = 1.28 μM; $[Tb^{3+}]_0$ = 28.6 μM), the initial intensity was unchanged. However, the intensity increased slowly, due to the presence in the PMS-elastase of ~4% active enzyme, causing the release of acetyltrialanine. Acetate ion at 1.92 mM caused a 5% increase in the intensity of Tb^{3+}·elastase (20% at 11 mM).

Determination of K_i for Acetyltrialanine. It was possible to follow the elastase-catalyzed hydrolysis of succinyltrialanine *p*-nitroanilide in 0.05 M Mops buffer, μ = 0.1, pH 6.58, at an elastase concentration very similar to the lowest enzyme concentration at which K_d could be determined by luminescence measurements. Kinetic constants for the hydrolysis of succinyltrialanine *p*-nitroanilide and K_i values for acetyltrialanine were determined in the presence and absence of Tb^{3+} and are listed in Table III.

Table III: Steady-State Kinetic Results for the Elastase–Succinyltrialanine *p*-Nitroanilide–Acetyltrialanine System[a]

$[Tb^{3+}]$ (μM)	$[E]_0$ (μM)	succinyltrialanine *p*-nitroanilide k_{cat} (s^{-1})	succinyltrialanine *p*-nitroanilide K_m (mM)	acetyltrialanine, K_i (mM)
	0.23	15.3 ± 1.0	2.4 ± 0.3	1.6 ± 0.1
30	0.31	12.7 ± 0.5	1.8 ± 0.2	1.4 ± 0.1

[a] At 25 °C in 0.05 M Mops buffer, μ = 0.1, pH 6.58.

Discussion

The excitation spectrum of Tb^{3+}·elastase (λ_{max} = 283 nm; shoulder at 292 nm) resembles closely the absorption spectrum of tryptophan (280 nm; 287 nm) with a small red shift. This confirms that energy transfer occurs from a tryptophan side chain to the bound Tb^{3+}. The emission spectrum shows some fine structure in the major band (540–550 nm) not previously reported for Tb^{3+} bound to a protein and not present in the emission spectra of Tb^{3+}·trypsin and Tb^{3+}·chymotrypsin run under similar conditions (de Jersey, Lahue, and Martin, unpublished experiments). The major band is partly resolved into a sharp peak at 543 nm and a second smaller peak at 547 nm. The magnitude of the enhancement of Tb^{3+} luminescence on binding to proteins is determined by the proximity of the Tb^{3+} to the aromatic group from which energy transfer occurs. In Tb^{3+}·elastase, the enhancement is 1.9×10^4. Trypsin and α-chymotrypsin each bind one Tb^{3+} with an association constant similar to but somewhat less than that measured here for elastase (de Jersey, Lahue, and Martin, unpublished experiments). The enhancements are 1.6×10^3 for trypsin (bovine) and 3.3×10^3 for α-chymotrypsin. X-ray structures have been determined for elastase (Sawyer et al., 1978), trypsin (Bode & Schwager, 1975), and α-chymotrypsin (Birktoft & Blow, 1972), but only in the trypsin case has a specific Ca^{2+} binding site been located in the crystals. By analogy with trypsin, Sawyer et al. (1978) have proposed that the Ca^{2+} in elastase is bound to the γ-carboxylate groups of Glu-70 and Glu-80. The greater enhancement of luminescence in Tb^{3+}·elastase presumably reflects a shorter distance between the bound Tb^{3+} and the tryptophan side chain from which energy transfer occurs (probably Trp-141). A more detailed comparison of the Tb^{3+} complexes of trypsin, α-chymotrypsin, and elastase will be published shortly from this laboratory.

In addition to the significance of the enhancement in terms of the structure of the complex, the large enhancement in Tb^{3+}·elastase has important bonuses in practical terms. Firstly, it permits measurement of metal ion binding at protein concentrations as low as 0.3 μM, several orders of magnitude lower than those required in other physical methods such as NMR, EPR, and equilibrium dialysis. This not only greatly reduces the amount of enzyme required but also reduces the possibility of protein–protein interactions. Secondly, since luminescence intensity varies as r^{-6}, where r is the distance between the energy donor and the bound Tb^{3+} (Martin & Richardson, 1979), the strong enhancement may be a sensitive monitor of even subtle conformational changes occurring at some distance from the Tb^{3+} binding site. Any structural change which alters the distance between the tryptophan side chain and the Tb^{3+} would obviously change the luminescence intensity ($I \propto r^{-6}$). At pH 6.58 with elastase concentrations below 7 μM, clean 1:1 binding of Tb^{3+} to elastase occurs, with $K_T = 3 \times 10^5$ M^{-1} (Table I). The failure to observe simple 1:1 binding of Tb^{3+} at higher enzyme concentrations indicates that considerable caution must be exercised when Tb^{3+} is used as a probe for Ca^{2+} to ensure that 1:1 complex formation is being observed. Dimicoli & Bieth (1977) measured Ca^{2+} and Gd^{3+} binding to elastase by an equilibrium dialysis procedure using $^{45}CaCl_2$ at an elastase concentration of 2×10^{-4} M, obtaining K values

of 2.2×10^4 M^{-1} for Ca^{2+} and 5×10^4 M^{-1} for Gd^{3+} (in 0.05 M acetate buffer, pH 5, at 35 °C). Given the quite different conditions, the agreement between the present results and those of Dimicoli & Bieth (1977) is reasonable and serves to establish strong Ca^{2+} and lanthanide binding to porcine elastase.

The binding constant found for Ca^{2+} in this research is ~90% that for Tb^{3+}, while Zn^{2+} and Mg^{2+} bind only 3 and 1.3% as strongly as Tb^{3+}. These results are consistent with a relatively large binding site (for which Mg^{2+} is too small) composed predominantly of oxygen donors (thus not favoring transition and other metal ions such as Zn^{2+}) only two of which are negatively charged (accounting for the nearly equal binding of similarly sized Ca^{2+} and Tb^{3+}). Under physiological conditions, therefore, elastase seems tailored to bind only Ca^{2+}. The calcium concentration in human pancreatic and duodenal secretions is ~3 mM (Altman & Dittmer, 1961). Since the dissociation constant of Ca^{2+} from elastase is of the order of 0.003 mM, elastase normally occurs as a Ca^{2+}·enzyme.

Acetyltrialanine, acetyltetraalanine, and trialanine cause a maximal increase in the luminescence intensity of Tb^{3+}·elastase of two- to threefold (Table II). The luminescence data are quantitatively explained by the binding of the peptide X to Tb^{3+}·elastase to give the ternary complex Tb^{3+}·E·X. The effect of the binding of X on the intensity of the bound Tb^{3+} could conceivably be achieved in two ways: (1) direct binding of the peptide to the Tb^{3+} (as a ligand, probably via the carboxylate ion, replacing a water molecule) or (2) binding of X at a site remote from the Tb^{3+} (such as the active site) which induces a slight change in the spatial arrangement of the tryptophan side chain engaged in energy transfer to Tb^{3+}.

The three peptides examined are all known to be competitive inhibitors of elastase (Table II), indicating that (2) above is a reasonable proposition. Three lines of evidence indicate that the luminescence effects of the peptides are not due to their binding at the active site of the enzyme. Firstly, K_d for acetyltrialanine is smaller by a factor of ~4 than K_i for acetyltrialanine as a competitive inhibitor of the specific elastase substrate succinyltrialanine *p*-nitroanilide, measured under the same conditions (Tables II and III). This suggests that there are two binding sites for the peptide in Tb^{3+}·elastase. Secondly, binding of acetyltrialanine to Tb^{3+}·elastase is unaffected by the presence of elastinal at concentrations sufficient to ensure that the active site is occupied by elastinal; i.e., acetyltrialanine binds to the E·elastinal complex just as well as to the free enzyme (Table II). Elastinal itself produces a small decrease in the intensity of luminescence of Tb^{3+}·elastase. This effect and the twofold increase caused by acetyltrialanine are strictly additive, again suggesting different binding sites for the two compounds. Thirdly, acetyltrialanine binds to PMS-elastase (in which the active-site serine residue has been modified by the phenylmethanesulfonyl group) just as well as to the native enzyme (Table II).

What then is the nature of the binding site represented by K_d? Since acetyltrialanine methyl ester has no effect on the luminescence of Tb^{3+}·elastase or Tb^{3+}·PMS-elastase, it is reasonable to propose that a free carboxylate ion is required to produce the intensity change and that the compounds causing the intensity increase do so by binding to a positively charged group in the enzyme, possibly the bound Tb^{3+}. The question remains as to how specific is the binding. A simple carboxylate, acetate, produces a small increase in luminescence intensity at a much higher concentration than is required of the alanine peptides. Elastase is known to have an extended active site, with the possibility of forming a considerable number of noncovalent bonds with peptide substrates (Atlas, 1975; Thompson & Blout, 1973). Perhaps the complexes of the alanine peptides with the bound Tb^{3+} are stabilized by noncovalent interactions with portions of the extended active site remote from the active-site serine.

K_d depends on the elastase concentration. Two possible explanations for this dependence are suggested: (1) carboxylate side chains in a second elastase molecule may compete for the bound Tb^{3+} with acetyltrialanine; (2) dimerization of the enzyme may cause an increase in K_d due to steric hindrance of binding of acetyltrialanine in a dimer; cf. the effect of dimerization of α-chymotrypsin on K_i values for competitive inhibitors (Nichol et al., 1972). Binding of acetyltrialanine to Tb^{3+}·PMS-elastase is also dependent on protein concentration (Table II), but to a lesser degree.

Tb^{3+} at concentrations sufficient to saturate the metal ion binding site has no significant effect on k_{cat} and K_m for the elastase-catalyzed hydrolysis of succinyltrialanine *p*-nitroanilide nor on K_i for acetyltrialanine (Table III). Similarly, Dimicoli & Bieth (1977) reported that Ca^{2+} and Gd^{3+} had no effect on the kinetic constants for hydrolysis or inhibition. These results also indicate that the metal ion binding site is unlikely to be close to the active site. While the present work establishes the existence of a second binding site for peptides on elastase (the K_d site) by using luminescence from Tb^{3+} bound at the metal ion binding site as a probe, it does not comment on whether the presence of a bound metal ion is a requirement for binding to the K_d site.

Our results emphasize the need for great caution in interpretation of data on enzyme systems obtained by physical methods using probes such as the one used here. Dimicoli & Bieth (1977) studied the ^{19}F NMR of the ternary complex elastase–(trifluoroacetyl)trialanine–Gd^{3+}, determining the effect of the Gd^{3+} ion on the relaxation times of the fluorine nuclei. The data were interpreted as indicating a distance of ~20 Å between the fluorine nuclei and the Gd^{3+} ion. Our results on the binding of acetyltrialanine to Tb^{3+}·elastase raise the possibility that (trifluoroacetyl)trialanine binds to the K_d site of Gd^{3+}·elastase. Indeed, if the carboxylate group of the peptide were liganded to the Gd^{3+}, the distance between the fluorine nuclei and the Gd^{3+} could approach 20 Å. However, additional experiments by Dimicoli and his co-workers seem to rule out this possiblity [Dimicoli et al. (1979) and references therein]. For example, their NMR and kinetic results indicate that the trifluoroacetyl peptides and their derivatives bind in a unique manner to elastase, that this binding is greatly strengthened by the trifluorocetyl group (vis-à-vis the acetyl group in acetyltrialanine), and that the binding is unaffected by Gd^{3+}. In studies with trifluoroacetyl peptide chloromethyl ketones, they have shown the existence of two binding sites: a nonproductive site and a productive site leading to covalent modification. These two sites probably differ from the two sites found in the present study in being exclusive rather than independent; i.e., binding at the productive (active) site prevents binding at the nonproductive site and vice versa. The much higher elastase concentration used in the NMR experiments than in the luminescence experiments should also be considered.

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Na^+,K^+-ATPase: Ligand-Induced Conformational Transitions and Alterations in Subunit Interactions Evidenced by Cross-Linking Studies†

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ABSTRACT: The membrane-bound Na^+,K^+-ATPase is an oligomer containing several α subunits (catalytic subunits) and several β subunits of unknown function. To obtain information on subunit interactions, we studied the effects of the enzyme's physiological ligands on the cross-linking of the α subunits in the presence of *o*-phenanthroline and Cu^{2+}. Two distinct cross-linking patterns were observed depending on the concentrations of the cross-linking reagents. (1) In the presence of 0.25 mM Cu^{2+} and 1.25 mM *o*-phenanthroline, cross-linking did not occur unless ATP was added. This ATP-induced formation of the cross-linked α,α dimer was stimulated by Na^+ and inhibited by K^+. The half-maximal effect was obtained at 2–5 μM ATP. Na^+ + ATP dependent dimer formation was accompanied by Na^+-dependent phosphorylation of the enzyme. The steady-state level of phosphoenzyme was attained within 10 s, while the level of the α,α dimer continued to rise up to 5 min. The formation of phosphorylated dimer and the conversion of this to unphosphorylated dimer by K^+ were demonstrated. ADP and the β,γ-methylene analogue of ATP did not induce dimer formation. The exposure of a set of sulfhydryl groups upon phosphorylation of α subunit and subsequent dimerization are suggested by the data. (2) In the presence of 0.25 mM Cu^{2+} and 0.5 mM *o*-phenanthroline, a cross-linked α,α dimer was obtained in the absence of Na^+, K^+, and ATP and under all ligand conditions except when K^+ + ATP was added. The half-maximal inhibitory effect of ATP on dimer formation in the presence of K^+ was obtained at ~0.2 mM ATP. In the presence of K^+, ADP and the β,γ-methylene analogue of ATP also inhibited dimer formation. These data suggest the existence of a conformational state of the α subunit with bound K^+ and ATP (at a low-affinity site) and with a set of occluded sulfhydryl groups. When either ligand is removed, the groups are exposed and dimerization occurs. Because the conformational transitions detected by the cross-linking studies are known to be closely related to Na^+ and K^+ translocation steps, the findings suggest that alterations in subunit interactions at a domain of two α subunits regulate the transport function of the enzyme.

The active transports of Na^+ and K^+ across the plasma membranes of most eucaryotic cells are carried out by Na^+,K^+-ATPase.[1] The early studies on the complex kinetics of the reactions catalyzed by the enzyme (Robinson, 1967) and on the different reactive states of the phosphorylated enzyme (Albers, 1967; Post et al., 1969) suggested the occurrence of a variety of ligand-induced conformational transitions of the enzyme, and this has been supported by a multitude of subsequent studies along similar lines (Glynn & Karlish, 1975; Albers, 1976). More direct evidence for such transitions has been obtained with the aid of several conformational probes (Hart & Titus, 1973; Jorgensen, 1975; Karlish et al., 1978; Karlish & Yates, 1978). Examination of this literature indicates, as expected, that the conformational states revealed by one probe may not be distinguished by another. It is clear, therefore, that if the correlation of the various conformational transitions with the transport function of the enzyme is to be attempted, different probes must be used.

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[1] Abbreviations used: Na^+,K^+-ATPase, sodium- plus potassium-dependent adenosinetriphosphatase (EC 3.6.1.3); Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; NaDodSO$_4$, sodium dodecyl sulfate.

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