# Binding of Terbium to Porcine Pancreatic Elastase. Ligand-Induced Changes in the Stability, the Maximum Luminescence Intensity, and the Circularly Polarized Luminescence Spectrum of the Complex†

Guy Duportail, Jean-Francois Lefevre, Patrick Lestienne, Jean-Luc Dimicoli, and Joseph G. Bieth*

ABSTRACT: The single $Ca^{2+}$ binding site on porcine pancreatic elastase [Dimicoli, J. L., & Bieth, J. (1977) *Biochemistry 16*, 5532] has been investigated by $Tb^{3+}$ luminescence measurements. This technique allowed us to determine the binding constant, $K_a$, of $Tb^{3+}$ to elastase under a variety of conditions. In addition, the maximum luminescence intensity ($L_m$) and the circularly polarized luminescence (CPL) measurements were used to detect conformational changes of elastase induced by various ligands. $K_a$ increases with pH, and this increase is correlated with the ionization of one group having a p$K$ of 6.4 and of other groups having larger p$K$ values. Below pH 5.0 and above pH 7.0, elastase undergoes important conformational alterations evidenced by $I_m$ and CPL measurements. The binding of $Tb^{3+}$ to elastase is entropy driven ($\Delta H$ = +10.7 kcal mol$^{-1}$ and $\Delta S$ = +56 eu). The conformation of elastase does not change with temperature between 5 and 35 °C. Turkey ovomucoid, human plasma $\alpha_1$-proteinase inhibitor, and $\alpha_2$-macroglobulin did not prevent the binding of $Tb^{3+}$ to elastase. The two latter proteins induce important conformational changes of elastase. After reaction with the irreversible inhibitor Boc-$Ala_4$-CMK, elastase still binds $Tb^{3+}$ with the same affinity as does free elastase. The reversible inhibitors $Ala_3$, $CH_3CO$-$Ala_3$, and $CF_3CO$-$Ala_3$ increased $K_a$ at pH 6.5 and decreased it at pH 8.1. The two former compounds induce very important conformational changes in the elastase molecule.

The pancreatic proteases trypsin and chymotrypsin as well as their zymogens are known to bind $Ca^{2+}$ ions (Epstein et al., 1974; Osborne et al., 1972). We have recently shown that porcine pancreatic elastase shares this property in binding a single $Ca^{2+}$ ion with an association constant of $2 \times 10^4$ M$^{-1}$ at pH 5.0 (Dimicoli & Bieth, 1977). Moreover, $Gd^{3+}$ competes with $Ca^{2+}$ for the binding site of elastase. We used the former ion as a paramagnetic probe to determine the distance between the ion binding site and the fluorines of a trifluoroacetylated oligopeptidic inhibitor. This led us to conclude that the metal ion binding site of elastase is composed of Glu-70 and Glu-80 (chymotrypsin sequence numbering system), i.e., the same residues as those of trypsin (Epstein et al., 1977).

Independently, Brittain et al. (1976) showed that $Tb^{3+}$ binds to elastase and so provides a luminescent probe for investigating the metal–enzyme interaction. These authors found that the emitted luminescence resulted from an energy transfer between a tryptophan residue and $Tb^{3+}$. Moreover, they reported that $Tb^{3+}$–elastase exhibits a clear-cut circularly polarized luminescence (CPL)[1] spectrum. Recording of such spectra as well as the maximal luminescence intensity ($L_m$) measurements provides information on the structural characteristics of the metal ion binding site and hence on the overall conformation of the protein bearing this site.

The conformation of elastase has been tested by intrinsic fluorescence (Wasi & Hofmann, 1968), differential spectroscopy (Jori et al., 1973), circular dichroism (Gorbunoff & Timasheff, 1972; Karibian et al., 1974), and optical rotatory dispersion (Kaplan et al., 1973; Karibian et al., 1974). These investigations were mainly focused on the influence of pH on elastase conformation. Very little is known about conformational changes induced by small peptides (Shotton et al., 1971), and the literature lacks information concerning structural changes induced by macromolecular elastase inhibitors.

This investigation extends our previous study of the metal ion binding site of elastase and is specifically aimed at probing the effect of pH, temperature, and low and high molecular weight ligands on the elastase conformation.

## Materials and Methods

Porcine pancreatic elastase and human plasma $\alpha_2$-macroglobulin were isolated as described previously (Dimicoli & Bieth, 1977; Meyer et al., 1975). Turkey ovomucoid isolated by the method of Feeney et al. (1967) was a gift of Dr. Gertler, University of Jerusalem, Israel. Human plasma $\alpha_1$-proteinase inhibitor isolated by the method of Pannell et al. (1974) was a gift of Dr. Travis, University of Georgia. Boc-$Ala_4$-CMK was synthetized as described previously (Dimicoli et al., 1979). $Ala_3$ and $CH_3CO$-$Ala_3$ came from Sigma, and $CF_3CO$-$Ala_3$ was prepared as described previously (Dimicoli et al., 1976).

Anhydrous $TbCl_3$ specially prepared for ion implantation came from Koch-Light. Water was purified by using the "Q system" of Millipore which yields water containing less than $10^{-12}$ M $Ca^{2+}$.

Irreversibly inhibited elastase was prepared as follows. Boc-$Ala_4$-CMK was dissolved in dimethylformamide, and 1 vol % of this solution was added to elastase dissolved in barbital–acetate buffer of appropriate pH (see below). The final concentrations were elastase = 5.5 $\mu$M and inhibitor = 50 $\mu$M. After 15 h at room temperature, the solutions were tested for residual enzymatic activity (Bieth et al., 1974). In all cases the inhibition was complete. These solutions were used directly for $Tb^{3+}$ binding experiments.

*Fluorescence measurements* were performed with a FICA Model 55 spectrofluorometer. The excitation and emission

† From the Laboratoire de Physique, ERA 551 du CNRS (G.D. and J.-F.L.), and the Laboratoire d'Enzymologie, FRA 47 de l'INSERM (P.L. and J.G.B.), Faculté de Pharmacie, Université Louis Pasteur B.P. 10, 67048 Strasbourg, France, and the Institut Curie, 91405 Orsay, France (J.-L.D.). *Received October 11, 1979.* This work was supported by grants from CNRS (ATP "Structures Chirales 77") and INSERM (CRL 77.1.086.3).

[1] Abbreviations used: CPL, circularly polarized luminescence; $L_m$, maximum luminescence intensity (i.e., luminescence intensity at saturating $Tb^{3+}$ concentration); Pipes, 1,4-piperazinediethanesulfonic acid; Boc, *tert*-butoxycarbonyl; CMK, chloromethyl ketone.

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Table I: Effect of Some Proteinase Inhibitors on the Association Constant ($K_a$) and the Maximum Luminescence Intensity ($L_m$) of the Elastase-$Tb^{3+}$ Complex

| proteinase inhibitors | pH | ionic strength | $K_a$ of elastase ($M^{-1} \times 10^{-4}$) | $K_a$ of complex ($M^{-1} \times 10^{-4}$) | $L_m$ elastase/$L_m$ complex |
|---|---|---|---|---|---|
| $\alpha_1$-proteinase inhibitor | 6.3[a] | 0.1 | 3.6 | 2.9 | 1.8 |
| $\alpha_1$-proteinase inhibitor | 6.3[a] | 0.2 | 2.5 | 1.8 | 2.0 |
| $\alpha_2$-macroglobulin | 6.5[b] | 0.05 | 29 | 2.7 | 0.33 |
| ovomucoid | 7.0[b] | 0.05 | 33 | 28 | 0.82 |

[a] 1 volume of 0.05 M Tris-HCl, pH 8.0 (the solvent of $\alpha_1$-proteinase inhibitor), + 3 volumes of 0.05 M acetate + NaCl to obtain the desired ionic strength. [b] Barbital-acetate buffer as in the legend to Figure 1.

wavelengths were 290 and 544 nm, respectively (Brittain et al., 1976). Elastase was dissolved in buffer at a concentration of 5 to 6 $\mu$M. The luminescence was measured after successive additions of very small aliquots of $TbCl_3$ dissolved in the same buffer (but see also legend to Figure 1). Fresh stock solutions of $TbCl_3$ were prepared daily because precipitation occurs even at pH <7.0. When the volume of added $TbCl_3$ solution was not negligible with respect to the total volume, the luminescence intensity was corrected to account for the dilution. The corrections were usually small. The intrinsic fluorescence of elastase was small and was substracted from the total luminescence.

The protein proteinase inhibitors were dissolved in the same buffers as elastase (see Table I). Elastase and inhibitor solutions were mixed in a way such that the molar ratio of inhibitor to enzyme was 1. The luminescence intensity was corrected in order to take into account the screen effect due to the absorption of the inhibitors (Burstein, 1968)

$$L_{cor} = L_{obsd}\frac{A_e + A_i}{A_e} \times \frac{1 - 10^{-A_e}}{1 - 10^{-(A_e+A_i)}}$$

where $L$ is the total luminescence and $A_e$ and $A_i$ are the absorbances of elastase and inhibitor, respectively. In order to minimize this correction, we used 2-mm optical path cuvettes.

*Circularly polarized luminescence* (CPL) was measured with an instrument constructed in our laboratory according to the general principle described by Steinberg & Gafni (1972). The main modifications were in data acquisition, which was performed with a minicomputer (computer automation, 16K words). The light source was a 600-W xenon–mercury lamp. The wavelength of excitation was 295 nm and was selected by a Bausch & Lomb high-intensity monochromator with a bandwidth of 0.5 nm. The wavelengths of fluorescence emission were selected by a Jobin-Yvon monochromator (H20 UV model) with a bandwidth of 0.5 nm. A Schott UG II glass filter and a 2 M $NaNO_2$ solution in a 1-cm cell were introduced to cut off stray light with a wavelength above 360 nm in the exciting beam and with a wavelength shorter than 390 nm in the fluorescence beam, respectively.

The emission anisotropy factor is defined as (Steinberg & Gafni, 1972)

$$g_{em} = \frac{I_L - I_R}{I_L + I_R} = \frac{2\Delta I}{I}$$

where $I_L$ and $I_R$ are the observed intensities of left and right circularly polarized emission, respectively. The reported values of $g_{em}$ are accurate within the limits of ±10%.

All other experimental details may be found under Results.

## Results

*Measurement of the Association Constant of the Elastase–$Tb^{3+}$ Complex.* In agreement with previous studies (Brittain et al., 1976), we found that the luminescence of $Tb^{3+}$ is considerably enhanced by elastase. Experiments performed with varying excitation wavelengths confirmed also that energy transfer occurs through a tryptophan residue. The luminescence intensities ($L$) of mixtures of constant amounts of elastase and increasing amounts of $Tb^{3+}$ were used to determine the association constant ($K_a$) of $Tb^{3+}$–elastase under a variety of conditions. When $K_a$ was sufficiently low ($K_a \leq 2 \times 10^4$ $M^{-1}$), double-reciprocal plots according to

$$\frac{1}{L} = \frac{1}{L_m} + \frac{1}{L_m K_a[Tb^{3+}\ \text{total}]} \qquad (1)$$

were used where $L_m$ is the maximal luminescence intensity. Equation 1 assumes that there is only one $Tb^{3+}$ binding site on elastase. This assumption is valid, as will be shown later. When the binding was tighter, Scatchard plots were used. The concentrations of elastase–$Tb^{3+}$ complex were determined from the experimentally measured $L$ and $L_m$ values and the known concentration of elastase by assuming again that the stoichiometry is 1:1. The error on $K_a$ is about ±10%.

Addition of excess $Ca^{2+}$ to a mixture of elastase and $Tb^{3+}$ resulted in a complete quenching of the luminescence. This is in accord with the competition noticed previously between $Ca^{2+}$ and another lanthanide, $Gd^{3+}$ (Dimicoli & Bieth, 1977). This demonstrates that the three metal ions bind at a single and same site.

In order to check the validity of our method of determination of $K_a$, we have measured the association constant of $Ca^{2+}$–elastase by competition with $Tb^{3+}$. Let $L_1$ be the luminescence of a $Tb^{3+}$–elastase mixture and let $L_2$ be the luminescence of the same mixture after addition of a quantity of $Ca^{2+}$ equimolar to that of $Tb^{3+}$. Under the condition where the concentration of bound ions is negligible with respect to the concentration of total ions, it may be shown that $K_a(Tb^{3+})/K_a(Ca^{2+}) = L_2/(L_1 - L_2)$. The mean value of this ratio obtained by using three different concentrations of ions was 1.6 in 0.05 M acetate buffer, pH 5.0, at 32 °C, and the values of $K_a$ ($Tb^{3+}$) and $K_a$ ($Ca^{2+}$) were $3.3 \times 10^4$ and $2 \times 10^4$ $M^{-1}$, respectively. The latter result compares favorably with the value of $2.2 \times 10^4$ $M^{-1}$ obtained previously under nearly identical conditions (Dimicoli & Bieth, 1977).

*Effect of pH and Temperature on $K_a$, $L_m$, and CPL of $Tb^{3+}$–Elastase.* The effect of pH on $K_a$ was studied with a buffer containing the same ions (barbital and acetate) at any pH in order to avoid nonspecific $Tb^{3+}$ ion interactions. The results are shown in Figure 1. $K_a$ increases strongly with pH up to pH 6.5. Its value levels off then up to pH 7.5 and increases again at pH 8.1, where it was so high that no precise measurements could be made ($K_a > 2 \times 10^7$ $M^{-1}$). The slope of the line relating log $K_a$ to pH is unity, suggesting the participation of a single group with an apparent p$K$ of 6.4. The fact that $K_a$ is higher at pH 8.1 than at pH 7.5 suggests, however, the involvement of another ionizing group.

The maximum luminescence intensity of the $Tb^{3+}$–elastase complex decreases strongly below pH 5.0 and above pH 7.0, suggesting that conformational changes of elastase occur outside these limits of pH (Figure 1). This was confirmed by recording CPL spectra of $Tb^{3+}$–elastase at various pH values (see, for instance, Figure 2). Between pH 5.0 and 7.0, the three $g_{em}$ values did not change significantly. However, at pH 4.0 the CPL spectrum was almost flat and at pH 8.1 it was considerably altered (see Table III).

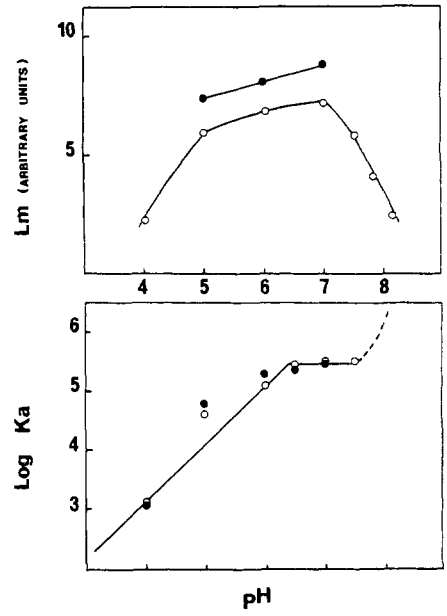


FIGURE 1: pH dependency of $K_a$ and $L_m$ of the $Tb^{3+}$–elastase complex at 25 °C. The buffers were mixtures of 5 mM sodium barbital, 5 mM sodium acetate, and 40 mM sodium chloride. For pH ≥7, the stock solutions of $TbCl_3$ were prepared in the pH 6.5 buffer. (○) Free elastase; (●) elastase inactivated with Boc-$Ala_4$-CMK.

The van't Hoff plot shown in Figure 3 indicates that the binding of $Tb^{3+}$ to elastase is endothermic, as already noticed for the binding of $Ca^{2+}$ (Dimicoli & Bieth, 1977). The following thermodynamic quantities were found: $\Delta H = +10.7$ kcal $mol^{-1}$ and $\Delta S = +56$ eu. The $g_{em}$ values of the CPL spectra did not change significantly between 5 and 35 °C.

*Influence of Protein Proteinase Inhibitors on $K_a$, $L_m$, and CPL of $Tb^{3+}$–Elastase.* Turkey ovomucoid, human plasma $\alpha_1$-proteinase inhibitor, and $\alpha_2$-macroglobulin are proteins known to form very tight complexes with elastase (Gertler & Feinstein, 1971; Pannell et al., 1974; Bieth et al., 1970). Under our experimental conditions (elastase = 6 μM) the 1:1 complexes are undissociated. None of these proteins was able to prevent the binding of $Tb^{3+}$. Moreover, $\alpha_1$-proteinase inhibitor and ovomucoid did not change significantly the association constant (Table I). The former protein alters, however, to some extent the conformation of elastase since it decreases $L_m$ by a factor of 2. The most dramatic effects are observed with $\alpha_2$-macroglobulin which decreases the binding constant by a factor of 10 and increases $L_m$ by a factor of 3 (see Figure 4).[2]

The above conformational changes of elastase were confirmed by CPL spectral recordings. Figure 2 shows that $\alpha_2$-macroglobulin and $\alpha_1$-proteinase inhibitor markedly change the shape of the CPL spectrum whereas ovomucoid produces only small effects.

*Influence of Synthetic Elastase Inhibitors on $K_a$, $L_m$, and CPL of $Tb^{3+}$–Elastase.* The irreversible complex formed by reacting elastase with Boc-$Ala_4$-CMK had about the same affinity as free elastase for $Tb^{3+}$ at various pH values (Figure 1). This inhibitor induces probably a slight conformational change of elastase since it increases $L_m$ by 20–30% between pH 5.0 and 7.0. However, it does not change significantly the CPL spectrum of $Tb^{3+}$–elastase.

Table II: Effect of Synthetic Elastase Inhibitors on the Association Constant ($K_a$) and the Maximum Luminescence Intensity ($L_m$) of the Elastase–$Tb^{3+}$ Complex at 25 °C

| inhibitors | $K_a$ ($M^{-1} \times 10^{-4}$) at pH 6.5[a] | $L_m$ elastase/$L_m$ complex pH 6.5[a] | $L_m$ elastase/$L_m$ complex pH 8.1[a] |
|---|---|---|---|
| none | 4.4 | | |
| $Ala_3$ (133 mM) | 8.0 | 0.52 | 1.25 |
| $CH_3CO$-$Ala_3$ (20 mM) | 7.0 | 0.50 | 0.80 |
| $CF_3CO$-$Ala_3$ (0.2 mM) | 7.0 | 0.66 | 1.00 |

[a] 10 mM Pipes buffer; ionic strength was adjusted to 0.1 with KCl except for $Ala_3$ where it was adjusted to 0.2. The pH was brought to the desired value with HCl after dissolution of the inhibitors.

The effect of three reversible inhibitors of increasing potency was also investigated (Table II). To ensure maximal saturation of elastase, these compounds were used at concentrations $\simeq 20K_i$. At pH 6.5 the inhibitors increased both $K_a$ and $L_m$. At pH 8.1, $L_m$ did not vary significantly but the $Tb^{3+}$–elastase affinity decreased strongly. However, no precise measurements of $K_a$ could be made due to the rapid formation of $Tb(OH)_3$.

Among the three inhibitors, only $Ala_3$ and $CH_3CO$-$Ala_3$ change significantly the emission anisotropy factors of $Tb^{3+}$–elastase (Table III). The most important effects of these two inhibitors are on the 538-nm emission band. None of the other ligands tested in this investigation produced such dramatic effects. It is also worthwhile to notice that $Ala_3$ and $CH_3CO$-$Ala_3$ seem to prevent the important conformational change of elastase which occurs at pH 8.1.

## Discussion

*Metal Ion Binding Site of Elastase.* Although at least two residues of the metal ion binding sites of porcine trypsin and elastase are probably identical (i.e., Glu-70 and Glu-80), the two metal ion binding sites must be composed of different subsites or have different conformations since they bind metal ions with considerably different affinities. For instance, the $K_a$ of $Tb^{3+}$–trypsin is $8.8 \times 10^2$ $M^{-1}$ at pH 6.3, 25 °C (Epstein et al., 1974) whereas that of $Tb^{3+}$–elastase is $2.9 \times 10^5$ $M^{-1}$ at pH 6.5, 25 °C. The above view is strengthened by the fact that trypsin binds $Ca^{2+}$, $Gd^{3+}$, and $Tb^{3+}$ with widely different affinities (Epstein et al., 1974) whereas elastase binds these cations with very similar constants. The two structurally related proteases share, however, a common property: their binding of lanthanides is accompanied by an unfavorable positive enthalpy change which is overcompensated by a large positive entropy difference (Epstein et al., 1977). This strongly suggests that in both cases the ion–protein interaction is highly specific and involves multiple nonionic interactions in addition to electrostatic ones [see also Stroud et al. (1971)].

The pH dependency of $K_a$ shows that the binding of $Tb^{3+}$ to elastase is controlled by an ionizing group with an apparent p$K$ of 6.4. This value is similar to that found by others for the chymotrypsinogen–$Ca^{2+}$ system (i.e., p$K$ = 7.0) (Osborne et al., 1972). The group which ionizes with a p$K$ of 6.4 is certainly not one of the two Glu residues of the metal binding site since such a high p$K$ would imply that the carboxylates lie in a highly hydrophobic environment, a fact which is contradicted by the available X-ray data (Shotton & Hartley, 1970). The observed increase of $K_a$ with pH might result from an alteration of the ion binding site of elastase induced by a neighboring ionizing group.

[2] The luminescence of $Tb^{3+}$ is enhanced by $\alpha_2$-macroglobulin alone, suggesting the formation of a $Tb^{3+}$–macroglobulin complex. This luminescence was substracted from the total luminescence of the ternary $Tb^{3+}$–elastase–macroglobulin complex (Figure 4a). The Scatchard plot was constructed by using these corrected values (Figure 4b). When the $Tb^{3+}$ binding experiment was repeated with a twofold molar excess of $\alpha_2$-macroglobulin over elastase and the appropriate corrections were made, the $K_a$ value did not change.

Table III: Effect of Synthetic Elastase Inhibitors on the Emission Anisotropy Factor ($g_{em}$) of $Tb^{3+}$-Elastase at 25 °C

| inhibitors | $g_{em}$ at 538 nm × $10^3$ | | $g_{em}$ at 544 nm × $10^3$ | | $g_{em}$ at 548 nm × $10^3$ | |
|---|---|---|---|---|---|---|
| | pH 6.5 | pH 8.1 | pH 6.5 | pH 8.1 | pH 6.5 | pH 8.1 |
| none | −14.4 | −4.8 | +17.6 | +28.0 | −14.0 | −15.9 |
| $Ala_3$ (133 mM) | −27.0 | −25.6 | +24.8 | +21.5 | −17.0 | −12.9 |
| $CH_3CO$-$Ala_3$ (20 mM) | −30.0 | −15.4 | +24.8 | +16.3 | −16.2 | −12.9 |
| $CF_3CO$-$Ala_3$ (0.2 mM) | −19.2 | −3.9 | +14.3 | +20.8 | −14.9 | −14.2 |
| Boc-$Ala_4$-CMK[a] | −13.0 | | +17.8 | | −14.0 | |

[a] Irreversible Boc-$Ala_4$-elastase complex was prepared as described under Materials and Methods.

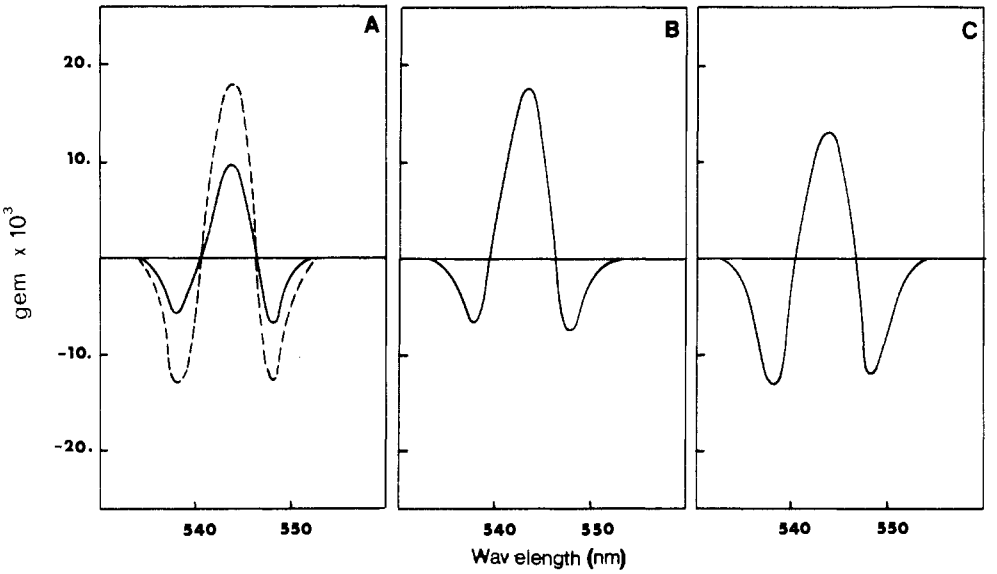


FIGURE 2: CPL spectra of $Tb^{3+}$–elastase (---) nd $Tb^{3+}$–elastase complexed with protein proteinase inhibitors (—). (A) $\alpha_1$-Proteinase inhibitor; (B) $\alpha_2$-macroglobulin; (C) ovomucoid. The s ectrum obtained in the presence of $\alpha_2$-macroglobulin has been corrected for the luminescence due to the binding of $Tb^{3+}$ to $\alpha_2$-macroglobulin (13% of total luminescence intensity) and for the intrinsic fluorescence of $\alpha_2$-macroglobulin (9% of total luminescence intensity).

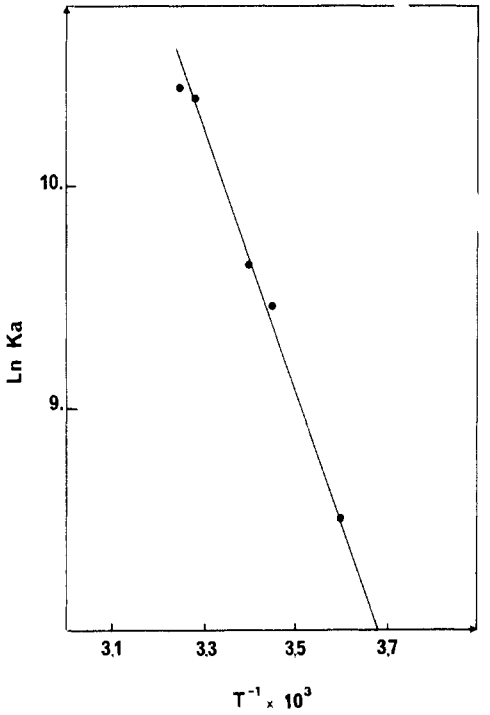


FIGURE 3: Temperature dependency of the association constant $K_a$ of the $Tb^{3+}$–elastase complex at pH 5.0 (50 mM acetate buffer).

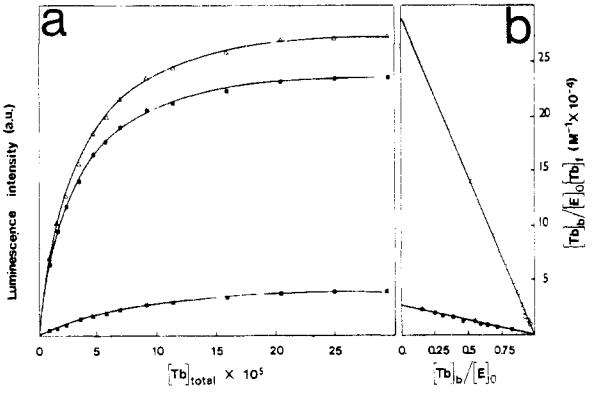


FIGURE 4: (a) Binding of $Tb^{3+}$ to the $\alpha_2$-macroglobulin–elastase complex (Δ) and to $\alpha_2$-macroglobulin alone (■). The corrected curve (●) was constructed by substracting the luminescence data of curve ■ from those of curve Δ. Conditions: pH 6.5; ionic strength = 0.05; 25 °C; elastase = $\alpha_2$-macroglobulin = 4 μM. (b) Scatchard plots for the association of $Tb^{3+}$ with free (○) and $\alpha_2$-macroglobulin-bound elastase (●).

None of ligands used in this study was able to prevent the binding of $Tb^{3+}$ to elastase. Except for $\alpha_2$-macroglobulin, all of these compounds bind at the active center of elastase. This confirms that the metal ion binding site is remote from the active site (Dimicoli & Bieth, 1977). $\alpha_2$-Macroglobulin does not bind at the active center of elastase (Bieth et al., 1970). Nevertheless, it does not prevent the binding of $Tb^{3+}$ but the association constant is 10-fold lower than for free elastase. This decrease in affinity results probably from the important change of the elastase conformation induced by the macroglobulin (Figure 2). The structures of the binding sites of the two elastase molecules bound to $\alpha_2$-macroglobulin (Bieth et al., 1970) must be very similar since no abnormality was detected in the $Tb^{3+}$ saturation curve.

*Conformation of $Tb^{3+}$–Elastase.* The observed luminescence of the complex is due to an energy transfer from a tryptophan residue of elastase to the bound $Tb^{3+}$ ion. For this transfer to occur, the donor and acceptor must be in very close contact. As a consequence, small variations in their distance will change the value of $L_m$. Variations of $L_m$ thus reflect conformational changes of elastase. Another sensitive conformational parameter is of course the CPL spectrum. The shape of this spectrum (Figure 2) is similar but not identical with that depicted previously (Brittain et al., 1976). The differences between our $g_{em}$ values and those of Brittain et al. must be ascribed to the use of different bandwidths for the monochromators. In addition, we wish to point out that the spectrum of $Tb^{3+}$–elastase is simpler than that of $Tb^{3+}$–trypsin in which three positive and two negative peaks have been reported (Epstein et al., 1977). Changes of $L_m$ are usually paralleled by changes of one or several anisotropy factors except for elastase saturated with $CF_3CO$-$Ala_3$ or irreversibly inhibited by Boc-$Ala_4$-CMK where $L_m$ increases whereas the anisotropy factors remain unchanged. One may thus conclude that at least for elastase $L_m$ is a more sensitive parameter to detect conformational changes than CPL spectra.

The conformational change of elastase at pH 4.0 (Figure 1) is probably the same as that detected by other physical techniques and which is accompanied by an irreversible denaturation of the enzyme (Wasi & Hofmann, 1968; Jori et al., 1973). At pH 8.1, another important conformational change is evident (Table III). Previous circular dichroism experiments were unable to detect a transconformation of elastase in the pH range 5.0–8.5 (Gorbunoff & Timasheff, 1972; Karibian et al., 1974). However, at pH 10.6, a small conformational change in elastase could be detected and was attributed to the disruption of the ion pair formed between N-terminal Val ($pK$ = 9.7) and Asp-194 (Karibian et al., 1974). This view is supported by the fact that in chymotrypsin the deprotonation of the N-terminal Ile residue ($pK$ = 8.5) and hence the disruption of the salt bridge are accompanied by an important conformational change in the enzyme (Hess, 1971). At first sight, the transconformation of elastase which we have diagnosed at pH 8.1 or even at pH 7.7 (Figure 1) does not seem to be governed by the N-terminal Val since at pH 7.7 and 8.1 this residue is still 99 and 97.5% protonated. We are nevertheless tempted to ascribe this transconformation to the deprotonation of Val since the reversible inhibitors $Ala_3$ and $CH_3CO$-$Ala_3$ seem to prevent this structural change and since a similar hindrance of pH-induced transconformation has been found with chymotrypsin substrates or inhibitors (Hess, 1971; Garel & Labouesse, 1970).

There are many reports on conformational changes of trypsin or chymotrypsin induced by low molecular weight substrates or inhibitors (Orr & Elmore, 1977; Brittain et al., 1976; Garel & Labouesse, 1970; Garel, 1975) but very little is known about such effects in elastase. X-ray crystallographic studies have demonstrated that the binding of $Ala_3$ to elastase is accompanied by a discrete structural modification of the active center of the enzyme (Shotton et al., 1971). This modification propagates probably far beyond the active site since $Ala_3$ induces changes of $L_m$, $K_a$ (Table II), and anisotropy factors (Table III). The increases of $L_m$ and $g_{em}$ produced by $Ala_3$ are in agreement with previous results (Brittain et al., 1976). We could, however, not duplicate the report that $Ala_3$ and $CH_3CO$-$Ala_3$ quench the luminescence at pH 8.1 (Brittain et al., 1976).

The substrate (inhibitor) binding site of porcine elastase is composed of at least seven subsites numbered $S_5$ to $S_{2'}$ (Atlas, 1975). $Ala_3$, which brings about an important conformational change, occupies subsites $S_3$ to $S_1$ (Shotton et al., 1971). It is interesting to note that the occupancy of one more subsite ($CH_3CO$-$Ala_3$–elastase complex) does not hinder this conformational alteration whereas the occupancy of two more subsites (Boc-$Ala_4$–elastase complex) prevents it almost completely (Figure 1 and Tables II and III). $CF_3CO$-$Ala_3$ is 100 times more potent an inhibitor than $CH_3CO$-$Ala_3$ (Dimicoli et al., 1976). In addition, recent studies have indicated that $CF_3CO$-peptides do not bind at the classical subsites of the active center of elastase (Dimicoli et al., 1979). The findings of the present investigation confirm that $CF_3CO$-$Ala_3$ behaves differently from $CH_3CO$-$Ala_3$ since it does not significantly change the anisotropy factors of $Tb^{3+}$–elastase (Table III).

Of the three protein proteinase inhibitors tested in the present study, only plasma $\alpha_1$-proteinase inhibitor and $\alpha_2$-macroglobulin produced important changes of elastase conformation. The negative effect of turkey ovomucoid may be compared to the negative effect of chicken ovomucoid on the conformation of trypsin (Brittain et al., 1976). Circular dichroism indicates protein transconformation upon complex formation between $\alpha_1$-proteinase inhibitor and proteases (Saklatvala et al., 1976). The question as to which one of the two proteins changes in conformation, however, could not be answered by this technique. The present study shows that, at least, the protease is involved. It is interesting to notice that $\alpha_1$-proteinase inhibitor and $\alpha_2$-macroglobulin, which bind elastase at different sites (only the former protein blocks the active center of the enzyme), also induce different conformational changes since the former protein decreases $L_m$ while the latter increases it. This is the first report of an $\alpha_2$-macroglobulin-induced transconformation of a protease. This structural change may account for the change of the enzyme's kinetic parameter upon binding of the macromolecule.

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# In Vivo Replication of Hepatic Deoxyribonucleic Acid of Rats Treated with Dimethylnitrosamine: Presence of Dimethylnitrosamine-Induced $O^6$-Methylguanine, $N^7$-Methylguanine, and $N^3$-Methyladenine in the Replicated Hybrid Deoxyribonucleic Acid†

Samuel E. Abanobi, Amedeo Columbano,‡ Richard A. Mulivor,§ Srinivasan Rajalakshmi, and Dittakavi S. R. Sarma*

ABSTRACT: Experiments were designed to determine whether some chemical lesions such as $O^6$-methylguanine, $N^7$-methylguanine, and $N^3$-methyladenine induced in rat liver DNA by the hepatocarcinogen dimethylnitrosamine permit replication in vivo. For this purpose, [$^{14}$C]dimethylnitrosamine was administered to methylate the parental strand of liver DNA. Four hours later, a time period when the carcinogen cannot be detected in either the liver or the blood, rats were subjected to partial hepatectomy in order to induce DNA replication. During the S phase, 5-bromo-2-deoxyuridine was administered to render the newly made strands heavy. The rebanded, hybrid, hepatic DNA of density 1.714 g/cm$^3$ and greater was pooled from the neutral cesium chloride gradient, dialyzed, and lyophilized. The hybrid DNA was then treated with $S_1$ nuclease to digest any single-stranded regions. The results obtained indicated the presence of $O^6$-methylguanine, $N^7$-methylguanine, and $N^3$-methyladenine in $S_1$ nuclease resistant, hybrid DNA. The results are interpreted to indicate that these chemical lesions permitted in vivo DNA replication.

Cell proliferation has often been implicated in cell transformation and the development of preneoplastic and neoplastic lesions with chemicals, radiation, and viruses (Todaro & Green, 1966; Borek & Sachs, 1968; Pound, 1968; Warwick, 1971; Rajewsky, 1972; Kakunaga, 1974; Rajalakshmi & Sarma, 1975; Craddock, 1976; Cayama et al., 1978; Ying & Sarma, 1979). Although the mechanism by which cell proliferation stimulates the induction of preneoplastic and neoplastic cell populations is not known, replication of carcinogen-damaged DNA prior to repair offers an attractive mechanism by which carcinogen-induced lesions in DNA can become fixed in the newly made DNA (Rajalakshmi & Sarma, 1975; Sarma et al., 1975; Craddock & Henderson, 1978). It is therefore important to determine whether DNA with carcinogen-induced lesions replicates in vivo. We had earlier shown that hepatic DNA damaged by liver carcinogens DMN[1] and *N*-hydroxy-2-(acetylamino)fluorene does replicate in vivo, generating stable DNA of normal size even though the parental strand had alkali-sensitive lesions, as measured by sedimentation analysis using alkaline sucrose gradients (Rajalakshmi & Sarma, 1975; Zahner et al., 1977). These results indicated that carcinogen-induced alkali-sensitive lesions in DNA permit DNA replication in vivo. However, since the interaction of carcinogens with DNA results in several chemical lesions (Irving, 1973; Sarma et al., 1975; Magee et al., 1975; Singer, 1975; Pegg, 1977; Miller, 1978), the question arises whether all lesions permit replication and, if they do, with what efficiency. In the current investigation, we have attempted to determine whether some specific methylated bases induced in liver DNA by DMN will permit replication of the DNA in vivo.

The experimental approach consisted of (a) labeling the chemical lesions in the parental strand of DNA with [$^{14}$C]-DMN, (b) inducing cell proliferation to stimulate replication

† From the Department of Pathology, University of Toronto, Medical Sciences Building, Toronto, Ontario, Canada, M5S 1A8. *Received August 9, 1979.* Supported in part by U.S. Public Health Service Research Grants CA 14689, CA 29538, and CA 21157 from the National Cancer Institute and from the National Cancer Institute of Canada. Initial stages of this work were carried out at the Fels Research Institute, Temple University School of Medicine, Philadelphia, PA 19140.

‡ Present address: Cattedra di Patologia Generale I, Universita di Cagliari 09100, Italy.

§ Present address: Department of Human Genetics, University of Pennsylvania, Philadelphia, PA.

[1] Abbreviations used: DMN, dimethylnitrosamine; m$^7$G, $N^7$-methylguanine; m$^6$G, $O^6$-methylguanine; m$^3$A, $N^3$-methyladenine; BrdUrd, 5-bromo-2-deoxyuridine; CsCl, cesium chloride; EDTA, tetrasodium ethylenediaminetetraacetate; PCA, perchloric acid.

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