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NMR AND ENZYMATIC INVESTIGATION OF THE INTERACTION BETWEEN ELASTASE AND SODIUM TRIFLUOROACETATE

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Summary

At pH 5.5, sodium trifluoroacetate is a potent competitive inhibitor of porcine elastase (K_i = 2.6 mM) and human leukocyte elastase (K_i = 9.3 mM). For both enzymes the K_i increases strongly with pH. Sodium fluoride is inactive on pancreatic elastase and sodium acetate is a weak inhibitor of this enzyme. Trifluoroethanol inhibits both enzymes but is less active than trifluoroacetate in acidic pH conditions. Bovine trypsin and α-chymotrypsin are resistant to the action of sodium trifluoroacetate and trifluoroethanol. The interaction between sodium trifluoroacetate and pancreatic elastase is also demonstrated by ^{19}F NMR spectroscopy. Trifluoroacetyltrialanine is able to displace trifluoroacetate from its complex with pancreatic elastase. In addition, a method using turkey ovomucoid for the active site titration of leukocyte and pancreatic elastase is described.

Pancreatic elastase belongs to the family of serine proteinases. Its three-dimensional structure has been determined and a great deal of work has been devoted to the elucidation of its specificity and mechanism of action (for a recent review see ref. 1). Among these studies, an NMR investigation has revealed that trifluoroacetyl peptides are much more potent inhibitors than the corresponding acetyl peptides [2]. For instance, the K_i of trifluoroacetyl trialanine (7.9 μM) is 1/140 of that of acetyl trialanine. On the other hand, a detailed NMR investigation of the elastase · trifluoroacetyl trialanine complex [3] revealed that there is only one molecule of inhibitor bound per molecule of enzyme and that the value of the dissociation rate constant, k_{off} does not change within a wide range of enzyme and inhibitor concentrations. These results suggest that pancreatic elastase prossesses a specific binding site for the trifluoroacetyl group.

In order to check this hypothesis in a different way, and to evaluate the con-

tribution of the trifluoroacetyl group to the binding energy of trifluoroacetyl peptides, we have performed an enzymatic and NMR study of the interaction between porcine pancreatic elastase and sodium trifluoroacetate. The effect of this anion has also been studied on human leukocyte elastase and bovine trypsin and α-chymotrypsin. In addition, the action of trifluoroacetate has been compared to that of acetate, fluoride and trifluoroethanol.

Material and Methods

Porcine pancreatic elastase and human leukocyte elastase were isolated by known procedures [4,5]. The protein concentration of solutions of these enzymes was determined using the following molar extinction coefficients: $5.2 \cdot 10^4\ M^{-1} \cdot cm^{-1}$ for pancreatic elastase [4] and $2.9 \cdot 10^4\ M^{-1} \cdot cm^{-1}$ for leukocyte elastase [5]. The active site titration of the two elastases was performed with turkey ovomucoïd, a gift from Dr. Gertler. Increasing amounts of this inhibitor were added to constant amounts of enzyme. After 10 min of preincubation at 25°C, the activities were measured with trialanine-*p*-nitroanilide [6]. All reagents were dissolved in 200 mM Tris · HCl buffer pH 8.0. The molarity of the turkey ovomucoïd solution was determined using a molar extinction coefficient of $1.27 \cdot 10^4\ M^{-1} \cdot cm^{-1}$ [7]. The compositions of the reaction media were: elastase (pancreatic or leukocyte): 1 μM; inhibitor: variable; substrate: 2 mM.

The inhibition constants (K_i) were determined by measuring the effect of increasing amounts of inhibitor on the activity of constant amounts of enzyme using two concentrations of the substrate succinyltrialanine-*p*-nitroanilide [8]: 0.5 and 3 mM for pancreatic elastase and 0.5 and 1.5 mM for leukocyte elastase. Stock solutions of substrate and inhibitors were made up in the appropriate buffers. Pancreatic and leukocyte elastases were dissolved in 200 mM Tris · HCl buffer, pH 8.0, and distilled water respectively. The enzymatic reactions were started by adding a small aliquot of enzyme solution (1—3% of total reaction volume) to the inhibitor + substrate mixture. The final enzyme concentrations were 10 nM and 0.35 μM for pancreatic and leukocyte elastase respectively. The reaction rates were measured at 410 nm using a Beckman 24 K model spectrophotometer equipped with thermostated cell holder (25°C). The inhibition constants were determined using Dixon plots [9].

The source, active site titrations and substrates of bovine trypsin and chymotrypsin were the same as described elswhere [10]. The composition of the reaction media were: 50 nM trypsin, 1.5 mM benzoyl-L-arginine-*p*-nitroanilide 50 mM Tris · HCl pH 8.0 and 5% (v/v) dimethyl formamide on the one hand and 1 μM chymotrypsin, 1.5 mM succinyl-L-phenylalanine-*p*-nitroanilide, 300 mM Tris · HCl pH 8.0 and 5% (v/v) dimethylformamide on the other hand. Stock solutions of the enzymes were prepared in 1 mM HCl containing 20 mM $CaCl_2$. Activity measurements were performed as in the case of elastase.

The NMR spectra were recorded at 100 MHz (^1H) and 94 MHz (^{19}F) using a Varian XL 100 spectrometer operating in the Fourier transform at a sample temperature of 34°C. Trifluoroacetic acid contained in a coaxial capillary tube was used as external reference for ^1H NMR (the proton of the carboxyl function) and for ^{19}F NMR (the fluorines of the trifluoromethyl group).

Results and Discussion

1. Active site titration of pancreatic and leukocyte elastase

Previous studies have shown that turkey ovomucoid inhibits porcine pancreatic and human leukocyte elastase [7,11]. Preliminary investigations showed that the K_i are about 5 and 10 nM respectively. In order to use a natural inhibitor as an active site titrant, the inhibition curve must be linear and this condition can only be satisfied if the total enzyme concentration is 100 times (or more) higher than K_i [12]. We used therefore a 1 μM concentration of both elastases. In order to get measurable reaction rates, we used trialanine-*p*-nitroanilide which is much less sensitive than succinyltrialanine-*p*-nitroanilide [6,8].

As can be seen in Fig. 1, the inhibition is linear with both elastases. Using the appropriate molar extinction coefficients at 280 nm of elastases and turkey ovomucoid [4,5,7] we found that our pancreatic and leukocyte preparations are composed of 100 and 90% active enzyme, respectively.

2. Enzymatic investigation of the interaction between pancreatic elastase and sodium trifluoroacetate

The Dixon plot [9] shown in Fig. 2a gives a K_i of 62 mM for the inhibition of pancreatic elastase by sodium trifluoroacetate at pH 8.0. The inhibition is purely competitive since the Cornish-Bowden plot [13] shown in Fig. 2b yields lines which can be considered as parallel within the limits of experimental errors.

The inhibition of elastase by the trifluoroacetate anion has been studied at different pH values in order to see whether the K_i decreases with pH as in the case of trifluoroacetyl-tripeptides [2]. As shown in Fig. 3, sodium trifluoroacetate does not behave like peptidic inhibitors since its K_i increases considerably with pH. For instance between pH 5.5 and 10.5, the dissociation constant increases by a factor of about 100. The inhibition was found to be purely competitive in all cases.

In the acidic pH region, the small trifluoroacetate anion is thus a relatively

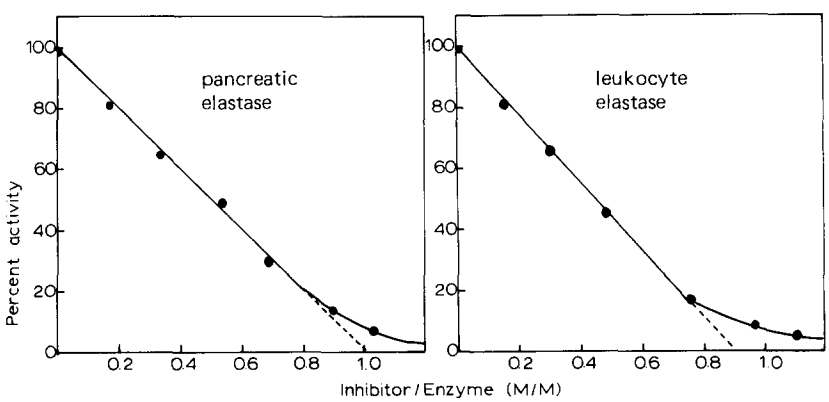

Fig. 1. Active site titration of porcine pancreatic and human leukocyte elastases (1 μM) with turkey ovomucoid. The molarities indicated on the abscissa scales have been calculated with the molar extinction coefficients at 280 nm given in the text.

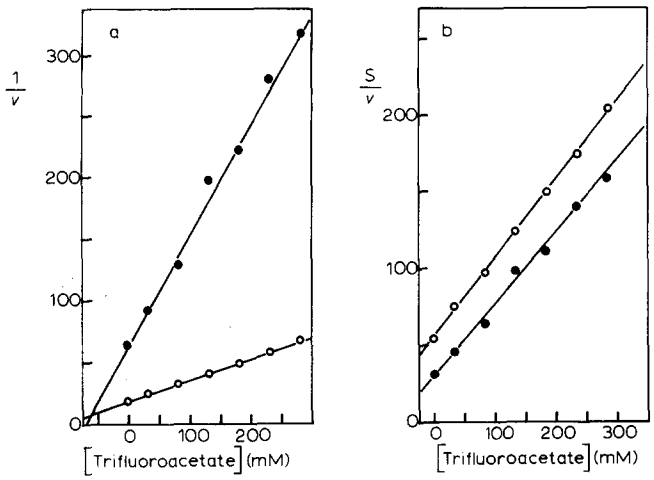

Fig. 2. Dixon plot (a) and Cornish-Bowden plot (b) of inhibition of porcine pancreatic elastase by sodium trifluoroacetate at pH 8.0, 25°C. The substrate concns. are: 0.5 mM (●———●) and 3 mM (○———○). The rate v is in arbitrary units.

potent inhibitor of pancreatic elastase (Table I). For instance, at pH 5.5 its K_i (2.6 mM) is nearly identical to that of a series of acyl dipeptides investigated by Dzialoszynski and Hofmann [14] at the same pH. On the other hand, it is interesting to note that at this pH, the sum of $-\Delta G^0$, the free energies of binding * of trifluoroacetate (3.53 kcal · mol^{-1}) and Ala_3 (1.84 kcal · mol^{-1} taken from ref. 14) is almost equal to the free energy of binding of trifluoroacetyl-Ala_3 (5.93 kcal · mol^{-1} taken from ref. 2). This suggests strongly that the increased affinity of trifluoroacetyl peptides with respect to acetyl peptides results mainly from a strong interaction of the trifluoroacetyl substituent itself with a specific binding site of the enzyme.

The unfavorable effect of pH on the affinity of the trifluoroacetate anion might be due to an electrostatic repulsion between the carboxyl group of trifluoroacetate and negatively charged groups of the enzyme which titrate between pH 5.5 and 10.5. From the shape of the curve relating pK_i to pH (Fig. 3) it may be inferred that more than one group exerts its effect on K_i.

3. NMR investigation of the interaction between pancreatic elastase and sodium trifluoroacetate

At p^{2}H 5.0, in the presence of 1 mM elastase, the ^{19}F resonance of trifluoroacetate is shifted downfield. The intensity of the shift decreases with the concentration of trifluoroacetate (Fig. 4a).

Since the variations of the chemical shifts were always lower than 15 Hz we had to determine the influence of elastase on the magnetic susceptibility of the solutions because the reference is external [15]. We have observed that the ^{1}H resonances of tetradeuterotrimethylsilyl propionate and of tetramethylammonium chloride dissolved in 50 mM d^4 acetate buffer, p^{2}H 5.0 or in 50 mM Tris

* Calculated with the classical formula $-\Delta G^0 = RT \ln(1/K_i)$.

TABLE I

INHIBITION OF PORCINE PANCREATIC AND HUMAN LEUKOCYTE ELASTASE BY SODIUM TRIFLUOROACETATE AND TRIFLUOROETHANOL AT 25°C

The buffers used were the same as those given in the legend to Fig. 2.

Inhibitor	pH	K_i pancreatic elastase (mM)	K_i leukocyte elastase (mM)
Sodium trifluoroacetate	8.0	62	200
	6.5	8	
	5.5	2.6	9.3
Trifluoroethanol	8.0	93	34
	6.5	130	54

buffer p^2H 8.0 have a chemical shift which does not depend upon the elastase concentration even if the latter is as high as 1.2 mM. This result excludes the possibility of an interaction between these two differently charged compounds and the enzyme which would yield identical effects on their ^1H resonances and which, in addition, would be exactly compensated by a magnetic susceptibility effect due to the presence of enzyme. The most plausible explanations are either that the assumed interaction between elastase and these two compounds does not lead to a modification of the chemical shifts of their resonances or that the

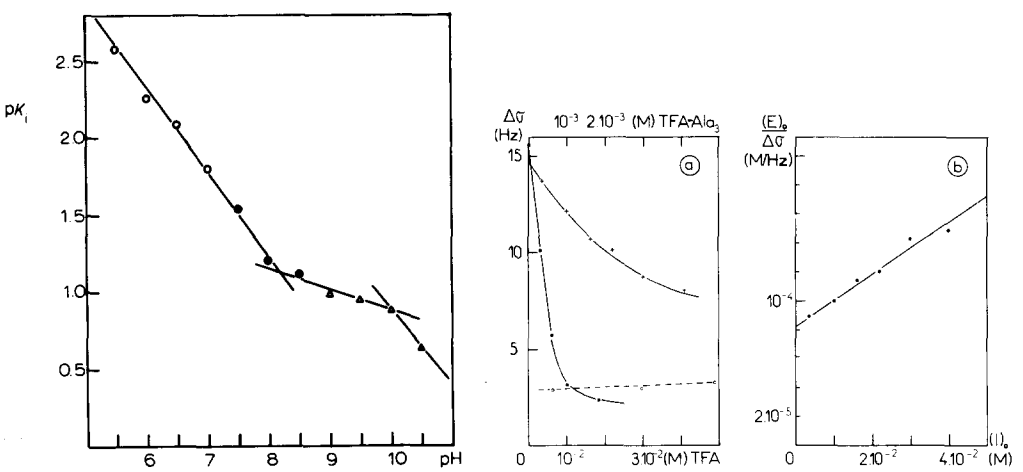

Fig. 3. pH dependence of the inhibition constant K_i of the porcine pancreatic elastase · trifluoroacetate complex at 25°C. The buffers are: (○) 0.2 M KH_2PO_4 + 0.2 M Na_2HPO_4, (●) 0.2 M Tris + HCl, (△) 0.2 M glycine + NaOH.

Fig. 4. (a) Fluorine chemical shift variations of sodium trifluoroacetate (TFA) in 50 mM d^4 acetate buffer p^2H 5.0, (+) in the presence of 1 mM pancreatic elastase and varying concn. of trifluoroacetate (●) in the presence of constant concn. of elastase (1.3 mM) and trifluoroacetate anion (5.2 mM) and varying concn. of trifluoroacetyl trialanine (○) in 50 mM Tris p^2H 8.0 in the presence of 0.7 mM elastase and varying concn. of trifluoroacetate. (b) Fluorine chemical shift variations of sodium trifluoroacetate in 50 mM d^4 acetate buffer p^2H 5.0 in the presence of 0.58 mM elastase and varying concns. of trifluoroacetate. The data are plotted in accordance with Eqn. 1. (note: all chemical shift variations are downfield with respect to the resonance of free trifluoroacetate.)

elastase induced modification of the magnetic susceptibility is not high enough to change significantly the chemical shifts measured. Since magnetic susceptibility is proportional to the magnetic field, it will be negligible a fortiori for ^{19}F NMR.

We have therefore analyzed the elastase induced variations of the chemical shifts of trifluoroacetate at p^2H 5.0 by assuming the formation of a reversible elastase · trifluoroacetate complex. One single band of chemical shift was observed for various concentrations of trifluoroacetate. This indicates that the exchange between free and bound inhibitor is fast in the NMR time scale [15]. On the other hand, the concentration of trifluoroacetate (I^0) was always higher than that of elastase (E^0) so that the concentration of complex could be neglected with respect to (I^0). Under these conditions, it may be easily demonstrated that:

$$\frac{(E^0)}{\Delta\sigma} = \frac{1}{\Delta\sigma_{EI}} [(I^0) + K_i] \qquad (1)$$

where $\Delta\sigma$ and $\Delta\sigma_{EI}$ are the variations of chemical shift observed in the free and complexed inhibitor respectively and K_i is the dissociation constant of the complex. A plot of $(E^0)/\Delta\sigma$ vs. (I^0) yielded a straight line from which values of 1000 Hz and 72 mM were found for the chemical shift and dissociation constant of the complex respectively.

Under identical conditions (i.e $p^2H = 5.0$), the ^{19}F resonance of trifluoroethanol was shifted downfield by 2 Hz and this shift did not depend upon the trifluoroethanol concentration but depended upon the elastase concentration. This indicates that trifluoroethanol forms loose and non-specific complexes with elastase at p^2H 5.0.

This non specific effect was assumed to be the same whatever the trifluoromethylated compound. Hence it was used to correct the spectroscopic data ontained with the trifluoroacetate. With this correction the relationship between $(E^0)/\Delta\sigma$ and (I^0) was again linear (Fig. 4b). Values of 570 Hz and 47 mM were found for the chemical shif and the dissociation constant of the complex respectively. The latter value is higher than that determined by the enzymatic method. It must however be kept in mind that the values obtained by NMR spectroscopy may be relatively imprecise because the chemical shifts are low (15 Hz) and the estimation of the non-specific effects may be inaccurate. In addition, it is difficult to compare two K_i values obtained with such widely differing enzyme concentrations.

Addition of the specific inhibitor trifluoroacetyl trialanine [2] to the mixture of elastase and trifluoroacetate at p^2H 5.0 resulted in a decrease of the shift of the resonance of trifluoroacetate. When the concentration of peptide was equal to that of elastase the resonance was only 2 Hz downfield with respect to the resonance of free trifluoroacetate (Fig. 4a). The peptidic inhibitor displaces thus the trifluoroacetate anion from its complex with elastase. This suggests strongly that trifluoroacetate binds to a unique site of the enzyme and that this site is the same as the binding site of the trifluoroacetyl-Ala_3. On the other hand, the substrate succinyltrialanine-*p*-nitroanilide is also able to displace the trifluoroacetate anion since the inhibition is purely competitive. It may therefore be inferred that the trifluoroacetate-binding site is identical with

or close to one of the subsites occupied by the substrate, i.e. subsites S_4 to S'_1 (according to the nomenclature of Schechter and Berger [16]).

At p^2H 8.0 the downfield shift of trifluoroacetate was only 3 Hz in the presence of 0.7 mM enzyme and the intensity of the shift did not depend upon the trifluoroacetate concentration up to a value of 50 mM. This is in agreement with the high K_i value found by enzymatic rate measurements.

4. Effect of other anions and trifluoroethanol on the activity of pancreatic elastase

Visser and Blout [17] have reported that the esterolytic activity of elastase is inhibited to about 50% by 0.2 M sodium fluoride. With our assay system, we found no inhibition by this salt up to a concn. of 0.56 M.

At pH 8.0, sodium acetate had little effect (50% inhibition with a 2 M concn.) whereas at pH 5.5 its inhibitory activity was more pronounced. At this pH the K_i was 100 mM.

Trifluoroethanol inhibited pancreatic elastase with a K_i of 93 mM at pH 8.0. Unlike what has been found with trifluoroacetate, the decrease of pH did not increase the affinity since at pH 6.5 the K_i was only 130 mM (Table I). In both cases the inhibition was competitive. Since organic solvents usually inhibit elastase [18] the action of ethanol was investigated as a control. As shown in Fig. 5a, the inhibitory effect of trifluoroethanol is distinctly more pronounced than that of ethanol.

The importance of fluorine atoms is evidenced by the fact that acetate is a very poor inhibitor compared to trifluoroacetate. However, the trifluoromethyl group alone is not sufficient since trifluoroethanol forms very loose complexes with pancreatic elastase as shown by NMR and enzymatic studies. For instance, at pH 6.5 trifluoroacetate has a K_i of 8 mM whereas trifluoroethanol has a K_i of 130 mM (Table I). The carbonyl group of trifluoroacetate plays therefore an important role. It is probable that the electron-withdrawing nature of the trifluoromethyl group renders the carbonyl group more susceptible to combine with nucleophilic residues of the enzyme.

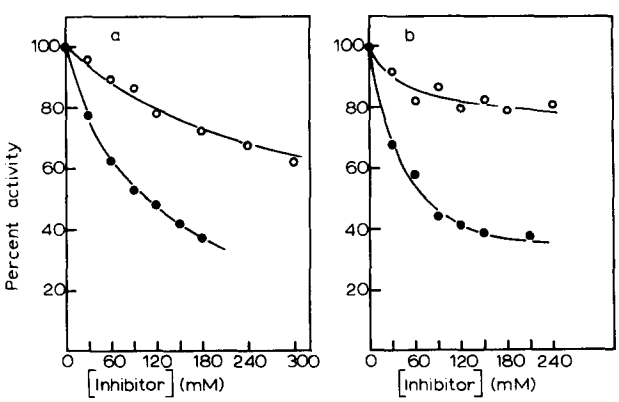

Fig. 5. Influence of ethanol (○) and trifluoroethanol (●) on the activity of porcine pancreatic elastase (a) and human leukocyte elastase (b). Activity measurements were performed in 0.2 M Tris · HCl pH 8.0, 25°C with 1 mM succinyltrialanine-*p*-nitroanilide.

5. Influence of sodium trifluoroacetate and trifluoroethanol on leukocyte elastase

Trifluoroacetate was found to be 3—4 times less potent on leukocyte elastase than on pancreatic elastase and again its inhibitory activity decreased with pH (Table I). On the other hand, trifluoroethanol was found to be a relatively good inhibitor of the leukocyte enzyme. In all cases the inhibition was competitive. The effects of ethanol and trifluoroethanol are compared in Fig. 5b. Again the latter compound is much more active than the former.

6. Influence of sodium trifluoroacetate and trifluoroethanol on trypsin and α-chymotrypsin

Both proteases were insensitive of the action of trifluoroethanol. The effect of trifluoroacetate on trypsin was rather complex: between 0 and 200 mM trifluoroacetate the activity was slightly enhanced (maximum = 22%) then it was depressed and returned to its original value. Similar concentrations of sodium chloride were without effect.

The activity of α-chymotrypsin was enhanced by trifluoroacetate: 65% activation was observed with a 600 mM concentration of trifluoroacetate. The effect was however nonspecific since sodium chloride gave the same extent of activation in agreement with former results (e.g. ref. 19).

The trifluoroacetate anion is therefore a rather specific inhibitor of pancreatic and leukocyte elastase.

Acknowledgements

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