Trifluoroacetylated Peptides as Substrates and Inhibitors of Elastase: A Nuclear Magnetic Resonance Study†

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ABSTRACT: Trifluoroacetyl di- and tripeptides have been synthesized in order to investigate their interactions with elastase by proton and fluorine magnetic resonance. These substituted peptides behave as substrates or inhibitors of the enzyme, depending upon their length. They are hydrolyzed with production of trifluoroacetic acid and unsubstituted parent peptides exclusively. The amino acid specificity observed and the absence of hydrolysis in the presence of an enzyme substituted at the serine residue of the active site indicate that the trifluoroacetic hydrolysis occurs at this site. It requires the fixation of the C-terminal amino acids at the two S′ subsites, as does the peptidic hydrolysis of unsubstituted or acetylated oligoalanines. Trifluoroacetyl tripeptides exhibit a much higher affinity for the protein, as compared with the unsubstituted or acetylated peptides as well as compared with the trifluoroacetyl dipeptides, and they act as powerful inhibitors of the enzyme. The inhibitory binding mode has been shown to involve the fixation of the trifluoroacetyl group at subsite S_4 or in its vicinity, allowing for the cooperative fixation of the C-terminal alanine at S_1 and the accommodation of a trans proline at S_2.

Elastase is a pancreatic serine proteinase which exhibits a rather strict specificity for the hydrolysis of peptide bonds on the carboxylic side of glycine and alanine residues (Narayanan and Anwar, 1969). The efficiency of the enzyme-catalyzed hydrolysis of peptide, ester, and amide bonds in synthetic oligopeptides depends upon the number of amino acids of the substrate (Atlas et al., 1970; Thompson and Blout, 1970). Five subsites, labeled S_1 to S_5, and two subsites, labeled S_1' and S_2', have been identified for the binding of amino acids positioned respectively at the N-terminal and C-terminal ends relative to the serine-containing catalytic site (Atlas, 1975; Thompson and Blout, 1973; Schechter and Berger, 1967). The nature and the specificity of these subsites have been investigated biochemically using synthetic substrates and inhibitors and by x-ray diffraction techniques for some enzyme–inhibitor complexes (Shotton et al., 1972). This last technique is not applicable to extended investigations and the former ones are limited to the measurements of rate and equilibrium constants for the catalytic process.

Nuclear magnetic resonance offers the possibility of overcoming part of these limitations since it can be very sensitive to molecular interactions, even in the absence of catalytic activity, such as at low pH values or in enzyme–inhibitor complexes (Roberts and Jardetzky, 1970; Gerig and Reinheimer, 1970; Gammon et al., 1972). On the other hand, the observation of naturally occurring nuclei, such as the protons, or of nuclei specifically substituted within chemical groups, such as ^{19}F in trifluoromethyl or trifluoroacetyl groups, provides a direct analysis of the molecular interactions contributing to the binding of substrates or inhibitors (Dahlquist and Raftery, 1968). This paper presents such an investigation of the binding modes of trifluoroacetylated peptides to elastase.

Materials and Methods

Elastase. Porcine elastase was prepared from a pancreatic extract "Trypsin 1-300" (Nutritional Biochemicals Corp., Cleveland, Ohio) and its purity was checked by electrophoresis on polyacrylamide gel at pH 4.3 (Shotton, 1970). The activity and the stability of the enzyme were assayed optically using both a natural substrate, Remazol brillant blue elastine (Rinderknecht et al., 1968), and a synthetic one, succinyltrialanine-*p*-nitroanilide (Bieth et al., 1974). The autolysis of a 1 mM solution of the enzyme at pH 8, 0.05 M Tris[1] buffer, and at pH 5, 0.05 M acetate buffer, was less than 25% and negligible, respectively, after 8 h at 34 °C. This stability was sufficient for the NMR investigation of inhibitors in the pH range of activity of the enzyme.

The serine at the active site was substituted by a *p*-fluorophenylsulfonyl group by exposure of a 0.6 mM solution of the enzyme during 2 h at pH 8 and room temperature to a fourfold excess of the fluoride salt, eliminated afterward by dialysis. The treated enzyme was stable for at least 8 h at 34 °C but it exhibited a residual activity of approximately 5%.

The proteins were stored after lyophilization in water and solubilized in heavy water just before use at corrected pD values of 5 or 8 (deuterated acetate (0.1 M) and deuterated phosphate (0.1 M), respectively). The concentration of the enzyme, in a range of 10^{-4} to 10^{-3} M, was measured optically (ϵ_{280nm} 5.23×10^4 M^{-1} cm^{-1}).

Trifluoroacetyl Peptides. N-Trifluoroacetyl peptides containing a C-terminal alanine have been prepared from the unsubstituted peptides (purchased from Sigma Co.) following the method of Shallenberg and Calvin (1955). The purity was checked by fluorine and proton NMR. They were stable at pD 5 and slowly hydrolyzed at pD 8 with a maximum rate ob-

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[1] Abbreviations are standard for amino acids and peptides; L isomers are considered exclusively unless specified; TFA, trifluoroacetyl N-substituent; INDOR, Internuclear Double Resonance; Tris, tris(hydroxymethyl)aminomethane; CAT, computer of averaged transients; CW, continuous wave; TTS, tetradeuteriotrimethylsilylpropionate.

TABLE I: Proton Chemical Shifts of the CH_α and the Methyl Resonances of Alanines in Oligopeptides and in Their Acetyl and Trifluoroacetyl Derivatives at pD 8.[a]

	3-CH_α	2-CH_α	1-CH_α	3-CH_3	2-CH_3	1-CH_3
Ala			378.8			147.9
Ac-Ala			413.0			133.8
TFA-Ala			423.6			144.5
Ala-Ala		397.9	416.5		151.2	136.4
Ac-Ala-Ala		431.4	415.1		137.5	133.8
TFA-Ala-Ala		447.5	416.5		147.6	134.5
Ala-Phe		382.0			139.0	
TFA-Ala-Phe					138.5	
Ala-Ala-Ala	399.2	435.0	413.3	150.6	138.3	133.6
Ac-Ala-Ala-Ala	431.2	435.2	414.0	139.7	138.3	133.6
	(−4.2)[b]	(−2.7)	(+22.1)	(−4.2)	(−0.3)	(+8.4)
TFA-Ala-Ala-Ala	447.1	434.8	414.4	148.0	140.5	133.5
	(−3.4)	(−1.7)	(+22.5)	(−2.7)	(−1.0)	(+8.7)
TFA-Gly-Ala-Ala					141.2	134.5
					(−2.2)	(+7.9)
TFA-Gly-Pro-Ala						134.5

[a] The shifts at 100 MHz are referenced to the methyl resonance of tetradeuteriotrimethylsilylpropionate. The residues are numbered starting from the C terminal. [b] The figures in parentheses are the shifts of the resonances at pD 1.5, corresponding to neutral peptides, relative to the shifts at pD 8, at which the peptides are anionic.

served for TFA-Gly-Ala-Ala (15% hydrolysis in 150 min at 34 °C).

NMR. The NMR spectra were recorded at 100 MHz (^1H) and 94 MHz (^{19}F) using a Varian XL-100 spectrometer. The temperature of the 10-mm diameter sample tubes was 34 °C. In some instances, the spectra were accumulated in a C-1024 CAT using the CW mode of functioning or were recorded in the Fourier transform mode in order to improve the sensitivity. The proton chemical shifts were referenced to internal tetradeuteriotrimethylsilylpropionate (TTS) which was found to exhibit no significant chemical shift modification in the presence of the enzyme. For the whole range of elastase concentrations, the proton resonance of internal TTS was not shifted with respect to that of trifluoroacetic acid which was contained in a coaxial capillary tube. An hypothesis has been made that this external reference could be used in the fluorine experiments without need for susceptibility correction. Thus, this ^{19}F external reference was used throughout this work since internal trifluoroacetic acid exhibits a low-field shift due to direct interaction with the enzyme.

The CH_α proton resonances of the various amino acids were assigned by pH titration. Then homonuclear decoupling using the INDOR technique allowed us to assign the methyl doublet of the corresponding alanines (Table I). The large background absorption of the protein in the proton experiments carried out in the presence of elastase was eliminated by recording the difference spectra. The fluorine resonances of the TFA group in the substituted peptides appear as a doublet or a triplet, due to an heteronuclear coupling with the CH_α proton(s) ($^5J_{FH}$ = 0.7 Hz). However, proton noise decoupling was used in most of the experiments in order to improve the sensitivity.

Kinetics and Equilibrium Analysis. In conditions of fast or intermediate rates of chemical exchange, the chemical shift δ of some resonance characteristic of the substrate is a function of the enzyme and substrate concentrations E_0 and I_0 (Gammon et al., 1972):

$$\delta = \left(\frac{E_0 + I_0 + K_I - \{(E_0 + I_0 + K_I)^2 - 4E_0I_0\}^{1/2}}{2I_0}\right)\delta_{EI} \quad (1)$$

where K_I is the dissociation constant and δ_{EI} is the chemical shift of the resonance in the complex.

The inhibitory power of the peptides can be measured kinetically by competitive inhibition with the synthetic substrate succinyltrialanine-*p*-nitroanilide at two different concentrations S_0; then the rate of hydrolysis, measured optically, is (Dixon, 1953):

$$v = \frac{k_{cat}E_0}{1 + K_m/S_0(1 + I_0/K_I)} \quad (2)$$

where k_{cat} and K_m are respectively the rate constant and the Michaelis constant for the hydrolysis of the substrate. In conditions of strictly competitive inhibition, the inhibition constant K_I is equivalent to the dissociation constant measured by NMR. The parameters K_I, δ_{EI}, k_{cat}, and K_m in eq 1 and 2 have been computed from the experimental data by using a program for nonlinear regression which provided the 95% confidence intervals.

Results

The Inhibition of the Hydrolysis of Succinyltrialanine-p-nitroanilide by Trifluoroacetylated Peptides. The kinetics of the hydrolysis of this synthetic substrate by the trifluoroacetylated peptides and by their nonsubstituted or acetylated analogues at pH 8 and 5 were characteristic of competitive inhibition. The inhibition constants derived from these optical investigations for the various peptides are considerably decreased upon trifluoroacetylation (Table II). This increase of the inhibitory power depends upon both the length of the oligopeptide and on the amino acid composition. When compared with the corresponding acetylated peptides, the inhibition constant of the trifluoroacetylated peptides is one order of magnitude smaller for the dipeptides and a factor of 100 smaller for the tripeptides (Table II). The maximum increase of affinity, observed for TFA-trialanine, corresponds to a gain of 3 kcal mol^{-1} for the binding energy. The substitution, however, of a glycine or a proline residue for the two N-terminal alanines, respectively, diminishes the inhibitory power of the trifluoroacetyl substitution. A similar effect was already observed for acetylated peptides. The inhibitory power of the

TABLE II: Interaction Parameters of Alanine Containing Peptides and Their Trifluoroacetyl Derivatives as Measured by NMR (Dissociation Constant and ^{19}F Induced Chemical Shift) and by Biochemical Inhibition (Inhibition Constant).[a]

	pH or pD	K_I NMR (M)	δ_{EI} (Hz)	K_I Inhib (M)
TFA-Ala-Ala	5	*b*	146 ± 129	
	8	Hydrolyzed		$2.9 \pm 0.5 \times 10^{-3}$
TFA-Pro-Ala	8	*c*		$\sim 1.5 \times 10^{-2}$
TFA-Ala-Phe	5	Hydrolyzed		
	8	Hydrolyzed		$1.8 \pm 0.3 \times 10^{-3}$
TFA-Gly-Pro-Ala	5	$1.1 \pm 0.4 \times 10^{-3}$	169 ± 20	$3.6 \pm 0.5 \times 10^{-4}$
	8	$3.7 \pm 3.6 \times 10^{-4}$	119 ± 20	$1.2 \pm 0.6 \times 10^{-4}$
TFA-Gly-Ala-Ala	5	$3.0 \pm 1.0 \times 10^{-4}$	136 ± 7	$4.0 \pm 1.0 \times 10^{-4}$
	8	*b*	98 ± 18	$5.5 \pm 2.0 \times 10^{-5}$
TFA-Ala-Ala-Ala	5	*b*	113 ± 46	$4.5 \pm 1.0 \times 10^{-5}$
	8	*b*	~30[d]	$7.9 \pm 2.0 \times 10^{-6}$
Ala-Ala	8			$\sim 10^{-1}$
Ala-Ala-Ala	8			$\sim 7 \times 10^{-3}$
Ac-Ala-Ala-Ala	8			$1.1 \pm 0.4 \times 10^{-3}$

[a] ^{19}F NMR measurements were carried out at 94 MHz in D_2O, 0.1 M acetate or phosphate buffer, at concentrations in enzyme of 3×10^{-4} to 10^{-3} M. The biochemical inhibition constants were measured optically in H_2O using succinyltrialanine-*p*-nitroanilide as substrate at concentrations from 2×10^{-8} to 2×10^{-6} M in elastase. Uncertainties are the 95% confidence intervals derived by nonlinear regression. [b] The computed values are not statistically significant with respect to the 95% confidence interval. [c] The NMR spectra of the two proline isomers are very little perturbed by elastase. [d] Computed assuming saturation of the enzyme.

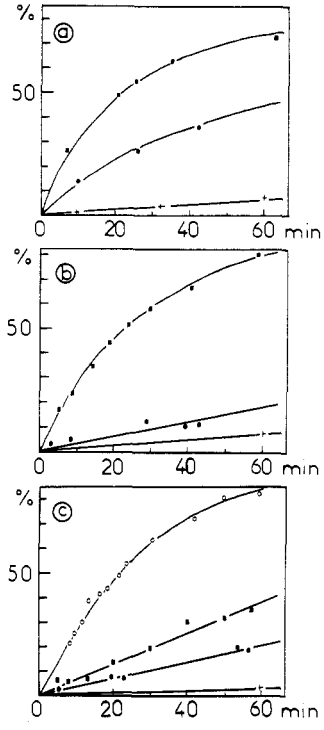

FIGURE 1: Time course of peptide hydrolysis by elastase, 1.6×10^{-4} M, as measured by NMR at pD 8 (0.1 M phosphate buffer) and 34 °C. (a) Hydrolysis of: $(Ala)_4$, 1.3×10^{-2} M (●); Ac-$(Ala)_4$, 1.14×10^{-2} M (■); and Ac-$(Ala)_3$, 1.2×10^{-2} M (+). The production of dialanine was measured by ^1H NMR. (b) Hydrolysis of: TFA-Ala-Phe, 6.1×10^{-3} M (■); and TFA-Gly-Ala-Ala, 6.6×10^{-3} M (●). There is also a slow hydrolysis of TFA-Gly-Ala-Ala in the absence of the enzyme (+). (c) Hydrolysis of TFA-Ala-Phe, 4.4×10^{-3} M (○). The reaction is inhibited by addition of TFA-Gly-Ala-Ala, 5×10^{-3} M (■), or in the presence of *p*-fluorotosylelastase (●). The spontaneous hydrolysis in similar conditions (+) is negligible. The hydrolysis of the trifluoroacetylated peptides was measured by ^{19}F NMR following the production of trifluoroacetic acid.

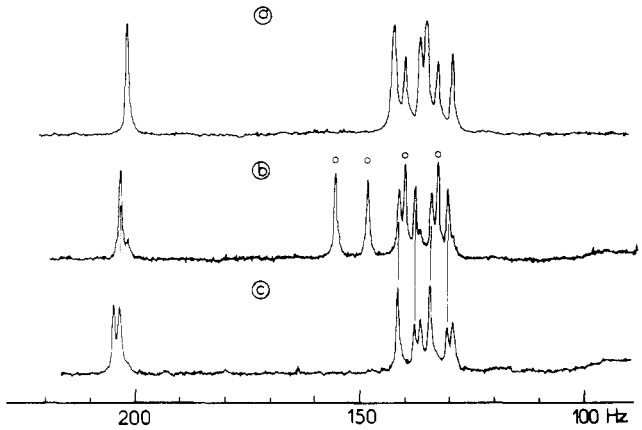

FIGURE 2: Proton NMR spectra of the methyl resonances of Ac-L-$(Ala)_4$, 5×10^{-3} M, at pD 8, alone (a), and after 48 h of hydrolysis at 24 °C in the presence of elastase, 1.6×10^{-4} M (b). The products of the reaction, which is already complete after 2 h, are identified as dialanine (○) and Ac-L-Ala-Ala. A small impurity is more apparent after hydrolysis. A spectrum of an equimolar mixture of the D and L isomers of Ac-DL-Ala-Ala is given for comparison (c). It exhibits no change in the presence of elastase for at least 48 h. The spectra, obtained at 100 MHz by accumulation in the Fourier transform mode, are referenced to internal tetradeuteriotrimethylsilylpropionate.

trifluoroacetylated dipeptides also depends upon the amino acid composition, being minimum for proline-containing peptides but not significantly altered by substitution of a C-terminal phenylalanine.

NMR Investigations of Elastase-Trifluoroacetylated Peptide Interactions. Under conditions of catalytic activity of the enzyme, i.e., in the presence of native protein at pD 8, the behavior of the NMR experiments is quite different for the unsubstituted or acetylated peptides, and for the trifluoroacetylated peptides.

The time evolution of the ^1H NMR spectra of the unsubstituted and acetylated oligoalanines shows one that they are hydrolyzed at a rate which depends upon the length (Figure 1)

$$Ac\text{-}Ala_4 > Ala_4 \gg Ac\text{-}Ala_3 \gg Ac\text{-}Ala_2 \simeq Ala_3 \simeq Ala_2 = 0$$

A dialanine, identified by its ^1H NMR spectrum (Figure 2) and corresponding to the C-terminal fragment, was observed

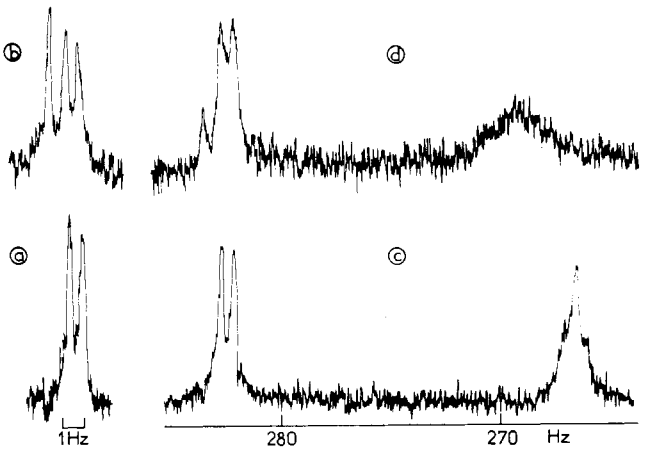

FIGURE 3: ^{19}F NMR spectra of trifluoroacetyl peptides in the absence (bottom) and in the presence (top) of elastase, 1.6×10^{-4} M, at pD 8. (left) TFA-Ala-Ala, 5×10^{-3} M (a), is hydrolyzed by elastase (b). The low-field resonance corresponds to a production of 40% trifluoroacetic acid after 25 min at 34 °C. (right) In the presence of an equimolar amount of TFA-Gly-Ala-Ala (c), the enzymatic hydrolysis of TFA-Ala-Ala is partly inhibited (d) (20% of hydrolysis after 35 min at 34 °C). The fluorine resonance of TFA-Gly-Ala-Ala is broadened upon interaction with elastase, but its integrated area is not modified. The spectra were recorded at 94 MHz. In the absence of proton decoupling, the fluorine resonances appear as a doublet for TFA-Ala-Ala, a triplet for TFA-Gly-Ala-Ala, and a singlet for trifuoroacetic acid. The chemical shifts are referenced to external trifluoroacetic acid.

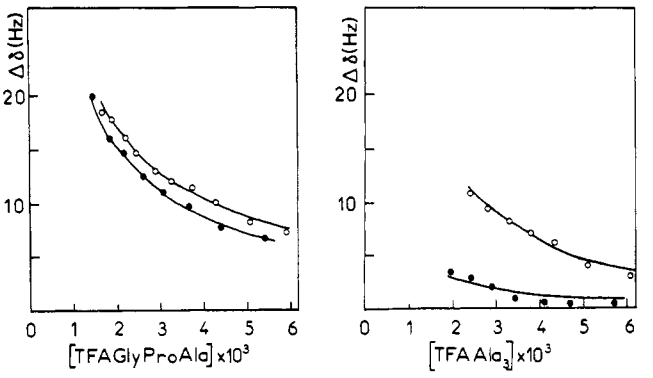

FIGURE 4: Fluorine chemical shift variations at 94 MHz of TFA-Gly-Pro-Ala and TFA-Ala-Ala-Ala upon titration of elastase (3.1×10^{-4} and 1.9×10^{-4} M, respectively) at pD 5 (open circles) and at pD 8 (full circles).

in every case as a reaction product together with a stoichiometric amount of Ac-Ala_2, Ala_2, or Ac-Ala, respectively.

^1H and ^{19}F show that trifluoroacetylated dipeptides are also hydrolyzed at a relatively fast rate with the appearance of trifluoroacetic acid, giving a narrow ^{19}F resonance line, and the corresponding unsubstituted dipeptide (Figure 3). The rate of hydrolysis, which is much faster than that of the corresponding acetyl dipeptide, parallels the known specificity of the S′ subsites

$$\text{TFA-Ala-Phe} > \text{TFA-Ala-Ala} \gg \text{TFA-Pro-Ala} \simeq 0$$

In the present experimental work, only pseudo-first-order rate constants could be measured by NMR, and these were for the trifluoroacetic hydrolysis of TFA-Ala-Phe and TFA-Ala-Ala 190 and 60 M^{-1} min^{-1}, respectively (1.6×10^{-4} M in elastase, 5×10^{-3} M in peptide). The spontaneous hydrolysis is therefore negligible. The pseudo-first-order rate of trifluoroacetic hydrolysis catalyzed by the enzyme is comparable to that of the peptidic hydrolysis of Ac-Ala_4 or Ala_4 (160 and 60 M^{-1} min^{-1}, respectively).

The spontaneous hydrolysis, however, is at least equivalent to that catalyzed by the enzyme in the trifluoroacetylated tripeptides which are very slowly hydrolyzed by elastase. But the trifluoroacetylated tripeptides do not exhibit any peptidic hydrolysis in contrast to the corresponding acetyl tripeptides. Furthermore, the trifluoroacetylated tripeptides inhibit the trifluoroacetic hydrolysis of the TFA-dipeptides (Figure 3), confirming the good inhibitor character of these compounds. As a matter of fact, the trifluoroacetylated tripeptides bind much more strongly to elastase than their dipeptide analogues. The line broadening and the chemical shifts of the resonances in the ^1H and ^{19}F NMR spectra, which are observed in the presence of the enzyme, are characteristic of fast or intermediate chemical exchange rate between bound and bulk trifluoroacetylated peptides. The contribution of the exchange process to the line widths does not allow any quantitative derivation since there are not yet detailed measurements of relaxation times. The chemical shift of the ^{19}F resonances induced by the protein varies upon titration of the enzyme by the TFA-tripeptides in excess (Figure 4), allowing the derivation of a value for the dissociation constant K_I accordingly to eq 1 (Table II). Some of these data suffer from large uncertainties, mainly for the strongest interactions which correspond to nearly complete saturation of the enzyme even at the early stages of the titration. Then, the dissociation constant can be neglected and the induced chemical shift can be estimated directly from concentration ratios. The dissociation constants derived from the NMR experiments compare favorably, within these limitations, with the biochemical inhibition constants derived spectrophotometrically under somewhat different conditions of solvent (H_2O, 2×10^{-1} M Tris buffer), temperature (26 °C), and enzyme concentration (2×10^{-8} M at pH 8 and 2×10^{-6} M at pH 5). On the other hand, there is no simple correlation between the amplitude of the induced shifts in the complexes and their dissociation constants.

Similar shifts and line broadening occur in the ^1H NMR spectra in the presence of elastase. Even with use of difference spectra, however, their analysis is restricted to the methyl resonances of alanine residues and of acetyl groups. The induced shifts are small and upfield (Figure 5). Although they do not allow us to derive confidently a value for the dissociation constant, they do offer a way of comparing the strength of the perturbation at various amino acids of the peptides: When an alanine residue is present as a N-terminal amino acid bound to the trifluoroacetyl or acetyl group, its methyl resonance always exhibits in the complex a shift and a line broadening larger than those of other alanine residues. The induced shift and broadening of the resonance of the C-terminal alanine are always larger than those of a central alanine, but are hardly observable when a proline residue is present at this position. In acetylated tripeptides, the acetyl group interacts with the protein, though it does not provide an affinity enhancement comparable to that due to the trifluoroacetyl substitution.

A particular behavior of the proline-containing peptides should be noted. They are always present in solution in water or in organic solvents in two isomeric forms. For example, the methyl resonance of the alanine neighbor to the proline in TFA-Pro-Ala at pD 5 appears as a minor, ca. 20%, doublet 1.4 Hz downfield from a major doublet. This phenomenon has already been observed in ^1H (Deber and Bovey, 1970) and ^{13}C (Thomas and Williams, 1972) NMR and was attributed to cis–trans isomerization of the peptide bond opposite to the alanine residue. The trans isomer is probably that favored in water as well as in polar solvents. This effect is still larger in the ^{19}F spectra where an upfield shift, up to 87 Hz, occurs for

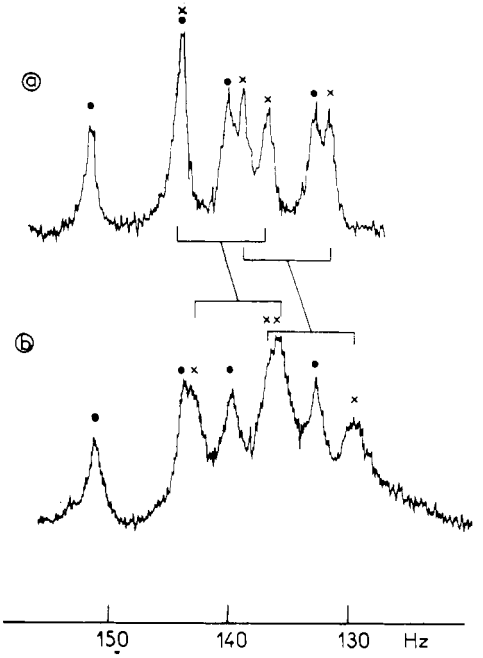

FIGURE 5: Proton NMR spectra at 100 MHz of the methyl resonances of alanines in an equimolar mixture (2.4×10^{-3} M), at pD 5, of TFA-Ala-Ala (dots) and TFA-Gly-Ala-Ala (crosses) (a) alone and (b) in the presence of 5×10^{-4} M elastase. Note the larger induced shifts and line broadening for the tripeptide in the second spectrum recorded by difference with the protein solution. High-field doublets correspond to the C-terminal alanines. The spectra are referenced to the methyl resonance of internal tetradeuteriotrimethylsilylpropionate.

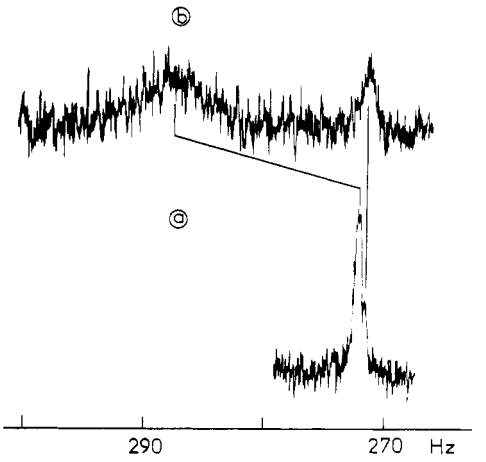

FIGURE 6: Proton noise decoupled ^{19}F NMR spectra at 94 MHz of TFA-Gly-Pro-Ala, 2.1×10^{-3} M, at pD 5 alone (a), and in the presence of elastase, 3.1×10^{-4} M (b). The enzyme induces a large difference in the chemical shifts, referenced to external trifluoroacetic acid, for the trans and the cis isomers of the proline containing peptide.

TABLE III: Dissociation Constants and Induced Chemical Shifts for Trifluoroacetyl Tripeptides Binding to *p*-Fluorotosylelastase as Measured by ^{19}F NMR at 94 MHz.

	pD	K_I (M)	δ_{EI} (Hz)
TFA-Gly-Pro-Ala	5	*a*	96 ± 12
	8	*a*	<5[b]
TFA-Gly-Ala-Ala	5	$9.6 \pm 9.4 \times 10^{-4}$	115 ± 36
	8	$2.7 \pm 1.0 \times 10^{-3}$	93 ± 18
TFA-Ala-Ala-Ala	5	*a*	98 ± 35
	8	*a*	~ 26[c]

[a] Most values of the dissociation constant were not statistically significant with respect to the 95% confidence intervals. [b] Observed value which could not be extrapolated for the complex due to the very low affinity for the enzyme. [c] Computed assuming saturation of the enzyme.

the major isomer. Only the major isomers interact significantly with the protein (Figure 6), probably favored by the trans conformation.

NMR may be advantageously carried out in order to investigate elastase–peptide interactions in conditions of enzyme inactivity. The trifluoroacetic hydrolysis of the TFA–dipeptides by *p*-fluorophenylsulfonylelastase is lowered even at pD 8 to a level corresponding, within the experimental errors, to the residual activity of the enzyme. Thus, the reaction should be normally catalyzed at the active center. The affinity of the substituted enzyme for some peptides, such as TFA-Gly-Ala-Ala, can be shown by NMR to be lower as compared with the native enzyme (Table III).

The absence of a strict correspondence between the values of the dissociation constants of the complexes, or the inhibition constants measured in conditions of enzyme activity, and the intensity of the induced shifts of the fluorine resonances in the complexes has already been noted. For example, among the three substituted peptides investigated, the larger shift observed for TFA-Gly-Pro-Ala corresponds to the lower affinity for elastase at pD 8. Such a lack of correspondence is further observed for the same peptide at two different pD values. The absence of pD effect, going from pD 8 to pD 5, for both the large induced shift and low affinity of native elastase for TFA-Gly-Pro-Ala contrasts with the decrease of affinity for TFA-Ala_3 parallel to an increase of the induced shift observed in similar conditions (Figure 4).

Discussion

Trifluoroacetylated peptides behave as substrates and inhibitors of pancreatic elastase. Dipeptides exhibit trifluoroacetic hydrolysis at an appreciable rate but are weak inhibitors of peptidic as well as of trifluoroacetic hydrolysis. The substituted tripeptides, containing a C-terminal alanine, however, are good competitive inhibitors of elastase which are hydrolyzed at a much slower rate. These two different behaviors should correspond to at least two distinct modes of binding of the peptides to elastase.

The productive binding mode observed almost exclusively for the dipeptides should correspond to the presence of the TFA group at S_1 and of the two amino acids at S_1' and S_2'. This is confirmed by the absence of peptidic hydrolysis and a relative initial rate of trifluoroacetic hydrolysis parallel to the known specific affinities of the S′ subsites (no accommodation of a proline at S_1', high affinity of S_2' for phenylalanine). This situation is quite similar to that observed for the peptidic hydrolysis of unsubstituted and acetylated peptides which always produces a C-terminal dipeptide. Occupancy of at least two S subsites is necessary in order to observe this production at an appreciable rate. The trifluoroacetic hydrolysis is certainly catalyzed by elastase at the serine active site since it is inhibited in the chemically modified enzyme and it exhibits a pH dependence similar to that for peptidic hydrolysis. Therefore, the rate of trifluoroacetic hydrolysis of the TFA-dipeptides can correspond either to a larger affinity of S_1 for the TFA group, as compared with an alanine residue, or to a higher catalytic efficiency for the hydrolysis of a pseudo-peptidic bond next to the TFA group. A methyl group fits precisely in the cavity observed at S_1, corresponding to the specific affinity for ala-

nine. This subsite must accommodate, with more difficulty, a bulky TFA group, and no important perturbation of the ^{19}F resonances has been observed in the NMR spectra. The TFA-dipeptides are, finally, poor competitive inhibitors of elastase, supporting the hypothesis of an increased rate of hydrolysis rather than a stronger binding.

The large inhibition of the enzyme induced by the TFA-tripeptides should correspond to a different and nonproductive binding mode. There are no reasons why the addition of a third amino acid, which should be bound at a hypothetical S_3' subsite, could increase the affinity so much and at the same time decrease the catalytic efficiency. The possibility of a different mode of simultaneous occupancy of S and S′ subsites is also improbable, considering the absence of peptidic hydrolysis. This nonproductive binding mode does not need to correspond to the binding of the polypeptidic backbone within the cleft of the protein. There could be a specific site outside of this cleft having a large affinity for the TFA group. The S subsites, however, appear as much better candidates to accommodate the TFA-tripeptides. The effect of amino acid substitution upon the inhibition constants measured biochemically, as well as upon the affinity constants measured by NMR, favors S_4 to S_1 as inhibitory binding subsites. According to this mode of fixation: the proline residue substituted for the central alanine can be bound at S_2 (Thompson and Blout, 1973) in at least one of its isomeric forms; the fixation of the C-terminal alanine corresponds to the well-known specificity of S_1; the trifluoroacetic group should be bound at S_4 or in the vicinity of this subsite.

Such a mode of binding is further suggested by the proton NMR spectra of the TFA-tripeptides (Figure 5). The methyl doublets of the two terminal alanines in TFA-Ala_3, and that of the C terminal one in TFA-Gly-Ala-Ala, are much more broadened in the presence of elastase than that of the central alanine. This observation is in agreement with the crystallographic data about elastase–trialanine (Shotton et al., 1972) which forms a complex with the methyl group of the central alanine pointing outward, in contrast to the two other methyl groups interacting directly with S_1 and S_3.

A similar possibility of nonproductive binding of the TFA-dipeptides should result in the presence of the C-terminal amino acid at S_2. This could still provide some steric hindrance of S_1 corresponding to the inhibition of the active site. The much larger affinity and inhibitory power of the TFA-tripeptides are not solely due to the affinity of S_1 for alanine. The observed differences in affinity indicate that there is a large cooperativity in the binding of the TFA group and of the C-terminal alanine. Such a site-to-site cooperativity through conformational change of the protein has already been proposed for a different case by Thompson (1974) for S_{4-5}–S_1 interactions, and described by Shotton et al. (1972) in elastase–inhibitor complexes. This interaction, which adds up to 3 kcal per mol to the binding energy of the TFA-tripeptides, is dependent both upon the amino acid composition and upon the pH. This latter dependence could be due to the titration of some of the groups of the enzyme, such as His-51 (located at the boundary of S_4), since the TFA-peptides are anionic in the whole range of investigated pH values.

Conclusion

The trifluoroacetylation of peptides considerably increases the possibility of interactions with elastase. New properties of substrates or of inhibitors depend upon the number and the nature of their amino acids.

In the productive binding mode, corresponding to trifluoroacetic hydrolysis, the trifluoroacetic group should be within the hydrophobic pocket which constitutes the S_1 subsite. Binding of two amino acids in the S′ subsites is necessary for efficient catalysis as observed for peptidic hydrolysis of unsubstituted or acetylated peptides. The latter, however, requires two amino acids to be present in the S subsites. The more efficient hydrolysis of the trifluoroacetyl-dipeptides was attributed to an increased catalytic rate with respect to the pseudo-peptidic bond rather than to a higher affinity of the TFA group for S_1. The presence of a strongly electronegative trifluoromethyl group should increase the electrophilicity of the neighbor carbonyl and facilitate its nucleophilic attack by the serine of the active center. Such an inductive model, already proposed in order to described the hydrolysis of trifluoroacetyl amino acids by pepsin (Hunkapiller and Richards, 1972), and the binding of trifluoroacetate inhibitors to acetylcholinesterase (Gentinetta et al., 1974), could be revised to take into account the polarizability of the carbonyl group (Holmes and Thomas, 1975).

The nonproductive mode of binding of the trifluoroacetyl group, most probably at S_4, is still more questionable. There is an evident lack of correlation between the affinity of the TFA-tripeptides and the intensity of the ^{19}F chemical shift induced by the protein at various pH values which suggests rather indirect physical mechanisms of interaction, including a long-range cooperativity with S_1. In any case, the site specificity of these different kinds of interaction is clearly indicated and is responsible for the inhibitor or substrate character of the trifluoroacetylated peptides.

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Regulation of a Metabolic System in Vitro: Synthesis of Threonine from Aspartic Acid†

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ABSTRACT: Six enzymes involved in the conversion of aspartate to threonine have been extracted from *Escherichia coli* and separated from each other. Two of these enzymes, aspartokinase and homoserine dehydrogenase, have also been partially purified from *Rhodopseudomonas spheroides*. In an attempt to determine whether small changes in the kinetic properties of individual enzymes are important to the regulation of metabolic flux through a coupled reaction system, the partially purified enzymes were recombined in a variety of ways under reaction conditions designed to resemble the in vivo situation. These conditions include: use of an entire metabolic system rather than a single reaction; high enzyme concentrations at the same relative concentrations as found in the cell; and low, steady-state concentrations of substrates and products. Metabolic flux was followed spectrophotometrically and the concentrations of aspartic semialdehyde, homoserine, *O*-phosphohomoserine, and threonine were measured. The results indicate that the threonine concentration is of major importance in regulating metabolic flux by inhibiting aspartokinase, the first reaction in the pathway. When threonine-insensitive aspartokinases were used, threonine concentrations reached higher levels and the rate of NADPH oxidation remained higher. The fact that neither aspartic semialdehyde nor homoserine accumulated as the threonine concentration increased and the lack of correlation between changes in metabolic flux and ADP/ATP or NADPH/NADP ratios indicate that more subtle forms of metabolic regulation, such as "reverse cascade", secondary feedback sites, or "energy charge", are of little regulatory importance in this isolated, metabolic system. The results also emphasize the need for caution in projecting in vivo control mechanisms from in vitro experiments.

For a number of years biochemists have inferred mechanisms of metabolic control from studies of the catalytic properties of purified enzymes. In gross terms the control mechanisms which have been devised are compatible with growth patterns and nutritional requirements of normal organisms and specific mutants. Synthesis of the aspartic acid family of amino acids (lysine, methionine, threonine, isoleucine, and *ms*-diaminopimelate) has been extensively studied because the highly branched nature of this pathway creates a complex regulatory problem and because different organisms appear to have evolved different mechanisms for regulating flux through this pathway (Datta, 1969). In coliform bacteria, metabolic control employs multiple aspartokinases (EC 2.7.2.4) under separate control (Cohen et al., 1969), while in *Rhodopseudomonas capulatus* (Datta and Gest, 1964) and *Bacillus polymyxa* (Paulus and Gray, 1964) a single aspartokinase is subject to concerted feedback inhibition. Two aspartokinases have been isolated from *Bacillus subtilis*, one subject to concerted feedback inhibition and the other inhibited by diaminopimelate (Rosner and Paulus, 1971). In *Bacillus licheniformis* and *Rhodopseudomonas spheroides* (Datta and Prakash, 1966; Gibson et al., 1962), control appears to be by a "reverse cascade" mechanism in which threonine inhibits homoserine dehydrogenase (EC 1.1.1.3), causing aspartic semialdehyde to accumulate and this aspartic semialdehyde (rather than threonine) inhibits aspartokinase.

In addition to the variety of control patterns observed within the aspartic acid family there have been reports of regulatory interactions between this metabolic family and other metabolic pathways. For example, Klungsoyr et al. (1968) have shown a relationship between lysine-sensitive aspartokinase activity and energy charge and have proposed an interaction between pathways of energy metabolism and amino acid synthesis. Baich and Hagan (1970) reported that hexose monophosphates inhibit a partially purified preparation of aspartokinase and interpreted this observation as indicating a communication between carbohydrate (energy) metabolism and protein synthesis. Kotre et al. (1973) have shown that homoserine inhibits *E. coli* glutamate dehydrogenase (EC 1.4.1.2) and suggested that this is another example of "reverse cascade" in which homoserine impedes aspartate formation by reducing the supply of the primary amino donor, glutamate.

Activation and inhibition patterns observed in experiments with single, purified enzymes may suggest opportunities for control but they can not demonstrate that any specific control

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