Biochemical Pharmacology, Vol. 34, No. 18, pp. 3315–3321, 1985.
Printed in Great Britain.

0006-2952/85 $3.00 + 0.00

FATTY ACID PEPTIDE DERIVATIVES AS MODEL COMPOUNDS TO PROTECT ELASTIN AGAINST DEGRADATION BY ELASTASES

W. HORNEBECK, E. MOCZAR, J. SZECSI* and L. ROBERT†

Laboratoire de Biochimie du Tissue Conjonctif, GR CNRS 40, Faculté de Médecine, Université Paris-Val de Marne, 8 rue de Général Sarrail, 94010 Créteil Cédex, France

(*Received* 25 *February* 1985; *accepted* 18 *April* 1985)

Abstract—Peptide sequences which fit the extended binding sites of porcine pancreatic elastase and human leukocyte elastase were covalently coupled to oleic acid. These compounds behave as competitive inhibitors towards both elastases. The coupling of fatty acid moiety to the peptide greatly decreases its inhibitor constant (K_i) vs human leukocyte elastase (K_i for Oleoyl(Ala)$_2$ProValine: 3.0 (10^{-6} M). It is less active on porcine pancreatic elastase (K_i for Oleoyl(Ala)$_2$ProAlanine: 3.8 10^{-4} M). The modifications of the carboxylic end group of the peptide to an aldehyde further greatly enhanced the inhibition capacity of the compound towards leukocyte elastase (K_i for Oleoyl(Ala)$_2$ProAlaninal: 0.7 μM).

Oleoyl peptide derivatives were seen to bind in a saturable fashion to purified insoluble elastin, and decreased the susceptibility of the macromolecule to hydrolysis by both pancreatic and leukocyte elastases.

As stoichiometric quantities of elastase (vs inhibitor) could not desorb ^{3}H-oleoyl(Ala)$_2$Pro-Val bound to insoluble elastin, it is postulated that oleoyl peptide derivatives may act as bifunctional agents. This contention was further strengthened by the comparison of the adsorption curves of elastase to untreated insoluble elastin and elastin saturated with oleoyl peptide derivatives respectively.

It was shown finally that Oleoyl(Ala)$_2$Pro-Valine was also capable of inhibiting elastases in their adsorbed form to insoluble elastin.

Active site directed inhibitors of elastases have been designed in order to control elastolysis associated with several connective tissue disorders [1]. For instance, and despite its toxicity, methoxy-succinoyl-L-alanyl-L-prolyl-L-valine-chloromethylketone (MeoSuc(Ala)$_2$Pro-ValCH$_2$Cl) an effective leukocyte elastase inhibitor, was successfully used in *in vivo* experiments against experimental emphysema induced in hamsters by the intratracheal instillation of purified human leukocyte elastase [2].

One of the major problems with the *in vivo* use of peptide chloromethylketones resides in their potential for reacting at other undesirable sites [3]. This disadvantage could be partly overcome by coupling the inhibitor to a suitable carrier that could be targeted to the desired site of action. With this end in view, human albumin microspheres were used as carriers as they were shown to be trapped in the pulmonary capillary bed after intravenous injection [4].

In the present study, insoluble elastic fibres were defined as the target. Because of the hydrophobic properties and the high affinity for lipids of the main constituants of these fibres, e.g. elastin [5–10], fatty acids were used as carriers coupled to peptide sequences known to bind effectively to the extended substrate binding site(s) of elastase(s) [11, 12].

As a model system, we synthesized several bifunctional inhibitors of this type and analysed *in vitro* their interactions both with purified elastases (porcine pancreatic elastase; human leukocyte elastase) and purified insoluble elastin from calf ligamentum nuchae.

EXPERIMENTAL

Oleic acid and amino acids were obtained from Sigma Chemicals (St. Louis, MO). Succinoyl-trialanine-paranitroanilide (Suc(Ala)$_3$Na) was purchased from Choay Laboratories (Paris, France).

Silica gel sheets for t.l.c. were from Merck-Darmstadt (F.R.G.). Tritiated borohydride NaB^{3}H$_4$ (spec. act.: 74 10^3 MBq/mmole) and 9.10 (n) – 3M oleic acid (spec. act.: 1600 MBq/mmole) came from CEA (Saclay, France). Porcine pancreatic elastase (Batch No. 4) was obtained from Whatman Chemicals (Maidstone, Kent, U.K.).

Leukocyte elastase was purified from human spleen essentially as described by Starkey and Barrett [13]. The enzyme preparation was homogenous as indicated by SDS polyacrylamide gel electrophoresis with a molecular weight of 27,000. Alternatively, we used a pure preparation kindly provided by Drs J. Bieth and C. Boudier (Université Louis Pasteur, Strasbourg, France). Stock solutions of active enzyme (1–3.10^{-5} M) were kept frozen at −20° in 1 mM acetic acid.

Insoluble elastin was purified from calf ligamentum nuchae by the hot alkali procedure [14, 15]. Its composition is similar with that of pure elastin (exempt of glycoproteins and collagen contaminants)

* Present address: M.T. A. Központi Kémiai Kutato Intézet Budapest.

† To whom correspondence should be addressed.

‡ Abbreviations used: P.P. elastase, porcine pancreatic elastase; H.L. elastase, human leukocyte elastase; Suc(Ala)$_3$Na, succinoyl-trialanine-paranitroanilide; t.l.c., thin layer chromatography.

as evidenced by amino acid analysis and the absence of hexoses and hexosamines in the preparation [15].

All other reagents were obtained from commercial sources and were of the purest grades available.

Optical rotation was measured on a Jobin-Yvon (France) electronic polarimeter, melting points on a Kofler hot bench (Prolabo, France).

SYNTHESIS OF FATTY ACID DERIVATIVES

All peptides were synthesized by the conventional mixed anhydride procedure [16]. Briefly, the tert-butyloxylcarbonyl protected amino acids were activated by isobutyl chloroformate in presence of *N*-methyl morpholine. The amino acid methyl esters were dissolved in a mixture of dimethylformamide–tetrahydrofuran (1/3 v/v). The peptide methyl esters were hydrolysed by sodium hydroxide in an aqueous solution. The elimination of the protecting group (tert-butyloxy) was carried out with HCl in dioxane. The purity of the peptides was controlled by t.l.c. in butan-1-ol/acetic acid water (4:1:1 by vol.) or butan-1-ol/acetic acid/water/pyridine (15:3:12:10 by vol.).

Amino acids were analysed on an LKB amino acids analyser (model 3201) after hydrolysis of peptides with 5.7 M HCl under vacuum for 24 hr at 105°. All peptides synthesized possessed the expected compositions (±6%).

Oleoyl-L-alanyl-L-alanyl-L-prolyl-L-valine

Ninety-eight milligrammes of L-alanyl-L-alanyl-L-prolyl-L-valine, HCl form (0.25 mmole) were dissolved in 1 ml of 90% aqueous ethanol containing 87 μl of triethylamine (0.264 mmole). After vigorous stirring, the mixture was kept at 4°, and 126 μl of oleoyl chloride (0.37 mmole) was added dropwise in 15 min. It was then stirred for 4 hr at room temperature and the solvent was evaporated under vacuum. One hundred microlitres of water was then added, the pH of the mixture was adjusted to 8.5 with triethylamine and the unreacted oleic acid was extracted with hexane.

The pH of the aqueous layer was brought to 4 and the oleoyl tetra-peptide was extracted by ethyl acetate. The solvent was evaporated under vacuum and the compound was crystallized from ethylacetate by addition of petroleum ether.

Oleoyl-L-alanyl-L-alanyl-L-prolyl-L-valine (yield 74 mg, 50%, m.p. 130–135° decomp. $\alpha_D^{22} = -72 \pm 3°$ (c 0.5 in ethanol)).

It was homogenous on t.l.c. $R_f = 0.3$ in chloroform–methanol, 9:1 v/v.

Anal. C, 65.5; H, 9,9:N, 9.2; *calc.* for $C_{34}H_{60}N_4O_6$C, 65,6; H, 9.9.; N, 9.0.

Oleoyl-L-alanyl-L-alanyl-L-prolyl-L-alanine (m.p. 162–164°, $\alpha_D^{22} = -76 \pm 3°$ c 0.5 methanol; *anal.* C, 64,6; H, 9.8; N, 9.4; *calc.* for $C_{33}H_{58}N_4O_6$ C, 64.7; N, 9.4) and *Oleoyl-L-alanyl-L-alanyl-L-proline* (m.p. 170–172°, $\alpha_D^{22} = -68 \pm 2°$; *anal.* C, 66.8; H, 10.4; N, 7.9; *calc.* for $C_{30}H_{55}N_3O_5$ C, 67.0; H, 10.3; N, 7.8) were prepared in an analogous manner. The amino acid analysis of the three oleoyl peptides gave respectively: Ala:Pro:Val: 2:1:0.9; Ala-Pro: 3:1; Ala-Pro 2: 0.9.

9.10 (n) ^3H oleoyl-L-alanyl-L-alanyl-L-prolyl-L-valine.

To 0.2 ml of a toluene solution of (9.10 (n) ^3H) oleic acid (spec. act.: 126 MBq/μl) 22.5 mg of oleoyl chloride (0.075 mmole) was added; the toluene was removed in a vacuum desiccator and the solution was equilibrated overnight. The oleoylation of 19.7 mg of L-alanyl-L-alanyl-L-prolyl-L-valine (0.05 mmole) was carried out with this solution, essentially as described above for the unlabelled product. ^3H oleoyl-L-alanyl-L-alanyl-L-prolyl-L-valine showed only one component on t.l.c. ($R_f = 0.3$ in chloroform–methanol, 9:1 v/v). Its specific radioactivity was 0.16 MBq/μmole.

Oleoyl-L-alanyl-L-alanyl-L-prolyl-L-alaninal. Oleoyl-L-alanyl-L-alanyl-L-proline 52.2 mg; 0.1 nmole was dissolved in dimethylformamide (1.08 ml) and cooled at −20° in a dry-ice acetone bath. *N*-ethyl morpholine (11 μl, 0.1 mmole) was added followed by isobutylchloroformate (13 μl, 0.1 mmole); the mixture was stirred for 2 min at −20° and 15.6 μl of alaninol (16 mg; 0.1 nmole) was added. The reaction mixture was evaporated. The residue was extracted successively with 0.4 M HCl, 0.5 M sodium carbonate and distilled water. The solvent was removed under vacuum and the residue crystallized from ethyl acetate m.p.: 80–83°. $\alpha_D^{20} = -72 \pm 3°$. *Anal.* C, 65.3; H, 9.9, N 9.1.

Loss at 100° 7.1, *calc.* for $C_{32}H_{59}O_5H_4$ $1/2C_2H_5COOH_3$: C, 65.5. H, 10.0, N, 9.1. The substance was homogenous on t.l.c. $R_f = 0.35$ with chloroform–methanol (9:1 by vol.). Amino acid analysis, Ala:Pro: 2:0.9.

Oleoyl-L-alanyl-L-alanyl-L-prolyl-L-alaninonol was oxidized as described by Thompson [17]. Briefly, 29 mg (0.05 mmole) of this compound was dissolved in 0.18 ml chloroform, 20 μl (0.3 mmole) of dimethylsulfoxide and 31 mg (0.15 mmole) of dicyclohexylcarbodiimide were added followed by 9.6 μl (0.15 mmole) of phosphoric acid consisting of successive additions of 1.2 μls aliquots over a 2 hr period and the solution was stirred for 4 hr. The chloroform and part of the dimethylsulfoxide were removed under vacuum at 30°.

The residue was dissolved in chloroform (1 ml) and left at −20° for 2 hr.

The formed dicyclo-hexyl urea precipitate was removed and the aldehyde was separated from other products by t.l.c. on Kieselgel plates (Merck, Damstadt, F.R.G.) with chloroform–methanol (9:1 by vol) as solvent.

The Schiff reagent reacting spot was extracted from the silica with chloroform, the solvent was evaporated under vacuum to give 4 mg (yield 15%) of a white amorphous substance $\alpha_D^{25} = -70 \pm 3°$ which was considered as oleoyl-L-alanyl-L-alanyl-L-prolyl-L-alaninal.

It was homogenous on t.l.c. with benzene–ethanol (95:5 by vol.) $R_f = 0.6$ and chloroform–methanol (9.1 by vol.) $R_f = 0.4$. However, it starts to decompose in chloroform or in aqueous solutions after 24 hr as evidenced by t.l.c. analysis.

Inhibition studies

Except otherwise specified, all other reactions

were performed in 100 mM Tris–HCl, 0.01% Brij 35 0.02% NaN_3 pH 8.1.

(a) *With Suc(Ala)$_3$Na as substrate.* The nature of the inhibition of the elastases by the listed compounds and their respective K_is were determined by the Dixon plot analysis [18] using Suc(Ala)$_3$Na as substrate [19]. In order to ascertain the type of inhibition, the Cornish–Bowden representation was also used [20].

A stock solution (125 mM) of Suc(Ala)$_3$Na was prepared in *N*-methyl-pyrrolidone. The substrate concentrations ranged from 0.125 to 1 mM; porcine pancreatic elastase (2–10 μM) and human leukocyte elastase (20–50 μM) were preincubated with the compounds for 15 min at 22° before recording the activity hydrolysing Suc(Ala)$_3$Na in a Beckman Acta III spectrophotometer at 410 nm (ΣM nitroanilide: 8800) at a constant temperature of 37°.

The K_is of these compounds were evaluated using Dixon plots [18] and least square analysis. All compounds, including unmodified peptides were dissolved in ethanol. Their final concentration in the assay volume (1 ml) never exceeding 2.5%. The effect of this low organic solvent concentration upon elastase activities was taken into consideration.

(b) *With ^{3}H ligamentum nuchae insoluble elastin as substrate.* Alternatively, we used ^{3}H ligamentum nuchae insoluble elastin as substrate as already described [21]. Different preparations gave specific activities in the range $0.1–0.4 \cdot 10^6$ cpm/mg.

Interaction of ^{3}H-labelled oleoyl peptides with insoluble elastin

Purified ligamentum nuchae insoluble elastin was first allowed to equilibrate with 100 mM Tris, 0.01% Brij 35, 0.02% NaN_3, pH 8.1 for 24 hr at 37°. Increasing amounts of dispersed elastin were incubated for 24 hr at 37° under mechanical stirring with constant amounts of ^{3}H-labelled oleoyl derivative (oleoyl-ala-ala-prolyl-valine, spec. act.: 1730 cpm/μmole, 64.4 μmoles); the tubes were then centrifuged at 10,000 *g* in a hematocrite centrifuge and the insoluble elastin pellet washed exhaustively with buffer in order to eliminate most of the unspecific binding. The elastin was then solubilized with 50–100 μg of porcine pancreatic elastase and 100 μl of the resulting hydrolysate were withdrawn for radioactivity determination in 10 ml of scintillation fluid Ready-Solv (Beckman, Scotland).

Elastase adsorption experiments

^{3}H-insoluble elastin samples were pretreated with an excess of unlabelled oleoyl(Ala)$_2$-ProVal as stated above. The residual pellet was then dispersed in 100 mM Tris–HCl, 0.02% NaN_3 pH 7.6 and increasing amounts of elastase(s) were then added (0.04–8 μM). The mixture was stirred for 2–3 min at room temperature, centrifuged at 10,000 *g* in a hematocrite centrifuge and the residual supernatant analysed for unbound elastase activity on Suc(Ala)$_3$Na.

Alternatively, elastase(s) were preadsorbed on to insoluble elastin [21] and the pellet dispersed in 100 mM Tris–HCl 0.01% Brij 35, 0.02% NaN_3 pH 8.1.

Excess amounts of inhibitor were then added and the resulting hydrolysis (as quantitated by the released ^{3}H-labeled peptides) was compared to those obtained when similar amounts of elastase and inhibitor were allowed to react during the same time prior to the addition of the substrate.

RESULTS

In preliminary experiments, we synthesized several fatty acid peptide derivatives such as trialanine peptides covalently linked through their N-terminal end to the carboxylic group of different fatty acids [22].

The inhibitory capacity of these compounds towards porcine pancreatic elastase was essentially similar to those of trialanine. In contrast, the fatty acid peptide derivatives markedly inhibited human leukocyte elastase. As was also reported for unmodified fatty acids [23], their inhibitory capacity was directly related to the hydrophobic character of the fatty acid moiety.

The introduction of an oleoyl moiety at the P_5S_5-interaction level only slightly affected their K_i values on porcine pancreatic elastase (K_i(Ala)$_2$ProVal = $2.1\ 10^{-3}$ M; K_i Ol(Ala)$_2$ProVal: $1.3\ 10^{-3}$ M). In contrast, coupling of an oleoyl moiety to a tetrapeptide which fits the extended binding site of human leukocyte elastase resulted in an 500-fold decrease of the K_i value.

According to the Dixon or Cornish-Bowden representations, oleoyl(Ala)$_2$ProVal behaves as a competitive inhibitor (Figs 1a–d). This favours the hypothesis that the hydrophobic binding site in

Table 1. Inhibitor constants (K_is as determined by Dixon plots analysis) of several fatty acid peptides and oleoyl peptide derivatives towards porcine pancreatic elastase and human leukocyte elastase

	Porcine pancreatic elastase	Human leukocyte elastase
(Ala)$_2$ProAla	$2.05\ 10^{-3}$ M	$1.8\ 10^{-3}$ M
(Ala)$_2$ProVal	$2.10\ 10^{-3}$ M	$1.5\ 10^{-3}$ M
Ol(Ala)$_2$ProAla	$3.8\ 10^{-4}$ M	$5.8\ 10^{-6}$ M
Ol(Ala)$_2$ProAlaninal	not det.	$0.7\ 10^{-7}$ M
Ol(Ala)$_2$ProVal	$1.3\ 10^{-3}$ M	$4.0\ 10^{-6}$ M
Ol(Ala)$_2$Pro	not det.	$12.0\ 10^{-6}$ M
Oleic Acid[23]	—	$9.0\ 10^{-6}$ M

not det.: not determined.

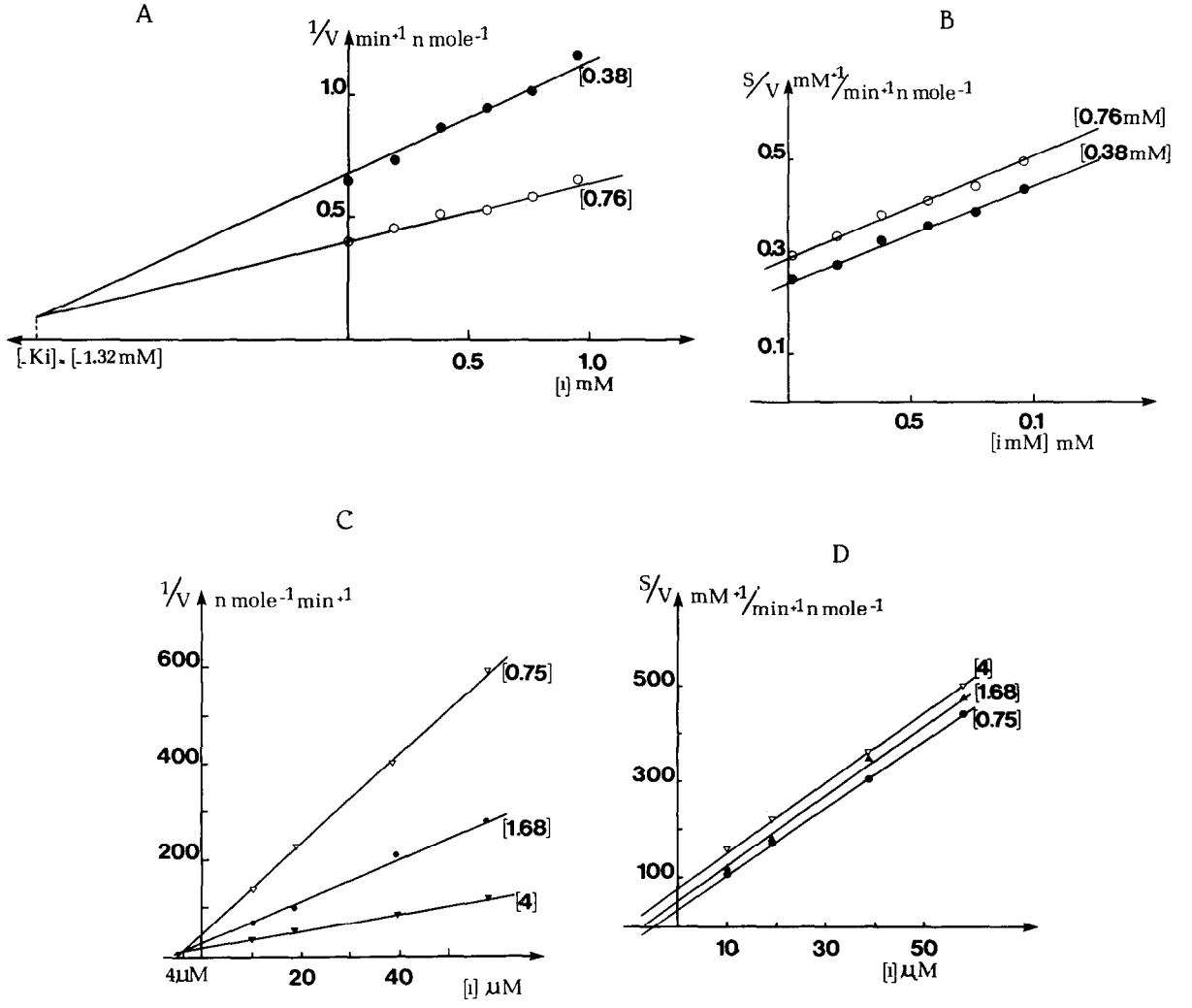

Fig. 1. Determination of the type of inhibition and inhibition constants (K_is) of Oleoyl(Ala)$_2$ProVal towards porcine pancreatic (A, C) and human leukocyte (B, D) elastases according to Dixon [18] (A, B) and Cornish-Bowden [20] (C, D). The enzyme and the inhibitor were preincubated for 15 min before determining the hydrolysis of Suc(Ala)$_3$NA at 410 nm (ε_M nitroaniline = 8800 in 100 mM Tris–HCl buffer, 0.01% Brij 35, 0.02% NaN_3 pH 8.1; temperature, 37°). The concentration of Suc(Ala$_3$)NA is indicated in brackets in the figure.

human leukocyte elastase is situated in the vicinity of the active center of the enzyme. The coupling of hydrophobic substances as fatty acids to this site greatly affected the catalytic activity of this enzyme since oleic acid and oleoyl(Ala)$_2$Proline behave as non competitive inhibitors in the 10^{-5} M range [23] (Table 1).

The K_i of 4 µM for Ol Ala-Ala-ProVal can be compared with that of 9 µM for free oleic acid [23], so that the addition of the peptide moiety is quite small in these conditions. However, modifying the carboxyl end group of the peptide to an aldehyde resulted in a nearly 10-fold reduction of the inhibition constant (K_i Ol(Ala)$_2$ProAlaninal: 0.7 µM) (see Table 1). Also and as previously noticed for other types of peptides, inhibitors of leukocyte elastase, peptides which contained a valine residue at P1(oleoyl(Ala)$_2$ProVal: K_i, 4 µM) were more efficient than peptides bearing an alanine residue at the same site (Oleoyl(Ala)$_2$ProAla; K_i = 5.8 µM) [12]. The adsorption of ^{3}H-oleoyl(Ala)$_2$ProVal on insoluble elastin was investigated as described in Material and Methods. Due to the limited solubility of this oleoyl-derivative, its concentration was kept constant through each set of experiments and the quantity of added micronized insoluble elastin was allowed to vary in the range 0.2–10 mg/ml.

In our experimental conditions, 1 mg of purified insoluble elastin could be saturated with 10.2 nmoles of oleoyl(Ala)$_2$ProValine.

The binding of oleoyl peptides to ^{3}H insoluble elastin considerably affected its rate of degradation by purified elastases as shown on Fig. 2A and 2B. A 40% decrease in elastolysis by porcine pancreatic elastase (Fig. 2A) is noticeable when 10 nmoles of either oleoyl(Ala)$_2$ProVal or oleoyl(Ala$_2$)ProAla were bound to 1 mg of elastin.

When bound to elastin, those lipopeptides exerted

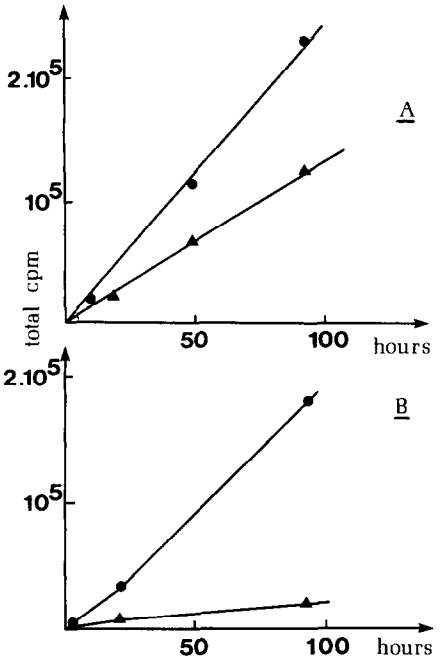

Fig. 2. Effect of preadsorption of several oleoyl peptide derivatives to insoluble elastin on its rate of hydrolysis by elastases. (A) Porcine pancreatic elastase (1 nM): 1 mg of ^3H insoluble elastin (spec. act.: 0.35 10^6 cpm/mg) was pretreated with 100 nmoles of Ol(Ala)$_2$ProValine (▲——▲). The kinetics of dissolution of these pretreated elastins were compared to those of a non-treated sample or treated with similar concentrations of Ala$_2$ProValine (●——●). (B) Human leukocyte elastase (142.5 ng, 5.27 nM); 1 mg of ^3H insoluble elastin (spec. act.: 0.35 10^6 cpm/mg) was pretreated with 80 nmoles of Ol(Ala)$_2$Provaline (▲——▲). The kinetics of the hydrolysis of these pretreated elastins were compared to those of insoluble elastin pretreated in similar conditions with 100 nmoles of Ala$_2$ProValine (●——●). Buffer: 100 mM Tris–HCl, 0.01% Brij 35, 0.02% NaN_3 pH 8.1; temperature: 37°.

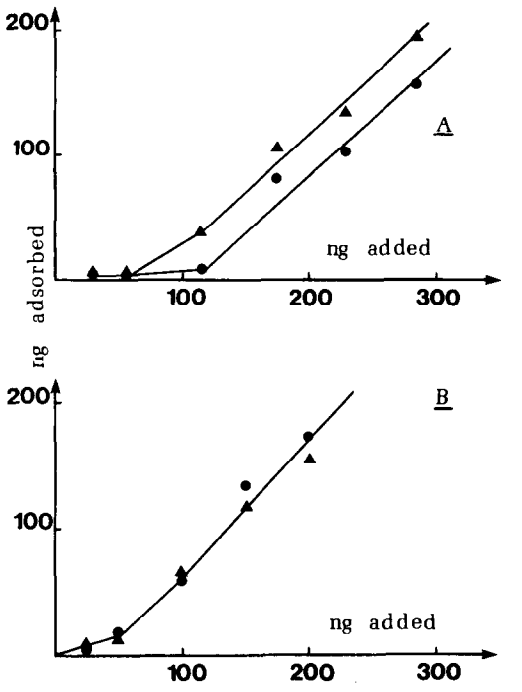

Fig. 3. Quantitative adsorption of elastases on to insoluble elastin and Ol(Ala)$_2$ProValine-insoluble elastin complexes. 10–12 nmoles of Ol(Ala)$_2$ProValine were adsorbed to 1 mg of insoluble elastin in the conditions listed in the text. The pellets were dispersed in 1 ml of 100 mM Tris–HCl pH 7.6 and increasing quantities of elastase(s) were added. The mixture is allowed to stand 2–3 min at room temperature and then centrifuged at 10,000 *g*. Aliquots of the supernatants were withdrawn to quantitate the non adsorbed elastase(s) on Suc(Ala)$_3$NA: (A) Human leukocyte elastase; (B) Porcine pancreatic elastase; ▲——▲, treated elastin; ●——●, untreated elastin.

a more pronounced inhibition on elastolysis by human leukocyte elastase than on porcine pancreatic elastase (Fig. 2B). Their effect appeared to be directly related to their respective K_is as determined on Suc(Ala)$_3$Na. In addition, when ^3H insoluble elastin was pretreated with oleoyl(Ala)$_2$Pro Alaninal in similar experimental conditions, it appeared to be completely refractory to the action of the same amounts of both elastases.

We further investigated the nature of inhibition of elastolysis by oleoyl peptide derivatives. Several interaction mechanisms could be put forward in order to explain the observed inhibition of degradation of insoluble elastin.

A higher affinity of these compounds to elastase relative to elastin, could result in a desorption of the inhibitor previously bound to insoluble elastin. To test this hypothesis, insoluble elastin saturated with ^3H-oleoyl(Ala)$_2$ProValine was incubated for short periods of time during which no appreciable elastolysis was noticed and the releasable radioactivity as well as the amount of the remaining oleoyl peptide bound to insoluble elastin was quantitated.

At different elastase(s) concentrations, up to stoichiometric amounts with respect to the bound inhibitor, no significant variations in the quantity of labelled oleoyl peptides remaining bound to elastin was observed. This finding rules out the above hypothesis concerning the desorption of the oleoyl peptides from insoluble elastin by the enzyme(s).

It was previously shown that the first step of elastin degradation catalyzed by elastases consisted in a rapid adsorption of the enzyme(s) on the insoluble elastin [21, 24]. We then studied the quantitative modification of this adsorption induced by the oleoyl peptide bound to insoluble elastin.

The enzyme concentrations used were of the same order of magnitude (10–200 ng) as those used for enzyme kinetic studies. Figure 3B indicates that insoluble elastin saturated with Ol(Ala)$_2$ProVal (10–12 nmoles/mg) bound porcine pancreatic elastase as efficiently as untreated samples.

The amount of adsorbed human leukocyte elastase was somewhat more important (10–20% increase depending on the concentration of enzyme added) on oleoyl(Ala)$_2$ProValine treated elastin than on the control elastin samples (Fig. 3A). It has to be emphasized that the saturation of elastin by this compound was capable of reducing by 30–50% the rate of degradation of insoluble elastin by porcine pancreatic elastase and by more than 80% it hydroly-

Table 2. Inhibition of elastase(s) in their free and adsorbed form to insoluble elastin by Oleoyl(Ala)$_2$ProValine

State of the protease	Porcine pancreatic elastase (cpm released	% inhib.)	Human leukocyte elastase (cpm released	% inhib.)
Free form	196,450	00	17,310	00
Free form + Ol(Ala)$_2$ProVal	125,310	36.2	3390	80.4
Adsorbed to elastin	126,410	00	7910	00
Adsorbed to elastin + Ol(Ala)$_2$ProVal	8740	30.9	5640	28.7

2.85 μg of either porcine pancreatic elastase or human leukocyte elastase were alternatively mixed with: (a) a solution of 0.386 mM of Ol(Ala)$_2$ProVal in 100 mM Tris–HCl, 0.01% Brij 35 0.02% NaN_3 pH 8.1 for 15 min; we then added 100 μl of a 10 mg/ml ^{3}H insoluble elastin dispersion. The mixture was incubated for 3 hr at 37° under mechanical shaking, and the tubes were centrifuged at 10,000 *g* for 10 min; the releasable labelled elastin peptides were quantitated in the supernatants; (b) 1 mg of ^{3}H insoluble elastin in 0.5 ml of 100 mM Tris–HCl pH 7.6 for 2–5 min; the tubes were centrifuged at 10,000 *g* for 15 min and the supernatant discarded. We then added 1 ml of 100 mM Tris–HCl 0.01% Brij 35, 0.02% NaN_3 pH 8.1 containing Ol(Ala)$_2$ProValine (0.38 mM). The tubes were incubated as above and the radiolysis determined.

sis by human leukocyte elastase. These results exclude the possibility that the inhibitory effect on elastolysis by preadsorbed oleoyl peptides was due to a reduction in the quantity of adsorbed enzyme(s).

High molecular weight natural inhibitors of elastase(s) including alpha$_1$ proteinase inhibitor (α_1PI) were known to inhibit partially elastase(s) when adsorbed previous to insoluble elastin [21]. Active site directed inhibitors as well as low molecular weight inhibitors were found more effective in this respect. Similar sets of experiments were performed in the present study in order to compare the capacity of oleoyl(Ala)$_2$ProValine to inhibit elastase(s), both in their free and adsorbed states to insoluble elastin (Table 2).

It could be evidenced that both forms of porcine pancreatic elastase were equally inhibited by the same amount of inhibitor. In contrast, human leukocyte elastase was found partially resistant to inhibition (28.7% compared to 80.4%) in its adsorbed state to elastin. This may favour the hypothesis that human leukocyte elastase binds preferentially to other sites on the elastin fibres that porcine pancreatic elastase.

DISCUSSION

In the present report, the capacity of oleoyl peptide derivatives to inhibit both pancreatic elastase and human leukocyte elastase was investigated. Human leukocyte elastase differs from porcine pancreatic elastase in its substrate specificity, having a broader activity for longer chain aliphalic amino acids at the bond cleaved (VAL > ALA). It was previously shown that human leukocyte elastase was inhibitable by *Cis* unsaturated fatty acids [23], and on this basis, it was suggested that part of the difference between these proteases resides in the presence of an unusual hydrophobic binding site on the leukocyte enzyme. Recent data established that modifying lysine residue (P_4S_4 site of interaction) to more hydrophobic desmosine-like structure as 2-picolinoyl derivatives, greatly increased the rate of hydrolysis of paranitroanilide substrates [25].

The synthesis of fatty acid peptide derivatives allowed us to confirm the presence of a hydrophobic binding site on the human leukocyte elastase molecule. It was also found that fitting the linked peptide chain to the extended binding site of the enzyme increased the inhibitory capacity of the oleoyl derivatives. Also modifying the carboxylic end group of the fatty acid peptide to an aldehyde further potentiated its inhibitory capacity. As an example, we found that Oleoyl(Ala)$_2$ProAlaninal was more effective than oleoyl(Ala)$_2$ProAlanine to inhibit human leukocyte elastase (K_i = 0.7 μM). According to this model, potent elastase inhibitors could be designed. We recently synthesized Oleoyl-(Ala)$_2$ProValCH_2Cl and compared its inhibitory potential to those of Oleoyl(Ala)$_2$ProVal towards purified rat leukocyte elastase (W. Hornebeck *et al.*, in preparation). It was found that the inhibitory capacity (inverse of the quantity of the compound which reduces by 50% the activity of 1 μg of rat leukocyte elastase), was about 10^5 greater than those of the unmodified lipopeptide. These substances also presented the major advantage to contain a reporter group for elastin. ^{3}H-labelled (in the fatty acid moiety) oleoyl peptides were synthesized and it could be demonstrated that oleoyl(Ala)$_2$ProVal bound in a saturable fashion to insoluble elastin.

Insoluble elastin treated with oleoyl peptide derivatives was found partly resistant to hydrolysis by elastase(s). The inhibition was more pronounced with human leukocyte elastase. These results contrast with the higher susceptibility of oleate-treated elastin to degradation by porcine pancreatic elastase [26]. The mechanism of the inhibition was further investigated and favour the proposition that oleoyl peptides act as bifunctional inhibitors. The fatty acid residues ensure the interaction of the compound with the elastic fibre and the peptide portion with the active site of the elastase. The advantage of such compounds is that they inhibit only elastases near or at the susceptible sites of the elastic fibres.

High molecular weight natural inhibitors (α_1Pi) were known to inhibit only partially elastase(s) when preadsorbed to insoluble elastin [21]. Active site directed inhibitors as well as low molecular weight inhibitors were found more effective in this respect.

A similar set of experiments was performed in the present study in order to compare the capacity of oleoyl(Ala)$_2$ProValine to inhibit elastase(s) both in their free and adsorbed states to insoluble elastin.

It could be evidenced that in both conditions, porcine pancreatic elastase was equally inhibited by

the same amount of inhibitor. In contrast, human leukocyte elastase was found partially resistant to inhibition (28.7% compared to 80.4%) in its adsorbed state to elastin. This may favour the hypothesis that human leukocyte elastase binds preferentially to other sites on the elastin fibres than porcine pancreatic elastase.

As several (or all) elastase-type proteases may well play other important biological roles than degrading elastic fibres [27], such substrate-targeted inhibitors could have a great advantage over other inhibitors which would not distinguish between elastases near or at the elastic fibre and at other sites.

Acknowledgements—Supported by C.N.R.S. (GR 40), INSERM (No. 825012), University Paris-Val de Marne and the Fondation de Recherches sur le Tissu Conjonctif (fellowship to Dr J. Szecsi). We are grateful to Dr Boudier and Dr J. Bieth (Université Louis Pasteur, Strasbourg) for the gift of purified human leukocyte elastase.

REFERENCES

1. J. C. Powers, *Ann. Rev. resp. Dis.* **127**, 54(s) (1983).
2. P. Stone, E. C. Lucey, J. D. Calore, G. J. Snider, C. Franzblau, M. J. Castillo and J. C. Powers, *Rev. resp. Dis.* **124**, 56 (1981).
3. V. Ranga, J. Kleinerman, J. Sorensen and J. C. Powers, *Am. J. Resp. Dis.* **124**, 613 (1981).
4. R. R. Martodam, D. Y. Twumasi, I. E. Liener, J. C. Powers, M. Nishino and G. Krejcarek, *Proc. natn. Acad. Sci. U.S.A.* **76**, 2128 (1979).
5. M. Szigeti, G. Monnier, B. Jacotot and L. Robert, *Connective Tissue Research* **1** 145 (1972).
6. D. M. Kramsch, C. Franzblau and W. Hollander, *J. clin. Invest.* **50**, 1666 (1971).
7. D. M. Kramsch and W. Hollander, *J. clin. Invest.* **52**, 236 (1973).
8. W. Hornebeck and S. M. Partridge, *Eur. J. Biochem.* **51**, 73 (1975).
9. M. Claire, B. Jacotot and L. Robert, *Connective Tissue Research* **4**, 61 (1976).
10. M. Moczar, E. Moczar and L. Robert, *Front. Matrix Biol.* **8**, 1974 (1980).
11. J. C. Powers, B. F. Gupton, A. D. Harley, N. Nishino and R. J. Withley, *Biochim. biophys. Acta* **485**, 156 (1977).
12. B. MacRae, K. Nakajima, J. Travis and J. C. Powers, *Biochemistry* **19**, 3973 (1980).
13. P. M. Starkey and A. J. Barrett, *Biochem. J.* **155**, 255 (1976).
14. A. I. Lansing, T. B. Rosenthal, M. Alex and E..W. Dempsey, *Anat. Rec.* **114**, 555 (1952).
15. L. Robert and W. Hornebeck, in *The Methodology of Connective Tissue Research* (Ed. D. A. Hall), pp. 81. Joyson, Oxford (1976).
16. G. W. Anderson, S. E. Zimmermann and F. M. Callahan, *J. Am. chem. Soc.* **89**, 5012 (1967).
17. R. C. Thompson, *Biochemistry* **12**, 47 (1973).
18. M. Dixon, *Biochem. J.* **55**, 170 (1953).
19. J. Bieth, B. Spiess and C. G. Wermuth, *Biochem. Med.* **11**, 350 (1974).
20. A. Cornish-Bowden, *Biochem. J.* **137**, 143 (1974).
21. W. Hornebeck and H. P. Schnebli, *Hoppe Seyler's Z. physiol. Chem.* **363**, 455 (1982).
22. M. Bihari-Varga, E. Moczar and E. S. Gruber, *Thermal. Annal.* **27**, 287 (1983).
23. B. M. Ashe and M. Zimmermann, *Biochem. biophys. Res. Commun.* **75**, 194 (1977).
24. B. Robert, W. Hornebeck and L. Robert, *Biochimie* **56**, 239 (1974).
25. P. M. Yasutake and J. C. Powers, *Biochemistry* **20**, 3675 (1981).
26. R. Jordan, N. Hewitt, H. Kagan and C. Franzblau, *Biochemistry* **13**, 3497 (1974).
27. W. Hornebeck, D. Brechemier, M. C. Bourdillon and L. Robert, *Connect. Tissue Res.* **8**, 245 (1981).