Inhibition of human leucocyte elastase by ursolic acid

Evidence for a binding site for pentacyclic triterpenes

Qi-Long YING,* Alex R. RINEHART,* Sanford R. SIMON*‡ and John C. CHERONIS†
*Department of Pathology, State University of New York at Stony Brook, Stony Brook, NY 11794, and †Cortech Inc., 6840N Broadway, Unit F, Denver, CO 80221, U.S.A.

INTRODUCTION

Human leucocyte elastase (HLE, EC 3.4.21.37) is a lysosomal proteinase stored in high quantities in the azurophilic granules of polymorphonuclear leucocytes (Dewald et al., 1975). For decades the enzyme has been the subject of intensive study because of its potential for tissue destruction in many disease states, such as pulmonary emphysema and rheumatoid arthritis (Janoff, 1985). Administration of exogenous elastase inhibitors has been proposed as a possible means of protecting tissues from proteolytic attack (Powers, 1983; Groutas, 1987). A number of inhibitors have been isolated from natural sources or synthesized de novo (Groutas, 1987; Bode et al., 1989); some of these have been proved to be effective in animal models (Powers, 1983; Schnebli, 1985; Hoskel et al., 1986). In an effort to find naturally occurring substances which may be utilized as prototype compounds for synthetic inhibitors, we have identified several plant triterpenoids with considerable inhibitory activity towards HLE. Ursolic acid, a pentacyclic triterpene acid, has the greatest potency in this class of compounds. The present paper provides evidence that the inhibition of HLE by the triterpenes is due to reversible binding to a site which lies within a portion of the extended substrate-binding domain of the enzyme.

MATERIALS AND METHODS

Chemicals

Ursolic acid and other pentacyclic triterpenes, except uvaol, were from Carl Roth (Karlsruhe, Germany), and were all of h.p.l.c. standard grade. Uvaol was from Sigma (St. Louis, MO, U.S.A.). The structures of these triterpenes are given in Fig. 1. Boc-Leu-ONp, MeO-Suc-Ala-Ala-Pro-Val-pNA, Suc-Ala-Ala-Ala-pNA and Suc-Ala-Ala-Pro-Phe-pNA were also from Sigma. Bz-Ile-Glu-Gly-Arg-pNA (S-2222) and pGlu-Pro-Val-pNA (S-2484) were from Kabi (Franklin, OH, U.S.A.). Elastin from bovine neck ligaments was from Elastin Products (Pacific, MO, U.S.A.) and was labelled with [14C]formaldehyde according to the procedure of Yu & Yoshida (1979) to a specific radioactivity of 160 nCi/mg. Dulbecco's modified phosphate-buffered saline [DPBS (per litre): KCl, 0.2 g; KH₂PO₄, 0.2 g; NaCl, 8 g; and Na₂HPO₄·7H₂O, 2.171 g; pH 7.2 at 25°C; Hazleton Biologics, Lenexa, KS, U.S.A.)] was used as the buffer throughout the study.

Enzymes

HLE from azurophilic granules was from Athens Research and Technology (Athens, GA, U.S.A.). a-Chymotrypsin (EC 3.4.21.1) and tosylphenylalaninechloromethane ("TPCK")-treated trypsin (EC 3.4.21.4) were from Sigma. Porcine pancreatic elastase (EC 3.4.21.11) was from Elastin Products. The active centre of HLE was titrated with Z-Ala-Ala-Pro-aza-Ala-ONp (Enzyme Systems Products, Livermore, CA, U.S.A.) as described by Powers et al. (1984).

Enzyme assays with synthetic substrates

The activity of HLE was routinely measured with substrate MeO-Suc-Ala-Ala-Pro-Val-pNA (Bieth et al., 1974) in DPBS.
containing 5% (v/v) dimethyl sulphoxide (DMSO), pH 7.2. Assays were performed on a ThermoMax Kinetic Microplate Reader (Molecular Devices Co., Palo Alto, CA, U.S.A.) in 96-well microplates at 25°C. For a typical assay, 50 μl of the substrate in DPBS was first mixed with inhibitor in 50 μl of DPBS containing 10% DMSO. Controls were diluted with DPBS/DMSO alone. The mixture was equilibrated at 25°C for 5 min before initiation of amylolysis by the addition of 50 μl of HLE in DPBS containing 5% DMSO. Release of p-nitroaniline was monitored at 405 nm. The rate of substrate hydrolysis was linear during the first 1 min of recording. Concentrations of p-nitroaniline released were calibrated with equal volumes of standard solutions of p-nitroaniline (Sigma) in the same buffer. HLE activity was also assayed with substrates pGlu-Pro-Val-pNA (Kramps et al., 1983) and Boc-Leu-ONp. Esterolysis of Boc-Leu-ONp was monitored on a computer-controlled LKB Ultraspec-4050 spectrophotometer equipped with reaction-rate software (Pharmacia–LKB, Piscataway, NJ, U.S.A.). The reaction mixtures contained 850 μl of DPBS and 25 μl each of the appropriate concentrations of substrate and inhibitor in DMSO in polystyrene cuvettes at 24°C, to which 100 μl of HLE in DPBS was added to start the reaction. Release of p-nitroaniline was monitored at 405 nm after a 1 min delay to allow the reaction to reach a steady state. After this lag, the reaction rate remained linear. Absorbance was recorded over the next 1 min and was converted into concentration of product according to Beer's law, by using an absorption coefficient (ε_max) of 1.27 × 10^4 M⁻¹ cm⁻¹, determined experimentally from a calibrated stock solution (Sigma).

Other serine proteinases were assayed with the following substrates: a-chymotrypsin, Suc-Ala-Ala-Pro-Phe-pNA (Del-Mar et al., 1979); trypsin, Bz-Ile-Glu-Gly-Arg-pNA (Aurell et al., 1977); and porcine pancreatic elastase, Suc-Ala-Ala-Ala-pNA (Bieth et al., 1974). The buffer used was 0.05 M Tris/HCl containing 0.15 M NaCl and 10% DMSO, pH 8.0.

**Enzyme assays with [14C]elastin**

Elastolysis was determined by using a method modified from that of Yu & Yoshida (1979). A volume of 100 μl of a 10 mg/ml suspension of elastin prepared by diluting our stock of [14C]elastin with unlabelled protein to give a final specific radioactivity of 32 nCi/mg was added to a 1.5 ml microcentrifuge tube. The suspension was diluted with 800 μl of DPBS and mixed with inhibitor in 50 μl of DMSO. Elastolysis was started by addition of 4.5 μg of HLE in 50 μl of DPBS. The reaction was carried out with constant gentle agitation at 37°C for 2 h, and quenched by addition of 400 μl of 25% (w/v) trichloroacetic acid. After centrifugation, labelled peptides in supernatants were counted for radioactivity.

**Kinetic analysis**

Kinetic data were analysed by Dixon (1953) and, when appropriate, Cornish-Bowden (1974) plots, which were generated by non-linear least-squares regression of the raw velocity data and transformation of the best fits into the appropriate forms (Enzfit; Elsevier–BIOSOFT, Cambridge, U.K.).

**RESULTS**

**Inhibitory activity**

Effects of ursolic acid on the amidolytic activity of HLE were first tested with MeO-Suc-Ala-Ala-Pro-Val-pNA as the substrate. Fig. 2 shows a Cornish-Bowden plot obtained for several different ursolic acid concentrations. Ursolic acid can be described as a competitive inhibitor, with a computed inhibition constant, K_i, of 4 μM. Interaction between ursolic acid and HLE appears to be rapid, since a linear rate of amidolysis had already been achieved within the instrument's 30 s dead time between mixing and commencement of data collection. Inhibition is reversible, as judged by partial recovery of amidolytic activity upon dilution of the enzyme–inhibitor mixture. More complete removal of the inhibitor by gel filtration on Sephadex G-25 resulted in full restoration of amidolytic activity. Since the inhibitor and the enzyme appear to form a reversibly dissociable complex which cannot progress further towards the catalytic step, the inhibition constant we have determined, 4 μM, can be considered as a true dissociation constant for ursolic acid from a binding site on HLE.

We next examined the inhibitory activity of ursolic acid using the insoluble substrate [14C]elastin. Fig. 3 shows that ursolic acid can effectively protect against degradation of [14C]elastin by HLE. Addition of 100 μM-ursolic acid inhibits elastolysis more than 90%; complete inhibition is observed with 200 μM-triterpene. However, this concentration exceeds the solubility of ursolic acid in the buffer we have employed, and the inhibitory mechanism may be complicated by the precipitation of the compound on the surface of the insoluble substrate.

**The inhibitory mechanism**

Previous kinetic and X-ray-crystallographic studies have established an extended substrate-binding domain in HLE which interacts with at least five substrate residues on the proximal side,
and two residues on the distal side, of the scissile peptide bond (Nakajima et al., 1979; Wei et al., 1988; Navia et al., 1989; reviewed by Bode et al., 1989). The tetrapeptide substrate, MeO-Suc-Ala-Ala-Pro-Val-pNA, is bound to five subsites within this extended binding domain, designated S₁-S₅. Since ursolic acid displays pure competitive inhibition with this substrate, we can assume that the binding site for the triterpene lies within these five subsites. In order to localize the binding site for ursolic acid more precisely, two shorter peptide substrates, namely pGlu-Pro-Val-pNA and Boc-Leu-ONp, were used. The interactions of these small peptides with the extended substrate-binding domain have not been previously reported. However, it is reasonable to assume that pGlu-Pro-Val-pNA interacts with subsites S₁, S₃, and S₅, and Boc-Leu-ONp with S₁ and S₄. Table 1 shows the effect of substrate length on the kinetics of inhibition by ursolic acid. With pGlu-Pro-Val-pNA as substrate, ursolic acid is still a competitive inhibitor. With Boc-Leu-ONp as a substrate, however, the mode of inhibition changes to non-competitive (Fig. 4).

This transformation points to the critical role of S₄ for binding of ursolic acid to HLE. The simplest explanation for the results is that substrates which occupy S₄ block ursolic acid from this binding site. The amino acid components of S₄ have been identified to be Val-216 and Phe-192 (Wei et al., 1988; Navia et al., 1989; Bode et al., 1989). It is likely that the binding site for ursolic acid and the S₄ subsite for substrate binding share at least some common elements of these residues.

We have considered the role of the carboxy group at position 28 of the ursolic ring system for binding to HLE. The activity of ursolic acid was assayed in the presence of high salt to probe the contribution of electrostatic interactions involving this group. Addition of up to 1 m-NaCl to the assay system does not change the mode of inhibition, but raises the value of $K_i$ from 4 μM to 13 μM. These results might be explained by a conformational distortion of the binding site caused by high salt. To exclude this possibility, we tested the inhibitory activity of uvaol, a pentacyclic triterpene alcohol with a structure identical with that of ursolic acid, except for a hydroxy group instead of a carboxylic group at position 28 (Fig. 1). Uvaol is also a competitive inhibitor, with an inhibition constant, $K_i$, of 16 μM, a value comparable with that of ursolic acid in high salt solution (Table 2). These results demonstrate that electrostatic interactions between the 28-carboxy group and a positively charged group on the enzyme contribute to binding. At the pH employed in this study, arginine residues are the only positively charged amino acid residues in the enzyme (Sinha et al., 1987). Their spatial distribution has been carefully determined by X-ray-crystallographic analysis.
Table 2. Effect of ionic strength on inhibition of HLE by triterpenes

The Table shows the effect of increasing salt concentration and reduction of the 28-carboxy group on inhibition of HLE by ursolic acid. The substrate MeO-Suc-Ala-Ala-Pro-Val-pNA was used in the determination of the inhibition constants as described in text. With this substrate, both ursolic acid and uvaol display competitive inhibition. The results are expressed as means ± S.D., with the numbers of determinations indicated in parentheses.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>$K_i$ (µM)</th>
</tr>
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<tbody>
<tr>
<td>Ursolic acid (12-urs-3β-ol-28-oic acid)</td>
<td></td>
</tr>
<tr>
<td>In DPBS/5% DMSO, pH 7.2</td>
<td>4.4±0.7 (3)</td>
</tr>
<tr>
<td>In DPBS/5% DMSO, pH 7.2, containing 1 M NaCl</td>
<td>13.3±0.6 (3)</td>
</tr>
<tr>
<td>Uvaol (12-urs-3β-28-diol)</td>
<td></td>
</tr>
<tr>
<td>In DPBS/5% DMSO, pH 7.2</td>
<td>15.7±1.5 (3)</td>
</tr>
</tbody>
</table>

Fig. 5. Diagrammatic representation of the binding of ursolic acid to the extended substrate-binding domain of HLE

Table 3. Inhibition constants for various triterpenes

Inhibition constants for pentacyclic triterpenes other than ursolic acid against HLE were determined as described in text. The assays were performed with MeO-Suc-Ala-Ala-Pro-Val-pNA as the substrate in DPBS/5% DMSO, pH 7.2, except for hederagenin, for which DPBS/10% DMSO, pH 7.2, was used. With this substrate, all the triterpenes display competitive inhibition. The results are expressed as means ± S.D., with the numbers of determinations indicated in parentheses.

<table>
<thead>
<tr>
<th>Triterpene</th>
<th>Systematic name</th>
<th>$K_i$ (µM)</th>
</tr>
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<tbody>
<tr>
<td>Oleanolic acid</td>
<td>12-Oleanen-3β-ol-28-oic acid</td>
<td>6.4±1.0 (3)</td>
</tr>
<tr>
<td>Erythrodial</td>
<td>12-Oleanen-3β-28-diol</td>
<td>17.3±0.6 (3)</td>
</tr>
<tr>
<td>Hederagenin</td>
<td>12-Oleanen-3β-23-diol-28-oic acid</td>
<td>62±5.5 (3)</td>
</tr>
<tr>
<td>18β-Glycyrrhetic acid</td>
<td>12-Oleanen-3β-ol-11-one-30-oic acid</td>
<td>185±5 (3)</td>
</tr>
</tbody>
</table>

(Wei et al., 1988; Navia et al., 1989). If an ursolic acid molecule interacts with HLE at subsite S2, the only arginine residue which would be accessible to the carboxy group at position 28 would be Arg-217. This residue has been identified as a component of subsites S3 and S4 (Wei et al., 1988; Navia et al., 1989; Bode et al., 1989).

On the basis of the data described above, we propose that the binding site for ursolic acid extends from a C-terminal limit at S3, covers S4 and S5, and reaches to Arg-217 in the N-terminal direction. Binding of ursolic acid to this site should be responsible for the observed inhibition. Fig. 5 shows a diagrammatic representation of the binding. The conformer of ursolic acid is drawn by inference from the X-ray-crystallographic structure of hederagenin (Roques et al., 1978), a closely related pentacyclic triterpene acid (Fig. 1).

Specificity

The effect of ursolic acid on amidolysis catalysed by α-chymotrypsin, trypsin and porcine pancreatic elastase was examined by using substrates Suc-Ala-Ala-Pro-Phe-pNA, Bz-Ile-Glu-Gly-Arg-pNA and Suc-Ala-Ala-Ala-pNA respectively. With these chromogenic oligopeptide analogues as substrates, ursolic acid behaves as a simple competitive inhibitor of α-chymotrypsin, trypsin and pancreatic elastase, but with much lower potency than that seen with HLE. The inhibition constants were determined to be 37.0±4.6 µM for α-chymotrypsin (n = 3), 157±8 µM for trypsin (n = 3), and 180±8 µM for pancreatic elastase (n = 3). These results indicate that the specificity of inhibition by ursolic acid is not strictly restricted to HLE, and it can be expected that other serine proteinases might also be inhibited by this compound.

Activity of other triterpenes

Table 3 lists the inhibition constants of other pentacyclic triterpenes against HLE. The four compounds in Table 3 are members of the β-amyrin group of triterpenes, ursolic acid and uvaol belonging to the α-amyrin group. Since these triterpenes all have closely related structures (Fig. 1), it is reasonable to assume that they are bound at the same site on HLE as ursolic acid. The contribution of electrostatic interactions to the overall free energy of binding of these inhibitors is apparent from comparison of the relative activities of oleanolic acid and erythrodial, which differ only in the replacement of a carboxy group by a hydroxy group. The results in Table 3 show that oleanolic acid is a stronger inhibitor than erythrodial, supporting the conclusion we have already drawn from comparison of the two corresponding α-amyrins, ursolic acid and uvaol, that binding of triterpene acids to HLE is strengthened through formation of a salt bridge involving the carboxy group of the inhibitor.

DISCUSSION

Ursolic acid can function either as a competitive inhibitor or as a non-competitive inhibitor of hydrolysis of peptide substrates by HLE, depending on the length of the substrate (Table 1). On the basis of this dependence on substrate size, we have concluded that the binding site for triterpenes overlaps subsite S4 of the extended substrate-binding domain of the enzyme. It is conceivable that the binding site lies some distance from the extended substrate-binding domain, but occupation of this site induces a conformational change which extends to S4. An allostERIC mechanism of this sort requires an appropriate set of structural interactions. There have been no reports of such allosteric interactions extending beyond the substrate-binding domain. However, there is both kinetic and structural evidence for an allosteric mechanism by which interactions of substrates or inhibitors with subsite S4 on the enzyme affect the catalytic function. Stein et al. (1987) have shown that interactions between subsite S4 on the enzyme and the residue at position P3 on a peptide substrate markedly affect the kinetics of acylation. The structural basis for the communication between subsite S4 and
the catalytic centre has been attributed to sharing of the side chain of Val-216 by both subsites S₁ and S₄ (Bode et al., 1989). Aside from these interactions between subsites S₁ and S₄, no other obvious conformational interactions can be deduced from the published X-ray-crystallographic data.

Some further understanding of the mechanism of inhibition of HLE by ursolic acid may be obtained by comparing the inhibition with that of the fatty acids. Cook & Ternai (1988) have recently proposed a binding site for oleic acid and related cis-unsaturated fatty acid analogues which lies within the extended substrate-binding domain of HLE. They suggest that the double bond of oleic acid interacts with subsite S₂. Given the nature of the acyl chain of the fatty acid extends to S₁ and the carbohydrate group is pointed towards the side chain of Arg-217. This putative binding site is very similar to the site we have described for ursolic acid, although we find no evidence for extension of the triterpenes towards S₂ and S₄ on the enzyme. Regardless of the precise structural basis of the interactions of these inhibitors with HLE, the present data show that the extended substrate-binding domain of this proteinase has the ability to bind hydrophobic ligands with very different structures. The functional significance of the ability of HLE to accommodate a diversity of hydrophobic structures is uncertain, but it is well known that the enzyme is able to hydrolyse a broad spectrum of proteins, from the major components of the extracellular matrix to many plasma proteins (Bieth, 1989). To bind and degrade various protein substrates, the extended substrate-binding domain should be able to accommodate molecules with different conformations. It seems reasonable to conclude that there is a common structural basis for the broad substrate specificity of HLE and its ability to bind a wide range of hydrophobic ligands which are not substrates, but which do affect substrate binding or catalysis. We implicate Arg-217 as playing a critical role in contributing to the free energy of binding of the negatively charged inhibitors to HLE, but the relative contributions of binding of such inhibitors to the S₁ subsite and their electrostatic interactions with Arg-217 to the detailed molecular mechanism for inhibition remain to be elucidated.

Ursolic acid is widely distributed in the plant kingdom (Simonsen & Ross, 1957). The compound is present in great abundance in the wax-like coatings of apples, pears and other fruits (Sando, 1923; Markley et al., 1935; Markley & Sando, 1937; Croteau & Fagerson, 1971). From the data of Fernandes et al. (1964) we calculate that one mature Bramley apple, for example, may contain over 50 mg of ursolic acid in its peel. Ursolic acid has also been isolated from many medicinal plants, and has a variety of pharmacological activities attributed to it, including anti-arthritis, anti-ulcer and anti-inflammatory activities (Iwu & Ohiri 1980; Gupta et al., 1981; Kosuge et al., 1985; Hirota et al., 1990). The reported anti-inflammatory activities of this compound prompted us to investigate its potential as an inhibitor of HLE. The present data demonstrate that the compound is an effective agent in protecting several synthetic peptide substrates and insoluble elastin against hydrolysis by HLE. Ursolic acid is even effective as an inhibitor of HLE-mediated degradation of 3H,32P-labelled extracellular matrix (Q.-L. Ying, A. R. Rinehart, S. R. Simon & J. C. Cheronis, unpublished work). However, these ‘in vitro’ results do not necessarily confirm that the pharmacological effects observed in vivo can be attributed to elastase inhibition. In the published studies employing animal models, the triterpene was administrated orally (Iwu & Ohiri, 1980; Gupta et al., 1981; Kosuge et al., 1985). Given the low solubility of ursolic acid in aqueous solution and the value of the inhibition constant, this compound may not reach a sufficiently high concentration at an inflammatory focus to inhibit the levels of HLE released by large numbers of recruited neutrophils (Bieth, 1984). Alternative mechanisms of action may contribute to the observed pharmacological effects. Such alternative mechanisms are even more likely to account for the efficacy of oleanolic acid, hederagenin and 18β-glycyrrhetic acid, which have also been reported to be effective anti-inflammatory agents (Finney & Somers, 1958; Gupta et al., 1969; Bhargava et al., 1970; Chaturvedi et al., 1976; Takagi et al., 1980). However, as a class of readily available natural products of limited toxicity, pentacyclic triterpenes can serve as parent compounds for a new class of HLE inhibitors with greater potency.

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