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X-ray crystal structure of the complex of human leukocyte elastase (PMN elastase) and the third domain of the turkey ovomucoid inhibitor

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Orthorhombic crystals diffracting beyond 1.7 A resolution, have been grown from the stoichiometric complex formed between human leukocyte elastase (HLE) and the third domain of turkey ovomucoid inhibitor (OMTKY3). The crystal and molecular structure has been determined with the multiple isomorphous replacement technique. The complex has been modeled using the known structure of OMTKY3 and partial sequence information for HLE, and has been refined. The current crystallographic R-value is 0.21 for reflections from 25 to 1.8 Å resolution. HLE shows the characteristic polypeptide fold of trypsin-like serine proteinases and consists of 218 amino acid residues. However, several loop segments, mainly arranged around the substrate binding site, have unique conformations. The largest deviations from the other vertebrate proteinases of known spatial structure are around Cys168. The specificity pocket is constricted by Val190, Val216 and Asp226 to preferentially accommodate medium sized hydrophobic amino acids at P1. Seven residues of the OMTKY3-binding segment are in specific contact with HLE. This interaction and geometry around the reactive site are similar as observed in other complexes. It is the first serine proteinase glycoprotein analysed, having two sugar chains attached to Asn159 and to residue 109.

Key words: human leukocyte elastase/ovomucoid inhibitor/serine proteinase/complex/crystal structure

Introduction

Human leuckocyte elastase (HLE) is a major protein of the azurophilic granules of human polymorphonuclear granulocytes. It is a serine proteinase with optimal activity around neutral pH. It degrades not only elastin, but also various other tissue proteins like collagens and proteoglycans, and plasma factors like fibrinogen, fibrin and anti-thrombin III. *In vivo* HLE presumably plays a role in the digestion of damaged tissues and bacterial degradation products.

The most important physiological inhibitor of HLE in human plasma is α_1-proteinase inhibitor (see for example, Travis and Salvesen, 1982; Löbermann *et al.*, 1984). Excessive leakage of the enzyme from leukocytes and/or reduced levels of natural inibitors in cases of α_1-proteinase inhibitor deficiency can lead to various types of tissue damage, as observed in pulmonary emphysema, cystic fibrosis, rheumatoid arthritis, adult respiratory distress syndrome and other diseases (reviewed by Janoff, 1985). Furthermore, HLE has been found to be involved in the pathogenesis of clotting disorders and other inflammatory processes (reviewed by Fritz *et al.*, 1984). Its importance as a pathogenic agent in various disease processes in humans has raised interest in searching for appropriate protein inhibitors and designing potent synthetic inhibitors (reviewed by Stein *et al.*, 1985).

HLE is said to be a strongly basic glycoprotein of mol. wt close to 30 000 daltons (Ohlsson and Olsson, 1974; Baugh and Travis, 1976). The heterogeneity of HLE observed by ion-exchange chromatography has been assigned to differences in the carbohydrate moiety which could amount to ~20% of the mol. wt. An amino-terminal sequence of HLE comprising the first 74 amino acids has been published (Travis *et al.*, 1980) and recently confirmed in essence (Heck *et al.*, 1985). Enzymatic studies with small peptidyl substrates as well as inhibitory studies with synthetic peptidyl inhibitors suggested an extended substrate binding site of HLE and showed a strong preference for valine as P1 residue (reviewed by Stein *et al.*, 1985).

We have obtained single crystals of HLE in complexes with different inhibitors (Bode, unpublished data). The complexes formed with the third domain of turkey ovomucoid inhibitor (OMTKY3: Bogard *et al.*, 1980) yielded crystals suitable for high resolution X-ray crystal analysis. An analogous chymotrypsin-OMTKY3 complex has recently been crystallized and analysed by Read *et al.* (1984) but not yet published in detail. We have almost completed the crystallographic analysis of the HLE-OMTKY3 complex. In this paper we present the main results of the analysis and describe the most important structural features of this complex. The full structure and experimental details will be communicated elsewhere.

Results

The numbering and identification of the HLE amino acid sequence used in this work is based on preliminary and partial chemical sequence data and on electron density interpretation. Although we feel confident about the correct assignment, the sequence must still be regarded as tenative. The chymotrypsinogen numbering scheme (Hartley and Shotton, 1969) is used. Inhibitor residues have an I after the sequence number (Kato *et al.*, 1978). According to the current X-ray model HLE consists of 218 amino acid residues. The single chain molecule starts with Ile16 which is involved in the internal salt bridge with Asp194, as observed in all trypsin-like serine proteinases. As shown in Figure 1, the last residue in the HLE chain observable in the electron density map is Gln243 which is completely defined by its side chain and by its carboxamide group. The main chain is currently only undefined at residue Asp147. For ~25 residues there is presently no adequate electron density of the side chains.

Figure 2 shows the α-carbon drawing of the current model of the HLE-OMTKY3 complex. Its OMTKY3 component differs from the same inhibitor as observed in the complex with the *Streptomyces griseus* proteinase B (SGPB) (Fujinaga *et al.*, 1982; Read *et al.*, 1983) and from the closely related third domain of silver pheasant analyzed in the free state (Bode *et al.*, 1985) only for ~0.4 Å if all α-carbons from Val7I to Cys56I

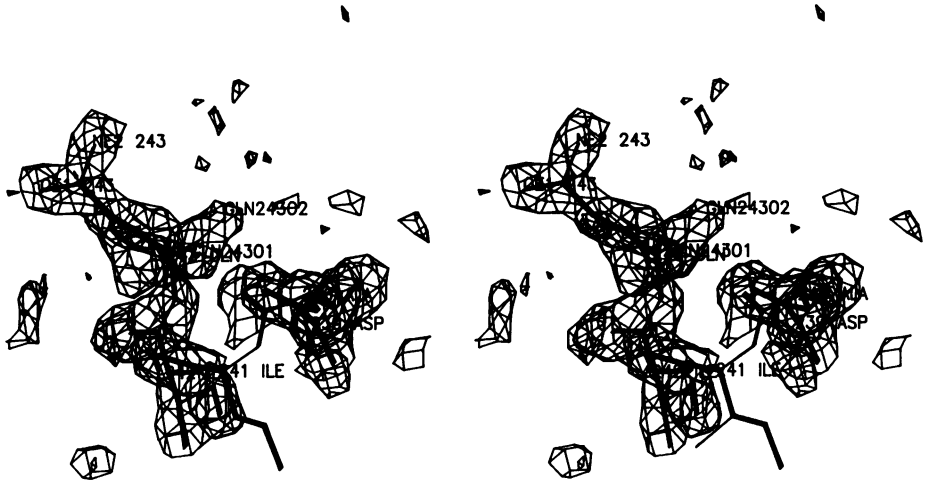

Fig.1. Section of the Fourier map around the carboxy terminus of HLE superimposed with HLE segment Asp239 to Gln243. Contour surface is at 1σ.

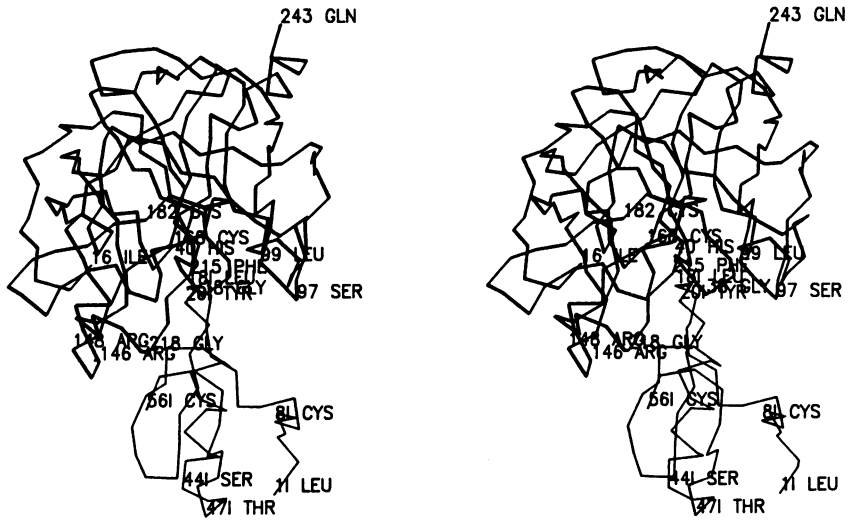

Fig. 2. α-Carbon drawing of the complex formed by HLE (bold connections) and OMTKY3 (thin connections).

are compared. The inhibitor component of the HLE-OMTKY3 complex is tilted with respect to the enzyme component compared with OMTKY3 in the SGPB complex if only the proteinases are optimally superimposed. A similar observation has been made in the chymotrypsin–OMTKY3 complex (Read *et al.*, 1984). The polypeptide chain of OMTKY3 in the HLE complex can unambiguously be traced from Ala2I to Cys56I, i.e. only the amino terminal Leu1I seems to be disordered. The amino-terminal segment to Cys8I is arranged in an extended conformation, mainly stabilized by interactions with a symmetry-related protein molecule. This is a rather flexible segment as it adopts various conformations in different crystal structures of ovomucoids and the pancreatic secretory trypsin inhibitor (PSTI) (Weber *et al.*, 1981; Papamokos *et al.*, 1982; Bolognesi *et al.*, 1982).

Only seven residues of the proteinase binding loop Pro14I(P5) to Tyr20I(P2′) are involved in specific, direct contacts with the enzyme. Quite weak van der Waals contacts are furthermore possible for Arg21I, Gly32I, Asn33I and Asn36I (mainly with the side chains of Phe192 and Leu143 of HLE). Favourable main chain–main chain hydrogen bonds are formed in the enzyme-inhibitor interface between Cys16I N and Val216 O, between Cys16I O and Val216 N, between Leu18I N and Ser214 O, between Leu18I O and Gly193 N and Ser195 N, and between Tyr20I N and Phe41 O.

Figure 3 gives a view of the S1-binding site showing the location of the P1 residue Leu18I between main chain segments 214–216 and 191–192 of the specificity pocket of HLE. As in the case of the porcine pancreatic elastase (PPE), the pocket is constricted towards its bottom by more bulky side chains of residues Val216, Val190 and Asp226. Due to a smaller residue 213 (Ala) and due to a changed geometry of the loop 216–220, however, the entrance of the pocket seems to be more open. The back of this pocket, around Gly189, is quite differently organized and contains several polar groups, including internally fixed solvent (water) molecules.

The side chain of the P2 residue Thr17I is in weak contact P1′ with the HLE side chains of Leu99 and His57. The disulfide bridge formed with Cys16I (P3) is in van der Waals contact with Phe192. The side chain of Ala15I (P4) runs parallel with Arg217. This would allow negatively charged residues at P4 and/or P5 to interact electrostatically in a favourable manner. Polar P1′ residues (whose side chains are not involved in an intra-residue hydrogen bond as is Glu19I in OMTKY3) could interact with the side chain of Asn61. Finally, the P2′ side chain of Tyr20I points towards Ile151 of HLE.

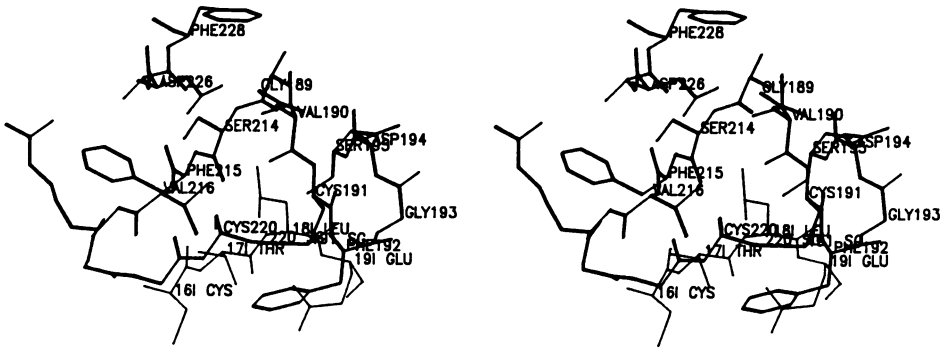

Fig. 3. Stereo view into the specificity pocket, showing HLE segments 189–195, 213–220 and 226–228 (thick lines) and part of the binding segment of OMTKY3 from Cys16I to Glu19I (thin lines).

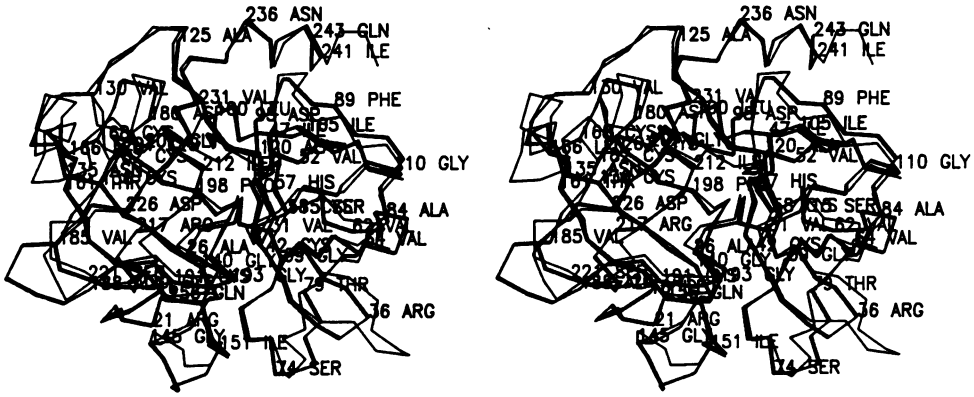

Fig. 4. α-Carbon drawing of the polypeptide chain of HLE (thick lines) optimally superimposed with PPE.

The polypeptide chain of the HLE component is superimposed in Figure 4 with the refined 1.65 Å model of PPE (E.Meyer, unpublished results). In its general chain fold HLE is a typical trypsin-like serine proteinase resembling bovine chymotrypsin (Cohen *et al.*, 1981; Tsukada and Blow, 1985), trypsin (Bode and Schwager, 1975), porcine kallikrein (Bode *et al.*, 1983), pancreatic elastase (Sawyer *et al.*, 1978) and the rat mast cell protease II (Reynolds *et al.*, 1985), the vertebrate serine proteinases so far known by their tertiary structures.

About 170 α-carbon atoms of the present HLE-model have clearly recognizable equivalent residues in chymotrypsin, pancreas elastase and rat mast cell protease (coordinates as obtained from the Brookhaven Protein Data Bank (Bernstein *et al.*, 1977). The root mean square deviation of these residues is ~0.8 Å [determined with OVRLAP (Rosmann and Argos, 1975; adopted by W.S.Bennett)]. The similarity with pancreas elastase is further emphasized by the common disulfide bridges 42–58, 136–201, 168–182 and 191–220.

At some sites the HLE chain deviates, however, from the related proteinases. On the basis of topological equivalences we propose an alignment relative to chymotrypsinogen with deletions at residues 37, 92, 149, 167, from 169 to 176 (eight deletions), from 203 to 206 (four deletions) and at the carboxy terminus (two deletions) and with insertions from 63A to 63C (3), from 168A to 168B (2) and at 220A. All of these changes in chain length occur in surface stretches or loops.

As can be seen in Figure 4, significant deviations are observed in the HLE molecule compared with PPE at residue 25, at the tight turn between Leu35 and Gly39 (four deletions), between Asn61 and Val64 (two insertions), in the 'calcium loop' especially between residues 76 and 78, from Phe89 to Leu100 (three deletions), around Gly131, in the 'autolysis loop' between Arg146 and Arg148, at the 'methionine loop' from Thr164 to Asn180 (11 deletions), from Leu184 to Ala188 (one deletion), from Asn202 to Gly207 (four deletions), from Arg217 to Gly219 (one deletion), from Ser221 to Leu223, and at the carboxy-terminus starting with Asp239 (two deletions).

The largest deviation occurs around the 'methionine loop' (Figure 5). There is no indication of an 'intermediate helix' which is normally observed in the vertebrate proteinases around Cys168. The HLE chain deviates from PPE beginning with Thr164. As a consequence Cys168 is, in contrast to its disulfide bridging partner Cys182, already considerably shifted (Figure 5). The loop segment between Cys168 and Cys182 is of much smaller size and quite different conformation. As shown by Figure 6, this short loop is well defined by appropriate electron density. Residue 180 topologically equivalent to Met180 in most other serine proteinases [except, e.g., the rat mast cell protease II (Woodbury *et al.*, 1978)], is in HLE asparagine (or aspartate). Its side chain is involved in a hydrogen bond with the guanidyl group of the spatially adjacent Arg177 (Figures 5 and 6).

In the present Fourier map two different carbohydrate chains seem to be covalently linked to residues 159 and 109. At the first site, Asn159-Val160-Thr161, two pyranose sugars are fully defined and a third to a minor extent by density (Figure 7). A 2-acetamido-2-deoxy-β-D-glucopyranose (labelled as residue 401 in Figure 7) is N-glycosidically attached to Asn159 and further 1,6-linked to a terminal α-L-fucopyranose (residue 402 in Figure 7) and probably 1,4-linked to another hexose (residue 403 in Figure 7). At the second site another 2-acetamido-2-deoxy-β-

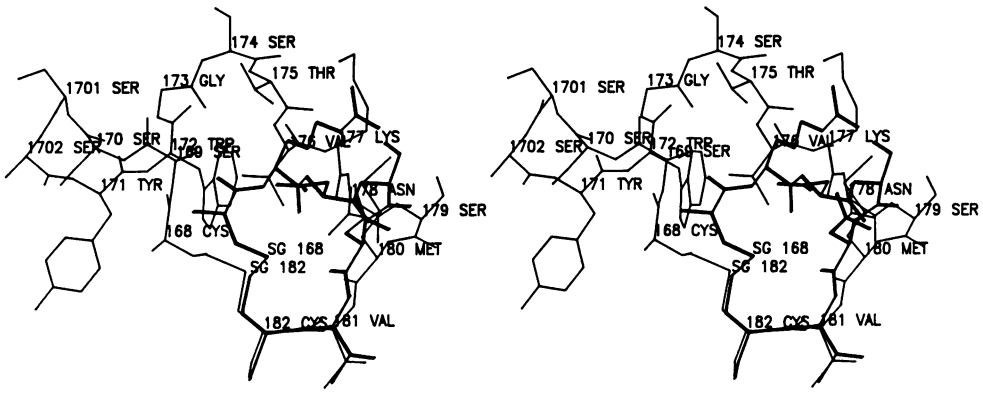

Fig. 5. HLE segment Cys168 to Cys182 (thick lines) optimally superimposed with the analogous segment of PPE.

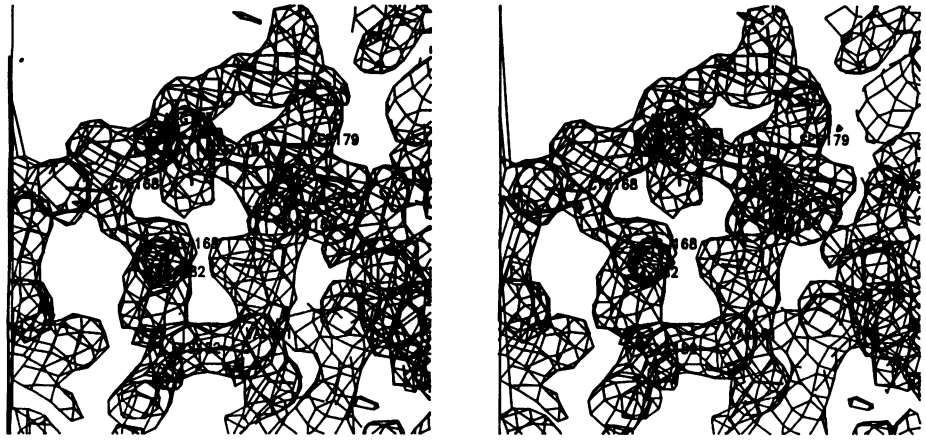

Fig. 6. Section of the Fourier map around loop segment Cys168-Cys182 superimposed with the HLE model. Contour surface is at 1σ.

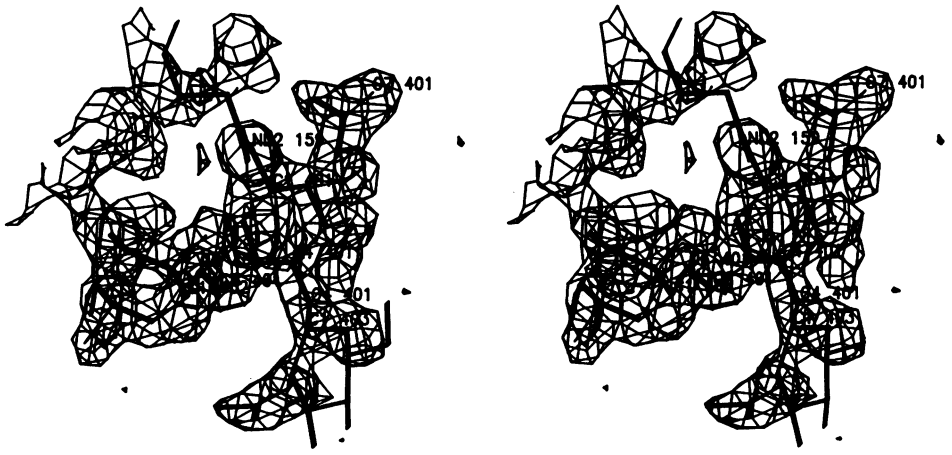

Fig. 7. Section of the Fourier map around the first carbohydrate attachment site at Asn159, superimposed by Asn159 and three adjacent, defined hexoses. Contour surface is at 1σ.

pyranose which is probably of a glucose type is attached to a branched amino acid residue 109 (presumably an asparagine) and furthermore α-1,6-linked to a fucose-like hexose and through its oxygen 4 to another poorly defined sugar.

The active site residues are similarly positioned as in the other vertebrate serine proteinases of known spatial structure and are engaged in very similar hydrogen bonding networks as observed in the analogous trypsin (Huber *et al.*, 1974), trypsinogen (Bolognesi *et al.*, 1982) and kallikrein (Chen and Bode, 1983) complexes formed with the bovine pancreatic trypsin inhibitor and PSTI, respectively. In particular, the side chain conformation of Ser195 and its interaction with the scissile peptide bond Leu18I-Glu19I of the inhibitor which is still intact are similar to those complexes. Thus this HLE−OMTKY3 complex can be most adequately described as a distorted Michaelis complex with a O^{γ} (Ser195) $-C^{1}$ (Leu18I) distance of 2.6 Å and ω_1 and χ_c values of 172° and −5° respectively. These values describe the distortion of the scissile peptide group. They indicate similar

distortion to other complexes but the magnitudes are smaller as the geometry of the peptide groups has been restrained to planarity at the present stage of refinement (for definitions and comparison see Marquart *et al.*, 1983).

Discussion

As HLE plays an important role in human pathophysiology, a more detailed knowledge of its molecular properties is desirable. The present crystal structure analysis in connection with the parallel sequence analysis is an important step forward. According to the present state of the analysis, HLE is a typical serine proteinase consisting of 218 amino acid residues and carrying two carbohydrate chains. This would correspond to a mol. wt for the protein moiety of ~23 000 daltons. The carbohydrate shows electron density to account for a mol. wt of ~1000 daltons, but is presumably larger. The intermolecular space in the crystals would allow the accommodation of longer carbohydrate chains. The much larger mol. wt values given in the literature are possibly adulterated by the carbohydrate which can lead to an overestimation in SDS–polyacrylamide gel electrophoresis.

HLE shows large deviations to the other vertebrate proteinases so far known by their spatial structure. We doubt that current strategies (Greer, 1981; Blundell *et al.*, 1983) of modelling proteins of unknown spatial structure by combining three-dimensional elements from known related structures would have had success in this case. As outlined above, considerable 'global' deviations are observed at surface loops around residues 36, 63, 95, 151, within the 168–182 loop, around the specificity pocket (around residue 186 and between 216 and 225), around residue 131 and at the carboxy terminus. With the exception of the last two, these sites are parts of loop segments which form the substrate binding site or are arranged around this binding site. Thus they strongly influence the specificity of HLE towards large protein substrates.

The HLE is a carbohydrate-containing serine proteinase. Unfortunately the identification of carbohydrate residues and their linkage resides predominantly on the electron density interpretation. This density is however very clear. One of the sugar chains is glucosamine-based, attached to Asn159 through an N-glycosidic linkage. For the second site at present there is good indication for another glucosamine-based carbohydrate likewise N-glycosidically linked to an asparagine residue. In conjunction with other known glycoprotein structures these structural elements will further improve our understanding of the prerequisites of protein glycosylation.

The molecular structure of HLE will further help to understand its specificity towards small synthetic and natural inhibitors and substrates. The mode in which peptidyl moieties bind to this enzyme are clearly sketched by the proteinase-binding loop of the OMTKY3 component. The preference for distinct amino acids at the different peptide sites can already be understood qualitatively.

The analysis of an HLE-ovomucoid complex seems to be particularly helpful in the design of suitable HLE inhibitors as in the laboratory of M.Laskowski,Jr. >100 closely related third domains have been prepared and characterized by their sequences. They exhibit a striking hypervariability of the proteinase-binding loop (Laskowski and Kato, 1980). The affinity and rate data for the interaction with HLE can be determined (Ardelt and Laskowski, 1983) and the corresponding HLE complexes modelled in order to rationalize the measured data in terms of structure. Such projects can be extended to other known Kazal-type inhibitors and, due to the observed similarity in the binding loops of most proteinase inhibitors, to other 'small' protein inhibitors of HLE, like eglin (Bode *et al.*, 1986). All these approaches will possibly help to design HLE-directed protein inhibitors with specifically adjusted properties.

Materials and methods

HLE was isolated from granule extracts of normal human granulocytes by a procedure previously described for elastase from porcine granulocytes (Geiger *et al.*, 1985). The sample used represents a pool of elastase peaks obtained from ion-exchange chromatography on carboxymethyl cellulose CM52. This sample is a single chain protein, which in polyacrylamide gel electrophoresis separates into three major and two minor fractions. As it has been shown to be essentially free of contaminating proteins, these differences are likely to reflect differences in glycosylation.

The complex of HLE with OMTKY3 was crystallized in the orthorhombic space group $P2_12_12_1$ with cell constants $a=73.05$ Å, $b=72.55$ Å, $c=52.45$ Å, from 0.45 to 0.5 M sodium citrate, pH 10, using the hanging drop vapour diffusion method. These crystals grow to sizes up to $0.3 \times 0.3 \times 0.5$ mm^3 and diffract beyond 1.7 Å resolution. X-ray intensity data were collected with Cu Kα radiation using films on rotation cameras at 2°C. From three native crystals, 62 000 reflections above the 1σ significance level were collected to 1.7 Å resolution and evaluated (Schwager *et al.*, 1975). They were merged and scaled yielding 21 000 unique reflections (corresponding to 75% of all data expected to 1.7 Å resolution). The final Rmerge [defined as $\Sigma I(i) - <I>/\Sigma I(i)$] is 0.083.

In an attempt to find orientation and translation of the elastase–inhibitor complex in the crystals, Patterson search techniques (Huber, 1965) were applied by using a synthetic model of chymotrypsin–OMTKY3 which had been constructed on the basis of the trypsinogen-PSTI complex (Bolognesi *et al.*, 1982) and OMSVP3 (Bode *et al.*, 1985), and the SGPB–OMTKY3 complex (Fujinaga *et al.*, 1982). Because these attempts failed the method of multiple isomorphous replacement (MIR) had to be used. Five useful heavy atom derivatives were found, which could successfully be interpreted and refined yielding 5500 multiple isomorphous phases with an overall figure-of-merit of 0.67 to 3 Å resolution. These MIR phases allowed us to calculate a 3 Å Fourier map which was further improved by solvent flattening (Wang, 1983). This phase-combined 3 Å Fourier map allowed the placement of the chymotrypsin–OMTKYY3 complex as a starting model of the HLE–OMTKY3 molecule. Both components of the complex were separately adjusted to this map in real space. Both polypeptide chains were then fitted to the Fourier map on a PS300 interactive display system using a modified version of FRODO (Jones, 1978). The partial sequence information available at the begin of modelling (a long amino-terminal segment, a shorter carboxy-terminal stretch and several short intervening segments) was useful in assignment. The model of the complex was refined by means of the energy constrained crystallographic refinement EREF (Jack and Levitt, 1978) in a cyclic manner with repetitive inspections of phase-combined Fourier maps at the display system. Electron density interpretation helped to align some of the missing peptides and to correct their current sequence. The corrected amino acid sequence, in turn, facilitated the single residue assignment in the spatial model. The current R-value (defined as $\Sigma(|Fobs| - |Fcalc|)/(\Sigma|Fobs|)$ for 20 000 data from 25 to 1.8 Å resolution is 0.21.

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