Structure of the proteinase inhibitor eglin c with hydrolysed reactive centre at 2.0 Å resolution

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The inhibition of serine proteinases by both synthetic and natural inhibitors has been widely studied. Eglin c is a small thermostable protein isolated from the leech, Hirudo medicinalis. Eglin c is a potent serine proteinase inhibitor. The three-dimensional structure of native eglin and of its complexes with a number of proteinases are known. We here describe the crystal structure of hydrolysed eglin not bound to a proteinase. The body of the eglin has a conformation remarkably similar to that in the known complexes with proteinases. However, the peptide chain has been cut at the 'scissile' bond between residues 45 and 46, presumed to result from the presence of subtilisin DY in the crystallisation sample. The residues usually making up the inhibiting loop of eglin take up a quite different conformation in the nicked inhibitor leading to stabilising contacts between neighbouring molecules in the crystal. The structure was solved by molecular replacement techniques and refined to a final R-factor of 14.5%.

Eglin c; Serine proteinase inhibitor; Hydrolysed reactive loop; Crystal structure

1. INTRODUCTION

Serine proteinases have an active site involving three catalytic residues: serine, histidine and aspartate. They fall into two main families: the trypsin, chymotrypsin and elastase group, and the subtilisin-like enzymes. The enzymes within each individual family have diverged from a common ancestor, but the two families show no similarity in sequence or three-dimensional structure and appear to have converged on the same solution for proteinase activity [1]. The serine proteinases have been extensively studied by a variety of biochemical and genetic techniques and their mechanism and specificity is well understood [2,3]. In addition the three-dimensional structures have been determined for several enzymes from both families, as well as their complexes with synthetic and naturally occurring protein inhibitors and for many site-directed mutants [4–7].

In spite of the difference in protein fold for the two families their binding sites show certain similarities. In both cases there are clefts on the surface of the enzyme with defined sites for a number of amino acid residues on each side of the scissile bond of the substrate [8]. The nature of these sites confers a different specificity on the various enzymes. Thus trypsin hydrolyses peptides on the carboxy side of arginine and lysine, chymotrypsin prefers large aromatic residues, and elastase small side

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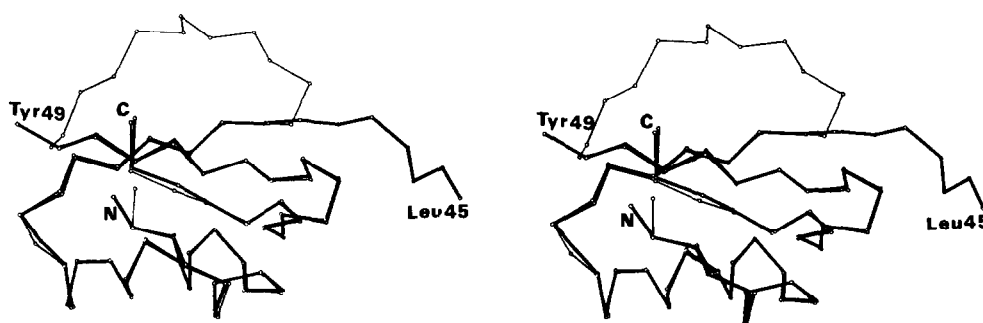
chains. In contrast subtilisins are relatively broad in their specificity [1].

Eglin c is a naturally occurring polypeptide from the leech Hirudo medicinalis [9]. It powerfully inhibits both chymotrypsin [10] and subtilisin [4,11], and in addition the leukocyte proteinases elastase and cathepsin G [12], through the formation of 1:1 complexes. It has thus attracted particular attention as a possible therapeutic agent against various pathogenic elastic tissue agents, blood clotting disorders and inflammatory processes. Native eglin contains 70 amino acids. The 6 or 7 N-terminal residues of native eglin appear to be cleaved off by many proteinases. The three-dimensional crystal structures of several complexes of eglin with members of both families have been determined. Only complexes with subtilisins are discussed below. Most of the eglin molecule makes up a central rigid body, with a hydrophobic core. The structure of this body is essentially the same in all complexes studied. The inhibitory loop, residues 40–50, points away from the surface of this body and mimics the substrate when bound to the active site of the proteinases. The peptide bond between residues 45 and 46 lies in the position of the scissile bond of the substrate. The body and the loop of eglin take up different relative conformations in the various complexes studied, the loop bending about hinges at each end.

The solution structure of eglin c was determined by NMR techniques [13]. It was attempted unsuccessfully for many years to crystallize eglin on its own [4], but only recently the wild-type structure of eglin was re-

Fig. 1. Stereo view of the Cα positions of intact eglin taken from the mesentericopeptidase complex (thin lines) superimposed on the cleaved inhibitor from the present study (thick lines). Residues 39 to 45 take up a completely different conformation in the nicked eglin, and the N-terminal residue moves slightly. The body of the molecule retains the same conformation as in the complex.

ported [14]. Eglin has been used to form complexes of proteinases to avoid autolysis of the enzymes. We have crystallized complexes with both thermitase and mesentericopeptidase and determined their structures [15,16]. Other groups have solved the structure of subtilisin Carlsberg [4,11] and thermitase [17] with eglin. More recently we tried to co-crystallize eglin with subtilisin DY, which differs by only 31 amino acids from Carslberg, out of 270 [18].

2. MATERIALS AND METHODS

Crystals of eglin c were grown at 16°C by hanging drop vapour diffusion [19] under the conditions described for the complex between eglin and subtilisin Carlsberg [4]. Eglin c (10 mg/ml) in 0.02 M acetic acid was mixed at a ratio of 1.2:1.5 with subtilisin DY (around 8 mg/ml) in 0.1 M sodium acetate, pH 6.0. Crystals were obtained in a small percentage of the experiments.

The space group of the crystals was $P4_3$ with unit cell parameters of a and $b = 42.0$ Å and $c = 35.9$ Å. As judged by this unit cell surprisingly the crystals appeared to contain no proteinase. This is a classic example of serendipity in crystallisation conditions; one would not imagine that addition of subtilisin DY would be necessary to obtain crystals of eglin. The asymmetric unit finally contains one molecule of hydrolysed eglin. The packing density parameter V_m was calculated to be 2.3 Å³/Dalton [20]. A single crystal mounted in a glass capillary was used to collect data on a conventional sealed tybe X-ray source with MoK_α radiation and a graphite monochromator. A MAR imaging plate detector was used to collect data up to 2.0 Å resolution. The reduced data set contains 4,131 reflections and shows a completeness of 98%, with 93% of those measured having intensities greater than three standard deviations. The R_{merge} defined as

$R(I) = \Sigma |I - <I>| \Sigma I$ is 6.0%.

The structure solution was achieved by molecular replacement with an eglin model from the mesentericopeptidase complex and was not straightforward. It was eventually achieved using a new rotation and translation program AMORE [21]. After adjustment of the position as a single rigid body, the model was initially refined using an automatic refinement procedure developed in EMBL Hamburg [22]. After this step the rebuilding of the model was performed by inspecting $2F_o - F_c$ synthesis and $F_o - F_c$ difference Fourier synthesis, and 'omit' maps where particular parts around the N terminus and in the region of the hydrolysed bond were omitted from phase calculation. All graphics work was carried out using the program FRODO [23] on an Evans and Sutherland PS 300 system. Finally the model was refined

using stereochemically restrained least squares minimisation [24] to an R factor of 14.5%. The mean temperature factor for all main chain atoms was 11.1 Å² and for all side chain atoms 15.5 Å². The r.m.s. errors in bond lengths compared to the target values was 0.024 Å. The torsion angles ω of the peptide planes have mean deviation of 2.8° with respect to the ideal value of 180°.

3. RESULTS AND DISCUSSION

Eglin c has no disulfide bonds but is even then very stable against denaturation by heat. The structure consists of a well defined rigid core, with an α-helix on one side, a four stranded β-sheet and a binding loop with high mobility. Eglin c is a member of the potato inhibitor family [25] where the homologous structure of the proteinase inhibitor CI-2 from barley seeds has been determined in complex with proteinases by X-ray crystallography [26]. The structure of eglin in the crystal is shown in Fig. 1, overlapped with intact eglin as in the mesentericopeptidase complex. A most striking feature is that the inhibitor has been cleaved at the scissile bond: 'This was the most unkindest cut of all' [27]. The body of eglin maintains the same structure as in the complex, with an r.m.s. difference in Cα positions of only 0.3 Å. This indicates high rigidity and high stability for the core of eglin: its structure is maintained even when the loop has been cleaved in the presence of proteinase. Residues 40 to 45 on the N-terminal side of the scissile bond have a well defined position in the crystal with clear electron density, Fig. 2, quite different from that in the intact loop in the complex. Residues 49 and 50 also take up well defined new positions. There is however no density for residues 46–48 and it is not clear whether these are disordered or have been cleaved from the rest of the peptide chain. Also there is no electron density for the first 6 residues at the N terminus as observed in other complex structures. This could be due to local disorder or to specific hydrolysis. In the recently reported free eglin c structure the N terminus was clearly visible in the electron density. Residues 40 to 45 point away from the body of eglin in the present struc-
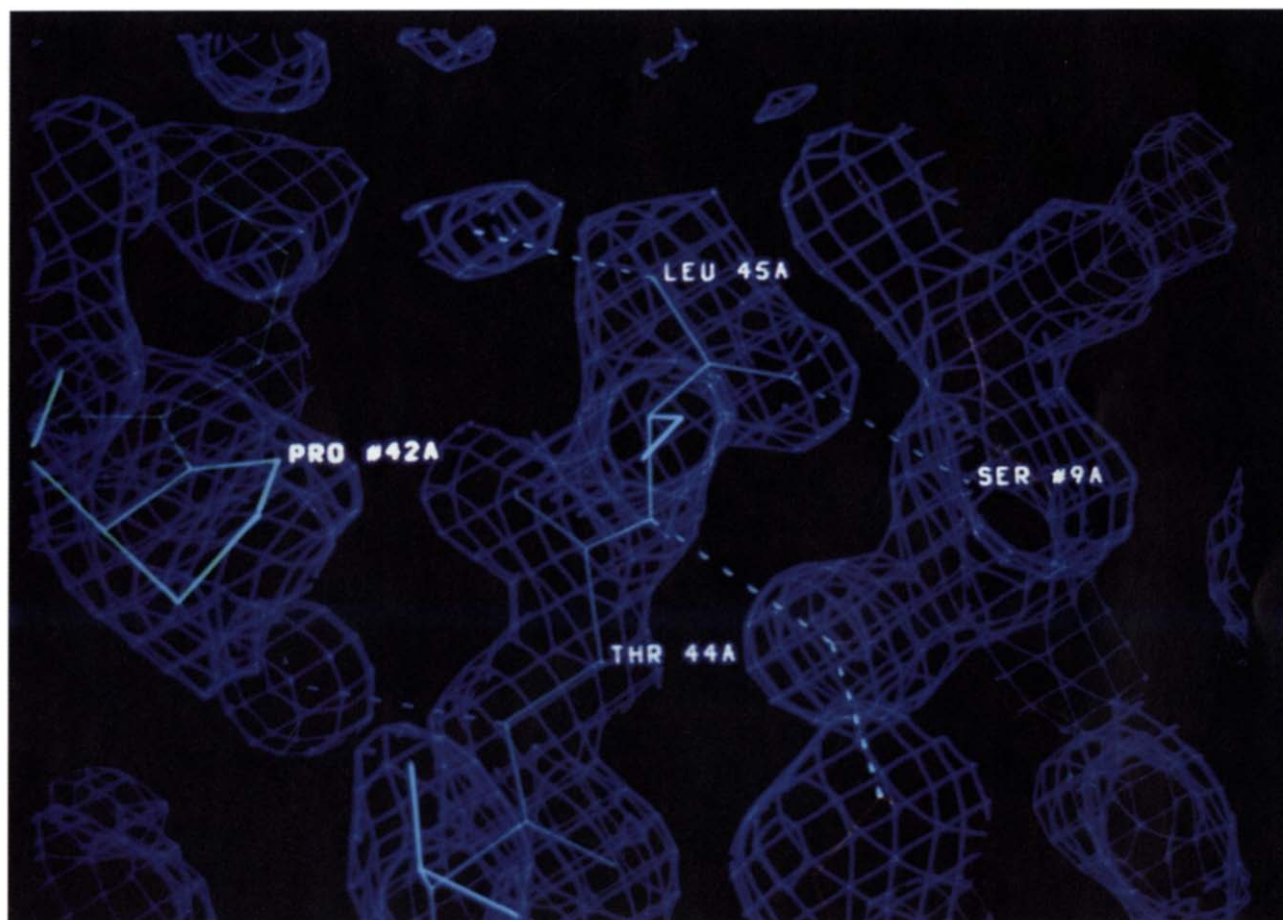
Fig. 2. Stereoview of the $(2F_o-F_o)$ electron density contoured at the 1.3σ level around residues 43 to 45 in the nicked eglin structure. These residues have good electron density and take up a well defined conformation packed between two neighbouring molecules in the crystal lattice.

ture, in an extended conformation. They lie close to the 4-fold screw axis and the side chains make extensive contacts with each other and with the cores of neighbouring eglin molecules in the crystal lattice, Fig. 3.

This may well confer stability on the particular conformation and thus facilitate crystallization. It is not clear why subtilisin DY appears to cleave eglin under conditions where highly homologous subtilisin Carlsberg and
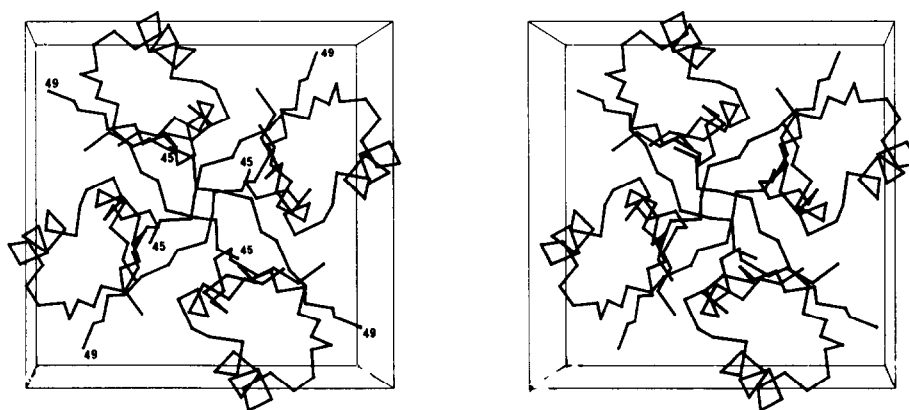

Fig. 3. Stereo picture of the $C\alpha$ positions of the four nicked eglin molecules in the unit cell viewed down the 4-fold axis. The extended arms, residues 39 to 45, pack around the axis against symmetry related eglin molecules.

also other proteinases from the subtilisin family do not. Experiments are planned to address this question.

REFERENCES

[1] Kraut, J., Robertus, J.D., Birktoft, J.J., Alden, R.A., Wilcox, P.E. and Powers, J.C. (1971) Cold Spring Harbor Symp. Quant. Biol. 36, 117–123.

[2] Wells, J.A. and Estell, D.A. (1988) Trends Biochem. Sci. 13, 291–297.

[3] Carter, P., Nilsson, B., Burnier, J.P., Burdick, D. and Wells, J.A. (1989) Proteins 6, 240–248.

[4] Bode, W., Papamokos, E. and Musil, D. (1987) Eur. J. Biochem. 166, 673–692.

[5] Bott, R., Ultsch, M., Kossiakoff, A., Graycar, T., Katz, B. and Power, S. (1988) J. Biol. Chem. 263, 7895–7906.

[6] Teplyakov, A., Kuranova, I.P., Harutyunyan, E.H., Vainshtein, B.K., Frömmel, C., Höhne, W.E. and Wilson, K.S. (1990) J. Mol. Biol. 214, 261–279.

[7] Betzel, Ch., Pal, G.P. and Saenger, W. (1988) Eur. J. Biochem. 178, 155–171.

[8] Markland, F. and Smith, E., in: The Enzymes (P. Boyer, Ed.) 3rd Edn., Vol. 3, Academic Press, New York, 1971, pp. 562–609.

[9] Schnebli, H.P., Seemüller, U., Fritz, H., Mascher, R., Liersch, M., Virca, G.D., Bodmir, J.L., Snider, G.L., Lucey, E.C. and Sione, P.G. (1985) Eur. J. Respir. Dis. 66, 66–70.

[10] Frigerio, F., Coda, A., Pugliese, L., Lionetti, C., Menegatti, E., Amiconi, G., Schnebli, H.P., Ascenzi, P. and Bolognesi, M. (1992) J. Mol. Biol. 225, 107–123.

[11] McPhalen, C.A., Schnebli, H.-P. and James, M.N.G. (1985) FEBS Lett. 188, 55–58.

[12] Seemüller, U., Fritz, H. and Eulitz, M. (I1981) Methods Enzymol. 80, 804–816.

[13] Hyberts, S.G., Goldberg, M.S., Havel, T.F. and Wagner, G. (1992) Protein Sci. 1, 736–751.

[14] Hipler, K., Priestle, J.P., Rahuel, J. and Gruetter, M.G. (1992) FEBS Lett. 309, 139–145.

[15] Dauter, Z., Betzel, Ch., Höhne, W.-E., Ingelman, M. and Wilson, K.S. (1988) FEBS Lett. 236, 171–178.

[16] Dauter, Z., Betzel, Ch., Genov, N., Pipon, N. and Wilson, K.S. (1991) Acta Crystallogr. B47, 707–730.

[17] Gros, P., Betzel, Ch., Dauter, Z., Wilson, K.S. and Hol, W.G.J. (1989) J. Mol. Biol. 210, 347–367.

[18] Peters, K., Pauli, D., Hacke, H., Boteva, R.N., Genov, N.C. and Fittkau, S. (1989) Curr. Microbiol. 18, 171–177.

[19] Davies, D.R. and Segal, D.M. (1971) Methods Enzymol. 22, 266–269.

[20] Matthews, B.W. (1968) J. Mol. Biol. 33, 491–497.

[21] Navaza, J. (1992) Proceedings of the Daresbury Study Weekend on Molecular Replacement, SERC Daresbury Laboratory, Warrington, UK.

[22] Lamzin, V.S. and Wilson, K.S. (1993) Acta Crystallogr. D., in press.

[23] Jones, T.A. (1978) J. Appl. Crystallogr. 11, 268–272.

[24] Hendrickson, W. and Konnert, J.H., in: Biomolecular Structure, Function, Conformation and Evolution (R. Srinivisan, Ed.) Vol. 1, 1980, pp. 43–57.

[25] Seemüller, U., Eulitz, M., Fritz, H. and Strohl, A. (1980) Hoppe-Seyler's Z. Physiol. Chem. 361, 1841–1846.

[26] McPhalen, C.A. and James, M.N.G. (1988) Biochemistry 27, 6582–6598.

[27] Shakespeare, W. (1600) Julius Caesar.