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X-ray crystal structure of the serine proteinase inhibitor eglin c at 1.95 Å resolution

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The crystal structure of eglin c, naturally occurring in the leech *Hirudo medicinalis*, is known from its complexes with various serine proteinases, but the crystallization of free eglin c has not yet been reported. A method is described for growing well-diffracting crystals of free eglin c from highly concentrated protein solutions (≈200 mg/ml). The space group of the orthorhombic crystals was determined to be $P2_12_12_1$ with unit cell parameters a = 32.6, b = 42.0, c = 44.1 Å. The structure of free eglin c was resolved at 1.95 Å resolution by Patterson search methods. The final model contains all 70 amino acids of eglin c and 125 water molecules. In comparison to the eglin structure known from its complexes with proteinases, only small differences have been observed in free eglin c. However, the reactive site-binding loop and a few residues on the surface of eglin have been found in different conformations due to crystal contacts. In contrast to the complex structures, the first seven amino acids of the highly flexible amino terminus can be located. Crystallographic refinement comprised molecular dynamics refinement, classical restrained least-squares refinement and individual isotropic atomic temperature refinement. The final R-factor is 15.8%.

Eglin *c*; Serine proteinase inhibitor; Crystal structure; Uncomplexed structure

1. INTRODUCTION

Eglin c, naturally occurring in the leech *Hirudo medicinalis* [1], is a 70 amino acid strong inhibitor of several serine proteinases, such as α-chymotrypsin, subtilisin, elastase and cathepsin G [2]. Because of its inhibitory potency towards the latter two proteinases, eglin c has attracted particular interest as a possible therapeutic agent for the treatment of pulmonary diseases and inflammatory processes [3]. The gene encoding for eglin c has been synthesized and cloned into *Escherichia coli* [4], and large quantities of *N*-acetylated eglin c have been expressed and purified for preclinical investigations.

Crystals of eglin c in complex with subtilisin Carlsberg have been reported in 1985/1986 by McPhalen et al. [5], Bode et al. [6] and Grütter et al. [7], and the structure has been resolved [5,6]. Since then, crystal structures of eglin c in complex with the proteinases thermitase [8] and α-chymotrypsin [9], as well as mutant eglins in complex with subtilisin Novo [10] have been published. The structures of free wild-type eglin c [11] and mutants of eglin c [12] were resolved by 2D NMR investigations, but no crystal structure of free eglin c has yet been been reported.

Eglin c is a member of the potato inhibitor I family of serine proteinase inhibitors according to the classification of Laskowski and Kato [13]. Like the chymotrypsin inhibitor 2 (CI-2) [14,15], another member of this proteinase inhibitor family isolated from barley seeds [16], eglin c consists of a twisted mixed parallel and antiparallel four-stranded β-sheet, flanked on one side by a α-helix and an extended reactive site binding loop on the other, which results in a wedge-like shape for these two molecules. In both inhibitors, the arrangement of secondary structure elements is strand–helix–strand–binding loop–strand–strand with the carboxy terminus located near the reactive site binding loop. Contrary to most serine proteinase inhibitors, eglin c and CI-2 lack disulfide bridges for binding loop stabilization. Instead, two neighbouring arginine side chains (Arg-51, Arg-53 for eglin c and Arg-65, Arg-67 for CI-2), protruding from β-strand 3 of the core towards the binding loop, have electrostatic and hydrogen-bonding interactions with residues on either side of the scissile bond (P2- and P1′-position of the binding loop according to the nomenclature of Schlechter and Berger [17]). In eglin c, the P1-position is occupied by a leucine residue (Leu-45), ensuring its high specificity towards elastase.

The structure is known for free CI-2 [15], for CI-2 in complex with subtilisin [14], for eglin, complexed with subtilisin [6,5,10], and now also for free eglin, making these two inhibitors interesting objects for investigations of the mechanism of serine proteinase inhibition proposed by Laskowski and Kato [13]. According to this hypothesis, the reactive site binding loop of the inhibitor binds to the enzyme in the manner of a good substrate (k_{on} = 10^6–10^7 $M^{-1}\cdot s^{-1}$). Similiar to a substrate,

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the inhibitor is cleaved, but at very low rates and with hydrolysis occuring only at a single peptide bond (termed the 'scissile bond'). The good inhibitory properties ($k_i = 10^{-10}$–10^{-12} M^{-1}) result from tight binding with very slow release of either cleaved or uncleaved inhibitor. As already pointed out by McPhalen et al. [15], the comparison of free and complexed inhibitor may help to elucidate the reasons why these molecules are inhibitors rather than good substrates. It was proposed [15] that binding loop stabilization keeps the carboxy- and amino-termini of the cleaved bond in close vicinity, facilitating the resynthesis of uncleaved inhibitor by the enzyme. A further important aspect is, that due to entropical reasons higher binding loop rigidity is accompanied by a higher gain of total free energy upon binding of the inhibitor to the enzyme [15]. This results in a more tight complex, and hence higher inhibitory potency of the inhibitor. In cases where disulfide bridges flank the scissile bond it is obvious that binding loop stabilization must always be present, even for the free inhibitor. It is an interesting question whether this is also true for the non-covalent stabilizing system provided by the core arginines in eglin c.

2. EXPERIMENTAL

2.1. *Crystallization*

Crystallization experiments were set up according to the hanging drop method. In a first step, large clusters of crystals were grown which were used for further seeding experiments: Highly purified lyophilized eglin c was dissolved in 200 mM acetic acid + 2.5% polyethylene glycol 10 000 (PEG 10 000) to a concentration of 130 mg/ml. The protein solution was immediately centrifuged in order to minimize the number of nucleation sites. 4 μl of this solution were taken for the hanging drop and were equilibrated against 600 ml of a buffer solution consisting of 200 mM ammonium acetate, in a range of pH 6.0–8.0, and 15–30% PEG 10 000. Crystals were grown at room temperature within 5 months. Best results were obtained with a reservoir buffer of pH 8.0, containing 30% PEG 10 000.

For the second crystallization step, lyophilized eglin c was dissolved in 5 mM hydrochloric acid to a concentration of 250 mg/ml. The drop, containing 3 μl of centrifuged protein solution and 1 μl of reservoir solution, was equilibrated against a buffer consisting of 200 mM potassium phosphate, pH 8.0, and 1–4% PEG 4 000. After one day of equilibration, the drops were seeded using single crystal fragments obtained from a cluster of crystals from the first crystallization step by cutting with a platinum wire. The crystals were grown at 4°C. They grew to a final size of 0.08×0.12×0.25 mm^3 within 4 weeks.

2.2. *Data collection*

X-ray diffraction data were collected using a FAST area detector (Enraf-Nonius, Delft, The Netherlands). Graphite-monochromated $Cu_{K\alpha}$ radiation was provided by an FR571 X-ray generator operated at 40 kV and 70 mA with an apparent focal spot of 0.3×0.3 mm^2. The capillary was mounted on a 3-circle κ goniostat and was rotated 100° around the ω-axis about an arbitrary crystal axis in steps of 0.1°/frame with an exposure time of 90 s/frame. Data were collected to 1.9 Å with a 2θ swing out angle of −15° and a crystal-to-detector distance of 40 mm. Crystal orientation determination, data collection and on-line data evaluation were performed employing the program MADNES [18]. The measured diffraction intensities were scaled using the programs ROTAVATA and AGROVATA of the CCP4 (Daresbury Laboratory) program package.

2.3. *Data evaluation*

The space group of the eglin crystals was determined to be $P2_12_12_1$ with unit cell parameters $a = 32.6$, $b = 42.0$, $c = 44.1$ Å. The asymmetric unit contains one eglin molecule ($V_m = 1.9$ Å^3/Da). 12 113 reflections were measured in the resolution range from 15 Å to 1.95 Å. They were reduced and merged resulting in 4,590 unique reflections with an R_{merge}, defined as $(\Sigma_{hkl}\Sigma_i|I-\bar{I}|)/(\Sigma_{hkl}\Sigma_i I)$, of 8.9% for the whole sphere and of 32.6% for the last shell from 2.00–1.95 Å. Data completeness is 97.1%.

Molecular replacement searches were performed using the eglin coordinates of the complex structure eglin c / subtilisin Carlsberg, which were generously provided by Dr. W. Bode [6]. Rotational search was started with an eglin model in which the amino terminus (residues 1–11), the binding loop (residues 39–50) and the C-terminus (residues 68–70) as well as 18 solvent exposed side chains were omitted. However, the whole eglin molecule with all 63 amino acids defined in the complex structure proved to be the better search model, yielding a clear peak in the rotation function map of the CCP4-program POLARRFN [19] for the Eulerian angles $\alpha = 119.5°$, $\beta = 124.2°$, $\gamma = 79.5°$. The solution of the rotation function was found to be very sensitive to slight changes of the outer Patterson cut-off radius. In the resolution range from 100 Å to 2.0 Å, the best signal-to-noise ratio was obtained for Patterson inner and outer cut-off radii of 0 Å and 15.0 Å, which yielded a solution peak 4.5 standard deviations (σ) above the mean and 1.47 times the highest noise peak. Computing the translation function TFSGEN [19] in the resolution range from 100 Å to 2.0 Å, a well-defined peak, 7.3 σ above the mean was obtained for the orthogonal coordinates 7.74, 14.57, 2.80 Å. After correct orientation of the model in the unit cell, the initial R-factor, defined as $(\Sigma_{hkl}|F_o-F_c|)/(\Sigma_{hkl}|F_o|)$, was calculated to be 50.8% for the eglin model comprising all 63 amino acids defined in the complex structure (resolution range from 10.0 Å to 3.00 Å).

Rotation and translation of the eglin molecule were followed by rigid body refinement employing the program COREL5 [20] in the resolution range from 10.0 Å to 7.0 Å. For this, the molecule was split into three domains comprising the residues 8–39, 40–49 and 50–70. The structure was further improved by manual fitting using the program FRODO [21] on an Evans and Sutherland PS390 system. In the region of the binding loop, the structure was improved with a $(F_o-F_c)\sigma_A$ map [22], which was calculated omitting residues 40–48.

Before starting restrained least-squares refinement, the R-factor between calculated and measured structure factors was 46.3% (resolution range from 10.0 to 3.0 Å). The first 34 refinement cycles using the program package TNT [23] were run with gradually increasing resolution and weight for the geometrical constraints, which resulted in an R-factor of 39.2% (10.0–1.95 Å resolution). After checking the structure on the computer graphics display system, it was submitted to molecular dynamics refinement, employing the program package XPLOR [24] which brought the R-factor down to 29.7% (resolution range from 10.0 to 1.95 Å. Individual isotropic atomic temperature factor refinement yielded an R-factor of 26.3%. Insertion of the seven amino-terminal amino acids, followed by 45 new refinement cycles improved the R-factor to 22.9% (6.0–1.95 Å resolution). In nine additional model building/TNT refinement rounds the structure was further improved and 125 water molecules were included. The final R-factor for the resolution range from 6.0 to 1.95 Å was calculated to be 15.8%.

3. RESULTS

The structure of free eglin c was compared with the highly resolved (1.2 Å) structure of eglin c bound in the complex with subtilisin Carlsberg[1] [6]. Except for the

[1] In the following, for simplicity, this eglin structure is referred to as 'bound eglin'.

Fig. 1.

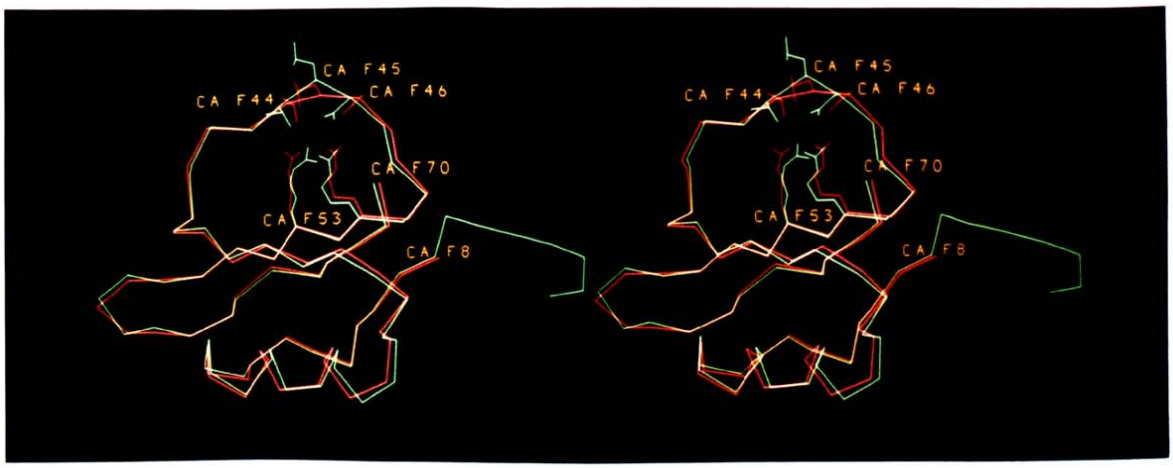

Fig. 3.

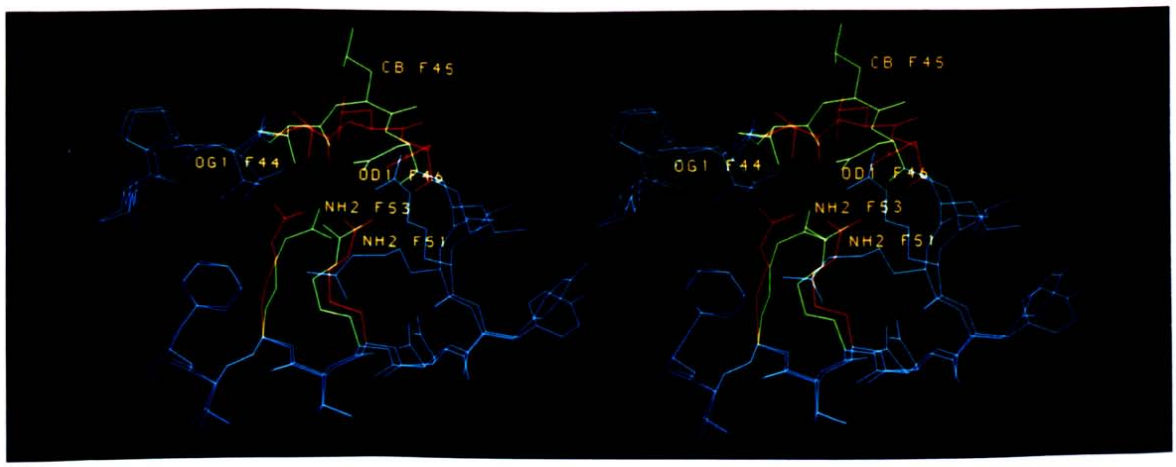

Fig. 4.

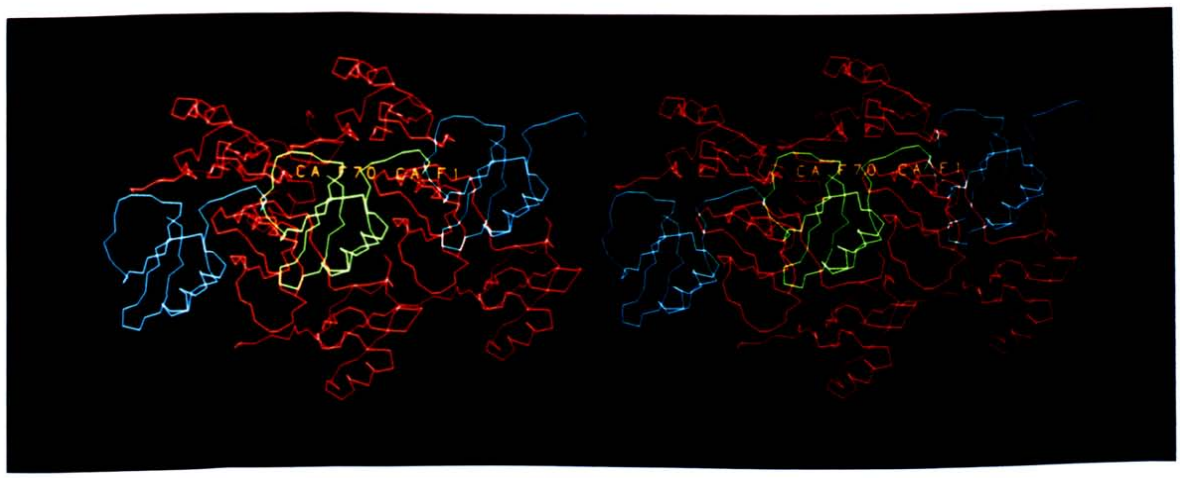

Fig. 1. Superposition of the C_α backbones of free (green) and bound eglin (red.). While the displacements in the core region are rather low, they are considerably larger for the residues flanking the P1-residue Leu-45 in the reactive site binding loop domain. The reactive site binding loop is chiefly stabilized by Arg-51, Arg-53, protruding from the core, and Thr-44, Asp-46, in the binding loop. The first seven amino acids are not defined in the structure of bound eglin.

Fig. 3. Conformational differences in the binding loop domain of free (green) and bound eglin (red.). The binding loop is chiefly stabilized by the two neighbouring argines from the core and the P2-and P1'-residue in the loop. For simplicity, all the other residues of both structures are shown in blue. While in the complex structures the loop conformation is fixed by the surface topology of the enzyme, in free eglin this is done by crystal contacts, which results in the loss of the interactions of Arg-51 and Arg-53 to the side chains of the P2- and P1'-residue.

Fig. 4. Position of free eglin c in the crystal lattice. The discussed molecule is shown in green, symmetry related molecules in red and blue. Contrary to the known eglin/proteinase structures, the highly flexible amino-terminus can be located. It occupies the only large solvent region in the crystal between neighbouring molecules and extends to the reactive site binding loop of an adjacent molecule.

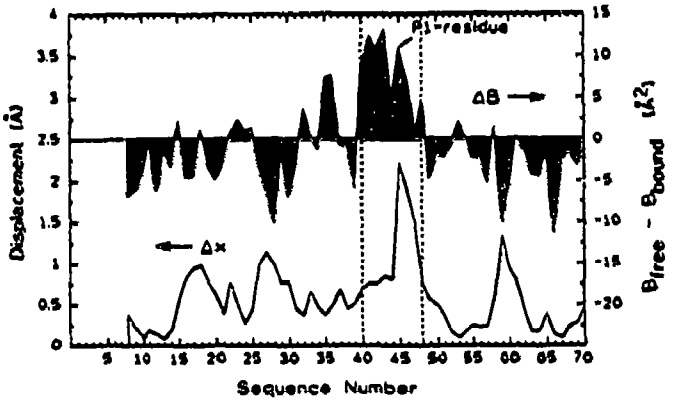

Fig. 2. Comparison of free and bound eglin. Upper curve: difference (in Å²) of the mean main chain B-factor vs. residue number. Lower curve: displacement (in Å) of the C_α-atoms as a function of the residue number. Except for the reactive site binding loop (marked by vertical lines), large atomic displacements are not correlated with high B-factors. The first seven residues are not defined in the bound structure.

binding loop region, the two structures are very similar (Fig. 1). After a least-squares fit using the C_α positions of the amino acids 8–70 of both structures, the RMS deviation between all C_α atoms was calculated to be 0.769 Å. While the deviations for the core amino acids are relatively low, indicating that the core is compact and cannot be easily distorted, the displacements in the binding loop region, especially for the P1-, P1'- and P2'-position, are considerably higher (Fig. 2). Omitting these three residues brings the RMS deviation for the remaining 60 C_α atoms down to 0.589 Å. The three next largest displacements (residues 18, 27, 59) were found in external loops. Interestingly, the high displacement for the residue Gly-59 is accompanied by considerably better atomic temperature factors and a flip of the peptide between this residue and the proceeding Pro-58 by 180° relative to the bound structure. The ϕ, ψ-angles for this peptide in free eglin c are similar to those observed in the structure of eglin c/subtilisin Carlsberg reported by McPhalen et al. [5] and eglin c/thermitase reported by Gros et al. [8].

Table I presents the main chain ϕ, ψ-torsional angles of the binding loop region of free eglin c and of eglin c in complex with several proteinases. Data for free and bound CI-2 are also shown. While the torsional angles, especially for the residues P3–P1', are quite similar for all the inhibitor/proteinase complexes shown, the ψ-angle of the P1-residue is significantly different in the structure of free eglin c with a change of ≈21 standard deviations from the mean calculated for 5 different structures of wild type and mutant eglin c in complex with subtilisin. While in complex with a proteinase, the conformation of the eglin binding loop is fixed by the surface topology of the active site of the enzyme, in the crystal structure of free eglin c this is done by crystal contacts. Fig. 3 shows the conformational differences observed for the binding loop of free and bound eglin c. In contrast to what McPhalen et al. [15] reported for the free CI-2, the reactive site binding loop of free eglin c is considerably better defined. Except for the first five amino acids, all averaged atomic temperature factors (B-factors) for main chain atoms are below 25 Å². As expected, the highest B-factor deviations relative to the bound structure have been observed for residues belonging to the reactive site binding loop, especially for the P1- and P3-residue, which are very well defined in the bound structure.

Except for McPhalen et al. [5], who could locate the residue Leu-7, in all eglin/proteinase structures reported so far, the eglin structure was only determined for the residues 8 to 70. The first 6 amino acids could not be located in any of the structures reported. In contrast, for free eglin all residues of the amino-terminal peptide segment from Thr-1 to Leu-7 can be located, although with some degree of uncertainty as illustrated by high temperature factors. The amino terminus occupies the only large solvent region in the crystal between neighbouring molecules, extending to the reactive site binding loop of an adjacent molecule (Fig. 4). Except for a salt bridge formed between the side chains of Glu-2 and Arg-53*, no interactions can be observed in the final model for the first four amino acids which only have poorly defined electron density. The N terminus of recombinant eglin c is acetylated; the acetyl group also shows only weak electron density.

As described previously [25], no obvious anchoring points can be located for the amino-terminal heptapeptide of eglin, and according to methods of secondary structure prediction [26,27] this peptide does not show a strong propensity for any specific structural element. Both considerations imply a high flexibility of the amino-terminus, which is consistent with structural features observed for free eglin c. The averaged atomic temperature factors for the first five residues are extremely high (> 60 Å²). As in bound eglin, the first amino-terminal amino acids which are fixed to the eglin core belong to the segment Lys-8–Phe-10, having inter-main-chain hydrogen bonds to the carboxy-terminal segments Pro-67–Val-69. Except for the peptide oxygen atom of Ser-5, which forms a hydrogen bond to the side chain of Lys-8, all amino-terminal amino acids have no contacts to the eglin core and hence in solution may be able to adapt any conformation compatible with steric restraints, leading to a high flexibility of the amino-terminus which makes it potentially accessible to proteinases. According to the investigations of Bode et al. [25] with subtilisin and Pugliese et al. [28] with chymotrypsin, the amino-terminal peptide is cleaved between Glu-6 and Leu-7 or Leu-7 and Lys-8. The cleavage occurs at very low rates by free enzyme during formation of the crystals. Hence the lack of the amino-terminal peptide cannot be observed, neither in a freshly formed complex between eglin c and subtilisin [25] nor at any stage during the purification process used in our

Table 1. Main chain conformational angles ϕ,ψ (degrees) of the eglin c and CI-2 reactive site binding loops

Structure	$P5_{(\phi,\psi)}$		$P4_{(\phi,\psi)}$		$P3_{(\phi,\psi)}$		$P2_{(\phi,\psi)}$		$P1_{(\phi,\psi)}$		$P1'_{(\phi,\psi)}$		$P2'_{(\phi,\psi)}$		$P3'_{(\phi,\psi)}$		$P4'_{(\phi,\psi)}$	
Eglin c	Ser-41		Pro-42		Val-43		Thr-44		Leu-45		Asp-46		Leu-47		Arg-48		Tyr-49	
CI-2	Thr-55		Ile-56		Val-57		Thr-58		Met-59		Glu-60		Tyr-61		Arg-62		Ile-63	
wt-egl/therm[1]	-65	136	-67	127	-129	152	-64	133	-99	50	-103	166	-108	129	-137	122	-91	-3
wt-egl/chym[2]	-104	126	-58	145	-142	163	-79	157	-102	50	-104	162	-161	117	-132	128	-77	10
wt-egl/subC[3]	-68	138	-71	140	-139	168	-62	143	-115	45	-97	169	-117	110	-121	112	-76	-2
wt-egl/subC[4]	-64	141	-76	138	-143	165	-65	151	-112	42	-96	176	-120	113	-119	115	-74	-2
wt-egl/subN[5]	-57	143	-81	137	-134	172	-71	133	-96	44	-105	174	-116	123	-120	122	-81	-9
L45R-egl/subN[6]	-60	149	-81	141	-136	171	-70	137	-90	42	-104	171	-114	127	-128	126	-83	-16
R53K-egl/subN[7]	-52	139	-81	128	-116	167	-84	127	-85	45	-103	172	-115	123	-127	130	-85	-9
Mean(egl/sub)	-58	142	-78	137	-134	169	-70	136	-100	44	-101	172	-116	119	-123	121	-80	-8
σ(egl/sub)	4.4	4.4	4.5	5.2	10.4	2.9	8.4	9.2	13.3	1.5	4.2	2.7	2.3	7.3	4.2	7.5	4.7	5.9
wt-egl (free)[8]	-60	143	-71	153	-132	163	-74	150	-82	12	-99	159	-111	128	-129	123	-78	-9
$\lvert\Delta_{egl}\rvert^{*}$[abs]	2	1	7	16	2	6	4	12	18	32	2	13	5	9	6	2	2	1
$\lvert\Delta_{egl}\rvert^{*}[\sigma]$	0.4	0.2	1.6	3.1	0.2	2.0	0.4	1.3	1.3	20.8	0.5	5.0	2.6	1.2	1.4	0.3	0.4	0.2
CI-2 (free)[9]	-80	130	-90	117	-103	166	-77	134	-63	27	-91	128	-93	117	-125	115	-63	-30
$\lvert\Delta_{CI2}\rvert^{\dagger}$[abs]	32	30	3	23	30	0	13	13	40	7	0	18	13	8	7	2	6	2
CI-2/subN[10]	-112	160	-93	140	-133	166	-64	147	-103	34	-91	146	-106	109	-118	113	-59	-28

[1] Complex structure wild type-eglin c / thermitase [8]
[2] Complex structure wild type-eglin c / bovine pancreatic α-chymotrypsin [9]
[3] Complex structure wild type-eglin c / subtilisin Carlsbeg [6]
[4] Complex structure wild type-eglin c / subtilisin Carlsbeg [5]
[5] Complex structure wild type-eglin c / subtilisin Novo [10]
[6] Complex structure L45R-eglin c / subtilisin Novo [10]
[7] Complex structure R53K-eglin c / subtilisin Novo [10]
[8] Present structure
[9] Structure of the free chymotrypsin inhibitor CI-2 [15]
[10] Complex structure CI-2 / subtilisin Novo [14]

* Difference between the mean conformational angles of eglin in complex with subtilisin and the conformational angles observed in the structure of free eglin. The difference is given in degrees and in units of the standard deviation.
† Difference (in degrees) between the conformational angles of CI-2 in complex with subtilisin Novo and free CI-2.

laboratory for wild-type and mutant eglins. Similar to eglin c, the chymotrypsin inhibitor CI-2 also has a long floppy amino-terminus, comprising the residues 1 to 18, which is subjected to multiple proteolytic cleavage during purification [16]. McPhalen et al. [15] report no electron density for the first eighteen residues at any stage of their refinement.

For a more thorough analysis of the structural differences between free and bound eglin, five different eglin/subtilisin structures have been compared (*subtilisin Carlsberg complexes with*: wild-type eglin c [6,5]; *subtilisin Novo complexes with*: wild-type eglin c, L45R-eglin c and R53K-eglin c [10]). The analysis reveals the following points: (i) the side chain of the first core amino acid Lys-8 and the side chains of two residues on the surface of the helix (Arg-22, His-28) have different conformations in most of the different eglin structures, seemingly due to crystal contacts; (ii) in all complexes,

Arg-48 has a fully extended conformation, forming a hydrogen bond with an enzyme residue. In contrast, in free eglin c the side chain of Arg-48 folds back and forms a salt bridge with the side chain of Asp[2]-33; (iii) the reactive site binding loops of the discussed eglin structures in complex with subtilisin show a well conserved conformation; the only observed conformational freedom is a bending at 'hinges' where the loop enters the core as previously described [10]. In contrast, the binding loop of free eglin c shows significant structural differences (Table I and Fig. 3). In comparison to the complex structures, the core-binding loop interactions are different in free eglin c. The side chain interactions Arg-51–Asp-46 (P1'-position) and Arg-53–Thr-44 (P2-position) are lost, resulting from a shift in opposite directions of the arginine head groups out of the plane defined by the binding loop, seemingly caused by crystal contacts (e.g. the Arg- 53–Glu-2* interaction).

4. DISCUSSION

The structural results confirm that the eglin core is rather rigid. The few observed significant structural differences affect only side chains of residues distributed on the surface of the eglin molecule and are caused by crystal contacts. In contrast, the transition from the free to the bound eglin structure is accompanied by a concerted conformational change in the binding loop, implying an induced fit, leading to the adaption of a structure complementary to the accessible enzyme surface. This is in agrement with the high flexibility of the reactive site binding loop observed by NMR investigations [11]. Under complex or cyrstal formation or both, only one of all possible binding loop conformations is 'frozen out'. Hence what is observed in the crystal structures, is that conformation which has adapted best to the local environment defined by the enzyme surface topology or the position of crystal neighbours. A further example for this induced fit mechanism is the bending at 'hinges' where the loop enters the core [10], which is observed when comparing different eglin/proteinase structures.

Similar to the free chymotrypsin inhibitor CI-2 [15], binding loop stabilization provided by the arginines is not fully preserved in the crystal structure of free eglin c. The electrostatic and hydrogen bonding interactions of the two protruding side chains of Arg-51 and Arg-53 to the side chains of the P1- and P2-residue are lost. Only the hydrogen bonds to the main chain peptide oxygen atoms of these residues remain unchanged. This implies that the binding loop stabilization provided by the arginines can be distorted easily (e.g. by crystal contacts) and that it may not always be present in full strength, unless the binding loop adapts to the surface topology of the enzyme upon formation of the complex. Investigations are in progress in which the formation of specific core-binding loop interactions is prevented by specifically designed mutants. Early results imply that binding loop stability affects the inhibitory properties, but not the velocity of binding to the enzyme. Moreover, the changed binding loop stabilization observed in free eglin c may be accompanied by higher loop flexibility which allows the inhibitor to interact with a wider range of enzymes [31] and thus might be an evolutionary advantage, albeit the higher flexibility leads to less tight binding to the enzyme. The total free energy of the system is reduced upon binding due to entropic reasons since the flexible binding loop is fixed by the enzyme in a rigid position leading to a reduction of internal degees of conformational freedom. However, the inhibition of eglin c towards subtilisin Carlsberg ($k_i = 1.5 \cdot 10^{-11}$ M)[1], bovine pancreatic α-chymotrypsin ($k_i = 2.7 \cdot 10^{-11}$ M)[1] and human leucocyte elastase ($k_i = 1.5 \cdot 10^{-11}$)[1] is quite strong, and in evolutionary terms, the flexibility to interact with a wider range of enzymes of comparable specificity [31] might overcome this energetical disadvantage [15].

In the crystal structure of free eglin, the amino-terminal hepta-peptide is found in an extended conformation. As predicted [25], this peptide does not seem to have a preference for a specific secondary structure and under natural conditions is most likely to be able to adapt any structural conformation compatible with steric restraints. While the long, floppy carboxy terminus of another low molecular weight serine proteinase inhibitor from the leech *Hirudo medicinalis* (hirudin) is of specific functional importance in thrombin inhibition [32,33], it is unknown whether the long floppy amino terminus of eglin c has any biological significance.

REFERENCES

[1] Seemüller, U., Meier, M., Ohlsson, K., Müller, H.P. and Fritz, H. (1977) Hoppe Seyler's Z. Physiol. Chem. 385, 1105–1117.
[2] Seemüller, U., Fritz, H. and Eulitz, M. (1981) Methods Enzymol. 80, 804–816.
[3] Schnebli, H.P. and Braun, N.J. in: Proteinase Inhibitors, (A.J. Barret and G. Salvesen, Ed.) Elsevier, Amsterdam, The Netherlands, 1986, pp. 613–627.
[4] Rink, K., Liersch, M., Sieber, P. and Meyer, F. (1984) Nucleic Acids Res. 13, 6369–6387.
[5] McPhalen, C.A., Schnebli, H.P. and James, M.N.G. (1985) FEBS Lett. 188, 55–58.
[6] Bode, W., Papamokos, E., Musil, D., Seemüller, U. and Fritz, H. (1986) EMBO J. 5, 813–818.

[2]According to DNA-sequence and mass spectroscopy, residue 33 is an aspartate and not an asparagine. Among the eglin c/proteinase structures deposited in the Brookhaven Data Bank (Upton, NY, USA), [8] (*1TEC*) has the right residue aspartate, [6] and [5] (*1CSE, 2SEC*) have the wrong residue asparagine.

[3]These values have been measured in our laboratory under identical conditions at pH 7.9, $T = 37°$ C. They were within the range reported by other authors [29].

[7] Grütter, M.G., Märki, W., Walliser, H.P. (1985) J. Biol. Chem. 260, 11436–11437.
[8] Gros, P., Fujinaga, M., Dijkstar, B.W., Kor, K.H. and Hol, W.G.J. (1989) Acta Crystallogr. Sect. B 45, 488–499.
[9] Bolognesi, M., Pugliese, L., Gatti, G., Frigerio, F., Coda, A., Antolini, L., Schnebli, H.P., Menegatti, E., Amiconi, G. and Ascenzi, P. (1990) J. Mol. Recogn. 3, 163–168.
[10] Heinz, D.W., Priestle, J.P., Ruhuel, J., Wilson, K.S. and Grütter, M.G. (1991) J. Mol. Biol. 217, 353–371.
[11] Hyberts, S.G. and Wagner, G. (1990) Biochemistry 29, 1465–1474.
[12] Wagner, G., Hyberts, S.G., Heinz, D.W. and Gütter, M.G. in: Proceedings of the Sixth Conversation in the Discipline Biomolecular Stereodynamics, (R.H. Sarma, Ed.) Adenine Press, Albany, New York, 1990.
[13] Laskowski Jr., M. and Kato, I. (1980) Annu. Rev. Biochem. 49, 593–626.
[14] McPhalen, C.A., Svendsen, I., Jonassen, I. and James, M.N.G. (1985) Proc. Natl. Acad. Sci. USA 82, 7242–7246.
[15] McPhalen, C.A. and James, M.N.G. (1987) Biochemistry 26, 261–269.
[16] Svendsen, I., Jonassen, I., Hejgaard, J. and Boisen, S. (1980) Carlsberg Res. Commun. 45, 389–395.
[17] Schechter, I. and Berger, A. (1967) Biochem. Biophys. Res. Commun. 27, 157–162.
[18] Messerschmidt, A. and Pflugrath, J.W. (1987) J. Appl. Cryst. 20, 306–315.
[19] Kabsch, W. (1988) J. Appl. Cryst. 21, 67–71.
[20] Sussman, J.L., Holbrook, S.R., Church, G.M. and Kim, S.H. (1977) Acta Crystallogr. A33, 800–804.
[21] Jones, T.A. (1978) J. Appl. Crystallogr. 11, 268–272.
[22] Read, R.J. (1986) Acta Crystallogr. A42, 140–149.
[23] Tonrud, D., Ten Eyck, L.F. and Matthews, B.W. (1987) Acta Crystallogr. Sect A 34, 489–501.
[24] Brünger, A.T., Kuriyan, J. and Karplus, M. (1987) Science 235, 458–460.
[25] Bode, W., Papamokos, E. and Musil, D. (1987) Eur. J. Biochem. 166, 673–692.
[26] Chou, P.Y. and Fasman, G.D. (1974) Biochemistry 13, 222–244.
[27] Robson, B. (1974) Biochem. J. 141, 853–867.
[28] Pugliese, L., Gatti, G., Bolognesi, M., Coda, A., Menegatti, E., Schnebli, H.P., Ascenzi, P. and Amiconi, G. (1989) J. Mol. Biol. 208, 511–513.
[29] Braun, N.J., Bodmer, J.L., Virca, G.D., Metz-Virca, G., Maschler, R., Bieth, J.G. and Schnebli, H.P. (1987) Biol. Chem. Hoppe-Seyler 368, 299–308.
[30] Ascenzi, P., Amiconi, G., Menegatti, E., Guarneri, M., Bolognesi, M. and Schnebli, H.P. (1988) J. Enzyme Inhibition 2, 167–172.
[31] Read, R.J. and James, M.N.G. in: Proteinase Inhibitors, ed. by (A.J. Barret and G. Salvesen, Ed.) Elsevier, Amsterdam, The Netherlands, 1986, pp. 301–336.
[32] Grütter, M.G., Priestle, J.P., Ruhuel, J., Grossenbacher, H., Bode, W., Hofsteenge, J. and Stone, S.R. (1990) EMBO J. 9, 2361–2365.
[33] Rydel, T.J., Ravichandran, K.G., Tulinsky, A., Bode, W., Huber, R., Roitsch, C. and Fenton, J.W. (1990) Science 249, 277–280.