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Mode of binding of E-64-c, a potent thiol protease inhibitor, to papain as determined by X-ray crystal analysis of the complex

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The three-dimensional structure of the E-64-c–papain complex has been determined by X-ray crystal analysis at 2.5 Å resolution (conventional $R = 26.9\%$). The structure determined indicates that: (i) the C2 atom of the oxirane ring of E-64-c is covalently bound by the S^{γ} atom of Cys-25 of papain; (ii) this covalent bond formation results in a configurational conversion of the oxirane C2 atom from the *S*- to the *R*-form; and (iii) extensive hydrogen bonding and hydrophobic interactions are responsible for the specific interaction of the E-64-c molecule with papain.

E-64-c; Papain; Enzyme-inhibitor complex; X-ray crystal analysis

1. INTRODUCTION

Proteases are important in the initiation, maintenance and termination of a wide variety of biological processes [1–3]. In view of the pivotal role of proteolysis in these processes, the development of specific and non-toxic protease inhibitors is an interesting challenge in drug design.

Thiol proteases, such as papain, cathepsin and Ca^{2+}-activated neutral protease, possess an essential, highly reactive thiol group at their active sites.

E-64-c [(+)-(2*S*,3*S*)-3-(1-[*N*-(3-methylbutyl)amino]leucylcarbonyl)oxirane 2-carboxylic acid] (**1**) is the active form of loxistatin (**2**), a potent cysteine protease inhibitor [4]. Loxistatin was designed as a clinically usable drug for the treatment of muscular dystrophy [5], based on the prototype E-64 (**3**), a natural product isolated from cultures of *Aspergillus japonicus* [6,7]. The chemical structures of **1–3** are shown in fig.1.

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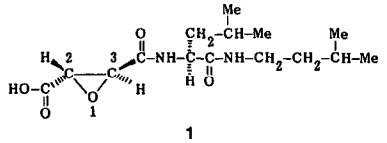
To facilitate the design of more potent and useful protease inhibitors, it is desirable to establish the mode of binding of existing inhibitors



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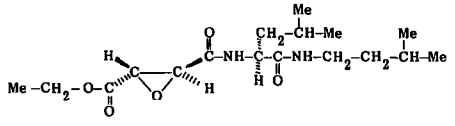

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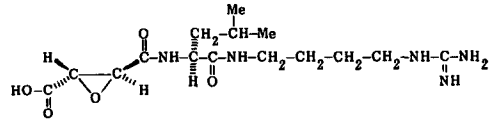

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Fig.1. Chemical structures of E-64-c (**1**), loxistatin (**2**) and E-64 (**3**).

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to proteases. Here, we report the mode of binding of E-64-c in the E-64-c–papain complex as determined by X-ray crystal analysis at 2.5 Å resolution.

2. EXPERIMENTAL

Twice-crystallized papain was prepared by the method of Kimmel and Smith [8] from the protease (from Sigma) and further purified as described by Sluyterman and Wijdenes [9]. The purified enzyme (9.1 mg/ml) in a solution containing 10% dimethyl sulfoxide, 0.1 M KCl, 50 mM cysteine and 10 mM EDTA (pH adjusted to 7.0) was treated with a 5 molar excess of E-64-c (dissolved in a minimal amount of dimethyl sulfoxide). This treatment led to complete inhibition of the enzyme, as confirmed by assaying caseinolytic activity according to standard methods [6,10]. The inhibited enzyme was thoroughly dialyzed against water.

For crystallization, the inhibited enzyme (E-64-c–papain complex) was dissolved to a concentration of 1.5% (w/v) in 0.1 M β-aminoethanol hydrochloride buffer (pH 9.2) containing 64% methanol-ethanol (2:1, v/v). Crystals of the complex were obtained within a few weeks using the sitting-drop method in a temperature-controlled room (20°C).

The crystals were mounted on a goniometer in the usual way with a small amount of mother liquid. X-ray diffraction data were collected to 1.9 Å resolution on a Rigaku AFC automatic diffractometer in an ω scan with Ni-filtered CuKα radiation. Data were treated employing corrections for Lorentzian, polarization and absorption effects.

The crystals were orthorhombic and belonged to space group $P2_12_12_1$. The unit-cell parameters are as follows: a = 42.90(1) Å, b = 95.51(4) Å, c = 49.99(2) Å, V = 2.048 × 10^5(1) Å^3 and Z = 4.

A total of 6962 reflections to a resolution limit of 2.5 Å were first used for the structure solution. The structure was solved by molecular replacement techniques using the atomic coordinates of 2-hydroxyethylthiopapain refined with 2.0 Å resolution data [11] as the starting structure. Several cycles were further refined by a stereochemically restrained least-squares procedure using the PROLSQ program [12]. Details of the analysis will be reported elsewhere.

3. RESULTS AND DISCUSSION

Atom fittings were carried out using the FRODO program [13,14] on an IRIS 3000 interactive computer graphics system. The E-64-c molecule could be easily identified on a $(2F_o - F_c)$ map. A stereoscopic view of the partial electron density map is shown in fig.2, where the map corresponds to a conventional R value of 0.269 using 6430 reflections above the 3σ (F_o) threshold. As demonstrated in fig.2, a clear-cut density map for the E-64-c molecule emerged. This map shows a well-defined continuous chain of positive density running from the catalytic site near Ser-24 right through the active-site groove and terminating in the vicinity of the Tyr-67 and Val-157 residues. A similar mode of binding has also been observed in the crystal structure of the papain–benzyloxycarbonyl-L-phenylalanyl-L-alanine chloromethyl ketone complex [15]. Fig.2 undoubtedly indicates that a covalent bond was formed between the C2 carbon atom of the E-64-c oxirane ring and the S^γ atom of Cys-25 of the papain active site; the C2–S^γ bond length was 1.87 Å. The entire conformation of the E-64-c molecule is best described as a flattened, slightly curved structure. Similar conformations have also been observed for the crystal structures of the loxistatin [16] and E-64 (Yamamoto, D. et al., unpublished) molecules themselves.

It is of interest that the configurational conversion took place at the C2 atom of the E-64-c oxirane ring, as shown in fig.3. The *S* configuration of the E-64-c molecule itself was converted to the *R* form by covalent bond formation with the S^γ atom of Cys-25. This implies that nucleophilic attack of the Cys-25 SH group occurs at the opposite side to the O atom of the oxirane ring plane, and attack from the same side causes electronic repulsion between the lone pairs of the Cys-25 S^γ and oxirane O atoms. This configurational conversion has also been demonstrated by ^{13}C-NMR spectroscopy [17].

A perspective view showing the possible interactions of E-64-c with the papain active site is shown in fig.4. The formation of seven hydrogen bonds was observed at the present refinement of the crystal structure. The $N^{\epsilon 1}$ (Gln-19), $N^{\delta 1}$ (His-159), N and O (Gly-66) and O (Asp-158) atoms participated in the formation of hydrogen bonds with the polar N and O atoms of E-64-c. The bond lengths (2.60–3.10 Å) are in a reasonable range. On the other hand, the 3-methylbutyl and leucyl side chains of E-64-c were stabilized by hydrophobic interactions with the neighboring Tyr-61 and -67, and Val-133, -157 and Asp-158 residues, respectively, as can be seen in fig.4.

Structure refinement using reflections to a resolution of 1.9 Å is now in progress.

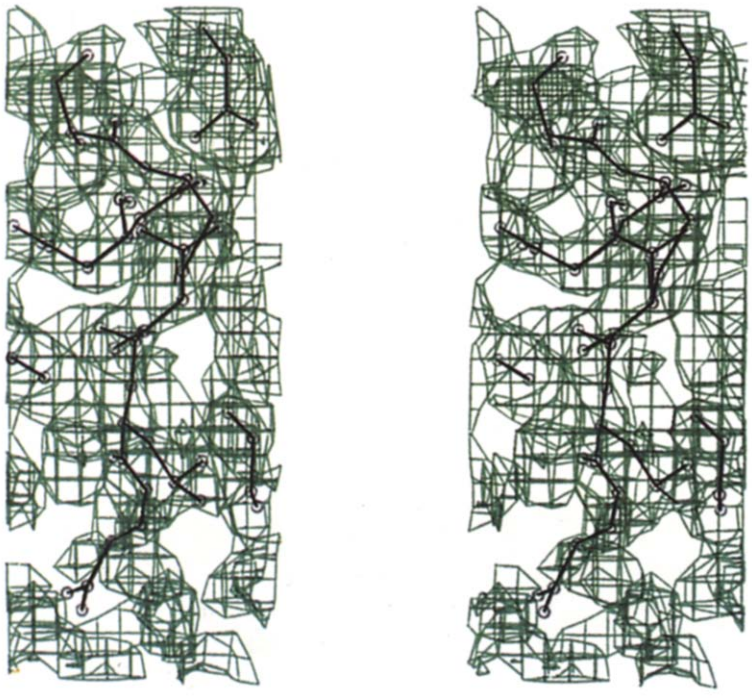

Fig.2. Stereoscopic view of fragment of an electron density contour map which corresponds to the binding of the E-64-c molecule (thick lines) to the active site of papain. Some nearest-neighbor amino acid residues are also shown by the stick bonds. The 3-methylbutyl terminal of E-64-c had relatively large thermal motions, and the corresponding contour is not shown at this electron density level.

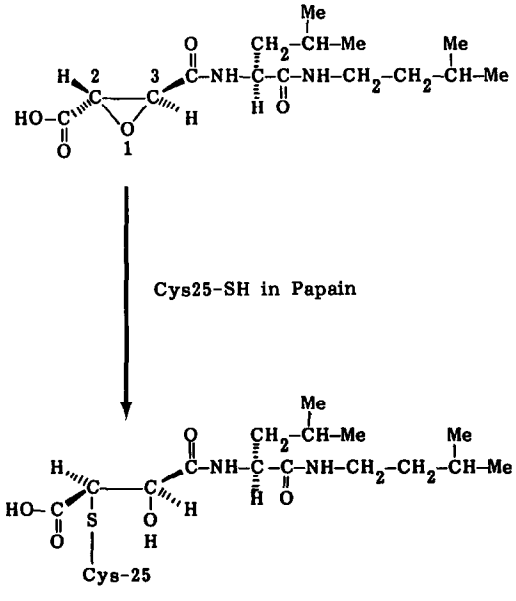

Fig.3. Configurational conversion around the C2 atom of the E-64-c oxirane ring caused by covalent bond formation with the S^{γ} atom of the Cys-25 residue of the active site of papain.

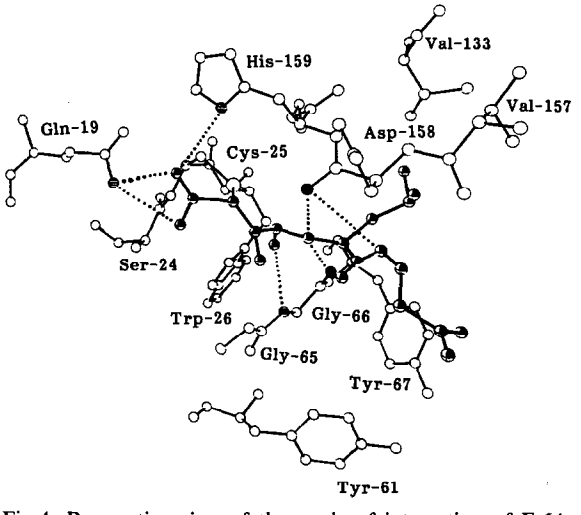

Fig.4. Perspective view of the mode of interaction of E-64-c with each amino acid residue forming the papain active site. Dotted lines represent possible formation of hydrogen bonds. The E-64-c molecule is shown in the thermal ellipsoidal style. Filled and shaded circles represent oxygen and nitrogen atoms of each amino acid residue participating in the hydrogen bond, respectively.

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