Molecular Dynamics Simulation of Papain–E-64 (*N*-[*N*-(L-3-*trans*-Carboxyoxirane-2-carbonyl)-L-leucyl]agmatine) Complex

Daisuke YAMAMOTO,*[,a] Hirofumi OHISHI,[a] Toshimasa ISHIDA,*[,a] Masatoshi INOUE,[a] Shigeyuki SUMIYA[b] and Kunihiro KITAMURA[b]

Osaka University of Pharmaceutical Science,[a] 2–10–65 Kawai, Matsubara, Osaka 580, Japan and Research Center of Taisho Pharmaceutical Co., Ltd.,[b] 1–403 Yoshino-cho, Ohmiya, Saitama 330, Japan. *Received December 14, 1989*

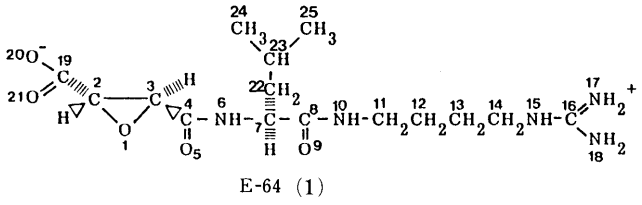
To investigate the possible binding mode of E-64 (*N*-[*N*-(L-3-*trans*-carboxyoxirane-2-carbonyl)-L-leucyl]agmatine), a potent cysteine protease inhibitor, to papain active site, molecular dynamics simulations were applied to two complex forms: *R*- and *S*- configurational forms of E-64 C2 atom for the covalent bond formation with the papain Cys-25 SH group. The tertiary structures of the papain–E-64 complexes were built by visual interactive modelling and the energy minimization technique, and were subjected to the dynamics simulations of 10 ps. Although no significant difference was observed between the potential energies of energy-minimized *R*- and *S*-complex forms, the molecular dynamics simulations suggested that the hydrogen bonding mode of the former form is more advantageous than that of the latter one. Comparing with the hydrogen bonds observed in the papain–E-64 complex crystal, it could be concluded that the present molecular dynamics simulation reflects well the three-dimensional structure concerning the interaction of E-64 with the papain active site. The conformational characteristics of E-64 and its possible interaction mode with papain were also discussed.

Keywords molecular dynamics simulation; E-64; papain; enzyme–inhibitor complex; interaction mode

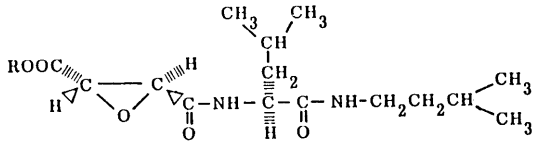
Introduction

Cysteine proteases containing a highly reactive cysteine residue at the active site are abundant in the body, and are responsible for much of the intracellular proteolysis,[1-3] so the means by which their activities are controlled is of great interest. In contrast with the vast amount of information that has been obtained on the serine protease–inhibitor interaction,[4] very little is known of the inhibitory mechanism of cysteine protease. Thus the knowledge of the enzyme–inhibitor interaction is valuable for considering the biological function of cysteine protease.

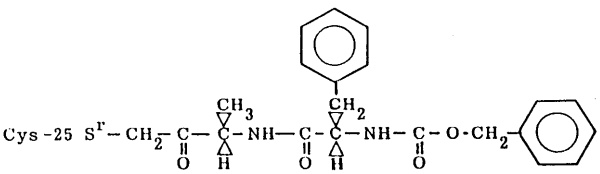
E-64 (**1**), *N*-[*N*-(L-3-*trans*-carboxyoxirane-2-carbonyl)-L-leucyl]-agmatine, which was first isolated from *Aspergillus japonicus*,[5,6] is one of the potent non-protein inhibitors for cysteine proteases.[7-9] Because of its potent inhibitory activity coupled with its low toxicity, this molecule becomes a lead compound for the drug development to suppress the elevated levels of cysteine protease activity associated with certain disease states such as muscular dystrophy.[10,11]



E-64 (1)



loxistatin (2): R= $-CH_2CH_3$
E-64-c (3): R= $-H$



ZPACK (4)

Chart 1

As a part of conformational studies of cysteine protease inhibitors, we recently analyzed the crystal and molecular structures of E-64[12] and its analogue loxistatin (**2**).[13] The conformations of both molecules showed a flattened and slightly curved structure, which is similar to that of *N*-benzyloxycarbonyl-phenylalanylalanine-methylketone (ZPACK, **4**), a substrate analogue covalently bound to the papain Cys-25 SH group.[14] This indicates that the molecular conformation of E-64 or loxistatin observed in their single crystal structures is to some extent reserved in the inhibitor–enzyme complex. In order to ascertain this possibility, the crystallographic and molecular dynamics simulation studies have also been carried out.

The preliminary X-ray crystal analysis[15] of the papain–E-64-c (**3**) complex at 2.5Å resolution suggested the covalent bonding of papain Cys-25 SH group to E-64-c C2 atom from the opposite side of its oxirane O1 atom, consequently causing the $S \rightarrow R$ configurational change of the C2 atom (see Fig. 1). However, the detailed molecular conformation of E-64-c and its interaction mode with papain have to wait until the structural refinement of the complex is accomplished adequately.

On the other hand, molecular dynamics (MD) simulation could be a valuable method for investigating the possible interaction of E-64 or its analogue with the papain active site, because it provides a lot of information about the conformational changes of the molecule coexisting with solvent molecules at an appropriate temperature.[16] This paper deals with the structural reason why the covalent bond formation with the papain Cys-25 SH group is taking place at the E-64 C2 atom with *R*-configuration, not with *S*-one, through the computational generation of possible

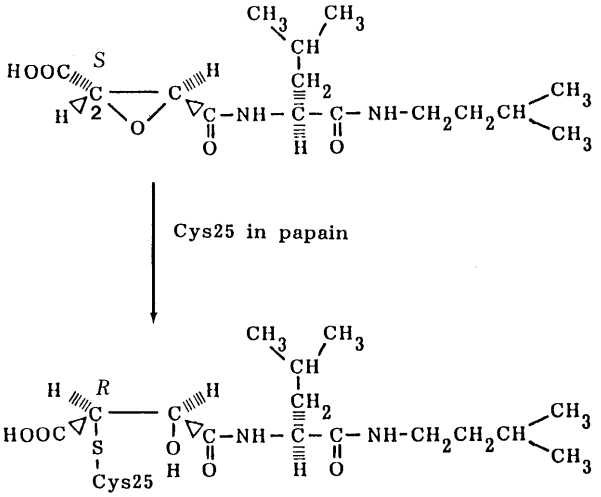


Fig. 1. Configurational Conversion Around the C2 Atom of the E-64 Oxirane Ring Caused by the Covalent Bond Formation with the S^{γ} Atom of the Cys-25 Residue of the Active Site of Papain

binding modes of E-64 with the papain active site.

Experimental

Generation of E-64–Papain Complex Structure First, using the graphics program QUANTA (Polygen Co., Ltd., MA, USA) on IRIS3130 workstation, visual model building of E-64–papain complex was carried out using the crystal structure of ZPACK–papain complex.[14] because the whole molecular conformation of E-64 is very similar to that of ZPACK. Concerning the covalent bonding between the E-64 C2 atom and the Cys-25 Sγ atom in papain, two kinds of modes, *i.e.*, *R*- and *S*-configurations, were considered.

Secondly, using the program AMBER 3.0[17] with the OPLS parameters,[18] the binding structures of the modeled (*R*)- and (*S*)-E-64–papain complexes, which consisted of amino acid residues within 10Å of the E-64 C7 atom, were energy-minimized by the conjugate gradient method, respectively, until each gradient was smaller than 1.0 kcal/mol/Å, where the distance dependent dielectric constant, $\varepsilon = R_{ij}$, and 8Å nonbonded cutoff were employed. The number of atoms included in each minimizations were 714 for (*R*)-E-64–papain complex and 707 for (*S*)-E-64–papain complex.

Molecular Dynamics (MD) Simulation of E-64–Papain Complex To each of the energy-minimized complexes, water molecules with TIP3P energy parameters[19] were added within the sphere of 16Å radius from the E-64 C7 atom, and they were restrained by a boundary force of 1.0 kcal/mol/Å^2 to prevent their leakage from the molecular system. The number of water molecules was 234 for (*R*)-E-64–papain system and 263 for (*S*)-E-64–papain system, respectively. The MD simulations were done with time steps of 0.002 ps, $\varepsilon = 1$ and a nonbonded cutoff of 8 Å. Then, the initial velocities for respective molecular systems were given from the Boltzmann's distribution of 10 K, and then the temperature of the respective systems, with a relaxation time of 0.5 ps, was elevated up to 310 K. In order to save computational time, furthermore, the SHAKE method[20] was used for hydrogen atoms within a 16 Å sphere and all atoms out of the sphere were fixed.

All numerical calculations were carried out on MicroVAX II and VAX 8300/VMS computers.

Results and Discussion

Energy-Minimized Structures of E-64–Papain Complexes The energy-minimized structures of (*R*)-E-64–papain and (*S*)-E-64–papain complexes are shown in Fig. 2a and 2b, respectively, where no unusual short contacts are observed. The potential energies of these structures were almost the same, that is, −621 kcal/mol for (*R*)-E-64–papain complex and −618 kcal/mol for (*S*)-E-64–papain complex. Comparative calculations showed that the binding of E-64 molecule to the papain S subsites[21] was much more energetically stable than the S′ subsites; the binding at S subsites has also been observed in the X-ray crystal analyses of papain–E-64[22] and –E-64-c[15] complexes. This is contrary to earlier predictions,[23,24] where the E-64 interacts with the S′ subsites of papain.

Table I. Probabilities (%) of Hydrogen Bond Formations between E-64 and Papain during Molecular Dynamics Simulation of 10 ps, along with Hydrogen Bonds Observed in the Crystal of E-64–Papain Complex

	E-64	Papain	Probability (%)
R-Form			
	O5	Gly-66 N	62.7
	O5	Trp-26 N	5.9
	N6	Asp-158 O	54.9
	N10	Gly-66 O	82.4
	N15	Tyr-61 O$^{\eta}$	19.6
	N17	Tyr-61 O$^{\eta}$	3.9
	O20	His-159 N$^{\delta}$	68.6
	O20	Gln-16 N$^{\varepsilon}$	19.6
	O21	His-159 N$^{\delta}$	11.8
	O21	Gln-19 N$^{\varepsilon}$	59.4
S-Form			
	O5	Gly-66 N	25.5
	O5	Trp-26 N	9.8
	N6	Asp-158 O	35.3
	N10	Gly-66 O	13.7
	N10	Asn-64 O	3.9
	N15	Tyr-61 O$^{\eta}$	21.7
	N15	Tyr-67 O$^{\eta}$	23.5
	N17	Tyr-61 O$^{\eta}$	15.7
	N18	Tyr-67 O$^{\eta}$	11.8
	O20	Ala-160 N	88.2
Crystal			Distance (Å)
	O5	Gly-66 N	2.88
	N10	Gly-66 O	3.04
	N17	Tyr-61 O$^{\eta}$	2.96
	N18	Tyr-67 O$^{\eta}$	2.93
	O20	His-159 N$^{\delta}$	2.90
	O21	Cys-25 N	2.95
	O21	Gln-19 N$^{\varepsilon}$	2.87

The possible hydrogen bonds are also shown in Fig. 2a and 2b. The difference between the hydrogen bonding modes would be due to the configurational difference of the E-64 C2 atom. Another characteristic difference could be observed in (1) the orientation of C3–O1 bond direction with respect to the backbone chain and (2) the hydrophobic interaction between the E-64 agmatine (C11–C14) and papain Tyr-61 and -67 residues.

Molecular Dynamics Simulation MD simulations covering 10 ps were carried out in order to examine conformational changes in respective binding sites of (*R*)-E-64–papain and (*S*)-E-64–papain complexes. Figure 2c and 2d show 10 ps snapshots of both the binding structures. It is important to note that these snapshots, though arbitrary selected, represent respective averaged structures during 10 ps MD simulations. In (*R*)-E-64–papain complex (Fig. 2c), little conformational change was observed in the papain binding site, when it was compared with the energy-minimized complex (Fig. 2a). On the contrary, a relatively large conformational deformation was found for (*S*)-E-64–papain, especially around the His-159 and Gln-19 residues of papain (compare Fig. 2b and 2d). Since both of the energy-minimized complex structures are quite similar to each other as seen in Fig. 2a and 2b, these

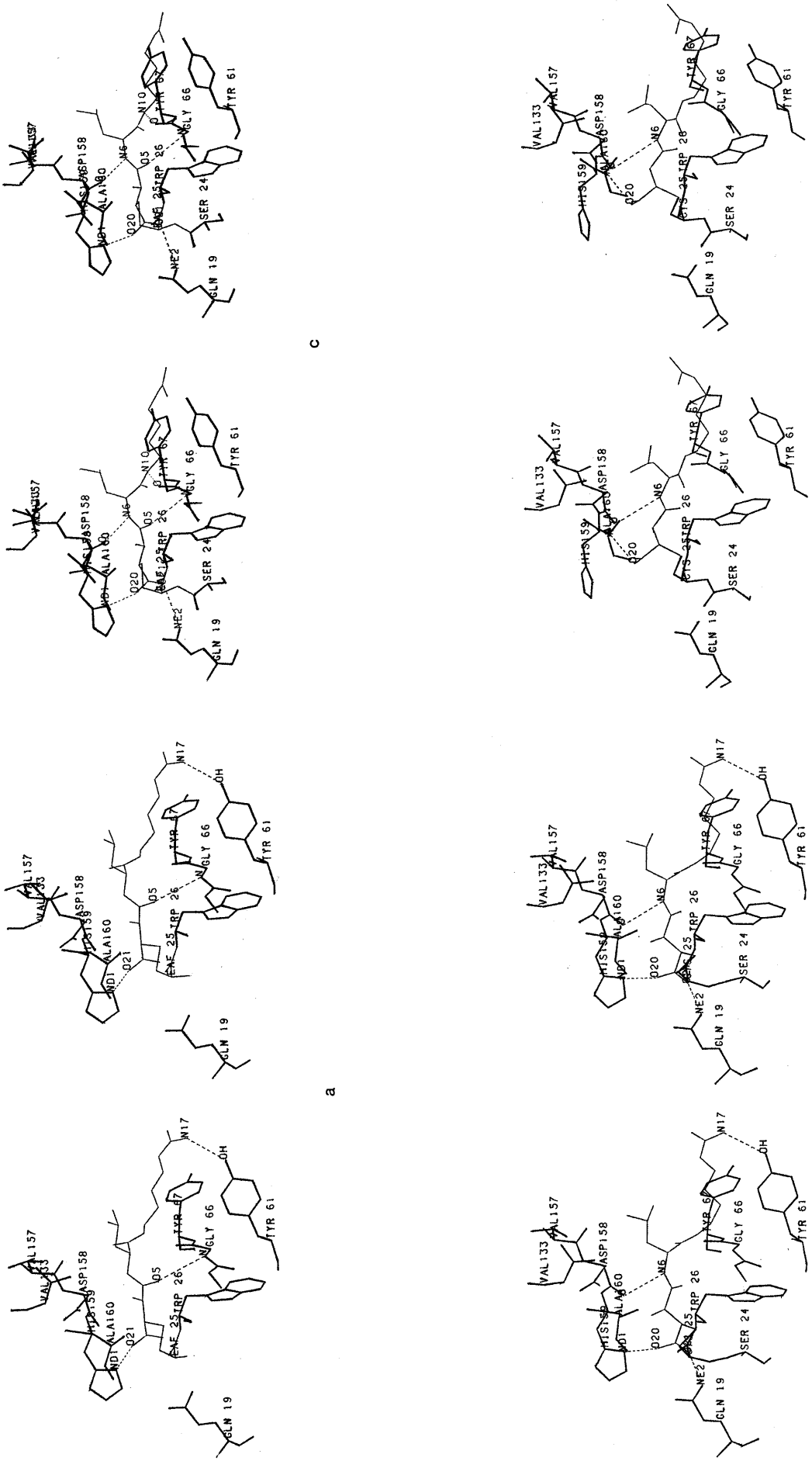


Fig. 2. Stereoviews of the Calculated Tertiary Structures of E-64–Papain Interaction Region

a and b are the energy-minimized structures of (*R*)-and (*S*)-E-64–papain complexes, respectively. Respective snapshots at MD simulations of 10 ps are shown in c and d. Water molecules are not shown to clarify the diagrams of c and d.

results suggest that the binding of (*S*)-E-64 brings unreasonable changes into papain binding site structure.

Table I lists the probabilities of hydrogen bond formations during MD simulations of 10 ps, where the hydrogen bonds observed in the (*R*)-E-64–papain complex crystal[22] are also listed for the comparison. The probability was calculated from the ratio of the atomic pair showing the hydrogen bond formation (donor...acceptor $\leqq$3.2Å) at each step of 10 ps MD simulation. Judging from Table I, it could be said that (1) the probabilities of respective atoms participating in the hydrogen bond formations are much higher for (*R*)-E-64–papain complex than (*S*)-one, (2) the hydrogen bonds of O5–Gly-66 N, N6–Asp-158 O and N10–Gly-66 O are stronger in the(*R*)-E-64–papain complex than (*S*)-one, and (3) the terminal carboxyl oxygen atoms in (*R*)-E-64 are tightly linked by the hydrogen bonds with His-159 N^{δ} and Gln-19 N^{ε} atoms, while those in (*S*)-E-64 are linked by only one hydrogen bond with Ala-160 N atom. Thus, the stereostructure in Fig. 2c reveals considerably the actual interaction mode between the E-64 and papain active site.

Possible Reasons for *R* Configurational Change of (*S*)-E-64 C2 Atom Accompanying a Covalent Bond Formation with Papain Cys-25 S^{γ} Atom As was stated, the (*R*)-E-64–papain complex is suggested to be of greater advantage for hydrogen bond formations with the polar atoms in the papain active site than the (*S*)-one. Thus the *R* configurational change of (*S*)-E-64 could be supposed to be due to the stereochemical requirement accompanying the interaction between the E-64 and papain active site. On the other hand, the following reason could also be considered. As is conceivable from the chemical structure of E-64 (Chart 1), the attachment of Cys-25 SH group to the E-64 C2 atom from the opposite side of oxirane O1 atom is energetically more favorable than from the same side by the electrostatic repulsion between the Cys-25 SH group and the oxiran O1 atom.

Conformational Comparison of E-64 Molecule Superimposition of E-64 conformation in MD simulation with that observed in its crystal structure is shown in Fig. 3; the comparison between the respective torsion angles is given in Table II. Although some of torsion angles are different, the whole conformations of E-64 are similar to each other, and could be characterized as having a flattened and slightly curved form. This means that the molecular conformation of E-64 observed in the single crystal is maintained well on binding with papain and represents the stereostructure suitable for joining with the papain active site. It is interesting to note in connection with this conformational similarity that the ZPACK also exhibits the conformational characteristics common to E-64,[12] and tight binding to papain could be determined by the whole conformation of ZPACK or the E-64 molecule. This would be useful for

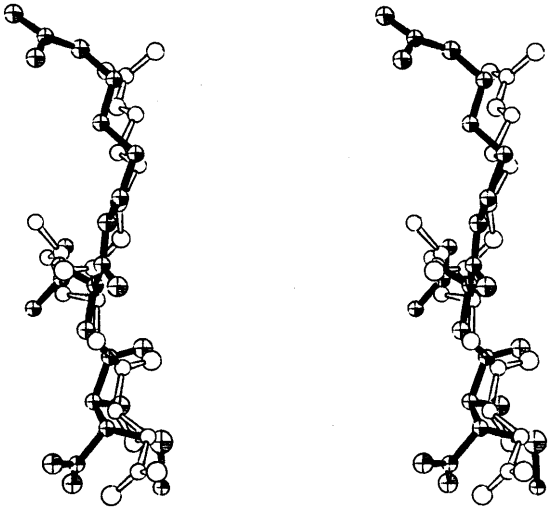


Fig. 3. Superposition of E-64 Molecular Conformations Observed in the Crystal Structure (Open Bonds) and the Present Molecular Dynamics Simulation (Filled Bonds)

The latter conformation corresponds to the MD simulation at 8.2 ps.

Table II. Torsion Angles (°) of E-64 Conformations in Its Crystal Structure and MD Simulation (at 8.2 ps) of Complex Structure with Papain

Bond sequence	MD simulation	Crystal	
		I	II
C3–C4–N6–C7	168.3	−174.1	−178.1
C4–N6–C7–C8	−81.2	−132.6	−115.5
N6–C7–C8–N10	140.2	128.1	118.9
N6–C7–C22–C23	−176.7	−177.1	−175.8
C7–C8–N10–C11	172.7	173.0	177.5
C8–N10–C11–C12	−74.3	−149.9	−93.5
N10–C11–C12–C13	−170.2	60.0	−53.8
C11–C12–C13–C14	173.1	175.2	−174.0
C12–C13–C14–N15	−171.7	176.7	84.9
C13–C14–N15–C16	−112.8	179.9	147.6
C14–N15–C16–N17	34.8	7.7	8.9

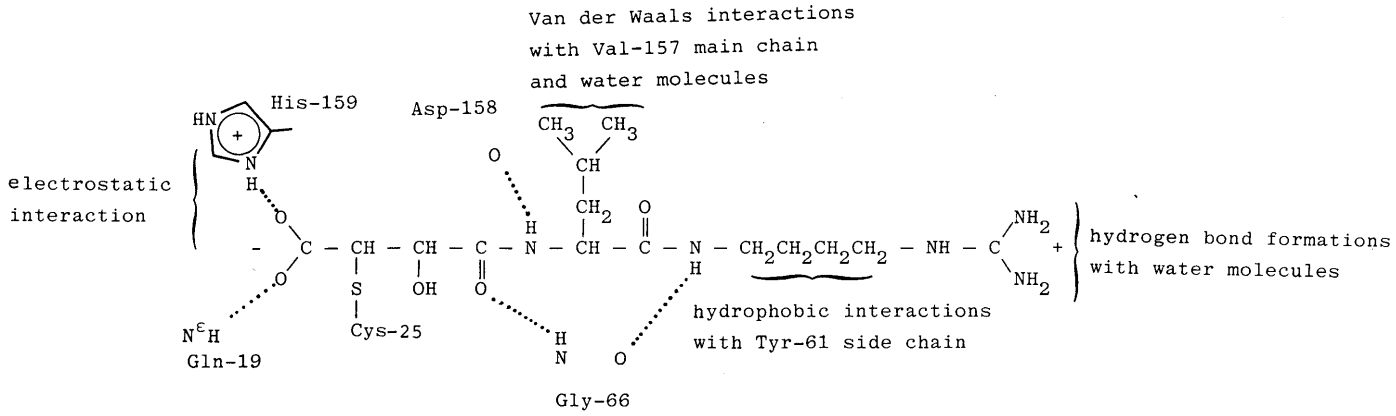


Fig. 4. Schematic Diagram Showing the Interaction of E-64 with Papain, Proposed by Molecular Dynamics Simulation

designing potent inhibitors of cysteine protease. It could explain a reason why, though the chemical structures of ZPACK and E-64 molecules are quite different from each other (see Chart 1), especially the directions of peptide bonds (–NH–CO– for ZPACK and –CO–NH– for E-64), both the molecules occupy the same S subsites. This is contrary to the previous prediction (S′ site occupation for E-64 and S-site for ZPACK[23,24]).

Possible Interaction Mode of E-64 with Papain A schematic diagram showing the interactions of E-64 with the papain active site is shown in Fig. 4, which is proposed as the most reasonable mode based on the averaged structure during the 10 ps MD simulation of (*R*)-E-64–papain complex.

In addition to the hydrogen bonds listed in Table I, this figure shows the importance of electrostatic and hydrophobic interactions for the potent inhibitory activity of the E-64 molecule. This interaction mode is essentially the same as previously predicted by us[12] and is not in conflict with the crystal structures of papain–E-64[22] and –E-64-c[15] complexes.

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