HYDROLYSIS OF ANGIOTENSIN II RECEPTOR BLOCKER PRODRUG OLMESARTAN MEDOXOMIL BY HUMAN SERUM ALBUMIN AND IDENTIFICATION OF ITS CATALYTIC ACTIVE SITES

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ABSTRACT:

In the present study, we investigated the esterase-like activity of human serum albumin (HSA) and the mechanism by which it hydrolyzes, and thereby activates, olmesartan medoxomil (CS-866), a novel angiotensin II receptor antagonist. CS-866 has previously been shown to be rapidly hydrolyzed in serum in which HSA appeared to play the most important role in catalyzing the hydrolysis. We found that the hydrolysis of CS-866 by HSA followed Michaelis-Menten kinetics. Compared with the release of p-nitrophenol from p-nitrophenyl acetate (PNPA), CS-866 showed lower affinity to HSA and a lower catalytic rate of hydrolysis. Thermodynamic data indicated that PNPA has a smaller value of activation entropy ($\Delta S$) than CS-866; consequently, PNPA is more reactive than CS-866. Ibuprofen and warfarin acted as competitive inhibitors of hydrolysis of CS-866, whereas dansyl-$\mathrm{L}$-asparagine, n-butyl-$p$-aminobenzoate, and diazepam did not. These findings suggest that the hydrolytic activity is associated to parts I and site II for ligand binding. All chemically modified HSA derivatives (Tyr-, Lys-, His-, and Trp-modifications) had significantly lower reactivity than native HSA; Lys-HSA and Trp-HSA had especially low reactivity. All the mutant HSAs tested (K199A, W214A, and Y411A) exhibited a significant decrease in reactivity, suggesting that Lys-199, Trp-214, and Tyr-411 play important roles in the hydrolysis. Results obtained using a computer docking model are in agreement with the experimental results, and strongly support the hypotheses that we derived from the experiments.

Ester prodrugs are hydrolyzed to their pharmacologically active metabolites after absorption. Esterases present in the small intestine, plasma, and liver are involved in this process. In most cases, intestinal esterases serve as the major enzymes in activation of prodrugs during the first pass through the gut after absorption. However, prodrugs that are relatively resistant to hydrolysis by intestinal esterases enter the blood circulation and are activated by serum (plasma) and liver esterases. The major hydrolyzing enzymes in serum are cholinesterase, arylesterase, carboxylesterase, and albumin. The relative importance of each serum esterase in prodrug activation varies among animal species and prodrugs.

Olmesartan medoxomil [CS-866: (5-methyl-2-oxo-1,3-dioxolen-4-yl)methoxy-4-(1-hydroxyl-1-methylethyl)-2-propyl-1-[4-[2-(tetrazol-5-yl)-phenyl][phenyl]methylimi-dazol-5-carboxylate] is a novel nonpeptide angiotensin II receptor antagonist that acts as an antihypertensive prodrug (Koike et al., 2001; Neutel, 2001; Brousil and Burke, 2003). After oral administration, CS-866 is rapidly de-esterified, producing an active acid metabolite, olmesartan (RNH-6270) (Fig. 1) (Koike et al., 2001; Neutel, 2001; Brousil and Burke, 2003). Hydrolysis of CS-866 in serum has been observed in several species, and comparison among five species has shown that hydrolytic activity is highest in rabbits, followed by dogs, mice, rats, and humans (Ikeda, 2000). Furthermore, it was found that differences in hydrolytic activity due to serum albumin are large compared to the combined activity of all serum components. Thus, HSA might make an important contribution to activation of CS-866 after oral administration.

In the present study, we examined the esterase-like activity of HSA and the mechanism of its hydrolysis of CS-866. First, the general properties of the hydrolytic reaction of HSA with CS-866 were determined, including the kinetics and thermodynamics, and compared with those of the hydrolytic reaction between HSA and p-nitrophenyl acetate (PNPA) (Means and Bender, 1975; Sakurai et al., 2004). Second, to characterize the effects of exogenous compounds on hydrolysis, we investigated changes in hydrolytic activity in the presence of various serum components.
presence and absence of various ligands. Then, we examined the importance of certain types of amino acid residues of HSA for the hydrolysis of CS-866, using chemical modification techniques. Recombinant HSA (rHSA) proteins with alterations of specific amino acid residues were prepared using site-directed mutagenesis techniques, to obtain detailed information about the contribution of those residues. Finally, computer docking models of CS-866 and HSA were constructed and were found to be consistent with the experimental results.

**Materials and Methods**

**Mammals.** HSA was donated by the Chemo-Sera-Therapeutic Research Institute (Kumamoto, Japan). HSA was defatted before use (Chen, 1967). CS-866 and RNH-6270 were donated by Sanyko Co., Ltd. (Tokyo, Japan). PNPA, succinic anhydride and n-butyl p-aminobenzoate (n-butyl p-AB) were purchased from Nakalai Tesque Co., Ltd. (Kyoto, Japan), trinitrobenzensulfonic acid was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), tetranitromethane was obtained from Aldrich Chemical Co. (Milwaukee, WI), and trinitrobenzene was obtained from Sumitomo Pharmaceutical Co. (Tokyo, Japan), diazepam was obtained from Sumitomo Pharmaceutical Co. (Osaka, Japan), tetriramthone was obtained from Aldrich Chemical Co. (Milwaukee, WI), and trinitrobenzensulfonic acid was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Dansyl-l-asparagine (DNSA), 2-hydroxyl-5-nitrobenzyl bromide, and diethyl pyrocarbonate were purchased from Sigma-Aldrich (St. Louis, MO). Restriction enzymes, T4 polynucleotide kinase, calf intestinal alkaline phosphatase, a DNA ligation kit, TaKaRa EX TaqDNA polymerase, and a site-directed mutagenesis kit (oligo-nucleotide-directed dual amber method) were obtained from Takara Shuzo Co., Ltd. (Kyoto, Japan). A DNA sequence kit was obtained from Applied Biosystems (Tokyo, Japan). The Pichia Expression kit was purchased from Invitrogen (Carlsbad, CA). All other chemicals were of analytical grade.

**Esterase-Like Activity Measurement.** Procedures for Michaelis-Menten Equation Runs. The reaction was started by adding CS-866 in 100% acetoniwater (5 μl) to preincubated HSA (120 μl, 75 μM), at a final concentration of 10 to 250 μM. Incubation proceeded for 10 min and was terminated by adding 500 μl of acetoniwater to the incubation mixture. We have checked that 4% acetoniwater has little effect on the reaction. After centrifugation for 1 min, a 30-μl aliquot of the deproteinized supernatant was subjected to high-performance liquid chromatography, and RNH-6270 was separated from CS-866 on an ODS column using the following conditions: column, YMC-Pack ODS-AM, AM-302, 150 × 4.6 mm i.d.; column temperature, 40°C maintained by a Hitachi 655A-52 column oven; a Hitachi L-6000 pump; a Hitachi FL detector L-7480 fluorescent monitor; a HITACHI D-2500 Integrator; mobile phase, acetoniwater/water/acetic acid, 40:60:0.1; wavelength, excitation = 260 nm, emission = 370 nm; flow rate, 1.0 ml/min.

The reaction between CS-866 and HSA took place at 4°C. Under that condition, Michaelis-Menten equation analysis can be applied.

\[
v = \frac{V_{\text{max}}[S]}{K_M + [S]}
\]

Here, [S] is the concentration of substrate. That is the case, because previous studies have revealed a linear relationship between 1/V and 1/S when plotted in a Lineweaver-Burk plot (Koike et al., 2001):

\[
\frac{1}{v} = \frac{1}{V_{\text{max}}} + \frac{K_M}{V_{\text{max}}[S]}
\]

**Procedures for Kinetic Runs.** Hydrolysis of CS-866 (5 μM) by HSA (at least a 5-fold excess concentration over the substrate) was performed using conditions able to avoid complications due to multiple reactive sites of albumin. Under such conditions, pseudofirst-order rate constant analysis can be performed. The pseudofirst-order rate constant for the release of RNH-6270 (kcat), the dissociation constant of the substrate-HSA complex (Ks), and the catalytic rate constants (kcat) were calculated as reported elsewhere (Sakurai et al., 2004).

**Thermodynamic Analysis.** Thermodynamic analysis of the HSA-catalyzed reaction was performed at temperatures ranging from 20°C to 40°C. We calculated the thermodynamic parameters, the free energy change for the initial reaction between enzyme and substrate (∆G0), the activation free energy for the rate-determining step (∆Gcat), the free energy difference for the reaction (∆G), the activation energy (∆Ea), the activation enthalpy change (∆H), and the activation entropy change (∆S), using previously published methods (Sakurai et al., 2004).

Effects of Ligands. HSA (120 μl, 75 μM) was preincubated, and the reaction was performed by adding CS-866 in 100% acetoniwater (5 μl) to the solution, at a final concentration of 100 to 250 μM, in the presence or absence of each ligand (at a final concentration of 0–600 μM) (Ikeda, 2000). Incubation proceeded for 10 min at 37°C, and the release of RNH-6270 was measured by high-performance liquid chromatography as described above.

**Chemical Modification of HSA.** Histidine Residues. Chemical modification of His residues was performed using diethyl pyrocarbonate (Roosemont, 1978). An average of 2.22 His residues was modified out of the total of 16 His residues.

Lysine Residues. Chemical modification of Lys residues was performed according to the method of Gounaris and Perlmann (1967). The modification ratio was calculated as described by Haynes et al. (1967). An average of 3.80 of the 59 Lys residues was modified.

Tyrosine Residues. Chemical modification of Tyr residues was performed as outlined by Sokolovsky et al. (1966). An average of 1.24 of the 18 Tyr residues was modified.

Tryptophan Residues. Chemical modification of the single Trp residue was performed at room temperature (Fehske et al., 1978). An average of 0.88 of the 1 Trp residue was modified.

Chemical modifications of specific amino acid residues (Tyr, Lys, His, and Trp) were performed with the assumption that the effects on other amino acid residues would be negligible. The secondary and tertiary protein structures of all the modified HSA were examined by circular dichroism measurements before use, and no significant difference was observed between the derivatives and native HSA (data not shown).

**Synthesis and Purification of rHSA Forms.** The recombinant DNA techniques used to produce wild-type rHSA and the single-residue mutants were
essentially the same as those described by Watanabe et al. (2001). A chimeric plasmid (pDB-ADH-L10-HSA-A) containing cDNA for the mature form of HSA and an L10 leader sequence was donated by Tonen Co. (Tokyo, Japan). The mutagenic primers used (underlined letters indicate mismatches) were as follows: 5'-CAAAACAGAGACTGCTGTGACGCTTCG-3' for K199A; 5'-GACATTGCTGAAGCAGCTGAGTACTGCTGTG-3' for W214A; 5'-CTACAGTGGCTGCCAGCACAAG-3' for Y411A.

The L10-HSA coding region was amplified by polymerase chain reaction using a forward and a reverse primer containing a 5'-terminus EcoRI site, and was cloned into the EcoRI-flanked pKF19k vector (Takara Shuzo Co., Ltd.), and mutagenesis was then performed. The mutation was confirmed by DNA sequencing of the entire HSA coding region using the dyeoxy chain termination method and an Applied Biosystems ABI Prism 310 Genetic Analyzer. To construct the HSA expression vector pHL-D2-HSA, an L10-HSA coding region with or without the desired mutation site was incorporated into the methanol-inducible pHL-D2 vector (Invitrogen). The resulting vector was introduced into the yeast species Pichia pastoris (strain GS115) to express rHSA. Secreted rHSA was isolated from the growth medium by precipitation with 60% ammonium sulfate at room temperature, and was then purified using a column of Blue Sepharose CL-6B (GE Healthcare, Little Chalfant, Buckinghamshire, UK). The eluted rHSA was deionized and then defatted using charcoal treatment.

The resulting protein exhibited a single band on an SDS/polyacrylamide gel, and all of the recombinant proteins migrated to the same position as native HSA (data not shown). Any secondary or tertiary structural differences between native (wild-type) and mutant rHSAs were analyzed by circular dichroism (data not shown). In the far-UV and near-UV regions, all HSAs exhibited the same characteristics as native HSA.

Docking of CS-866 to HSA. To dock CS-866 to HSA, we used the crystal structure of the HSA-myristate-S-warfarin complex (PDB ID 1H9Z; Petipas et al., 2001). The docking calculation of CS-866 to HSA was performed using SYBYL FlexX (Rarey et al., 1996). CS-866 docked at site I and site II. The residues within 5 Å from S-warfarin were defined as site I, and the residues within 5 Å from myristate-3 and -4 were defined as site II. During the docking calculation, the structure of HSA was kept rigid. The docking algorithm generated 275 and 209 different placements of CS-866 in site I and site II, respectively. All placements were evaluated using the scoring function of FlexX. For each site, because the top 10 placements exhibited nearly identical binding modes, we chose the placement with the best value as the candidate binding mode.

Refinement of Docking Models. To refine the docking models, the coordinates of CS-866 and the residues within 10 Å from CS-866 were optimized to reduce the root mean square of the gradients of potential energy coordinates of CS-866 and the residues within 10 Å from CS-866 were optimized to reduce the root mean square of the gradients of potential energy.

Statistics. Where possible, statistical analyses were performed using Student’s t test.

Results

Hydrolotic Kinetics. First, we were able to confirm that the hydrolysis of CS-866 by HSA followed Michaelis-Menten kinetics (data not shown). Table 1 shows the $K_M$, $V_{max}$, $k_{cat}$, and specificity constant ($k_{cat}/K_M$) values for the hydrolitic reaction. To elucidate the reactivity of CS-866, we compared the kinetic parameters for CS-866 with those determined for the release of p-nitrophenol from PNPA (Table 2) (Means and Bender, 1975; Sakurai et al., 2004). The $K_S$ value was found to be lower for CS-866, suggesting that PNPA has greater affinity than CS-866 for HSA (Fersht, 1998; Sakurai et al., 2004). The catalytic rate constant, $k_{cat}$, was also found to be greater for PNPA.

Thermodynamics. The relationship between the catalytic rate constants and temperature followed the Arrhenius equation. Accordingly, a linear relationship was found between $\ln k_{cat}$ and absolute temperature in degrees Kelvin (data not shown). The activation energy of the reaction, $E_a$, calculated from the Arrhenius plot (Fersht, 1998; Sakurai et al., 2004), was found to be 37.1 kJ mol$^{-1}$.

Using the HSA-hydrolis parameters, we compared energy changes and thermodynamic parameters between CS-866 and PNPA (Table 3). CS-866 had larger values of $\Delta G$ (96.3 kJ mol$^{-1}$) and $\Delta S$ ($-0.207$ kJ mol$^{-1}$ K$^{-1}$) than PNPA.

Effects of Ligands on Hydrolis. The site I-specific ligands warfarin, DNSA, and n-butyl p-AB were used as inhibitors to investigate, as compared with competition with the hydrolitic reaction (Yamasaki et al., 1996; Kragh-Hansen et al., 2002). Interestingly, warfarin inhibited hydrolis in a competitive manner, with a $K_i$ value of 155 μM in a Dixon plot (Fig. 2A). By contrast, neither n-butyl p-AB nor DNSA inhibited HSA-catalyzed hydrolis of CS-866 (Fig. 2, B and C).

The site II-specific ligands ibuprofen and diazepam were used to investigate, whether there is competition between that ligand-binding site and the catalytic site (Kragh-Hansen et al., 2002). Diazepam had no inhibitory effect, but competitive inhibition was observed with ibuprofen, with a $K_i$ value of 235 μM in a Dixon plot (Fig. 3A and 3B).

Effect of Chemical Modification on Hydrolis. Hydrolitic activities of the four specifically modified HSA derivatives (Tyr-, Lys-, His-, and Trp-HSA) were assayed (Fig. 4). Compared with native-type HSA, all modified HSA derivatives had significantly decreased hydrolitic activity ($p < 0.05$). Modification of Lys residues or of the single Trp residue resulted in the most pronounced reductions in catalytic reactivity.
Examination of Hydrolytic Activity Using Site-Directed Mutagenesis. Wild-type rHSA and the HSA single-residue mutants K199A, W214A, and Y411A were used to examine involvement of various amino acid residues in the hydrolysis. The hydrolytic activity of each rHSA was examined to elucidate the contribution of specific amino acid residues (Fig. 5). Compared with the wild-type rHSA, all mutant rHSAs showed significant decreases in catalytic activity. K199A exhibited a particularly marked decrease in catalytic activity \((p < 0.01)\), suggesting that Lys-199 plays a particularly important role in the hydrolysis. These results are in good agreement with those obtained with the chemically modified HSAs. The W214A and Y411A mutants showed a significant reduction in catalytic activity \((p < 0.05)\), indicating that the amino acid residues Trp-214 and Tyr-411 are also involved in the hydrolytic reaction.

Molecular Interaction of CS-866 and HSA in Docking Models. We obtained two docking models: model I for site I, and model II for site II. In model I, the binding of CS-866 was similar to that of warfarin (Fig. 6). The biphenyl moiety of CS-866 was bound to the hydrophobic pocket consisting of Leu-219, Leu-238, Val-241, Leu-260, Ala-261, Ile-264, Ile-290, and Ala-291. The 2-propyl-imidazole moiety and the propan-2-ol moiety were bound to the other hydrophobic pocket (Phe-211, Trp-214, Ala-215, Leu-219, and Leu-238). Hydrogen bonds to and electrostatic interactions with other residues are detailed in Table 4. Oxygen atoms of the vinylene carbonate moiety formed hydrogen bonds with side chains of Arg-218 and of Arg-222. Negative charges of the tetrazole moiety interacted electrostatically with side chains of Lys-199 and of Arg-257. The hydroxyl oxygen atom of the propan-2-ol moiety formed a hydrogen bond with the side chain of Lys-199, and the hydrogen atom formed a hydrogen bond with the side chain of His-242. The carbonyl oxygen atom of the ester moiety formed a hydrogen bond with the side chain of Lys-199. The ester moiety of CS-866 was in the vicinity of Glu-292. However, the side chain of Glu-292 was distant from the carbonyl carbon of the ester moiety, because the docking program, FlexX, cannot account for chemical reactions and remains rigid. We changed the torsion angles.
of the side chain of Glu-292 without steric hindrance as Glu-292 became capable of a nucleophilic reaction.

In model II, CS-866 was bound to site II using the pocket for myristate-4 (Fig. 7) (Curry et al., 1998). The pocket for myristate-3 was not occupied. The biphenyl moiety and the 2-propyl-imidazole moiety were bound to the hydrophobic pocket consisting of Leu-387, Pro-486 and Ala-490. The propan-2-ol moiety was surrounded by the hydrophobic residues (Leu-387, Leu-430, and Leu-453).

Table 5 shows hydrogen bonds and electrostatic interactions in site II. Negative charges of the tetrazole moiety interacted electrostatically with the side chain of Arg-485, and the tetrazole moiety formed a hydrogen bond with the side chain of Asn-391. The side chain of Ser-489 formed hydrogen bonds with the hydroxyl oxygen atom of the propan-2-ol moiety and the carboxyl oxygen atom of the ester moiety. The carboxyl oxygen atom of the ester moiety formed a hydrogen bond with the side chain of Lys-414. The carboxyl oxygen atom of the ester moiety formed a hydrogen bond with the side chain of Tyr-411.

**Discussion**

This antihypertensive prodrug, CS-866, is hydrolyzed in the serum. Hydrolysis of CS-866 in serum has been observed in several species, and comparison among five species has shown that hydrolytic activity is highest in rabbits, followed by dogs, mice, rats, and humans (Ikeda, 2000). Furthermore, we examined the activity due to serum albumin. It was found to be highest in humans, followed by rats, mice, rabbits, and dogs (data not shown). This indicates that the mechanisms of hydrolysis of CS-866 in serum differ among those species, and that HSA plays a more important role in producing RNH-6270 than other serum albumin species.
Fig. 6. Stereo drawings of ligands in site I. A, modeling structure for CS-866. The torsion angles of the side chain of Glu-292 were changed as Glu-292 becomes capable of a nucleophilic reaction. B, crystal structure for S-warfarin (PDB ID 1H9Z). Relaxed stereo viewing.
Thermodynamic Properties. The esterase-like activity of HSA is dependent on the catalytic rate constant, $k_{cat}$, and increases with a decrease in the activation free energy change, $\Delta G$. Thus, the magnitude of $\Delta G$, which is dependent on activation entropy change (\(\Delta S\)), as calculated from a thermodynamic analysis, can be regarded as an indicator of hydrolytic activity of HSA (Fersht, 1998; Sakurai et al., 2004). Because PNPA has lower $\Delta G$ and $\Delta S$ values than CS-866 (Table 3), PNPA exhibited greater affinity for HSA and a higher catalytic rate than CS-866 (Table 2). Hydrolysis reactions catalyzed by albumin have previously been found to have a particularly great entropy difference between the ground state (ES) and the transition state (ES*) (Sakurai et al., 2004). The active sites of HSA to which the substrate (the ester portion) for hydrolysis, and thus has a smaller entropy difference between the transition state (ES*) and the ground state (ES). This may be the reason why hydrolysis of PNPA proceeds more readily than hydrolysis of CS-866. That is, compared to CS-866, PNPA has a structure and orientation that are better suited to hydrolysis by HSA.

Relationship Between Ligand Binding Sites and Hydrolytic Active Sites. HSA is the most abundant protein in blood plasma and serves as a storage protein and transport protein for many endogenous and exogenous compounds (Peters, 1996; Kragh-Hansen et al., 2002). The unique capability of HSA to reversibly bind a large number of compounds is usually explained by the existence of a number of ligand-binding sites of HSA for CS-866 and the proteins’ ligand-binding sites was investigated in the present study.

There are interesting patterns of competition between site I and site II ligands for hydrolysis. Although warfarin, which is regarded as a typical ligand of site I of HSA, acts as a competitive inhibitor, this does not necessarily indicate that the HSA catalytic site for CS-866 is subsite Ia, because ibuprofen, a typical site II ligand, also exhibited evidence of competitive inhibition (Figs. 2A and 3B). These results suggest that substrate specificity of the esterase-like region and ligand-binding site of HSA is inconsistent. In other words, the catalytic site for CS-866 on HSA may recognize CS-866 in a manner different from that of the ligand-binding site.

Roles of Specific Amino Acid Residues. For proteins whose X-ray crystallographic structure is known, the role of each amino acid residue can be quantitatively determined using the amino acid displacement (site-directed mutagenesis) technique and information obtained from X-ray analysis.

The present chemical modification experiments indicate that Lys, Trp, and Tyr residues of HSA are important for hydrolysis of CS-866 by HSA, and that His residues are also involved (Fig. 4). These experiments were performed with mildly modified HSA, because, for example, only 1.24 of the Tyr residues and 3.8 of the Lys residues were modified. However, HSA has 59 Lys residues, and the numbers of Trp, Tyr, and His residues are 1, 18, and 16, respectively. Previous findings have demonstrated that Tyr-411 is most likely the reactive Tyr of HSA (Watanabe et al., 2000). It is also known that the reactivity of Lys-199 is high (Means and Bender, 1975). Furthermore, it is reported that this single Trp residue contributes to the esterase-like activity of HSA (Ozeki et al., 1980; Kurono et al., 1982). In an attempt to identify specific residues of importance for the hydrolysis of CS-866, we examined the activity of several rHSA, namely wild-type HSA and the single-residue mutants K199A, W214A, and Y411A.

Because we did not observe a great decrease of the hydrolytic activity of HSA for CS-866, even in the single-residue mutants K199A and Y411A, we conclude that the catalytic sites of HSA for CS-866 are not solely confined to the Lys-199 and Tyr-411 residues but, rather, involves several additional amino acid residues (Fig. 5).

The single Trp residue, Trp-214, is located close to Lys-199, as indicated by X-ray diffraction analysis, and is an element of a major interdomain cluster of hydrophobic residues (He and Carter, 1997; Sugio et al., 1999). The mutant W214A exhibited a significant decrease in hydrolytic activity (Fig. 5). In addition, the microenvironment around Trp-214 was investigated to obtain detailed information about the role of this residue in the hydrolysis. After incubation with CS-866 for 10 min, the relative fluorescence intensity of HSA decreased by more than half and the $\lambda_{max}$ was blue-shifted (data not shown). These results are consistent with a model indicating that the Trp-214 residue is involved in hydrolytic reaction. These limited data lead us to the idea that a double (or triple) mutation of Lys-199, Trp-214, and Tyr-411 could completely abolish the hydrolytic activity. Further investigations on this point are under way at this laboratory.

Structural Mechanism of Hydrolysis Based on Models. The present findings suggest that HSA has two catalytic sites for CS-866, for the following two reasons. Mutation at site I or site II diminishes but does not abolish the hydrolytic activity. The hydrolytic activity is inhibited by both warfarin (site I drug) and ibuprofen (site II drug).

In model I (Fig. 6), CS-866 occupied the binding site of warfarin; this is consistent with the results showing that warfarin inhibits the hydrolytic activity of HSA. In site I, the carboxyl oxygen atom of the ester moiety formed a hydrogen bond with the side chain of Lys-199, and this hydrogen bond could function as an oxygen hole. The importance of Lys-199 indicated by the model is consistent with the decreased hydrolytic activity of the K199A mutant and the HSA variant produced by chemical modification of Lys. The catalytic residue may be Glu-292; the distance between the oxygen atom of the side chain of Glu-292 and the carboxyl carbon of the ester moiety of CS-866 was 4.8 Å. The hydrophobic interaction between CS-866 and Trp-214 indicated by the model is consistent with the diminished
hydrolytic activity of the W214A mutant and the HSA variant produced by chemical modification of Trp.

Model II (Fig. 7) indicates that the mechanism of hydrolysis of CS-866 is almost the same as that found in previous studies for \( p \)-nitrophenyl esters, with the exception of the involvement of Arg-410 (Watanabe et al., 2000; Sakurai et al., 2004). Instead of Arg-410, Lys-414 was used to create an oxyanion hole. The distance between the hydroxyl oxygen atom of Tyr-411 and the carbonyl carbon of the

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**Fig. 7.** Stereo drawings of ligands in site II. A, modeling structure for CS-866; B, crystal structure for myristate-3 and -4 (PDB ID 1H9Z). Relaxed stereo viewing.
ester moiety of CS-866 was 3.3 Å, indicating that it is possible that Tyr-411 plays the role of a catalytic residue. The importance of Tyr-411 and Lys-414 is consistent with the decreased hydrolytic activity of the Y411A mutant and of the HSA variants produced by chemical modification of Tyr or Lys. In our model II, CS-866 was bound to the pocket for myristate-4 in site II. The binding pockets of the site II ligands ibuprofen and diazepam are unknown. If ibuprofen binds to the pocket for myristate-4, our model II provides a mechanism for inhibition of hydrolytic activity of HSA by ibuprofen.

The present findings indicate that hydrolysis of CS-866 by HSA is dependent on $\Delta S$. Another important factor is the orientation between the catalytic active site on HSA and the ester region of the substrate. There are differences between the catalytic active sites and the ligand-binding sites of HSA. Furthermore, the residues of Lys-199, Trp-214, and Tyr-411 play important roles in this catalytic reaction. All of these experimental findings are consistent with the docking model that we derived from computer simulation.

References


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