Role of Arg-410 and Tyr-411 in human serum albumin for ligand binding and esterase-like activity

Hiroshi WATANABE*, Sumio TANASE†, Keisuke NAKAJOU*, Toru MARUYAMA*, Ulrich KRAGH-HANSEN‡ and Masaki OTAGIRI*[1]

*Department of Pharmaceutics, Faculty of Pharmaceutical Sciences, Kumamoto University, 5-1 Oe-honmachi, Kumamoto 862-0973, Japan,
†Department of Biochemistry, School of Medicine, Kumamoto University, Honjo 2-2-1, Kumamoto 860-0811, Japan, and
‡Department of Medical Biochemistry, University of Aarhus, DK-8000 Aarhus C, Denmark

Recombinant wild-type human serum albumin (rHSA), the single-residue mutants R410A, Y411A, Y411S and Y411F and the double mutant R410A/Y411A were produced using a yeast expression system. The recombinant proteins were correctly folded, as they had the same stability towards guanidine hydrochloride and the same CD spectrum as HSA isolated from serum (native HSA). Thus the global structures of the recombinant proteins are probably very similar to that of native HSA. We investigated, by ultrafiltration and CD, the high-affinity binding of two representative site II ligands, namely ketoprofen and diazepam. According to the crystal structure of HSA, the residues Arg-410 and Tyr-411 protrude into the centre of site II (in subdomain 3A), and the binding results showed that the guanidino moiety of Arg-410, the phenolic oxygen and the aromatic ring of Tyr-411 are important for ketoprofen binding. The guanidino moiety probably interacts electrostatically with the carboxy group of ketoprofen, the phenolic oxygen could make a hydrogen-bond with the keto group of the ligand, and the aromatic ring may participate in a specific stacking interaction with one of or both of the aromatic rings of ketoprofen. By contrast, Arg-410 is not important for diazepam binding. The two parts of Tyr-411 interact favourably with diazepam, and probably do so in the same way as with ketoprofen. In addition to its unique ligand binding properties, HSA also possesses an esterase-like activity, and studies with *p*-nitrophenyl acetate as a substrate showed that, although Arg-410 is important, the enzymic activity of HSA is much more dependent on the presence of Tyr-411. A minor activity could be registered when serine, but not alanine or phenylalanine, was present at position 411.

Key words: binding site, drug–albumin interaction, enzyme activity, site-directed mutagenesis.

INTRODUCTION

Human serum albumin (HSA) is the most abundant protein in blood plasma, and serves as a depot protein and transport protein for many endogenous and exogenous compounds [1,2]. The ready availability of HSA has greatly helped workers to gain insight into the mechanisms of ligand–protein interactions. HSA is known to have at least two distinct binding sites for several physiologically important compounds and a large number of strongly bound drugs, namely site I (in subdomain 2A), also called the warfarin binding site, and site II (in subdomain 3A), also called the indole and benzodiazepine binding site [3,4]. It has been suggested that drugs that bind to site I are bulky heterocyclic molecules with a negative charge localized in the middle of the molecule, and that drugs that bind to site II are aromatic carboxylic acids with a generally extended shape carrying the negative charge on the carboxy group at one end of the molecule, away from the hydrophobic centre [4].

Most non-steroidal anti-inflammatory drugs (NSAIDs) and tranquillizers of the benzodiazepine group interact strongly with HSA. Interestingly, NSAIDs of the arylpropionic acid type, which possess a carboxylate at the end of an extended hydrophobic molecule (e.g. ketoprofen, ibuprofen and flufenamic acid), bind to site II [2,5]. By contrast, phenylbutazone, which does not possess a carboxylate, binds to site I [6]. However, a carboxy group does not seem to be obligatory for binding to site II, because diazepam, a basic drug that exists mainly in the unionized form at neutral pH, also binds with high affinity to this site [2].

He and Carter [7] examined in detail the binding chemistry of the ligand tri-iodobenzoic acid, which is an aromatic carboxylic acid. In site II, the carboxylate of tri-iodobenzoic acid interacts primarily with the guanidino moiety of Arg-410, and is within 4 Å (0.4 nm) of the phenolic oxygen of Tyr-411. According to the crystal structure of HSA, both residues protrude into the centre of site II.

In addition to its ligand binding capabilities, HSA possesses interesting enzymic properties. For example, Means and co-workers [8,9] have shown that the protein can act as an esterase towards *p*-nitrophenyl acetate, and that this activity results in acetylation of a tyrosine residue. The primary reactive site and the active residue have been suggested to be site II and Tyr-411 respectively. In contrast, site I does not seem to be involved [10]. In the crystal structure, the reactive group of Tyr-411 is in close proximity (2.7 Å) to the nitrogen atoms of Arg-410 [7], a finding which may explain the unusual reactivity of this residue towards nucleophilic substitution. The importance of Arg-410 and Tyr-411 for the different functions of HSA is also emphasized by the finding that these residues are highly conserved between HSA and other mammalian albumins [2,11–14].

In the present study, we have focused our research on the importance of Arg-410 and Tyr-411. First we constructed a yeast expression system to overproduce recombinant wild-type HSA (rHSA) and several mutants of the protein. Next we used the single mutants R410A, Y411A, Y411S and Y411F and the double mutant R410A/Y411A to elucidate, in a direct way, the impact of Arg-410 and Tyr-411 on the ligand binding properties of site II. As representative ligands we used ketoprofen and diazepam. Finally, the structural basis for the esterase activity of HSA was studied.

Abbreviations used: HSA, human serum albumin; rHSA, recombinant HSA; GdnHCl, guanidine hydrochloride; NSAID, non-steroidal anti-inflammatory drug; ΔG, free energy of unfolding of a protein.

[1] To whom correspondence should be addressed (e-mail otagirim@gpo.kumamoto-u.ac.jp).

MATERIALS AND METHODS

Reagents

The racemate of ketoprofen was a gift from the Kissei Pharmaceutical Co. (Matsumoto, Japan), and diazepam was donated by Nippon Roche K. K. (Tokyo, Japan). Guanidine hydrochloride (GdnHCl) and *p*-nitrophenyl acetate were purchased from the Nakalai Tesque (Kyoto, Japan). Restriction enzymes, T4 polynucleotide kinase, calf intestinal alkaline phosphatase, a DNA ligation kit, TaKaRa EX *Taq* DNA polymerase and a site-directed mutagenesis kit (oligonucleotide-directed dual amber method) were obtained from Takara Shuzo Co. Ltd (Kyoto, Japan). A DNA sequence kit was obtained from Perkin-Elmer Applied Biosystems (Tokyo, Japan). *Pichia* Expression kit was purchased from Invitrogen. Other chemicals used were obtained from commercial suppliers.

Synthesis and purification of rHSAs

Introduction of mutations into the HSA coding region

A chimaera plasmid (pJDB-ADH-L10-HSA-A) containing cDNA for the mature form of HSA along with an L10 leader sequence was a gift from Tonen Co. (Tokyo, Japan). Mutagenic primers synthesized (underlined letters indicate mismatches) were: 5′-CTATTAGTTGCTTACACCAAG-3′ for R410A; 5′-CTATTAGTTCGTGCCACCAAG-3′ for Y411A; 5′-CTATTAGTTCGTTCCACCAAG-3′ for Y411S; 5′-CTATTAGTTCGTTTCACCAAG-3′ for Y411F; and 5′-CGCTATTAGTTGCTGCCACCAAGAAAG-3′ for the R410A/Y411A double mutant. The L10–HSA coding region was amplified by PCR with a forward and a reverse primer carrying a 5′-terminal *Eco*RI site and cloned into the *Eco*RI-digested pKF19k vector (Takara Shuzo Co.), and mutagenesis was carried out. The mutation was confirmed by DNA sequencing of the entire HSA coding region using the dideoxy chain termination method with a Perkin–Elmer ABI Prism 310 Genetic Analyzer. The wild-type and the mutagenized inserts were released by digestion with *Eco*RI and recloned into the *Eco*RI-digested methanol-inducible pHIL-D2 vector (Invitrogen) to construct HSA expression vector pHIL-D2-HSA. All of the cloning procedures were conducted in accordance with the guidelines for research involving recombinant DNA molecules issued by the Ministry of Education, Science, Sports and Culture of Japan.

Expression of rHSAs

A pHIL-D2-HSA expression vector coding for a particular rHSA mutant was introduced into the yeast species *Pichia pastoris* (strain GS115) by the lithium chloride method. A yeast clone which contained the expression cassette stably integrated into the chromosomal DNA was isolated in each case. In the growth phase, the cells were grown to confluence in 100 ml of BMGY [1 % yeast extract, 2 % peptone, 100 mM potassium phosphate (pH 6.0), 1.34 % yeast nitrogen base with ammonium sulphate without amino acids, 4×10^{-5} % biotin, 1 % glycerol] in 1-litre flasks at 30 °C with shaking. The cells were grown to an attenuance of 2 at 600 nm after 30 h of incubation. In the induction phase, the growth-phase cells were harvested by centrifugation (1500 ***g***, 10 min, 4 °C), and cell pellets were resuspended in 1 litre of BMMY [1 % yeast extract, 2 % peptone, 100 mM potassium phosphate (pH 6.0), 1.34 % yeast nitrogen base with ammonium sulphate without amino acids, 4×10^{-5} % biotin, 1 % methanol] in 3-litre flasks, and returned to the 30 °C shaker. The cells were grown for an additional 96 h. To maintain induction, 100 % methanol was added (to give a final concentration of 0.5 % methanol) every 24 h.

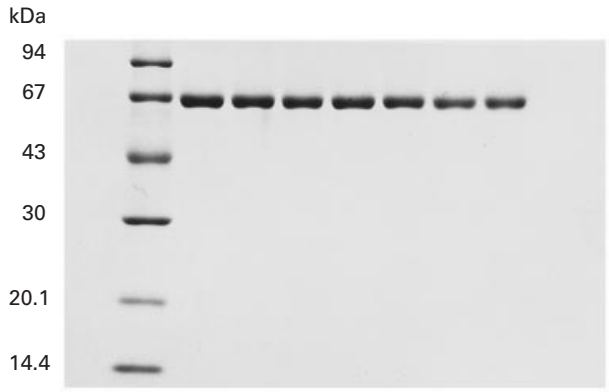

Figure 1 SDS/PAGE of native HSA and rHSAs

Lane 1 (numbering from the left), molecular mass markers; lane 2, wild-type rHSA; lane 3, mutant R410A; lane 4, mutant Y411A; lane 5, double mutant R410A/Y411A; lane 6, mutant Y411S; lane 7, mutant Y411F; lane 8, native HSA. The gels were stained with Coomassie Brilliant Blue.

Purification of rHSAs

The secreted rHSA was isolated from the growth medium as follows. The medium was brought to 60 % saturation with ammonium sulphate at room temperature. The temperature was then lowered to 4 °C and the pH was adjusted to 4.4, the isoelectric point of HSA [15]. The precipitated protein was collected by centrifugation (10000 ***g***, 20 min, 4 °C) and resuspended in distilled water. Dialysis was carried out for 48 h at 4 °C against 100 vol. of distilled water, followed by a further 24 h against 100 vol. of 200 mM sodium acetate buffer, pH 5.5. Afterwards, the solution was loaded on to a column of Blue Sepharose CL-6B (Amersham Pharmacia Co.). The column was washed with approx. 5 bed volumes of 200 mM sodium acetate buffer, pH 5.5, and then the rHSA was eluted with 20 mM sodium acetate buffer, pH 6.5, containing 1 M NaCl and 10 % (v/v) ethanol. The eluted rHSA was deionized and then defatted using charcoal treatment, as described by Chen [16]. The resulting protein exhibited a single band on an SDS/PAGE gel, and all the recombinant proteins migrated to the same position as native HSA (Figure 1). Sample purity was estimated by density analysis of Coomassie Brilliant Blue-stained protein bands on SDS/PAGE gels. Each protein sample was > 97 % pure. N-terminal amino acid sequences of the proteins were determined in a Perkin–Elmer ABI 477A protein sequencer.

Immunological experiments

The cross-reactivity of a polyclonal antiserum raised against native HSA with the various rHSAs was examined by ELISA [17]. Native HSA and all rHSAs had identical reactivity with the antiserum using the ELISA method.

CD measurements

Measurements were obtained using a JASCO J-720 type spectropolarimeter (JASCO, Tokyo, Japan) at 25 °C. For calculation of the mean residue ellipticity [θ], the molecular mass of the albumins was taken as 66.5 kDa. Far-UV and near-UV CD spectra were

recorded at protein concentrations of 1.5 μM and 15 μM respectively in 67 mM sodium phosphate buffer (pH 7.4). For both types of experiments, the final ketoprofen or diazepam concentration was 7.5 μM.

Solvent-induced denaturation of rHSAs employing GdnHCl

Samples of 1.5 mg/ml HSA or rHSA in 67 mM sodium phosphate buffer (pH 7.4) containing different concentrations of GdnHCl were incubated at 25 °C for 24 h prior to analysis, to ensure that equilibrium had been achieved. After equilibrium had been attained, CD measurements were carried out with a JASCO J-720 type spectropolarimeter. Ellipticity at 222 nm was employed. Assuming a two-state behaviour, the equilibrium denaturation profiles were analysed using a linear extrapolation method as follows. The equilibrium constant for unfolding in the presence of a denaturant, K_D, can be calculated from eqn (1):

$$K_D = (f_N - f_{obs})/(f_{obs} - f_D) \quad (1)$$

where f_{obs} is the observed ellipticity, and f_N and f_D (calculated from the respective baselines before and after denaturation) are the ellipticities of the native and denatured forms of albumin respectively. The value of K_D can be used to calculate the free energy of unfolding of the protein, ΔG (eqn 2):

$$\Delta G = -RT(\ln K_D) \quad (2)$$

In this equation, R is the gas constant and T is the absolute temperature. Now, since it has been found that ΔG in the presence of GdnHCl is linearly related to the concentration of GdnHCl ([GdnHCl]), ΔG_{H2O} (the apparent free energy of unfolding in the absence of denaturant) and m (the slope of the linear plot) can be calculated by plotting ΔG against [GdnHCl], as indicated in eqn (3):

$$\Delta G = \Delta G_{H2O} - m[\text{GdnHCl}] \quad (3)$$

This denaturation process induced by GdnHCl was confirmed to be a reversible process, because removal of GdnHCl by dialysis from the samples resulted in similar intensities on CD as for the native form of HSA and the rHSAs.

Ligand binding experiments

To study ligand binding to HSA and rHSA, we added ketoprofen or diazepam to a solution of 10 μM HSA or rHSA in 67 mM sodium phosphate buffer (pH 7.4) to give a final concentration of ketoprofen or diazepam of 5 μM. The unbound ligand fractions were separated using the Amicon MPS-1 micropartition system with YMT ultrafiltration membranes by centrifugation (2000 ***g***, 25 °C, 40 min). Adsorption of ketoprofen or diazepam on to the filtration membranes and apparatus were negligible. The concentration of unbound ligand was determined by HPLC. The HPLC system consisted of an Hitachi 655A-11 pump and an Hitachi 655A variable-wavelength UV monitor. LiChrosorb RP-18 (Cica Merck, Tokyo, Japan) was used as the stationary phase. The mobile phase consisted of 0.2 M acetate buffer (pH 4.5)/acetonitrile (3:2, v/v) for ketoprofen, and deionized water/acetonitrile (2:3, v/v) for diazepam. Ketoprofen was detected at 257 nm and diazepam was detected at 230 nm by means of UV monitoring. The unbound fraction (%) was calculated according to the following equation:

Unbound fraction (%) = [ligand concentration in filtered fraction/total ligand concentration (before ultrafiltration)] × 100

Determination of esterase-like activity

The reaction of *p*-nitrophenyl acetate with HSA and rHSAs was followed spectrophotometrically at 400 nm (JASCO Ubest-35 UV/visible spectrophotometer) by monitoring the appearance of *p*-nitrophenol. The reaction mixtures contained 5 μM *p*-nitrophenyl acetate and 20 μM protein in 67 mM sodium phosphate buffer (pH 7.4). Reactions were followed at 25 °C. Under these conditions, pseudo-first-order rate constant analysis could be applied, as described in previous reports [10,18], and apparent hydrolysis rate constants (k_{obs}) were calculated.

RESULTS AND DISCUSSION

Tertiary structure and protein stability of rHSAs

The N-terminal sequence of the rHSAs is SSDAHKSEVAHRFKDLGEEN. As compared with native HSA isolated from serum, the recombinant proteins have two extra amino acids derived from the L10 leader sequence [19], namely the two initial serine residues. SDS/PAGE analysis (Figure 1) revealed that the molecular masses of wild-type and mutant rHSAs are identical to that of native HSA. This finding excludes the possibility of a large truncation of the C-terminal ends.

In order to elucidate any structural differences between native HSA and the rHSAs, their secondary structures were analysed by far-UV CD. The CD spectra showed (Figure 2) that the rHSAs have the same secondary structure, i.e. they have the same content of α-helix and β-sheets. Furthermore, the different rHSAs have identical near-UV CD spectra (results not shown). Both the far-UV and near-UV CD spectra of the rHSAs were identical with those of native HSA. The identity of the far-UV and near-UV CD spectra provides evidence for the absence of any major differences in secondary and tertiary structure, so that the protein conformations are probably very similar.

The protein stability of native HSA and the rHSAs in the presence of GdnHCl as a denaturant was analysed by monitoring changes in the far-UV CD spectra. The fractions of the denatured state of native HSA and the rHSAs were calculated from ellipticities at 222 nm and plotted against denaturant concentration. The transition curves of native HSA and the rHSAs exhibited an apparent two-state denaturation behaviour (results not shown). Using these plots, various thermodynamic para-

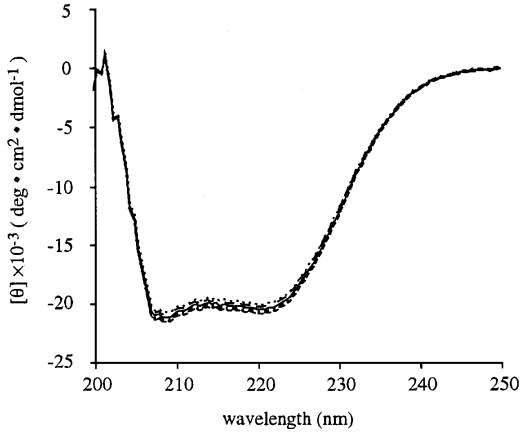

Figure 2 Far-UV CD spectra of rHSAs at 25 °C

The protein concentration was 1.5 μM in 67 mM sodium phosphate buffer (pH 7.4). Spectra are shown for wild-type rHSA, mutant R410A, mutant Y411A, double mutant R410A/Y411A, mutant Y411S and mutant Y411F.

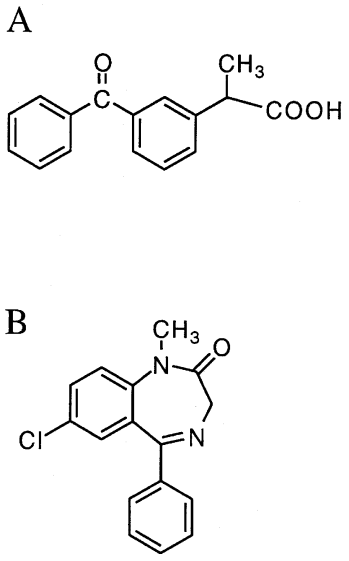

Figure 3 Chemical structures of ketoprofen (A) and diazepam (B)

Table 1 Binding of ketoprofen and diazepam to wild-type or mutant rHSAs at pH 7.4 and 25 °C

The sample solutions contained 5 μM ketoprofen or diazepam and 10 μM wild-type or mutant rHSA in 67 mM sodium phosphate buffer (pH 7.4). Values are means ± S.D. for three experiments.

	Free (unbound) fraction (%)	
rHSA	Ketoprofen	Diazepam
Wild type	7.2 ± 1.9	18.8 ± 2.2
R410A	35.8 ± 4.0	20.8 ± 2.0
Y411A	61.9 ± 1.2	72.7 ± 2.2
R410A/Y411A	92.7 ± 0.4	78.4 ± 5.7
Y411S	65.9 ± 0.8	62.1 ± 1.3
Y411F	39.1 ± 3.3	53.8 ± 0.6

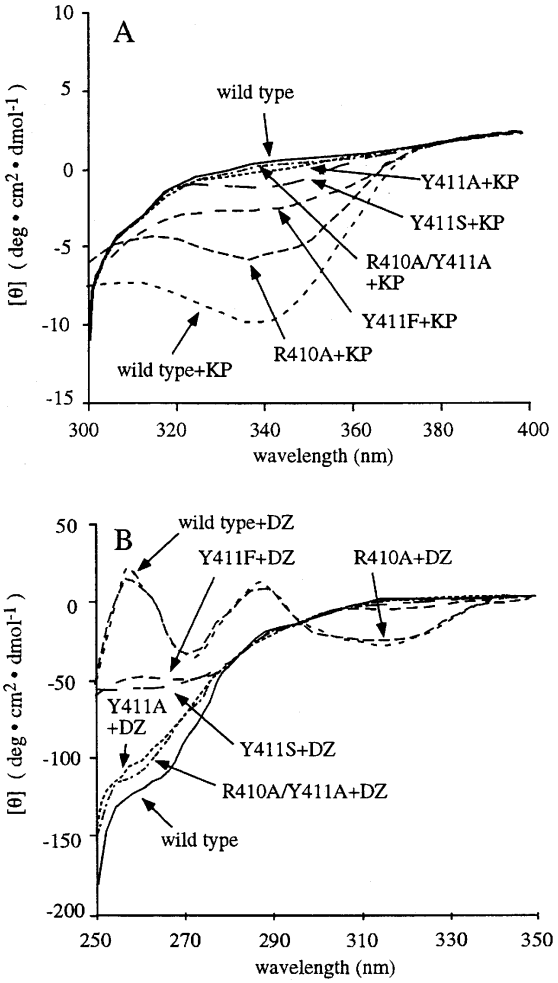

Figure 4 CD spectra of wild-type rHSA alone and of wild-type rHSA, mutant R410A, mutant Y411A, double mutant R410A/Y411A, mutant Y411S and mutant Y411F in the presence of ketoprofen (A) or diazepam (B) at 25 °C

The sample solutions contained 7.5 μM ketoprofen (KP) or diazepam (DZ) and 15 μM rHSA in 67 mM sodium phosphate buffer (pH 7.4).

meters characterizing the GdnHCl-induced denaturations were estimated. ΔG_{H2O} represents the apparent free energy of secondary structure in the absence of GdnHCl, the value of m is generally accepted as an index of the extent of exposure of nonpolar surfaces, and $[GdnHCl]_{\frac{1}{2}}$ is the concentration of GdnHCl at the mid-point of denaturation. The values of these thermodynamic parameters for the wild-type and the mutant rHSAs were essentially identical with those determined for native HSA (m = 10.5 kJ/mol per M; ΔG_{H2O} = 21.7 kJ/mol; $[GdnHCl]_{\frac{1}{2}}$ = 2.1 M). Thus we can conclude that substitution of Arg-410 and/or Tyr-411 has no detectable effect on the structural stability of HSA.

Ligand binding studied by ultrafiltration

In order to elucidate the impact of the side chains of Arg-410 and Tyr-411 on ligand binding, we examined by ultrafiltration the binding of two representative site II ligands, namely ketoprofen, which has a carboxylic acid moiety, and diazepam, which does not contain such a moiety (Figure 3, Table 1), to wild-type and mutant rHSAs. The characteristics of binding of ketoprofen and diazepam to wild-type rHSA were the same as to native HSA (results not shown). However, for ketoprofen binding, replacement of Arg-410 with alanine led to an increase in the unbound fraction from 7% to approx. 35%. Practically the same effect was seen following substitution of Tyr-411 with phenylalanine. Replacing Tyr-411 with the small amino acids alanine and serine resulted in unbound fractions of approx. 65% and, finally, having alanine in both positions 410 and 411 resulted in very low binding (the unbound fraction increased to > 90%). For diazepam binding, replacement of Arg-410 did not lead to a significant change in the unbound fraction. However, modification of Tyr-411 resulted in all cases in diminished binding. For the mutants Y411F and Y411S the unbound fraction increased to approx. 60%, and for the mutants Y411A and R410A/Y411A it increased to approx. 75%.

Ligand binding studied by CD measurements

The CD spectra of the unbound rHSAs in the UV region were identical with that of native HSA (results not shown). When

ketoprofen binds to HSA, a specific Cotton effect is induced in the protein [20,21]. Figure 4(A) shows the CD spectra obtained when ketoprofen was added to the different rHSAs. Binding of ketoprofen to wild-type rHSA resulted in a negative Cotton effect, with a maximum at about 340 nm. The magnitude of this characteristic Cotton effect was the same as in the case of native HSA (results not shown). Replacement of Arg-410 with alanine led to a decrease in the specific negative Cotton effect as compared with that induced in the wild-type rHSA. For the Y411A mutant and the R410A/Y411A double mutant, the effect was not generated or was only very small. Intermediate spectral changes were observed for the mutants Y411S and Y411F.

Binding of benzodiazepines to the main binding site of HSA induces optical activity in the symmetrical drug molecules [22–24]. However, in the case of diazepam binding, specific Cotton effects can be observed in the UV region for wild-type rHSA [and native HSA (results not shown)], with maxima at 255 nm and 285 nm and a minimum at 315 nm (Figure 4B). Replacement of Arg-410 with alanine did not appreciably change the spectrum induced by diazepam binding. In contrast, the effects introduced by diazepam binding in the Y411A mutant and the R410A/Y411A double mutant were much smaller, and these spectra were much more similar to that of the wild-type protein in the absence of ligands. Finally, intermediate effects were registered for the mutants Y411S and Y411F. In summary, the results obtained with CD measurements are in agreement with those from the ultrafiltration experiments.

Ligands such as medium-chain fatty acids, L-tryptophan, iopanoate, clofibrate, diazepam and other benzodiazepins, and NSAIDs of the type 2-arylpropionic acid, such as ketoprofen and ibuprofen, are known to bind with high affinity to site II of HSA. Maruyama et al. [25] investigated the binding characteristics of suprofen and its methyl ester derivative. They concluded that the carboxy group is necessary for the binding of suprofen to site II of HSA, and, based on experiments with chemically modified HSA, that one or more tyrosine residues is important for this binding. Rahman et al. [26] also showed the importance of a charged carboxy group for binding of carprofen and ketoprofen to site II. Our results propose that the carboxy group of the ligands binding to site II interacts ionically with the guanidino group of Arg-410. However, the guanidino group is not important for binding of ligands such as diazepam, which does not possess a carboxy group (cf. Figure 3).

He and Carter [7] discussed the binding chemistry of triiodobenzoic acid, as well as that of many other small aromatic carboxylic acids. In site II, the carboxylate interacts primarily with the guanidino moiety of Arg-410, and is within 4 Å of the oxygen of Tyr-411 [7]. Curry et al. [27] have determined the crystal structure of HSA complexed with five molecules of myristate at 2.5 Å. Two molecules of myristate (myristate 3 and myristate 4) are bound in site II. Interestingly, the carboxylate of myristate 4 interacted with Arg-410 and Tyr-411. Recently, Chuang et al. [5] investigated the binding site for ketoprofen on HSA by using a photoaffinity labelling technique. They found that the amino acid sequence of the peptide photolabelled by ketoprofen was CTESLVNRR, which corresponds to the amino acid sequence of HSA from Cys-477 to Arg-485. Furthermore they showed, by using a computer graphic docking model, that the carboxylate of ketoprofen (cf. Figure 3) interacted with Arg-410.

There are several reports concerning the exact location of the diazepam binding site on HSA [28–31]. Sjödin et al. [32] demonstrated that a part of the diazepam site must be located in a large trypsin-resistant fragment of HSA (residues 182–585), because this fragment is still able to bind diazepam, although the affinity is much weaker than with the intact protein. Furthermore, one tyrosine residue has been suggested to be a part of the diazepam binding site. This proposal is based on the finding that selective modification of HSA with tetranitromethane resulted in a pronounced decrease in diazepam binding [33]. Finally, Maruyama et al. [34] showed that the diazepam binding site and the other drug binding sites in site II may not be identical, but may be overlapping.

According to X-ray crystallographic studies Arg-410 and Tyr-411 protrude into the centre of site II, and the present results show in a direct way that these residues are important for ligand binding to site II of HSA. This is in accordance with results obtained with X-ray crystallography and other techniques. However, on the basis of the present work it is possible to differentiate between the importance of the two residues and to obtain rather detailed information about Tyr-411. Thus Arg-410 is important for the binding of ligands possessing a charged carboxy group, such as ketoprofen, probably by virtue of electrostatic interactions between the carboxylate of the ligand and the guanidino moiety of the residue. Removal of the phenolic oxygen of Tyr-411, as in the mutant Y411F, diminished binding to the same extent as removal of the guanidino group at position 410, indicating the existence of a hydrogen bond between the keto group of ketoprofen (Figure 3) and this phenolic oxygen of wild-type rHSA. However, the aromatic ring of Tyr-411 is also important for ketoprofen binding, because substitution of tyrosine with alanine increased the free fraction of the ligand even further. Relative to that situation, placing a small residue with a hydroxy group (i.e. serine) at position 411 did not improve binding. In summary, three elements have been identified as being important for high-affinity binding of ketoprofen, namely the guanidino moiety of Arg-410, a phenolic oxygen that is part of Tyr-411 and the aromatic ring of Tyr-411. The aromatic ring is probably important because it can make a specific stacking interaction with the aromatic rings of the ligand. The conclusion that Arg-410 and Tyr-411 are very important for binding is also supported by the finding that binding of ketoprofen to the double mutant was very low, as in this mutant the site is very open and lacks the three points of contact.

In contrast with the binding of ketoprofen, the guanidino group of Arg-410 is not important for the high-affinity binding of diazepam, which does not possess a carboxy group. However, for this ligand also the two components of the tyrosine residue at position 411 are important for binding, and they probably establish the same types of binding forces to diazepam as to ketoprofen. The reason for this proposal is that removal of the phenolic oxygen greatly increased the free fraction of diazepam, and replacing the residue with an alanine led to a further increase. In the case of diazepam binding, the hydroxy group of serine can to a certain extent compensate for the absence of the aromatic ring of Tyr-411. As for ketoprofen, the lowest binding was observed for the double mutant with the 'empty' site II.

In general, it seems possible that Arg-410 and Tyr-411 may play critical roles in modulating the affinity of HSA for a variety of ligands.

Analysis of esterase-like activity

We investigated the catalytic activity of the rHSAs towards *p*-nitrophenyl acetate (Table 2). The enzymic activity of wild-type rHSA was identical with that of native HSA (results not shown). For the R410A mutant, the catalytic activity was only approx. 12% of that of wild-type rHSA. This result shows that Arg-410 is closely involved in the esterase-like activity. Replacement of Tyr-411, the predicted nucleophile in the hydrolytic reaction of

Table 2 Hydrolysis rate constants (k_{obs}) for *p*-nitrophenyl acetate

The reaction mixtures contained 5 μM *p*-nitrophenyl acetate and 20 μM wild-type or mutant rHSA in 67 mM sodium phosphate buffer (pH 7.4), and reactions were followed at 25 °C. Values are means ± S.D. for three experiments. Significance of differences compared with wild type: $^*P < 0.001$.

rHSA	$10^3 \times k_{obs}$ (s^{-1})
Wild type	8.50 ± 0.11
R410A	1.06 ± 0.18*
Y411A	< 0.01†
R410A/Y411A	< 0.01†
Y411S	1.80 ± 0.14*
Y411F	< 0.01†

† Below the limit of detection.

HSA, with alanine or phenylalanine led to the complete abolition of hydrolytic activity towards *p*-nitrophenyl acetate. Of course, in the 'empty' R410A/Y411A double mutant the hydrolytic activity was also completely lost (Table 2). However, the presence of serine at position 411 resulted in approx. 21% activity, indicating that the presence of a hydroxy group and the nature of its immediate surroundings are crucial for the esterase activity of HSA.

Shaw identified a single reactive tyrosine residue (cited in [35]) and determined the amino acid sequence surrounding it in HSA (cited in [36]). By using the presently available complete sequence of HSA [37], that reactive tyrosine can be tentatively identified as residue number 411.

Means and Bender [8] found that the observed pH–(log rate constant) profile for the esterase reaction of HSA appears to correspond with that for the reaction of a basic group with a pK_a value around 8.7. The apparent limiting rate at high pH is several orders of magnitude greater than expected for most simple nucleophiles of this pK_a value. Thus the reaction is not only a simple nucleophilic displacement, but must be strongly facilitated by other features of the protein environment. Our data indicate that Arg-410 may contribute to the strongly nucleophilic feature of the phenolic oxygen of Tyr-411, as the guanidino moiety is in close proximity to the phenolic oxygen.

Assigning the esterase-like activity of HSA to Arg-410 and, in particular, Tyr-411 is supported by studies showing that the reaction is strongly inhibited by low concentrations of ligands such as medium-chain fatty acids and 2-arylpropanoic acid NSAIDs [2].

Concluding remarks

In conclusion, our results suggest that defining the role of Arg-410 and Tyr-411 in ligand binding to HSA may be important in understanding the structure and function of site II (in subdomain 3A). It is noteworthy that both residues are well conserved in evolution (conserved among species such as frogs and humans) [2], a finding which indicates a potential role for these amino acids in physiologically important HSA–ligand interactions. As observed for Arg-410 and Tyr-411, Arg-218 in site I (in subdomain 2A) is also highly conserved in evolution. Petersen et al. [15] speculated that these residues in subdomains 2A and 3A act as 'gatekeepers' by reducing the affinity of certain ligands, and that this capability is related to their conservation in evolution. However, our data show that Tyr-411 in particular is positively involved in the binding of ketoprofen and diazepam, which can be taken as typical representatives of the ligands that bind to site II. Finally, not only Tyr-411 but also Arg-410 is essential for the esterase-like activity of HSA.

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