Conformational stability and warfarin-binding properties of human serum albumin studied by recombinant mutants

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Correctly folded recombinant wild-type human serum albumin and the single-residue mutants K199A, W214A, R218H and H242Q were produced with the use of a yeast expression system. The changes in R218H resulted in a pronounced decrease in intrinsic fluorescence. Thermodynamic parameters for thermal denaturation of the present mutants and of five additional mutants have been determined, showing small increases in stability for two mutants (R218H and H242Q) and a larger decrease in stability for one (W214A). In the last of these, denaturation was a heterogeneous process starting at physio-

logical temperature. The high-affinity binding constant for warfarin at pH 7.4 was determined by fluorescence spectroscopy: there was a significant increase in affinity for binding of warfarin to H242Q and K199A and a smaller decrease in affinity for W214A and R218H. The findings show that Trp-214 is not as essential for the high-affinity binding of warfarin as has previously been thought.

Key words: conformational changes, high-affinity binding, protein stability, site-directed mutagenesis, thermal denaturation.

INTRODUCTION

Human serum albumin (HSA) is a single, non-glycosylated polypeptide that organizes to form a heart-shaped protein with approx. 67% α -helix but no β -sheet [1–3]. The protein is composed of three homologous domains (I–III); each domain has two subdomains (A and B) possessing common structural elements. The protein is able to bind reversibly a large number of endogenous and exogenous compounds [4,5]. The capability of binding aromatic and heterocyclic compounds depends largely on the existence of two major binding regions, namely Sudlow's site I and site II [6], which are located within specialized cavities in subdomains IIA and IIIA respectively [1,2]. Of these sites, site I seems to be the more versatile, because it can bind ligands that are chemically very different with a high affinity [5]. Several examples of independent, anti-co-operative and competitive binding of ligands to this region have been published and several attempts to subdivide the site have been made [7–9]. However, detailed molecular information on the site is still scarce. Sugio et al. [2] have studied the crystal structure of the unliganded forms of both HSA and wild-type recombinant HSA (rHSA) and found that site I is formed as a pocket, with the inside wall being formed by hydrophobic side chains and the entrance to the pocket being surrounded by positively charged residues.

In the present study we have made the recombinant mutants K199A, W214A, R218H and H242Q and wild-type rHSA by using the yeast species *Pichia pastoris*. These amino acid residues were chosen for mutation because X-ray crystallography has shown that they are important elements in forming site I: three of them are placed at the entrance (Lys-199, Arg-218 and His-242) and one is positioned in the lower, hydrophobic part (Trp-214) of the binding pocket [2]. The importance of the four

residues is also emphasized by the finding that they are highly conserved between human and other mammalian albumins [5]. Finally, Arg-218 is of interest because natural mutation of this residue to histidine [10,11] or proline [12,13] causes the clinically important syndrome familial dysalbuminaemic hyperthyroxinaemia. The mutants were synthesized with several purposes in mind. Because albumin is a flexible molecule, we wished to study the effect of the mutations on its structural properties and thermal stability. The widely used drug warfarin binds with high affinity to site I in subdomain IIA. However, details of this important interaction are not known. Therefore, in an attempt to obtain some detailed information on this binding site, we studied the high-affinity binding of warfarin to the recombinant proteins at physiological pH by fluorescence spectroscopy.

MATERIALS AND METHODS

Synthesis and purification of rHSA forms

The recombinant DNA techniques used in producing wild-type rHSA and four single-residue mutants were as described previously [14]. A chimaeric plasmid (pJDB-ADH-L10-HSA-A) having cDNA for the mature form of HSA along with an L10 leader sequence was a gift from Tonen Co. (Tokyo, Japan). The mutagenic primers used (underlined letters indicate mismatches) were 5'-CAAACAGAGACTCGCCTGTGCCAGTCTCC-3' for K199A, 5'-GAGCTTTCAAAGCAGCTGCAGTAGCTCGCCTG-3' for W214A, 5'-GGGCAGTAGCTCATCTGAGCCAGAG-3' for R218H, and 5'-TCTTACCAAAGTCCAAACGGGAATGCTGCC-3' for H242Q. The L10-HSA coding region was amplified by PCR with a forward and a reverse primer

Abbreviations used: HSA, human serum albumin; rHSA, recombinant HSA.

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carrying a 5'-terminal *EcoRI* site and cloned into the *EcoRI*-digested pKF19k vector (Takara Shuzo Co., Kyoto, Japan). Mutagenesis was performed with a site-directed mutagenesis kit (oligonucleotide-directed dual amber method) which was obtained from Takara Shuzo Co. The mutation was confirmed by DNA sequencing of the entire HSA coding region with the dideoxy chain termination method on a PerkinElmer ABI Prism 310 Genetic Analyzer. For constructing the HSA expression vector pHIL-D2-HSA, a L10-HSA coding region without or with the desired mutation site was incorporated into the methanol-inducible pHIL-D2 vector (Invitrogen Co., San Diego, CA, U.S.A.). The resulting vector was introduced into the yeast species *P. pastoris* (strain GS115) to express rHSA. Secreted rHSA was isolated from the growth medium by a combination of precipitation with 60 % (w/v) $(\text{NH}_4)_2\text{SO}_4$ and purification on a Blue Sepharose CL-6B column (Amersham Pharmacia Co., Uppsala, Sweden). Isolated protein was defatted by using the charcoal procedure described by Chen [15], deionized, freeze-dried and then stored at -20°C until use. The resulting albumins (treated with dithiothreitol) exhibited a single band on SDS/PAGE and all the recombinant proteins migrated to the same position as native HSA. Density analysis of protein bands stained with Coomassie Brilliant Blue showed that the purity of the recombinant albumins was more than 97 %.

Spectroscopic studies

All CD measurements were made with a Jasco J-720 spectropolarimeter (Tokyo, Japan). Far-UV and near-UV intrinsic spectra were recorded from 200 to 250 nm and from 250 to 350 nm respectively. For calculation of the mean residue ellipticity $[\theta]_{\text{mrw}}$, the molecular masses of the albumins were taken as 66.5 kDa.

Intrinsic fluorescence spectra were obtained on a Jasco FP-770 fluorescence spectrometer (Tokyo, Japan), with 1 cm quartz cells, thermostatically controlled devices and 5 nm excitation and emission bandwidths. The albumins were excited at 295 nm; the spectra were corrected for buffer baseline fluorescence.

Thermal denaturation measurements

CD melting curves were determined by monitoring the changes in the dichroic intensity at 222 nm as a function of temperature. For thermal denaturation experiments in the range 298–358 K, a heating rate of 1.0 K/min was applied with a Jasco PTC-348 thermostat (Tokyo, Japan). The midpoint of denaturation (melting temperature, T_m) as well as the van't Hoff enthalpy (ΔH_m) were determined with essentially the same method as that of Budisa et al. [16].

Differential scanning calorimetry was performed on a MicroCal MC-2 ultrasensitive DSC (MicroCal, Northampton, MA, U.S.A.) with heating rates of 1.0 K/min and a sample concentration of 0.1 mM in 67 mM sodium phosphate buffer, pH 7.4. The calorimetric reversibility of the thermally induced transition was checked by reheating the protein solution in the calorimetric cell after cooling from the first run; it was observed that heating to above 358 K caused irreversible denaturation of the proteins.

Ligand binding experiments

Potassium warfarin was donated by Eisai Co. (Tokyo, Japan), and fluorescence measurements were made on the Jasco FP-770

fluorimeter (Tokyo, Japan). The fluorophores of warfarin were excited at 320 nm and the fluorescence emission intensity was measured at the maximum (approx. 380 nm). When a warfarin molecule binds to HSA, the intensity of this fluorescence emission is enhanced approx. 10-fold over that for unbound warfarin. To calculate binding constants for the protein–warfarin interactions, three separate titrations were performed and evaluated as described by Maes et al. [17]. For estimating the molar fluorescence of 100 % protein-bound warfarin, only data points at a protein-to-warfarin molar ratio higher than 10:1 were used, to keep the error of the extrapolation under 1 % [18]. All fluorescence intensities were corrected for inner filter effects and for buffer baseline fluorescence.

At the molar ratios between warfarin and albumin (not more than 0.4) used in this study, the binding results were well characterized by assuming binding to the high-affinity site only. Therefore primary binding constants (K) were estimated by fitting the experimental data to the following simple equation with a non-linear least-squares computer program (MULTI):

$$r = K[L_t]/(1 + K[L_t])$$

where r is the number of moles of ligand bound per mole of protein, and $[L_t]$ is the concentration of unbound ligand.

RESULTS

Structural characterization of rHSA forms

N-terminal sequencing for 20 cycles revealed that approximately half of the recombinant proteins had two additional serine residues derived from the L10 leader sequence [14] and that the remaining had zero, one or three extra serine residues in comparison with HSA isolated from serum. SDS/PAGE analysis revealed that the molecular masses of the wild-type and mutant rHSA forms were identical with that of HSA. Taken together, these findings exclude the possibility of large truncations or extensions of the N-termini and C-termini.

To obtain information on secondary and tertiary protein structures, CD measurements were performed in the far-UV and

Figure 1 Intrinsic fluorescence spectra of rHSA forms at pH 7.4 and 25 °C

The proteins were excited at 295 nm and their concentration was 2 μM in 67 mM sodium phosphate buffer. From top to bottom, spectra are shown for mutant K199A, mutant H242Q, wild-type rHSA, mutant R218H and mutant W214A. The spectra are averages of three measurements.

Figure 2 Thermal denaturation of rHSA forms

Fractions of denatured protein were calculated from CD data monitored at 222 nm. The protein concentration was 20 μ M in 67 mM sodium phosphate buffer, pH 7.4. Results are shown for wild-type rHSA (■), mutant K199A (●), mutant W214A (▲), mutant R218H (□) and mutant H242Q (○). Results are averages for three determinations.

near-UV regions. All rHSA forms display the same characteristics in the far-UV region, including minima near 209 and 222 nm, indicating the same content of α -helix and β -sheets. The rHSA forms also had similar spectral characteristics in the near-UV region, including minima near 262 and 268 nm, indicating that the mutants adopted structures comparable with that of wild-type rHSA. Furthermore, the ellipticities of the four mutants were the same. Both the far-UV and near-UV CD spectra of the wild-type rHSA were identical with those of HSA.

HSA has a single tryptophan residue, Trp-214, located almost centrally in helix 2 of subdomain IIA [1]. To obtain detailed information on the environment of this residue and on the exposure of the fluorophore to the aqueous solvent, tryptophan emission spectra were obtained after excitation at 295 nm. As seen in Figure 1, the spectra obtained for the K199A and H242Q mutants were both very similar to that of wild-type rHSA (which was identical with that of HSA). In contrast, the relative fluorescence intensity of R218H was approximately half, and

$\lambda_{\max}$ was slightly blue-shifted from 346 to 343 nm. Finally, the mutant W214A had a very low fluorescence, which most probably originated from tyrosine residues.

It is possible that the fluorescence spectra could be affected by minor changes in environment, such as differences in solvation.

Structural stability

The impact of the mutations on the structural properties of albumin was also investigated by studying the thermal denaturation of the proteins. Figure 2 shows that denaturation of the mutants K199A, R218H and H242Q is apparently a two-state transition. The steepness of the curves for these three mutants is comparable with that of the curve for wild-type rHSA (and HSA). In contrast, the transition profile for W214A is less steep; denaturation is initiated at physiological temperature, 10–15 °C lower than the initiation temperatures of the other proteins. The form of the denaturation curve for W214A indicates a less co-operative unfolding process [19]. Furthermore, the curve indicates that the thermal denaturation of this mutant is more heterogeneous. Therefore we also studied the thermal denaturation of W214A and wild-type rHSA by differential scanning calorimetry; the results of this method gave clear supporting evidence for heterogeneous denaturation of the mutant. However, for the sake of comparison the denaturation of W214A was also analysed in terms of a two-state process; the thermodynamic parameters calculated from the curves in Figure 2 are given in Table 1. Both negative and positive values for ΔT_m and $\Delta(\Delta H_m)$ were estimated, changing in parallel, i.e. if ΔT_m was negative, then $\Delta(\Delta H_m)$ was also negative. The results indicate that R218H and H242Q were more stable than wild-type rHSA and that K199A was less resistant to temperature increases. However, the most evident changes were again found for W214A, which had a T_m 3.0 K lower than that of wild-type rHSA and, as a consequence of the less co-operative denaturation [19], a much lower ΔH_m .

The thermal denaturation of five mutants substituted in Arg-410 and/or Tyr-411 [14] was also studied by CD. These residues are elements in Sudlow's binding site II in subdomain IIIA. In all cases, the forms of the denaturation curves were comparable to that of wild-type rHSA; only marginal changes were found in the thermodynamic parameters (Table 1).

Table 1 Thermodynamic parameters for thermal denaturation of rHSA forms

The protein concentration was 20 μ M in 67 mM sodium phosphate buffer, pH 7.4. ΔT_m is T_m for the mutant minus T_m for wild-type rHSA; $\Delta(\Delta H_m)$ is ΔH_m for the mutant minus ΔH_m for wild-type rHSA. Results are means $\pm$ S.D. for three experiments.

rHSA	T_m (K)	ΔT_m (K)	ΔH_m (kJ/mol)	$\Delta(\Delta H_m)$ (kJ/mol)
Wild-type	333.7 $\pm$ 0.1		312.9 $\pm$ 10.6	
K199A	331.9 $\pm$ 0.1	−1.8	259.4 $\pm$ 13.5	−53.5
W214A	330.7 $\pm$ 0.1	−3.0	134.2 $\pm$ 10.1	−178.7
R218H	335.3 $\pm$ 0.2	1.6	380.9 $\pm$ 15.2	68.0
H242Q	335.2 $\pm$ 0.1	1.5	357.9 $\pm$ 18.4	45.0
R410A	333.2 $\pm$ 0.3	−0.5	304.9 $\pm$ 11.4	−8.0
Y411A	332.9 $\pm$ 0.2	−0.8	299.1 $\pm$ 17.53	−13.8
R410A/Y411A	332.8 $\pm$ 0.1	−0.9	294.7 $\pm$ 12.8	−18.2
Y411S	334.0 $\pm$ 0.2	0.3	316.8 $\pm$ 17.2	3.9
Y411F	333.0 $\pm$ 0.2	−0.7	302.1 $\pm$ 10.9	−10.8

Table 2 High-affinity binding of warfarin to rHSA forms at pH 7.4 and 25 °C

The albumin concentration was 10 μ M; the total concentration of warfarin varied from 0.5 to 4 M. The experiments were performed in 67 mM sodium phosphate buffer. Results are means $\pm$ S.D. for three experiments.

rHSA	$10^{-5} \times$ Association constant (M^{-1})
Wild-type	3.63 ± 0.22
K199A	15.90 ± 0.75
W214A	1.71 ± 0.13
R218H	1.13 ± 0.23
H242Q	22.17 ± 0.86

Warfarin binding studied by fluorescence measurements

The binding of warfarin to all the albumins at pH 7.4 was quantified by fluorescence spectroscopy; the association constants calculated are given in Table 2. The high-affinity binding constant for wild-type rHSA, and that for HSA, is $3.6 \times 10^5 M^{-1}$. This value is in good agreement with published values [$(3.3\text{--}7.4) \times 10^5 M^{-1}$] [5,9,20]. In comparison, the binding constants determined for H242Q and K199A were about 6-fold and 4-fold higher respectively. However, the affinities of R218H and W214A for warfarin were decreased approx. 3-fold and 2-fold respectively. The result obtained with the R218H mutant is in accordance with the findings of Petersen et al. [20], who studied warfarin binding to the same mutant by fluorescence spectroscopy and equilibrium dialysis and reported a 4–5-fold decrease in affinity. The effects of the amino acid substitutions show that the side chains of Trp-214 and Arg-218 are important

for warfarin binding, whereas the side chains of Lys-199 and His-242 seem to interfere with binding.

DISCUSSION

Mutation-induced changes in protein conformation and stability

The present study shows that single-residue mutations can modify the stability of albumin and that the effects are more pronounced for mutations in subdomain IIA than for mutations in subdomain IIIA (Table 1) [14]. Because the effects of the mutations at site II on protein stability are only marginal (Table 1), we discuss here the mutations at site I.

X-ray crystallographic analyses of HSA and wild-type rHSA have shown that the A and B subdomains assemble through hydrophobic helix-packing interactions primarily involving helices 2, 3 and 8 [21]. The four mutations at site I are all placed in or close to helix 2 or 3 of the centrally placed subdomain IIA (Figure 3). They could therefore all have an effect on the helix packing of this subdomain and thereby on the conformation and stability of the whole molecule. This was indeed so, because we found minor increases in thermal stability for R218H and H242Q, a small decrease in stability for K199A and a larger decrease in stability for W214A (Table 1).

The X-ray crystallographic analyses [21] also revealed that albumin is assembled by hydrophobic and salt-bridge interactions between subdomains IA, IB and IIA on the one side and subdomains IIB, IIIA and IIIB on the other. Among the potential salt bridges is one formed by Arg-218 and Asp-451, because in the crystal structure these two residues are only approx. 3.65 Å apart (Figure 3). Breaking of this ionic interaction is most probably part of the reason why the mutation of Arg-218 to histidine affects not only albumin's thermal stability but also its intrinsic fluorescence (Figure 1). The latter finding can be

Figure 3 Stereoscopic schematic drawing of the primary warfarin binding site of HSA

The Figure was drawn with Molscript [26] based on sequence 1UOR deposited in the Brookhaven Protein Data Bank. Helices 1, 2 and 3 are the three N-terminal helices of subdomain IIA.

explained as follows. Breaking the salt bridge between Arg-218 and Asp-451 (perhaps supplemented by the removal of the positive charge at position 218) results in transfer of the tryptophan residue to a more hydrophobic environment, where it comes in closer contact to quenching residues. These residues could be histidine (strong quencher), glutamine, asparagine, methionine and/or arginine (moderate quenchers) [22].

Trp-214 is also important for holding together the two halves of the heart-shaped albumin, because the residue is an element in a major interdomain cluster of hydrophobic residues [21]. Apparently, replacement of tryptophan with alanine implies a significant decrease in protein stability, because the mutation results in a marked reduction in thermal stability (Table 1). Actually, mutant W214A has both the lowest T_m and ΔH_m of the nine mutants examined. Furthermore, in contrast with the other mutants and wild-type rHSA, thermal denaturation of W214A was a heterogeneous process. This observation can be explained by assuming that in this mutant domain II, for example, denatures at a significantly lower temperature than the other two domains.

In contrast with K199A and W214A, mutants R218H and H242Q had greater stability. This finding provides useful information for the design of recombinant albumin for clinical applications.

Warfarin binding

Structural details of the interaction between warfarin and albumin are not known. However, it is widely accepted that warfarin binds with high affinity to a region in subdomain IIA [5,21]. Analysis of the crystal structure of the protein has revealed that this binding region is formed as a pocket, with the inside wall being formed by hydrophobic side chains and the entrance to the pocket being surrounded by positively charged residues [2]. Because the residues mutated in the present study are elements of the general binding pocket (Figure 3), they could all modify warfarin binding. The results showed that this was indeed true, but the effects differed both qualitatively and quantitatively.

Trp-214 is located in the hydrophobic part of the binding pocket; mutating this residue to alanine decreased the association constant approx. 2-fold (Table 2). Thus Trp-214 could be part of the high-affinity binding site of warfarin, but it does not seem to be essential for binding. The importance of the tryptophan residue for warfarin binding has also been evaluated by chemical protein modifications. Fehske et al. [23] 'selectively and completely' modified Trp-214 with 2-hydroxy-5-nitrobenzyl bromide and *o*-nitrophenylsulphenyl chloride and observed that in both cases warfarin binding was strongly decreased. These results led the authors to conclude that Trp-214 is an important part of the drug-binding site. The stronger effect observed by Fehske et al. [23] could have been caused by the presence of the bulky modifiers and/or by major conformational changes of albumin introduced when preparing the HSA derivatives. If Trp-214 is indeed an element of the binding site it most probably interacts hydrophobically with the non-polar part of the warfarin molecule.

Arg-218 is located at the entrance to the binding pocket (Figure 3); replacing that residue with histidine diminishes the binding constant approx. 3-fold (Table 2). Thus in HSA this arginine residue contributes positively to binding warfarin. At pH 7.4, warfarin is negatively charged because its acidic enol group has a pK_a of 5.05 in aqueous solution [24]. Therefore in the native protein there is most probably an ionic interaction between warfarin and Arg-218.

Lys-199 and His-242 are placed close to each other at the opening of the binding region (Figure 3); the inter-residue distance is only 3.4 Å. Rather surprisingly, mutation of one of the two residues increases drug binding significantly; the increment in the association constant for K199A and H242Q is approx. 4-fold and 6-fold respectively (Table 2). Thus when both Lys-199 and His-242 are present, they seem to impede the access of warfarin to the binding pocket.

He and Carter [1] examined in detail the binding chemistry of the ligand 2,3,5-tri-iodobenzoate; they found that the carboxy group of this ligand interacts with Lys-199 (and Arg-222 and Arg-257) and lies within approx. 4.0 Å of His-242. Thus in this example the two residues interact favourably with the ligand. In contrast with warfarin binding, Trp-214 and Arg-218 are not important for the binding of 2,3,5-tri-iodobenzoate. Furthermore, binding of L-thyroxine is partly blocked by Arg-218, because mutation of the residue to a histidine results in a more than 10-fold increment in the binding constant [10,11]. Thus the detailed construction of the binding sites for warfarin, 2,3,5-tri-iodobenzoate and L-thyroxine differs. This finding and the conformational studies lend support to the view that the binding region of subdomain IIA (Sudlow's site I) is large, flexible and harbours several individual ligand-binding sites that are sometimes independent of each other but in other cases influence each other [25].

This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports, and Culture of Japan (10557245; 11694298) and by the Sasakawa Scientific Research Grant from The Japan Science Society.

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Received 4 September 2000/28 March 2001; accepted 19 April 2001