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Binding of Warfarin, Salicylate, and Diazepam to Genetic Variants of Human Serum Albumin with Known Mutations

ULRICH KRAGH-HANSEN, STEPHEN O. BRENNAN, MONICA GALLIANO, and OSAMU SUGITA

Institute of Medical Biochemistry, University of Aarhus, DK-8000 Aarhus C, Denmark (U.K.-H.); Molecular Pathology Laboratory, Department of Pathology, Christchurch School of Medicine, Christchurch, New Zealand (S.O.B.); Department of Biochemistry, University of Pavia, I-27100 Pavia, Italy (M.G.); Department of Laboratory Medicine, Niigata University School of Medicine, Niigata 951, Japan (O.S.)

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SUMMARY

Possible effects of single point mutations on the ligand-binding capabilities of human serum albumin (Alb) were investigated by studying the interactions between the strongly bound drugs warfarin, salicylate, and diazepam and five structurally characterized genetic variants of the protein. Equilibrium dialysis data, obtained with the variants and normal serum Alb, revealed pronounced reductions in high affinity binding of all three ligands to Alb Canterbury (313 Lys→Asn) and to Alb Parklands (365 Asp→His). By contrast, unchanged binding of the drugs was found in the case of Alb Verona (570 Glu→Lys). Different effects on binding were observed for the other two variants. Salicylate was the only drug bound with a lower affinity to Alb Niigata (269 Asp→Gly), whereas binding of both salicylate and diazepam to Alb Roma (321 Glu→Lys) were moderately reduced. In about half of the cases of diminished binding, the primary association constant was reduced by 1 order of magnitude, giving rise to an increase in the unbound fraction of the drugs of 500% or more at therapeutically relevant molar ratios of drug and protein. Changes in protein charge seem to be of only minor importance for reduced binding. More likely, conformational changes in the 313–365 region of the proteins are the main cause for diminished binding of these diverse ligands, which probably have different high affinity binding sites. The specific reduction in salicylate binding after modification of residue 269 may be due to conformational changes at or close to the salicylate binding site.

The metabolism, distribution, and excretion of endogenous as well as exogenous compounds can be greatly influenced by binding to plasma proteins, most notably Alb, which is able to bind extensively a great number of different compounds in a reversible manner (1, 2). Therefore, if the binding capabilities of serum Alb are impaired, this could influence the biological fate of endogenous compounds and the pharmacokinetics of tightly bound drugs. Such an impairment could perhaps take place if the normal (wild-type) serum Alb (Alb A) is replaced, partially or completely, by a genetic variant of the protein. In the present study we have examined this possibility by studying high affinity binding of three strongly bound and widely used drugs to genetic variants of human serum Alb and comparing the bindings with those to Alb A isolated from the same persons and to a commercial preparation of the protein (also proposed to be Alb A). The variants have previously been characterized in detail (3–7) and possess single mutations in different parts of the protein molecule, namely 269 Asp→Gly (Alb Niigata), 313 Lys→Asn (Alb Canterbury), 321 Glu→Lys (Alb Roma), 365 Asp→His (Alb Parklands), and 570 Glu→Lys (Alb Verona).

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The unique binding properties of serum Alb seem to be based on the existence of separate regions for high affinity binding of ligands (1, 2, 8, 9). However, only little is known about the structure and the molecular localization of these regions. Serum Alb with known mutations may be a valuable tool in this respect, because they can be regarded as "perfect" protein modifications in the sense that only one amino acid residue, of known type and position, is altered, as compared with Alb A. This is in contrast to chemically modified proteins, because covalently bound reagents are seldom specific, i.e., more than one kind of amino acid residue is usually modified (1). In an attempt to shed light on the above-mentioned issues, we have, among the great number of strongly bound drugs, chosen warfarin, salicylate, and diazepam, because these ligands have been suggested as marker ligands for different binding regions of human serum Alb (1, 2, 8, 9).

Materials and Methods

Chemicals. The human serum Alb variants were isolated from serum or plasma samples and purified as previously described (3–7). Alb A was also isolated from the samples, except in the case of the plasma containing Alb Parklands. Commercial human serum Alb (97% pure according to the manufacturer) was obtained in a lyophilized form

ABBREVIATION: Alb, albumin.

from AB Kabi (Stockholm, Sweden). All Albs were delipidated according to the procedure described by Chen (10) but with the following modifications. The protein solutions were acidified by addition of H_2SO_4, and charcoal was replaced by hydroxyalkoxypropyl-dextran (Sigma Chemical Co., St. Louis, MO). The ratio between the amount of dextran derivative and protein was 10:1 (w/w). Control experiments, performed by adding [9,10-^{3}H(N)]palmitic acid (New England Nuclear, Boston, MA) and unlabeled fatty acid to charcoal-defatted Alb and afterwards defatting by the dextran derivative, showed a residual content of palmitate of 7.6 ± 1.3%. Protein concentrations were determined by the method of Lowry *et al.* (11).

The ligands [^{14}C]warfarin (46 mCi/mmol) and [2-^{14}C]diazepam (54 mCi/mmol) were purchased from Amersham International (Amersham, Buckinghamshire, England) and [7-^{14}C]salicylic acid (58.2 mCi/mmol) was bought from New England Nuclear. The purity of the various batches were controlled and, when necessary, improved by thin layer chromatography using the media recommended by the manufacturers. Unlabeled warfarin was a Sigma product, salicylic acid was supplied by Merck (Darmstadt, West Germany), and diazepam was donated by Dumex Ltd. (Copenhagen, Denmark).

Equilibrium dialysis studies. The binding experiments were carried out at 20° with media containing 33 mM sodium phosphate buffer, pH 7.4. Samples with varying ligand concentrations, with and without a constant concentration of serum Alb (0.1 or 0.2%, w/v), were prepared. Gentamicin sulfate (donated by Essex Pharma A/S, Farum, Denmark) was added to all media in a final concentration of 20 μg/ml in order to prevent bacterial growth. The degree of ligand binding was determined by a Dianorm equilibrium dialyser (Dianorm-Geräte, München, West Germany) with half-cell volumes of 250 μl. The dialysis membranes were made from natural cellulose and had a molecular weight cutoff of 5000 (Diachema dialysis membranes). Sample aliquots of 200 μl containing protein or protein plus ligand were pipetted into the left side of the cells, and samples of 200 μl containing ligand or buffer alone were pipetted into the right side of the cells. As references, representing 100% unbound ligand, cells were prepared with ligand dissolved in buffer in one side of the cells and buffer in the other sides. After filling, the equilibrium dialysers were placed in a temperature-controlled waterbath, and the cells were rotated for 17–18 hr about their horizontal axis at a speed of 12 rpm. After that period of time, the half-cells were emptied, and the concentrations of ligand in the Alb-free media were determined by liquid scintillation counting. Binding percentages were calculated from the concentrations of ligand in the right side media of Alb-containing cells and their corresponding reference cells.

Control experiments with reference solutions on one side of the dialysis membranes and buffer on the other side showed that the membranes were fully permeable for all three ligands under the present conditions, that ligand adsorption to membranes and cell walls was negligible, and that equilibrium was established within the period of time used. Gentamicin does not bind to serum Alb, and complex formation between the ligands and gentamicin could be excluded, because the light absorption spectra (200–400 nm) of warfarin, salicylate, and diazepam were unaffected by the presence of the antibiotic. As measured by the method of Lowry *et al.* (11), protein leakage could be neglected. No fluid shifts from the right sides of the cells to the left sides, owing to the presence of protein in the latter compartments, were observed at the present protein concentrations (15 or 30 μM).

Calculations. For all ligands, binding to one high affinity site was studied. The association constants (K) were calculated by converting the binding percentages to the number of mol of ligand bound/mol of Alb ($\bar{\nu}$) and fitting these by least squares to the concentration of unbound ligand ($[L]_f$), according to the following equation:

$$\bar{\nu} = \frac{K \cdot [L]_f}{1 + K \cdot [L]_f}$$

Results

Binding of warfarin. High affinity binding of this anticoagulant to defatted preparations of different Alb variants, Alb A, and commercial human serum Alb is shown in Fig. 1, A–E. It is seen that the bindings to all Alb forms, except for the low binding to Alb Canterbury (Fig. 1B) and to Alb Parklands (Fig. 1D), are similar. For the unreduced bindings the primary association constant (K) was calculated to be 2.5×10^5 M^{-1}. This value is comparable to those previously published (12, 13). By contrast, warfarin binding to Alb Canterbury (Fig. 1B) is characterized by a K value that is approximately 1 order of magnitude lower (3.5×10^4 M^{-1}). Drug binding to Alb Parklands (Fig. 1D) is also reduced ($K = 6.5 \times 10^4$ M^{-1}) as compared with binding to the commercial protein. Therefore, binding of warfarin to Alb Parklands is probably also lower than that to endogenous Alb A, which was not available in this case.

In Fig. 1F, the positions of the various mutations have been

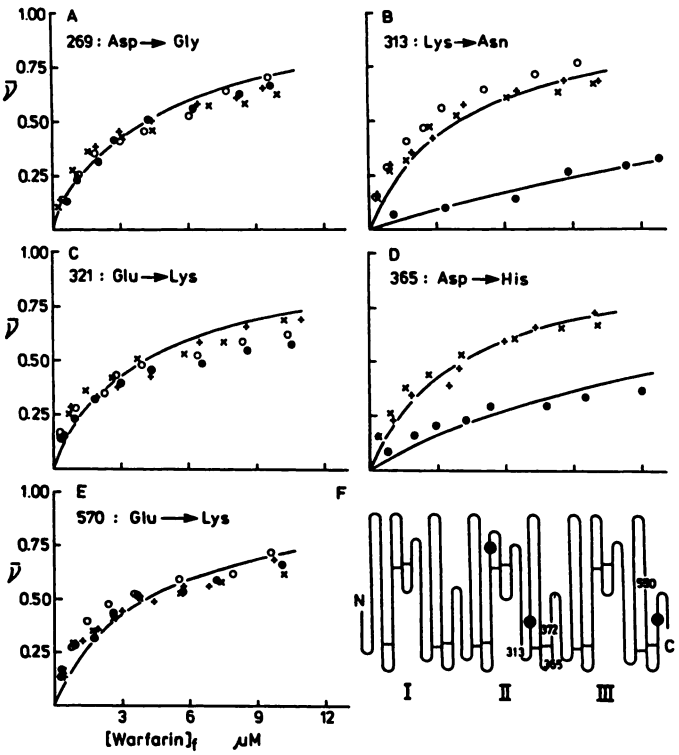

Fig. 1. A–E, Binding of warfarin to different genetic variants of human serum Alb (as indicated in the panels), Alb A, and commercial human serum Alb. The equilibrium dialysis was performed at 20°, by placing drug plus serum Alb variant (●), Alb A (○), or commercial protein (×) in the left side of the cells and buffer, pH 7.4, in the corresponding right side and by placing commercial serum Alb in the left side of the cells and drug in the corresponding right side (+). The binding curves are least squares fits of the equation given in Materials and Methods. The association constants calculated are given in the text. Warfarin concentrations varied from 3.0 to 30.0 μM, while the serum Alb concentration was kept constant at 15 μM. $\bar{\nu}$ represents the average number of mol of drug bound/mol of protein. The data are representative results from one to three experiments. F, The polypeptide chain and the disulfide bonds (*small horizontal bars*) of human serum Alb shown according to the model of Brown (14). The positions of the mutations (cf. A–E) are indicated in the model either as *closed circles* (mutations not affecting binding) or as the *number* of the position involved (mutations affecting binding). In the panel, positions 372 and 550 represent Alb Naskapi and Alb Mexico-2, respectively. The *roman numerals* indicate the three domains of the protein.

indicated in the model of human serum Alb originally proposed by Brown (14). Substitution of Asp 269 by Gly (Alb Niigata), Glu 321 by Lys (Alb Roma), or Glu 570 by Lys (Alb Verona) does not affect high affinity warfarin binding and these positions have been marked by *closed circles* in the figure. Positions 313 (Alb Canterbury) and 365 (Alb Parklands), which did affect warfarin binding, have been shown by their *numbers*. Positions 372 and 550 have also been indicated by their *numbers*, because Blumberg and co-workers (15, 16) have reported a small but significant decrease in warfarin binding to Alb Naskapi (372 Lys→Glu) and Alb Mexico-2 (550 Asp→Gly).

Binding of salicylate. High affinity binding of salicylate to the various serum Alb preparations is illustrated in Fig. 2, A–E. With this marker ligand, large variations in the association constant for the single primary binding site were found. The K value for drug binding to the commercial protein is 8.5×10^4 M^{-1}. This value is lower than those previously published, namely 1.9×10^5 M^{-1} (12) and 4.6×10^5 M^{-1} (17). However, it should be noted that these previous values were obtained at much higher protein concentrations (4.0 and 2.5%, respectively) and by using a slightly different procedure.

The association constants for salicylate binding to endogenous Alb A (Fig. 2, A–C and E) are generally higher, on an average 68%, than that characterizing binding to commercial serum Alb, whereas most mutations give rise to a decrease in salicylate binding. The K value of endogenous Alb A varied somewhat depending on the source of protein, 1.3×10^5 M^{-1} (Fig. 2A), 1.5×10^5 M^{-1} (Fig. 2B), 1.0×10^5 M^{-1} (Fig. 2C), and 1.9×10^5 M^{-1} (Fig. 2E) for Alb A isolated from serum or plasma samples also containing Alb Niigata, Alb Canterbury, Alb Roma, and Alb Verona, respectively. This variation probably reflects differences in isolation procedures.

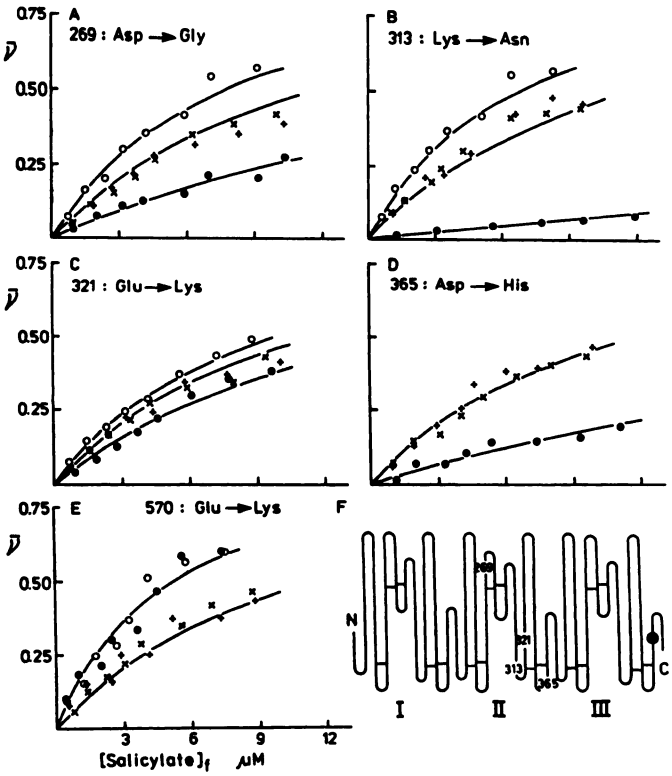

Fig. 2. A–E, Binding of salicylate to genetic variants of human serum Alb (●), Alb A (○), and commercial protein (×, +). The drug concentration varied from 2.5 to 25.0 μM, while the protein concentration was kept constant at 15 μM. The data are representative results from one to three experiments. F, A model of human serum Alb in which the positions of the mutations are indicated either as a *closed circle* (mutation not affecting binding) or as the *number* of the position involved (mutations affecting binding). For further information, see the legend to Fig. 1.

Salicylate binding to Alb Niigata (Fig. 2A) is reduced when compared both with binding to endogenous Alb A and with binding to the commercial protein. The K value (3.5×10^4 M^{-1}) is only one fourth of that found for binding to the corresponding Alb A. However, the most pronounced effect of a mutation observed in this study is that of salicylate binding to Alb Canterbury (Fig. 2B). In this case, the K value is reduced from 1.5×10^5 M^{-1} to only 9.0×10^3 M^{-1}. In comparison, the decrease in drug binding to Alb Roma is small, namely from a K value of 1.0×10^5 M^{-1} to 6.0×10^4 M^{-1} (Fig. 2C). Binding of salicylate to Alb Parklands is weaker ($K = 2.3 \times 10^4$ M^{-1}) than binding to the commercial serum Alb. Therefore, it is probably also much weaker than that to Alb A. The mutation of Alb Verona is the only one in this study not affecting binding of salicylate (Fig. 2E).

The results obtained with salicylate are summarized in Fig. 2F. In the model, the mutations resulting in a decreased drug binding are indicated by *numbers*, whereas the single mutation not affecting binding is represented by a *closed circle*.

Binding of diazepam. Under the present conditions, binding of this marker ligand also takes place at one site of human serum Alb irrespective of the preparation used (Fig. 3, A–E). In all cases, the K value for high affinity binding of the drug to commercial serum Alb was calculated to be 2.2×10^4 M^{-1}. This value is surprisingly low, when compared with those obtained at high protein concentrations, 4.7×10^5 M^{-1} (17) and 6.6×10^5 M^{-1} (12).

By analogy with salicylate binding, binding of diazepam to endogenous Alb A is stronger than that to the commercial preparation. The K value is, except in one case, 1.2×10^5 M^{-1} (Fig. 3, A, C, and D). The exception is Alb A isolated from the plasma sample also containing Alb Canterbury. In that example, the K value is somewhat higher, namely 1.8×10^5 M (Fig. 3B).

Neither diazepam binding to Alb Niigata (Fig. 3A) nor that to Alb Verona (Fig. 3E) is changed as compared with binding to endogenous Alb A. By contrast, drug binding to the other variants, having mutations placed in between the former two in the protein sequence, is diminished. The K value for binding to Alb Roma (Fig. 3C) is moderately reduced, namely to 7.5×10^4 M^{-1}. Binding both to Alb Canterbury (Fig. 3B) and to Alb Parklands (Fig. 3D) is reduced to the same low level as binding to the commercial protein ($K = 2.2 \times 10^4$ M^{-1}).

In Fig. 3F the mutations affecting high affinity binding of diazepam are indicated by *numbers* and those without any detectable effect are shown by *closed circles*.

Discussion

Clinical aspects. The genetic variants of human serum Alb used in this study all differ from the normal (wild-type) form of the protein by only a single amino acid residue. The results revealed that such single point mutations can significantly reduce high affinity binding of specific ligands. In several cases, the binding was reduced to an extent that suggested probable consequences for drug administration. High affinity binding of warfarin to Alb Canterbury serves as a good example of this.

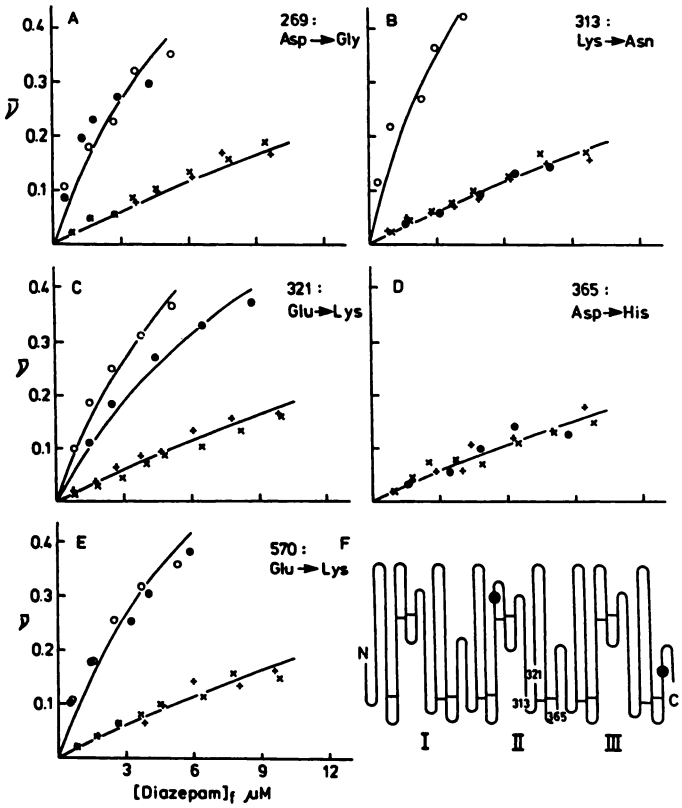

Fig. 3. A–E, Binding of diazepam to genetic variants of human serum Alb (●), Alb A (○), and commercial protein (×, +). The drug concentration varied from 2.4 to 24.2 μM, while the protein concentration was kept constant at 30 μM. The data are representative results from one to three experiments. F, A model of human serum Alb in which the positions of the mutations are indicated either as *closed circles* (mutations not affecting binding) or as the *number* of the position involved (mutations affecting binding). For further information, see legend to Fig. 1.

The association constant for binding of the drug to Alb A is 2.5 × 10^5 M^{-1}, whereas the constant for warfarin binding to the variant isolated from the same person is only 3.5 × 10^4 M^{-1}. If the molar ratio between total warfarin and serum Alb in plasma is 1 to 2, a clinically relevant situation, the reduced association constant will result in an increase of the unbound fraction of the drug from 1.2% to 7.5%, in other words an increment of more than 500% of the biologically active form of warfarin. Although this effect is probably smaller in heterozygote persons, the use of warfarin as an anticoagulant could result in unexpected hemorrhagic episodes in persons carrying Alb Canterbury. For the same serum Alb variant, an even greater reducing effect was observed on high affinity binding of salicylate (Fig. 2B) and diazepam (Fig. 3B).

Another clinically relevant finding is the high affinity binding of salicylate and diazepam, but not of warfarin, to Alb A isolated directly from the various patients. This is stronger than binding to a commercial lot of human serum Alb, also proposed to be Alb A. Although the explanation(s) for these differences are unknown at present, they are probably not due to the presence of reversibly bound endogenous compounds such as fatty acids, because all serum Alb samples used were delipidated in order to remove these and other lipophilic ligands. One possible explanation could be that the commercial preparation, to a greater extent than Alb A from the patients, was modified covalently, for example by glycation or by blockade of the free sulfhydryl group through the formation of mixed disulfides. Alternative or additional reasons could be that the isolation procedure for the commercial protein, in contrast to the other preparations of serum Alb, involved the use of ethanol precipitation and that the commercial protein had been heated to 60° for 10 hr by the manufacturer. Finally, the commercial protein could be contaminated with proteins binding the ligands very poorly.

Molecular aspects. All the genetic variants used in this study have point mutations that result in a changed charge on the albumin molecule. In the case of Alb Canterbury, the net negative charge was increased (313 Lys→Asn). Therefore, because the ligands are negatively charged or possess electronegative centers at physiological pH the possibility exists that the decreased ligand binding to this variant could be explained by an electrostatic effect. However, although Lys-313 seems to be very important for high affinity binding of warfarin, salicylate, and diazepam, it is not likely that the residue directly takes part in the formation of all of these high affinity binding sites. Previous binding studies, performed in two different laboratories (8, 13), have revealed independent high affinity binding of warfarin and diazepam to human serum Alb. Furthermore, the high affinity binding sites of warfarin and salicylate are not identical (13). Therefore, the pronounced reductions in ligand binding found in the case of Alb Canterbury are most probably the results of conformational changes of the protein molecule and/or a long range electrostatic effect.

In the case of the four remaining genetic variants, a decrease and not an increase of the negative charge has taken place. In two of these, an acidic residue is replaced by a neutral one, namely Asp by Gly in position 269 (Alb Niigata) and Asp by His in position 365 (Alb Parklands). Furthermore, in Alb Roma and Alb Verona, a negatively charged Glu is replaced by a positively charged Lys in position 321 and in position 570, respectively. For these four variants, the examples of decreased ligand binding cannot be explained by an electrostatic effect. Therefore, the altered binding properties of these variants must be caused by conformational changes of the protein molecule, either directly at the different high affinity binding sites or indirectly through conformational changes at more distant sites of the proteins.

Figs. 1F, 2F, and 3F show that the mutation in position 570 does not affect the binding of any of the three ligands. The explanation for this finding could be that the amino acid residue is placed relatively close to the C-terminus and far away from the binding sites. Furthermore, the mutation seems to be placed in a position without far-ranging effects on protein conformation. If that is the case, how far from the C-terminus is it then possible to place a mutation without affecting ligand binding? The answer will probably depend on the ligand in question, but it is of interest in this respect to note that a small but significant decrease in warfarin binding was observed (15, 16) when replacing Asp in position 550 by Gly.

The most pronounced effects on binding were all the result of a mutation in the second domain of serum Alb. The region 313–365 seems to be especially important. At present it is not clear whether the amino acid residues in the positions mentioned here take part directly in forming one or more of the high affinity binding sites for warfarin, salicylate, or diazepam or whether the effects observed are brought about by more or less localized conformational changes of the human serum Alb

molecule. However, one assignment can perhaps be made, because only binding of salicylate was reduced by modifying residue 269.

Acknowledgments

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Send reprint requests to: Ulrich Kragh-Hansen, Institute of Medical Biochemistry, University of Aarhus, DK-8000 Aarhus C, Denmark.