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HIGH-AFFINITY BINDING OF WARFARIN, SALICYLATE AND DIAZEPAM TO NATURAL MUTANTS OF HUMAN SERUM ALBUMIN MODIFIED IN THE C-TERMINAL END

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Abstract—High-affinity binding of warfarin, salicylate and diazepam to four natural mutants of human serum albumin was studied by equilibrium dialysis at pH 7.4. The mutants Alb Milano Fast and Alb Vanves possess single amino acid substitutions close to the C-terminus, namely 573 Lys → Glu and 574 Lys → Asn, respectively. By contrast, Alb Catania and Alb Venezia are chain termination mutants in which several amino acids have been changed or deleted. Binding of warfarin to the variants was lower than binding to normal (wild-type) albumin (Alb A). The association constants were 73% (Alb Milano Fast, Alb Vanves and Alb Catania) or 67% (Alb Venezia) of that determined for Alb A. The results obtained with salicylate were more dependent on the type of mutation. The constants were either comparable to the normal value (Alb Catania) or reduced to 64% (Alb Milano Fast), 71% (Alb Vanves) or 43% (Alb Venezia) of that value. Diazepam binding to the variants was normal, except for binding to Alb Venezia in which case the association constant was reduced to 76% of that calculated for Alb A. The results are in accordance with the view that warfarin, salicylate and diazepam bind to three different high-affinity sites. It is proposed that the sites for warfarin and salicylate are situated rather close to each other in domain II, and that these high-affinity sites are relatively susceptible to conformational changes of the protein. By contrast, the primary diazepam site is placed closer to, or within, domain III of albumin and seems to be less affected by conformational changes in the protein molecule.

The three-dimensional structure of crystalline albumin (Alb A) is now known at a resolution of 4 Å [1]. Although knowledge of this crystal structure map is a major step forward in the understanding of this important transport protein, X-ray crystallographic studies have not yet given detailed information about the molecular basis for the unique ligand binding capabilities of albumin. Therefore, other methods are usually resorted to when trying to locate and characterize high-affinity binding sites in the protein. These methods are based on the use of dissolved albumin and include ligand labelling followed by identification of the labelled site and ligand binding to chemically modified albumins or to fragments of the protein (for reviews, see Refs 2, 3). However, the results obtained by these approaches are often ambiguous owing to significant changes in the conformation of either protein or ligand.

In the present work, structurally characterized genetic variants of Alb A in a purified and delipidated form were used in an attempt to identify type and position of amino acid residues of importance for high-affinity binding of the widely used and strongly albumin-bound drugs warfarin, salicylate and diazepam. The majority of the albumin variants so far characterized are caused by mutations grouped in three short segments of the molecule: in the propeptide and the amino terminal end, and in the C-terminal portion of domains II and III. This apparent clustering of the mutations may reflect a folding of the polypeptide chain that makes these regions more exposed to the solvent and, therefore, potentially important for the ligand binding of albumin. We have utilized four different variants which all possess molecular changes in one of these regions, namely the C-terminal tailpiece. In two of the albumins the change was a single amino acid substitution, namely 573 Lys → Glu in Alb Milano Fast [4] and 574 Lys → Asn in Alb Vanves [5]. In Alb Catania the amino acids in positions 580–582 have been changed and those in positions 583–585 have been deleted [6], and in Alb Venezia the amino acids in positions 572–578 and 579–585 have been substituted and deleted, respectively [7]. Binding data, obtained by equilibrium dialysis, revealed that in several cases drug binding to the variants was less than binding to normal albumin.

MATERIALS AND METHODS

Chemicals. Fresh samples of serum from individuals with Alb A and an albumin variant were donated by Dr F. Porta, Ospedale di Circolo, Varese, Italy (Alb Milano Fast), Dr J. M. Fine, Institut National de Transfusion Sanguine et Immunohematologie, Paris, France (Alb Vanves), Dr C. Petrini, Ospedale S. Carlo Borromeo, Milano, Italy (Alb Catania) and Dr F. Gonano, Ospedale

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Gervasutta, Udine, Italy (Alb Venezia). Alb A and the different variants were taken from the same subjects and isolated and purified in the same laboratory as described previously [4–7]. A commercial preparation (pooled fraction) of human serum albumin proposed to be Alb A (97% pure according to the manufacturer) was bought in a lyophilized form from AB Kabi (Stockholm, Sweden). All proteins were delipidated according to the procedure described by Chen [8] but with the following modifications. The albumin solutions were acidified by adding H_2SO_4 instead of HCl, and charcoal was replaced by hydroxyalkoxypropyl-dextran (Sigma Chemical Co., St Louis, MO, U.S.A.). Sulphuric acid was preferred to hydrochloric acid because SO_4^{2-}, in contrast to Cl^-, does not bind to albumin, and the dextran derivative was used instead of charcoal because defatting by the former gives a better protein recovery (Kragh-Hansen, manuscript in preparation). The ratio between the masses of dextran derivative and protein was 10:1. Control experiments, performed by adding tracer amounts of [U-^{14}C]palmitic acid (Amersham International, Amersham, U.K.; sp. radioact. 828 Ci/mol) to the protein solutions before defatting revealed a residual content of fatty acids of 7.0 ± 1.0% (N = 10) after treatment with the dextran derivative. After defatting the albumins were freeze-dried and then stored at −20° until use. Protein concentrations were determined by the method of Lowry *et al.* [9].

The compound [7-^{14}C]salicylic acid (56.1 Ci/mol) was bought from New England Nuclear (Boston, MA, U.S.A.), whereas [^{14}C]warfarin (56 Ci/mol) and [2-^{14}C]diazepam (53.8 Ci/mol) were purchased from Amersham International. The amount of radiochemical impurities which do not bind to albumin was estimated by determining the residual concentration of the radioactively labelled compounds at infinite albumin concentration [10]. They were found to be 0% (salicylic acid), 0.2% (warfarin) and 0.1% (diazepam) in the batches used in the present work. Unlabelled sodium salicylate (≥99.5% pure) was supplied by Merck (Darmstadt, Germany), crystalline warfarin was obtained from Sigma and pure diazepam was donated by Dumex Ltd (Copenhagen, Denmark).

Equilibrium dialysis. Ligand binding was examined at 20° with media buffered with 33 mM sodium phosphate pH 7.4. Each experiment involved the use of one drug, commercial albumin, endogenous Alb A and all of the four genetic variants. Throughout the work Alb A isolated from the person also carrying Alb Vanves was used as a representative of normal, endogenous albumin. Samples with various concentrations of unlabelled and ^{14}C-labelled ligand and a constant concentration of protein (0.1%, w/v) were prepared. Salicylate and warfarin were added, with magnetic stirring, to the solutions as small volumes (5–10 μL) of concentrated stock solutions consisting of ligand dissolved in buffer (salicylate) or 0.1 M NaOH (warfarin). The final solutions of warfarin changed less than 0.05 pH unit. The stock solutions of diazepam were prepared in ethanol, and in this case the small volumes (10 μL) of stock solution were evaporated to dryness under a stream of nitrogen before an albumin solution was added.

The degrees of ligand binding were determined with a Dianorm equilibrium dialyser (Dianorm Geräte, München, Germany) with half-cell volumes of 250 μL. The dialysis membranes were made from natural cellulose and had a molecular mass cut-off of 5000 Da (Diachema dialysis membranes). Sample aliquots of 175 μL containing protein and ligand were pipetted into the left side of the cells, and samples of 175 μL of buffer alone were pipetted into the right side of the cells. After being filled, the equilibrium dialysers were placed in a temperature-controlled water bath, and the cells were rotated around their horizontal axis at a speed of 12 rpm for 17–18 hr. After this period, the half-cells were emptied, and the concentration of ligand in both the albumin-free and the albumin-containing media was determined by liquid scintillation counting of aliquots of 100 μL. No quenching of the radioactivity of the ^{14}C-labelled drugs by the proteins was observed.

Control experiments with ligand dissolved in buffer on one side of the dialysis membranes and buffer on the other side showed that equilibrium was established for all three drugs within the experimental period. As measured by the method of Lowry *et al.* [9], protein leakage could be neglected.

Calculations. The radioactivity of drug-containing solutions which had not been dialysed was taken to represent the known concentrations of total ligand. The concentrations of bound and free ligand were calculated by using the radioactivity of aliquots taken from the albumin-containing half-cells, representing bound plus free ligand, and from the corresponding albumin-free half-cells, which represent free ligand.

In the present study, low ligand to protein molar ratios were utilized. Therefore, only high-affinity drug binding was assumed to take place, and the association constants (K) were calculated by using the following equation:

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

where $\bar{\nu}$ represents the average number of moles of ligand bound per mole of protein, and $[L]_f$ is the concentration of the free form of the ligand. Graphical Scatchard analysis of the experimental data was also performed (not shown), as outlined by Pedersen *et al.* [11]. In this procedure, association constants were determined by extrapolation to zero of the x-values. Since comparable binding constants were obtained by the two different methods, the above-mentioned assumption of solely high-affinity binding is valid.

Statistical differences between ligand binding to endogenous Alb A and to the other albumin preparations were tested by Student's t-test.

RESULTS

The genetic variants

Table 1 summarizes the molecular characteristics of the four genetic variants of Alb A used in the present work. It is seen that both Alb Milano Fast

Table 1. Molecular characteristics of the albumin mutants used in the present study

Name of variant	Position of the amino acid(s) mutated	Amino acid changed from	Amino acid changed to	Change in net charge*	Reference
Alb Milano Fast†	573	Lys	Glu	−2	4
Alb Vanves	574	Lys	Asn	−1	5
Alb Catania‡	580	Gln	Lys	+1	6
	581	Ala	Leu		
	582	Ala	Pro		
	583	Leu	—§		
	584	Gly	—		
	585	Leu	—		
Alb Venezia	572	Gly	Pro	−1	7
	573	Lys	Thr		
	574	Lys	Met		
	575	Leu	Arg		
	576	Val	Ile		
	577	Ala	Arg		
	578	Ala	Glu		
	579	Ser	—§		
	580	Gln	—		
	581	Ala	—		
	582	Ala	—		
	583	Leu	—		
	584	Gly	—		
	585	Leu	—		

* Difference between the net charge of the variants and that of Alb A at pH 7.4.
† Formerly called Alb Mi/Fg [4].
‡ Formerly called Alb Ge/Ct [6].
§ The amino acids in the positions indicated and those placed closer to the C-terminus are deleted.

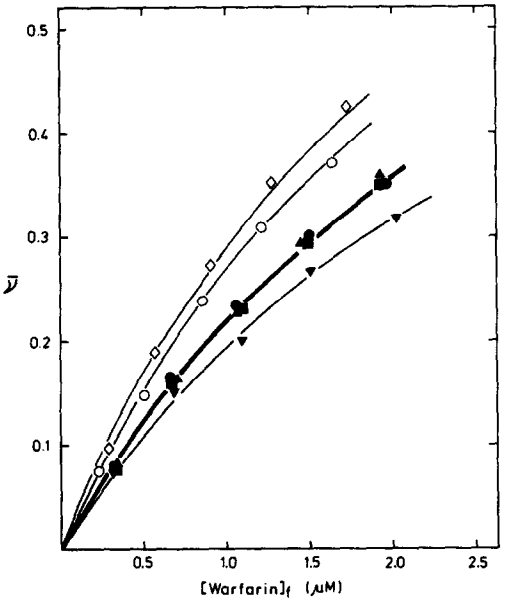

Fig. 1. High-affinity binding of warfarin to endogenous Alb A (○), commercial Alb A (◇), Alb Milano Fast (■), Alb Vanves (●), Alb Catania (▲) and Alb Venezia (▼) as determined by equilibrium dialysis. The results are single determinations from a representative experiment. The binding curves were constructed by using the equation given in the Materials and Methods, and the association constants calculated are included in Table 2. The symbols $\bar{\nu}$ and [Warfarin]$_f$ represent the average number of moles of ligand bound per mole of albumin and the concentration of free warfarin, respectively.

and Alb Vanves differ from normal albumin by a single amino acid. In the former protein, Lys-573 has been modified to a Glu, and in the latter Lys-574 has been substituted by an Asn. It is likely that the amino acid changes are the results of single base mutations in the structural gene [4, 5]. In Alb Catania and Alb Venezia the molecular modifications are more extensive, and they consist of a shortening of the polypeptide chain in the C-terminal end as well as a replacement of several amino acids in the new C-terminal ends (cf. Table 1). These proteins are the only examples reported so far of albumin mutants with a shortened polypeptide chain and the pronounced modifications in the structures could not be caused by point mutations in the albumin gene. Alb Catania seems to be the result of a base deletion followed by a frame shift in the structural gene [12]. The synthesis of Alb Venezia can be explained as a skipping of exon 14 followed by partial proteolytic degradation of the translation product by serum carboxypeptidase B [12].

Originally, the variants were detected on the basis of an electrophoretic mobility different from that of Alb A. Table 1 gives the reason for the abnormal electrophoretic mobilities by showing the charge by which the four mutants deviate from normal albumin. At pH 7.4, Alb Catania is less negative than Alb A, whereas the other variants possess more negative charges than the normal protein.

Binding of warfarin

High-affinity binding of warfarin to defatted preparations of endogenous Alb A, of commercial human serum albumin, also proposed to be Alb A,

Table 2. High-affinity binding of drugs to different albumin preparations

Protein	Association constants ($10^5\ M^{-1}$)		
	Warfarin	Salicylate	Diazepam
Endogenous Alb A	3.3 ± 0.6	1.4 ± 0.2	1.7 ± 0.4
Commercial Alb A	3.6 ± 0.6	1.3 ± 0.2	1.8 ± 0.4
Alb Milano Fast	2.4 ± 0.4*	0.9 ± 0.1*	1.7 ± 0.3
Alb Vanves	2.4 ± 0.6†	1.0 ± 0.3†	1.5 ± 0.3
Alb Catania	2.4 ± 0.6†	1.2 ± 0.2	1.8 ± 0.4
Alb Venezia	2.2 ± 0.5*	0.6 ± 0.1*	1.3 ± 0.3†

In each experiment the binding of various concentrations of one ligand to a constant concentration (15 μM) of the two preparations of Alb A and of the four genetic variants was examined. The total concentrations of ^{14}C-labelled plus unlabelled ligand were varied over the following concentration ranges: warfarin, 2.0–8.9 μM; salicylate, 2.8–15.9 μM; diazepam, 4.0–16.0 μM. The media were buffered with 33 mM sodium phosphate, pH 7.4, and the temperature was 20°.

The values given in the table are means ± SD of four or five experiments.

* $P < 0.01$, † $P < 0.10$. All comparisons were made to the results obtained with endogenous Alb A.

and of the four different albumin variants is shown in Fig. 1. From this representative experiment it is seen that in all cases drug binding to the two different preparations of Alb A was higher than to the variants. Ligand binding to the two preparations of Alb A seemed to be somewhat different, because warfarin binding to the endogenous protein was slightly lower than that to commercial albumin. It is also apparent from the Figure that ligand binding to the variants to some extent depended on the mutation. Alb Venezia possessed the lowest affinity for warfarin, whereas drug binding to the Albs Milano Fast, Vanves and Catania was similar and intermediate to the binding levels for Alb Venezia and endogenous Alb A.

In order to compare the binding in a more quantitative way association constants were calculated from this and other experiments performed in principally the same manner (Table 2). The primary association constants for warfarin binding to endogenous Alb A and commercial Alb A were $3.3 \pm 0.6 \times 10^5$ and $3.6 \pm 0.6 \times 10^5\ M^{-1}$, respectively. However, according to calculations performed by Student's *t*-test these values were not significantly different ($P > 0.30$). The small and insignificant difference between the binding constants is probably due to differences in source and processing of the proteins. These might have led to slight variations in protein conformation.

The association constants determined for warfarin binding to the variants were all significantly lower than that found for endogenous Alb A. The constants calculated for binding to the Albs Milano Fast, Vanves and Catania were, on average, reduced to 73% of that determined for endogenous Alb A, and the constant characterizing warfarin binding to Alb Venezia was reduced to 67%.

Binding of salicylate

The association constants determined for high-affinity binding of salicylate to the two different preparations of Alb A are very similar (Table 2). It is also evident that the effects on salicylate binding of the different protein mutations are more distinct that those found for binding of warfarin (and diazepam, see below). Drug binding to Alb Catania is not significantly different from binding to Alb A. By contrast, binding to the other three genetic variants is reduced to different extents. The average binding constant is reduced to 71% for Alb Vanves, 64% for Alb Milano Fast and 43% for Alb Venezia.

Binding of diazepam

Diazepam was least affected by the present amino acid substitutions of albumin. Binding to all the proteins, except for Alb Venezia, can be described by similar association constants ranging from 1.5×10^5 to $1.8 \times 10^5\ M^{-1}$ (cf. Table 2). Alb Venezia was the only mutant in this study with a reduced affinity for the drug, and the primary association constant was 76% of that determined for Alb A (Table 2).

DISCUSSION

Molecular aspects

In the present study the ligand binding properties of two single point mutants and two chain termination variants of Alb A were examined by studying the high-affinity binding of warfarin, salicylate and diazepam. In eight of the 12 ligand–variant combinations, binding to the variants was different from binding to normal albumin, whereas in the four remaining cases, the association constants were not significantly different from those determined for binding to Alb A. In all of the examples of modified binding, the association constant was decreased. No cases of enhanced binding to the variants were found.

The examples of reduced ligand binding can be caused by different molecular mechanisms. *A priori*, the possibility exists that the reductions in binding are the result of electrostatic effects, because, at a physiological pH, the ligands are negatively charged

(warfarin and salicylate) or possess electronegative centres (diazepam), and because all the variants have net charges which are different from that of Alb A (cf. Table 1). However, as illustrated by the following examples, it is very unlikely that the decreases in binding can be explained by electrostatic effects alone. First, the binding of warfarin to Alb Catania is reduced and not increased even though this variant possesses a net charge less negative than that of Alb A (cf. Table 1). Second, if electrostatic effects were the sole cause of the modified bindings, Alb Milano Fast should bind the ligands with the lowest affinity, since this variant is the one most negatively charged (cf. Table 1). However, as seen from Table 2, the ligands bind without exception most weakly to Alb Venezia. Therefore, the diminished binding is most probably the result of steric alterations and/or conformational changes in the albumin molecule, either directly at the binding sites or indirectly through changes elsewhere in the protein.

The effects of different single point mutations on high-affinity binding of warfarin, salicylate and diazepam are illustrated in Fig. 2. The information is given in a qualitative way with the two-dimensional model of albumin proposed by Brown [13]. The numbers in the figure represent positions in the amino acid sequence where substitutions have been found to be associated with reduced ligand binding, whereas the closed circles indicate substitutions which do not affect drug binding. The information about positions 573 (Alb Milano Fast) and 574 (Alb Vanves) is from the present study (Table 2). The results obtained with the variants Alb Niigata (269 Asp → Gly), Alb Canterbury (313 Lys → Asn), Alb Roma (321 Glu → Lys), Alb Parklands (365 Asp → His) and Alb Verona (570 Glu → Lys) are all taken from a previous publication [14]. Finally, the mutations 372 (Alb Naskapi) and 550 (Alb Mexico-2) have been included in Fig. 2A, because warfarin has been reported to bind less firmly to these genetic variants [15, 16]. It is apparent from the Figure that the ligands are affected differently by the variants. For example, diazepam binding (Fig. 2C) is only affected by three of the amino acid changes, whereas the binding of warfarin (Fig. 2A) and especially of salicylate (Fig. 2B) is reduced in most of the cases. In addition, one example of specific reduction in binding can be given, namely binding of salicylate to Alb Niigata. The differences in the effects of the single point mutations on ligand binding indicate that the three ligands bind to different high-affinity sites in the albumin molecule. This proposal is in agreement with results of competition studies which revealed that warfarin, salicylate and diazepam bind to three different primary sites of Alb A [2, 17, 18].

Figure 2A reveals that binding of warfarin is affected by single substitutions far apart from each other in the amino acid sequence of albumin. The question now arises as to which of these residues, if any, takes part in forming the binding site. In trying to answer this, the work of Bos *et al.* [19, 20] on high-affinity binding of warfarin to Alb A and to large fragments thereof is of great interest. The fragments corresponded to domains I and II (residues

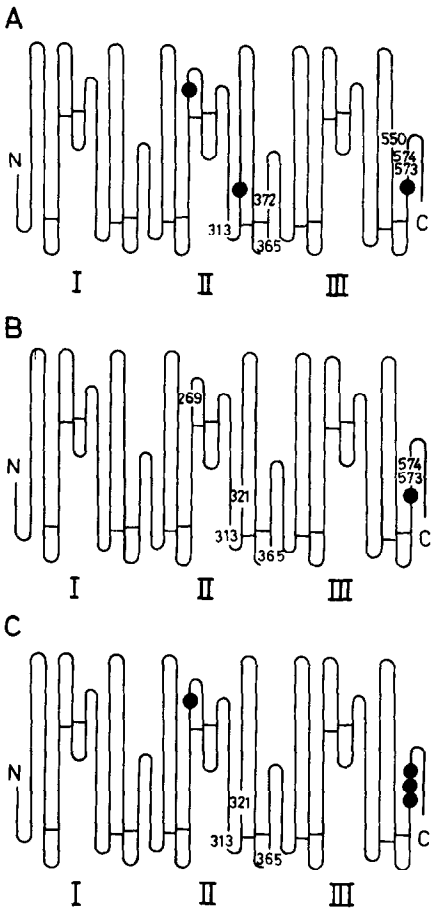

Fig. 2. The polypeptide chain and the disulfide bonds (small horizontal bars) of Alb A are shown according to the model of Brown [13]. The roman numerals indicate the three domains of the protein, and N and C denote the N-terminal and the C-terminal ends of albumin, respectively. The effects of different single point mutations on high-affinity binding of warfarin (A), salicylate (B) and diazepam (C) are shown in a qualitative way, namely by giving the number of the position involved in cases of reduced ligand binding, and a closed circle when the mutation does not affect binding. The effects of chain termination variations on drug binding are discussed in the text.

1–387) and domains II and III (residues 198–585), and were produced by peptic and tryptic cleavage, respectively. On the basis of results obtained with circular dichroism, equilibrium dialysis and stopped-flow fluorescence measurements the authors concluded that the primary warfarin site is placed in domain II. This proposal could imply that the residues in positions 313, 365 and 372 are relatively close to the binding site, whereas the amino acids in positions 550, 573 and 574 are far away from the site. The magnitude of the diminishing effects of the different mutations on binding seems to support such a proposal, because the amino acid substitutions in positions 313 and 365 reduce the association constant for warfarin to about 20% of that determined for Alb A [14], whereas the substitutions in positions

573 and 574 only reduce the constant to about 70% of the normal value (Table 2). Furthermore, it should be noted that binding of warfarin is unaffected by a replacement of glutamic acid with lysine in position 570 (Fig. 2A).

Where in domain II then is warfarin bound with a high affinity? More information is still needed before this question can be answered in detail. However, it is of interest to note that drug binding to the variants Alb Niigata (269 Asp → Gly) and Alb Roma (321 Glu → Lys) is normal. Placing the high-affinity binding site for warfarin in domain II also implies that the reducing effects of the mutations in positions 550, 573 and 574 on ligand binding are most probably indirect.

The high-affinity binding of salicylate is even more affected than warfarin binding by the mutations, since six of the seven amino acid substitutions studied so far are associated with diminished drug binding (Fig. 2B). As was the case with warfarin, the magnitude of the reductions in binding are dependent on the position of the mutation. In general, the mutations placed in domain II all reduce the binding constant to a greater extent than the two mutations placed in domain III. In addition, it is noted that salicylate binding to Alb Verona (570 Glu → Lys) is normal [14]. Therefore, the results obtained with genetic variants of albumin seem to be in agreement with a proposal previously put forward stating that the high-affinity binding sites for warfarin and salicylate are placed quite close to each other in a common binding region in the albumin molecule [21].

In contrast to the high-affinity binding of warfarin and salicylate, the binding of diazepam is only affected by relatively few mutations, all of which are placed in region 313–365 (Fig. 2C). This finding probably implies that the diazepam site is composed of amino acid residues placed rather close to each other in the amino acid sequence of albumin and/or the site is less susceptible to conformational changes in the protein. Bos *et al.* [22] have tried to locate the high-affinity binding site for diazepam in Alb A by investigating the interaction between the drug and the same large peptic and tryptic fragments as in the study on warfarin binding. By using circular dichroism and equilibrium dialysis the authors found that at least the main part of the primary site is located in domain III. Thus, an assignment of the binding site to the N-terminal part of domain III or to that section of the protein plus the C-terminal part of domain II seems to agree very well with both the binding results of the fragmentation studies and the results obtained with the genetic variants.

In summary, the different high-affinity binding sites for warfarin and salicylate seem to be placed close to each other in domain II of the albumin molecule. The sites are rather sensitive to conformational changes in the protein, because binding to them can be reduced by amino acid substitutions taking place in domain III far from the tentative placing of the two binding sites. This finding also shows that domains II and III do not act as independent entities in the albumin molecule. The fact that the chain termination variants, especially Alb Venezia, bind warfarin and salicylate less firmly than Alb A (Table 2) is a further indication of important interactions between the two domains. Diazepam binds to a separate high-affinity site which seems to be placed closer to, or within, domain III. Although this binding site appears to be less sensitive to conformational changes in the albumin molecule, diazepam binding is also diminished by the pronounced molecular changes which have taken place in Alb Venezia. The reduced association constants of this variant for ligands bound primarily to sites in domains II and III far from the C-terminal end suggest that this region plays an important role in the binding of serum albumin. A possible explanation for this finding could be that the hydrophobic tailpiece, which in Alb Venezia is substituted by a shorter hydrophilic segment [7] of a disordered conformation [12], is necessary for a tight folding of the C-terminal region [7]. The latter conformation seems also to be important for the stability of the molecule, as Alb Venezia accounts for only 30% of the total albumin content in the heterozygous subjects carrying this albumin variant [7].

Clinical aspects

Can the presence *in vivo* of the variants studied in this work have an impact on the pharmacokinetics of warfarin, salicylate or diazepam? In an attempt to shed light on this question the effect on the free drug concentrations was calculated after diminishing the association constants to 70%, as an example. When doing so, it was assumed that the molar ratio between total drug and albumin in plasma is 1:2, and that the association constants determined for binding to endogenous Alb A under the present conditions (Table 2) also apply to the physiological concentration of the protein (approximately 600 μM [2, 3]). The calculations revealed that the free fraction of the drugs increased by as much as 40–42%. Therefore, although this effect is probably smaller in heterozygote subjects, several of the present ligand–variant combinations could result in adverse drug reactions.

In a review, Tárnoky [23] gave the incidence of heterozygote carriers of albumin variants as 1:1000–1:10,000 in Europe and in populations of European descent. However, more recently Wagner [24] has examined blood samples from about 38,000 individuals for alloalbumins by isoelectric focussing and he found a frequency of 1:400. Perhaps introduction of still more sensitive electrophoretic techniques (e.g. hybrid isoelectric focussing [24]) will reveal that isoforms of albumin are even more common than reported by Wagner. Thus, bisalbuminemia could have a greater clinical and pharmacological importance than hitherto believed.

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