High-affinity binding of laurate to naturally occurring mutants of human serum albumin and proalbumin

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Binding of laurate (n-dodecanoate) to genetic variants of albumin or its proprotein and to normal albumin isolated from the same heterozygous carriers was studied by a kinetic dialysis technique at physiological pH. The first stoichiometric association constant for binding to proalbumin Lille (Arg⁻² → His) and albumin (Alb) Roma (Glu³²¹ → Lys) was increased to 126 % and 136 % respectively compared with that for binding to normal albumin, whereas the constant for Alb Maku (Lys⁵⁴¹ → Glu) was decreased to 80 %. In contrast, normal laurate-binding properties were found for as many as nine other albumin variants with single amino acid substitutions. Because the net charges of all these mutants were different from that of normal albumin, the results suggest that the examples of modified laurate binding are not caused by long-range electrostatic effects. Rather, the three positions mentioned are located close to different binding sites

for the fatty acid anion. The most pronounced effect was observed for the glycosylated Alb Casebrook, the binding constant of which was decreased to 20 %. Binding to the glycosylated Alb Redhill was also decreased, but to a smaller extent (68 %). These decreases in binding are caused by partial or total blocking of the high-affinity site by the oligosaccharides, by the negative charges of the oligosaccharides, and/or by conformational changes induced by these bulky moieties. Laurate binding to two chain-termination mutants (Alb Catania and Alb Venezia) was normal, indicating that the C-terminus of albumin is not important for binding. By using different preparations of normal albumin as controls in the binding experiments, it was also possible to compare the effect of various methods for isolation and defatting on laurate binding.

INTRODUCTION

Human serum albumin (denoted here by the prefix Alb) consists of a single polypeptide chain and is synthesized in and exported from the liver as a non-glycosylated protein. Albumin is quantitatively the most important transport and depot protein in the circulation. This circumstance is caused by two factors: first, its high concentration in most fluids (the concentration in plasma is approx. 0.6 mM [1], and in the interstitial fluids it varies between 0.2 and 0.5 mM [2]); secondly, albumin is able to bind a great number of chemically diverse endogenous and exogenous ligands reversibly [1,3,4]. However, the binding properties of albumin for a particular ligand can be influenced by a number of factors, e.g. by simultaneous binding of other ligands or by chemical modification of the protein. In the present work we have studied the effect of genetically determined structural changes on the ability of albumin to bind non-esterified fatty acids. As a representative of these physiologically important ligands, we have chosen the medium-chain fatty acid laurate (n-dodecanoate), the binding of which has previously been studied in detail [5], and references therein).

So far more than 50 structurally different genetic variants of human serum albumin or proalbumin (denoted by the prefix proAlb) have been identified [1,6]. These variants can be divided into three categories, namely single amino acid substitutions, glycosylated proteins and chain-termination mutants. Examples

of all these types of mutation were represented in the 14 different albumin and proalbumin variants used in the present investigation of high-affinity laurate binding. Ligand binding was quantified by using radioactively labelled laurate in a kinetic dialysis technique [5]. As well as being of physiological importance, the results presented here help to pinpoint specific binding sites, because all the variants used had been previously characterized by protein and/or DNA sequencing.

MATERIALS AND METHODS

Chemicals

Four different types of human serum albumin preparation were used in this study. (1 and 2) The genetic variants and their normal (wild-type) counterpart, endogenous Alb A, were isolated from serum from heterozygous carriers by ion-exchange chromatography. For details of the isolation procedures and about the structural characterization of the variants, see the references listed in Table 1. After isolation, the albumins were checked by electrophoresis; no denaturation or significant (no more than 5 %) cross-contamination was detected. The proteins were desalted, freeze-dried and then transported to Aarhus, Denmark, where they were delipidated by treatment with a hydroxy-alkoxypropyl dextran (Sigma Chemical Co., St. Louis, MO, U.S.A.) in dilute H₂SO₄, pH 3.0, at 2–3 °C [7]. After defatting,

the proteins were dialysed extensively against deionized water, freeze-dried and stored at -20°C until use. In the binding studies, the variants and their corresponding Alb A were always examined in the same experiment. Thus the albumins from the same donor were treated in exactly the same way, from the time that the blood samples were taken until the binding experiments were performed. (3) Fraction V albumin (96–99% pure) was obtained from Sigma (A-1653) and delipidated by using the dextran derivative. (4) Essentially fatty acid-free albumin (at least 96% pure) was also a product of Sigma (A-1887), and it had been delipidated commercially by the charcoal method of Chen [8].

Lauric acid (more than 99.5% pure) was from Fluka Chemie AG (Buchs, Switzerland) and $[1-^{14}\text{C}]$ lauric acid (approx. 50 Ci/mol) was from Amersham International (Little Chalfont, Bucks., U.K.). The amount of radiochemical impurities not bound to albumin was determined as previously described [9] and was found to be approx. 0.1% of the total radioactivity. Because the dialysis exchange method is relatively insensitive to radiochemical impurities [9], corrections for the small amounts of impurities were considered unnecessary. Aqueous solutions of laurate, with and without radioactively labelled laurate, were prepared as described before [10].

Other chemicals such as acids, bases and salts were also of the best grades commercially available. Throughout the study, only doubly deionized water was used.

Dialysis exchange of laurate

Two types of experiment were performed, and in both cases the medium used was 66 mM sodium phosphate, pH 7.4. In the first type, solutions of 25 μM fatty acid anion, with or without $[^{14}\text{C}]$ laurate included, were made in buffer alone and kept at 25°C . At time zero, 1.0 ml samples with and without radioactively labelled ligand were pipetted into the left and right compartments respectively of the Teflon dialysis cells. The dialysis equipment, Dianorm-GD-1, was from Diachema AG (Munich, Germany), and each cell had a volume of 1.36 ml on each side of the cellulose membrane (Diachema type 10.14, molecular-mass cut-off 5.0 kDa). After closure, the apparatus was placed in a temperature-controlled water bath at 25°C and the cells were rotated around their horizontal axis at 12 rev./min. After 10, 20, 30 or 40 min the half-cells were emptied and 800 μl portions from the left and right compartments were used for liquid-scintillation counting in a Packard Tri-Carb liquid-scintillation spectrometer.

In the second type of experiment, solutions of different concentrations of laurate (5.2–25.0 μM) were prepared with or without $[^{14}\text{C}]$ laurate included. However, these solutions also contained a constant quantity (approx. 30 μM) of albumin, its concentration determined by dissolving a weighed amount of the freeze-dried protein in the desired volume of buffer and ligand. At time zero, 1.0 ml samples with and without radioactively labelled fatty acid anion were pipetted into the left and right compartments respectively of the dialysis cells. These cells were allowed to rotate in the water bath for a known time of approx. 19 h. After the half-cells had been emptied, aliquots of 800 μl were taken for liquid-scintillation counting.

As the laurate solutions were made in NaOH, the pH of the final solutions was checked at room temperature with a combination electrode, type GK 2401 C, from Radiometer Danmark A/S (Copenhagen, Denmark). To prevent bacterial growth, all solutions contained 10 mg/l gentamicin sulphate. Control experiments revealed that the antibiotic did not interfere with laurate binding. In all experiments, each dialysis cell was used to define a single data point.

Data analysis

In all cases, the time of dialysis was insufficient to establish equilibrium with respect to radioactively labelled laurate. Therefore it is possible to calculate, from the experiments performed without protein, a rate constant, k , for net transfer of unbound laurate through the dialysis membrane from the following relationship [5]:

$$\ln \left[\frac{Q_l - Q_r}{Q_l + Q_r} \right] = -kt \quad (1)$$

The terms Q_l and Q_r stand for radioactivity in the samples taken from the left and right compartments respectively after dialysis for t min. Now, knowing k for unbound ligand, it is possible to determine the concentration of unbound fatty acid anion, c , in the albumin-containing solutions [5]:

$$\ln \left[\frac{Q_l - Q_r}{Q_l + Q_r} \right] = -kt \frac{c}{C} \quad (2)$$

In eqn. (2) C denotes the known concentration of total laurate. Performing experiments with various concentrations of total ligand give connected values of c and r ; the latter parameter is the number of moles of ligand bound per mole of protein. Except for the glycosylated variants (69.5 kDa), the molecular masses of the albumins were set equal to 67 kDa. Applying the following equation to the different sets of binding data, r and c , allows a calculation of stoichiometric association constants for laurate binding, i.e. K_1 , K_2 , ..., K_N :

$$r = \frac{K_1 c + 2K_1 K_2 c^2 + \dots + NK_1 K_2 \dots K_N c^N}{1 + K_1 c + K_1 K_2 c^2 + \dots + K_1 K_2 \dots K_N c^N} \quad (3)$$

The term N represents the maximum number of laurate molecules that can be bound per albumin molecule.

An estimate of the first stoichiometric constant, K_1 , can be obtained by a simple graphical approach. Dividing eqn. (3) by c leads to:

$$\frac{r}{c} = \frac{K_1 + 2K_1 K_2 c + \dots + NK_1 K_2 \dots K_N c^{N-1}}{1 + K_1 c + K_1 K_2 c^2 + \dots + K_1 K_2 \dots K_N c^N} \quad (4)$$

From eqn. (4) it is seen that if $c \rightarrow 0$ (or $r \rightarrow 0$) then $r/c \rightarrow K_1$ [5]. In practice, the experimental data are plotted on a Scatchard plot, r/c against r , and K_1 is obtained by extrapolation of r to 0 (see Figure 1).

Calculations of rate constants, k , and binding constants, K_1 , were performed by an iterative procedure with the Sigma Plot software from Jandel Scientific, which enables linear regression with confidence intervals.

RESULTS

The genetic variants

Table 1 summarizes the molecular characteristics of the 14 genetic variants of albumin and proalbumin used in this work. For the sake of comparison, the Table also includes the variants Alb Niigata and Alb Redhill, because their laurate-binding properties were investigated at an earlier stage of this study [25].

Proalbumin is an albumin molecule to which the propeptide, Arg-Gly-Val-Phe-Arg-Arg, is still bound at the N-terminus.

Table 1 Molecular characterization and laurate-binding properties of genetic variants of human serum albumin and proalbumin

The positions of proalbumin are from —6 to —1 (the juxtaposition to albumin itself), and those of albumin are from 1 to 585. The parameter K_1 is the first stoichiometric binding constant.

Variant name	Reference	Molecular change	$10^6 K_1$ (M^{-1})	
			Variant	Alb A
Lille	[11]	Arg ⁻² → His	16.1	12.8
Tradate-2	[6]	Lys ²²⁵ → Asn	7.5	6.6
Herborn	[12]	Lys ²⁴⁰ → Glu	10.9	9.8
Niigata*	[13]	Asp ²⁶⁹ → Gly	9.5	9.0
Caserta	[6]	Lys ²⁷⁶ → Asn	10.8	11.2
Tagliacozzo	[14]	Lys ³¹³ → Asn	12.9	13.1
Roma	[15]	Glu ³²¹ → Lys	12.5	9.2
Sondrio	[16]	Glu ³³³ → Lys	13.9	13.6
Kashmir	[17]	Glu ⁵⁰¹ → Lys	12.2	12.6
Ortonovo	[18]	Glu ⁵⁰⁵ → Lys	13.2	12.1
Maku	[19]	Lys ⁵⁴¹ → Glu	9.6	11.9
Milano Fast	[20]	Lys ⁵⁷³ → Glu	10.1	11.2
Redhill*	[21]	Arg ⁻¹ retained, Ala ³²⁰ → Thr, glycosylated at Asn ³¹⁸	6.4	9.4
Casebrook	[22,23]	Asp ⁴⁹⁴ → Asn, glycosylated at Asn ⁴⁹⁴	2.5	12.4
Catania	[24]	583–585 deleted, 580–582 substituted	9.2	9.8
Venezia	[24]	579–585 deleted, 572–578 substituted	10.2	9.5

* Results from [25], in which another type of equilibrium dialysis cell was used.

Normally this protein does not occur in detectable amounts in the circulation because the propeptide is cleaved off within the liver cells. However, replacement of Arg⁻² (as in proAlb Lille) or Arg⁻¹ prevents the proteolytic cleavage of the propeptide but not the secretion of the protein, in which case proalbumin variants, in contrast with wild-type proalbumin, can be isolated from the serum. The sequence of the propeptide shows that even though Arg⁻² has been replaced by His, the net charge of proAlb Lille is less negative than that of Alb A at physiological pH.

Most albumin variants are single amino acid substitutions. The non-glycosylated forms of these have net charges that differ from that of Alb A as follows: Alb Roma, Alb Sondrio, Alb Kashmir and Alb Ortonovo are all +2 variants, Alb Niigata is a +1 variant, Alb Tradate-2, Alb Caserta and Alb Tagliacozzo are —1 variants, and Alb Herborn, Alb Maku and Alb Milano Fast are —2 variants. Thus all types of changes in net charge are represented in this work. All of the variants are from two of the three domains [4] of albumin; those with mutations in the region 225–333 are domain II variants, and those with mutations in the region 501–573 are domain III variants. Only a few albumins have been found with mutations in domain I [6], and because they also are difficult to get in sufficient amounts it was not possible to include any of them in this study.

It is uncommon for an amino acid substitution to result in the formation of an oligosaccharide attachment sequence as happened in both Alb Redhill and Alb Casebrook. The structure of the oligosaccharide bound to Alb Casebrook has been determined [23], whereas that of Alb Redhill is still largely unknown, but both carbohydrates are negatively charged owing to the presence of sialic acid [21–23]. Alb Redhill is unique because it is the only example so far known of an albumin variant with two mutations. One is Ala³²⁰ → Thr, which leads to glycosylation of Asn³¹⁸; the other is Arg⁻² → Cys, which results in abnormal hydrolysis of the prepropeptide and thereby to the formation of albumin still possessing an Arg residue at position —1 [21].

Figure 1 Binding of laurate to Alb Casebrook (○) and to different control preparations of Alb A

The preparations of normal protein were commercially charcoal-defatted albumin (Δ), commercial albumin defatted by the dextran method (∇) and the corresponding endogenous albumin defatted by the dextran derivative ($\square$). The linear regression lines were generated by the method of least squares, and the intercepts with the ordinate give K_1 . The symbols r and c represent the moles of ligand bound per mole of albumin and the concentration of unbound ligand respectively. The concentration of total laurate varied from 5.2 to 25.0 μM ; the protein concentration was kept constant at 30 μM . The medium was 66 mM sodium phosphate, pH 7.4, and the reaction temperature was 25 °C. The results are from a single experiment.

Finally, Table 1 includes two chain-termination mutants, Alb Catania and Alb Venezia. Alb Catania is three residues shorter than Alb A, and the three last residues at the new C-terminus are changed from Gln-Ala-Ala to Lys-Leu-Pro. The reason for these molecular changes is a deletion of nucleotide C-15985 followed by a frame shift in the structural albumin gene [24]. The molecular changes of Alb Venezia are even more pronounced. The variant has been shortened by seven residues, and the new C-terminus is changed from Gly-Lys-Lys-Leu-Val-Ala-Ala to Pro-Thr-Met-Arg-Ile-Arg-Glu. These modifications are caused by a skipping of exon 14 in the Alb Venezia mRNA, followed by partial proteolytic degradation of the translation product by serum carboxypeptidase B [24].

Laurate binding to the variants and endogenous Alb A

Fatty acid binding was quantified by the dialysis exchange method [5] because it is better suited for the determination of very low concentrations of unbound ligand, and because it is less sensitive to any unbound radiochemical impurities than, for example, equilibrium dialysis. The binding data obtained were analysed with a Scatchard plot. From the illustrative examples shown in Figure 1, it is apparent that the results can be well described by linear regression lines, and the intercepts of these lines with the ordinate give K_1 [see eqn. (4)]. It is also apparent from Figure 1 that all r values are less than 1. This was done in an attempt to focus on the high-affinity binding of laurate. The experiments with a particular variant and its corresponding endogenous Alb A were performed as many times as the material allowed, i.e. from one to four times.

From Table 1 it is apparent that most of the natural mutations

did not greatly affect laurate binding. For nine of the eleven single amino acid substitutions, K_1 for the variant was 90–114 % of that calculated for the corresponding endogenous Alb A. In addition, and rather surprisingly, the extensive modifications that have taken place in the two chain-termination variants did not affect the affinity for laurate. Thus K_1 for Alb Catania and Alb Venezia were 94 % and 107 % respectively of the K_1 values determined for their corresponding Alb A.

In five cases, K_1 for the variant was changed by 20 % or more. Thus the binding constant for proAlb Lille was increased to 126 %. Furthermore two of the single amino acid substitutions resulted in pronounced, but inversely directed, effects on fatty acid binding: K_1 for Alb Roma was increased to 136 %, whereas that for Alb Maku was decreased to 80 %. However, the most pronounced effects were observed with the two glycosylated variants. As illustrated in Figure 1, laurate binding to Alb Casebrook was much lower than binding to Alb A, and K_1 was diminished to only 20 % (see Table 1). Previously, similar binding experiments performed with smaller dialysis cells had shown that K_1 for Alb Redhill is reduced to 68 % [25].

Laurate binding to Alb A

If the results reported for Alb Niigata and Alb Redhill are not taken into account, then the results given in Table 1 are based on a total of 28 experiments. All of these experiments included the same two controls, namely laurate binding to commercially charcoal-defatted albumin and laurate binding to commercial albumin defatted by the dextran method. Although these two commercial preparations have been isolated from pooled plasma, both are proposed as being Alb A. The results showed that the affinity for laurate of the former preparation was always higher than that of the latter (see Figure 1); the average K_1 values ($\pm$ S.D.) were $(20.7 \pm 2.7) \times 10^6 \text{ M}^{-1}$ and $(13.1 \pm 1.7) \times 10^6 \text{ M}^{-1}$ respectively. Charcoal-defatted albumin also binds diazepam with a higher affinity than the dextran-defatted protein. In contrast, the two types of albumin preparation bind warfarin, Ca^{2+} and Ni^{2+} equally well [7]. The differences in the binding properties of laurate and diazepam are not caused by a different delipidation efficiency of the two procedures but are due to differences in protein conformation after delipidation [7].

On the basis of the present information it is possible to make another comparison, that is laurate binding to two different albumin preparations both defatted by the dextran derivative. One is commercial albumin isolated from pooled plasma by precipitation with cold alcohol; the other is albumin isolated from individual serum samples by ion-exchange chromatography. The association constant for fatty acid binding to the former is, as mentioned above, $(13.1 \pm 1.7) \times 10^6 \text{ M}^{-1}$, and that for the latter preparation is $(11.1 \pm 1.7) \times 10^6 \text{ M}^{-1}$. Thus the laurate-binding properties of albumin are not influenced by the differences in these two isolation procedures.

DISCUSSION

The laurate-binding properties of 14 structurally different genetic variants of human serum albumin and proalbumin were studied by a kinetic dialysis technique. In most cases the variants bound the fatty acid anion to the same extent, within experimental error, as normal endogenous albumin isolated from the same heterozygous carriers. However, in four examples the natural mutations implied modified high-affinity binding of the ligand. These four mutants were proAlb Lille, the single-amino-acid-substituted Alb Roma and Alb Maku and the glycosylated

variant Alb Casebrook. In addition, earlier studies have revealed that another glycosylated variant, Alb Redhill, also binds laurate with an altered affinity [25].

Detailed thermodynamic examinations have shown that binding of the first three or four molecules of laurate is greatly influenced by electrostatic attraction between the carboxy group of the ligand and positively charged groups of the albumin molecule [5,10]. The present examples of modified binding are in full accordance with this finding. Thus proAlb Lille and Alb Roma have net charges at physiological pH that are more positive than Alb A, and they both bind laurate with an increased affinity (K_1 is increased to 126–136 %). Furthermore Alb Maku, Alb Redhill and Alb Casebrook are more negatively charged than Alb A, and these variants bind the fatty acid anion with a lower affinity than does the normal protein (K_1 is decreased to 20–80 %).

With the exception of the mutation $\text{Ala}^{320} \rightarrow \text{Thr}$ in Alb Redhill, the substitutions of all the genetic variants studied are located on the surface of the albumin molecule and are exposed to the solvent [4]. Therefore the finding of normal laurate binding to as many as nine of the variants with a single amino acid substitution (Figure 2) indicates that the overall net charge of albumin is not itself a decisive factor for binding of this ligand. This finding also implies that the examples of modified binding should not be explained by such a crude mechanism.

The positions of the three single amino acid substitutions affecting laurate binding are located far from each other in the albumin molecule. This is seen in Figure 2, which shows the polypeptide chain as proposed by Brown and Shockley [27]. Consulting Figure 7 of Carter and Ho [4] reveals that the positions are also widely separated in the three-dimensional structure of crystalline albumin. Despite these remote positions, the possibility exists that the three mutations could modify, through long-range electrostatic effects or conformational changes, the binding properties of the same laurate-binding location. However, these possibilities are also rendered improbable by the many examples of variants with normal laurate-binding properties. Rather, the three single amino acid substitutions affecting binding are placed at or close to different laurate-binding locations of albumin. Such a proposal is especially supported by the finding of modified binding to albumin substituted in position 321 but normal binding to the albumins modified in position 313 or 333 (Table 1).

It is generally assumed that medium-chain fatty acid anions bind with a high affinity to domain III (Figure 2) of normal albumin [1,3,4,29]. Therefore the finding that a replacement of a positively charged Lys residue in position 541 by a negatively charged Glu (Alb Maku) results in a decreased affinity for laurate indicates this residue to be important for high-affinity binding of the ligand. However, because the decrease in binding is relatively small, it is probable that, although Lys^{541} is important for binding, it is not a crucial element in forming the high-affinity binding site. The increased binding to proAlb Lille and Alb Roma indicates that laurate binding can also take place in such a way that residues close to the N-terminus and close to position 321 respectively are of relevance (Figure 2). If it is assumed that laurate binding to Alb A takes place with high affinity in domain III, then the two other binding regions mentioned represent sites of a lower affinity. However, the presence of the modified propeptide and the mutation of Glu^{321} respectively improve the binding capabilities of these regions to such an extent that the sites now contribute much more to the total binding, or have perhaps even become high-affinity binding sites.

The proposal of the existence of a low-affinity binding site for laurate at the N-terminus of normal albumin seems to be in

Figure 2 Locations of single amino acid substitutions in albumin and proalbumin variants

The Figure shows the amino acid sequences of human serum albumin (residues 1–585) and proalbumin (residues –6 to –1). The sequences are shown in the one-letter code and are based on the genomic sequence analysis of Minghetti et al. [26]. The layout of the polypeptide chain and the arrangement of the 17 disulphide bonds (small horizontal bars) are those of Brown and Shockley [27]. The division of albumin into domains is in accordance with Figure 1 in Carter and Ho [4]: I, residues 1–199; II, 200–391; III, 392–585. The three open squares and nine filled circles represent positions of amino acid substitutions that affected and did not affect respectively laurate binding. The two X's denote the positions of glycosylation in Alb Redhill (position 318) and Alb Casebrook (position 494). The modifications of the two chain-termination variants, Alb Catania and Alb Venezia, are given in the text. The bold circles indicate the locations of single amino acid substitutions in genetic variants not included in this work. The illustration is a modified version of Figure 1 of Madison et al. [28].

accordance with the ^1H -NMR spectroscopic studies of Oida [29]. This author observed that low-affinity binding, in contrast with high-affinity binding, of laurate and other medium- and long-chain fatty acid anions resulted in changes in C2-chemical shifts of mobile histidine residues in human serum albumin. These histidine residues could be His³ and/or His⁹ (Figure 2).

Cistola et al. [30] have studied the binding of ^{13}C -enriched saturated fatty acid anions to BSA by ^{13}C -NMR spectroscopy. They proposed the existence of three high-affinity binding sites for laurate and other fatty acid anions of a longer chain length and suggested that the positively charged triplets Arg¹⁴³-Arg¹⁴⁴-His¹⁴⁵, Arg³³⁴-Arg³³⁵-His³³⁶ and Lys⁵³²-His⁵³³-Lys⁵³⁴ are essential components of these three sites. The latter tripeptide is identical with the segment 534–536 of the human protein [1,4] and forms the C-terminus of helix h8(III) [4]. In comparison, Lys⁵⁴¹, which is modified in Alb Maku, is located in the short loop connecting the helices h8(III) and h9(III) [4]. Placing Lys⁵⁴¹ in the vicinity of the high-affinity site for laurate binding is therefore in accordance with the putative location proposed by Cistola et al. [30]. The second triplet, identical with segment 336–338 in Alb A [1,4], is associated with the C-terminus of helix h8(II); Arg³³⁴ (and Arg³³⁶) and Arg³³⁵ (and Arg³³⁷) are within the helix, and His³³⁶ (and His³³⁸) is placed just outside the helix [4]. Glu³³³ is located in the C-terminal half of the same helix, but an exchange of that residue for a Lys (Alb Sondrio) did not affect laurate binding (Table 1). Thus the present piece of information does not point to helix h8(II) as an important element in forming a (high-affinity) binding site for laurate. The first triplet, identical with 144–146 in Alb A [1,4], is located with two residues in the C-terminal part of helix h8(I) and one residue adjacent to the helix [4]. At present no comments can be made on the proposal of Cistola et al. [30], because no variants with substitutions in domain I were available for this study.

The most pronounced effects on laurate binding were observed in the case of the glycosylated variants Alb Redhill and Alb

Casebrook (Table 1). The strongly decreased binding to Alb Casebrook could be explained by the oligosaccharide's being attached to the protein in such a position that it can, partly or totally, block the proposed high-affinity binding site in domain III. This proposal seems to be in accordance with the finding of normal binding of laurate to the two chain-termination variants Alb Catania and Alb Venezia, which indicates that the primary laurate site is associated with the middle and/or the N-terminal part of domain III (see below). Such a location of the site could also explain the diminished binding to Alb Redhill, because in that case the oligosaccharide, even though it is anchored to an amino acid residue in domain II, could approach the high-affinity binding site for laurate. The decreased ligand binding could also be caused by simple electrostatic repulsion between the protein-bound oligosaccharide and the fatty acid anion. Structural analyses have shown that the carbohydrate bound to Alb Casebrook is an $\alpha(2,6)$ -disialylated complex biantennary glycan [23]. The structure of the carbohydrate attached to Alb Redhill is different [22], but has not yet been determined in detail. However, it has a size similar to that bound to Alb Casebrook (2.5 kDa) and also contains two sialic acid residues [21]. Finally, conformational changes of the two proteins, induced by the presence of the oligosaccharides, could contribute to the decreases in binding. That conformational changes have indeed taken place in the variants, compared with endogenous Alb A, has been rendered probable by electrophoretic examinations [21,22].

The molecular characteristics of the two chain-termination variants differ in several ways from that of Alb A. First, the net charge of Alb Catania and Alb Venezia are +1 and –1 respectively, compared with the net charge of Alb A at physiological pH. Secondly, because the polypeptide chains have been shortened by three (Alb Catania) and seven residues (Alb Venezia), and because both variants have a proline residue in their new C-terminal, the last helix [h10(III)] [4] is more or less abolished in these proteins. Thirdly, the hydrophilicity profiles of

the C-terminal ends of the variants are very different from that of Alb A [31]. Therefore, taking into account the fact that laurate is assumed to bind to domain III with high affinity [1,3,4,29], it is surprising that both Alb Catania and Alb Venezia bind laurate with normal affinity. The explanation for these findings could be that the laurate-binding site does not involve amino acid residues very close to the C-terminus of albumin. This proposal is in agreement with the finding of unaltered binding properties of Alb Milano Fast, which is modified at position 573. Alternatively, or in addition, the presence of intact disulphide bridges in this region of the albumin molecule (Figure 2) has a high stabilizing effect on the domain.

From Figure 1 it is seen that all r values are less than 1 and that the different sets of binding data can be well described by linear regression lines. The experimental protocol leading to this type of situation was chosen in an attempt to focus on high-affinity binding alone. However, even under these conditions it is likely that the albumin solutions are not mixtures of two components only, namely protein molecules without ligand and protein molecules to which one molecule of ligand is bound. Rather, the solutions are more complex mixtures composed of ligand-free albumin and albumin molecules binding one, two or perhaps more ligand molecules [32]. The exact composition is dependent on the binding constants used in the theoretical calculations. However, at the present low r values, albumin molecules binding zero, one or two ligand molecules are expected to be the dominant species. How could such a heterogeneity in binding influence the interpretation of the results? If a one-to-one combination of albumin and laurate is the most abundant complex, the interpretation of the results given in Table 1 should follow the lines given above. Simultaneous binding of two ligand molecules could take place close to each other. For this type of binding, the molecular discussion presented will also be valid, but it would deal with locations binding two laurate molecules instead of one. Finally, the possibility exists that a major part of albumin binds two laurate molecules at separate sites. Because the effect of the single amino acid substitutions seems to be of a limited extent on the albumin molecule, the latter combination most probably represents a situation in which the binding of one ligand molecule could be affected and the binding of the other molecule is unaffected. In this example, a modified binding would be qualitatively the same as that observed for the overall binding, but the quantitative effects of the mutations would be underestimated. In conclusion, even though different albumin-laurate combinations probably exist in our media, at least the qualitative interpretation of the experimental findings given above should be valid.

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