Characterization of the binding sites for dicarboxylic acids on bovine serum albumin

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Dicarboxylic acids are prominent features of several diseases, including Reye's syndrome and inborn errors of mitochondrial and peroxisomal fatty acid oxidation. Moreover, dicarboxylic acids are potentially toxic to cellular processes. Previous studies [Tonsgard, Mendelson & Meredith (1988) *J. Clin. Invest.* **82**, 1567–1573] demonstrated that long-chain dicarboxylic acids have a single high-affinity binding site and between one and three lower-affinity sites on albumin. Medium-chain-length dicarboxylic acids have a single low-affinity site. We further characterized dicarboxylic acid binding to albumin in order to understand the potential effects of drugs and other ligands on dicarboxylic acid binding and toxicity. Progesterone and oleate competitively inhibit octadecanedioic acid binding to the single high-affinity site. Octanoate inhibits binding to the low-affinity sites. Dansylated probes for subdomain 2AB inhibit dodecanedioic acid binding whereas probes for subdomain 3AB do not. In contrast, low concentrations of octadecanedioic acid inhibit the binding of dansylated probes to subdomain 3AB and 2AB. L-Tryptophan, which binds in subdomain 3AB, inhibits hexadecanedioic acid binding but has no effect on dodecanedioic acid. Bilirubin and acetylsalicylic acid, which bind in subdomain 2AB, inhibit the binding of medium-chain and long-chain dicarboxylic acids. Our results suggest that long-chain dicarboxylic acids bind in subdomains 2C, 3AB and 2AB. The single low-affinity binding site for medium-chain dicarboxylic acids is in subdomain 2AB. These studies suggest that dicarboxylic acids are likely to be unbound in disease states and may be potentially toxic.

INTRODUCTION

Dicarboxylic acids (DCAs) are formed after the ω -oxidation of monocarboxylic acids by a cytochrome *P*-450-coupled enzyme pathway (see review by Mortensen, 1984). Although the formation of DCAs was described more than 50 years ago (Verkade *et al.*, 1933), their role in mammalian fatty acid metabolism is poorly understood. Only 5–10% of circulating non-esterified fatty acids in ketotic rats are metabolized through this pathway (Björkhem, 1978). However, DCAs have recently been recognized to be a prominent feature of several diseases in humans (Tonsgard, 1986; Rocchiccioli *et al.*, 1986; Vianey-Liand *et al.*, 1987). In Reye's syndrome as much as 55% of the circulating non-esterified fatty acids are DCAs. In this illness 85–90% of the DCAs are long chain, of length C_{14} – C_{18} (Tonsgard, 1986). These long-chain DCAs are potentially toxic to mitochondrial function (Mortensen & Gregersen, 1982; Passi *et al.*, 1984; Tonsgard & Getz, 1985; Kimura, 1986). Since alterations in mitochondrial structure and function are prominent features of both Reye's syndrome and Zellweger syndrome (Goldfischer *et al.*, 1973; DeVivo, 1978; Kelley, 1983), understanding the regulation of DCA metabolism may be important in these as well as other diseases where DCAs accumulate.

The binding of monocarboxylic fatty acids to albumin and the intracellular fatty acid-binding proteins modulates the transport and metabolism of these fatty acids. Moreover, binding of a potentially toxic ligand to albumin protects against the toxic effects of the ligand, since toxicity is proportional to the concentration of unbound ligand (Spector, 1975). Our initial observations (Tonsgard & Getz, 1985) indicated that long-chain DCAs in patient serum exert toxic effects on isolated mitochondria even in the presence of defatted albumin, suggesting that DCAs are largely unbound in patients. In order to test this

hypothesis, we examined the affinity and capacity of albumin for DCAs. We demonstrated that albumin has a single higher-affinity site for long-chain DCAs of K_d 1–2 μ M and between one and three lower-affinity binding sites of K_d 20–60 μ M, depending on the chain length of the DCA. Low concentrations of oleic acid displace long-chain DCAs from the higher-affinity albumin binding site; DCA is displaced from the lower-affinity sites only when the oleate/albumin ratio approaches 3:1 (Tonsgard *et al.*, 1988). We undertook the present study to characterize further the DCA-binding sites on the albumin molecule in order to understand the potential effects of drugs and other ligands on DCA binding and toxicity.

EXPERIMENTAL

Monocarboxylic and dicarboxylic acids

Unlabelled monocarboxylic acids and DCAs were from Sigma Chemical Co. (St. Louis, MO, U.S.A.), Applied Science (Warrenville, IL, U.S.A.), Foxboro Analabs (North Haven, CT, U.S.A.) and Ultra Scientific (North Kingston, RI, U.S.A.). The purity of the unlabelled acids was assessed by g.l.c. (Tonsgard, 1986) and was found to be at least 99.5%. 3 H-labelled dicarboxylic acids and [14 C]octanoic acid were from Amersham (Arlington Heights, IL, U.S.A.). The 3 H-labelled dicarboxylic acids were prepared by Amersham by reaction with tritiated water at a very high specific radioactivity. The chemical purity of the labelled compounds was assessed by g.l.c. and found to be at least 97%. The radiopurity of the labelled compounds was assessed by t.l.c. (Tonsgard *et al.*, 1988) and was at least 93.4%.

Potassium salts of a mixture of labelled and unlabelled monocarboxylic acid or DCA were made as described previously (Tonsgard *et al.*, 1988).

Abbreviations used: DCA, dicarboxylic acid.

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Albumin

Essentially fatty acid-free (less than 0.005 %, w/w) crystalline BSA was from Sigma Chemical Co. and further purified as described by Spector *et al.* (1969). The monocarboxylic fatty acid content of the purified albumin was determined by g.l.c. as described previously (Tonsgard *et al.*, 1988) and found to be less than 0.02 mol of fatty acid/mol of albumin.

Modification of albumin

Purified defatted albumin (0.2 mM) was acetylated with 1 mM-acetylsalicylic acid in 0.10 M-sodium phosphate buffer at pH 6.8 and 7.6 as described by Hawkins *et al.* (1969) at 37 °C. The albumin solution was then dialysed for 48 h at 4 °C against multiple changes of 0.15 M-NaCl containing 0.01 M-sodium salicylate and then against 0.1 M-sodium phosphate buffer, pH 7.4. The albumin was then freeze-dried and stored at 4 °C. In other experiments, albumin was acetylated with acetic anhydride at pH 8.0 as described by Fraenkel-Conrat (1957). Acylation of amino groups was assessed by the ninhydrin test (Fraenkel-Conrat, 1957). Thiol-group-modified BSA was purchased from ICN Biochemicals (Costa Mesa, CA, U.S.A.). The thiol groups were modified by reaction with L-cysteine (Miles Laboratories, Kankakee, IL, U.S.A.). The degree of thiol-group modification was determined to be at least 95 % as performed by the manufacturer.

Equilibrium dialysis

Binding of DCAs to defatted BSA was determined by using equilibrium dialysis as described previously (Tonsgard *et al.*, 1988). Binding was assessed with various concentrations of DCA (0.05–1.5 mM) in a near-physiological salt solution containing 116 mM-NaCl, 4.9 mM-KCl and 16 mM-sodium phosphate buffer, pH 7.4. DCA was added to one side of the chamber and defatted albumin in the same salt solution was added to the other side to a final concentration of 0.05 mM. Because octadecanedioic acid is less soluble than the other dicarboxylic acids, binding of this acid was assessed with 0.01 mM-albumin and 0.010–0.200 mM-octadecanedioic acid. Experiments were performed in triplicate. The recovery of radioactivity was calculated to be between 88 % and 101 %. The data shown are the results of between two and five separate experiments.

Competition experiments

The binding sites for DCAs were identified by using equilibrium dialysis and competition with ligands having well-characterized albumin binding sites as described below.

Progesterone was purchased from Steraloids (Wilton, NH, U.S.A.) and [4-¹⁴C]progesterone from Amersham. A mixture of labelled and unlabelled progesterone was dissolved in ethanol and diluted with buffer. The final ethanol concentration in the experiments was 0.25 %. The chemical purity and radiopurity of the progesterone was assessed by t.l.c. on silica-gel plates developed in dichloromethane/acetone (4:1, v/v) and found to be 99.0 % and 98.3 %. Preliminary equilibrium-dialysis experiments with 100 µM-progesterone incubated with 5 µM-albumin for various times demonstrated that progesterone equilibrates within 28 h. The amount of bound progesterone was determined from the distribution of ¹⁴C radioactivity (c.p.m.) between the two compartments of the dialysis chamber. The recovery of progesterone was 75–80 %. Some progesterone was found to adhere to the sides of the dialysis chambers, as has been previously noted (Moll & Rosenfield, 1977). Although the amount of bound progesterone must be regarded as an approximation, equilibrium dialysis is the preferred technique for assessment of progesterone binding (Westphal, 1969).

Competition experiments with octanoic acid were performed as described previously (Tonsgard *et al.*, 1988).

Bilirubin was obtained from ICN Biomedicals (Costa Mesa, CA, U.S.A.). Bilirubin was dissolved in dimethyl sulphoxide (4 mg/ml) and stored at –20 °C. On the day of the experiment the concentration of the bilirubin stock solution was checked spectrophotometrically at 455 nm (Tietz, 1976). The bilirubin stock solution was then dissolved in 0.1 M-Na₂CO₃ containing 60 µM-albumin. The bilirubin solution was then further diluted to a bilirubin concentration of 100 µM and defatted albumin was added (final concentration 50 µM) (Tietz, 1976). The final dimethyl sulphoxide concentration in the experiments was 0.5 %. Bound and unbound bilirubin were separated by the use of 1 ml Sephadex G-25 spin columns described previously (Tonsgard *et al.*, 1988). Bilirubin was measured spectrophotometrically at 455 nm. The recovery of bilirubin at this concentration was 96 %. With a 50 µM-bilirubin and -albumin concentration, the molar ratio of bilirubin bound to albumin was 0.72:1.

L-[methylene-¹⁴C]Tryptophan was purchased from Amersham and unlabelled L-tryptophan was purchased from Sigma Chemical Co. A mixture of labelled and unlabelled L-tryptophan was dissolved in phosphate buffer. In experiments with 50 µM-albumin the final tryptophan concentration in the dialysis chamber was 0.5 mM, and in experiments with 5 µM-albumin the final concentration was 0.1 mM. Binding of L-tryptophan was assessed from the partitioning of ¹⁴C radioactivity (c.p.m.) as described previously (Tonsgard *et al.*, 1988).

Acetylsalicylic acid was purchased from Sigma Chemical Co. and [carboxy-¹⁴C]acetylsalicylic acid from New England Nuclear (Boston, MA, U.S.A.). A mixture of labelled and unlabelled acetylsalicylic acid was dissolved in ethanol and diluted with dialysis buffer. The final concentration of ethanol in the experiments was 0.6 %. The acetylsalicylic acid solution was assessed by t.l.c. on silica-gel G plates with hexane/acetic acid/chloroform (17:3:2, by vol.). The chemical purity and radiopurity were found to be 99 % and 98.8 % respectively. Competition experiments between acetylsalicylic acid and DCAs for binding to albumin were performed with the use of two different approaches. Albumin was preincubated with acetylsalicylic acid as described above at various pH values. Then the albumin was dialysed and freeze-dried (Hawkins *et al.*, 1968). [³H]DCA binding was assessed by equilibrium dialysis with the albumin preincubated with acetylsalicylic acid. Alternatively a mixture of unlabelled and ¹⁴C-labelled acetylsalicylic acid in phosphate buffer was added to the side of the equilibrium-dialysis chamber containing albumin, and [³H]DCA was added to the other side of the chamber (Tonsgard *et al.*, 1988). Preliminary experiments demonstrated that acetylsalicylic acid equilibrates within 24 h. The amount of bound acetylsalicylic acid was determined from the distribution of ¹⁴C radioactivity (c.p.m.) between the two compartments of the dialysis chamber. The recovery of acetylsalicylic acid was between 86 % and 101 %.

Dansyl-amino acids bind in either subdomain 2AB or 3AB (see Fig. 1). Dansylglycine, dansylsarcosine, dansylproline and dansylglutamine were purchased from Sigma Chemical Co. With the exception of dansylglycine, the probes are believed to have a single binding site (Sudlow *et al.*, 1975). The probes were dissolved in warm methanol and buffer. The final concentration of methanol in the experiments was 0.1 %. A molar ratio of fluorescent probe to albumin less than 1:1 was used to ensure that most of the probe was bound, as described by Sudlow *et al.* (1976). In experiments with dansylglycine the ratio of probe to albumin was 1:20, as described by Sudlow *et al.* (1976), in order to focus on the principal dansylglycine-binding site. Control experiments in which various concentrations of DCA were incubated with dansyl probes in the absence of albumin demonstrated that the

dansylated compounds only fluoresce when bound to albumin. Displacement of the probe was determined by comparing the fluorescence of the probe in the presence of albumin and in the absence of competitor to the fluorescence in the presence of albumin and various concentrations of DCA measured on a Perkin-Elmer LS-5B luminescence spectrometer with the excitation and emission wavelengths suggested by Sudlow *et al.* (1975, 1976). However, in some instances addition of DCA to solutions containing BSA and probe initially increased fluorescence. Therefore, in order to assess the binding of dansyl-amino acid and displacement by DCA, we also directly measured the displacement of dansylated amino acids by using equilibrium dialysis with ^3H -labelled dansyl-amino acids and unlabelled DCAs. ^3H -labelled dansyl-amino acids were synthesized as described by Oray *et al.* (1983) from L-[2,3,4,5- ^3H]proline and L-[G- ^3H]glutamine (Amersham) and dansyl chloride (Sigma Chemical Co.). The reaction products were identified and separated by h.p.l.c. after comparison with authentic standards. The concentrations of ^3H -labelled dansyl-amino acids were determined spectrophotometrically by using the molar absorption coefficient $\epsilon 4.5 \times 10^{-3} \text{ M}^{-1} \cdot \text{cm}^{-1}$.

Analysis of binding

Binding isotherms were analysed by the damping Gauss-Newton algorithm with the use of a non-linear least-squares-fit computer program (Tongsgard *et al.*, 1988). The points in the Figures are experimental data and the lines are the best fit of the data to the theoretical equations. Dissociation constants are expressed as the means $\pm$ s.d..

The binding of DCAs to albumin was analysed by using the equation of reversible saturable binding to one, two or three classes of non-interacting equivalent sites, i.e. the equation of a Langmuir isotherm. For the case of one class of sites

$$K_d = (FA_r S_t) / C_x = [FA_r (S_t - FA_b)] / FA_b$$

where K_d is the dissociation constant (micromolar), FA_r is the concentration of free DCA (micromolar), FA_b is the concentration of bound DCA (micromolar), C_x is the concentration of DCA-site complex (micromolar) and S_t and S_b are the concentrations of free and total binding sites respectively. The molar ratio of bound DCA to albumin can be calculated from S_t and the albumin concentration and is an estimate of the number of DCA-binding sites (Tongsgard *et al.*, 1988). We used the Akaike information coefficient to ascertain whether the best fit of the data could be obtained by assuming one, two or three classes of binding site (Yamaoka *et al.*, 1981).

RESULTS

Higher-affinity long-chain dicarboxylic acid-binding site

Previous investigations demonstrated that albumin has at least two distinct classes of binding sites for DCAs (Tongsgard *et al.*, 1988). Long-chain DCAs have a single higher-affinity binding site and compete for one of the three subdomains that are the primary binding sites of long-chain monocarboxylic acids, subdomains 1C, 2C and 3C (Fig. 1) (Brown & Shockley, 1982). We examined the effect of competition with the steroid progesterone in order to localize further the higher-affinity DCA-binding site. Progesterone binds to Trp-214 in subdomain 2C (Brown & Shockley, 1982; Swaney & Klotz, 1970). In the presence of $100 \mu\text{M}$ -[$1\text{-}^{14}\text{C}$]progesterone (Fig. 2) the apparent K_d for octadecanedioic acid is higher at $13.9 \pm 3.0 \mu\text{M}$ (compared with $1.9 \pm 1.1 \mu\text{M}$ in the absence of competition), but the predicted number of binding sites for octadecanedioic acid is unchanged,

i.e. 4.4 ± 0.2 and 4.4 ± 0.8 mol/mol of albumin in the absence and in the presence of progesterone respectively. Fig. 2 also shows the expected desorption of progesterone, suggesting that progesterone and octadecanedioic acid bind in subdomain 2C.

Localization of the dodecanedioic acid-binding site

Medium-chain-length DCAs have a single lower-affinity binding site (Tongsgard *et al.*, 1988). [$1\text{-}^{14}\text{C}$]Octanoic acid ($450 \mu\text{M}$)

Fig. 1. Model of the albumin molecule

The drawing of the albumin molecule is taken from the model proposed by Brown & Shockley (1982).

Fig. 2. Competition of progesterone with octadecanedioic acid binding to albumin

Binding of [^3H]octadecanedioic acid was assessed in the presence of $5 \mu\text{M}$ -albumin incubated with ($\circ$) and without ($\triangle$) $100 \mu\text{M}$ -[$1\text{-}^{14}\text{C}$]progesterone. $\square$, Bound progesterone.

Table 1. Effect of ligands on dodecanedioic acid binding to albuminResults are means $\pm$ S.D.

Competing ligand	Dissociation constant (μ M)	Bound DCA (mol/mol of BSA)	No. of data points
None	87.4 $\pm$ 6.6	1.1 $\pm$ 0.01	20
Octanoate (450 μ M)	170.2 $\pm$ 50.9	0.2 $\pm$ 0.03	20
Bilirubin (100 μ M)	7.8 $\pm$ 17.4	0.2 $\pm$ 0.04	15
Tryptophan (500 μ M)	96.3 $\pm$ 17.4	1.0 $\pm$ 0.08	18
Thiol-group-modified BSA	120.4 $\pm$ 19.4	1.2 $\pm$ 0.04	20
Acetylsalicylic acid (600 μ M), pH 7.6	325.2 $\pm$ 68.0	1.0 $\pm$ 0.03	25

Fig. 3. Effect of dodecanedioic acid on the binding of fluorescent probes to albumin

The inhibition of binding of fluorescent probes to albumin was assessed in the presence of various concentrations of dodecanedioic acid spectrophotometrically. The concentration of albumin was 50 μ M and the concentration of dansyl-amino acid was 2.5 μ M. Dansylsarcosine ($\square$) binds in subdomain 3AB and dansylglycine ($\circ$) binds in subdomain 2AB.

inhibits dodecanedioic acid binding. When a single molecule of octanoic acid is bound to the BSA molecule, the binding of dodecanedioic acid is essentially eliminated (Table 1). This confirms previous studies (Tonsgard *et al.*, 1988) that suggested

Fig. 4. Competition of octanoic acid with hexadecanedioic acid binding to albumin

Binding of [3 H]hexadecanedioic acid was assessed in the presence of 50 μ M-albumin with ($\circ$) and without (Δ) 450 μ M-[1- 14 C]octanoic acid.

that the low-affinity binding site for medium-chain DCAs is in the subdomains that bind medium-chain-length monocarboxylic acids (1AB, 2AB and 3AB; Fig. 1).

We also examined the effect of bilirubin and tryptophan, which bind in subdomains 2AB and 3AB respectively (Brown & Shockley, 1982), on dodecanedioic acid binding. When albumin is incubated with 100 μ M-bilirubin and various concentrations of dodecanedioic acid, 0.72 mol of bilirubin/mol of albumin is bound and 0.8 mol of dodecanedioic acid/mol of albumin is displaced (Table 1). In contrast, L-tryptophan, which binds in subdomain 3AB (Brown & Shockley, 1982), has no effect on dodecanedioic acid binding (Table 1).

The drug-binding sites of albumin in subdomains 2AB and 3AB have been characterized by their affinity for different dansylated amino acids (Sudlow *et al.*, 1975, 1976). Dodecanedioic acid inhibits dansylglycine binding in subdomain 2AB but has little effect on dansylsarcosine binding in subdomain 3AB (Fig. 3). These studies support the results of the competition studies with bilirubin and tryptophan, indicating that dodecanedioic acid binds in subdomain 2AB.

Localization of the lower-affinity long-chain dicarboxylic acid-binding sites

Previous studies (Tonsgard *et al.*, 1988) suggested that the medium-chain monocarboxylic acid-binding domains, 1AB, 2AB and 3AB (Fig. 1) also bind long-chain DCAs with K_d values for DCAs in the region of 20 μ M. Hexadecanedioic acid and octa-

Table 2. Effect of different ligands on the binding of long-chain dicarboxylic acidsResults are means $\pm$ S.D.

DCA	Competing ligand	Dissociation constant (μ M)		Bound DCA (mol/mol of BSA)	No. of data points
		K_{d1}	K_{d2}		
Hexadecanedioic acid	None	2.5 $\pm$ 1.4	27.8 $\pm$ 6.1	4.4 $\pm$ 0.3	35
	Octanoic acid	0.9 $\pm$ 0.7	34.5 $\pm$ 3.3	3.3 $\pm$ 0.1	38
	Acetylsalicylic acid	2.3 $\pm$ 1.1	70.2 $\pm$ 3.0	4.0 $\pm$ 0.1	24
	L-Tryptophan	1.9 $\pm$ 0.3	454.5 $\pm$ 25.3	3.8 $\pm$ 0.3	22
Octadecanedioic acid	None	1.9 $\pm$ 1.7	18.4 $\pm$ 15.3	4.6 $\pm$ 1.1	30
	Thiol-group-modified BSA		1.4 $\pm$ 0.3	4.7 $\pm$ 0.9	23

Fig. 5. Effect of octadecanedioic acid on the binding of dansyl-amino acids to albumin

The binding of 2.5 μM ^3H -labelled dansyl-amino acids was assessed by equilibrium dialysis using 5 μM -albumin and various concentrations of octadecanedioic acid. The Figure indicates the concentration of bound dansylglutamine ($\circ$), which binds in subdomain 2AB, and the concentration of bound dansylproline ($\square$), which binds in subdomain 3AB.

Fig. 6. Competition between L-[methylene- ^{14}C]tryptophan and ^3H hexadecanedioic acid binding to albumin

Binding of various concentrations of hexadecanedioic acid to 50 μM -albumin was assessed by equilibrium dialysis in the presence ($\circ$) and in the absence ($\square$) of 700 μM -L-tryptophan. The experimental data were found to fit best to an equation for two classes of binding sites, in which one of the binding sites showed co-operative binding. The equation used was:

$$FA_b = \frac{(S_1)(FA_t)^n}{K_{d1} + FA_t^n} + \frac{(S_2)(FA_t)}{K_{d2} + FA_t}$$

where n is the degree of co-operativity of the first binding sites and the dissociation constants are K_{d1} and K_{d2} . Other terms were defined previously. Non-linear least-squares analysis of the data yields $n = 1.8$.

decanedioic acid have between three and four of these lower-affinity binding sites (Table 2). When albumin binds a single molecule of octanoic acid, the observed saturation of hexadecanedioic acid is decreased from 4.4 ± 0.3 to 3.3 ± 0.1 mol/mol of albumin (Table 2). Octanoic acid has little effect on the K_d (Table 2). Octanoic acid inhibits hexadecanedioic acid binding after the molar ratio of hexadecanedioic acid to albumin exceeds

Fig. 7. Effect of aspirin (acetylsalicylic acid) on dodecanedioic acid binding to albumin

Binding of ^3H dodecanedioic acid was assessed in the presence of 50 μM -albumin preincubated with ($\triangle$) and without ($\circ$) 600 μM -acetylsalicylic acid.

1:1, i.e. 50 μM (Fig. 4). This suggests that octanoic acid and the secondary DCA-binding sites are in the same subdomains.

In order to localize further the lower-affinity long-chain DCA-binding sites we examined the effect of competition with ligands for subdomains 2AB and 3AB. Octadecanedioic acid inhibits binding of dansylglutamine and dansylproline to subdomains 2AB and 3AB respectively (Fig. 5). Displacement of dansylproline (3AB) is apparent at low concentrations of octadecanedioic acid. However, as shown in Fig. 5, octadecanedioic acid does not begin to displace dansylglutamine (2AB) until the ratio of octadecanedioic acid to albumin approaches 3:1 (i.e. 15 μM and 5 μM respectively). Double-label experiments with L-[^{14}C]tryptophan (which binds to subdomain 3AB) and ^3H hexadecanedioic acid demonstrate that L-tryptophan does not interfere with binding of the first molecule of hexadecanedioic acid (Fig. 6), which we have assigned to subdomain 2C. However, L-tryptophan does shift the K_d for binding the remaining DCA (Table 2). We also found co-operativity between two of these lower-affinity binding sites (Fig. 6). Taken together, the experiments with dansylglutamine, dansylproline and L-tryptophan indicate that long-chain DCA binds in subdomains 2AB and 3AB. Moreover, the more pronounced effect of long-chain DCA on the binding of dansylproline (3AB) and the apparent shift in K_d of long-chain DCA that occurs with L-tryptophan suggests that one or two molecules of long-chain DCA are preferentially bound in subdomain 3AB and that long-chain DCA is only bound in subdomain 2AB when other sites are saturated.

Effect of acetylsalicylic acid on dicarboxylic acid binding

We examined the effect of acetylsalicylic acid on DCA binding (Fig. 7 and Table 1) because acetylsalicylic acid has a well-characterized binding site in subdomain 2AB of human serum albumin (Walker, 1976) and because use of acetylsalicylic acid predisposes children to Reye's syndrome (Starko *et al.*, 1980; Pinsky *et al.*, 1988). At pH 7.4, acetylsalicylic acid acetylates several peptides, including Lys-199 in subdomain 2AB in human serum albumin (Walker, 1976; Hawkins *et al.*, 1969). At acidic pH, the degree of acetylation is reduced but both acetylsalicylic acid and salicylic acid reversibly bind to subdomain 2AB (Hawkins *et al.*, 1968; Pinckard *et al.*, 1970).

The binding of dodecanedioic acid to BSA is markedly inhibited by acetylsalicylic acid (Fig. 7 and Table 1); the K_d for

albumin increases almost 4-fold, but the predicted saturation is not significantly affected. We found little difference in the competition between acetylsalicylic acid and dodecanedioic acid within the pH range 6.8–7.6. The degree of acylation of potential amino groups on BSA after incubation with acetylsalicylic acid was between 32 % and 43 % in this pH range, as measured by the ninhydrin test. In addition, acetylsalicylic acid inhibits the binding of hexadecanedioic acid (Table 2).

Effect of acylation or thiol-group modification of albumin

We also examined the binding of medium-chain and long-chain DCA binding to thiol-group-modified BSA and to BSA that was acylated with acetic anhydride. When 97.2 % of the available sites of albumin are acetylated, DCAs do not bind to albumin. In contrast, modification of 95 % of the thiol groups of albumin does not affect dodecanedioic acid binding and increases the affinity of octadecanedioic acid for albumin.

DISCUSSION

We proposed previously (Tonsgard *et al.*, 1988) that the single higher-affinity DCA-binding site on albumin is in a subdomain that is a primary binding site for long-chain monocarboxylic acids and that low-affinity DCA-binding sites are in subdomains that bind medium-chain monocarboxylic acids and drugs (Brown & Shockley, 1982). The current studies support those hypotheses. Progesterone, which binds in subdomain 2C (Swaney & Klotz, 1970), competitively inhibits the binding of octadecanedioic acid. This finding, in conjunction with previous competition studies with oleic acid, suggests that the higher-affinity long-chain DCA-binding site is in subdomain 2C. Specific probes for subdomains 1C and 3C are not available to confirm this localization. Octanoic acid displaces dodecanedioic acid and hexadecanedioic acid. Displacement of DCA is not complete because octanoic acid binding is spread over three potential binding sites (1AB, 2AB and 3AB). Dodecanedioic acid and octadecanedioic acid also inhibit the binding of fluorescent probes to the drug-binding sites (2AB and 3AB).

Localization of the binding sites reveals differences in the binding of medium-chain and long-chain DCAs. The competition studies with long-chain DCAs and fluorescent probes (Fig. 5) demonstrate that at low DCA/albumin molar ratios long-chain DCA displaces the probe from subdomain 3AB but does not affect the binding of dansylglutamine to subdomain 2AB. Dansylglutamine is only displaced when albumin is almost saturated with DCA. In contrast, competition studies with medium-chain DCAs (Fig. 3) demonstrate that dodecanedioic acid displaces dansyl-amino acid from subdomain 2AB but has little effect on the binding of the probe to subdomain 3AB. The minor effects on binding of dansylsarcosine to subdomain 3AB may be due to conformation changes induced by the binding of dodecanedioic acid and do not suggest significant binding of the DCA in that subdomain. From these studies we conclude that long-chain DCAs bind first to the high-affinity site in subdomain 2C, and then to subdomain 3AB. As albumin becomes saturated with long-chain DCAs, an additional molecule of long-chain DCA is bound in subdomain 2AB. Medium-chain-length DCAs, however, bind exclusively to subdomain 2AB. No specific ligands for subdomain 1AB are available. Therefore we cannot exclude the possibility that long-chain DCAs are bound in subdomain 1AB. However, the competition experiments with L-tryptophan (subdomain 3AB) indicate that L-tryptophan competes with the binding of two molecules of hexadecanedioic acid. Since in the absence of competing ligands albumin only binds four molecules

of hexadecanedioic acid, the competition experiments with L-tryptophan taken together with the other competition studies suggest that the binding sites for hexadecanedioic acid are in subdomains 2C, 3AB and 2AB. The possibility of more than one molecule of ligand binding to a subdomain is supported by our finding of co-operativity in the binding of hexadecanedioic acid in competition with tryptophan and by previous work by Berde *et al.* (1979), who proposed that albumin bound two molecules of *cis*-parinaric acid in anti-parallel fashion in a single subdomain. Peters (1985) suggests that domain 3 may be able to unfold slightly in order to accommodate more than one ligand.

The competition between DCAs and acetylsalicylic acid tends to confirm the localization of the DCA-binding sites. Acetylsalicylic acid acylates Lys-199 in subdomain 2AB in human serum albumin and to a lesser extent other lysine residues (Hawkins *et al.*, 1969; Walker, 1976). In addition, acetylsalicylic acid and salicylic acid bind reversibly in subdomain 2AB of human serum albumin (Pinckard *et al.*, 1970). BSA does not have a lysine at amino acid position 199, although there are several lysine residues within loop 4 of subdomain 2AB, including Lys-220, which is included in the binding site for bilirubin.

The differences in the binding of long-chain and medium-chain DCA are not due to differences in the *pK* values since at physiological pH, DCAs are more than 99 % deprotonated (Weast & Astle, 1981). It is more likely that the differences in the binding of medium-chain and long-chain DCA are due to the hydrophobic properties of the long-chain molecules: as the chain length increases, hydrophobicity increases and binding increases. The experiments with acetylated albumin modification suggest that electrostatic interactions may also be important in binding DCAs.

Our observations indicate that steroids, bilirubin, monocarboxylic acids and drugs either compete against or inhibit DCA binding. These other ligands are present in patients with Reye's syndrome (Mamunes *et al.*, 1974; Pollack *et al.*, 1975; Starko *et al.*, 1980). Exposure to aspirin occurs in as many as 92 % of patients with Reye's syndrome (Pinsky *et al.*, 1988). The inhibition of the binding of medium-chain-length DCA to albumin by acetylsalicylic acid therefore provides one explanation for the prominent medium-chain dicarboxylic aciduria in Reye's syndrome. Moreover, the ratio of the concentration of serum monocarboxylic acids to the serum concentration of albumin in Reye's syndrome approaches and in some cases exceeds 3:1 (Pollack *et al.*, 1975). Reye's-syndrome sera contain elevated concentrations of both long-chain and medium-chain monocarboxylic acids (Mamunes *et al.*, 1974) in addition to concentrations of DCAs as high as 0.4 mM (Tonsgard, 1986). Since monocarboxylic acids and DCAs compete for binding and monocarboxylic acids have an affinity for albumin that is one to two orders of magnitude greater than that of DCAs (Spector, 1975), it is likely that a significant fraction of DCAs is unbound in patients with Reye's syndrome. This is of potential importance since DCAs, particularly long-chain DCAs, have toxic effects on mitochondrial structure and function (Passi *et al.*, 1984; Tonsgard & Getz, 1985; Kimura, 1986). Alterations in mitochondrial structure and function are characteristic, if not central, features of the illnesses in which the accumulation of long-chain DCAs occurs, namely Reye's syndrome and diseases of peroxisomal metabolism. Therefore it is possible that unbound DCAs could contribute to the pathogenesis of these illnesses.

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