The fatty acid analogue 11-(dansylamino)undecanoic acid is a fluorescent probe for the bilirubin-binding sites of albumin and not for the high-affinity fatty acid-binding sites

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1. The fluorescent fatty acid probe 11-(dansylamino)undecanoic acid (DAUDA) binds with high affinity to bovine and human serum albumin (BSA and HSA) at three sites. 2. The K_d of the primary binding site could not be determined; however, the two secondary sites appeared to be equivalent, with an apparent K_d of 8×10^{-7} M for both BSA and HSA. 3. The spectral characteristics of DAUDA when bound to the primary site of the two albumins were different, with HSA producing a greater fluorescence enhancement and emission maximum at a shorter wavelength (480 nm) than for BSA (495 nm). 4. Displacement studies indicated that the DAUDA-binding sites were not equivalent to the primary long-chain fatty acid-binding sites on albumin, but corresponded to the bilirubin sites. Fatty acyl-CoAs also bind to the bilirubin sites, as do medium-chain fatty acids. 5. The solubility, stability and spectral properties of DAUDA make it an excellent probe for investigating the bilirubin-binding sites of albumin, particularly HSA.

INTRODUCTION

The fluorescent fatty acid probe 11-(dansylamino)undecanoic acid (DAUDA) has been shown to bind with high affinity to rat liver fatty acid-binding protein (FABP), and this binding is accompanied by a 60-fold fluorescent enhancement and a 50 nm blue-shift in emission-wavelength maximum (Wilkinson & Wilton, 1986), consistent with the probe being located in a nonpolar environment within the protein. The properties of this probe make it particularly suitable for detecting, quantifying and evaluating the binding properties of rat liver FABP (Wilkinson & Wilton, 1986, 1987a,b; Sheridan *et al.*, 1987; Wilton, 1989), to which it binds with 1:1 molar stoichiometry (Wilkinson & Wilton, 1987b).

Serum albumin is the best-known protein that binds long-chain fatty acids, and is remarkable in terms of both the number of binding sites and the variety of different nonpolar ligands that are transported in addition to fatty acids (Brown & Shockley, 1982). These additional ligands include the physiologically relevant compounds lysophosphatidylcholine and bilirubin. In this paper I have investigated the binding of DAUDA to serum albumin, and I have demonstrated that this fatty acid analogue does not bind to the high-affinity fatty acid-binding sites of albumin, but binds instead to the separate bilirubin-binding sites on the protein.

The stability, solubility and spectral characteristics of DAUDA make it a particularly effective probe for investigating the primary high-affinity bilirubin-binding site on human serum albumin (HSA). This site is of importance in preventing high levels of free bilirubin in the blood, which in the newborn can cause bilirubin encephalopathy and associated neurological disorders. There is therefore clinical interest in understanding the binding of bilirubin to albumin and the effects of drugs and other competitive ligands on this binding.

EXPERIMENTAL

Materials

Essentially fatty acid-free BSA and HSA were obtained from Sigma as A0281 and A3782 respectively. DAUDA was obtained from Molecular Probes, Junction City, OR, U.S.A., or synthesized by treating dansyl chloride with 11-aminoundecanoic acid.

Assay procedure

All assays were performed in 0.1 M-potassium phosphate buffer, pH 7.4, at 25 °C by using a Perkin-Elmer LS3B fluorescence spectrometer. Binding and displacement assays were performed at an excitation wavelength of 350 nm and an emission wavelength of 500 nm. All spectra are uncorrected. DAUDA and oleic acid (1 mM) were added in methanol, and the final methanol concentration in the assay was kept below 2% (v/v). Bilirubin (1 mM) was prepared in 0.2 M-NH₃.

RESULTS AND DISCUSSION

Spectral properties of DAUDA when bound to BSA or HSA

When a molar excess of either BSA or HSA was titrated into a fixed concentration (1 μ M) of DAUDA, a limiting value of fluorescence enhancement was reached when all the DAUDA became bound to the protein. The fluorescence emission spectra for DAUDA bound to either BSA or HSA are shown in Fig. 1. In both cases there is a considerable fluorescence enhancement and a blue-shift of the fluorescence emission maximum compared with the spectral properties of the probe in buffer. This is indicative of the fatty acid probe binding at a non-polar site on the protein. The spectral change was more dramatically shown with HSA, where the emission maximum of the bound probe was

Fig. 1. Emission spectra of DAUDA when bound to BSA or HSA

To DAUDA ($1 \mu\text{M}$) in 0.1 M-potassium phosphate buffer, pH 7.4, was added either (a) BSA or (b) HSA at a concentration of $7 \mu\text{M}$, and the fluorescence emission spectrum was recorded. The spectrum of DAUDA in buffer was also recorded (c). Excitation was at 350 nm.

at 480 nm, compared with 495 nm for BSA, indicating that there are significant differences in the non-polar binding pocket for this probe between the two proteins. For DAUDA binding to HSA, a fluorescence enhancement at 480 nm of nearly 100-fold was observed.

As a polarity guide, the fluorescence spectrum of DAUDA ($1 \mu\text{M}$) was determined in several organic solvents, and the emission maximum was determined (shown in parentheses). The solvents were ethanol (505 nm), butan-1-ol (500 nm), chloroform (485 nm), ethyl acetate (480 nm), diethyl ether (470 nm) and cyclohexane (455 nm).

Stoichiometry of binding of DAUDA to BSA and HSA

Fixed concentrations of HSA and BSA were titrated with DAUDA, and the resulting binding curves are presented as Scatchard plots (Fig. 2). In order to quantify the amount of bound ligand, the fluorescence intensity of a known concentration of bound DAUDA was determined by adding excess albumin. For the calculations of the Scatchard plot it had to be assumed that all binding sites for DAUDA showed similar fluorescence-enhancement properties. A similar assumption had to be made when investigating the interaction of other fluorescent fatty acid probes with albumin (Sklar *et al.*, 1977; Berde *et al.*, 1979a).

Fig. 2. Scatchard analysis of the binding of DAUDA to BSA and HSA

To the albumin solution ($1.4 \mu\text{M}$) were added samples ($1\text{--}5 \mu\text{l}$) of 0.75 mM -DAUDA, and the fluorescence was recorded. The proportions of bound and free probe were calculated, and the data were subjected to Scatchard analysis. It was assumed that the maximal fluorescence achieved in the presence of excess albumin represents 100% binding and that the amount of DAUDA bound is proportional to the relative intensity. The plots for BSA (●) and HSA (■) are shown: $\bar{v}$, number of mol of DAUDA bound per mol of albumin; L , concn. of free ligand.

The Scatchard plots (Fig. 2) highlight the characteristics of binding of DAUDA to the albumins. Both BSA and HSA showed an apparent binding stoichiometry of 3, with the second and third sites having apparently very similar affinities, giving K_d values of approx. $8 \times 10^{-7} \text{ M}$. The first high-affinity site could not be accurately quantified, but a K_d value below 10^{-7} M was indicated.

Jacobsen (1969) quantified the binding of bilirubin to HSA, and identified one high-affinity binding site (K_d about 10^{-8} M) and two similar sites of lower affinity (K_d about 10^{-6} M). The above results highlight the similarity between the binding of DAUDA and bilirubin to albumin.

Although the primary binding site for bilirubin is now well established, it is less clear from the literature as to whether there are one or two secondary binding sites for bilirubin. In this context it may be relevant that, after rapid saturation of albumin with DAUDA, a stirred solution showed a partial loss of fluorescence over several minutes that could be explained by slow ligand-induced conformational changes resulting in the possible loss of one DAUDA-binding site (D. C. Wilton, unpublished work).

Displacement of albumin-bound DAUDA by long-chain fatty acids and bilirubin

In order to gain information as to the precise nature of the DAUDA-binding site on albumin, the ability of different types of ligand to displace albumin-bound DAUDA was investigated. In order to carry out this investigation, sufficient DAUDA was added to BSA and HSA to saturate the primary binding site.

The results, which are shown in Fig. 3, clearly demonstrated that in fact DAUDA was binding to the primary bilirubin-binding site and not to the high-affinity fatty acid-binding sites of albumin. Thus when the albumin-bound DAUDA was titrated with bilirubin there was an immediate loss of fluorescence, due

Fig. 3. Displacement of DAUDA bound to the primary high-affinity binding site of albumin by other ligands

DAUDA (1.5 nmol) was added to albumin (1.4 nmol) and the fluorescence was recorded (100%). Up to 10 μ l of a 1 mM solution of ligand was added, and the fluorescence was recorded. The percentage of original fluorescence was calculated for (a) BSA and (b) HSA. The ligands used were: ●, oleic acid; ▼, bilirubin; ■, oleoyl-CoA; ▲, octanoic acid.

to displacement of bound DAUDA, and nearly 50% displacement was achieved at a bilirubin concentration equal to that of the DAUDA, indicating that the two ligands bind with similar affinity to the bilirubin-binding site. However, the data cannot exclude the theoretical possibility that the bilirubin and DAUDA bind to separate but interacting sites on the protein.

However, when oleic acid was used to displace bound DAUDA, there was minimal displacement initially until about 3 mol of oleic acid had been added per mol of albumin, after which point DAUDA displacement occurred. This result indicates that the oleate was binding first to the three high-affinity fatty acid-binding sites on albumin (Brown & Shockley, 1982; Cistola *et al.*, 1987a), and only after these three sites were saturated did DAUDA displacement occur. Moreover, the bilirubin results strongly suggest that the additional oleic acid is binding to the bilirubin-binding sites.

The displacement studies with HSA (Fig. 3) show an identical phenomenon, except that there appears to be a smaller difference in the affinities of the primary and secondary binding sites for long-chain fatty acids, because significant displacement of DAUDA by oleic acid occurs before saturation of the primary fatty acid-binding sites.

The capacity of the medium-chain fatty acid octanoic acid to cause immediate displacement (Fig. 3) is consistent with the concept that medium-chain fatty acids bind preferentially at a separate site from the high-affinity long-chain fatty acid-binding sites, and that this site may be equivalent to the primary bilirubin-binding site. It is apparent from the displacement curves that octanoic acid binds with lower affinity than bilirubin.

The fluorescent probe 5-(dimethylamino)naphthalene-1-sulphonic acid is the parent fluorophore of DAUDA. This probe has been shown to bind to BSA and HSA at two binding sites, with K_d values of 2×10^{-7} M and 3×10^{-6} M. The probe was most readily displaced by medium-chain fatty acids, but was not significantly displaced by long-chain fatty acids at molar ratios of fatty acid to albumin of up to 3 (Doody *et al.*, 1982). Hence this probe shows similar characteristics to DAUDA, but binds to albumin with slightly lower affinity.

The results with DAUDA parallel previous work on bilirubin

binding to albumin, where it was observed that albumin-bound bilirubin was only displaced by long-chain fatty acids after the addition of 2 mol of fatty acid per mol of protein (Woolley & Hunter, 1970; Berde *et al.*, 1979a). Similarly, the binding of a monoionic spin-labelled probe to albumin, which also bound at the bilirubin sites, was not displaced until 3 mol of long-chain fatty acids were added to the system (Soltys & Hsia, 1978). On the other hand, medium-chain fatty acids appear to bind to this site, and readily displaced the spin-labelled probe (Soltys & Hsia, 1978).

For HSA, spectral studies using the fluorescent polyene fatty acid parinaric acid have demonstrated that the high-affinity sites for fatty acids are remote from the bilirubin sites (Berde *et al.*, 1979a). A similar situation has been shown to exist for α -fetoprotein (Berde *et al.*, 1979b).

Although fatty acyl-CoAs are not normally physiological ligands for albumin, many assays *in vitro* make use of the capacity of albumin to bind fatty acyl-CoAs to lower the free concentration of these potentially inhibitory detergents. When the ability of oleoyl-CoA to displace bound DAUDA from BSA was tested (Fig. 3), unlike oleic acid, immediate displacement was observed, indicating that this ligand was binding at the high-affinity bilirubin-binding site of albumin. Care must therefore be taken in interpreting results involving albumin-bound fatty acyl-CoAs (Pauly & McMillin, 1988), especially if drugs and other ligands are employed that bind to the bilirubin sites.

General discussion

The ability of albumin to discriminate between long-chain fatty acids and bilirubin contrasts with the situation found with rat liver FABP, where both ligands and also haem bind at the same site (Vincent & Muller-Eberhard, 1985). For liver FABP, the crystal structure may now be confidently predicted to be that of a 'clam-shell-like' molecule containing a non-polar binding pocket presumably able to accommodate long-chain fatty acids, bilirubin and haem. This three-dimensional structure has already been identified for two members of the FABP family, namely intestinal-FABP (Sacchettini *et al.*, 1988, 1989) and the P2 protein from myelin (Jones *et al.*, 1988).

Albumin, on the other hand, is predicted to contain three domains, produced by the parallel alignment of six α -helices. Three primary long-chain-fatty-acid-binding sites are proposed, in which the long-chain fatty acids are inserted into the centre of the tube formed by the α -helices (Brown & Shockley, 1982). Presumably the steric constraints of the high-affinity fatty acid-binding sites on albumin do not allow the more bulky DAUDA to bind at these sites, whereas the less bulky fluorescent fatty acid probe parinaric acid may be accommodated (Sklar *et al.*, 1977; Berde *et al.*, 1979a). Secondary sites for long-chain fatty acids are also proposed, which share identity with medium-chain-fatty-acid- and bilirubin-binding sites (Brown & Shockley, 1982). Direct support for this basic model comes from more recent n.m.r. studies (Cistola *et al.*, 1987a,b) and from the low-resolution three-dimensional structure of HSA (Carter *et al.*, 1989).

The exclusion of DAUDA from the primary long-chain fatty acid-binding sites, yet its ability to bind with high affinity to the secondary sites, make it a very useful probe for the study of these secondary sites, which are the primary binding sites for a number of important ligands, including bilirubin and medium-chain fatty acids. The binding of ligands to the high-affinity bilirubin site of albumin with concomitant displacement of bound bilirubin is of particular interest in drug therapy, especially in the newborn, since displacement of bilirubin can have damaging effects on the newborn. The binding of DAUDA to HSA and its displacement by competing ligands provides a simple method of assay for drugs that may bind to the primary bilirubin site of albumin. DAUDA should be particularly effective in this respect, because not only does it show a nearly 100-fold fluorescence enhancement when bound to the primary bilirubin-binding site of HSA but also DAUDA is both relatively soluble and chemically stable.

The binding of DAUDA to FABP and its subsequent displacement by long-chain fatty acids has been utilized as the basis for a continuous fluorescence displacement assay for enzymes that liberate long-chain free fatty acids, in particular phospholipase A₂ and triacylglycerol lipase (Wilton, 1990). The studies described above provide the explanation for why albumin cannot be effectively used in the displacement assay. The fatty acids released on enzymic hydrolysis do not initially cause significant displacement of DAUDA from albumin, as shown in

Fig. 3. Hence there is no significant change in fluorescence until all the primary fatty acid-binding sites on albumin are saturated.

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REFERENCES

- Berde, C. B., Hudson, B. S., Simoni, R. D. & Sklar, L. A. (1979a) *J. Biol. Chem.* **254**, 391–400
- Berde, C. B., Nagai, M. & Deutsch, H. F. (1979b) *J. Biol. Chem.* **254**, 12609–12614
- Brown, J. R. & Shockley, P. (1982) in *Lipid-Protein Interactions* (Jost, P. C. & Griffith, O. H., eds.), vol. 1, pp. 26–68, John Wiley and Sons, New York
- Carter, D. C., He, X.-M., Munson, S. H., Twigg, P. D., Gernert, K. M., Broom, B. & Miller, T. Y. (1989) *Science* **244**, 1195–1198
- Cistola, D. P., Small, D. M. & Hamilton, J. A. (1987a) *J. Biol. Chem.* **262**, 10971–10979
- Cistola, D. P., Small, D. M. & Hamilton, J. A. (1987b) *J. Biol. Chem.* **262**, 10980–10985
- Doody, M. C., Gotto, A. M. & Smith, L. C. (1982) *Biochemistry* **21**, 28–33
- Jacobsen, J. (1969) *FEBS Lett.* **5**, 112–114
- Jones, T. A., Bergfors, T., Sedzik, J. & Unge, T. (1988) *EMBO J.* **7**, 1597–1604
- Pauly, D. F. & McMillin, J. B. (1988) *J. Biol. Chem.* **263**, 18160–18167
- Sacchettini, J. C., Gordon, J. I. & Banaszak, L. J. (1988) *J. Biol. Chem.* **263**, 5815–5819
- Sacchettini, J. C., Gordon, J. I. & Banaszak, L. J. (1989) *J. Mol. Biol.* **208**, 327–339
- Sheridan, M., Wilkinson, T. C. I. & Wilton, D. C. (1987) *Biochem. J.* **242**, 919–922
- Sklar, L. A., Hudson, B. S. & Simoni, R. D. (1977) *Biochemistry* **16**, 5100–5108
- Soltys, B. J. & Hsia, J. C. (1978) *J. Biol. Chem.* **253**, 3023–3028
- Vincent, S. H. & Muller-Eberhard, U. (1985) *J. Biol. Chem.* **260**, 14521–14528
- Wilkinson, T. C. I. & Wilton, D. C. (1986) *Biochem. J.* **238**, 419–424
- Wilkinson, T. C. I. & Wilton, D. C. (1987a) *Biochem. J.* **242**, 913–917
- Wilkinson, T. C. I. & Wilton, D. C. (1987b) *Biochem. J.* **247**, 485–488
- Wilton, D. C. (1989) *Biochem. J.* **261**, 273–276
- Wilton, D. C. (1990) *Biochem. J.* **266**, 435–439
- Woolley, P. V. & Hunter, M. J. (1970) *Arch. Biochem. Biophys.* **140**, 197–209

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