Comparative studies of the binding of some ligands to human serum albumin non-covalently attached to immobilized Cibacron Blue, or covalently immobilized on Sepharose, by column affinity chromatography

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A comparative study of the ligand-binding properties of human serum albumin was performed by the technique of affinity chromatography with the protein attached to immobilized Cibacron Blue F3GA (Blue Sepharose), or covalently immobilized on Sepharose. The binding strength of octanoate, decanoate and dodecanoate is much weaker when human serum albumin is attached to immobilized Cibacron Blue, indicating that the binding sites for fatty acids are involved in the attachment of human serum albumin to immobilized Cibacron Blue. The results revealed additional alterations of the ligand binding when human serum albumin was attached to immobilized Cibacron Blue, involving sites outside of the binding domains of fatty acids. Thus the stereoselective binding of L-tryptophan was abolished, and the resolution of the warfarin enantiomers was impaired. However, the binding strength of warfarin and salicylic acid was rather close to the values observed with human serum albumin covalently immobilized on Sepharose. It is suggested that the availability of the binding sites for L-tryptophan, warfarin and salicylic acid is partially blocked by the complex between albumin and the dye without direct participation in the complex-formation. An alternative interpretation involves an allosteric mechanism brought about by complex-formation between serum albumin and the immobilized Cibacron Blue.

The triazine dye Cibacron Blue F3GA interacts with many proteins, and this property has been utilized by numerous workers to purify proteins. Binding of a protein to immobilized triazine dyes has previously been taken as evidence that the protein contains a site or domain that specifically binds dinucleotides (Thompson *et al.*, 1975; Stellwagen, 1977). However, a number of experiments indicate that binding to immobilized triazine dyes is by no means a diagnostic probe for such sites (Stellwagen, 1979). Thus proteins such as interferon and human serum albumin, which are not known to possess dinucleotide-binding sites, have also been shown to bind to immobilized Cibacron Blue.

It has been shown that the strength of binding to immobilized Cibacron Blue is not the same for serum albumin of different species. Thus human serum albumin is bound to a far greater extent than is serum albumin of species such as rabbit, horse, dog or chicken (Leatherbarrow & Dean, 1980; Naval *et al.*, 1982). It has been proposed that the mechanism of interaction between human serum albumin and Cibacron Blue involves the binding sites of bilirubin (Leatherbarrow & Dean, 1980).

Further investigations have shown that pre-saturation with long-chain fatty acids significantly increased the dissociation constants of human serum albumin bound to immobilized Cibacron Blue, whereas pretreatment with bilirubin did not have such an effect (Metcalf *et al.*, 1981). The results indicate that the binding sites for long-chain fatty acids are utilized for the non-covalent binding of human serum albumin to immobilized Cibacron Blue.

In view of this finding, it would be interesting to elucidate whether other binding sites or domains on human serum albumin are influenced, in addition to those for long-chain fatty acids, when human serum albumin is bound to immobilized Cibacron Blue. Such an investigation has now been performed by studying the binding of some ligands to human serum albumin attached to immobilized Cibacron Blue. The results are compared with those obtained with human serum albumin covalently bound to Sepharose. In these experiments, the technique of column affinity chromatography was used, as described previously (Lagercrantz *et al.*, 1979, 1981). In these previous studies, the ligand binding

of some fatty acids, steroids and drug substances to serum albumin covalently immobilized on Sepharose was studied by column affinity chromatography. Samples of radioactively labelled ligands were applied to columns of serum albumin immobilized by coupling to Sepharose. Elution was performed by displacement with pure buffer. The strongest association constant, K_1 , of the stepwise multiple equilibria was evaluated with zonal chromatography. The high resolution of the method allowed the study of stereoselective binding of the enantiomers of warfarin and tryptophan to serum albumin from some different species (Lagercrantz *et al.*, 1981).

In the present investigation the binding to human serum albumin attached to immobilized Cibacron Blue, or covalently attached to Sepharose, was studied for tryptophan, salicylic acid, warfarin, octanoate, decanoate and dodecanoate. In addition, the binding properties of a dinucleotide to human serum albumin covalently attached to Sepharose were studied as an actual check of the statement that human serum albumin does not possess any binding sites for dinucleotides.

Experimental

Serum albumin

Human serum albumin, essentially free from fatty acids, i.e. less than 0.005% according to the manufacturer, was obtained from Sigma (product no. A1887, lot 110F-9350).

Radiochemicals

The radiochemicals were obtained from Amersham International, Centre d'Energie Atomique or New England Nuclear.

Measurements of radioactivity

The measurements were performed as described by Lagercrantz *et al.* (1979, 1981).

Affinity chromatography

Blue Sepharose (Pharmacia Fine Chemicals) (4 g) was washed with water and 0.04 M-sodium phosphate buffer, pH 7.4, as recommended by the manufacturer. The gel (about 16 ml) was loaded into a column of diameter 1.6 cm. Phosphate buffer (0.04 M), pH 7.4, was used in all of the experiments, and was passed through the column for 2 h (40 ml/h) before a weighed amount of human serum albumin dissolved in buffer was applied on the column.

The radiochemicals to be chromatographed on serum albumin immobilized on Blue Sepharose were used as supplied, without further addition of non-labelled carriers. The amount of substances labelled with ^{14}C corresponded to 1–10 nmol for each compound. The radioactivity corresponded to about 0.05 μCi (1.85 kBq). The labelled compounds were

applied to the column dissolved in a small volume of buffer (approx. 200 μl), and eluted with pure buffer. Each eluted fraction was collected during 2.5 min, and the rate of flow was 40 ml/h. Generally, single runs were made for each separate ligand.

The volume V_A^* in eqns. (1a) and (1b) (see below), i.e. the elution volume of ligand A with no specific interaction between ligand A and serum albumin, was determined by elution of $^3\text{H}_2\text{O}$. Zonal chromatography on covalently immobilized human serum albumin was performed as described by Lagercrantz *et al.* (1979, 1981).

Results

The strongest association constants, K_1 , were evaluated from zonal chromatography in our earlier experiments by use of eqn. (1a) (Lagercrantz *et al.*, 1979, 1981):

$$\bar{V}_A - V_A^* = V_s \cdot [\bar{X}] \cdot K_1 \quad (1a)$$

In this equation $\bar{V}_A$ is the elution volume of ligand A, V_A^* is the elution volume of A with no specific interaction between A and immobilized serum albumin, V_s is the volume of the gel, and $[\bar{X}]$ is the molar concentration of serum albumin on the gel.

The product $V_s \cdot [\bar{X}]$ in eqn. (1a) is equivalent to the molar amount of serum albumin just applied and immobilized on the column. Consequently, eqn. (1a) can be rewritten:

$$\bar{V}_A - V_A^* = m \cdot K_1 \quad (1b)$$

where $m = V_s \cdot [\bar{X}]$.

Eqn. (1b) was tested and found valid by zonal chromatography of [^{14}C]salicylic acid. A plot of $\bar{V}_A - V_A^*$ against m , evaluated from the applied amount of human serum albumin, and assuming the M_r of serum albumin to be 66000 (cf. Putnam, 1965), gave rise to a straight line starting from the origin (Fig. 1). The values of K_1 for [^{14}C]salicylic acid were very nearly the same for all four m values in Fig. 1, i.e. $K_1 = 3.10 \times 10^4 \text{ M}^{-1}$.

Although all albumins have been found to bind to Blue Sepharose, human serum albumin is bound to a far greater extent than are albumins of other species. The apparent association constant for the complex between Blue Sepharose and human serum albumin was reported to be between 2.4×10^4 and $4.7 \times 10^4 \text{ M}^{-1}$ (Angal & Dean, 1977) and to be $3.9 \times 10^4 \text{ M}^{-1}$ (Naval *et al.*, 1982).

At first, it might appear to be impossible to study the binding of ligands to human serum albumin immobilized on Blue Sepharose when the association constants are equal to or larger than the association constants for the complex between Blue Sepharose and albumin. However, ligand binding to human serum albumin immobilized on Blue Sepharose can be evaluated when the association constants are nearly three orders of magnitude larger

than that of the complex between Blue Sepharose and human serum albumin. For small amounts of serum albumin, eqn. (1b) is assumed to be valid. According to the manufacturer, the number of coupled dye molecules on a column of Blue Sepharose prepared from 4 g of starting material corresponds to $40\mu\text{mol}$. With $V_s \cdot [\bar{X}]$ equal to this value, and K_1 for the complex between Blue Sepharose and human serum albumin taken to be $4 \times 10^4 \text{ M}^{-1}$, the elution volume $\bar{V}_A - V_A^*$ is equal to

Fig. 1. Affinity chromatography of [^{14}C]salicylic acid on a column of Blue Sepharose (Cibacron Blue F3G-A covalently attached to Sepharose CL-6B) with non-covalently attached human serum albumin

$\bar{V}_A$ is the elution volume of [^{14}C]salicylic acid; V_A^* is the elution volume with no specific interaction between the ligand and serum albumin; $V_s \cdot [\bar{X}]$ is the amount of human serum albumin immobilized on the column (in μmol), i.e. m in eqn. (1b). The results with four different amounts of serum albumin are shown. The elution was performed with 0.04 M-phosphate buffer, pH 7.4.

1.6 litres by use of eqn. (1b). With $V_s \cdot [\bar{X}]$ taken to be $0.1\mu\text{mol}$ (6.6 mg) of serum albumin and the same columns of Blue Sepharose, an elution volume $\bar{V}_A - V_A^*$ of 1.6 litres corresponds to an association constant of $1.6 \times 10^7 \text{ M}^{-1}$. By an increase of the amount of Blue Sepharose and a decrease in serum albumin immobilized, still larger constants might be evaluated.

The maximum value of the elution volume $\bar{V}_A - V_A^*$ for the ligands studied here was almost an order of magnitude lower than the critical value of 1.6 litres. In accordance with the above considerations, no leakage with loss of serum albumin could be detected in any of the experiments. It should be emphasized that the salt concentration of the buffer solution must be kept as low as possible, since the complex between Blue Sepharose and serum albumin is weakened by a high salt concentration (cf. Stellwagen, 1977, 1979). Thus elution with 1.5 M-KCl is used for complete removal of serum albumin from columns of Blue Sepharose. The condition of the column and the amount of serum albumin immobilized are easily checked by running a ligand with a known value of K_1 , such as [^{14}C]salicylic acid.

Table 1 gives the K_1 values obtained with DL-[^{14}C]tryptophan, [^{14}C]salicylic acid, *rac*-[^{14}C]warfarin, [^{14}C]octanoate, [^{14}C]decanoate and [^{14}C]dodecanoate by zonal chromatography on human serum albumin immobilized on Blue Sepharose (eqn. 1b), together with the corresponding values obtained with human serum albumin covalently bound to Sepharose 4B. {The standard deviations of the K_1 values observed with serum albumins covalently attached to Sepharose did not exceed $\pm 10\%$, and were generally about $\pm 5\%$ (Lagercrantz *et al.*, 1979, 1981). The K_1 values obtained with human serum albumin immobilized on

Table 1. Association constants, K_1 , obtained by zonal chromatography on immobilized human serum albumin evaluated by use of eqns. (1a) and (1b)

For comparison, the corresponding values are quoted from the literature for some of the ligands when bound to human serum albumin in solution.

Radioligand	$K_1 (\text{M}^{-1})$		
	Immobilized on Blue Sepharose (eqn. 1b)	Covalently immobilized on Sepharose (eqn. 1a)	Free in solution
D-[^{14}C]Tryptophan	$0.18 \times 10^{4*}$	$0.13 \times 10^{4\dagger}$	$1.6 \times 10^{4*}$ (McMenamy & Oncley, 1958)
L-[^{14}C]Tryptophan		$1.10 \times 10^{4\dagger}$	
[^{14}C]Salicylic acid	3.11×10^4	$6.6 \times 10^{4\dagger}$	2.8×10^4 (Kober <i>et al.</i> , 1978)
R-[^{14}C]Warfarin	$4.6 \times 10^{5*}$	$3.3 \times 10^{5\dagger}$	$2.1 \times 10^{5*}$ (Kober <i>et al.</i> , 1978)
S-[^{14}C]Warfarin		$4.4 \times 10^{5\dagger}$	
[^{14}C]Octanoate	1.1×10^4	7.0×10^5	2.4×10^6 (Ashbrook <i>et al.</i> , 1975)
[^{14}C]Decanoate	4.9×10^5	8.0×10^6	
[^{14}C]Dodecanoate	1.6×10^6	5.4×10^7	

* Enantiomers not resolved.

† From Lagercrantz *et al.* (1981).

Blue Sepharose exhibited standard deviations of the same order of magnitude: for [^{14}C]salicylic acid, $K_1 = 3.11 \pm 0.05 \times 10^4 \text{ M}^{-1}$; for [^{14}C]warfarin, $K_1 = 4.63 \pm 0.39 \times 10^5 \text{ M}^{-1}$; i.e. ± 1.6 and $\pm 8.4\%$ respectively.} For comparison, the corresponding association constants are quoted from the literature for some of the ligands when bound to human serum albumin in solution.

Without human serum albumin immobilized on the columns of Blue Sepharose the radioligands were eluted with the buffer front, indicating that no interaction occurred with the matrix or the covalently attached Cibacron Blue. The analogous finding is also valid for the matrix used in the covalent immobilization of serum albumin.

Human serum albumin immobilized on Blue Sepharose is not able to resolve the enantiomers of tryptophan, which are eluted as a single component (see Table 1). The K_1 value is low and rather close to that observed for D-[^{14}C]tryptophan on covalently immobilized human serum albumin. The resolution of the warfarin enantiomers is also impaired for human serum albumin immobilized on Blue Sepharose, and could only be traced in some runs. However, the K_1 value of warfarin is very close to the value obtained for the enantiomers resolved with human serum albumin covalently immobilized on Sepharose 4B. The K_1 value obtained with [^{14}C]salicylic acid is not very different for the two methods of immobilization.

The three fatty acids investigated behave very differently for the two methods of immobilization. As shown in Table 1, the association constants K_1 are 20–70 times smaller when human serum albumin is immobilized on Blue Sepharose.

The study of ligands with K_1 values larger than those of the present series would require much smaller amounts of serum albumin attached to the solid phase, and would also give rise to elution volumes at or above the critical limit for elution of the attached serum albumin in the case of Blue Sepharose. Owing to these considerations and the relatively low solubility in the buffer solution, bilirubin and fatty acids of a chain length of more than 12 carbon atoms have not been investigated.

No binding could be detected in an experiment performed with nicotinamide-[U- ^{14}C]adenine dinucleotide and human serum albumin covalently immobilized on Sepharose 4B, as this substance was eluted with the buffer front. This result confirms the general statement that human serum albumin does not contain any binding sites for dinucleotides.

Discussion

The much weaker binding of octanoate, decanoate and dodecanoate indicates that the binding sites for fatty acids are involved in the attachment of human

serum albumin to immobilized Cibacron Blue. This finding is consistent with the results obtained by Metcalf *et al.* (1981), who reported an increased dissociation of human serum albumin from immobilized Cibacron Blue after presaturation with long-chain fatty acids.

The results obtained with the affinity-chromatographic technique revealed additional alterations of the ligand binding, these involving sites outside the binding domains of fatty acids. The abolition of the stereoselective binding of L-tryptophan, the impaired resolution of the warfarin enantiomers and the somewhat decreased binding of salicylic acid might be connected with a partial steric blocking of the actual sites by the albumin–Cibacron Blue complex without a direct involvement of the latter sites in the complex binding. An alternative interpretation consists of allosteric changes of the binding sites for tryptophan, warfarin and salicylic acid brought about by the attachment of serum albumin to immobilized Cibacron Blue.

Finally, it should be emphasized that the attachment of human serum albumin to immobilized Cibacron Blue cannot be recommended for the study of ligand binding, since the binding properties are very much changed by the interaction between the protein and the dye substance.

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