Drug-binding properties of rat α_1 -foetoprotein

Binding of warfarin, phenylbutazone, azapropazone, diazepam, digitoxin and cholic acid

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(Received 29 December 1983/Accepted 21 March 1984)

As part of an investigation into whether α_1 -foetoprotein (α_1 -FP) plays the same transport role in foetal serum as albumin does in the adult, the binding properties of both proteins were compared with respect to the binding of a series of compounds known to be bound by albumin's specific drug-binding sites. The binding of warfarin, phenylbutazone, azapropazone, diazepam, digitoxin and cholic acid by rat α_1 -FP and serum albumin was studied by equilibrium dialysis at 4°C. Rat α_1 -FP was shown to have neither albumin's high-affinity site II (diazepam as marker) nor its site III (digitoxin and cholic acid as markers). High-affinity binding by α_1 -FP was found for the specific markers (warfarin, phenylbutazone, azapropazone) of albumin's drug-binding site I. However, instead of albumin's one high-affinity site/molecule, a mean value of 0.5 site/molecule was obtained with rat α_1 -FP. Charcoal treatment at neutral pH of rat serum albumin did not affect its measured binding properties, but treatment of the α_1 -FP led to an increased affinity for warfarin, phenylbutazone and azapropazone without a change in the measured number of sites, indicating competition for binding at this site by (an) endogenous ligand(s). These results are discussed in terms of the structures of the two proteins and with respect to the physiological implications of the differences found.

The distribution and pharmacological actions of drugs are affected by the degree of their protein binding in serum, since the free fraction of a drug has a high turnover and corresponds to the active fraction (Sellers & Koch-Weser, 1977). Thus the serum binding proteins constitute a circulating reservoir for the drug, which is able to buffer the variations in concentration of the active compound.

During pregnancy, when the drugs are administered to the mother, the foetus almost always receives a certain quantity of drug, which may have unfortunate effects on its development. The binding of certain drugs to plasma proteins is lower in the foetus and the newborn than in the adult (Krasner *et al.*, 1973). Therefore, for a given total concentration of drug, there may be a higher free

serum drug concentration in the foetus and the neonate than in the adult, causing an enhanced pharmacological (and eventually toxic) effect. Consequently the serum transport of drugs in the foetus and the newborn is an important problem for the clinician. Although this transport is generally only estimated by the determination of the free and bound fractions of the drug of the whole serum, it appears of much more interest to attempt to determine the binding properties of the individual serum proteins towards a given drug (number of binding sites and affinity).

In the adult the main transport protein is albumin, which has a small number of relatively specific drug-binding sites (Fehske *et al.*, 1981). In the foetus, much of the serum albumin appears to be replaced by α_1 -FP, known to be a transport protein for biomolecules (Grigorova *et al.*, 1977; Ruoslahti, 1979a; Hervé *et al.*, 1982), but the drug-binding properties of this foetal protein have not been studied.

Abbreviation used: α_1 -FP, α_1 -foetoprotein.

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In this laboratory the problem of the drug-binding properties of α_1 -FP has been approached by using the rat as an experimental animal. This seems justified, since the serum drug-binding properties in humans and rats are very similar (Lázniczek *et al.*, 1982) and one would expect similar binding characteristics with human and rat α_1 -FP, since the two proteins present 67% homology in their primary sequence (Morinaga *et al.*, 1983).

In order to compare the drug-binding properties of rat α_1 -FP with those of rat serum albumin, a variety of drugs were used that are known to be specifically bound to sites I, II and III on the albumin molecule: warfarin, phenylbutazone and azapropazone (site I); diazepam (site II); digitoxin and cholic acid (site III).

Experimental

Binding proteins

α_1 -FP was prepared from foetal rat serum of 19 (± 2) day-old rat embryos (Charles Rivers CD strain) by an immunoabsorption method previously described (Cittanova *et al.*, 1974). This method permits the recovery of a protein with a purity greater than 97%. The protein used was from a pool of 50 preparations (each of serum from 19 rat embryos).

Rat serum albumin (prepared from fraction V rat serum) was obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. (no. 30F 9331) and was shown by electrophoretic analysis on 12% (w/v)-polyacrylamide gels to be 96% albumin. The protein concentration of α_1 -FP and serum albumin solutions were checked spectrophotometrically at 280 nm using $A_{1\text{cm}}^{1\%}$ values of 6.8 and 6.4 respectively, assuming M_r values of 70 000 for rat α_1 -FP (Grigorova *et al.*, 1977) and 66 000 for rat serum albumin.

Charcoal treatment at neutral pH

For some experiments, the rat α_1 -FP and serum albumin used in the equilibrium-dialysis studies were treated with activated charcoal (Hervé *et al.*, 1981). Protein solutions (with a concentration not exceeding 1 mg/ml) were incubated for 30 min at 37°C with Norit A charcoal (3.3 mg/ml of solution) and dextran T 70 (Pharmacia, Uppsala, Sweden) (0.33 mg/ml of solution) at pH 7.4. The mixtures were then centrifuged for 20 min at 4°C and 20 000 *g* (r_{av} , 10.80 cm) and the supernatants were collected. This permits the removal of aromatic compounds but not fatty acids (Chen, 1967). Electrophoresis in 12% (w/v) polyacrylamide gels of rat serum albumin before and after charcoal treatment, compared with bovine albumin preparation known to contain dimers (batch no. 33;

Miles, Elkhart, IN, U.S.A.), showed no significant change in the extent of albumin polymerization; dimers represented less than 4% of the protein.

Buffers

All solutions were prepared in 0.1 M-sodium phosphate buffer, pH 7.4, containing 50 mg of gelatin/litre (used to avoid adsorption of ligand to dialysis membranes without significantly binding ligand itself). Because of the poor solubility of digitoxin in aqueous medium, the same buffer containing 10% (v/v) ethanol (grade A; Merck, Darmstadt, Germany) was used for the binding measurements with this ligand. Previous experiments have shown that ethanol, when used at concentrations not exceeding 10%, does not denature α_1 -FP nor albumin, nor significantly disturb equilibrium experiments (Desfosses *et al.*, 1983). When necessary, the drugs were dissolved initially in a small volume of 0.1 M-NaOH. The final pH of all drug solutions was in the pH range 7.4–7.6.

Ligands

Phenylbutazone (Ciba-Geigy, Basel, Switzerland) and azapropazone (Siegfried A.G., Zofingen, Switzerland) were generously given by Doctor W. E. Müller (Institute of Pharmacology, University of Mainz, Mainz, Germany). Diazepam was generously given by the Roche Laboratory (Neuilly/Seine, 92521 France). Warfarin, digitoxin and cholic acid were purchased from Sigma Chemical Co. Aza[U- ^{14}C]propazone (17.9 $\mu\text{Ci/mg}$) was generously given by Dr. W. E. Müller and [U- ^{14}C]phenylbutazone (19.9 $\mu\text{Ci/mg}$) was generously given by Dr. W. Riess and Dr. W. Küng (Ciba-Geigy, Basel, Switzerland). [^{14}C]Warfarin (177 $\mu\text{Ci/mg}$) and [2- ^{14}C]diazepam (200 $\mu\text{Ci/mg}$) came from The Radiochemical Centre, Amersham, Bucks., U.K. [G- ^3H]Digitoxin (18.2 mCi/mg) and [N-2,4- ^3H]cholic acid (38.5 mCi/mg) were obtained from New England Nuclear Corp., Boston, MA, U.S.A. The radiochemical purity of the drugs was higher than 98% as determined by t.l.c.

Equilibrium dialysis

Binding experiments were performed, with rat serum albumin and rat α_1 -FP and the ligands listed above, by the method of Meyer & Schellman (1962). Dialysis cells with a total volume of 200 μl were separated into two equal compartments by a Visking membrane (18/32; Union Carbide Corp., Chicago, IL, U.S.A.), which had previously been washed with boiling distilled water containing 5% (w/v) Na_2CO_3 and 2.2% (w/v) EDTA to remove metal-ion impurities. One side of the cells contained 80 μl of the protein solution, at the same

concentration in all the cells (10–20 μM , depending on the ligand concerned). The other side of the cell contained the same volume of ligand at different concentrations (1–1000 μM), with a constant amount of radioactivity $[(1-4.2) \times 10^3 \text{ Bq}]$, depending on the ligand]. It should be noted that concentrations were no higher than 80 μM for digitoxin and 100 μM for diazepam, because of the low solubilities of these ligands in aqueous media.

Equilibrium was reached at 4°C after gentle shaking for 20–30h, depending on the ligand tested. The radioactivity in 50 μl samples from each compartment was then determined and scintillation counting was carried out with an Inter technique SL 4000 counter, with Picofluor 15 (Packard IC, Downers Grove, IL, U.S.A.) as scintillant.

Evaluation of the binding data

The binding data obtained with the different drugs were analysed as described by Scatchard (1949). The equation

$$\frac{\bar{r}}{[D]} = nk - \bar{r}k$$

was used, where $\bar{r}$ is mol of bound drug/mol of protein, n the number of binding sites, k the association constant for the drug–protein complex and $[D]$ the concentration of free drug. The apparent intrinsic association constant for proteins with one class of sites was determined by weighted linear-regression analysis. The apparent association constants for proteins with more than one class of sites [as described by the equilibrium equation (Klotz & Hunston, 1971)] were calculated from a polar transformation of experimental points in a Scatchard plot and an iterative non-linear least-squares analysis (Kim, 1970). A Wang 2200 calculator was used for these calculations. For some plots it was necessary to fix the number of low-affinity binding sites to avoid divergence and so no s.d. is given for n_2 .

Results and discussion

The calculated values of the binding results for all drugs tested are shown in Table 1, and typical Scatchard plots for rat α_1 -FP are shown in Fig. 1.

Rat serum albumin

For warfarin, phenylbutazone, azapropazone and cholic acid, rat serum albumin has two sets of binding sites: one high-affinity site and three of much lower affinity. However, the low solubilities of diazepam and digitoxin in aqueous media only permitted investigation of the higher-affinity binding sites: one such site was found. The existence of

Table 1. Binding parameters for rat serum albumin and α_1 -FP with warfarin, phenylbutazone, azapropazone, diazepam, digitoxin and cholic acid

Protein	Rat α_1 -FP					
	Before charcoal treatment			After charcoal treatment		
Drug	Rat serum albumin			Rat α_1 -FP		
	(results unchanged after charcoal treatment)					
	n_1	n_2	k_2 (M^{-1})	n_1	k_1 (M^{-1})	k_2 (M^{-1})
Warfarin	0.95 ± 0.07	$(0.83 \pm 0.08) \times 10^6$	2.84 ± 0.11	0.45 ± 0.02	$(0.76 \pm 0.03) \times 10^6$	0.47 ± 0.02
Phenylbutazone	1.00 ± 0.12	$(0.46 \pm 0.04) \times 10^6$	3.43 ± 0.09	0.34 ± 0.07	$(0.15 \pm 0.04) \times 10^6$	$(0.52 \pm 0.04) \times 10^4$
Azapropazone	1.54 ± 0.07	$(0.55 \pm 0.04) \times 10^6$	3.43 ± 0.55	1.70 ± 0.05	$(0.73 \pm 0.11) \times 10^4$	0.41 ± 0.03
Diazepam	0.87 ± 0.01	$(0.32 \pm 0.01) \times 10^5$	$< 10^{3*}$	0.24 ± 0.08	$(0.13 \pm 0.06) \times 10^6$	0.44 ± 0.07
Digitoxin	1.66 ± 0.52	$(0.26 \pm 0.01) \times 10^5$	$< 10^{3*}$	2.61 ± 0.14	$(0.40 \pm 0.10) \times 10^4$	1.27 ± 0.16
Cholic acid	0.87 ± 0.16	$(0.26 \pm 0.06) \times 10^6$	3.49 ± 0.12	$< 10^3$	$< 10^3$	$< 10^3$

* Binding parameters could not be measured because the poor solubility of these ligands in aqueous media.

Fig. 1. Scatchard plots of the binding of warfarin by rat α_1 -FP

The binding of warfarin (0.001–1 mM) by 0.01 mM-rat α_1 -FP before (○) and after (●) treatment of the protein with charcoal at neutral pH is shown. Equilibrium dialyses between two 80 μ l volumes were carried out at 4°C in 0.1 M-sodium phosphate buffer, pH 7.4, containing 50 mg of gelatin/litre, for at least 20 h. The binding parameters calculated from the curves are shown in Table 1 and the component binding plots making up each curve, as calculated from the binding parameters, are shown. $\bar{r}$ is the relative binding saturation (mol of warfarin bound/mol of α_1 -FP) and $[\text{warfarin}]_{\text{free}}$ is the concentration of the free drug. Each point is the mean result for four triplicate experiments.

a second set of binding sites of lower affinity for diazepam (Kragh-Hansen, 1983) and digitoxin could not be excluded.

The binding results obtained here with rat serum albumin agree with those previously reported for drug binding by human serum albumin, for example in the studies on the site-I markers warfarin, phenylbutazone and azapropazone by Fehske *et al.* (1979a, 1980, 1982) and by Kober & Sjöholm (1980), and on the site-III markers by Burke *et al.* (1971) (cholic acid), Lukas & de Martino (1969) and Brock (1976) (digitoxin). For the site-II marker diazepam, our results were an order of magnitude lower than those found by Kragh-Hansen (1983) [those we reported previously with rat serum albumin (Hervé *et al.*, 1982) for the other site-II marker, tryptophan, were approxi-

mately the same as those found by Kragh-Hansen (1983) with human albumin].

Though reported to be bound at the same site(III), albumin's affinity for cholic acid was some 10-fold greater than that for digitoxin. This difference could be due to different binding forces being involved (Lukas & de Martino, 1969), digitoxin binding being an endothermic, hydrophobic reaction with a protein conformational change, and binding of the more hydrophilic cholic acid being exothermic, with no major change in protein conformation.

Rat α_1 -FP

No significant binding of diazepam, digitoxin or cholic acid was found with rat α_1 -FP.

The results with diazepam confirm the absence of albumin's site II (specific for benzodiazepines and indole analogues; Sudlow *et al.*, 1975; Fehske *et al.*, 1979b) from rat α_1 -FP, previously shown by the absence of indole binding by the foetal protein (Hervé *et al.*, 1982).

Digitoxin and cholic acid are markers for albumin's binding site III, which thus also seems to be absent from rat α_1 -FP.

On the one hand the differences in primary sequence between rat serum albumin and α_1 -FP may explain the absence of these binding sites from the foetal protein. Four amino acid residues from very different parts of albumin's primary structure are involved in binding site II (Fehske *et al.*, 1981), and, in the mouse, only two of these amino acids (Arg-145 and Tyr-411) are present in the α_1 -FP primary structure (Ohno, 1981). On the other hand, α_1 -FP, unlike albumin, is glycosylated: the related murine α_1 -FP has two glycan molecules per molecule (Gorin *et al.*, 1981), and these may mask the binding regions of the foetal protein.

The absence of these two sites from rat α_1 -FP is of pharmacological as well as physiological importance. The replacement of much serum albumin by α_1 -FP in the foetus will diminish the serum binding of the corresponding molecules, whose activities (and/or toxic effects) will thus be increased and whose half-lives will be shortened.

Two sets of binding sites were found for warfarin (Fig. 1), phenylbutazone and azapropazone binding by rat α_1 -FP: there was a fractional number of high-affinity sites, but with the same order of affinity as that of albumin, and also, like albumin, lower-affinity sites were found.

A similar decrease in the number of α_1 -FP-binding sites for a given ligand as compared with albumin have been found in this laboratory for anilidonaphthalenesulphonate (Grigorova *et al.*, 1980) and thyroid hormones (Hervé *et al.*, 1982). Rat α_1 -FP exists as a heterogeneous population of subspecies, only one of these having the same

electrofocusing behaviour as albumin (Grigorova *et al.*, 1977). Thus it is possible that the decreased fractional number of binding sites on the foetal protein represents a situation in which less than half the molecules of the protein bind these drugs at the same number of sites (one) as albumin, the remaining molecules not binding them at all. The differences between the foetal isoproteins may, again, be due either to differences in primary structure, to the sugar composition, or to the positioning of the glycan molecules. It may now be, in evolutionary terms, that both explanations are valid, since the masking of the sites would remove the functional constraints on mutations in the genetic sequences corresponding to maintenance of the integrity of the binding areas, unless they are also involved in maintaining the architecture of the molecule.

Charcoal treatment

The rat serum albumin and α_1 -FP were necessarily purified by different methods. Endogenous ligands bound to the proteins might thus be removed to different extents during purification, possibly leading to differences in estimated binding properties. After the removal of remaining endogenous ligands by charcoal, under conditions unlikely to harm the proteins themselves, the estimated binding properties should be directly comparable.

Charcoal treatment of albumin did not give rise to any significant change in the binding of the ligands tested (Table 1).

Even after charcoal treatment, rat α_1 -FP did not bind diazepam, digitoxin or cholic acid, showing that the sites II and III were not simply blocked by endogenous ligand(s).

Though the binding parameters for the lower-affinity sites for phenylbutazone and azapropazone were only slightly modified by charcoal treatment, and were unaltered in the case of warfarin, the apparent association constants for the high-affinity binding of these drugs by rat α_1 -FP were all increased, the fractional number of high-affinity sites remaining virtually unchanged. The increases in the rat α_1 -FP affinities for these drugs were very different and not directly related to the absolute affinities, being 2.4-fold for warfarin, 8-fold for phenylbutazone and 7-fold for azapropazone.

Two sets of conclusions could be drawn from these results. Firstly, the differences in the relative increases in binding affinity indicate that these drugs do not occupy the α_1 -FP site I in the same way. Fehske *et al.* (1982), showing that albumin's lone tryptophan residue appears to be implicated in warfarin binding but not that of phenylbutazone and azapropazone, have suggested that this site is

composed of two overlapping sites. Such an arrangement of the α_1 -FP site would explain the results obtained here, and it may be relevant that mouse α_1 -FP, like albumin, has one tryptophan residue (Ohno, 1981). Secondly, the increases in affinity indicate the presence of an endogenous ligand at site I that is removed by the charcoal. The charcoal would be expected to remove hydrophobic polycyclic ligands. We have previously reported (Hervé *et al.*, 1981) the presence and some physical properties of an endogenous diffusible chromophore (not a fatty acid, bilirubin or oestrogen) that is extracted from rat α_1 -FP by charcoal. Tillement *et al.* (1978) have also described the existence of endogenous ligands and their clinical importance with respect to drug binding in physiological and pathological states.

Concluding remarks

The degree of primary sequence homology (35%) (Ohno, 1981) and the structural homologies (Grigorova *et al.*, 1977) between rat α_1 -FP and serum albumin would lead one to expect similarities and differences in binding properties such as have been found here.

Other similarities already reported include those for the binding of bilirubin (Ruoslahti *et al.*, 1979b; Berde *et al.*, 1979), fatty acids (Hsia *et al.*, 1980), aflatoxin (Evrain *et al.*, 1978), anilino-naphthalene-sulphonate (Grigorova *et al.*, 1980) and thyroid hormones (Hervé *et al.*, 1982). For the drugs bound at the (albumin) site I, the decrease in the number of α_1 -FP binding sites, compared with albumin, would lead to a decrease in their plasma binding. This would be even more evident for the drugs bound at the albumin sites II and III, not found at all with rat α_1 -FP. The effect on plasma binding of the site-I drugs would be further aggravated by the presence of (an) endogenous ligand (s) acting as (a) potent competitor(s) for their binding by α_1 -FP.

Thus in order to appreciate the drug-transporting role of α_1 -FP in foetal serum, it is necessary to extend this comparative type of study to other classes of drugs and to investigate potential competition by such endogenous ligands as oestrogens, bilirubin and fatty acids, compounds known to be strongly bound by rat α_1 -FP.

This work forms part of the Doctoral Thesis of F. H. We are grateful to Dr. W. E. Müller, Dr. W. Riess and Dr. W. Küng and to the Roche Laboratory, for generously providing gifts of chemicals. This work was supported by the Centre National de la Recherche Scientifique, Paris, France.

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