Participation of the lone tryptophan residue of rat α -foetoprotein in its drug-binding sites

Comparison with rat serum albumin

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The participation in drug binding of the lone tryptophan residue of rat α -foetoprotein (α -FP) and serum albumin, the two main transport proteins of foetal serum, has been studied by two different techniques. Firstly, the effect on phenylbutazone and warfarin binding of the chemical derivatization of the lone tryptophan residue of both proteins by 2-nitrophenylsulphonyl chloride (NPS) was studied. Secondly, the effect of phenylbutazone binding on the intrinsic fluorescence of the tryptophan residue of rat α -FP and albumin was investigated. The specific modification of the proteins by NPS did not affect the binding of warfarin by rat α -FP and albumin, but greatly decreased the affinity of the high-affinity sites of rat α -FP for phenylbutazone, though the numbers of these sites were not significantly changed. However, for albumin a similar decrease in the affinity constant appeared to be due to the reaction conditions. The spectrofluorimetric studies showed that the lone tryptophan residue of α -FP and albumin was quenched by phenylbutazone binding, and the quenching paralleled the fractional saturation of the high-affinity site only in the case of albumin. The effect of phenylbutazone binding on the intrinsic fluorescence of rat α -FP indicated that the lone tryptophan residue of this foetal protein is not in the same molecular environment as that of albumin, not participating directly in the high-affinity site for phenylbutazone, and the effect may be via some induced conformational change in rat α -FP. These results also confirm our previous suggestion that the high-affinity sites for phenylbutazone and warfarin are different on the rat α -FP molecule. The results seem to indicate that this is also the case for albumin, but confirmation is necessary.

INTRODUCTION

Rat α -foetoprotein (α -FP) and serum albumin, the two main transport proteins of foetal serum, have been shown to present very strong structural analogies (Jagodzinski *et al.*, 1981) and, in particular, they contain only one tryptophan residue in their primary sequences (Versée & Barel, 1978). Serum albumin's lone tryptophan residue appears to be involved in the binding of the drugs warfarin and phenylbutazone (Maes *et al.*, 1979; Fehske *et al.*, 1982), which seem to occupy the same high-affinity binding site on that protein. We have recently demonstrated (Hervé *et al.*, 1984) that rat α -FP also strongly binds the drugs warfarin and phenylbutazone, but at two different high-affinity sites with a negative-co-operativity mechanism acting between these sites (Hervé *et al.*, 1986). We have therefore investigated the possibility that the foetal protein's lone tryptophan residue was also involved in either phenylbutazone or warfarin binding. For this, two techniques have been used. Firstly, the effect on phenylbutazone and warfarin binding of the specific chemical modification of rat α -FP's lone tryptophan residue by NPS has been studied. Secondly, the effect of phenylbutazone binding on the intrinsic fluorescence of the protein's tryptophan residue has been investigated. As a reference, the same experiments were carried out with rat serum albumin.

The specificities of the drug-binding sites of rat α -FP

and serum albumin are discussed in the light of these results.

MATERIAL AND METHODS

Rat α -FP was purified as previously described (Cittanova *et al.*, 1974), and rat serum albumin was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Before their modification by NPS or spectrofluorimetric measurements the two proteins were treated with charcoal at neutral pH as previously described (Hervé *et al.*, 1984) in order to remove endogenous ligand(s) which could interfere in the binding studies. The concentrations of rat α -FP and serum albumin were determined by the method of Lowry *et al.* (1951) with bovine serum albumin grade A as standard. NPS, *N*-acetyltryptophanamide and warfarin were purchased from Sigma. Phenylbutazone and [14 C]phenylbutazone (19.9 μ Ci/mg) were generously given by Ciba-Geigy Ltd. (Basel, Switzerland). [14 C]Warfarin (177 μ Ci/mg) came from Amersham International (Amersham, Bucks., U.K.). Binding experiments and spectroscopic measurements were carried out in 0.1 M-sodium phosphate buffer, pH 7.40.

Modification of tryptophan by NPS

The lone tryptophan residue of rat α -FP and serum albumin was modified by NPS by the method of Boccu

Abbreviations used: α -FP, α -foetoprotein; NPS, 2-nitrophenylsulphonyl chloride.

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et al. (1970). To rat α -FP (7 mg/ml) and serum albumin (8 mg/ml) solutions in 20% acetic acid, NPS dissolved in 100% acetic acid was added in order to have a molar excess of NPS over protein of 7 in the case of rat α -FP and 5 in the case of rat serum albumin. After 5 h incubation at 4 °C with constant stirring, the mixtures were centrifuged for 10 min at 4 °C and 12000 rev./min ($r_{av.} = 10.80$ cm). The excess free NPS was then removed by chromatography on a Bio-Gel PS (Pharmacia) column (1.5 cm $\times$ 44 cm) in distilled water at pH 7.40. The number of NPS-modified amino acids per molecule of protein was determined spectrophotometrically in an 80% acetic acid medium at 365 nm for the NPS using the molar absorption coefficient of 4000 cm⁻¹·M⁻¹ for covalently bound NPS (Scoffone *et al.*, 1968) and by the method of Lowry *et al.* (1951) for the proteins. To determine the effect of the acetic acid on the proteins they were treated in the same way but without the addition of NPS. Also, in order to determine whether the reaction with NPS caused protein polymerization, electrophoresis in 12% (w/v) polyacrylamide gel in Tris/glycine buffer, pH 8.60, of the chemically modified rat α -FP and serum albumin was performed.

Since sulphonyl halides react selectively with tryptophan, and to a lesser extent with cysteine, in proteins, we have investigated the possibility that NPS could have reacted with one or both of the two cysteine residues of rat α -FP or with the one of rat serum albumin using the alkaline-cleavage method described by Boccu *et al.* (1970).

Binding experiments with warfarin and phenylbutazone

The binding experiments with rat α -FP or serum albumin that had been allowed to react with NPS were performed by equilibrium microdialysis as previously described (Hervé *et al.*, 1984, 1986). These experiments were also carried out with the acetic acid-treated controls. The concentrations of the proteins were 8–20 μ M, depending on the ligand. The drug concentrations varied from 2.5 to 1000 μ M, with a constant amount of radioactivity (0.37×10^3 Bq for phenylbutazone and 0.41×10^3 Bq for warfarin). Equilibrium was reached after 24 h agitation at 4 °C. The binding parameters of α -FP and albumin were determined by computer as previously described (Hervé *et al.*, 1984).

Spectrofluorimetric measurements

Fluorescence was measured with a FICA 55,000 MK II absolute differential spectrofluorimeter equipped with thermally regulated cells (20 °C). A solution of phenylbutazone (0.5 mM) was prepared in 0.01 M-sodium phosphate buffer, pH 7.40. Portions (2–188 μ l) of this solution were added sequentially to 2 ml of rat α -FP (4.5 μ M) or serum albumin (5.4 μ M) solutions to obtain molar drug/protein ratios in the range from 0 to 8.57 in the case of α -FP and 0 to 8.82 in that of albumin. After each addition of drug the fluorescence emission spectrum was recorded ($\lambda_{excitation} = 287$ nm) and the maximum emission determined. The maximum emission wavelengths were in the ranges 325–330 nm for rat α -FP and 335–340 nm for serum albumin. All the values of the fluorescence emission of α -FP and albumin, with and without addition of phenylbutazone, were related to the fluorescence emission at 355 nm of a solution of *N*-acetyltryptophanamide (10 μ M) taken as a reference in order to annul any variations of the fluorescence-emission intensities due to instrumental drift. The slope of the standard curve of fractional quenching of *N*-acetyltryptophanamide fluorescence by the same range of phenylbutazone concentrations (slope = 5631 ± 82 M⁻¹ phenylbutazone) was used as a correction factor for quenching of fluorescence by self-absorption. Quenching (Q) was defined as:

$$Q = 1 - F/F_0$$

where F_0 and F are the corrected fluorescence values of the protein solutions and the protein/phenylbutazone mixtures respectively. It was not possible to carry out the same experiments with warfarin, since this drug is itself fluorescent and absorbs at the emission wavelength of tryptophan. Therefore the number of parameters to be accounted for was beyond the precision of the method.

Theoretical calculations

For the spectroscopic study the concentrations of phenylbutazone bound to each of the two site classes of rat α -FP and serum albumin for each reagent concentrations, given the binding parameters for each of the proteins (Table 1), were calculated by using an iterative computer program developed in this laboratory.

Table 1. Binding parameters for rat serum albumin (RSA) and α -FP with warfarin and phenylbutazone before (Hervé *et al.*, 1984) NPS treatment, after 20% acetic acid treatment and after NPS treatment of the proteins

n_1 , n_2 , k_1 and k_2 are respectively the numbers and apparent intrinsic association constants of the high- and low-affinity sites respectively.

Protein	Drug . . .	Warfarin			Phenylbutazone		
	Parameter . . .	n_1	k_1 (M ⁻¹)	$n_2 \cdot k_2$ (M ⁻¹)	n_1	k_1 (M ⁻¹)	$n_2 \cdot k_2$ (M ⁻¹)
RSA							
Before NPS treatment		0.95 ± 0.07	(0.83 ± 0.08) × 10 ⁶	(0.43) × 10 ⁵	1.00 ± 0.12	(0.46 ± 0.04) × 10 ⁶	(0.62) × 10 ⁵
After 20% acetic acid treatment		0.70 ± 0.08	(0.97 ± 0.14) × 10 ⁶	(0.13) × 10 ⁵	1.12 ± 0.07	(0.17 ± 0.02) × 10 ⁶	(0.13) × 10 ⁵
After NPS treatment		0.72 ± 0.12	(0.98 ± 0.22) × 10 ⁶	(0.41) × 10 ⁵	1.65 ± 0.17	(0.10 ± 0.02) × 10 ⁶	(0.10) × 10 ⁵
α-FP							
Before NPS treatment		0.47 ± 0.02	(1.81 ± 0.10) × 10 ⁶	(0.13) × 10 ⁵	0.41 ± 0.03	(1.19 ± 0.19) × 10 ⁶	(0.35) × 10 ⁵
After 20% acetic acid treatment		0.53 ± 0.02	(3.53 ± 0.17) × 10 ⁶	(0.47 × 10 ⁵	0.46 ± 0.08	(1.29 ± 0.74) × 10 ⁶	(0.14) × 10 ⁵
After NPS treatment		0.47 ± 0.03	(2.07 ± 0.32) × 10 ⁶	(0.05) × 10 ⁵	0.53 ± 0.05	(0.39 ± 0.07) × 10 ⁶	(0.09) × 10 ⁵

Fig. 1. (a) Quenching of tryptophan fluorescence in rat serum albumin (●) and α -FP (▲) in the presence of bound phenylbutazone and (b) relative fluorescence quenching of serum albumin (●) and α -FP (▲) as a function of the drug/protein molar ratio

Concentrations were: rat serum albumin, $5.4 \mu\text{M}$; α -FP, $4.5 \mu\text{M}$; and phenylbutazone, $0.5\text{--}43 \mu\text{M}$ with albumin and $0.5\text{--}41 \mu\text{M}$ with a α -FP in 0.1 M -sodium phosphate buffer, pH 7.40. The activation and emission wavelengths were 287 nm and 340 nm for albumin and 287 nm and 330 nm for α -FP. Fluorescence intensities at 20 °C were related to the emission at 355 nm by *N*-acetyltryptophanamide ($10 \mu\text{M}$) and corrected for self-absorption. Bandwidths

RESULTS

It appeared that, under the experimental conditions used, the NPS reacted specifically with the lone tryptophan residue of rat α -FP and serum albumin, since no difference in the number of modified amino acids was found after alkaline cleavage of mixed aromatic/aliphatic disulphide which might have formed between NPS and free cysteine residues of the two proteins (Boccu *et al.*, 1970). This indicates that only the tryptophan residues of rat α -FP and albumin were labelled. Determination of the number of tryptophan residues modified by NPS with rat α -FP and serum albumin (1.17 and 1.39 respectively) confirmed the presence of only one residue per molecule of these proteins.

Electrophoresis of the modified proteins shows that NPS treatment does not cause dimerization of either.

The passage from an acid to a neutral pH after acid treatment of α -FP leads to a significant degree of denaturation (70–75% loss), whereas the NPS-treated α -FP was much more resistant to denaturation (55–60% loss), indicating that the NPS moiety had a protective or buffering effect. In any case denatured protein was removed by centrifugation before further experimentation.

Binding parameters of rat α -FP and serum albumin for warfarin and phenylbutazone after NPS treatment

The binding parameters obtained for the binding of the two drugs by the α -FP and albumin NPS derivatives are given in Table 1, where they are compared with those of the untreated proteins and the acid-treated controls. It can be seen that the modification of the lone tryptophan residue of rat α -FP or albumin does not significantly modify the binding parameters of either protein for warfarin. An increased affinity for warfarin was found with the acid-treated control for α -FP, but the protection against acid denaturation afforded by the NPS derivative probably also impeded this acid effect. For rat albumin, NPS treatment decreased the association constant for the high-affinity class of sites for phenylbutazone approx. 5-fold. However, a partial decrease in affinity was found with the acetic acid controls. This decrease is not simply due to protein denaturation, since the binding parameters of albumin for warfarin remained unaffected. For rat α -FP, NPS treatment led to a strong decrease (3.1-fold) in the high-affinity association constant for phenylbutazone, the number of these sites remaining unaltered [$n_1 = 0.4$, which may indicate a functional and molecular heterogeneity of the foetal protein (Hervé *et al.*, 1984)]. In contrast with that of albumin, the acid treatment of α -FP did not significantly modify the binding of phenylbutazone.

Fluorescence measurements

Fig. 1(a) shows that, in the presence of increasing phenylbutazone concentrations, the intrinsic fluorescence

were 7.5 nm. The horizontal lines show the minimum fluorescence for albumin (—) [Q_{max} (maximum quenching) = 0.82] and α -FP (----) (Q_{max} = 0.29) as calculated from reciprocal plots of the data. Experimental values were the means for four separate experiments. (b) Initial slopes are shown for the two quenching curves (—, albumin; ----, α -FP).

Fig. 2. Comparison between percentage maximum fluorescence quenching and the theoretically calculated fractional saturation of the high(—●)— and low(---▲)—affinity binding sites by phenylbutazone with rat serum albumin (●) and α -FP (▲).

A straight line with a slope of 1 indicates a direct relationship.

of rat α -FP and albumin progressively decreased. For serum albumin the successive spectra show a shift in emission maximum from 340 nm, the tryptophan maximum, to shorter wavelengths. This indicates that, in albumin, the tryptophan is normally in a highly polar environment which is rendered less polar by phenylbutazone binding. On the other hand, for α -FP the emission maximum (325 nm) of the successive spectra was not modified, suggesting that α -FP's tryptophan residue is normally in a much less polar environment than that of albumin. At low phenylbutazone/rat α -FP and phenylbutazone/albumin molar ratios (Fig. 1a) the fluorescence steadily decreased to a limit value, indicating a saturation of the drug-binding sites of rat α -FP and albumin. Maximum quenching values were determined by extrapolating the reciprocals of the mean corrected values by the method of Wallach *et al.* (1970). The quenching was much higher for albumin ($Q_{\max.} = 0.82$) than for α -FP ($Q_{\max.} = 0.29$). The initial slopes of the stoichiometric binding plots in Fig. 1(b) show that quenching rose twice as fast with α -FP as with albumin, possibly correlating with the fact that α -FP has only half the high-affinity sites of albumin (Hervé *et al.*, 1984). The intercepts ($n_1 + 1/k_1[P_r]$, where $[P_r]$ is the total protein concentration) of these initial slopes with $Q_{\max.}$ gave values of 1.02 and 1.74 for α -FP and albumin, which, in terms of the high-affinity parameters in Table 1, would have been expected to be approx. 0.6 and 1.4 respectively if bound phenylbutazone directly quenches the tryptophan. This is only an indication, since low-affinity binding is not considered.

Plots of the degree of fluorescence quenching relative to the theoretically calculated fractional saturation of each of the site classes for the two proteins are shown in Fig. 2. A direct relationship is shown by a straight line with a slope of 1. The Figure clearly shows that

quenching closely parallels the degree of occupancy of albumin's high-affinity site by phenylbutazone (slope = 0.86). For α -FP, however, this parallelism was not found, though quenching seemed more closely correlated with the degree of occupancy of the high-affinity sites than with that of the low-affinity sites.

DISCUSSION

Our previous studies on the specificities of the drug-binding sites of rat α -FP (Hervé *et al.*, 1986) have shown that the foetal protein contains, like albumin (Kragh-Hansen, 1985), only a small number of specific binding sites for biomolecules and drugs, with negative or positive co-operative effects acting between them. Rat α -FP and albumin have the particularity of containing only a single tryptophan residue, and the study of its participation in phenylbutazone and/or warfarin binding by the two proteins is thus possible. This study was carried out by using two different techniques: (1) specific chemical modification of the this residue in rat α -FP and albumin and (2) a study of its fluorescence quenching by phenylbutazone. From the results obtained it is obvious that both proteins present certain analogies. The warfarin binding by α -FP and albumin remains unaltered after NPS treatment, but this treatment did affect phenylbutazone binding. Similarly, phenylbutazone binding quenched the intrinsic tryptophan fluorescence of both proteins.

For rat serum albumin the NPS treatment greatly decreased the affinity for phenylbutazone, though a partial decrease was also found after treatment with acetic acid. Nevertheless the decrease in affinity for phenylbutazone after NPS derivatization was significantly greater than that after acid treatment. There exists a highly reactive lysine residue in albumin (Lys-199) (Bucala *et al.*, 1982) thought to be implicated in the warfarin/phenylbutazone binding site (Fehske *et al.*, 1981), and the decrease in affinity after acid treatment may be due to modification of this lysine residue [maybe via an acetylation (Walker, 1976)]. For rat α -FP, derivatization of the tryptophan residue by NPS did diminish high-affinity binding of phenylbutazone, and this was not due to the acid treatment.

The spectrofluorimetric studies showed that phenylbutazone quenches the intrinsic fluorescence of the tryptophan residues of rat α -FP and albumin, though to different extents. These results indicate that the lone tryptophan residue of these proteins is linked to phenylbutazone binding. Whereas our spectrofluorimetric results suggest a direct implication of albumin's lone tryptophan residue in the phenylbutazone-binding site, confirming those of Maes *et al.* (1979), who produced thermodynamic evidence for π - π electronic interactions between the drug and human serum albumin's lone tryptophan residue, this is not so for rat α -FP. The absence of a direct correlation between fluorescence quenching and site occupancy shows that some more complex phenomenon exists. Further, the emission wavelength of α -FP's tryptophan residue indicates that it is in a more hydrophobic environment than that of albumin and therefore may be more deeply buried in the protein. We have previously reported (Hervé *et al.*, 1986) a number of co-operativity effects involving the phenylbutazone high-affinity site and other binding sites on rat α -FP, which indicates that

high-affinity binding of phenylbutazone leads to a conformational change in the protein. It could be this which produces a change in the tryptophan residue's environment. Since such conformational changes, thermodynamically, are reciprocal, it is then not surprising that modification of the tryptophan by NPS has an effect of the phenylbutazone-binding site of the foetal protein.

The results after chemical modification of the two proteins (warfarin binding unaffected and phenylbutazone binding affected) support our previous proposition (Hervé *et al.*, 1986) that the two drugs occupy two different high-affinity sites on the foetal protein. They also support the proposal of Madsen & Ellis (1981), who worked on human serum albumin, that warfarin and phenylbutazone occupy two separate binding sites on the albumin molecule. However, our results disagree with the finding of Fehske *et al.* (1982) that NPS treatment of human serum albumin principally decreases warfarin binding by the protein. They also contradict the suggestion by those authors, on the basis of competitive-binding determinations, that the two drugs occupy the same binding area on the protein. The comparison of experimental displacements with theoretical displacement curves, as previously carried out by Hervé *et al.* (1986), could settle this question.

It therefore may be concluded that although phenylbutazone binding is inhibited by tryptophan derivatization of α -FP and albumin and also leads to quenching of the tryptophan fluorescence of the two proteins, the mechanisms are not the same in both proteins. The lone tryptophan residue of serum albumin appears to participate in the phenylbutazone-binding site, but that of α -FP seems to be elsewhere in the molecule. In this case, the two molecules would display a functional similarity, despite a structural difference.

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