Rat α -Foetoprotein

PURIFICATION, PHYSICOCHEMICAL CHARACTERIZATION, OESTROGEN-BINDING PROPERTIES AND CHEMICAL MODIFICATION OF THE THIOL GROUP

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1. Rat α -foetoprotein, an oestrogen-binding foetal globulin, was isolated in large quantities from amniotic fluid and serum by preparative electrophoresis on polyacrylamide slab gels or by chromatography on an immunoabsorbent column. Subsequently the two electrophoretic forms of this protein were separated by electrophoresis on the same medium. 2. Both forms were found to show identical binding with oestradiol. From the extrinsic fluorescence of the bound dye 8-anilinonaphthalene-1-sulphonic acid it was shown that the polarity of the binding site is practically identical for both forms. One residue of tryptophan was determined for both forms. The two electrophoretic variants display the same amount of secondary structure as demonstrated by circular dichroism. 3. The affinity of total α -foetoprotein for oestradiol as a function of pH was studied by using a Sephadex G-25 gel-equilibration method. Maximal binding occurred at pH 8.5. Only a fractional number of binding sites per molecule could be measured at pH 7.4, whereas at higher pH the number of sites was very close to unity. There was no significant effect of pH on the value of the association constant ($K_a = 4.3 \times 10^7 \pm 1.2 \times 10^7 \text{ M}^{-1}$). 4. Displacement experiments of bound labelled oestradiol with various steroids have permitted investigation of the specificity of α -foetoprotein. This foetal globulin binds rather strongly compounds that display the rigid structure of the oestratriene skeleton (oestradiol, oestrone). Diminished binding for diethylstilboestrol and a diethylstilboestrol affinity label was observed. No binding was measured with a more flexible structure such as hexoestrol [4,4'-(1,2-diethylethane-1,2-diyl)bisphenol]. 5. Chemical modification of cysteine residues of α -foetoprotein with two alkylating reagents [iodoacetic acid and 8-[N-(iodoacetylaminomethyl)amino]naphthalene-1-sulphonic acid] has very little effect on the oestrogen binding. It is suggested that the oestrogen-binding site does not contain a cysteine residue. From the kinetics of alkylation and from the fluorescence properties of the chemically bound thiol reagent 8-[N-(iodoacetylaminomethyl)amino]naphthalene-1-sulphonic acid, it was demonstrated that the very-slow-reacting thiol group is probably located in a non-polar region of the molecule.

α -Foetoprotein is a serum glycoprotein present in relatively high concentration in foetal and neonatal sera and in the amniotic fluid of many species. It is found in only trace amounts in adults but reappears in pathological states such as hepatomas, teratomas or liver injury (Abelev, 1971; Masseyeff, 1972; Ruoslahti *et al.*, 1974). The biological role of this protein is not clearly understood although activities involving strong oestrogen binding and an immunosuppression were reported (Arnon *et al.*, 1973; Aussel *et al.*, 1973; Swartz *et al.*, 1974; Laurent *et al.*, 1975; Benassayag *et al.*, 1975; Versée & Barel, 1977; Murgita & Tomasi, 1975).

Several purification procedures for α -foetoprotein have been published, affinity chromatography being used on anti-(α -foetoprotein) or anti-immunoabsorbents (Watabe, 1974; Cittanova *et al.*, 1974), on concanavalin A and *Ricinus communis* agglutinin columns (Pagé, 1973; Kerckaert *et al.*, 1977; Bayard

et al., 1977) and preparative slab-gel electrophoresis (Kerckaert *et al.*, 1975). As was shown for many other glycoproteins, the molecular heterogeneity of rat α -foetoprotein observed on Sepharose-linked concanavalin A and on polyacrylamide-gel electrophoresis may be due to variations in the type and amount of carbohydrate covalently attached to the polypeptide chain (Watanabe *et al.*, 1975; Kerckaert *et al.*, 1977).

Differences in oestrogen affinity between the electrophoretic variants of rat α -foetoprotein have been described (Benassayag *et al.*, 1975). On the contrary the two variants separated by their affinity for concanavalin A–Sepharose display identical binding of oestradiol (Soloff *et al.*, 1976). A clear explanation of the existence of some molecular forms that display high or low affinity for oestrogens is therefore not available at present.

The similarity of the physicochemical properties of α -foetoprotein and albumin suggested that this

protein may be evolutionarily related to albumin (Grigorova *et al.*, 1977; Barel & Versée, 1977). Immunological cross-reactions between the reduced and alkylated forms of human α -foetoprotein and albumin, and preliminary sequence work (Ruoslahti & Engvall, 1976; Ruoslahti & Terry, 1976), suggest that both proteins are believed to have originated as a result of duplications of a common ancestral gene with subsequent divergent development. Therefore it occurred to us that complementary information concerning the physical and chemical properties of α -foetoprotein would be of interest for comparison with albumin. In addition to this physicochemical approach a better knowledge of the oestrogen-binding properties of this foetal antigen was also needed.

Materials and Methods

Materials

Oestradiol, oestrone, testosterone, diethylstilboestrol, hexoestrol [4,4'-(1,2-diethylethane-1,2-diyl)biphenol], 8-anilino-naphthalene-1-sulphonic acid, methanesulphonic acid, 3-(2-aminoethyl)indole and sodium dodecyl sulphate were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. 8-[N-(Iodoacetyl-aminoethyl)amino]naphthalene-1-sulphonic acid was obtained from Aldrich Europe, Beerse, Belgium. *N*-Ethylmaleimide and iodoacetic acid were from Fluka, Buchs, Switzerland, and quinine sulphate was from BDH Chemicals, Poole, Dorset, U.K. Iodo[2-¹⁴C]acetic acid (sp. radioactivity 50 mCi/mmol), [4-¹⁴C]oestradiol (sp. radioactivity 50 mCi/mmol), [2,4,6,7-³H]oestradiol (sp. radioactivity 100 Ci/mmol), [2,4,6,7-³H]oestrone (sp. radioactivity 100 Ci/mmol) and [2,4,6,7-³H]testosterone (sp. radioactivity 100 Ci/mmol) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. All other reagents and chemicals were reagent grade. Rat serum albumin (Sigma) was purified on passage through a column of Sephadex G-100.

Purification of α -foetoprotein

At the appropriate gestational age (15–19 days), amniotic fluid and foetal plasma were collected from Wistar rat fetuses. The purification procedure we followed was essentially the same as those previously described by Cittanova *et al.* (1974) and Kerckaert *et al.* (1975). Briefly it consists in an initial step where haemoglobin is removed from the serum by batchwise adsorption on CM-cellulose in 0.01 M-potassium phosphate buffer, pH 6.0.

The second purification step was achieved either by (a) preparative slab-gel electrophoresis on polyacrylamide or (b) affinity chromatography on an immuno-adsorbent column of Sepharose coupled

with the rabbit antibodies against rat adult serum proteins.

The apparent molecular weight of α -foetoprotein in sodium dodecyl sulphate/polyacrylamide gels was determined by the method of Weber & Osborn (1969).

Amino acid composition

Protein samples were hydrolysed in 6M-HCl at 110°C *in vacuo* for 24, 48 and 72 h. The amino acid mixtures were analysed in a Jeol JLC-5AH analyser. Total cysteine residues were determined as carboxymethylcysteine after reduction with 2-mercaptoethanol in 8M-urea and carboxymethylation with iodoacetic acid. Tryptophan was determined separately by the fluorimetric method of Pajot (1976). Hexosamines were also determined on the amino acid analyser after hydrolysis of the protein with 6M-HCl or with 4M-methanesulphonic acid containing 0.2% 3-(2-aminoethyl)indole for 24 h at 110°C.

Circular-dichroism measurements

Spectra were recorded on a Cary 61 dichrograph at room temperature in 1.0 and 10 mm path-length silica cells. Protein concentrations varied from 0.1 to 1.0 mg/ml in 0.01 M-phosphate buffer at pH 7.4 and 8.5. The experimental curves were analysed by the method of Chen *et al.* (1972) to compute the percentage of α -helix, β -pleated sheet and random coil present.

Fluorescence measurements

Fluorescence measurements were performed at room temperature in an Aminco-Bowman SPF spectrofluorimeter. The excitation wavelength of 8-anilino-naphthalene-1-sulphonic acid-protein complexes was 350 nm. The quantum yields were calculated as described previously (Barel *et al.*, 1971). The emission wavelengths were not corrected for response of the photomultiplier.

Chemical modifications

The carboxymethylcysteine derivative of α -foetoprotein was obtained after alkylation with labelled iodoacetic acid (iodo[2-¹⁴C]acetic acid, sp. radioactivity 50 mCi/mmol) at pH 8.5 in the absence of denaturing agents, with the method described by Gurd (1972). The excess of reagents was removed by either extensive dialysis or passage through Sephadex G-25. The stoichiometry of incorporation was calculated from uptake of radioactivity. The thiol groups of α -foetoprotein were also allowed to react with *N*-ethylmaleimide at physiological pH and in the absence of denaturing agents as described by Riordan & Vallee (1972). The free thiol group was chemically modified with a fluorescent thiol reagent 8-[N-(iodoacetyl-aminoethyl)amino]naphthalene-1-

sulphonic acid (Hudson & Weber, 1973). Usually 5–10 mg of protein was dissolved in 1 ml of 0.1 M-Tris/HCl buffer, pH 8.5, and allowed to react in the dark with a 2 M excess of 8-[N-(iodoacetyl aminoethyl)-amino]naphthalene-1-sulphonic acid for either 24 h at 5°C or 4–6 h at 25°C. The labelled protein was purified on a column of Sephadex G-25. The amount of 8-[N-(iodoacetyl aminoethyl)amino]naphthalene-1-sulphonic acid chemically bound to α -foetoprotein was determined by measuring $A_{342.5}$ of the solution, with an extinction coefficient of $6300 \text{ M}^{-1} \cdot \text{cm}^{-1}$ for 8-[N-(iodoacetyl aminoethyl)amino]naphthalene-1-sulphonic acid (Hudson & Weber, 1973).

Binding studies

The interaction between α -foetoprotein and various ligands were studied by two methods: (a) an equilibrium dialysis technique on Sephadex G-25 as described by Pearlman & Crpy (1967) and Goertz *et al.* (1973) and (b) a DEAE-cellulose filter disc assay as described by Santi *et al.* (1973) and Khan & Rosner (1977).

Results and Discussion

Purification of α -foetoprotein

Complete purification of rat α -foetoprotein was achieved by two different methods: passage through an immunoabsorbent column and preparative electrophoresis on polyacrylamide slab gels. Examination of both preparations on analytical polyacrylamide-gel electrophoresis revealed the typical microheterogeneity of this protein (two bands: 2/3 slow-moving form and 1/3 fast-moving form). Both preparations were also submitted to sodium dodecyl sulphate/polyacrylamide-gel electrophoresis; this time a single band corresponding to an apparent mol.wt. of 71000 ± 3000 was revealed. No precipitation arc was observed after diffusion against a rabbit antiserum against adult rat serum proteins. These results show that our preparations were essentially free of contaminating proteins such as albumin, transferrin and globulins. By preparative slab-gel electrophoresis, we were able to prepare sufficient amounts of α -foetoprotein weekly (about 10–20 mg).

Amino acid analyses

The amino acid composition as well as the amino sugars are shown in Table 1, together with the recent data reported by Kerckaert *et al.* (1975). One tryptophan residue per molecule of protein was determined by fluorescence of the fully denatured protein in 6 M-guanidine hydrochloride. There was no difference in the tryptophan content between the two forms of α -foetoprotein. Our data on the number of trypto-

phan residues for total α -foetoprotein disagree with the result of Grigorova *et al.* (1977). These authors reported a much higher content of tryptophan (about 4.0 mol of residues/mol). Taking a possible sequence homology between rat albumin and α -foetoprotein into account, we found in an analogous way also one tryptophan residue for albumin. This last result agrees with the data obtained by Feldhoff & Peters (1976), who studied the tryptophan content of several albumins.

Our amino acid composition data are, within experimental error, in agreement with the results of Kerckaert *et al.* (1975), with the exception of glycine, which was significantly lower than the value reported by others. The reason for this difference with glycine is unknown, but may be due to the amino acid adsorbed on α -foetoprotein from the glycine-containing electrophoresis buffer that is used for the purification. Our preparations were extensively

Table 1. Amino acid composition and content of amino sugars of total rat α -foetoprotein

The values are numbers of residues per molecule of α -foetoprotein. The mol.wt. is 71000; 65700 for the peptide portion and 5300 for the carbohydrate content. Values represent averages of 24, 48 and 72 h hydrolyses. The labile amino acids were back-extrapolated to time 0 to correct for decomposition. Total cysteine plus half-cysteine were determined as S-carboxymethylcysteine. Tryptophan was separately determined by the spectrofluorimetric method of Pajot (1976). Abbreviation: N.D., not determined.

Amino acid and amino sugar	Residues/molecule (mol integer)	
	Proposed	Data of Kerckaert <i>et al.</i> (1975)
Aspartic acid	58 $\pm$ 1	51
Threonine	26 $\pm$ 1	30
Serine	39 $\pm$ 1	49
Glutamic acid	83 $\pm$ 6	91
Proline	26 $\pm$ 4	25
Glycine	30 $\pm$ 2	41
Alanine	45 $\pm$ 4	49
Valine	30 $\pm$ 4	25
Methionine	9 $\pm$ 1	11
Isoleucine	24 $\pm$ 1	25
Leucine	61 $\pm$ 3	54
Tyrosine	14 $\pm$ 1	14
Phenylalanine	22 $\pm$ 2	25
Histidine	13 $\pm$ 1	17
Lysine	43 $\pm$ 2	41
Arginine	20 $\pm$ 1	20
Cysteine + half-cysteine	28 $\pm$ 2	23
Tryptophan	1	N.D.
N-Acetylglucosamine	8	8
N-Acetylgalactosamine	1	—

Fig. 1. Scatchard plot of the binding of oestradiol to total α -foetoprotein

Equilibrium constants for steroid-protein interactions at 25°C were obtained by equilibrium dialysis with Sephadex G-25 in a batchwise fashion. The buffer used was 0.1M-potassium phosphate, pH7.4 and 8.5. The concentration of α -foetoprotein was maintained fixed (100nM). The oestradiol concentration ranged from 2nM to 1000nM. The association constant and stoichiometry of binding of α -foetoprotein at (a) pH7.4 and (b) pH8.5 were respectively $4.6 \times 10^7 \text{ M}^{-1}$ and 0.26 and $5.3 \times 10^7 \text{ M}^{-1}$ and 1.08. S_b and S_u are ligand bound and unbound; P_t is total protein concentration.

dialysed and passed through Sephadex G-25. It is of interest that the amino acid analyses of α -foetoprotein from different species (human, rat, mouse and rabbit) show a striking similarity.

Binding activity with oestrogens

The association constant and the stoichiometry of binding sites for the interaction of oestradiol with pure α -foetoprotein were determined from Scatchard (1949) plots (Fig. 1).

The effect of pH on the oestrogen binding is shown in Fig. 2. Only a fractional number of binding sites could be determined at pH7.4 (0.4mol/mol of protein), whereas at higher pH a stoichiometry of one binding site was observed. The pH optimum for binding is 8.0–8.5, depending on the buffer system, and from pH8.0 to more alkaline pH values the number of binding sites decreases. Relatively little work has been devoted to the influence of pH on steroid-albumin interactions. However, Romeu *et al.* (1976) have recently shown a similar strengthening of the association of various steroids with bovine serum albumin, when the pH rises from 5.0 to 9.0. The decrease of steroid binding above pH8.0–8.5 may be due to some structural transition by analogy with the well-known 'neutral transition' observed by Zurawski & Foster (1974). However, a possible role of some tyrosine residue, such as deprotonation of the phenol,

Fig. 2. Effect of pH on the binding of α -foetoprotein to oestradiol

Stoichiometry of binding, n (mol of oestradiol/mol of protein), as well as the association constant for the interaction of oestradiol protein at 25°C, were determined from Scatchard plots. The association constant remained, within experimental error, constant between pH6.5 and 9.0. ○, 0.1 M-Barbital/HCl buffer; ●, 0.1 M-phosphate buffer.

could also be involved. There was no effect of pH on the association constant. K_a remained constant from pH7.5 to 9.0 ($K_a = 2.0(\pm 1.0) \times 10^7 \text{ M}^{-1}$ in barbital/HCl buffer and $4.3(\pm 1.2) \times 10^7 \text{ M}^{-1}$ in phosphate buffer).

The Scatchard plots always gave, within experimental error, straight lines (Fig. 1), which suggests that α -foetoprotein displays only a single class of binding sites. In contrast, Benassayag *et al.* (1975) reported curvature in the Scatchard plot at very low steroid concentration with the possible existence of a second class of binding sites with high affinity ($K_a = 2 \times 10^{10} \text{ M}^{-1}$), but low stoichiometry (less than 0.1 mol/mol of protein).

It is clear from Figs. 1 and 2 that the number of binding sites at physiological pH (pH7.4) is a fractional number (0.4mol). It is logical to assume that α -foetoprotein is capable of binding various other compounds such as fatty acids, drugs and dyes. α -Foetoprotein was extensively dialysed or passed through Sephadex G-25 at pH3.0. These treatments seemed to increase the number of binding sites (from 0.4 to 0.6mol). Considering this very small (if significant) increase in binding, it is logical to assume that the presence of some endogenous compounds on the surface of α -foetoprotein interferes very little with the steroid-binding capacity.

The two electrophoretic variants of α -foetoprotein showed, within experimental error, identical binding characteristics at pH8.0 in phosphate buffer (identical

Fig. 3. Displacement of bound [^3H]oestradiol with various ligands

For this experiment the gel-equilibration assay (Goertz *et al.*, 1973) with Sephadex G-25 was used in 0.1 M-phosphate buffer, pH 8.5 and 25°C. A constant amount of [^3H]oestradiol was added to each assay tube (50 pmol). The protein concentration in the assay tube was also kept constant (150 pmol). Various amounts of the different unlabelled ligands were added as shown (from 50 pmol to 5000 pmol). From the radioactivity of the external volume, S_b , the amount of labelled oestradiol bound to α -foetoprotein, is calculated. The amount of bound labelled oestradiol in the absence of any inert ligand was taken as 100% binding capacity. The residual binding was plotted against the total amount of unlabelled ligand. $\circ$, Hexoestrol; $\triangle$, diethylstilboestrol; $\square$, acetyldiethylstilboestrol; $\bullet$, oestradiol; $\times$, diethyl-iodoacetylstilboestrol; $\blacktriangledown$, oestrone.

stoichiometry of binding and association constant). Benassayag *et al.* (1975) reported quantitative differences in binding parameters of the two forms (slow fraction shows 0.8 mol of binding site/mol and the fast fraction shows a very low value, 0.1 mol of binding site/mol). The association constant for the binding of oestrone with α -foetoprotein was of comparable magnitude ($K_a = 2 \times 10^7 \text{ M}^{-1}$ and stoichiometry of binding is 0.7 mol/mol of protein at pH 8.5). Under similar experimental conditions no binding with testosterone could be detected. These data confirm the results of Aussel *et al.* (1973), Swartz *et al.* (1974) and Laurent *et al.* (1975).

From the DEAE-cellulose filter-disc assay an association constant of $0.7 \times 10^7 \pm 0.3 \times 10^7 \text{ M}^{-1}$ and a stoichiometry of 0.5 ± 0.2 may be calculated by Scatchard (1949) analysis at pH 7.4. These values are in reasonably good agreement with those obtained by the real equilibration technique on Sephadex G-25 (see Fig. 1 for comparison).

Displacement of bound oestradiol with various ligands

To determine the interactions of several other steroids and an affinity label with α -foetoprotein, experiments on the displacement of radioactive oestradiol were performed. The results of the interactions with hexoestrol, diethylstilboestrol, acetyldiethylstilboestrol and iodoacetyldiethylstilboestrol are shown in Fig. 3 as a percentage of residual binding as a function of added amount of ligand. As controls, displacement of radioactive oestradiol with unlabelled oestradiol and oestrone were included for comparison.

Oestrone and oestradiol are equally effective in displacing bound oestradiol, whereas the affinity of diethylstilboestrol, acetyldiethylstilboestrol and iodoacetyldiethylstilboestrol for this protein is significantly lower. From these figures it was possible to compute a value of $7 \times 10^5 \text{ M}^{-1}$ for the affinity constant of α -foetoprotein for diethylstilboestrol. These results confirm the experiments of Laurent *et al.* (1975), who have used the quenching of bound 8-anilinonaphthalene-1-sulphonic acid fluorescence when various steroids are added to the medium to compute a degree of interfering action. Hexoestrol was unable to displace oestradiol. The lack of displacement capacity of hexoestrol can be possibly explained in terms of more or less total flexibility of this molecule. The lower affinity of diethylstilboestrol compared with that of oestradiol (a decrease in affinity by a factor of 50) can be correlated with some restricted rotational freedom of the two side chains. It is worth noting that both oestradiol and oestrone possess the rigid oestratriene skeleton necessary for maximum binding.

Extrinsic fluorescence of the complex between 8-anilinonaphthalene-1-sulphonic acid and α -foetoprotein

8-Anilinonaphthalene-1-sulphonic acid is used as a fluorescent probe for hydrophobic sites of albumin (Daniel & Weber, 1966). Upon binding to α -foetoprotein, the dye becomes fluorescent with an emission peak near 465 nm (Laurent *et al.*, 1975). This extrinsic fluorescence was used to investigate possible conformational differences between both electrophoretic forms of α -foetoprotein. The fluorescence properties of the two α -foetoprotein-dye complexes are summarized in Table 2. Both forms show, within experimental error, identical extrinsic fluorescence characteristics (quantum yield and wavelength of maximal fluorescence). This suggests that the environment of the 8-anilinonaphthalene-1-sulphonic acid-binding site on both electrophoretic forms of α -foetoprotein is essentially the same. Laurent *et al.* (1975) also showed that competition exists between 8-anilinonaphthalene-1-sulphonic acid and various steroids for the same binding site.

Table 2. *Quantum yield and wavelength of maximum fluorescence of the α -foetoprotein-8-anilidonaphthalene-1-sulphonic acid complex and of 8-anilidonaphthalene-1-sulphonic acid in water*

The fluorescence spectra were recorded at 25°C in 0.01 M-phosphate buffer, pH 7.4. Excitation wavelength was 350 nm. The fluorescence intensities were referred to that of a standard solution of quinine sulphate to correct for instrumental instabilities and to enable the calculation of quantum yields. The α -foetoprotein derivatives were studied at the same concentration, 3 μ M, in the presence of 5 μ M-8-anilidonaphthalene-1-sulphonic acid.

Compound	Quantum yield	Position of spectral maximum (nm)
Slow α -foetoprotein-8-anilidonaphthalene-1-sulphonic acid complex	0.036 ± 0.004	469
Fast α -foetoprotein-8-anilidonaphthalene-1-sulphonic acid complex	0.043 ± 0.004	469
8-Anilidonaphthalene-1-sulphonic acid*	0.010 ± 0.004	555

* Data of Turner & Brand (1968).

Chemical modification of the free thiol groups with various alkylating reagents

Alkylation of the free cysteine residues of α -foetoprotein was carried out with labelled iodoacetic acid. As shown in Fig. 4, the reaction proceeds extremely slowly at pH 7.4 and 25°C. After 24 h only 0.2–0.3 mol of iodoacetic acid was incorporated. The extrapolated value obtained through a double reciprocal plot indicated 1.2 mol of label/mol of protein. As would be expected, the reaction occurs more rapidly at pH 8.5 and higher temperature (37°C); after only 8 h reaction, the alkylation was complete, 1.05 ± 0.05 mol of iodoacetic acid/mol being incorporated.

Amino acid analysis of the chemically modified protein primarily showed the presence of carboxymethylcysteine and some traces of *N*^ε-dicarboxymethyl-lysine, as modified residues. The occurrence of any alkylation of histidine or methionine residues could not be revealed.

When the alkylation with iodoacetic acid was carried out at pH 8.5 in the presence of a saturating concentration of oestradiol (about 37 μ M), the amount of incorporated labelled iodoacetic acid was essentially the same (0.98 mol of alkylating reagent incorporated/mol of protein), which suggests that oestradiol has no protection effect in carboxymethylation of the free thiol group.

The present data confirm the result of Grigorova

Fig. 4. *Reaction of α -foetoprotein with iodo[14 C]acetic acid* α -Foetoprotein (0.15 μ mol) was incubated at 25°C with 15.6 μ mol of iodo[2- 14 C]acetic acid (sp. radioactivity 0.24 Ci/mol) in 11 ml of 0.1 M-phosphate buffer, pH 7.4, in the dark and under N_2 . At indicated times portions (2 ml) were removed from the reaction mixture, cooled in an ice bath, passed through a column of Sephadex G-25 and counted for radioactivity.

et al. (1977), who determined only 0.35 mol of cysteine/mol of protein by the method of Ellman (1959).

The second reagent used, *N*-ethylmaleimide, is also very specific for cysteine residues. With radioactive *N*-ethylmaleimide it was possible to measure the formation of *S*-(ethylsuccinimido)cysteine from the uptake of 14 C after passage through a column of Sephadex G-25. The rate of *N*-ethylmaleimide incorporation was extremely slow. After 6 h reaction in 0.1 M-potassium phosphate, pH 7.4 and 37°C, only 0.1 mol of cysteine residues was alkylated. The presence of 2 M-urea in the reaction medium had no effect on the degree of modification of cysteine with the reagent.

The available evidence confirms that the unique cysteine residue of α -foetoprotein reacts very slowly with two alkylating reagents. It was postulated that the thiol group is partially blocked with reversible alkylating reagents and/or partially oxidized by air. It has been shown that native albumin is also a mixture of mercaptalbumin, which has one free thiol group/molecule, and non-mercaptalbumin, which has none (Andersson, 1969).

The third alkylating derivative used was a fluorescent thiol reagent, 8-[*N*-(iodoacetyl-aminoethyl)-amino]naphthalene-1-sulphonic acid, which was first described by Hudson & Weber (1973). This compound combines the reactivity of acetamide toward thiol groups with the fluorescence properties of naphthalenesulphonic acid. When a 2 M excess of 8-[*N*-(iodoacetyl-aminoethyl)-amino]naphthalene-1-sulphonic acid was allowed to react with native α -foetoprotein, only 0.3 ± 0.2 mol of label was incorporated/mol.

To activate the thiol group of α -foetoprotein, a pretreatment with 5mM-dithiothreitol was carried out before alkylation. Under these conditions 0.8 ± 0.2 mol of label was readily incorporated/mol. As shown in Fig. 5, the emission spectrum of 8-[N-(iodoacetyl-aminoethyl)amino]naphthalene-1-sulphonic acid in water is rather low and 'red-shifted'. When this compound was chemically incorporated into α -foetoprotein, a substantial increase of quantum yield was observed with a 'blue shift' of maximal fluorescence.

Table 3 contains the emission maxima and quantum yields of 8-[N-(iodoacetyl-aminoethyl)amino]naphthalene-1-sulphonic acid in water, in *NN*-dimethylformamide and chemically bound to α -foetoprotein. From the wavelength of maximum fluorescence (465 nm) and increase of quantum yield, it is reasonable to assume that the micro-environment of the bound thiol dye is non-polar.

Carboxymethylation of the free thiol group of α -foetoprotein with iodoacetic acid yields a mono-alkylated derivative. This derivative does not react with 8-[N-(iodoacetyl-aminoethyl)amino]naphthalene-1-sulphonic acid, as shown by the absence of absorption at 342.5 nm and fluorescence at 465 nm. This result shows that only the free thiol group of α -foetoprotein reacts with this fluorescent probe and that this very-slow-reacting cysteine is located in a rather hydrophobic part of the molecule.

Effect of chemical modification on the oestrogen-binding property

The effect of alkylation with iodoacetic acid and 8-[N-(iodoacetyl-aminoethyl)amino]naphthalene-1-sulphonic acid on the oestrogen-binding capacity is shown in Table 4. The introduction of a negative charge (carboxymethylation) or a rather bulky substituent 8-[N-(iodoacetyl-aminoethyl)amino]naphthalene-1-sulphonic acid] has very little effect on stoichiometry and association constant.

In contrast with what preliminary work has suggested (Versée & Barel, 1977), it is assumed that the cysteine residue is not implicated in the oestrogen-binding site. When all the disulphide bridges of this protein were reduced and alkylated, a complete loss of binding was observed. This treatment, however, is drastic and it is normal to observe a complete lack of binding when all the disulphide bridges are opened.

Circular-dichroism spectra

The circular-dichroism spectrum of α -foetoprotein in the far (200–250 nm) and near (260–310 nm) u.v. is shown in Figs. 6(a) and 6(b).

As shown by Aubert *et al.* (1977), this protein shows two minima at 208 and 222 nm. The near-u.v.

Fig. 5. Emission spectra of 8-[N-(iodoacetyl-aminoethyl)amino]naphthalene-1-sulphonic acid in water and covalently bound to α -foetoprotein

The concentration of the free fluorescent thiol reagent in water was $16 \mu\text{M}$ (—). The concentration of α -foetoprotein was $24 \mu\text{M}$; 0.8 ± 0.2 mol of reagent/mol was chemically bound to the protein (----). The spectra were recorded in 0.1M-phosphate buffer, pH 7.4 and 25°C . The excitation wavelength was 350 nm.

Table 3. Quantum yield and wavelength of maximum fluorescence of 8-[N-(iodoacetyl-aminoethyl)amino]naphthalene-1-sulphonic acid in water, in *NN*-dimethylformamide and chemically bound to α -foetoprotein

The concentration of 8-[N-(iodoacetyl-aminoethyl)amino]naphthalene-1-sulphonic acid in water and in *NN*-dimethylformamide was $16 \mu\text{M}$. The concentration of α -foetoprotein in 0.1 M-phosphate buffer, pH 7.4, was $24 \mu\text{M}$ (0.8 ± 0.2 mol of 8-[N-(iodoacetyl-aminoethyl)amino]naphthalene-1-sulphonic acid/mol chemically bound to the protein).

Compound	Solvent	Quantum yield	Wavelength of maximal fluorescence (nm)
8-[N-(Iodoacetyl-aminoethyl)amino]naphthalene-1-sulphonic acid	Water	0.04	530
8-[N-(Iodoacetyl-aminoethyl)amino]naphthalene-1-sulphonic acid	<i>NN</i> -Dimethylformamide	0.71	463
8-[N-(Iodoacetyl-aminoethyl)amino]naphthalene-1-sulphonic acid- α -foetoprotein	Water	0.27	465

Table 4. Interaction of oestradiol with native and modified α -foetoprotein

Association constant and stoichiometry of binding were obtained through Scatchard plots as described in Fig. 1. The alkylation of α -foetoprotein with iodoacetic acid gave 1.1 ± 0.1 mol of *S*-carboxymethylcysteine/mol of protein and with 8-[*N*-(iodoacetylaminomethyl)amino]naphthalene-1-sulphonic acid, 0.8 ± 0.2 mol of dye chemically incorporated per mol of protein. The buffer used for the binding assay was 0.1 M-phosphate, pH 8.5.

Protein	Stoichiometry of binding	$10^7 \times$ Association constant (M^{-1})
Control	1.0	3.5
Carboxymethylated	0.7	1.1
Labelled with 8-[<i>N</i> -(iodoacetylaminomethyl)amino]naphthalene-1-sulphonic acid	0.7	2.9

Fig. 6. Circular-dichroism spectra of native and denatured α -foetoprotein

(a) Spectrum in the near u.v. with 1.0 mg of protein/ml in a 1 cm cell. (b) Spectrum in the far u.v. with 0.1 mg of protein/ml in a 0.1 cm cell. Solutions were in 0.01 M-phosphate buffer, pH 8.5. Spectra for native α -foetoprotein (—) and α -foetoprotein renatured from 6M-urea at pH 8.0 are superimposed (---); ---, α -foetoprotein in 2M-urea at pH 8.0; -.-.-, α -foetoprotein in 6M-urea at pH 3.0.

spectrum displays a rather large band with two small minima centred at 260 and 270 nm, which presumably correspond to contributions of phenylalanine, cysteine and tyrosine chromophores. In excellent agreement with Aubert *et al.* (1977), the spectra obtained for the two electrophoretic variants were identical within experimental error. Analysis of the circular-dichroism data of Chen *et al.* (1972) indicates about 52% as a helical and 18% as a β structure. The percentage of α -helix obtained for various serum albumins is roughly the same (40–60%). In addition to sequence homology, both proteins show similar amounts of secondary structure.

The circular-dichroism data in the far u.v. do not agree with a previous report from our laboratory

(Versée & Barel, 1976), where, based on a lower ellipticity, only 30–35% in the helix form was calculated for α -foetoprotein purified by immuno-adsorbent columns. This difference between the helix content of both α -foetoprotein preparations may be significant; a possible denaturation or degradation might have occurred during passage through the immuno-adsorbent. However, the identical circular-dichroism spectra of α -foetoprotein in the presence or the absence of 2M-urea rather discounts this explanation.

A major loss of secondary structure occurs at pH 3.0 in 6 M-urea. The denaturation is reversible after stepwise dialysis against potassium phosphate, the circular-dichroism spectrum of the native protein being fully restored.

Addition of a saturating concentration of oestradiol has no effect on the near- and far-u.v. spectra of total, slow and fast forms of α -foetoprotein.

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