High affinity of α -foetoprotein for arachidonate and other fatty acids

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Fatty acid analysis of purified bovine α -foetoprotein showed it to contain 2.7 mol of fatty acid/mol of α -foetoprotein. Purified α -foetoprotein focused at isoelectric point 4.8. Removal of bound ligands from α -foetoprotein by charcoal treatment changed its isoelectric point to 5.2. This change could be reversed by addition of exogenous fatty acids to the defatted α -foetoprotein. Albumin isolated from the same foetal calf serum source as α -foetoprotein contained 1.4 mol of fatty acid/mol of protein. α -Foetoprotein and albumin contained comparable amounts of fatty acids with 14 to 18 carbon atoms, but α -foetoprotein contained 16 times as much of the long-chain polyunsaturated fatty acids as albumin. α -Foetoprotein was found to have slightly higher affinity for palmitate and linoleate and severalfold higher affinity for arachidonate than albumin. These findings suggest that α -foetoprotein may play a role in the foetal metabolism of the long-chain polyunsaturated fatty acids.

α -Foetoprotein is a major foetal plasma protein produced in large quantities in the foetal liver and yolk sac. In adults, the concentration of α -foetoprotein is very low except in cases of primary liver cancer and teratocarcinoma (for review see Sell & Becker, 1978; Ruoslahti & Seppälä, 1979). The biological role of α -foetoprotein is not known.

Homologies have been found between the amino acid sequence of cyanogen bromide fragments of α -foetoprotein and the sequence of albumin (Ruoslahti & Terry, 1976), suggesting that α -foetoprotein could have functions similar to those of albumin. Recent results on the binding of various small-molecular-weight ligands to α -foetoprotein have provided support for this possibility. Purified human α -foetoprotein has been found to bind copper (Aoyagi *et al.*, 1978a) and bilirubin (Ruoslahti *et al.*, 1976; Aoyagi *et al.*, 1978b; Berde *et al.*, 1979). The affinity of α -foetoprotein for bilirubin is lower than that of albumin (Ruoslahti *et al.*, 1976). This and the high concentration of albumin make it somewhat unlikely that the binding of bilirubin by α -foetoprotein would play an important physiological role.

Human α -foetoprotein has also been found to contain fatty acids (Parmelee *et al.*, 1978). This has prompted us to compare the relative avidities of α -foetoprotein and albumin in binding of fatty acids. In the present paper we show that bovine α -foetoprotein contains more long-chain polyunsaturated fatty acids than bovine foetal albumin and that

α -foetoprotein shows a higher affinity for arachidonate than albumin.

Material and methods

Proteins

α -Foetoprotein was purified from foetal calf serum (Flow Laboratories, Rockville, MD, U.S.A.) by using immunoabsorbents and gel filtration. This procedure and the purity controls used have been described previously (Ruoslahti, 1978). The concentrations of bovine α -foetoprotein were determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. Fatty acids were removed from the α -foetoprotein with charcoal at pH 3, as described for albumin (Chen, 1967).

Adult and foetal albumin were prepared by the method of Evenson & Deutsch (1978) from bovine serum and from foetal calf serum that had been depleted of α -foetoprotein by immunoabsorption respectively.

Extraction and analysis of fatty acids

The fatty acids were extracted from α -foetoprotein and albumin essentially as described by Parmelee *et al.* (1978). Heptadecanoate ($C_{17:0}$) was added to the proteins as an internal standard and a mixture of n-propanol/n-heptane/0.5 M- H_2SO_4 (40:10:1, by vol.) was used for extraction.

Fatty acids were methylated as described by Brian *et al.* (1972) and analysed with a Hewlett-Packard type 5830 A gas chromatograph equipped with flame-ionization detectors and a peak integrator. Identification of fatty acids was on the basis of comparative retention times of standards and unknowns. The known amounts of n-heptadecanoate ($C_{17:0}$) added to all samples permitted conversion of area-percentage and nanomole-percentage data into absolute amounts of fatty acid.

Isoelectric focusing

α -Foetoprotein was focused in a 110ml column (LKB, Uppsala, Sweden) using pH4–6 ampholines

(LKB) and a sucrose gradient by standard procedures.

Equilibrium dialysis

The binding of fatty acids to defatted α -foetoprotein and foetal albumin was studied by the method of Spector *et al.* (1969). Utilizing double-compartment vials described by them, 3.2ml of n-heptane containing known amounts of fatty acid was allowed to equilibrate for 20h at 37°C with 0.8ml of protein (0.5mg/ml) in phosphate-buffered salt solution, pH 7.4, and 0.8ml of the phosphate-buffered salt solution only. The radiolabelled fatty

Table 1. Fatty acid in α -foetoprotein and albumin

Results are means $\pm$ S.D. n is the number of determinations made for each protein. α -Foetoprotein and albumin came from two independent batches of foetal calf serum.

Fatty acid	Fatty acid content (mol/mol of protein)		
	α -Foetoprotein ($n = 4$)	Foetal albumin ($n = 2$)	Commercial albumin ($n = 2$)
$C_{14:0}$	0.03 ± 0.02	0.04 ± 0.00	0.03 ± 0.00
$C_{15:0}$	0.01 ± 0.00	0.01 ± 0.00	—
$C_{16:1}$	0.07 ± 0.01	0.09 ± 0.00	0.04 ± 0.00
$C_{16:0}$	0.42 ± 0.03	0.41 ± 0.00	0.24 ± 0.01
*	0.02 ± 0.01	0.02 ± 0.01	0.01 ± 0.01
$C_{18:2}$	0.15 ± 0.01	0.08 ± 0.01	0.06 ± 0.01
$C_{18:1}$	0.52 ± 0.02	0.44 ± 0.02	0.32 ± 0.02
$C_{18:0}$	0.20 ± 0.01	0.24 ± 0.01	0.20 ± 0.00
$C_{20:5}$	0.09 ± 0.01	—	—
$C_{20:4}$	0.35 ± 0.02	0.05 ± 0.01	—
$C_{20:3}$	0.12 ± 0.01	0.02 ± 0.01	—
$C_{22:6}$	0.49 ± 0.05	0.01 ± 0.01	—
$C_{22:5}$	0.20 ± 0.01	0.01 ± 0.01	—
Total	2.66 ± 0.14	1.42 ± 0.06	0.90 ± 0.02

* An unidentified component that chromatographed at the position of $C_{17:1}$ fatty acid.

Fig. 1. Isoelectric-focusing profile of purified non-defatted bovine α -foetoprotein (a), defatted α -foetoprotein (b) and defatted bovine α -foetoprotein that was reconstituted with 2 molar equivalents of arachidonate (c)

■, A_{280} ; ····, pH.

acids [^3H]palmitate, [^{14}C]linoleate and [^3H]arachidonate (99% pure according to the manufacturer; New England Nuclear, Boston, MA, U.S.A.) used as markers were extracted three times with hexane-extracted phosphate-buffered salt solution to remove water-soluble impurities before use (Huntley *et al.*, 1977). Non-radioactive fatty acids were from Sigma (St. Louis, MO, U.S.A.) and were used without further purification. The initial concentration of fatty acid in the n-heptane phase was varied between 10 nM and 10 mM in the equilibrium dialysis. The vials were gassed with N_2 and prevented from exposure to light. The association constants and molar binding ratios between the fatty acids and the proteins were analysed by Scatchard plots (Scatchard, 1949).

In one series of experiments, the double-compartment vials were used to allow α -foetoprotein and albumin to compete for the binding of arachidonate (Huntley *et al.*, 1977). These analyses were carried out under the same conditions as the Scatchard analysis. Three initial concentrations (23 μM , 3.8 μM and 0.75 μM) of arachidonate were used in the n-heptane phase.

Results

Presence of fatty acids in α -foetoprotein

Analysis of the propanol/heptane extracts of α -foetoprotein indicated that purified α -foetoprotein contains fatty acids (Table 1). The molar ratio of fatty acids to protein was 2.7 for α -foetoprotein and 1.4 for foetal albumin. α -Foetoprotein and albumin from two batches of foetal calf serum were analysed with similar results. Commercial albumin (Cohn fraction V; Sigma) contained only 0.90 mol of fatty acid/mol of protein with no C_{20} or C_{22} fatty acids present. No fatty acids were found in α -foetoprotein treated with charcoal.

Effect of the presence of fatty acids on the isoelectric point of α -foetoprotein

A main protein peak, at pI 4.8, was obtained when bovine α -foetoprotein was submitted to isoelectric focusing (Fig. 1a). The peak was shifted to pI 5.2 after treatment with charcoal (Fig. 1b). Reconstitution of pI 5.2-form α -foetoprotein with a 2-fold molar excess of arachidonate converted the α -foetoprotein back into the pI 4.8 form (Fig. 1c).

Table 2. Association constants and molar binding ratios for binding of fatty acids to α -foetoprotein and albumin. Each value is the result of Scatchard analysis of an experiment where nine fatty acid concentrations were used. Two (or three) sets of experiments were performed with each fatty acid. The results for the individual experiments are shown separately.

Fatty acid	α -Foetoprotein		Albumin	
	Association constant (M^{-1})	Molar binding ratio of fatty acid to protein	Association constant (M^{-1})	Molar binding ratio of fatty acid to protein
$\text{C}_{16:0}$	2.1×10^7	(2.0)	1.4×10^7	(3.5)
	2.4×10^7	(2.1)	1.3×10^7	(3.2)
$\text{C}_{18:2}$	2.1×10^7	(1.8)	0.9×10^7	(2.7)
	1.8×10^7	(2.2)	0.7×10^7	(2.9)
$\text{C}_{20:4}$	8.1×10^7	(1.8)	1.6×10^7	(3.4)
	9.1×10^7	(1.6)	1.3×10^7	(2.8)
			1.6×10^7	(2.9)

Table 3. Competition by α -foetoprotein and albumin for the binding of arachidonate. Molar binding ratios for binding of arachidonic acid to α -foetoprotein ($\bar{V}$ α -foetoprotein) and albumin ($\bar{V}$ albumin) were measured at different initial concentrations of arachidonate in the n-heptane phase. Experiment I was carried out with two independent preparations of foetal albumin. Adult albumin was used in Experiment II. Each value is the mean $\pm$ S.D. for six determinations in Experimental I and three determinations in Experiment II.

Experiment	Initial concentration of arachidonate in the n-heptane phase (M)	$\bar{V}$ α -foetoprotein		
		$\bar{V}$ α -foetoprotein	$\bar{V}$ albumin	$\bar{V}$ albumin
I	2.3×10^{-5}	1.07 ± 0.04	1.19 ± 0.08	0.90
	3.8×10^{-6}	0.66 ± 0.07	0.27 ± 0.04	2.44
	7.5×10^{-7}	0.23 ± 0.01	0.05 ± 0.01	4.60
II	2.3×10^{-5}	1.18 ± 0.09	1.32 ± 0.18	0.91
	3.8×10^{-6}	0.73 ± 0.04	0.29 ± 0.02	2.48
	7.5×10^{-7}	0.24 ± 0.02	0.05 ± 0.01	4.98

Fig. 2. Scatchard plot of data for binding of linoleate to defatted bovine α -foetoprotein and to foetal albumin. $\bar{V}$ is mol of fatty acid/mol of protein and c is the concentration of unbound fatty acid in the aqueous phase. ●, α -foetoprotein; ○, albumin.

Binding of fatty acids to α -foetoprotein

Equilibrium dialysis revealed binding of fatty acids to α -foetoprotein with a higher affinity than to albumin (Table 2). The greatest difference was found with arachidonate. A difference in the binding properties of α -foetoprotein and albumin was also evident in the number of high-affinity binding sites for fatty acids. α -Foetoprotein had two and albumin three binding sites/mol of protein. The slope of the lower part in the Scatchard plots indicated that both α -foetoprotein and albumin may also have secondary binding sites for fatty acids (Fig. 2). Direct comparison of α -foetoprotein and albumin by allowing them to compete for the binding of arachidonate showed that α -foetoprotein bound more arachidonate than albumin at low concentrations of fatty acid (Table 3).

Discussion

Our results show that purified bovine α -foetoprotein contains fatty acids. These fatty acids seem to be tightly bound to α -foetoprotein. Fatty acids co-purified with α -foetoprotein through a procedure involving several separation steps and the presence or absence of fatty acid influenced the isoelectric point of α -foetoprotein. These results confirm and extend similar findings made previously with human α -foetoprotein (Parmelee *et al.*, 1978). Albumin also contains fatty acids and focuses at different pH

values depending on the presence or absence of fatty acids (Evenson & Deutsch, 1978; Basu *et al.*, 1978). However, α -foetoprotein and albumin are not identical with respect to their fatty acid-binding properties. Bovine α -foetoprotein and albumin contained about equal amounts of the C_{14} – C_{18} fatty acids, whereas α -foetoprotein had about 16 times as much of fatty acids with the C_{20} and C_{22} unsaturated fatty-acid chains as did albumin. We also found α -foetoprotein to have a 6 times higher affinity for arachidonate and a slightly higher affinity for palmitate and linoleate than albumin. Human α -foetoprotein also contains more of the highly unsaturated long-chain fatty acids than foetal albumin (Parmelee *et al.*, 1978). In contrast, Berde *et al.* (1979), who used a fluorescent fatty acid probe, reported that human α -foetoprotein binds polyunsaturated long-chain fatty acids with an affinity similar to that of albumin. Whether the difference between their results on human and ours on bovine α -foetoprotein is due to a species variation or depends on differences in the methods used in the two studies is not yet clear. However, it is noteworthy that the variation in association constants and molar binding ratios reported in the present paper between bovine α -foetoprotein and albumin is in agreement with the differences in the fatty-acid contents of these two proteins. Also, we confirmed the higher affinity of α -foetoprotein for arachidonate by using an assay where α -foetoprotein and albumin were competing for binding of arachidonate from the same source. In spite of the presence of only two high-affinity binding sites in α -foetoprotein as opposed to three in albumin, α -foetoprotein bound up to 5 times more arachidonate at low concentrations of the ligand.

The binding of fatty acids by the human and bovine α -foetoproteins seems to be shared by at least two other species, since radioactive arachidonate has been found to become associated with α -foetoprotein (as well as with albumin) in foetal porcine serum (Ingvarsson & Carlsson, 1978), and fatty acids have been found to compete with oestrogen for binding to rat α -foetoprotein, which, unlike the α -foetoproteins of non-rodent species, has a strong affinity for oestrogens (Benassayag *et al.*, 1979). It seems possible that α -foetoprotein is involved in the foetal metabolism of fatty acids, especially the long-chain polyunsaturated ones. Since these fatty acids are precursors of biologically highly active compounds such as prostaglandins (Samuelsson, 1972), α -foetoprotein may play a very significant functional role in the foetus.

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