THE BINDING OF FATTY ACIDS TO CYTOPLASMIC PROTEINS: BINDING TO Z PROTEIN IN LIVER AND OTHER TISSUES OF THE RAT: *

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SUMMARY: A cytoplasmic binding protein (Z protein) has now been shown to have a high affinity for fatty acids. This protein is present in the cytoplasm of rat liver, myocardium, skeletal muscle, intestinal mucosa, adipose tissue and kidney. Oleic acid was reversibly bound to Z with a Km of 2.8×10^{-6}M; flavispidic acid competitively inhibited this process. Relative binding of fatty acids to Z increased with chain length and decreased with unsaturation. Triolein, cholesterol and several bile salts were not bound by Z protein.

INTRODUCTION: The intestinal absorption and subsequent intracellular transport and metabolism of fatty acids may involve one or more receptor proteins (1,2). Y and Z, two cytoplasmic proteins isolated and purified from rat liver, have been shown to bind bilirubin and various dyes when administered *in vivo* or added to liver homogenate *in vitro* (3-6). The present studies reveal that Z protein binds fatty acids, particularly oleic acid, with high affinity. These findings suggest that the function of Z protein is related to fatty acid transport and metabolism in the liver and other tissues in which the protein occurs.

MATERIALS: Hexanoic acid -1-^{14}C, n-decanoic acid -1-^{14}C, lauric acid -1-^{14}C, myristic acid -1-^{14}C, stearic acid -9-10-^{3}H, linoleic acid -1-^{14}C, linolenic acid -1-^{14}C and glyceryl tri(oleate -1-^{14}C) were purchased from Amersham/Searle. Palmitic acid -1-^{14}C, oleic acid -4-5-^{3}H and cholesterol -4-^{14}C were obtained from New England Nuclear. Chenodeoxycholate -24-^{14}C and glycocholate -^{14}C (u) were a gift from Dr. A. F. Hofman of the Mayo Clinic. Unlabeled fatty acids and sodium taurocholate were obtained from Sigma Chemical Co.;

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sodium bromsulphalein (BSP) from Hynson, Westcott and Dunning; and β-flavispidic acid-N-methylglucaminate from Dr. Åhö, Turku, Finland.

METHODS: The binding of various fatty acids, cholesterol, bile salts and BSP to proteins of a 110,000 x g supernatant fraction of various tissues was studied. Male Sprague-Dawley rats (250-350gm) were anesthetized with ether; blood was withdrawn by intracardiac puncture and the following organs were rapidly removed, rinsed and perfused with isotonic saline, blotted, weighed and homogenized in 0.25M sucrose, 0.01M phosphate buffer to yield a 33% homogenate (w/v): liver, spleen, kidney, heart, lungs, intestinal mucosa, epidydimal fat and skeletal muscle. A supernatant fraction was prepared by centrifugation at 110,000 x g for 120 minutes at 2°C in a model L Spinco ultracentrifuge. Aliquots of supernatant representing 2 g of tissue were mixed with constant trace amounts of the various labeled ligands ($4x10^{-9}$ moles in 50μl of dioxane-propylene glycol 2:1 v/v) at 4°C, shaken and allowed to stand for 30 minutes before upward-flow chromatography on a Sephadex G-75 column (2.5 x 43cm) as previously described (3). Aliquots (5ml) of serum were treated similarly. In some experiments, 3μ moles of BSP was added and chromatography was performed on Sephadex G-100 to obtain better protein resolution. The presence of Z protein in the "Z fraction" of the various tissue supernates was established by the following observations: (a) electrophoresis of Z fraction on neutral SDS acrylamide gel revealed a single band with the same electrophoretic mobility as purified Z protein (5); (b) on gelfiltration of liver supernatant, BSP and fatty acid binding occurred in identical fractions; (c) anti-Z-Ig G, but not control Ig G, precipitated 70-80% of protein-bound BSP or oleic acid in "Z fraction", and (d) flavispidic acid-N-methylglucaminate, previously shown to displace organic anions from Z protein (3), inhibited fatty acid binding.

To quantitate the binding affinity of oleic acid to Z protein and characterize the inhibitory effect of flavispidic acid, "Z fraction" was prepared from 100ml of liver supernatant (40g liver) by chromatography on

Sephadex G-100. Dioxane-propylene glycol (0.1ml) containing various concentrations of labeled oleic acid was added to 0.5ml of 0.01M PO_4 buffer containing 3mg of "Z fraction". Phosphate buffer (0.1ml) containing 2.5mg of flavispidic acid was added to alternate samples. All samples were brought to a final volume of 1.0ml and pH 7.4 with PO_4 buffer, and were chromatographed on Sephadex G-75 (1.7 x 28cm). Final concentration of flavispidic acid was $6x10^{-7}$M. In some experiments, oleic acid was introduced as a micellar solution with sodium taurocholate (final concentration 10mM). The results obtained were analyzed according to the method of Lineweaver-Burk (7).

Protein concentrations in eluted fractions were determined by absorbance at 280mμ and by the Lowry method. BSP concentration was determined by absorbance at 580mμ after alkalinization with IN NaOH. DPM's in each fraction were determined be counting 1ml aliquots in 10ml of Bray's solution in a Packard liquid scintillation counter (Model 3375). Quench correction was by external standardization.

RESULTS: Binding of oleic acid ($4x10^{-9}$ moles) and BSP ($3x10^{-6}$ moles) to the proteins in a 110,000 x g liver supernatant after chromatography on Sephadex G-100 is shown in Figure 1. Three binding peaks (A,B and C) were detected. Peak A contained primarily albumin as determined by acrylamide gel electrophoresis, and bound 15% and 2% of added oleic acid and BSP respectively. Peak B

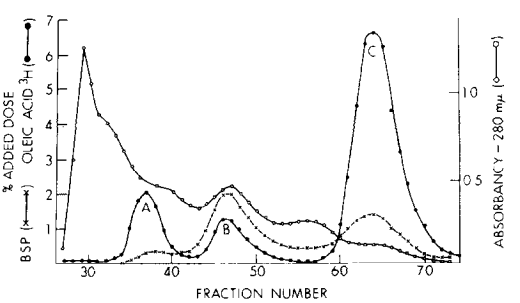

Fig. 1 Binding of oleic acid -^{3}H and sodium bromsulphalein (BSP) to the proteins in rat liver supernatant after chromatography on Sephadex G-100. The results shown were compiled from separate experiments in which only one ligand was added to liver supernatant.

comprised mainly Y protein as determined by acrylamide gel electrophoresis and immunoprecipitation, and contained 5% and 15% of the oleic acid and BSP respectively. In contrast, 41% of added oleic acid and only 10% of BSP were present in Peak C which was identified as Z protein. Relatively large amounts of BSP were added as no significant binding to Z protein was obtained with trace amounts comparable to those used for fatty acids.

The binding pattern of different fatty acids, triolein, cholesterol, chenodeoxycholate and glycocholate were studied in the same manner using Sephadex G-75 chromatography. Each of the fatty acids studied was primarily bound to Z

TABLE I

Binding of Fatty Acids to Z Protein in Rat Liver Supernatant*

FATTY ACID		% of Added Fatty Acid Recovered in Z Fraction**	% of Added Fatty Acid per mg Z Protein***
n-Hexanoic	6:0	0.02	0.01
Decanoic	10:0	1.8 ± 0.2	0.3 ± 0.1
Lauric	12:0	2.0 ± 0.5	0.3 ± 0.1
Myristic	14:0	6.9 ± 1.3	1.7 ± 0.4
Palmitic	16:0	14.6 ± 2.0	3.6 ± 0.2
Stearic	18:0	15.8 ± 4.1	3.6 ± 0.5
Oleic	18:1	40.7 ± 2.6	8.6 ± 0.2
Linoleic	18:2	11.7 ± 1.2	2.8 ± 0.4
Linolenic	18:3	10.0 ± 1.0	1.9 ± 0.3

* All values represent the mean ± S.E.M. See text for procedural details.

** Z fraction corresponds to peak C in Fig. 1.

*** Protein concentration was determined in the fraction containing the highest percentage of fatty acid. As only Z protein was detected in this fraction on SDS acrylamide gel electrophoresis, the results were expressed as % of added fatty acid per mg Z protein.

protein. The percentage of fatty acid bound as well as the percentage bound per mg of protein in the peak tube are shown in Table I. No significant binding of triolein or either bile salt was found. Cholesterol was bound entirely in a high molecular weight protein fraction in the void volume of the column.

Binding of oleic acid to Z protein was inhibited or reversed by addition of other fatty acids, BSP or flavispidic acid to liver homogenate or 110,000 x g supernatant in-vitro. To study further the characteristics of this reversible association, the binding of various concentrations of oleic acid to "Z fraction" alone was studied using Sephadex G-75 (Figure 2). V max (binding capacity) was 1.1×10^{-9} moles/mg Z; Km (binding affinity) was 2.8×10^{-6}M. The experiment was repeated in the presence of flavispidic acid (6×10^{-7}M) which competitively inhibited fatty acid binding (Figure 2).

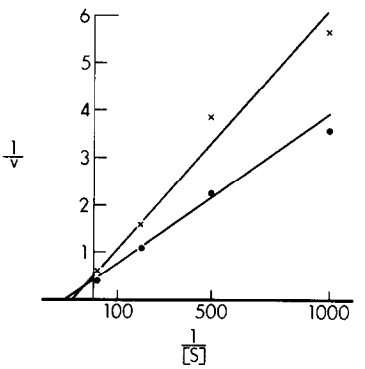

Fig. 2 Lineweaver-Burk plot of oleic acid binding to "Z fraction" in the presence (x ——— x) and absence (o ———^o) of flavispidic acid (6×10^{-7}M).
V = moles oleic acid $\times 10^{-9}$/ mg Z protein.
S = molar concentration.
The units on the ordinate are $\times 10^{9}$ and the units on the abscissa are $\times 10^{3}$.

Significant binding of oleic acid to Z protein was also found in supernatants of myocardium, skeletal muscle, intestinal mucosa, kidney and adipose tissue (Table II). The binding of oleic acid per mg of Z protein was greatest in liver and myocardium. In the supernatant of each tissue studied, including those without detectable fatty acid binding in the Z region, 14-62% of fatty acid added

TABLE II

Binding of Oleic Acid ^{3}H to Z Protein in Rat Tissue Supernatants*

ORGAN	% of Added Oleic Acid Recovered in Z Fraction **	% of Added Oleic Acid per mg Z Protein ***
Liver	40.7 ± 2.6	8.6 ± 0.2
Myocardium	13.8 ± 2.2	1.8 ± 0.3
Skeletal Muscle	2.1 ± 0.2	0.7 ± 0.1
Intestinal Mucosa	6.7 ± 0.2	0.7 ± 0.1
Adipose Tissue	4.3 ± 0.4	0.5 ± 0.1
Kidney	2.8 ± 0.5	0.4 ± 0.2

* All values represent mean ± S.E.M. See text for procedural details.

** "Z fraction" corresponds to peak C in Fig. 1.

*** Protein concentration was determined in the fraction containing the highest percentage of oleic acid. As only Z protein was detected in this fraction on SDS acrylamide gel electrophoresis, the results were expressed as % added oleic acid per mg Z protein.

was associated with proteins with molecular weights in excess of 30,000. The binding of fatty acid by serum was limited to albumin.

DISCUSSION: This study demonstrates the binding of fatty acids to specific cytoplasmic proteins in various mammalian tissues. A possible physiological role for these proteins in the transport and metabolism of fatty acids should be considered. This is particularly the case with regard to Z protein which binds most of the oleic acid in liver supernatant in-vitro. Experiments in progress confirm that fatty acids are rapidly transferred from plasma into liver in-vivo, (8) and demonstrate a binding pattern in supernatant fractions which is qualitatively similar to that found in-vitro, with the exception that 5-10% of supernatant radioactivity is bound to a high molecular weight protein (approximately 150,000 M.W.), possibly the sterol carrier protein described by Ritter and Dempsey

(9). Thin layer chromatographic studies reveal that the radioactivity in this fraction is predominantly esterified fatty acids, either triglyceride or cholesterol ester. Oleic acid binding to Z protein in the supernatant of tissues which actively metabolize fatty acids, such as myocardium, skeletal muscle, intestinal mucosa, adipose tissue and kidney also suggests a physiological role for Z. The property of Z protein to bind selectively free fatty acids and not triolein, cholesterol or bile salts in-vitro makes Z a suitable candidate for a fatty acid carrier protein. In addition, inhibition of fatty acid binding to Z by flavispidic acid and other organic anions, suggests a possible intracellular mechanism to regulate fatty acid metabolism and also provides an experimental approach to study the function of Z protein in-vivo. A role for Z in membrane transport of fatty acids is also possible in view of the ability of flavispidic acid to displace BSP from liver plasma membranes in-vitro (10).

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