Enterocytes located in the pig distal small intestine (ileum) contain a cytosolic protein that is homologous to two proteins that are also synthesized in these cells: intestinal and "liver" fatty acid-binding proteins (I- and L-FABP, respectively). To begin to investigate the functional interrelationships of these three proteins, we compared their patterns of tissue-specific expression and developmental regulation in the mouse. Blot hybridization analyses of RNA prepared from 12 adult tissues revealed that this mRNA was confined to the small intestine. Unlike I- and L-FABP, mRNA, which are most abundant in the proximal jejunum, this mRNA is most abundant in the ileum. While I- and L-FABP, gene transcription commences in late fetal life coincident with initial cytodifferentiation of the mouse gut epithelium, the ileal gene is activated later, at the suckling/weaning transition (postnatal day 12). The ileal location and developmental pattern of expression suggested that this protein may play a role in the intracellular transport of bile salts in the ileal epithelium. To test this hypothesis, we expressed the porcine ileal peptide (PIP) in Escherichia coli, purified it to apparent homogeneity, and analyzed its binding properties for bile acids and fatty acids using 13C NMR spectroscopy. Like I-FABP, PIP binds palmitate and oleate with a 1:1 molar stoichiometry. However, unlike I-FABP, PIP binds chenodeoxycholate. All crystals are orthorhombic in the P2,2,2, space group. The unit cell dimensions are a = 36.15 Å, b = 50.13 Å, and c = 67.18 Å.

The polarized absorptive cell of the small intestine, or enterocyte, contains at least two abundant ~15-kDa cytosolic fatty acid-binding proteins (FABP,). These have been named intestinal (I-) FABP, and liver (L-) FABP, based on their initial site of isolation. Transcription of the mouse and rat I-FABP, gene (fabpl) is restricted to the intestine (Sweetser et al., 1988a) while the L-FABP, gene (fabpl) is expressed in both enterocytes and hepatocytes (Sweetser et al., 1988b). These unlinked (Sweetser et al., 1987b) but homologous genes are activated during late fetal life (Hauft et al., 1989; Rubin et al., 1989). Both the I-FABP, and L-FABP, genes exhibit differences in their expression from the duodenum to ileum (Sweetser et al., 1988a, 1988b) that are established at the time of their initial activation (Hauft et al., 1989; Rubin et al., 1989) and perpetuated through adulthood.

The precise functions of I- and L-FABP, have not yet been defined. They are thought to be part of a multifunctional cytoplasmic transport system involved in the uptake and delivery of fatty acids to sites of metabolic processing (reviewed in Sweetser et al., 1987a).

Rat I-FABP, has been expressed in Escherichia coli (Lowe et al., 1987). It has been crystallized with and without palmitate, and the structures of the holoprotein and apoproteins have been solved to high resolution (Sacchettini et al., 1989a, 1989b). I-FABP, consists of 10 antiparallel β-strands in a +1,+1,+1... motif, and these strands are organized into two orthogonal β sheets. The overall conformation of the protein resembles a clamp shell (hence the term β-clamp). The bound ligand is located within the interior of the protein. Its carboxylate group forms part of a unique five-member hydrogen bonding network involving the ε-arginine group of a buried Arg, the side chain of a Gln residue, and two ordered solvent molecules. The hydrocarbon tail is maintained in a disnecit, bent conformation with slight left-handed twist, by the side chains of a number of aromatic and hydrophobic residues. The fatty acid-binding pocket also contains several ordered solvent molecules. X-ray crystallographic studies of the apoprotein indicate that removal of ligand has little effect on the overall structure of I-FABP, (Sacchettini et al., 1989b). The position occupied by the bound ligand is replaced by additional, ordered solvent molecules. The orientation of sev-

**Developmental and Structural Studies of an Intracellular Lipid Binding Protein Expressed in the Ileal Epithelium**

James C. Sacchettini‡‡, Sherrie M. Hauft‖, Sandra L. Van Camp‡, David P. Cistola‡, and Jeffrey I. Gordon‡‡**

From the Departments of ‡‡Biochemistry and Molecular Biophysics, ‥Pediatrics, and **Medicine, Washington University School of Medicine, St. Louis, Missouri 63110.

(Received for publication, June 13, 1990)
eral residues which overlie a potential solvent-accessible open-
ing to the interior of the protein are altered, suggesting that
solvent movement may represent an important part of the
binding mechanism

Rat L-FABP, has also been expressed in E. coli (Lowe et al.,
1984). Although bovine (Pahler et al., 1985) and rat (Winter et al., 1990) have been crystallized, their structures have
not yet been solved. Primary sequence comparisons suggest that I- and L-FABPs are likely to share a similar
overall conformation (Jones et al., 1988). However, compara-
tive 13C NMR studies of E. coli-derived rat I- and L-FABP,
have revealed several key differences in their fatty acid-
binding stoichiometries and mechanisms (Cistola et al., 1989).
Each mol of L-FABP, binds at least 2 mol of fatty acid,
whereas I-FABP, binds a maximum of 1. In addition, the
carboxylate groups of bound fatty acid are ionizable and
solvent accessible in L-FABP, unlike that in I-FABP, indi-
cating differences in the locations of the polar head groups of the
bound lipid. Based on these structural differences and
physiological considerations, we hypothesized that I-FABP,
may be specifically involved in the uptake of luminal fatty
acids into the enterocyte and their delivery to sites of triac-
glycerol resynthesis while L-FABP, may be responsible for
the trafficking of a wider variety of lipid ligands including
fatty acids, monoacylglycerols, lysophospholipids, and bile
salts (Cistola et al., 1989).

The ileal epithelium of pigs contains an ~15-kDa protein
originally known as porcine ileal polypeptide (PIP, see Wider
et al., 1984; Borststrom et al., 1986). Immunochemical
studies revealed that the protein is confined to villus-associated
enterocytes (Borststrom et al., 1986). In vitro studies
using purified PIP suggested that it stimulates gastric parietal
cell H+ production. Since entry of nutrients into the intestine
results in stimulation of gastric acid secretion, these results
led to the proposal that PIP was an intestinal phase hormone
or enteroxytin. The protein was therefore renamed gastro-
trpin. When the primary structure of porcine gastrotropin
was determined by Edman degradation (Walz et al., 1988) and
from the nucleotide sequence of a cloned cDNA (Gantz et al.,
1989), it became apparent that the 127-residue protein bore
striking sequence similarities to L-FABP,. Translation of in
vitro transcribed “gastrotropin” mRNA in a cell free system
containing canine pancreatic microsomes indicated that the
protein does not contain signals for co-translational entry
into the secretory pathway (Gantz et al., 1989). Furthermore,
biochemical studies revealed that neither the intact protein nor
a carboxyl-terminal amidated tridecapeptide spanning resi-
dues 56-78 stimulated acid secretion in gastric fistula rats,
inhibited pentagastrin-stimulated acid secretion in these an-
imals, or altered acid production in isolated canine gastric
parietal cells (Gauta et al., 1989). Together, these data indi-
cated that PIP (gastrotropin) is unlikely to represent a se-
creted enteroxyn. In the current study, we initiated a series of experiments
designed to determine the function of this protein in ileal
enterocytes. We note that, in the mouse, the gene encoding
this ileal protein has a pattern of tissue-specific and devel-
mental regulation which is very distinct from that of the I
and L-FABP, genes. In addition, a prokaryotic expression
vector was used to direct efficient synthesis of PIP in E. coli.
13C NMR studies of the protein’s ligand specificity and bind-
ing stoichiometry revealed that, like I- and L-FABP, it is
able to bind fatty acids. However, unlike I-FABP, PIP is also
able to bind bile salts. The data suggest that this ileal protein
may be uniquely adapted for cytoplasmic trafficking of fatty
acids through ileal enterocytes.

EXPERIMENTAL PROCEDURES

Analysis of the Tissue-specific Expression and Developmental
Regulation of the Mouse Ileal Lipid Binding Protein Gene

Dissection of Tissues—Adult (70-80-day-old) male C57BL/6J mice
were maintained under a strictly controlled light cycle (lights on at
0600 and lights off at 1800) and standard enriched chow diet (4400
calories/100 g). Animals (n = 6) were killed by cervical dislocation at similar times of the
day. Brain, heart, lung, skeletal muscle, spleen, kidney, pancre-
creas, testis, liver, stomach, plus seven different portions of the
intestinal tract were then quickly removed and frozen in liquid
nitrogen. Regional dissection of the gut was accomplished as follows.
The duodenum was considered to be the most proximal 5 cm of small
bowel (measured from the pylorus). The remainder of the small
intestine was divided into three equal length segments that were
designated proximal jejunum, distal jejunum, and ileum. The cecum
was separated from the colon which, in turn, was divided into two
equal length portions.

Tissues were also obtained from fetal C57BL/6J mice. The morning
that vaginal plugs were noted was designated as the 1st day of
pregnancy. Gestation mice were subsequently killed by cervical dislo-
cation at the 15th, 17th, and 19th days of gestation. Four tissues
(liver, stomach, intestine, and colon) were harvested from littersmates
(n = 5-8 animals), and subsequently frozen in liquid nitrogen before
isolation of RNA.

Postnatal patterns of expression of this gene were surveyed in mice
that were killed on the 1st, 4th, 8th, 12th, 16th, 20th, 24th, 28th,
35th, and 50th days after parturition. The entire small intestine was
harvested from these animals (n = 4 littersmates on the 1st to 20th
postnatal days, n = 2 littersmates on days 28, 35, and 50).

Isolation of Total Cellular RNA—RNA was extracted from (poled)
tissues using guanidine isothiocyanate and further purified by cen-
trifugation through cesium chloride gradients (Chirgwin et al., 1979).
The integrity of the RNA preparations was established by denaturing
methylmercury-agarose or formamide-agarose gel electrophoresis
(Thorpe, 1980).

RNA Blot Hybridization—Dot blots were constructed using a tem-
plate manifold and nitrocellulose filters (Bancroft and Bancroft, 1982;
Levin et al., 1987). Four concentrations of each RNA sample (0.5, 1,
2, and 3 μg) were applied to the filter. (Note that yeast tRNA was
added to these samples prior to denaturation so that the total con-
centration of RNA was identical for all “dots” on a blot). Dot blots
were probed with a 32P-labeled (Feinberg and Vogelstein, 1983) 390-
base pair Hindll DNA probe and RNA was transferred to GeneScreen
Plus filters (Du Pont). The blots were probed using hybridization and
washing stringencies suggested by the manufacturer.

The relative concentrations of the ileal lipid binding protein mRNA
in preparations of tissue RNAs were determined by laser densitometry
of filter autoradiographs (Levin et al., 1987). Only signals in the linear
range of film sensitivity were used. This linearity was determined
using a reference set of adult mouse total intestinal RNA standards
(concentration = 30 ng to 3 μg) that were included on each filter.

Expression and Purification of the Porcine Ileal Lipid Binding
Protein from E. coli

Construction of a Prokaryotic Expression Vector That Allows In-
ducible Expression of the Porcine Protein—We had previously sub-

Downloaded from http://www.jbc.org/ by guest on January 22, 2018
cloned a 570-bp EcoRI fragment encoding gastrinotropin (PPI) into pGEM2 (Gantz et al., 1989). Unique Neol and HindIII restriction sites were introduced at the start and stop codons of the cDNA using the polymerase chain reaction (Siakal et al., 1988) and the following two mutagenic oligodeoxynucleotides:

\[ 5'\text{-CTCCGGCGCAGTGGCCCTCCTGCGG-3'}\text{-mutagenic oligo} \]

\[ 3'\text{-AGAGAAGCGGTAGCGGATGACCC-5'}\text{-template DNA} \]

Following 10 cycles of amplification, the resulting fragment was digested with Neol and HindIII and subsequently purified by agarose gel electrophoresis. The 390-bp product was ligated to Neol/HindIII-digested pMON5840, a derivative of pMON5615 (Ollins et al., 1988; Duronio et al., 1990). Recombinant plasmid DNA was prepared from selected ampicillin-resistant colonies of strain JM101. Restriction analysis was used to initially establish that the desired three base changes had been successfully engineered. The inset fragment from the plasmid was sequenced in its entirety using the dideoxynucleotide method (Sanger et al., 1977). Only the co-polymerization changes in nucleotide sequence were noted. This plasmid, pMON5840-ILBP, was then used to transform E. coli strain MG1655 which is supE (Sacchetti et al., 1990).

Expression and Purification—Cells containing pMON5840-ILBP were grown to an OD600 of 32 in SBLH media (Sadler et al., 1974) supplemented with 100 μg/ml ampicillin. Growth to this high density was achieved using a 4-liter fermenter (Lab-Line, Melrose Park, IL) and a protocol detailed in Sadler et al. (1974). The temperature of the culture was maintained at 37 °C. Expression of the porcine protein was induced by adding naldixic acid to a final concentration of 150 μg/ml. Cells were incubated for an additional 2 h at 37 °C and then harvested by centrifugation (10,000 × g for 30 min at 4 °C). The high density 4-liter fermentation produced 280 g (wet weight) of cell paste.

Precipitated proteins were collected by centrifugation at 5,000 × g and dialyzed against a buffer consisting of 20 mM potassium phosphate, 0.05% sodium azide, 1 mM EDTA (final pH = 7.4-7.6) to remove cellular debris. Phenylmethylsulfonyl fluoride was added to the supernatant to a final concentration of 14.4 g/liter and stirring the mixture for 10 min at 4 °C. The high density 4-liter fermentation produced 280 g (wet weight) of cell paste, which was quickly frozen and stored at −80 °C. It was subsequently thawed, suspended in a solution of 50 mM Tris, 20% sucrose (final pH = 8.0), and then lysed at 4 °C in a French press under 1,500-2,000 p.s.i. The lysate was then filtered through a 0.22-μm filter at 10,000 × g for 30 min (at 4 °C) to remove cellular debris. Phenylmethylsulfonyl fluoride was added to the supernatant to a final concentration of 1 mM. DNA was removed from the solution by adding protamine sulfate (Sigma) to a final concentration of 14.4 g/liter and stirring the mixture for 10 min at room temperature. The precipitated DNA was removed by centrifugation (using the conditions described above). A concentrated ammonium sulfate solution (176 g/liter) was added to the supernatant (while it was stirred on ice) so that its final concentration was 176 g/liter (30% of saturation). Precipitated proteins were collected by centrifugation at 5,000 × g for 20 min and the supernatant brought to 60% saturation with solid ammonium sulfate. Following centrifugation, the soluble proteins were collected and dialyzed against a buffer containing 20 mM potassium phosphate, 0.05% sodium azide, 1 mM EDTA (final pH = 7.4), 0.1 M 1-13C-mercaptoethanol, 0.05% sodium azide. It was subsequently concentrated to 40 mg/ml using an Amicon ultrafiltration device fitted with a YM-5 membrane. Alternatively, the dialyzed protein solution was incubated with an equimolar amount of 13C-enriched sodium azide for 5 min at 25 °C and then concentrated.

The hinging drop vapor diffusion method was employed to produce single crystals of the protein with or without bound chenodeoxycholic acid. Crystals were prepared by mixing 5 μl of the protein solution and 5 μl of 50% polyethylene glycol 4000 (J.T. Baker Chemical Co.) on a silanized glass cover slip. The coverslips were then inverted and sealed over a solution of 50% polyethylene glycol 4000 using a 24-well tissue culture plate (Linbro, Flow Laboratories, McLean, VA). The plates were left undisturbed for 23 °C. The solution that had not been incubated with chenodeoxycholic acid produced crystals with two different morphologies: needle shaped and parallelepiped. The needle-shaped crystals grew to a maximum size of 2.0 × 0.05 × 0.05 mm while the parallelepiped type reached a final size of 0.2 × 0.2 × 0.2 mm. The chenodeoxycholic acid-containing protein produced crystals of the same morphology. These crystals were 0.1 mm thick. The maximal dimension across their face was 0.6 mm.

The space group and unit cell parameters of the crystalline ileal lipid binding protein were determined using precession photography. Parallelepiped shaped crystals were sealed in quartz capillaries and mounted on a Supper precession camera. This camera was attached to a three fine focus, single crystal x-ray generator. The generator is set to 1.9 kilowatts. Precession photographs were taken of all three principal zones from two separate crystals.

The unit cell parameters were verified and refined with data collected from a single protein crystal on a Rigaku AFC6S diffractometer (Molecular Structures, Houston, TX) using a least squares algorithm supplied with the software. The high resolution (2.5 Å or greater) reflections from the crystals were used in the refinement.

Chenodeoxycholic acid/PPI co-crystals were prepared for data collection, as described above, at the University of California, San Diego Area Detector Facility. Prior to data collection the principal zones of
the crystal were found and the crystal's unit cell was determined to be isomorphous to those protein crystals which were crystallized in the absence of chenodeoxycholate.

RESULTS AND DISCUSSION

Analysis of Tissue-specific Expression and Developmental Regulation—To gain insights about the function of this protein, we first compared its patterns of tissue-specific and developmental expression with that of I- and L-FABP<sub>i</sub>. We selected the mouse for these studies because there is a wealth of information about the cell-specific and developmental stage-specific expression of the mouse L-FABP<sub>i</sub> and I-FABP<sub>i</sub> genes.

Marked regional differences in mRNA accumulation occur along the duodenal-to-ileal axis of the adult gut, as illustrated in Fig. 1. For L-FABP<sub>i</sub>, the highest mRNA levels occur in the proximal jejunum. The L-FABP<sub>i</sub> gene is expressed in hepatocytes and in two types of differentiated intestinal epithelial cells: enterocytes and a small subpopulation of serotonin-producing enteroendocrine cells (Sweetser et al., 1988b; Roth et al., 1990). Developmental analyses (Rubin et al., 1989; Hau et al., 1989) indicate that transcriptional activation of the L-FABP<sub>i</sub> gene occurs at the same stage of fetal development in both intestine and liver, i.e., during the last few days of gestation. Its transcriptional activation coincides with the time of conversion of the poorly differentiated, stratified gut epithelium to a monolayer, composed of four terminally differentiated cell types, that overlies nascent villi (i.e. fetal days 16-18). Studies in transgenic mice indicate that cis-acting elements located between nucleotides -4000 and +21 mediate these developmental stage-specific, region-specific, and cell-specific patterns of L-FABP<sub>i</sub> gene expression (Sweetser et al., 1988b; Hau et al., 1989).

The I-FABP<sub>i</sub> gene is only expressed in adult mouse intestinal epithelial cells (enterocytes and goblet cells; Sweetser et al., 1988a). Marked regional differences are established and maintained along both the proximal-to-distal (Fig. 1) and crypt-to-villus axes of the gut. Functional mapping studies of the rat I-FABP<sub>i</sub> gene in transgenic mice indicate that nucleotides -1178 to +23 contain all the cis-acting elements necessary for appropriate geographic and cell-specific expression of the gene. The I-FABP<sub>i</sub> gene, like the L-FABP<sub>i</sub> gene, is initially activated in late fetal life with a distinct geographic pattern of expression that mimics the pattern present in adult animals (Rubin et al., 1989).

Figs. 1 and 2 summarize the results of our blot hybridization analysis of the pattern of accumulation of the mouse homolog of PIP (gastrotropin) mRNA in the developing and adult gastrointestinal tract. The mRNA was only detected in intestine. None of the other adult C57BL/6J tissue RNA samples screened (liver, stomach, pancreas, brain, kidney, spleen, testis, skeletal muscle, heart, or lung) produced any detectable signal. Its regional distribution along the proximal-to-distal axis of the gut was quite distinct from that of I- and L-FABP<sub>i</sub> mRNA (Fig. 1). The porcine cDNA reacted with a single mRNA species of ~600 bases (data not shown) which was confined to the distal jejunum and ileum. Levels of mRNA were highest in the ileum: its concentration in distal jejunal RNA was less than 10% of that documented in ileal RNA (see Fig. 1). The developmental pattern of expression also differed from that of the L- and I-FABP<sub>i</sub> genes. No mRNA was detected in fetal stomach, small intestine, colon, or liver RNA harvested during the 15th-19th day of fetal life: the time that activation of the L- and I-FABP<sub>i</sub> genes occurs (data not shown). The ileal gene remained "silent" not only during fetal life but throughout the suckling period (postnatal days 1-12, see Fig. 2). The mRNA was first detectable in small intestinal RNA prepared at the beginning of the suckling-weaning transition (postnatal days 12-14). Thereafter, steady state levels rose progressively during the weaning period (completed by postnatal day 28) and early adulthood.

Several conclusions were drawn from these data. First, activation of the ileal-specific gene at the suckling/weaning transition may reflect the effects of glucocorticoids (plasma concentrations of free corticosterone are low through postnatal day 12 and then rise abruptly) or thyroxine (reviewed in Henning, 1987). Ontologic changes in the expression of a number of intestinal genes at the suckling/weaning transition.
have been shown to be influenced by these hormones. This ileal-specific gene may represent a useful model (marker) for studying developmental changes that occur during the transition from a high fat, low carbohydrate diet of mother's milk to a high carbohydrate, low fat chow diet (Fernando-Warnakulasuriya et al., 1983; Frost et al., 1982). Second, this ileal-specific gene also provides a model for identifying cis-acting elements that restrict gene expression to the distal half of the small bowel. This model is particularly attractive since two homologous genes (L- and I-FABP) are expressed in the small bowel but in more proximal regions. Comparative functional mapping studies of these three genes using transgenic mice may provide further insights about the mechanisms which regulate their expression along the proximal-to-distal axis of the gut. Third, the patterns of developmental and regional expression of this gene suggest that its protein product has a function which is distinct from that of I- and L-FABP, and that such a function may be uniquely adapted to the metabolic needs of ileal epithelial cells (enterocytes). Active and passive transport of bile acids (salts) across the distal small intestinal epithelium is an important part of the enterohepatic circulation. Since the developmental regulation and regional expression of this ileal-specific gene correlate with that of the active transport system for bile salts (Little and Lester, 1980; Moyer et al., 1986), it is possible that this protein may play a role in ileal bile salt reabsorption (see Walz et al., 1988).

Therefore, two potential functions of PIP were investigated: (i) fatty acid binding, because of its sequence similarities to I- and L-FABP, and (ii) bile acid binding, because of its potential role in the transport of bile acids (salts) across the distal small bowel epithelium. Our approach for investigating these two possible functions was to first produce large quantities of the mammalian protein in E. coli and then to investigate its interactions with these lipids in vitro using 13C NMR. As noted in the Introduction, we have used a similar strategy to monitor and compare the interactions of 13C-enriched long chain fatty acids with E. coli-derived rat I- and L-FABP, (Cistola et al., 1989). The utility of this approach is based on the high degree of resolution of lipid carboxyl resonances from other protein resonances and the high sensitivity of carboxyl resonances to inter-molecular factors such as hydrogen bonding, hydration, degree of ionization (pH), involvement in charge-charge interactions, and proximity to ring currents. However, a limitation of this approach is the requirement for milligram quantities of protein, and a suitable source of protein had to be obtained.

Expression of PIP in E. coli—The porcine cDNA was inserted into pMON5840, the same prokaryotic expression vector we had previously employed for efficient synthesis of rat I-FABP, in E. coli (Sacchettini et al., 1990). The important features of the vector are outlined in Fig. 3A. Transcription is controlled by a nalidixic acid-inducible recA promoter (Ollins et al., 1988). A sequence from the gene 10 leader of bacteriophage T7 that serves to enhance the translation of a number of foreign mRNAs (perhaps by base pairing to a portion of E. coli 16 S rRNA; Ollins et al., 1988; Ollins and Rangwala, 1989) is interspersed between the recA promoter and the initiator methionine codon of the porcine ileal cDNA. The recombinant plasmid was placed in a supE' strain of E. coli (MG1655). We have previously shown that overexpression of I-FABP in supF' strains (e.g. JM101) can be accompanied by readthrough of its stop codon (Sacchettini et al., 1990).

SDS-polyacrylamide gel electrophoresis of E. coli lysates prepared from a strain containing the recombinant DNA revealed that nalidixic acid treatment (Feinstein et al., 1983) resulted in the appearance of a distinct band of the expected mass (15 kDa) that was not present in cells containing the vector without insert. This protein was subsequently purified from the lysates using a protocol similar to that employed for isolation of rat I-FABP (Sacchettini et al., 1989a): sequential ammonium sulfate precipitation followed by ion exchange and gel filtration chromatography (Fig. 3B). The final yield of purified protein from the 4-liter high density fermentation was 14 mg/g wet weight of cells. Edman degradation confirmed the identity of the protein and revealed that it lacked its initiator Met. This was not surprising given the fact that the protein contains an Ala at position 2. Proteins with Ala at position 2 are susceptible to digestion by E. coli methionylaminopeptidase (Hirel et al., 1989). The pl of the purified protein was 7.4, which is distinct from that of I-FABP, (pl = 6.0).

13C NMR Spectroscopy of the E. coli-derived Porcine Ileal Protein—To determine whether PIP binds fatty acids, 13C NMR spectra were collected for samples containing carboxyl 13C-enriched oleate or palmitate and PIP at different fatty acid-protein mole ratios. The carboxyl/carbonyl region of the natural abundance 13C NMR spectrum for PIP without added oleate is shown in Fig. 4A. The cluster of partially overlapped resonances between 168 and 182 ppm corresponds to natural abundance carbonyl carbons in the polypeptide backbone and to carboxyl carbons in aspartate and glutamate side chains (Cistola et al., 1989). Upon addition of 13C-enriched oleate (total fatty acid:protein mole ratio = 1:1), a prominent, narrow resonance appeared at 182.6 ppm (Fig. 4B). With increasing amounts of 13C-enriched oleate (to 2:1, 3:1, and 4:1 mole ratios), an additional broad resonance at ~179.6 ppm was also observed (Fig. 4, C–E). The latter was also noted in samples containing no protein, and was assigned to the carboxyl carbons of oleate/oleic acid in a liquid crystalline bilayer phase (see Cistola et al., 1986, 1988a, 1989). The narrow resonance at 182.6 ppm was assigned to oleate bound to PIP. To assess whether oleate interactions with PIP were specific or nonspecific, similar spectra were accumulated for samples containing increasing amounts of 13C-enriched oleate and hen white lysozyme (Fig. 4, F–J). Lysozyme was chosen

Comparison of these two strains prior to and after nalidixic acid induction indicated that expression of PIP had no discernible effects on their growth kinetics.
bound oleate was detected only in samples containing a fatty
one nonprotein carboxyl/carbonyl resonance was observed
with increasing amounts of added oleate. This very broad
counterpart in resonance at -179.8 ppm was essentially identical to its
acids, it is readily available, and its molecular mass is very
close to that of PIP (-14 kDa). As shown in Fig. 4, F-J, only
one nonprotein carboxyl/carbonyl resonance was observed with increasing amounts of added oleate. This very broad resonance at -179.8 ppm was essentially identical to its counterpart in panels C-E and was assigned to unbound bilayer-phase oleate/oleic acid. The spectra indicated no detectable binding of oleate to lysozyme, and suggested that the observed interactions between oleate and PIP were likely to occur at a mole ratio of greater than 1 (Fig. 4, C-E).4 Second, the intensity (area) of the resonance at 182.6 ppm, relative to that of PIP and lysozyme were 17 mg/ml. A pulse interval of 2.0 s with 90° pulses, a spectral width of 25,000 Hz, and 32 K time domain points were used for spectral processing. Number of spectral accumulations: 24,064 (A), 15,104 (B), 14,336 (C), 9,344 (D), 16,256 (E), 23,296 (F), 9,728 (G), 14,848 (H), 16,128 (I), and 12,800 (J). Different vertical scaling factors were used in these spectra and, therefore, peaks heights cannot be directly compared for different panels in this figure.

The stoichiometry of binding of oleate to PIP was approxi-
mately 1:1, based on the following observations. First, unbound oleate was detected only in samples containing a fatty acid:protein mole ratio greater than 1 (Fig. 4, C-E).4 Second, the intensity (area) of the resonance at 182.6 ppm, relative to natural abundance protein resonances, did not increase when oleate was added. The chemical shift values for oleate carboxyl resonances are indicated above each peak. The concentra-
tion of PIP and lysozyme were 17 mg/ml. A pulse interval of 2.0 s with 90° pulses, a spectral width of 25,000 Hz, and 32 K time domain points were used for spectral processing. Number of spectral accumulations: 24,064 (A), 15,104 (B), 14,336 (C), 9,344 (D), 16,256 (E), 23,296 (F), 9,728 (G), 14,848 (H), 16,128 (I), and 12,800 (J). Different vertical scaling factors were used in these spectra and, therefore, peaks heights cannot be directly compared for different panels in this figure.

as a control protein because it is not known to bind fatty
acids, it is readily available, and its molecular mass is very
close to that of PIP (-14 kDa). As shown in Fig. 4, F-J, only
one nonprotein carboxyl/carbonyl resonance was observed with increasing amounts of added oleate. This very broad resonance at -179.8 ppm was essentially identical to its counterpart in panels C-E and was assigned to unbound bilayer-phase oleate/oleic acid. The spectra indicated no detectable binding of oleate to lysozyme, and suggested that the observed interactions between oleate and PIP were likely to occur at a mole ratio of greater than 1 (Fig. 4, C-E).4 Second, the intensity (area) of the resonance at 182.6 ppm, relative to that of PIP and lysozyme were 17 mg/ml. A pulse interval of 2.0 s with 90° pulses, a spectral width of 25,000 Hz, and 32 K time domain points were used for spectral processing. Number of spectral accumulations: 24,064 (A), 15,104 (B), 14,336 (C), 9,344 (D), 16,256 (E), 23,296 (F), 9,728 (G), 14,848 (H), 16,128 (I), and 12,800 (J). Different vertical scaling factors were used in these spectra and, therefore, peaks heights cannot be directly compared for different panels in this figure.

The stoichiometry of binding of oleate to PIP was approxi-
mately 1:1, based on the following observations. First, unbound oleate was detected only in samples containing a fatty acid:protein mole ratio greater than 1 (Fig. 4, C-E).4 Second, the intensity (area) of the resonance at 182.6 ppm, relative to natural abundance protein resonances, did not increase when oleate was added. The chemical shift values for oleate carboxyl resonances are indicated above each peak. The concentration of PIP and lysozyme were 17 mg/ml. A pulse interval of 2.0 s with 90° pulses, a spectral width of 25,000 Hz, and 32 K time domain points were used for spectral processing. Number of spectral accumulations: 24,064 (A), 15,104 (B), 14,336 (C), 9,344 (D), 16,256 (E), 23,296 (F), 9,728 (G), 14,848 (H), 16,128 (I), and 12,800 (J). Different vertical scaling factors were used in these spectra and, therefore, peaks heights cannot be directly compared for different panels in this figure.

The stoichiometry of binding of oleate to PIP was approxi-
mately 1:1, based on the following observations. First, unbound oleate was detected only in samples containing a fatty acid:protein mole ratio greater than 1 (Fig. 4, C-E).4 Second, the intensity (area) of the resonance at 182.6 ppm, relative to natural abundance protein resonances, did not increase when oleate was added. The chemical shift values for oleate carboxyl resonances are indicated above each peak. The concentration of PIP and lysozyme were 17 mg/ml. A pulse interval of 2.0 s with 90° pulses, a spectral width of 25,000 Hz, and 32 K time domain points were used for spectral processing. Number of spectral accumulations: 24,064 (A), 15,104 (B), 14,336 (C), 9,344 (D), 16,256 (E), 23,296 (F), 9,728 (G), 14,848 (H), 16,128 (I), and 12,800 (J). Different vertical scaling factors were used in these spectra and, therefore, peaks heights cannot be directly compared for different panels in this figure.

The stoichiometry of binding of oleate to PIP was approxi-
mately 1:1, based on the following observations. First, unbound oleate was detected only in samples containing a fatty acid:protein mole ratio greater than 1 (Fig. 4, C-E).4 Second, the intensity (area) of the resonance at 182.6 ppm, relative to natural abundance protein resonances, did not increase when oleate was added. The chemical shift values for oleate carboxyl resonances are indicated above each peak. The concentration of PIP and lysozyme were 17 mg/ml. A pulse interval of 2.0 s with 90° pulses, a spectral width of 25,000 Hz, and 32 K time domain points were used for spectral processing. Number of spectral accumulations: 24,064 (A), 15,104 (B), 14,336 (C), 9,344 (D), 16,256 (E), 23,296 (F), 9,728 (G), 14,848 (H), 16,128 (I), and 12,800 (J). Different vertical scaling factors were used in these spectra and, therefore, peaks heights cannot be directly compared for different panels in this figure.

To assess the ionization behavior and solvent accessibility
of fatty acids bound to PIP, the sample represented by Fig. 4K was titrated with 1 N KOH or HCl, and 13C NMR spectra were accumulated at -0.5 unit pH intervals. The chemical shift values of the oleate carboxyl resonances as a function of pH are plotted in Fig. 5. Oleate bound to PIP (filled circles) exhibited a partial ionization shift between pH 6.7 and 5.5, with an estimated apparent pK, value < 5. Below pH 5.5, oleate dissociated from PIP and its corresponding NMR resonance became undetectable. This ionization and dissociation behavior was very similar to that observed for fatty acids bound to L-FABP (Cistol et al., 1988a, 1989), but distinct from that of L-FABP (Fig. 5, open circles). The ionization shift and apparent pK, value near 5 suggested that the carboxylate groups of oleate bound to PIP were solvent-accessible. Unbound bilayer-phase oleate (Fig. 5, filled squares) exhibited an ionization shift and approximate pK, value between 7.5 and 8.0, in agreement with previous results (Cistol et al., 1988a, 1988b).
We also used $^{13}$C NMR spectroscopy to determine if PIP binds bile acids in vitro. Very little is known about intracellular trafficking of bile acids in enterocytes and no cytosolic carriers of bile acids have been previously identified. Approximately 90 to 95% of the bile acids that pass through the small intestine are reabsorbed via both active and passive transport mechanisms as part of the enterohepatic circulation (reviewed in Dietschy, 1968; Hofmann, 1984; Erlinger, 1987). In humans, the primary bile acids are cholic acid (3α,7α,12α-trihydroxy-5β-cholanoic acid) and chenodeoxycholic acid (3α,7α-dihydroxy-5β-cholanoic acid). These bile acids are conjugated with glycine or taurine in the liver to form N-acyl conjugates and secreted into the bile. They then traverse the proximal to distal axis of the small bowel and are either reabsorbed without modification or are deconjugated by bacterial enzymes in the ileum prior to reabsorption. In addition, small amounts of secondary bile acids are also formed by bacterial modification of the primary bile acid pool: 7α-deoxycholic acid to deoxycholic acid (3α,12α-dihydroxy-5β-cholanoic acid) and chenodeoxycholic acid (3α-monohydroxy-5β-cholanoic acid). About 50% of the deoxycholic acid is reabsorbed while lithocholic acid is largely insoluble and poorly reabsorbed.

Given these considerations, we examined the interaction of chenodeoxycholate with PIP. An NMR sample containing PIP was titrated with carboxyl $^{13}$C-enriched chenodeoxycholate and spectra were collected at mole ratio increments of 1 (Fig. 6, A–E). Two $^{13}$C resonances corresponding to the carboxyl carbon of chenodeoxycholate were observed at -180.9–181.0 and 183.7–183.8 ppm. The intensities of both resonances increased with increasing mole ratio of bile acid to protein. An additional broader resonance was observed at ~179.5 ppm (Fig. 6E) when $^{13}$C-enriched oleate was added to the sample containing 4:1 bile acid to protein (Fig. 6D). (The final oleate:protein mole ratio in Fig. 6E = 1:1.) This resonance corresponded to unbound oleate (see above).

To compare the bile salt binding properties of PIP to those of a FABP homologue from intestine, a parallel experiment was run under essentially identical conditions, except that E. coli-derived I-FABP, was used, rather than PIP (Fig. 6, F–J). With increasing amounts of added bile salt, only one chenodeoxycholate carboxyl resonance was observed at 183.2 to 183.8 ppm (Fig. 6, A–E). Two $^{13}$C resonances corresponding to the carboxyl carbon of chenodeoxycholate were observed at -180.9–181.0 and 183.7–183.8 ppm. The intensities of both resonances increased with increasing mole ratio of bile acid to protein. An additional broader resonance was observed at ~179.5 ppm (Fig. 6E) when $^{13}$C-enriched oleate was added to the sample containing 4:1 bile acid to protein (Fig. 6D). (The final oleate:protein mole ratio in Fig. 6E = 1:1.) This resonance corresponded to unbound oleate (see above).

NMR ionization curves for chenodeoxycholate carboxyl resonances in samples containing PIP or I-FABP, are shown in Fig. 5. The resonance corresponding at 181.0 ppm in Fig. 6D exhibited a nearly complete titration curve with an apparent pK, of ~7 (Fig. 5, inverted triangles). The resonances at 183.8 in Fig. 6, D and I, also exhibited ionization shifts, but with estimated apparent pK, values near 5 (Fig. 5, filled and open triangles, respectively).

Several important differences were noted between the spectra obtained for samples containing PIP or I-FABP, First, the former exhibited a resonance at ~181.0 ppm (Fig. 6, A–E) that was not observed for samples containing chenodeoxycholate and I-FABP, (Fig. 6, F–J). Second, the presence of chenodeoxycholate blocked binding of oleate to PIP, but not to I-FABP. This was evidenced by the presence of unbound bilayer-phase oleate in the PIP sample (179.5 ppm, Fig. 6E) and protein-bound oleate in the I-FABP, sample (181.4 ppm, Fig. 6I).

![Fig. 6. Carboxyl/carboxyl region of proton-decoupled $^{13}$C NMR spectra for samples containing carboxyl $^{13}$C-enriched chenodeoxycholate and PIP (A–D), chenodeoxycholate, oleate, and PIP (E), chenodeoxycholate and I-FABP, (F–I), and chenodeoxycholate, oleate, and I-FABP. All spectra were accumulated at pH 7.4 and 37 °C. For the spectra shown in panels A–E, a sample of PIP in phosphate buffer was titrated with $^{13}$C-enriched chenodeoxycholate in 1 mole ratio increments and $^{13}$C NMR spectra were accumulated at each increment. To the sample at 4:1 mole ratio (D), $^{13}$C-enriched oleate was added to a final fatty acid:protein mole ratio of 1:1. In panels F–I, a parallel experiment was performed with I-FABP, instead of PIP. The number of transients are as follows: 9,656 (A), 11,520 (B), 7,424 (C), 20,480 (D), 9,472 (E), 6,912 (F), 6,056 (G), 7,188 (H), 6,144 (I), and 3,072 (J). All other sample and spectral conditions were as described in the legend to Fig. 4.](http://www.jbc.org/)

Fig. 6J). In the spectrum shown in Fig. 6E, no resonance was observed at the characteristic chemical shift for oleate bound to PIP (182.6 ppm; see Fig. 4). Taken together, these results suggested that the resonance at ~181.0 in Fig. 6, A–E, represents a specific binding interaction between chenodeoxycholate and PIP.

The resonance at ~183.8 ppm in Fig. 6 could not be definitively assigned at this time, since its chemical shift does not correlate with values for chenodeoxycholate in defined physicochemical environments (Cabrall et al., 1986). This resonance was observed in spectra for samples containing chenodeoxycholate and either PIP or I-FABP, but not in protein-free samples or in samples containing lysozyme. The nearly identical chemical shifts (~183.8 ppm) and ionization behavior (apparent pK, < 5; open versus filled triangles in Fig. 5) suggested that this resonance is equivalent for samples containing PIP versus I-FABP. It may represent unbound chenodeoxycholate in an undefined lipid aggregate or chenodeoxycholate in a "nonspecific" aggregate with protein. However, the possibility that this resonance represents binding of chenodeoxycholate to the binding pocket of I-FABP, can be ruled out, since the presence of chenodeoxycholate does not block fatty acid binding to this pocket (Fig. 6J). Similarly, it seems unlikely that this resonance at 183.8 ppm in Fig. 6, A–E,
represents chenodeoxycholate interactions with the binding pocket of PIP.

The NMR results are summarized in Table I. From these results, we conclude the following. First, like I- and L-FABP, PIP can bind long-chain fatty acids (palmitate and oleate). The fatty acid binding stoichiometry for PIP is that of I-FABP (1:1), but unlike that of L-FABP, which exhibits a binding stoichiometry of at least 2:1. In contrast, the carboxyl chemical shift of bound fatty acid, 182.6 ppm, is more similar to those for L-FABP. Second, unlike I-FABP, the PIP appears to bind bile salts in a specific manner. This specific interaction is characterized by a unique 13C carboxyl resonance at 181.0 ppm and the competition of bile acids and fatty acids for binding to PIP.

Crystalization and Preliminary X-ray Diffraction Studies of E. coli-derived Porcine Ileal Lipid Binding Protein—It is remarkable that the enteroctyotic population of the intestine synthesizes three abundant cytosolic proteins which can bind fatty acids in vitro, each with a distinctive regional distribution. The ileal lipid binding protein, I-FABP, L-FABP, and L-FABP belong to a family of intracellular proteins which currently contains 10 known members (reviewed in Jones et al., 1988; Sacchettini et al., 1990). Alignment of the primary structures of rat I-FABP and the P2 protein of bovine peripheral nerve reveals that they share ~27% identity and ~51% homology (Jones et al., 1988). Superimposition of their three-dimensional α-carbon backbone structures discloses a root mean square deviation of less than 1.6 Å (Sacchettini et al., 1990). The similarity in the secondary and tertiary structures of rat I-FABP, and bovine P2, despite their relatively low degree of primary sequence identity, suggests that many or all members of the intracellular lipid binding protein family may have a high degree of overall conformational "equivalence" (Jones et al., 1988). However, as seen from comparative studies of lipid interactions with FABP homologues from intestine, significant variations must occur in the conformations of their lipid binding sites.

Previous sequence comparisons of I-FABP, L-FABP, and PIP suggested that an additional 6 residue sequence is present in I-FABP, that is not represented in either of the other two homologous proteins (Gantz et al., 1989). This "insertion" occurs between Ala^26 and Tyr^39 of PIP, allowing us to place Gly^199 and Gly^201 of the ileal protein in a comparable position to Gly^196 and Gly^198 of I-FABP. The deleted sequences in the porcine ileal lipid binding protein and I-FABP, are predicted to occur at a turn between two β-strands in I-FABP, shortening each strand by ~8 residues. Based on inspection of our

### Table I

<table>
<thead>
<tr>
<th>Features</th>
<th>PIP</th>
<th>I-FABP&lt;sub&gt;e&lt;/sub&gt;</th>
<th>L-FABP&lt;sub&gt;e&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Binds fatty acids&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Yes&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Yes&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Yes&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Binds bile acids&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Yes&lt;sup&gt;b&lt;/sup&gt;</td>
<td>No&lt;sup&gt;c&lt;/sup&gt;</td>
<td>NA&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Binding stoichiometry for fatty acids</td>
<td>1:1</td>
<td>1:1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2:1&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Carboxyl chemical shift of bound fatty acid&lt;sup&gt;d&lt;/sup&gt;</td>
<td>182.6 ppm</td>
<td>181.4 ppm</td>
<td>182.2 ppm</td>
</tr>
<tr>
<td>Ionization behavior of bound fatty acids&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Ionizable; pK&lt;sub&gt;app&lt;/sub&gt; ~ 5</td>
<td>Nonionizable; pK&lt;sub&gt;app&lt;/sub&gt; ~ 4</td>
<td>Ionizable; pK&lt;sub&gt;app&lt;/sub&gt; ~ 5&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Bile acid blocks fatty acid binding</td>
<td>Yes&lt;sup&gt;c&lt;/sup&gt;</td>
<td>No&lt;sup&gt;c&lt;/sup&gt;</td>
<td>NA&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>Palmitate and oleate have been examined.

<sup>b</sup>Results from Cistola et al. (1988a, 1989).

<sup>c</sup>Chenodeoxycholate has been examined.

<sup>d</sup>Not assessed.

<sup>e</sup>Chemical shift values at pH 7.2 and 37 °C.

2-Å model of I-FABP<sub>e</sub> (Sacchettini et al., 1989a), we proposed that these two shortened strands could result in a “wider” opening between their two β-sheets without producing a disruption in overall conformation of the β-clam (Gantz et al., 1989).

Jones et al. (1988) observed that the δ-guanidinium groups of Arg<sup>120</sup> and Arg<sup>126</sup> in the P2 protein interact with the carboxylate group of its bound fatty acid. Our previous alignment of I-FABP, L-FABP, and the porcine ileal lipid binding protein indicated that Arg<sup>120</sup> of the ileal protein and Arg<sup>122</sup> of L-FABP correspond to Arg<sup>129</sup> of I-FABP (Gantz et al., 1989). However, Arg<sup>126</sup> of I-FABP<sub>e</sub> (which forms a salt bridge with the carboxylate group of palmitate), is not conserved in either of the other two proteins: L-FABP, is predicted to contain a Thr at this position while PIP has an Ala. The functional significance of these proposed structural differences is not known.

To begin to determine the structural basis for the differences in ligand-protein interactions observed in our NMR binding studies of I-FABP, and PIP, we identified conditions for crystallization of the E. coli-derived ileal protein before and after its exposure to chenodeoxycholate (see “Experimental Procedures”). Each protein solution yielded two types of crystals, needle shaped and parallel-piped, using polyethylene glycol 4000 as the precipitant. The space group and unit cell dimensions of the parallel-piped crystals formed without chenodeoxycholate were determined by precession photographic techniques and diffractometry. The crystals were orthorhombic in the P2<sub>1</sub>2<sub>1</sub>2<sub>1</sub> space group. The unit cell parameters are a = 36.15 Å, b = 50.13 Å, c = 67.18 Å (Table II). Each unit cell contains four molecules of the protein. The crystals that formed after exposure of the protein to chenodeoxycholate were isomorphous with the parallel-piped shaped crystals ob-

### Table II

| Properties of crystals of E. coli-derived porcine ileal lipid binding protein |
|---------------------------------|----------|----------|----------|
| Unit cell dimensions            |          |          |          |
| a = 36.15 Å                     | b = 50.13 Å | c = 67.18 Å |
| Unit cell properties            |          |          |          |
| Volume = 121,743 Å<sup>f</sup> |          |          |          |
| Space group = P2<sub>1</sub>2<sub>1</sub>2<sub>1</sub> orthorhombic |          |          |          |
| Number of molecules/cell = 4    |          |          |          |
| V<sub>v</sub>, dalton = 1.8      |          |          |          |
| % solvent (v/v) = 39            |          |          |          |
| Crystal properties              |          |          |          |
| Maximum resolution = 2 Å        |          |          |          |
tained in the absence of this ligand. This was established when we collected a data set of PIP-chenodeoxycholate crystals using an area detector. The maximum resolution of the crystals was 2.0 Å. Data were collected to 2.1 Å. The unit cell parameters were identical to those described above.

The morphology and small size of the needle-shaped crystals made handling very difficult. To date, we have only been able to obtain precession photographs of their principal axis. These photographs show a P2_12_12 unit cell with a = 39.1 Å, b = 39.5 Å, c = 78.0 Å.

Solution of the three-dimensional structure of the crystal line E. coli-derived porcine ileal protein with and without bound fatty acid or bile acid should yield important insights about the details of its interaction with these ligands. While much work remains to be done, I-FABP, L-FABP, and this ileal protein offer a potentially powerful model system for applying NMR and x-ray crystallographic techniques to de-
vise insights into the individual roles played by these three cytoplasmic proteins in the trafficking of their ligands within enterocytes. Together they may form a multifunctional cyto-

lipid transport system that serves different physiologic roles in different regions of the duodenal-to-ileal and crypt-to-villus axes of the gut.

REFERENCES

Dietzchy, J. M. (1968) J. Lipid Res. 9, 297–309
Developmental and structural studies of an intracellular lipid binding protein expressed in the ileal epithelium.
J C Sacchettini, S M Hauft, S L Van Camp, D P Cistola and J I Gordon


Access the most updated version of this article at http://www.jbc.org/content/265/31/19199

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/265/31/19199.full.html#ref-list-1