Identification of the Bile Acid-binding Site of the Ileal Lipid-binding Protein by Photoaffinity Labeling, Matrix-assisted Laser Desorption Ionization-Mass Spectrometry, and NMR Structure*

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The ileal lipid-binding protein (ILBP) is the only physiologically relevant bile acid-binding protein in the cytosol of ileocytes. To identify the bile acid-binding site(s) of ILBP, recombinant rabbit ILBP photolabeled with 3-azi- and 7-azi-derivatives of cholytyraine was analyzed by a combination of enzymatic fragmentation, gel electrophoresis, and matrix-assisted laser desorption ionization (MALDI)-mass spectrometry. The attachment site of the 3-position of cholytyraine was localized to the amino acid triplet His¹⁰⁰-Thr¹⁰¹-Ser¹⁰² using the photo reactive 3,3-azi-derivative of cholytyraine. With the corresponding 7,7-azi-derivative, the attachment point of the 7-position could be localized to the C-terminal part (position 112–128) as well as to the N-terminal part suggesting more than one binding site for bile acids. By chemical modification and NMR structure of ILBP, arginine residue 122 was identified as the probable contact point for the negatively charged side chain of cholytyraine. Consequently, bile acids bind to ILBP with the steroid nucleus deep inside the protein cavity and the negatively charged side chain near the entry portal. The combination of photoaffinity labeling, enzymatic fragmentation, MALDI-mass spectrometry, and NMR structure was successfully used to determine the topology of bile acid binding to ILBP.

Bile acids undergo an enterohepatic circulation involving the small intestine, the liver, and the kidney (1–4). This organotropism of bile salts is established by specific bile salt transport systems including active Na⁺/bile acid cotransporters in the apical membrane of ileocytes and proximal renal cells as well as the basolateral membrane of hepatocytes (5–9). Passive transporters in the basolateral membrane of intestinal and renal cells (10) as well as soluble binding proteins in the cytoplasm of hepatocytes, ileocytes, and renal proximal tubule cells and in blood complement enterohepatic cycling of bile acids. The carrier proteins for bile acids involved in their enterohepatic circulation were identified by our laboratory (11–23) using a set of photoreactive analogues of conjugated and unconjugated bile acids (11, 24–26). Recently, the active Na⁺/bile acid cotransporters in hepatocytes and ileocytes were identified and characterized by expression cloning (27, 28). Functional analysis of the Na⁺/bile acid cotransport systems in ileocytes and hepatocytes by target size analysis revealed that these transport systems are in their functional states complexes composed of several subunits (29–31). The rabbit ileal Na⁺/bile acid cotransport system revealed a functional molecular mass of 451 ± 35 kDa (31) being probably composed of four integral membrane proteins of 93 kDa (identified as dimers of the Na⁺/bile acid cotransporting protein (19)) and four cytoplasmically attached 14-kDa peripheral membrane proteins (19, 22, 31). The 14-kDa protein, the ileal lipid-binding protein (ILBP),1 has originally been identified as a predominant bile acid binder in the cytosol of ileocytes (21, 32–39). Photoaffinity labeling of intact rabbit ileal tissue revealed that ILBP is the only physiologically relevant bile acid-binding protein in the ileal cytosol (40). ILBP specifically interacts with the ileal bile acid transporter protein IBAT at the cytoplasmatic face of the ileocyte, suggesting a direct function in active ileal bile acid absorption (19, 22, 31). Recently we demonstrated a paradoxical binding behavior of ILBP. Photoaffinity labeling of ILBP by 3- or 7 diazirino-derivatives of cholytyraine in the presence of competing natural bile acids led to a stimulation of label incorporation rather than to an expected inhibition (41). This indicates that bile acids are able to increase the affinity of ILBP for bile acids thus making a positive substrate-load feedback regulation mechanism for the active uptake of bile acids in the ileum probable.

Thus, the interaction of bile acids with ILBP and the molecular identification of its bile acid-binding site(s) are important for the understanding of intestinal bile acid absorption. ILBP belongs to the family of intracellular lipid-binding proteins including the family of fatty acid-binding proteins (FABPs) (42–47). Whereas tertiary structures of several FABPs by x-ray crystallography have been published (48–52), no crystal structure of ILBP has been reported so far. The tertiary structure of recombinant porcine ILBP has been determined by homonuclear two-dimensional NMR spectroscopy (53). It was shown that bile acids can bind in the interior of the protein between two β-sheets, and two different modes for bile acid binding to ILBP were suggested from the NMR analysis as follows: 1) an orientation of the bile acid inside the protein cavity with the negatively charged side chain located deep inside the binding

1 The abbreviations used are: ILBP, ileal lipid-binding protein; FABP, fatty acid-binding protein; IBAT, ileal bile acid transporter; MALDI, matrix-assisted laser desorption ionization; MALDI-TOF, matrix-assisted laser desorption ionization-time of flight; NOE, nuclear Overhauser enhancement; NOESY, two-dimensional NOE; PVDF, polyvinylidene difluoride; RMSD, root mean square deviation; PAGE, polyacrylamide gel electrophoresis; SA, sinapinic acid; HCCA, 4-hydroxy-cyanylimidic acid; ATZ-aa, anilinothiazolinone-amino acid; PITC, phenylisothiocyanate; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.
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**EXPERIMENTAL PROCEDURES**

**Materials**

Photoaffinity labeling was performed with the photolabile bile acid analogues 2-3,3-azo-7α,12α-dihydroxy-5β-chol-24-oylaminoethanesulfonic acid, 2-3,3-azo-7α,12α-dihydroxy-5β-[125I]chol-24-oylaminoethanesulfonic acid (specific radioactivity 5.9 Ci/mmol), 2-7,7-azo-3α,12α-dihydroxy-5β-chol-24-oylaminoethanesulfonic acid, and 2-7,7-azo-3α,12α-dihydroxy-5β-[3H]chol-24-oylaminoethanesulfonic acid (specific radioactivity 20.25 Ci/mmol) synthesized as described (11, 24, 25). Acrylamide, N,N'-methylene bisacrylamide, and Serva Blue R-250 were from Serva (Heidelberg, Germany), and the pH was adjusted with 10–50 mM phenylglyoxal in 10 mM Tris/Hepes buffer (pH 7.4). The final concentration of ILBP was 70 pmol/l.

**Antibodies**

Purified recombinant rabbit ILBP (250 μg) or a dendrimeric peptide containing 8 times the sequence 90–102 of rabbit ILBP were mixed with complete Freund’s adjuvant (0.5 ml) and phosphate-buffered saline to a total volume of 1 ml. Equal portions of this mixture were injected at two positions (50 μg/ml muscle) and in the tail. Eggs induce the antibody response about 3 weeks after the injection were collected, and the antibody fraction was obtained from the yolks using a kit (EGGstract IgY Purification System) from Promega (Madison WI) according to protocols supplied by the vendor.

**Photoaffinity Labeling of Recombinant Rabbit ILBP**

Recombinant rabbit ILBP was adjusted with 10 mM Tris/Hepes buffer (pH 7.4), 100 mM NaCl, 100 mM mannitol to a protein concentration of 0.5 mg/ml and incubated in the dark for 5 min with radiolabeled 3,3-azo- or 7,7-azo-derivatives of cholytaurine or with 1 or 2 mM of unlabeled compounds (for concentration see legends to the figures). Photocross-linking was performed by irradiation at 350 nm in a Rayonet-RPR-100 photochemical reactor (The Southern Ultraviolet Co., Hamden, CN) equipped with 4 RPR-3500 Å lamps. Subsequently, noncovalently bound photoreactive bile acids were removed by gel permeation chromatography using prefiltered PD-10 columns or by precipitation of proteins with chloroform/methanol (54). Subsequently, photolabeled ILBP was immediately used for further analysis.

**Chemical Modification of Arginine Residues with Phenylglyoxal**

Freshly prepared rabbit ileal cytosol or recombinant rabbit ILBP were incubated at a protein concentration of 2 mg/ml for 1 h at 20 °C with 10–50 mM phenylglyoxal in 10 mM Tris/Hepes buffer (pH 7.4), 300 mM mannitol. Control samples were treated identically without addition of phenylglyoxal. Subsequently, proteins were separated from phenylglyoxal by gel permeation chromatography using PD-10 columns using the above buffer as eluent. After determination of protein concentration, the protein samples were immediately used for photoaffinity labeling experiments.

**SDS Electrophoresis and Detection of Radioactivity**

SDS-PAGE was performed as described previously (16, 22). Electrophoretic separation of peptide fragments was performed in Tris/Tricine gels (16, 5%) according to Schagger and von Jagow (55). Detection of radioactivity in gels, nitrocellulose sheets, or PVDF membranes was performed by slicing of the lanes into 2-mm pieces, digestion of proteins with tissue solubilizer Biolute S, and subsequent liquid scintillation counting.

**Blotting**

Proteins were bound to nitrocellulose membranes (0.2 μm trans-blot transfer medium from Bio-Rad) or to PVDF membranes by electrophoretic transfer from SDS-PAGE gels in a transblot cell (Bio-Rad) using 25 mM Tris, 192 mM glycine, 33% methanol (pH 8.3) as transfer buffer. Blotting conditions were 300 mA for 3 h, followed by 400 mA for another 0.5 h. Antibodies bound to protein on the membrane were detected using the Western-Light Chemiluminescent Detection Kit from Serva (Heidelberg, Germany).

**Amino Acid Sequence Analysis**

A sequence analyzer ABI 467 with pulsed liquid technique was used. The automatic procedure follows the Edman chemistry. The chemical process of protein sequencing as used in the pulsed liquid Protein Sequencer began by drying a solution (100 pmol to 2 nmol/20 μl) of the sample on a glass fiber filter disc (0.8 cm), precelyced with Biobrene®. The disc was then placed in the reaction vial in the machine following manufacturer’s instructions. A small quantity of a solution of phenylisothiocyanate (PITC) in heptane was delivered to the disc and washed with heptane, and the remaining solvent was blown off. An argon gas stream saturated with trimethylamine was passed through the filter to create the basic environment necessary for PITC to react with the amino group of the N terminus of the protein. Then the excess PITC on the filter was washed out using ethyl acetate, and the filter was dried. A few microliters of trifluoroacetic acid were then added to the filter to cleave the derivatized N-terminal amino acid from the rest of the protein. The cleavage process was accelerated by the cyclization of the phenylisothiocyanate adduct that resulted in the generation of an anilinothiazoline-amino acid (ATZ-AA) derivative. The ATZ-AA was then extracted from the filter (i.e., separated from the shortened protein) with ethyl acetate and collected in the conversion flask. The ethyl acetate was evaporated, and aqueous trifluoroacetic acid was added to induce the heat-assisted isomerization, whereby the unstable ATZ-AA was converted to the more stable phenylisothionyhydantoin-derivative. The latter was analyzed on-line using a sensitive high pressure liquid chromatography system. Subsequent cycles were initiated by a new delivery of PITC to the glass fiber disc.

**Enzymatic Fragmentation of Recombinant ILBP for MALDI-Mass Spectrometry**

Endoproteinase GluC—25 μg of ILBP labeled with the 3,3-azo- or 7,7-azo-derivatives of cholytaurine were digested in 45 μl of 25 mM ammonium carbonate buffer (pH 7.8) by adding 5 μl of a solution of 1.25 μg of endoproteinase GluC from Staphylococcus aureus V8 in 5 μl of the above buffer, and subsequent incubation for 8 h at 37 °C. The final concentration of ILBP was 35 pmol/l. Trypsin—25 μg of ILBP labeled with 3,3-azo- or 7,7-azo-derivatives of cholytaurine were digested in 16 μl of 25 mM Tris/HTC buffer (pH 8.5) by adding 1.25 μg of trypsin from bovine pancreas in 9 μl of the above sample followed by incubation for 16 h at 37 °C. The final concentration of ILBP was 70 pmol/μl.

**Sample Preparation for MALDI-Mass Spectrometry—20 μl of the GluC digestion solution were freeze-dried and redissolved in 10 μl of a solution of 0.1% trifluoroacetic acid and 30% acetonitrile. This solution was mixed with 10 μl of a saturated solution of recrystallized 4-hydroxy-α-cyanocinnamic acid (HCCA) in 0.1% of trifluoroacetic acid, 30% acetonitrile. 1 μl corresponding to approximately 9 pmol were spotted on a stainless steel target for crystallization at room temperature and subsequently used for MALDI measurements. The tryptic digest solution was diluted 1:8 with a saturated solution of HCCA in 0.1% trifluoroacetic acid, 30% acetonitrile. 1 μl corresponding to approximately 9 pmol were spotted on a stainless steel target for crystallization at room temperature and used subsequently for MALDI measurements.**

**MALDI-Mass Spectrometry**

For mass spectrometry a Bruker Time-of-Flight (Biflex®) instrument equipped with a 337 nm N2-UV laser, delayed extraction, reflector, and FAST pulser for post-source decay experiments was used. The decay of parent ions during the field-free drift obeys certain rules enabling us to deduce the sequence of a peptide including attached labels. The cleavage occurs mainly at the amide bond between two amino acids leaving N- or C-terminal daughter ions behind. Depending on where the positive charge is located after the breakage, b or y series are generated with the N or C terminus intact, respectively. For more details see Ref.
The instrument was calibrated with cytochrome c when used in positive linear mode. For the analysis of enzymatically generated peptides calibration was done with angiotensin II and ACTH-clip (18–39) and used in positive reflector mode. Laser attenuation was set to threshold.

NMR Structure of Recombinant Human ILBP

NMR Spectroscopy—All NMR experiments were performed at 32 °C using a 2–3 mM concentration of protein (unlabeled, 15N and 15N/13C labeled, respectively) in a buffer solution of 50 mM potassium phosphate buffer (pH 6.0). The NMR data were collected on a Bruker DRX 600 spectrometer equipped with a multichannel interface and a triple resonance probe (1H, 13C, and 15N) with X, Y, and Z gradients. The data were processed on an Indigo 2 station (Silicon Graphics) using the programs XWINNMR (57) and AURELIA (58) from Bruker.

Structure Calculations—Structures were calculated with the distance geometry program DIANA (59, 60) using the software package SYBYL, version 6.5 (SYBYL Molecular Modeling Package, Version 6.5, Tripos, St. Louis, MO) on SGI workstations (Indigo 2, Power Challenge). A set of 100 conformations was calculated employing the REDAC (61) strategy (redundant dihedral angle constraints) and the standard minimization parameters. Overall, six REDAC cycles were performed. A set of 10 structures with the lowest target function value was subjected to restrained energy minimization using the Tripos 6.0 force field (62). For the free ILBP the average RMSD of the 10 structures is 94 ± 6 pm for all backbone atoms (N, Cα, and Cβ) and 147 ± 11 pm for all heavy atoms. For the complex the average RMSD for all backbone atoms (N, Cα, and Cβ) is 81 ± 15 and 144 ± 12 pm for all heavy atoms.

RESULTS

Enzymatic Fragmentation of Recombinant Rabbit ILBP after Photoaffinity Labeling with 2-(3,3-azo-7α,12α-dihydroxy-5β-[7β,12β-3H]cholan-24-oylamino)ethanesulfonic acid. Six portions of 60 μg of recombinant rabbit ILBP were incubated each with 2.12 μCi (1 μCi) of 2-(3,3-azo-7α,12α-dihydroxy-5β-[7β,12β-3H]cholan-24-oylamino)ethanesulfonic acid in 80 μl of 10 mM Tris/Hepes buffer (pH 7.4), 100 mM mannitol, 100 mM NaCl at 20 °C for 5 min in the dark followed by irradiation at 350 nm for 10 min. After precipitation with chloroform/methanol protein was redissolved in SDS sample buffer, and enzymatic digestion was performed with 500 μg/ml GluC at 37 °C for 90 min. After addition of SDS sample buffer including 4 mM iodoacetamide, 4 mM phenylmethylsulfonyl fluoride, 4 mM EDTA, the sample was heated to 95 °C for 5 min, and subsequently, peptides were separated by SDS-PAGE on 16.5% gels (36 μg of peptides per lane) followed by blotting to PVDF membranes, staining, determination of distribution of radioactivity, and sequence analysis of radioactive peptide bands (Table I). A, stained peptides after SDS-PAGE and blotting to PVDF membranes. Lane A, marker proteins; lane B, rabbit ILBP; lane C, rabbit ILBP photolabeled with 2-(3,3-azo-7α,12α-dihydroxy-5β-[7β,12β-3H]cholan-24-oylamino)ethanesulfonic acid and digestion with endoproteinase GluC. B, distribution of radioactivity after slicing in 2-mm pieces and subsequent liquid scintillation counting.
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Table I

| Peptide band | Amount (pmol) | Amino acid sequence determined |
|--------------|---------------|-------------------------------|
| 11           | 300           | 41–58 ff                      |
| 13           | 600           | 41–59 ff                      |
| 14           | 560           | 41–58 ff                      |
| 17           | 1270          | 72–90 ff                      |
| 20           | 390           | 72–88 ff                      |

Table II

| Peptide band | Amount (pmol) | Amino acid sequence determined | Radiolabeling |
|--------------|---------------|-------------------------------|---------------|
| 5            | 40            | N terminus                    | -             |
| 8            | 6             | 2–24 ff                       | -             |
| 9            | 5             | 18–30 ff                      | -             |
| 10           | 30            | 41–56 ff                      | -             |
| 12           | 100           | 41–57 ff                      | +             |
| 13           | 65            | 41–70 ff                      | ++            |
| 16           | 140           | 72–107 ff                     | ++            |
| 17           | 100           | 72–100 ff                     | ++            |
| 18           | 60            | 41–59 ff                      | +++           |

Recombinant rabbit ILBP was photolabeled as described under "Experimental Procedures" and legend to Fig. 2. After precipitation with chloroform/methanol, protein was dissolved in 100 µl of 62.5 mM Tris/HCl buffer (pH 6.8), 0.2% SDS, 0.5% 2-mercaptoethanol, 0.001% bromphenol blue and mixed with 100 µl of a freshly prepared solution of endoproteinase GluC in 62.5 mM Tris/HCl buffer (pH 6.8) to a final protein concentration of 500 µg/ml. Digestion was performed for 90 min followed by SDS-PAGE on 16.5% gels and blotting to PVDF membranes. The determination of a second interaction point of the bile acid molecule with ILBP would allow an unequivocal determination of the spatial orientation of a bile acid molecule during binding to ILBP.
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Fig. 3. Enzymatic fragmentation of recombinant rabbit ILBP by chymotrypsin after photoaffinity labeling with 2-(7,7-azo-3a,12α-dihydroxy-5β-[3β-3H]cholan-24-oylamino)ethanesulfonic acid. Five portions of 40 μg of recombinant rabbit ILBP were incubated each in 150 μl of 10 mM Tris/Heps buffer (pH 7.4), 100 mM mannitol, 100 mM NaCl with 0.33 μM (1 μCi) 2-(7,7-azo-3a,12α-dihydroxy-5β-[3β-3H]cholan-24-oylamino)ethanesulfonic acid at 20 °C for 5 min in the dark followed by irradiation at 350 nm for 10 min. After precipitation with chloroform/methanol protein was re-dissolved in 100 μl of SDS sample buffer. 80 μl of this solution was mixed with 80 μl of a freshly prepared solution of chymotrypsin in 62.5 mM Tris/HCl buffer (pH 6.8) and sequence analysis of radioactive bands (Table III).

Recombinant rabbit ILBP was photolabeled and digested as described in legend to Fig. 3.

| Peptide band | Amount (pmol) | Amino acid sequence (sequence 90–102) | Immunoreactivity to epitope-specific antibody | Radio-labeling |
|--------------|---------------|---------------------------------------|-------------------------------------------|----------------|
|              |               |                                       |                                           |                |
| 1            | 40            | N terminus of chymotrypsin            | –                                         | –              |
| 2            | 30            | N terminus of ILBP 1–25 ff            | –                                         | –              |
| 3            | 20            | N terminus of ILBP 1–20 ff            | +                                         | –              |
|              | 20            | 65–85                                 |                                           |                |
| 4            | 130           | N terminus 1–32 ff                    | +++                                      | +++           |
| 5            | 700           | 76–117 ff                             | +++                                      | +++           |
| 6            | 115           | 81–110 ff                             | ++                                       | +++           |

Recombinant rabbit ILBP was photolabeled and digested as described in legend to Fig. 3.

between the bile acid ligand and ILBP, we have submitted recombinant ILBP to photoaffinity labeling with nonradiolabeled azi-derivatives of cholytaurine at concentrations of 1 or 2 mM and subsequently analyzed the cross-linked probes by MALDI-mass spectrometry.

Fig. 4A demonstrates that the recombinant ILBP photoaffinity labeled with 2-(7,7-azo-3a,12α-dihydroxy-5β-cholan-24-oylamino)ethanesulfonic acid exerted 3 major mass peaks of m/z 14293.8, 14452.4, and 14659.7 corresponding to the products des-methionyl-ILBP (14293.8) (Fig. 4A, peak I), formyl-methionyl-ILBP (14452.49) (Fig. 4A, peak II), and formyl-methionyl-ILBP/matrix adduct (14659.7) (Fig. 4A, peak III). Expression of proteins in Escherichia coli usually leads to a mixture of expressed proteins as follows: N-formyl-methionyl protein which can be transformed into the mature N-methionyl protein by a peptide deformylase (EC 3.5.1.27); the N-terminal methionine can be removed by a methionine aminopeptidase (EC 3.4.11.18) to the des-methionyl protein (63). Recombinant ILBP obtained in E. coli is a mixture of formylmethionyl-ILBP, des-methionyl-ILBP, and ILBP. Owing to the small mass difference of 28 Da, ILBP and formyl-methionyl-ILBP could not be resolved by MALDI-mass spectrometry. Irradiation of recombinant ILBP alone at 350 nm for 10 min had no influence on the mass spectrum. However, upon performing the ultraviolet irradiation in the presence of 1 mM 2-(7,7-azo-3a,12α-dihydroxy-5β-cholan-24-oylamino)ethanesulfonic acid an additional peak at m/z 14803.9 (Fig. 4A, peak IV) was detectable corresponding to the photocross-linked product of des-methionyl-ILBP with the photolabile bile acid analogue. Under the conditions used, only a small portion (estimated <5%) of the recombinant ILBP was covalently modified; nevertheless, the direct detection of the photocross-linked protein was possible by MALDI-mass spectrometry. By using the photoreactive isomer 2-(3,3-azo-7α,12α-dihydroxy-5β-cholan-24-oylamino)ethanesulfonic acid, similar results were obtained. After labeling in the presence of 1 mM of the 3-azi-derivative of cholytaurine a mass peak at m/z 14769.72 was detectable in addition to the mass peak of des-methionyl-ILBP at m/z 14269.9 (Fig. 4B, peak IV). For the formylated ILBP at mass peak m/z 14430.7 (Fig. 4B, peak II) also a satellite peak at m/z 14929.6 (Fig. 5B, peak V) was detectable indicating the incorporation of the photoprobe into formyl-ILBP.

MALDI-Mass Spectrometry of Peptide Fragments Obtained from Recombinant ILBP Photolabeled with 2-(7,7-Azo-3α,12α-dihydroxy-5β-cholan-24-oylamino)ethanesulfonic Acid—As a next step to the identification of the ligand contact points, the photomodified recombinant ILBP was digested with 1.25 μg of endoproteinase Gluc at a concentration of 35 pmol/μl. The resulting mixture of peptides was analyzed in the MALDI-TOF mode with unlabeled recombinant ILBP as a control (Table IV). The only additional peaks observed in the labeled probe were m/z 2319.29, 2799.12, and 2218.18 (Fig. 5A). The first two peaks can be explained as adduct peaks to m/z 1822.06 and 799.39 with a difference of 497.23 and 497.36, respectively. The peak at m/z 2218.18 is a double-charged peptide (aa 88–128) containing the C terminus, which is barely visible in the control. All other peaks appear also in the control. To confirm these findings post-source decay experiments (see “Experimental Procedures”) were made. In the case of the parent ion m/z 2319.29 the first significant fragment was at m/z 1823.16, which is parent ion minus label. This showed clearly that the 2319.29 peak is related to the 1822.06 peak, which is a fragment of the ILBP sequence from position 112–128, the C-terminal part. The position of the label could not be determined further since the label was split off completely during post-source decay experiments. Taking a closer look to the N terminus position of the label of the parent ion m/z 1296.75, we could show by post-source decay experiments that the lowest b fragment ion is at m/z 716.8, which is the normal b2 fragment (219.61) plus label. So the label is positioned at the N-terminal alanine or phenylalanine residue of peptide fragment 2–8.

MALDI-Mass Spectrometry of Peptide Fragments Obtained from Recombinant ILBP Photolabeled with 2-(3,3-Azo-7α,12α-dihydroxy-5β-cholan-24-oylamino)ethanesulfonic Acid—MALDI-mass spectrometry of recombinant ILBP photolabeled with the 3-azi-derivative of cholytaurine and subsequent en-
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doproteinase GluC digestion revealed a peptide fragment at m/z 1748.26 corresponding to amino acid sequence 88–103. An additional peak at m/z 2245.86 represents the cross-linking of the 3-diazirine photoprobe (m/z 5497.6) to peptide fragment 88–103 (m/z 1748.26) (Fig. 5B) indicating the attachment point of the 3-position of a bile acid to the sequence region 88–103 of ILBP. After trypsin digestion of recombinant ILBP photolabeled with 3-azi-cholyltaurine, an additional peak at m/z 2243.57 occurred (Fig. 6 and Table V). This peak probably is the cross-linking product of the photoprobe of peptide fragment m/z 1746.32 corresponding to amino acid sequence 91–105. Another additional peak occurs at m/z 2046.45, which is aa 91–108, a fragment with one omitted splitting position behind Lys105, probably due to steric hindrance from the label. In the control this steric hindrance is not existent and hence peak 2046.45 cannot be observed. Consequently there should be also an adduct peak to this longer fragment (aa 91–108) but probably intensity is too low and therefore is not detectable. Overlapping these two findings from GluC and trypsin digestions, the labeled peptide sequence with the attachment point of the 3-position of the bile acid moiety must be between position 91 and 103 (VVANFPNYQHTSE). For a further refinement of the attachment site of the 3-azi-derivative of cholyltaurine to the ILBP-peptide backbone the fragments m/z 1746.32 (aa 91–105) and m/z 2243.57 (aa 91–105, cross-linked to 3-azi-cholyltaurine) were selected by the FAST pulser of the MALDI instrument from the other tryptic peptide ions and submitted to sequence analysis. The parent ion m/z 2243.57 with the
sequence, KIESTHQYNPFNAVV, including the attached label produced predominantly y-series, e.g. y*6 to y*15 (Table VI and Fig. 7). Below y*6 the signals were barely detectable. So, since we could see only y*6 to y*15, the label must be attached to y*6 or lower. The sequence with the attached label therefore narrows down to KIESTH, because this and all higher fragments showed up with label. By taking into consideration that the GluC digestion resulted also in a labeled fragment (aa 88–103) ending at glutamic acid 103, we could narrow down to Glu103-GluC digestion resulted also in a labeled fragment (aa 88–103) containing in Table V.

Numbers refer to peaks assigned in Table V.

**NMR Structure of ILBP**—As an independent method to determine the ligand-binding site of ILBP, we have performed a NMR structure of recombinant human ILBP. The resonance assignment of free ILBP and of its complex with cholytaurine was performed following the standard procedure described by Wüthrich (64). The sequential assignment based on sequential NOEs in the homonuclear NOESY spectra and in the three-dimensional NOE-symmetry experiment in combination with a three-dimensional triple resonance 1H,13C–15N–1H correlation spectroscopy experiment. Most of the side chain resonances were assigned using the three-dimensional 1H–13C–15NOESY-heteronuclear multiple quantum coherence spectroscopy experiment. The theoretical NOE and HN–HN correlation spectroscopy experiment was performed following the standard procedure described by Wüthrich (64). The sequential assignment based on sequential NOEs in the homonuclear NOESY spectra and in the three-dimensional NOE-symmetry experiment in combination with a three-dimensional triple resonance 1H,13C–15N–1H correlation spectroscopy experiment.
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TABLE V

| Fragment | aa         | Sequence                  | $M_1 (M+H)^+$ | $M_2 (M+H)^+$ | Additional peaks ($M+H^+$) |
|----------|------------|---------------------------|---------------|---------------|-----------------------------|
| T1       | 2–6        | AFTGK                     | 523.28        |               |                             |
| T2       | 7–13       | FEMESEK                   | 899.37        | 899.15        |                             |
| T3       | 14–20      | NYDEFMK                   | 946.39        | 946.25        |                             |
| T4       | 21–31      | LGLPSDVVEK                | 1169.67       | 1169.62       |                             |
| T5       | 32–33      | SE                        | 262.14        |               |                             |
| T6       | 34–36      | NIK                       | 374.23        |               |                             |
| T7       | 37–42      | IVTEIK                    | 702.43        | 702.25        |                             |
| T8       | 43–63      | QDQGFETSHSSGQIMT          | 2437.05       | 2437.21       |                             |
| T9       | 64–68      | FTIGK                     | 565.33        | 565.20        |                             |
| T10      | 69–78      | ESEIQTFGGK                | 1095.52       | 1095.24       |                             |
| T11      | 79         | K                         | 147.11        |               |                             |
| T12      | 80–81      | FK                        | 294.17        |               |                             |
| T13      | 82–90      | AVNMEGK                   | 904.45        | 904.16        |                             |
| T14      | 91–105     | VVANFNYQHTSEIK            | 1746.87       | 1746.77 (4)   | 2243.56 (4*)                |
| T14–15   | 91–108     | VVANFNYQHTSEIKGD         | 2047.03       |               | (2046.45)                  |
| T15      | 106–108    | GDK                       | 319.15        |               |                             |
| T16      | 109–122    | LVEVSSIGVYTER             | 1508.79       | 1508.7        |                             |
| T17      | 120–125    | VSK                       | 333.21        |               |                             |
| T18      | 126        | R                         | 175.11        |               |                             |
| T19      | 127–128    | LA                        | 203.13        |               |                             |

TABLE VI

Measured fragment ions (Fig. 7) of parent ion 2243.58 selected from the spectrum of peptides shown in Fig. 8 obtained by trypsin cleavage of recombinant rabbit ILBP photolabeled with 2-(3,3-azo-7-dihydroxy-5-cholan-24-oylamino)-ethanesulfonic acid

In the PSD spectrum Y-series were dominating. Y* are fragments with label. Photolabeling and cleavage was described in legend to Fig. 6. ND, not detectable.

| No. | Sequence | y   | y*  |
|-----|----------|-----|-----|
| 1   | K        | ND  | ND  |
| 2   | KI       | 261.18 | ND | ND  |
| 3   | KIE      | 390.17 | ND | ND  |
| 4   | KIES     | 477.31 | ND | ND  |
| 5   | KIEST    | 578.05 | ND | ND  |
| 6   | KIESTH   | 715.17 | 1213.01 | ND | ND  |
| 7   | KIESTHQ  | 843.24 | 1341.8 | ND | ND  |
| 8   | KIESTHQY | 1006.23 | 1503.65 | ND | ND  |
| 9   | KIESTHQYN| ND  | 1618.13 | ND | ND  |
| 10  | KIESTHQYNP| 1217.13 | 1715.12 | ND | ND  |
| 11  | KIESTHQNPFF| 1364.07 | 1861.79 | ND | ND  |
| 12  | KIESTHQNPFFN| ND    | ND   | ND  | ND  |
| 13  | KIESTHQNPFFNAV| ND | ND  | ND  | ND  |
| 14  | KIESTHQNPFFNAV| 2047.21 | ND | ND  | ND  |
| 15  | KIESTHQNPFFNAV| 1746.94 | 2245.69 | ND | ND  |

A total of 1681 relevant (nontrivial) NOE distance constraints was derived from the NOESY data of the free protein. These constraints consisted of 216 intraresidue, 457 sequential, and 196 medium range (2–4 residues apart) and 812 long range (5 or more residues apart). Together with 28 constraints for the experimentally determined hydrogen bonds (and 19 for redundant hydrogen bonds), these data were used as input for distance geometry calculations using the program DIANA (59, 60). The secondary structure of ILBP consists of two short α-helices and 10 β-strands. The 10 antiparallel β-strands of human ILBP are arranged in two nearly orthogonal β-sheets forming a so-called β-clam structure (48) with a gap between β-strands D and E. The two α-helices close this β-barrel on one side. The global fold of human ILBP is typical for all lipid-binding proteins including the large family of FABP. After the addition of choyltaine, the chemical shifts of almost all amino acids changed significantly. These circumstances required a more or less complete new assignment following the same strategy as described above for the free protein. This time a slightly larger number of relevant NOE distance constraints was derived from the NOESY spectra consisting of 228 intraresidue, 517 sequential, and 184 medium range and 903 long range (total 1832). Despite the changes in chemical shifts, the structure of ILBP remains almost unchanged upon complexation with choyltaine. In addition to NOEs among protons belonging to the protein, a large number of intermolecular NOEs between protons of certain amino acids and protons of choyltaine were observed. All these amino acids are placed in the interior part of the protein forming a large binding cavity for the bile acid. To determine the structure of the complex, the structure with the lowest target function from the DIANA calculations was chosen to be energy-minimized. The bile acid was docked manually in the binding cavity of the protein. After a further energy minimization, the intermolecular constraints were added to the constraint file, and a restrained molecular dynamics simulation was performed. The system was equilibrated at 300 K for 50 ps, after then the trajectory was sampled at 300 K in 50-ps intervals for 500 ps. These 10 frames from the trajectory were energy-minimized and used for further analysis.
Identification of the Bile Acid-binding Site of ILBP

DISCUSSION

The enterohepatic circulation of bile acids plays a pivotal role for the regulation of serum cholesterol levels. Responsible for the efficient reabsorption of bile acids in the terminal ileum is the Na⁺/bile acid cotransport protein IBAT and the ileal lipid-binding protein ILBP specifically associated with IBAT (19, 22, 31). ILBP is the dominant binding protein for bile acids in the cytosol of the ileocyte (40). A possible central role of ILBP for the enterohepatic circulation of bile acids can also be deduced from the findings that the expression of ILBP in ileocytes is influenced by the presence of bile constituents (66), and bile acids have been identified as natural ligands for the farnesoid X receptor nuclear transcription factor (67–69). Accordingly, bile acids greatly stimulate via farnesoid X receptor the expression of ILBP in the ileocyte (67). Owing to this important function of ILBP for bile acid enterohepatic circulation, we attempted to localize the bile acid-binding site(s) in the ILBP protein. An unequivocal three-dimensional structure of the interaction of ILBP with bile acids is not yet known because all crystallization attempts to generate crystals suited for a high resolution structure determination of ILBP failed so far. A NMR structure of porcine ILBP was not able to determine unequivocally the orientation of the bile acid molecule during binding to ILBP leaving two different modes of bile acid binding open (53). We therefore have used a novel approach to identify the topology of bile acid binding to ILBP by using a combination of photoaffinity labeling, enzymatic degradation, and MALDI-mass spectrometry. A set of photoreactive bile acid analogues carrying the identical photoreactive group at different positions in the molecule should allow an unequivocal determination of the orientation of a bile acid during binding to ILBP as follows. (i) 2-(3, 3-Azo-7α,12α-dihydroxy-5β-[7β,12β-3H]cholan-24-oylamino)ethanesulfonic acid carries a carbene-generating diazirine function at position 3 of the steroid nucleus replacing the α-oriented 3-hydroxy group of natural bile acids. This compound behaves in any aspect of the enterohepatic circulation like natural bile acids (25). Recently, we could clearly show that the 3-hydroxy group of bile acids is not necessary for molecular recognition and transport by the Na⁺/bile acid cotransport systems (70, 71). Photoaffinity labeling of recombinant rabbit ILBP with this photoprobe localized the attachment point beyond amino acid 72. By MALDI-TOF mass spectrometry of peptide fragments obtained by endoproteinase GluC or trypsin digestion, the amino acid sequence His100-Thr1018-Ser102 was precisely identified as the contact point of the 3-position of the cholytaurine derivative with ILBP. (ii) The isomer carrying the photoreactive diazirine function at position 7 gave less clear results. With the radiolabeled 2-(7,7-azo-3α,12α-dihydroxy-5β-[3β-3H]cholan-24-oylamino)ethanesulfonic acid peptide fragments beyond position 72 as well as peptides of the N-terminal half of ILBP were identified. MALDI-TOF mass spectrometry localized binding of the 7-azi-derivative of cholytaurine to sequence 112 to 128. (iii) after chemical modification of the arginine residues in ILBP by phenylglyoxal treatment, photoaffinity labeling of ILBP by radiolabeled azi-derivatives of cholytaurine was significantly reduced demonstrating a specific interaction of the negatively charged bile acid side chain with one of the 3 arginine residues at positions 33, 122, or 126.

The combination of photoaffinity labeling, enzymatic fragmentation, and MALDI-mass spectrometry with resulting allocation of the attachment site of the 3-position of cholytaurine

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Fig. 8. Effect of phenylglyoxal treatment on photoaffinity labeling of ILBP. Freshly prepared cytosol from rabbit ileum (150 μg of protein) or cytosol pretreated with 10 mM phenylglyoxal (150 μg of protein) was incubated at 20 °C in the dark for 5 min with 0.847 μM (1 μCi) of 2-(3,3-azo-7α,12α-dihydroxy-5β-[7β,12β-3H]cholan-24-oylamino)ethanesulfonic acid in 200 μl of 10 mM Tris/Hepes buffer (pH 7.4), 100 mM mannitol, 100 mM NaCl followed by irradiation at 350 nm for 10 min. After precipitation with chloroform/methanol proteins were separated by SDS-PAGE on 12% gels followed by slicing of the tracks in 2-mm pieces and determination of the distribution of radioactivity by liquid scintillation counting after digestion of proteins with tissue solubilizer Biolute S. Upper panel, control. Lower panel, after treatment with 10 mM phenylglyoxal prior to photoaffinity labeling.

Fig. 9. Stereochemical view of cholytaurine binding to human ILBP obtained by NMR structure. The bile acid molecule is shown in white and the amino acid triplet Glu100, Thr101, Ser102 in yellow.
to amino acid sequence His$^{100}$-Thr$^{101}$-Ser$^{102}$ defines for the first time the topology of bile acid binding to ILBP with an orientation of the steroid ring moiety penetrating deep inside the protein cavity. To define the interaction of the negatively charged bile acid side chain with one of the 3 arginine residues 33, 122, or 126, we performed an NMR structure of human ILBP in the presence of cholyltaurine. Fig. 9 shows the binding of cholyltaurine to ILBP as determined by NMR structure. The bile acid molecule (white) completely fills the complementary binding cavity. The 3$\alpha$-hydroxy group specifically interacts with the amino acid triplet Glu$^{100}$-Thr$^{101}$-Ser$^{102}$ shown in yellow, confirming the results obtained by photoaffinity labeling and MALDI-mass spectrometry. The space-filling complementarity of the bile acid molecule and its binding cavity does not allow a significant rotation of cholyltaurine around its length axis thereby explaining the specific and exclusive labeling of amino acid sequence 100–102 by the 3-diazirine derivative of cholyltaurine. This binding mode of bile acids to ILBP with the steroid nucleus located deep inside the ILBP molecule excludes an interaction of the negatively charged side chain with arginine residues 33 and 126 that are oriented at the surface of ILBP interacting with the surrounding water (Fig. 10). In contrast, arginine residue 122 near the entry portal of the bile acid-binding cavity is in proximity to the negatively charged bile acid side chain. Therefore, modification of this arginine residue by phenylglyoxal probably accounts for the decreased photoaffinity labeling of ILBP indicating a decreased bile acid binding affinity. Modeling of the inner surface of the bile acid ligand-binding site clearly indicates a space-filling complementarity to the bile acid molecule with a characteristic curvature allowing an optimal steric binding of cis-configured rings A and B (Fig. 9). The 7$\alpha$-hydroxy group would have within a distance of 7 Å access to amino acids Thr$^{39}$, Ile$^{41}$, Trp$^{92}$, His$^{52}$, or Gin$^{58}$. The N-terminal peptide fragments of ILBP found after photo-labeling with 2-(7,7-azo-3$\alpha$,12$\alpha$-dihydroxy-5$\beta$-cholan-24-oylamino)ethanesulfonic acid could therefore result from an interaction of the 7-position with the ligand-binding site. The amino sequences 72–87 and 112–128 being identified as attachment sites of the 7-diazirine analogue of cholyltaurine do not have access to the bile acid molecule bound in the binding cavity interacting at the 3-position with amino acids 100–102. Fig. 11 shows that the amino acid sequence 112–128 (shown in green) is located at the surface of the ILBP molecule, and none of these amino acids can have direct contact with the 7-position of the bile acid molecule in the ligand-binding cavity. Similarly, amino acid sequences 72–87 are located outside of the binding cavity at the surface of the ILBP opposite to amino acid sequences 112–128 (Fig. 12); owing to the impossible rotation of
the bile acid molecule around its length axis, the 7-position cannot have contact to these amino acids in position 78–87. Consequently, the labeling of the amino acid sequences 78–87 and 112–128 by the 7-diaryazine analogue of cholate was suggested as we have proven in the present paper. The structure of porcine ILBP in complex with cholylglycine was published in Endocrinology 102, 1147–1154.

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Addendum—After submission of this manuscript, a refined NMR structure of porcine ILBP in complex with cholate was published (65). The identification of the bile acid with the steroid ring deep inside the binding cleft was suggested as we have proven in the present paper. The NMR structure proposed an interaction of the 3α-hydroxyl group with amino acid residue Glu110 and of the bile acid side chain with Lys77, but a resolved resolution of the interaction points between cholate and ILBP could not be obtained. This clearly demonstrates the power of the approach we have used for the determination of the attachment site of a ligand to its binding protein by a combination of photoaffinity labeling, enzymatic fragmentation, MALDI-mass spectrometry, and NMR structure.

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