Identification of a Ligand-binding Site in the Na+/Bile Acid Cotransporting Protein from Rabbit Ileum*

Received for publication, May 22, 2001, and in revised form, June 26, 2001
Published, JBC Papers in Press, July 10, 2001, DOI 10.1074/jbc.M104665200

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Reabsorption of bile acids occurs in the terminal ileum by a Na+/dependent transport system composed of several subunits of the ileal bile acid transporter (IBAT) and the ileal lipid-binding protein. To identify the bile acid-binding site of the transporter protein IBAT, ileal brush border membrane vesicles from rabbit ileum were photofinity labeled with a radioactive 7-azi-derivative of cholyltaurine followed by enrichment of IBAT protein by preparative SDS gel electrophoresis. Enzymatic fragmentation with chymotrypsin yielded IBAT peptide fragments in the molecular range of 20.4–4 kDa. With epitope-specific antibodies generated against the C terminus of the smallest peptide fragment carrying both the C terminus and the covalently attached radiolabeled bile acid derivative. This clearly indicates that the ileal Na+/bile acid cotransporting protein IBAT contains a bile acid-binding site within the C-terminal 56–67 amino acids. Based on the seven-transmembrane domain model for IBAT, the bile acid-binding site is localized to a region containing the seventh transmembrane domain and the cytoplasmic C terminus. Alternatively, assuming the nine-transmembrane domain model, this bile acid-binding site is localized to the ninth transmembrane domain and the C terminus.

Enterohepatic circulation of bile acids with a specific and highly efficient extraction of bile acids from the intestinal lumen, portal blood, and the primary filtrate in the kidney occurs by Na+/dependent transport systems localized in the apical membrane of ileocytes and renal proximal tubule cells and the basolateral membrane of hepatocytes (1, 2). The functional ileal and hepatic Na+/bile acid cotransport systems are protein complexes composed of several transporter protein subunits (3, 4). The ileal Na+/bile acid cotransport system with a molecular mass of 451 ± 35 kDa contains additionally several subunits of the cytoplasmically attached ileal lipid-binding protein (ILBP)1 (3, 5, 6). The mammalian Na+/bile acid cotransport proteins have 347–362 amino acids with 35–37% identity and 46–48% similarity between the ileal and the hepatic transporter (Refs. 7–12 and GenBank™ accession numbers 254357 and AJ131361). Originally a topology model with seven transmembrane domains has been suggested (2), but novel investigations with in vitro translation approaches favor a topology model with nine transmembrane domains (13). Investigations of the substrate specificity of the ileal and hepatic Na+/bile acid cotransport proteins using radiolabeled bile acid analogues in 10 mM Tris/Hepes buffer (pH 7.4), 300 mM mannitol were incubated with radiolabeled bile acid analogues in 10 mM Tris/Hepes buffer (pH 7.4), 100 mM NaCl, 100 mM mannitol at 20 °C for 10 min in the dark. Photocross-linking was achieved by ultraviolet irradiation at 350 nm for 10 min using a Rayonet RPR-100 photochemical reactor (Southern Ultraviolet Company, Hamden, CT) equipped with four RPR 3500 Å lamps. Afterward, the vesicle suspensions were diluted with ice-cold 10 mM Tris/Hepes buffer (pH 7.4), 300 mM mannitol, and washing was repeated twice.

**SDS Gel Electrophoresis—SDS-PAGE was carried out in vertical slab gels (20 × 17 × 0.15 cm) using an electrophoresis system LE 2/4 (Amersham Pharmacia Biotech) with gel concentrations of 9–15% at a ratio of 97.2% acrylamide and 2.8% N,N'-methylene bisacrylamide or for analytical purposes, in precasted NOVEX gels (4–12%, 12%, or 15% (Invitrogen), using an electrophoresis system XCell II from Novex. Electrophoretic separation of peptide fragments was performed in Tris/Tricine gels (16.5%) according to Schägger and von Jagow (18). After electrophoresis the gels were fixed in 12.5% trichloroacetic acid followed by preparative SDS gel electrophoresis. Enzymatic fragmentation with chymotrypsin yielded IBAT peptide fragments in the molecular range of 20.4–4 kDa. With epitope-specific antibodies generated against the C terminus of the smallest peptide fragment carrying both the C terminus and the covalently attached radiolabeled bile acid derivative. This clearly indicates that the ileal Na+/bile acid cotransporting protein IBAT contains a bile acid-binding site within the C-terminal 56–67 amino acids. Based on the seven-transmembrane domain model for IBAT, the bile acid-binding site is localized to a region containing the seventh transmembrane domain and the cytoplasmic C terminus. Alternatively, assuming the nine-transmembrane domain model, this bile acid-binding site is localized to the ninth transmembrane domain and the C terminus.

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‡ The abbreviations used are: ILBP, ileal lipid-binding protein; IBAT, ileal bile acid transporter; PAGE, polyacrylamide gel electrophoresis; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

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by staining with Serva Blue R-250 and determination of radioactivity by slicing of the gel lanes in 2-mm pieces, digestion of proteins with tissue solubilizer Biolute S, and liquid scintillation counting using a Scintillator Quickszint 501. Alternatively, proteins or peptides were transferred to nitrocellulose sheets.

Preparative SDS Gel Electrophoresis—Photolabeled monomeric and dimeric IBAT proteins were enriched by preparative SDS gel electrophoresis. Usually, 25 samples of rabbit ileal brush border membrane vesicles (100 μg of protein) were photolabeled each with 0.70 μM (1 μCi) 2-(7,7-azo-3a,12a-dihydroxy-5β-[3H])cholon-24-oxyamino-ethanesulfonic acid in 10 mM Tris/Hepes buffer (pH 7.4), 100 mM NaCl, 100 mM mannitol. After three washings, the membrane proteins (2.5 mg of membrane protein) were solubilized in 1000 μl of sample buffer (62.5 mM Tris/HCl (pH 6.8), 2% SDS, 5% 2-mercaptoethanol, 10% glycerol, 0.001% bromophenol blue). After centrifugation, the sample was applied to the separation gel of a preparative 7.5% SDS gel (diameter, 28 mm; length of separation gel, 5 cm). Electrophoresis was performed at 500 V (40 mA, 6 W), and the eluate was fractionated into 1.5-ml fractions. 10-μl aliquots were removed for determination of the distribution of radioactivity, and 10 μl of each fraction were precipitated (19) for analysis of protein composition by SDS-PAGE on 9% precasted gels followed by Serva Blue R-250 staining or blotting and detection of IBAT proteins with anti-IBAT antibodies. The protein in 70 μl of those fractions immunoactive for the monomeric 46 kDa IBAT was performed with KIBMAL-3 or KIBMAL-1 antibodies. Immunodetection of peptide fragments containing the C terminus of proteins with anti-IBAT antibodies. The protein in 70 μl of those fractions immunoactive for the monomeric 46 kDa IBAT protein were pooled and used for further analysis by enzymatic fragmentation.

Enzymatic Fragmentation—Brush border membrane vesicles or fractions from preparative SDS gel electrophoresis containing enriched IBAT protein were delipidated with chloroform/methanol (19) and redissolved in 3 μl of Tris/HCl buffer (pH 6.8), 0.2% SDS, 0.5% 2-mercaptoethanol/0.005% bromophenol blue. Enzymatic fragmentation was carried out by adding 5 μl of freshly prepared solutions of chymotrypsin (0.5–100 ng/ml) trypsin (1–100 ng/ml), endoproteinase Glu C (2–100 ng/ml) in the above buffer and subsequent incubation at 30 °C for 1 h. The reactions were stopped by addition of SDS sample buffer containing 4 μl EDTA, 4 μl iodoacetamide, 4 μl phenylmethylsulfonyl fluoride and heating for 5 min to 95 °C followed by subsequent peptide analysis by SDS-PAGE.

Blotting—Proteins and peptides were bound to nitrocellulose membranes (0.2 μm trans-blot transfer medium from Bio-Rad) by electrophoretic transfer from SDS gels in a transblot cell (Bio-Rad) using 25 mM Tris, 192 mM glycine, 33% methanol (pH 8.3) as transfer buffer. Blotting was performed at 300 mA for 3 h and 400 mA for another 0.5 h. Immunodetection of peptide fragments containing the C terminus of rabbit IBAT was performed with KIBMAL-3 or KIBMAL-1 antibodies at a dilution of 1:10000 and 1:10000 for the secondary anti-guinea pig antibody using the Western Light chemiluminescent detection kit from Serva (Heidelberg, Germany). Subsequently, the blots were sliced into 2-mm fragments and reswollen with 100 μl of water, and protein was digested by addition of 0.4 ml of Biolute S for 12 h. After the addition of 4 ml of Scintillator Quickszint 501 radioactivity was measured by liquid scintillation counting.

RESULTS

Strategy to Localize the Bile Acid-Binding Site of the Ileal Bile Acid Transporter Protein IBAT—The three-dimensional quantitative structure activity relationship pharmacophore model generated for mammalian Na+/bile acid cotransporters revealed the following specific interactions of a bile acid molecule with the transporter ligand-binding site (15): (i) the methyl groups 18 and 21 and the five-membered ring D of the steroid nucleus occupy two of the three hydrophobic binding sites; (ii) the negatively charged side chain maps the hydrogen bond acceptor site; (iii) the α-olefine of the hydrogen group at position 7 or 12 acts as a hydrogen bond donor and (iv) the 3α-hydroxy groups of cis-configured ring A do not show a specific interaction with any of the five specific binding features of the Na+/bile acid co-transporter.

From these findings we decided to use the photoreactive 7-diazirine analogue of cholytaurine, 2-(7,7-azo-3a,12α-dihydroxy-5β-cholyl-3H)cholon-24-oxyamino-ethanesulfonic acid, to map the ligand-binding site caused by the specific interaction of the 7 position of bile acids with the transporter protein. The following strategy was used to localize bile acid-binding sites of the ileal Na+/bile acid cotransporter: (i) photoaffinity labeling of the intact functional Na+/bile acid transport system in rabbit ileal brush border membrane vesicles with 2-(7,7-azo-3a,12α-dihydroxy-5β-[3β-3H]cholon-24-oxyamino)-ethanesulfonic acid; (ii) enrichment of the radiolabeled monomeric 46-kDa and dimeric 93-kDa forms of IBAT protein by preparative SDS gel electrophoresis; (iii) enzymatic fragmentation of the enriched IBAT protein; (iv) separation of peptide fragments by electrophoresis; and (v) identification of radiolabeled peptides with epitope-specific antibodies raised against the C terminus. As epitope-specific antibodies we have chosen antibodies generated against the C-terminal 23 or 51 amino acids of rabbit IBAT. If the bile acid-binding site would be localized near to the N terminus, peptide fragments containing the C terminus of molecular masses close to intact IBAT would show a lack of radioactivity. In contrast, with a localization of the bile acid-binding site near to the C terminus rather small peptides containing both the radiolabeled attached bile acid and the C terminus are expected. The identification and determination of the molecular masses of those peptide fragments containing both the immunoreactive C terminal and the radiolabel of the covalently cross-linked bile acid therefore allows the localization of attachment site of the 7 position of the bile acid to the IBAT protein.

Enzymatic Fragmentation of the Rabbit Ileal Na+/Bile Acid Cotransporter—For a precise localization of the bile acid-binding site on the IBAT amino acid sequence, a spectrum of clearly defined peptide fragments covering a broad range of molecular masses is necessary. We therefore investigated as peptidases endoproteinase Glu C (V8 protease), trypsin, chymotrypsin, thermolysin, subtilisin, and papain. Several clearly separated C-terminal peptides in the molecular mass range of 20.4–4 kDa could be generated by chymotrypsin, whereas with trypsin and endoproteinase Glu C less complex peptide patterns with fragments of 42, 32, and 6 kDa for trypsin and 7.5 kDa for endoproteinase Glu C were obtained. Papain digestion resulted in the generation of peptides of 39.6, 35, 32.8, and 13.3 kDa. With thermolysin and subtilisin no defined peptide fragments containing the C terminus were obtained. These fragmentation studies showed that the highest precision for localization of a bile acid-binding site would be possible by enzymatic fragmentation with chymotrypsin because chymotrypsin cleavage sites are distributed along the entire sequence of IBAT (Fig. 1).

Immunodetection with KIBMAL-1 antibodies generated against the C-terminal 51 amino acids yielded similar labeling pattern as with KIBMAL-3 antibodies, indicating that the antibodies KIBMAL-1 (terminal 51 amino acids) and KIBMAL-3 (terminal 23 amino acids) primarily recognize epitopes localized in the C-terminal 23 amino acids (Fig. 2). Peptide fragments with cleavage sites within the terminal 23 amino acids as possible with chymotrypsin probably will escape detection by KIBMAL-3 antibodies. It is therefore appropriate to assume that the probable amino acid sequence of a peptide fragment can be deduced from its molecular mass by comparison with the calculated mass of peptide fragments starting from the C terminus to the particular enzymatic cleavage site.

Enzymatic Fragmentation of Rabbit Ileal Brush Border Membrane Vesicles by Chymotrypsin after Photoaffinity Labeling with 2-(7,7-azo-3a,12α-dihydroxy-5β-[3β-3H]cholon-24-oxyamino)-ethanesulfonic Acid—As a next step rabbit ileal brush border membrane vesicles photoaffinity-labeled with 2-(7,7-azo-3a,12α-dihydroxy-5β-[3β-3H]cholon-24-oxyamino)-
ethanesulfonic acid were digested with 40 or 80 ng/ml chymotrypsin followed by separation of the peptide fragments by SDS-PAGE using the Schägger & Von Jagow system (18) to allow an optimal separation and resolution of small molecular mass peptide fragments. Fig. 3A (upper panel) shows the labeling pattern of ileal brush border membrane vesicles with incorporation of the photoprobe predominantly into three polypeptides of 14 kDa, the ileal lipid-binding protein, and the monomeric and dimeric forms of IBAT of 46 and 93 kDa, respectively. Treatment with 80 ng/ml chymotrypsin generated several radiolabeled peptide fragments of 17.2, 11, 7.7, 7.2, and 6.2 kDa with highest incorporation of the radiolabeled bile acid analogue into the polypeptides of 7.7 and 7.2 kDa (Fig. 3A). To determine whether the radiolabeled chymotryptic peptides are derived from the photolabeled ileal Na+/bile acid cotransport system composed of several subunits of IBAT and ILBP, comparable labeling experiments were performed with brush border membrane vesicles obtained from rabbit jejenum. Fig. 3B (upper panel) shows labeling of only one peptide of 87 kDa assumed to be a component of a passive uptake system for bile acids in the upper small intestine (20). After digestion with 80 ng/ml chymotrypsin, a completely different peptide was obtained with no prominently labeled peptide fragments in contrast to ileal brush border membrane vesicles (Fig. 3B). These findings clearly demonstrate that the radiolabeled peptide fragments obtained by chymotrypsin treatment of photolabeled rabbit ileal brush border membrane vesicles arose from the protein components of the ileal Na+/bile acid cotransport system, IBAT and ILBP.

Chymotrypsin Treatment of the Rabbit Na+/Bile Acid Cotransporter Enriched by Preparative SDS Gel Electrophoresis after Photoaffinity Labeling with 2-[(7,7-azo-3a,12a-dihydroxy-5β-[3H]cholan-24-oylamino)-ethanesulfonic acid—To determine the attachment site of bile acids to the ileal Na+/bile acid cotransporter, rabbit ileal brush border membrane vesicles were photoaffinity labeled with 2-[(7,7-azo-3a,12a-dihydroxy-5β-[3H]cholan-24-oylamino)-ethanesulfonic acid, and subsequently the membrane proteins were fractionated by preparative SDS gel electrophoresis. Aliquots of each fraction were submitted to SDS-PAGE, and the ileal Na+/bile acid cotransporting protein was detected by Western blotting using IBAT-specific antibodies as well as by determination of the incorporation of the radiolabeled photo-cross-linked bile acid by slicing of the gels and subsequent liquid scintillation counting. Fig. 4A shows the Coomassie Blue stains of the fractions eluted in the 46-kDa range from the preparative SDS gel, and in Fig. 4B the corresponding immunostains using the IBAT antibody KIBMAL-1 or KIBMAL-3 are shown. The intensity of the IBAT-positive signals of 46 and 93 kDa correlated with the incorporation of the radiolabeled bile acid cotransporter enriched by preparative SDS gel electrophoresis aggregates to a dimer (fractions 6, 8, and 10); in contrast to ileal brush border membrane vesicles (Fig. 3B). These findings clearly demonstrate that the radiolabeled peptide fragments obtained by chymotrypsin treatment of photolabeled rabbit ileal brush border membrane vesicles arose from the protein components of the ileal Na+/bile acid cotransport system, IBAT and ILBP.

Chymotrypsin cleavage sites of the rabbit ileal Na+/bile acid co-transporting protein.
The fractions containing the 46-kDa radiolabeled IBAT protein were submitted to enzymatic fragmentation by chymotrypsin using high resolution gels with separation distances greater than 12 cm. To allow a precise alignment of radiolabeled bands to C-terminal positive peptide fragments, the peptides were blotted after electrophoresis to nitrocellulose sheets; the nitrocellulose lanes were split into two halves, one being used for immunoblotting with KIBMAL antibodies, and subsequently, after indication of the Western positive bands, both stripes were cut into 2-mm pieces and counted for radioactivity.

Fig. 5 shows the congruent results from two independently performed experiments starting from different vesicle preparations. 25 portions of ileal brush border membrane vesicles (100 µg of protein) were photolabeled with 0.7 µM (1 µCi) of 2-(7,7-azo-3α,12α-dihydroxy-5β-[3H]cholan-24-oylamino)-ethanesulfonic acid. After preparative SDS-PAGE, the protein fractions containing the immunoreactive 46-kDa IBAT protein were digested with 75 ng/ml chymotrypsin. After SDS-PAGE and immunoblotting, the nitrocellulose sheets were cut into 2-mm pieces, and the distribution of radioactivity was determined by liquid scintillation counting. The experiment shown in Fig. 5A reveals that nine radiolabeled polypeptides of 20.4, 18.9, 17.1, 12.0, 10.3, 7.6, 6.6, 5.3, and 4.0 kDa were clearly separated, and 16 peptides containing the C terminus of 20.6, 19.3, 18.1, 17.4, 15.0, 12.3, 11.1, 10.5, 9.8, 9.2, 8.8, 8.3, 7.9, 7.4, and 6.9 kDa are visible after immunostaining with KIBMAL-1 antibodies. Fig. 5B shows the results obtained from an independently executed experiment (a total of five independent experiments were performed leading to nearly identical results). After electrophoretic separation seven radioactively labeled polypeptides of 19.6, 17, 12.4, 10.3, 7.0, 5.5, and 4 kDa were visible.
Bile Acid-binding Site of the Ileal Na\(^+\)/Bile Acid Transporter

**Fig. 4.** Enrichment of photolabeled IBAT protein by preparative SDS gel electrophoresis. 25 samples of 100 µg of rabbit ileal brush border membrane vesicles suspended in 20 µl of 10 mM Tris/Hepes buffer (pH 7.4), 300 mM mannitol were photolabeled each with 50 µl 10 mM Tris/Hepes buffer (pH 7.4), 100 mM NaCl, 100 mM mannitol containing 0.7 µM (1 µCi) 2-(7,7-azo-3α,12α-dihydroxy-5β-[3β-[3H]cholane-24-oylamino)-ethanesulfonic acid. After washing, the samples were pooled, and the membrane proteins (2.4 mg of protein) were fractionated by preparative SDS gel electrophoresis. Aliquots from the individual fractions (1.5 ml) were used for Coomassie staining (10 µl), immunoblotting with KIBMAL-3 antibodies (10 µl), and determination of the distribution of radiolabeled bands (70 µl). A, Coomassie staining. B, immunostaining with KIBMAL-3 antibodies. C, distribution of radioactivity in the 46-kDa IBAT protein.

were clearly detectable (Fig. 5B). Immunostaining identified peptides of 20.4, 19.5, 17.9, 17.0, 14.9, 12.1, 11.0, 10.3, 9.7, 9.1, 8.4, 7.8, 7.4, and 7.0 kDa reacting with C-terminal antibodies. From these experiments it can be deduced that the smallest peptide fragment of rabbit IBAT containing both the C terminus and the covalently photocross-linked bile acid at position 7 has a molecular mass of 6.6–7 kDa. Because KIBMAL-1 and KIBMAL-3 antibodies gave comparable immunostaining patterns, the chymotryptic peptide fragments detected with KIBMAL antibodies probably end at position 347 (the intact C terminus), 344, or 338. Table I lists the theoretical molecular masses of the possible chymotryptic peptides ending at position 347, 344, or 338. The radiolabeled peptides of 6.6–7 kDa reacting with C-terminal antibodies accordingly start downstream of position 280 (Table II). Accordingly, the attachment site of the 7-azi-derivative of cholytaurine to the IBAT transporter is localized to the C-terminal 67–56 amino acids, *i.e.* the C-terminal 19–16% of the amino acid sequence of the ileal bile acid transporter protein.

**DISCUSSION**

The ileal Na\(^+\)/bile acid cotransport system is a major regulator for serum cholesterol homeostasis and has become an area of increasing pharmacological interest. In earlier studies we could demonstrate by chemical modification of amino acid residues that vicinal cysteine groups and lysine residues are essential for the function of the ileal Na\(^+\)/bile acid cotransporting protein (21). We succeeded in the development of a predictive pharmacophore model for mammalian Na\(^+\)/bile acid co-transporters, allowing the definition of a novel model for the interaction of bile acids with the transporter protein (14, 15). Site-directed mutagenesis of the amino acids in a transporter protein is an appropriate way to identify those amino acids residues of importance for the function of the transporter. The identification of functionally essential amino acid residues does not, however, imply that they are part of the ligand-binding site. For an unequivocal localization of the ligand binding a covalent cross-linking of the ligand to the peptide backbone and subsequent determination of the cross-linking position is necessary. The ileal Na\(^+\)/bile acid cotransport system in intact ileal tissue is a heteromeric protein complex of 451 kDa composed of several transporter units and associated ileal lipid-binding proteins (3, 6). Because the structure and composition of the ileal Na\(^+\)/bile acid cotransport system in recombinant cell lines is not known and because functional differences of transporters found in intact tissues and recombinant cell lines were reported (22), we decided to photolabel the ileal Na\(^+\)/bile acid cotransporter *in situ* using freshly isolated rabbit ileal brush border membrane vesicles with subsequent determination of the attachment site. Originally we attempted to purify the photolabeled transporter to homogeneity and to apply a novel technology we have developed and successfully used for ILBP (23) with enzymatic fragmentation and sequence analysis of the labeled peptides by matrix-assisted laser desorption ionization mass spectrometry. This approach was, however, not applicable to the ileal Na\(^+\)/bile acid cotransporting protein caused by inherent experimental limitations and characteristics of IBAT: (i) The amount of the transporter protein in the ileocyte brush border membrane is extremely low. With increasing purity the transporter protein aggregates to macro-molecular protein complexes, and the yield of protein after each purification step was very low (from 80 mg of brush border membrane protein we could isolate a minute amount of pure
Fig. 5. Chymotrypsin fragmentation of the enriched 46-kDa IBAT protein after photoaffinity labeling of rabbit ileal brush border membrane vesicles with 2-(7,7-azo-3a,12a-dihydroxy-5β-[3β-3H]cholan-24-olamino)-ethanesulfonic acid and subsequent preparative SDS gel electrophoresis. Protein in fractions from preparative SDS gel electrophoresis (1 ml) containing the 46-kDa IBAT protein photolabeled with 2-(7,7-azo-3a,12a-dihydroxy-5β-[3β-3H]cholan-24-olamino)-ethanesulfonic acid was precipitated with chloroform/methanol and subsequently submitted to enzymatic digestion with 70 ng/ml chymotrypsin at 30 °C for 60 min. After SDS-PAGE peptides were blotted to nitrocellulose sheets followed by immunodetection with KIBMAL-1 antibodies and determination of the distribution of radioactivity by slicing of the blots into 2-mm pieces and following liquid scintillation counting. A and B represent two independently performed experiments starting with different ileal brush border membrane vesicle preparations.

TABLE I
Alignment of chymotryptic peptides containing the C terminus of the rabbit ileal Na\(^+\)/bile acid cotransporter to molecular masses of amino acid sequences

| N terminus of the peptide (X) | Molecular mass of peptide fragments | X → 347 | X → 344 | X → 338 |
|-----------------------------|-----------------------------------|---------|---------|---------|
| 164                         | 20891                             | 20428   | 19638   |
| 183                         | 18868                             | 18496   | 17706   |
| 188                         | 18186                             | 17813   | 17023   |
| 218                         | 15168                             | 14796   | 14005   |
| 222                         | 14677                             | 14305   | 13515   |
| 229                         | 13779                             | 13407   | 12617   |
| 235                         | 13116                             | 12744   | 11954   |
| 240                         | 12579                             | 12206   | 11417   |
| 244                         | 12156                             | 11784   | 10994   |
| 245                         | 11991                             | 11619   | 10894   |
| 254                         | 10980                             | 10607   | 9818    |
| 255                         | 10799                             | 10426   | 9637    |
| 280                         | 7998                              | 7626    | 6836    |
| 287                         | 7175                              | 6802    | 6012    |
| 289                         | 6910                              | 6538    | 5748    |
| 291                         | 6644                              | 6272    | 5482    |
| 295                         | 6139                              | 5767    | 4977    |
| 298                         | 5774                              | 5402    | 4612    |
| 302                         | 5296                              | 4923    | 4133    |

TABLE II
Alignment of radioactively labeled chymotryptic peptides containing the C terminus of rabbit IBAT photolabeled with 2-(7,7-azo-3a,12a-dihydroxy-5β-[3β-3H]cholan-24-olamino)-ethanesulfonic acid to C-terminal amino acid sequences

| Peptide found | C-terminal peptides |
|---------------|---------------------|
|               | X → 347 (intact)    | X → 344 | X → 338 |
| X → 347       | 20.4 164 164 154    |
| X → 344       | 19.2 183 183 178   |
| X → 338       | 18.9 188 188 188   |
|               | 17.1 188 188 188   |
|               | 17.0 188 188 188   |
|               | 12.4 240 240 229   |
|               | 12.0 245 244 235   |
|               | 10.3 255 255 255   |
|               | 10.0 254 254 254   |
|               | 9.0 255 255 255    |
|               | 7.6 280 280 280    |
|               | 7.0 289 287 280    |
|               | 6.8 289 287 280    |
|               | 6.6 289 287 280    |

IBAT protein resulting in one very faint band after SDS-PAGE; estimated amount, <1–10 ng of protein. (ii) For detectable satellites representing the cross-linked product of the bile acid and the representative bile acid binding protein in matrix-
assisted laser desorption ionization mass spectrometry, a concentration of ≥0.5 mM for the photoreactive bile acid and ≥0.1 mg/ml for the bile acid binding protein is necessary (23); such a concentration of functionally intact IBAT protein cannot be achieved by using brush border membrane vesicles, transfected cells, or intact ileal tissue. (iii) A principle alternative would be the use of high expression systems such as Pichia pastoris or Baculovirus, which may generate the necessary amounts of transporter protein. After purification these transporters may be incorporated into proteoliposomes, which could deal as the starting material for the identification of the ligand-binding site. A prerequisite for such an approach is the functional reconstitution of the transporter protein into proteoliposomes. Unfortunately, however, all attempts to functionally reconstitute the ileal Na+/bile acid cotransporter failed, even with protocols successfully used for a reconstitution of the hepatic canalicular bile acid transporter (24) or the H+/oligopeptide transporter from intestine (25).

We therefore followed the strategy to photolabel the transporter protein in its natural functional state and subsequently determine the attachment site by the identification of those peptide fragments containing both the C terminus and the covalently attached radioactive bile acid have a molecular mass of 6.6–7 kDa. This means that the attachment site of the 7-position of a bile acid is localized on a peptide fragment of the C-terminal 67–56 amino acids downstream of position 280 (7678.9 Da) or possibly even downstream of positions 289 (6911 Da) or 291 (6644 Da). The identification of an attachment site of the 7 position of 7-azi-cholyltaurine to the C terminus does not mean that the ligand-binding site is exclusively built up by the C-terminal 56–67 amino acids. It is probable that the ligand-binding site is formed by several transmembrane domains from different regions of the IBAT sequence. What we could show here is that the 7 position of cholyltaurine during binding to IBAT preferably interacts with a domain comprising the C-terminal 56–67 amino acids. The labeling of other peptides not containing the C terminus may indicate the binding of bile acids to other sequences that either are part of the primary ligand-binding site or are representative of secondary binding sites similar to the behavior of ILBP (23). According to the seven-transmembrane domain model, the identified bile acid-binding site would be localized in a peptide fragment containing the seventh transmembrane domain and the cytoplasmic C terminus (Fig. 6A). Based on the newer topology assuming nine transmembrane domains, the bile acid-binding site of the Na+/bile acid cotransporter would be harbored in a peptide sequence consisting of the ninth transmembrane domain and the cytoplasmic C terminus (Fig. 6B).
Independently on the topology model, the binding site would not involve a large extracellular peptide loop but only, if at all, a very small extracellular peptide sequence of less than 8 (seven-transmembrane model) or 10 (nine-transmembrane model) amino acids. Such an attachment of the 7 position of cholytaurine to an amino acid sequence downstream of position 280 or even 291 is in line with our previous investigations by topological photoaffinity labeling of the ileal Na\(^+\)/bile acid cotransport system (5). With membrane-impermeable dimeric bile acid analogues that specifically inhibit the ileal bile acid transporter from the luminal side without being translocated across the brush border membrane, the cytoplasmically attached ILBP could be labeled, suggesting that the bile acid-binding site is located closely to the cytoplasmic side of the transporter protein rather than to an extracellular loop (5, 26).

The localization of the bile acid-binding site to the C-terminal part of the transporter protein is in accordance with further indirect evidence. We demonstrated earlier that the inactivation of the ileal Na\(^+\)/bile acid cotransporter by thiol-modifying agents can be prevented by performing the chemical modification in the presence of bile acids (21). Recently it was demonstrated that the inactivation of the human ileal Na\(^+\)/bile acid cotransporter by membrane-impermeable methanethiosulfonates disappeared after site-directed mutagenesis of Cys\(^{270}\) to Ala\(^{270}\), indicating that this conserved cysteine residue is responsible for inhibition of bile acid transport activity by thiol agents (27). Because inactivation of the wild-type human ileal bile acid transporter was prevented by increasing concentrations of cholytaurine, it was suggested that cysteine 270 is located within or near the bile acid-binding site (27). In agreement with a location of the bile acid-binding site to the C-terminal part are also findings from mutations of the human bile acid transporter. Mutations at position 290 (P290S), 262 (T262M), or 243 (L243P) led to a complete loss of transporter function without changes in the expression of the mutant transporters, indicating that these mutations interfere with the bile acid binding or transport process (9, 28). Future studies with the expression of truncated forms of the ileal Na\(^+\)/bile acid transporter and attempts for two- or three-dimensional crystals of these modified transporters should allow further refinement of the bile acid-binding site.

Acknowledgments—We thank Meike Scharnagl and Nadine Goricke for excellent secretarial assistance.

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