Molecular Mechanisms in Bile Formation

Peter J. Meier and Bruno Stieger

Active canalicular secretion of bile salts and non-bile salt organic anions represents the major driving force of hepatic bile formation. The most important carriers involved have now been cloned on both the basolateral and canalicular sides of hepatocytes. Elucidation of their structure, transport properties, and regulation is an important step forward in the ultimate understanding of the molecular physiology of bile formation.

Continuous bile formation is an important function of the liver, and bile is used as a vehicle for the secretion of bile acids and the excretion of lipophilic endo- and xenobiotics. It is an osmotic process that is driven by active secretion of predominantly organic solutes into the minute channels of bile canaliculi, followed by passive inflow of water, electrolytes, and nonelectrolytes (e.g., glucose) across hepatocytes and tight junctions. Bile salts are quantitatively the major constituents of bile and are circulated in the enterohepatic circulation between the liver and the small intestine with high efficiency. Bile is also rich in phospholipids (predominantly phosphatidylcholine) that form mixed vesicles and micelles with bile salts and thereby provide a means for the disposal of highly lipophilic endogenous and exogenous compounds, such as cholesterol, from the body. Canalicular bile is produced by the highly polarized hepatocytes that localize a distinct set of transporters at their basolateral (sinusoidal) and apical (canalicular) plasma membrane domains (Fig. 1) (8, 11). Primary hepatic bile fluid is then modified by the bile ductular and gall bladder epithelial cells before it reaches the small intestine. Because active canalicular secretion of bile salts represents the major driving force of hepatic bile formation, and because the canalicular bile salt export pump (bsep) of mammalian liver exhibits apparent Michaelis-Menten constant ($K_m$) values for taurocholate between 15 and 56 µM. The system is electrogenic, with a sodium-to-taurocholate stoichiometry of >1. In addition to sodium/bile salt cotransport, sodium-independent bile salt transport pathways are also present at the basolateral plasma membranes of hepatocytes (8, 9, 11). These sodium-independent bile salt uptake systems have been less well understood of the molecular physiology of bile formation.

Basic mechanisms of hepatic bile formation

Bile salts are concentrated up to 1000-fold in bile compared with portal blood plasma and the hepatocyte cytosol. This vectorial and concentrative transport process requires a polar arrangement of the hepatocellular carriers involved (Fig. 1). The basolateral plasma membrane of the hepatocytes faces the space of Disse, which is in direct contact with the portal blood via the fenestrae of the endothelial cells lining the sinusoids. The basolateral plasma membrane represents the first barrier to be crossed by cholephilic substances, i.e., substances destined to be secreted or excreted into bile. The canalicular plasma membrane of hepatocytes limits the bile canaliculi that form the exit site for all cholephilic compounds from the hepatocytes. The canalicular plasma membrane is separated from the basolateral plasma membrane by tight junctions. They form the only anatomical barrier between the portal blood and the primary bile fluid produced by hepatocytes and are hence of great importance for the maintenance of the concentration gradients between blood and bile.

Hepatocellular uptake of bile salts across the basolateral plasma membrane occurs against a concentration gradient and is driven predominantly by a secondary active sodium-dependent transport system (8, 9, 11). This transport system has been well characterized in a variety of experimental systems and exhibits apparent Michaelis-Menten constant ($K_m$) values for taurocholate between 15 and 56 µM. The system is electrogenic, with a sodium-to-taurocholate stoichiometry of >1. In addition to sodium/bile salt cotransport, sodium-independent bile salt transport pathways are also present at the basolateral plasma membranes of hepatocytes (8, 9, 11). These sodium-independent bile salt uptake systems have been less well characterized.
characterized compared with the sodium-coupled uptake carrier, a fact that is reflected by the broad range of reported apparent $K_m$ values between 9 and 130 $\mu$M. However, based on numerous cis-inhibition studies in the intact perfused rat liver and in isolated hepatocytes, the sodium-independent bile salt uptake systems are highly versatile and can transport a wide variety of amphipathic substrates, including non-bile salt organic anions, organic cations, neutral steroids, and small peptides. After their basolateral uptake into hepatocytes, bile salts have to reach the canalicular plasma membrane for their secretion into bile (8). The mechanisms involved in the transcellular movement of bile salts across hepatocytes is poorly understood. With physiological bile salt loads, a considerable fraction of the bile salts is bound to intracellular binding proteins, including Y' (glutathione-S-transferases, 3-hydroxysteroid dehydrogenase) and fatty acid-binding proteins. In addition, free intracellular bile salts reach the canalicular cell pole rapidly by diffusion. At high bile salt loads, bile salts increasingly partition into intracellular organelles, such as the endoplasmic reticulum, the Golgi apparatus, and other membrane-bound compartments.

Finally, bile salts are secreted across the canalicular plasma membrane, which represents the rate-limiting step in overall transport of bile salts from blood into bile. Canalicular bile salt secretion appears to be modulated predominantly by the ATP-dependent transport system called bsep, whereas sulfates and ethereal glucuronides of bile salts are transported by a second ATP-dependent transport system (8, 11). In addition to bile salts, the continuous canalicular excretion of non-bile salt organic anions such as reduced glutathione (GSH) is an important driving force for the generation of bile salt-independent bile flow within bile canaliculi.

**Basolateral sodium-dependent bile salt transporter**

The basolateral sodium-dependent bile salt cotransporter has been cloned from rat and human liver by expression cloning in *Xenopus laevis* oocytes (8, 9, 11). The rat liver sodium-dependent taurocholate-cotransporting polypeptide (ntcp) (Fig. 1) consists of 362 amino acids with two NH$_2$-linked glycosylation sites near its NH$_2$ terminus and seven putative transmembrane domains. Its apparent molecular mass is ~51 kDa in isolated rat liver basolateral plasma membrane vesicles. It is localized exclusively at the basolateral plasma membrane of differentiated hepatocytes and is only expressed in mammalian hepatocytes. During rat development, ntcp can first be detected between days 18 and 21 of gestation. Functionally, ntcp transports all physiological bile salts, although its transport activity is highest for glycine- and taurine-conjugated dihydroxy and trihydroxy bile salts (9). In addition, ntcp can also transport estrogen conjugates such as estrone-3-sulfate to some extent (9). As indicated by previous studies in intact hepatocytes, ntcp mediates electroneutral sodium-taurocholate cotransport with an apparent sodium-taurocholate stoichiometry of 2:1 (9). The gene encoding ntcp is located on rat chromosome 6q24 and spans 16.5 kb, with 5 exons being separated by 4 introns (11). The human NTCP consists of 349 amino acids and shows a 77% amino acid identity with the rat liver ntcp. Its functional properties are very similar to the rat protein and its gene has been fine-mapped to chromosome 14q24.1-24.2 (9).

**Basolateral sodium-independent bile salt and organic anion transporters**

In rat liver, basolateral sodium-independent bile salt uptake is mediated by the two organic anion-transporting polypeptides oatp1 and oatp2 (Fig. 1). Oatp1 consists of 670 amino acids and represents a glycoprotein with 12 putative membrane-spanning domains. It has an apparent molecular mass of ~80 kDa in isolated basolateral liver plasma membranes (8, 9, 11). Oatp1 is a polyspecific transporter that accepts a wide variety of lipophilic substrates, albeit with different
affinities (Table 1), including bile salts, organic anionic dyes such as sulfobromophthalein (BSP), steroid conjugates, leukotriene C4 and other glutathione conjugates (7), the MRI contrast agent gadoxetate (Gd-EOB-DTPA, or gadolinium-ethoxybenzyl-diethylenetriamine-pentaacetic acid), neutral steroids such as ouabain, the dipeptidic angiotensin-converting enzyme inhibitors enalapril and temocaprilat, the peptidomimetic thrombin inhibitor CRC 220, the mycotoxin ochratoxin A, and the organic cation N-propylajmalinium (Table 1) (9). For many of these substrates, oatp1-mediated uptake appears to be the predominant, if not the only, uptake pathway in rat hepatocytes, as evidenced by the similarity of the $K_m$ values (Table 1). Most probably, oatp1 can function as an anion exchanger, with bicarbonate (14) and/or glutathione (7) as possible counteranions. Hence, glutathione efflux, which occurs continuously from hepatocytes along its in-to-out concentration gradient, could provide an efficient driving force for oatp1-mediated substrate uptake into hepatocytes (7). Antisense experiments in Xenopus laevis oocytes revealed that oatp1 may account for most of the sodium-independent bile salt uptake but for only ~50% of BSP uptake of rat liver (8). Developmentally, oatp1 expression precedes expression of ntcp, and its mRNA can be detected already at day 16 of gestation in the developing rat liver. In addition to its basolateral localization in hepatocytes, oatp1 is also expressed at the apical brush border of choroid plexus and renal proximal tubular (S3 segment) epithelial cells.

Oatp2 was originally cloned from a rat brain cDNA library (12). However, oatp2 is also highly expressed in liver and kidney (12). It consists of 661 amino acids and exerts a 77% identity to oatp1. Like oatp1, oatp2 also has 12 putative transmembrane-spanning domains and represents a glycoprotein with a molecular mass of ~80 kDa. In addition, oatp2 is also a polyspecific transporter with similar apparent $K_m$ values to oatp1 for the bile salts taurocholate (35 $\mu$M) and cholate (46 $\mu$M) and the estrogen conjugates 17β-estradiol glucuronide (3 $\mu$M) and estrone-3-sulfate (11 $\mu$M). However, oatp2 transports ouabain with a higher affinity ($K_m$ ~ 470 $\mu$M) than oatp1 (see Table 1) and mediates unique high-affinity transport of digoxin ($K_m$ ~ 0.24 $\mu$M) (12). Furthermore, on the basis of recent studies in cRNA-injected Xenopus laevis oocytes, oatp2 neither mediates transport of BSP nor gadoxetate. Hence, although both members of the Oatp gene family of membrane transporters exhibit polyspecific and partially overlapping substrate specificities, oatp1 appears to prefer amphipathic organic anions, whereas oatp2 has an extended substrate preference for neutral compounds. Whether additional differences between oatp1 and oatp2 also exist with respect to transport of amphipathic organic cations is presently under further investigation.

An OATP has also been isolated from human liver. It consists of 670 amino acids and exhibits a 67% amino acid identity with the rat liver oatp1 (9). Although its structural features are similar to the rat oatsps, the cloned human OATP transports bile salts and organic anions to a considerably lower extent than oatp1. Furthermore, in cRNA-injected Xenopus laevis oocytes, OATP-mediated initial transport rates were highest for the organic cation N-propylajmalinium and the peptidomimetic drug CRC 220 (9). In addition, more recent studies indicate that OATP mediates unique high-affinity transport of amphipathic organic anions, whereas oatp2 has an extended substrate preference for neutral compounds. Whether additional differences between oatp1 and oatp2 also exist with respect to transport of amphipathic organic cations is presently under further investigation.

### Table 1. Apparent $K_m$ values of organic anion-transporting polypeptide 1 (oatp1)-mediated organic substrate transport and comparison with sodium-independent substrate uptake in rat hepatocytes

<table>
<thead>
<tr>
<th>Oatp1-expressing Xenopus laevis oocytes and CHO cells</th>
<th>Rat Hepatocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Taurocholate</td>
<td>27–52</td>
</tr>
<tr>
<td>Cholate</td>
<td>54</td>
</tr>
<tr>
<td>Glycocholate</td>
<td>54</td>
</tr>
<tr>
<td>Taurochenodeoxycholate</td>
<td>7</td>
</tr>
<tr>
<td>Taouroursodeoxycholate</td>
<td>13</td>
</tr>
<tr>
<td>Sulfochromophthalein</td>
<td>1–3</td>
</tr>
<tr>
<td>17β-estradiol glucuronide</td>
<td>4</td>
</tr>
<tr>
<td>Estrone-3-sulfate</td>
<td>11</td>
</tr>
<tr>
<td>Dehydroepiandrosteronsulfate</td>
<td>9</td>
</tr>
<tr>
<td>Leukotriene C4</td>
<td>0.3</td>
</tr>
<tr>
<td>Dinitrophenylglutathione</td>
<td>408</td>
</tr>
<tr>
<td>Gadoxetate</td>
<td>3300</td>
</tr>
<tr>
<td>Enalapril</td>
<td>316–337</td>
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<tr>
<td>Temocaprilat</td>
<td>47</td>
</tr>
<tr>
<td>Ouabain</td>
<td>1700–3000</td>
</tr>
<tr>
<td>CRC 220</td>
<td>30–57</td>
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<tr>
<td>Ochratoxin A</td>
<td>17–29</td>
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</table>

Values are Michaelis-Menten constant values ($K_m$), expressed in micromoles.
transport of the bulky lipophilic organic cations methylquinine \( (K_m \sim 5 \, \mu M) \) and methylquinidine \( (K_m \sim 26 \, \mu M) \). These results support the notion that OATP does not represent the human homologue of oatp1 or oatp2 but rather represents a separate member of the Oatp-transporter gene family with a high transport preference for bulky lipophilic organic cations. Hence, although sodium-independent transport of bile acids is an intrinsic feature of oatp1, oatp2, and OATP, each of these polyspecific oatps exhibits additional substrate preferences that may even be specific for certain substrates, such as gadoxetate for oatp1, digoxin for oatp2, and methylquinine and methylquinidine for the human OATP.

Canalicular ATP-dependent bile salt transporters

Canalicular secretion of monovalent bile salts is mediated by an ATP-dependent bsep (Fig. 1). Bsep is a liver-specific canalicular protein of 1321 amino acids with an apparent molecular mass of 160 kDa in isolated canalicular rat liver plasma membrane vesicles (cLPM) (4). It is related to the multidrug resistance (mdr) family of ATP-binding cassette (ABC) proteins and identical to the sister of P-glycoprotein (spgp). When expressed in Sf9 cells, bsep mediated saturable ATP-dependent taurocholate uptake into isolated Sf9 cell vesicles with an apparent \( K_m \) value of 5.3 \( \mu M \). This value is similar to the \( K_m \) of ATP-dependent taurocholate uptake in isolated cLPM (2.1 \( \mu M \)). Identical results were also obtained for the dihydroxy bile salts taurochenodeoxycholate (bsep 2.0 \( \mu M \) and cLPM 2.0 \( \mu M \)) and tauroursodeoxycholate (bsep 5.6 \( \mu M \) and cLPM 5.6 \( \mu M \)). Furthermore, the rank order of maximal velocity \( (V_{\text{max}}) \) values was similar in bsep-expressing Sf9 cell vesicles and in cLPM, namely taurochenodeoxycholate \( > \) taurocholate \( > \) tauroursodeoxycholate. And finally, various bile salts inhibited ATP-dependent taurocholate uptake to a similar extent in cLPM and in bsep-expressing Sf9 cell vesicles. The same is also true for cyclosporin, which inhibited bsep-mediated taurocholate transport with an inhibition constant \( (K_i) \) value of 0.3 \( \mu M \), which is very similar to the \( K_i \) value previously determined in isolated cLPM (0.2 \( \mu M \)). This close similarity of the kinetic transport parameters and inhibition pattern of ATP-dependent bile salt transport between cLPM and bsep-expressing vesicles supports the concept that the isolated bsep represents the only canalicular bile salt export pump in rat liver. However, it cannot be excluded that, in other species, additional bsep-related bile salt export pumps may be present. Most interestingly, the human BSEP appears to be defective in the liver of patients with the type 2 form of hereditary progressive familial intrahepatic cholestasis (PFIC). The defective gene has been mapped to chromosome 2q24, and identification of the gene seems imminent.

In contrast to monoanionic bile salts, divalent sulfated and glucuronidated bile salts are excreted into bile by the multidrug resistance-associated protein 2 (mrp2) or canalicular multispecific organic anion transporter (cMOAT) (Fig. 1) (11). Important substrates of mrp2 include sulfotaurilithocholate, bilirubinmonoglucuronide and diglucuronide, 17β-estradiol glucuronide, glutathione conjugates, leukotriene \( C_4 \) and glutathione disulfide (GSSG). Whether mrp2 can also mediate canalicular excretion of GSH is not yet entirely clear. Failure to express mrp2 at the canalicular membrane, as is the case in the transport mutant TR/EHBR rat strains (11), results in conjugated hyperbilirubinemia and forms the basis of the hereditary Dubin-Johnson syndrome in humans (11).

Finally, it is important to note that canalicular bile salt secretion must be closely coordinated with canalicular phospholipid secretion to prevent bile salt-induced toxic damage to bile ductular cells. The major protective biliary phospholipid is phosphatidylcholine, which is transferred in an ATP-dependent manner from the inner to the outer leaflet of the canalicular membrane by the phosphatidylcholine flippase mdr2 (Fig. 1). Intracanalicular bile salts preferentially associate with phosphatidylcholine to form vesicles and mixed micelles. This close association of intracanalicular bile salts with biliary phospholipids decreases the intrabiliary concentration of free monomeric bile salts and thus reduces toxic bile salt damage to the bile ductular epithelial cells. Failure to express mdr2 (MDR3 in humans) results in progressive intrahepatic cholestasis and biliary cirrhosis.

Regulation of hepatic bile salt transporters

Studies in isolated hepatocytes have shown that cAMP rapidly increases the \( V_{\text{max}} \) of basolateral sodium-dependent bile salt uptake. This acute increase of the basolateral bile salt uptake capacity is partly due to a recruitment of ntcp from an intracellular pool and their insertion into the basolateral plasma membrane (10). An additional stimulation of sodium-dependent bile salt uptake may relate to the fact that incubation of isolated hepatocytes with cAMP leads to a hyperpolarization of hepatocytes, which in turn may acutely stimulate the electrogenic uptake of bile salts into hepatocytes (5). Interestlingly, chronic abolition of the transcellular bile salt flow by bile diversion or increasing transcellular bile salt transport by streptozotocin-induced diabetes showed no changes in the expression of ntcp, indicating that hepatic bile salt fluxes do not modulate the expression of ntcp (6). In contrast, intra- and extrahepatic cholestasis causes a rapid downregulation of ntcp expression on both the mRNA and protein levels (11). This rapid downregulation of ntcp may result from the accumulation of bile components within hepatocytes. Interestingly, the promoter region of ntcp contains a sequence that is identical to a bile salt-responsive element in the promoter of the cholesterol 7α-hydroxylase gene (11). Furthermore, evidence has recently been presented that endotoxin-induced cholestasis in rats leads to a decrease of hepatocyte nuclear factor 1 and footprint B-binding protein, which represent two critical transcription factors for ntcp and thus may be responsible for the transcriptional downregulation of the ntcp gene in cholestasis (15). In contrast to cholestasis, transcriptional ntcp expression is upregulated in postpartum female rats. This

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effect is dependent on the hormone prolactin and mediated by signal transducer and activator of transcription 5 (stat5) via the prolactin receptor (11). Considerably less is known about the acute and chronic regulation of basolateral oatp expression, but extrahepatic cholestasis induced by ligation of the common bile duct in rats also leads to a downregulation of oatp1 (2).

At the canalicular pole of hepatocytes, an acute increase in bile salt and organic anion secretion can be induced by an acute increase of the bile salt load and/or activation of protein kinase A (1). This effect is most probably due to a rapid insertion of ATP-dependent carriers into the canalicular plasma membrane from a subcanalicular vesicular compartment (3). This assumption is further supported by the fact that, after stimulation of protein kinase A, the expression of mrp2 is indeed expressed at a higher level in canalicular membranes of primary cultured rat hepatocyte couplets (13). Interestingly, bsep is also located at subcanalicular vesicles to a significant extent, as evidenced by Immunogold labeling studies (4). These subcanalicular vesicles might constitute an intracellular regulatory pool of canalicular bile salt transporters that can be rapidly recruited and inserted into the canalicular plasma membrane under increased bile salt load conditions, thereby leading to a rapid increase of the canalicular bile salt transport capacity. Although acute and chronic cholestasis may also lead to downregulation of canalicular ATP-dependent efflux pumps (11), their cholestasis-induced decrease in expression appears to be less pronounced compared with the basolateral bile salt carriers and may even be transient for bsep during chronic bile duct obstruction.

Conclusions and perspectives

With the recent cloning of bsep and the identification of the human BSEP gene, it appears that the major transporters involved in hepatocellular bile salt secretion have now been identified. This progress makes more detailed studies regarding the acute and chronic regulation of bile secretory processes possible in the near future. These studies should include transcriptional and posttranscriptional regulatory processes, transporter trafficking within hepatocytes, and structure/activity relationships under various physiological and pathophysiological conditions. Furthermore, the mechanisms involved in the intracellular transport and compartmentalization of bile salts should be investigated in more detail. Since cholangiocytes play an important role in the final modification of bile fluid, it is important to elucidate the exact relationship (cross talk) between hepatocytes and cholangiocytes in the overall process of bile formation. For example, there is increasing evidence that hepatocytes release ATP into the bile canaliculi, possibly via mdr-related ATP-channels, and that the biliary ATP and/or its degradation products may activate purinergic receptors at the apical membrane of cholangiocytes. And finally, the elucidation of the molecular mechanisms involved in the canalicular excretion of cholesterol into bile remains a major challenge. Is spontaneous flipping from the inner to the outer leaflet of the canalicular membrane followed by bile salt- and phospholipid-induced partitioning into mixed vesicles and micelles sufficient, or does canalicular cholesterol flipping require the involvement of a protein-dependent process? The near future should find definite answers to most of these questions and thereby lead to a more complete understanding of the physiology of hepatic bile formation.

References