Identification of Multiple Binding Sites for Substrate Transport in Bovine Organic Anion Transporting Polypeptide 1a2

Xiaoxiao Liu, Jiujiu Huang, Yongxue Sun, Kai Zhan, Zheren Zhang, and Mei Hong

College of Life Science and Guangdong Provincial Key Laboratory of Protein Function and Regulation in Agricultural Organisms (X.L., J.H., K.Z., M.H.), and College of Veterinary Medicine (Y.S., Z.Z.), South China Agricultural University, Guangzhou, China

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ABSTRACT

Organic anion transporting polypeptides (OATP) have been extensively recognized as key determinants of absorption, distribution, metabolism, and excretion of various drugs because of their broad substrate specificity, wide tissue distribution, and the involvement of drug-drug interaction. As the first cloned human OATP, OATP1A2 has been found to transport a wide spectrum of endogenous and exogenous compounds. Bovine Oapt1a2 shared high homology with the human transporter and is considered as its functional ortholog. In the present study, we expressed bovine Oatp1a2 in human embryonic kidney 293 cells and found that, unlike human OATP1A2, the transport of estrone-3-sulfate (E-3-S) exhibited biphasic saturation kinetics. The $K_m$ values were 0.25 ± 0.08 and 46.6 ± 18.5 μM, and $V_{max}$ values were 24.5 ± 4.4 and 375 ± 142 pmol/mg protein/min for high- and low-affinity sites, respectively, suggesting the presence of multiple binding sites. Further study on other Oatp1a2 substrates showed that the high affinity component for E-3-S is responsible for the interaction with taurocholate, bromsulphthalein, and rifampicin and is sensitive to proton concentration change, whereas the low affinity binding site is only involved in the binding of the antitumor drug methotrexate and had no response to change of pH.

Introduction

The organic anion-transporting polypeptides (human OATPs; rodents Oatps; gene symbol SLCO) are a family of transporters that mediate sodium-independent transport of a wide spectrum of structurally independent compounds (Hagenbuch and Gui, 2008). Substrates of OATPs are mainly amphipathic organic molecules, including bile salts [cholate, taurocholate (Tau), taurochenodeoxycholate, tauro-ursodeoxycholate, and glycocholate] (Kullak-Ublick et al., 1994; Eckhardt et al., 1999), bromsulphophthalein (BSP) (Kullak-Ublick et al., 1994), hormones and their conjugates [estrone-3-sulfate (E-3-S), estradiol-17β-glucuronide, dehydroepiandrosterone sulfate, aldosterone, and cortisol] (Bossey et al., 1996a; Kanai et al., 1996; Eckhardt et al., 1999), thyroid hormones (T3, rT3, and T4) (Abe et al., 1998; Eckhardt et al., 1999; Pizzagalli et al., 2002), and eicosanoids (PG2E and LTC4) (Li et al., 1998; Cattori et al., 2001), and various kinds of drugs. Besides these charged compounds, they also transport uncharged drugs, such as glycosides digoxin (Noel et al., 1997) and ouabain (Bossey et al., 1996b). The first discovered Oatp family member was rat Oatp1a1 that was cloned from rat liver (Jacquemin et al., 1994). Thus far, 40 members of the OATP/SLCO superfamily have been identified in human, rat, and mouse (Tamai, 2012). In terms of the evolutionary relationship, many Oatps of rat and mouse are proposed to be orthologs to each other, but not to human OATPs (Geyer et al., 2004). On the other hand, several Oatps were cloned from domestic animals, such as cattle and dog, that showed high homology with their human counterparts (Geyer et al., 2004; Gui and Hagenbuch, 2010).

Because of their broad substrate specificity, wide tissue distribution, and the involvement of drug-drug interaction, it has been proposed that human OATPs may contribute to the influx transport of oral drug delivery (Tamai, 2012) and that regulation of OATPs may have a profound effect on the absorption and bioavailability of drugs. OATP1A2 was the first cloned OATP family member in human being (Kullak-Ublick et al., 1995). It has been detected in various tissues, including the brain, liver, kidney, and small intestine (Kullak-Ublick et al., 1995; Steckelbroeck et al., 2004; Glaeser et al., 2007). OATP1A2 has a broad spectrum of substrates, including endogenous compounds, such as bile acids, steroid hormones and their conjugates, thyroid hormones, and various drugs, such as fexofenadine, ouabain, and the cyanobacterial toxin microcystin (Jacquemin et al., 1994; Kullak-Ublick et al., 1995; Cvetkovic et al., 1999; Eckhardt et al., 1999; Gao et al., 2000). Because of the extent of its substrate specificity and expression in organs important for drug disposition and response, OATP1A2 is believed to play an important role in drug absorption, distribution, metabolism, and excretion (Lee et al., 2005). Bovine Oatp1a2 (bOatp1a2) was cloned in 2004, and functional characterization of Oatp1a2 expressed in Xenopus laevis oocytes demonstrated that this transporter protein shows functional homology to human OATP1A2 (Geyer et al., 2004). In the present study, we expressed bovine Oatp1a2 in human embryonic kidney (HEK)293 cells and found that, unlike human OATP1A2, the transport of estrone-3-sulfate exhibited biphasic saturation kinetics. Moreover, several known substrates or inhibitor of OATPs only inhibited the high affinity site but had no effect on the low affinity site, except the antitumor drug methotrexate (MTX). Comparison of the computer-predicted model of bovine Oatp1a2 and human
OATP1A2 implicated that the secondary structure of these transporters may differ.

Materials and Methods

Materials. [3H]E-3-S and [3H]taurocholic acid were purchased from PerkinElmer Life Sciences (Waltham, MA). Reagents and enzymes for molecular biology and cell culture were obtained from Invitrogen (Carlsbad, CA). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO), except where otherwise stated.

Cell Culture and Generation of Stable Clones Expressing Bovine SLCO1a2. HEK293 cells were grown at 37°C and 5% carbon dioxide in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen). Confluent cells in 6-well plate were transfected with pcDNA3.1 plasmid or pcDNA3.1 plasmid containing SLCO1a2 cDNA and were selected with 0.5 mg/ml of G418. Single colonies were picked up in 96-well plates after 10 days and transferred to 60-mm cell culture dishes for further propagation. Total RNA was isolated from cells using Trizol reagent. Equal amount of RNA was subjected to reverse transcription reaction, and the resulting cDNA was used for PCR. Actin was used as an internal control. Uptake of 100 nM E-3-S and 5 μM Tau was measured at 37°C and pH of 6.0. Uptake activity was expressed as fold change, compared with cells expressing the empty vector pcDNA3.1. The results represent data from three experiments, with triplicate measurements for each sample. The results shown are means ± S.E.M. (n = 3).

Fig. 1. Establishment of stable clones expressing bovine Oatp1a2. (A) Reverse-Transcription PCR of different bOatp1a2-expressing clones. (B) Uptake of E-3-S and taurocholate for different bOatp1a2-expressing clones. HEK293 cells were transfected with pcDNA3.1 or pcDNA3.1 plasmid containing SLCO1a2 cDNA and were selected with 0.5 mg/ml of G418. Single colonies were picked up in 96-well plates after 10 days and transferred to 60-mm cell culture dishes for further propagation. Total RNA was isolated from cells using Trizol reagent. Equal amount of RNA was subjected to reverse transcription reaction, and the resulting cDNA was used for PCR. Actin was used as an internal control. Uptake of 100 nM E-3-S and 5 μM Tau was measured at 37°C and pH of 6.0. Uptake activity was expressed as fold change, compared with cells expressing the empty vector pcDNA3.1. The results represent data from three experiments, with triplicate measurements for each sample. The results shown are means ± S.E.M. (n = 3).

Fig. 2. Time course for uptake of E-3-S and Tau by bOatp1a2. Uptake of 100 nM E-3-S and 5 μM Tau was measured at 37°C and pH of 6.0; transport reaction was terminated at different time points as indicated. Net uptake was obtained by subtracting the uptake of cells with empty vector from bOatp1a2-expressing cells. The results represent data from three experiments, with triplicate measurements for each sample. The results shown are means ± S.E.M. (n = 3).
RNA Isolation and Reverse-Transcription Polymerase Chain Reaction. Total RNA was isolated from cells using Trizol reagent. Equal amount of RNA was subjected to reverse transcription reaction, and the resulting cDNA was used for polymerase chain reaction (PCR). Gene-specific primers for SLCO1a2 (F: gccaaatttgaaaactctcct, R: agggatggcagataagcaaa) and internal control actin (F: tgggcatgggtcagaaggat, R: gtgtggacttgggagaggac) were designed using DNAStar software (Madison, WI), and mRNA sequences were used as primer templates to avoid detection of genomic DNA.

Uptake Assay. Cells in a 48-well plate were used for transport measurement. To each well, uptake solution (125mM NaCl, 4.8mM KCl, 5.6mM D-glucose, 1.2mM KH2PO4, 25mM HEPES or MES 2-(N-morpholino) ethanesulfonic acid, 1.2 mM CaCl2, and 1.2 mM MgCl2 [pH 6.0 or 7.4] and [3H]E-3-S or [3H]Taurocholic acid) was added, and the uptake was stopped at different time points by addition of ice-cold phosphate-buffered saline (PBS) solution. The uptake solution was then aspirated, and the well was rapidly washed with ice-cold PBS solution three times. The cells were then solubilized in 0.2 M NaOH, neutralized in 0.2 M HCl, and the radioactivity of the cell lysate was measured using a liquid scintillation counter Triathler-Hidex (Hidex, Finland). The uptake count was standardized by the amount of protein in each well.

Statistical Analysis. Data statistical analysis was performed using Student’s t test. Differences between means are regarded as significant if \( P < 0.05 \).

Results

Establishment of Cell Lines Stably Expressing Bovine SLCO1a2. To investigate the functional characteristics of boOatp1a2, we first established cell lines that stably expressed boOatp1a2. Four cell lines resistant to G418 were selected and analyzed with reverse-transcription PCR. As shown in Fig. 1A, all four cell lines showed a band around molecular mass of 1.2Kb, which is corresponded to the size expected from the gene-specific primers used, whereas cells transfected with empty vector pcDNA3.1 did not show any signal. To further characterize the transport activity of these clones, uptake of prototypic substrates E-3-S and Tau was investigated. Consistent with the results obtained from reverse-transcription PCR, clone SLCO1a2-2 had the highest transport function for both E-3-S and Tau and was therefore used for further studies (Fig. 1B).

Kinetic Analysis for Uptake of E-3-S and Tau in boOatp1a2-Expressing Cells. Uptake of E-3-S and Tau was linear up to 2 minutes and 5 minutes, respectively (Fig. 2). Therefore, we analyzed the kinetic parameters of bovine Oatp1a2 and human OATP1A2 on uptake of estrone-3-sulfate and taurocholate.

![Fig. 3. Eadie-Hofstee plot for uptake of estrone-3-sulfate (A) and taurocholate (B) by boOatp1a2 and estrone-3-sulfate (C) and Tau (D) by human OATP1A2. Uptake of E-3-S and Tau was measured at concentrations range from 0.01 (0.05 \( \mu \)M for OATP1A2) to 50 \( \mu \)M and from 0.5 to 100 \( \mu \)M, respectively. Uptake was conducted at 37°C for 1 minute with cells expressing empty vector, boOatp1a2, or OATP1A2. Net uptake was obtained by subtracting the uptake of cells with empty vector from boOatp1a2- or OATP1A2-expressing cells. The results represent data from three experiments, with triplicate measurements for each sample. The results shown are means ± S.E.M. (n = 3).](604.png)

![TABLE 1 Kinetic parameters of bovine Oatp1a2 and human OATP1A2 on uptake of estrone-3-sulfate and taurocholate](604.png)

<table>
<thead>
<tr>
<th>Species</th>
<th>Substrate</th>
<th>( K_m ) ( \mu )M</th>
<th>( V_{max} ) pmol/normalized protein/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine</td>
<td>Estrone-3-sulfate</td>
<td>0.25 ± 0.08</td>
<td>24.5 ± 4.4</td>
</tr>
<tr>
<td></td>
<td>Taurocholate</td>
<td>46.6 ± 18.5</td>
<td>375 ± 142</td>
</tr>
<tr>
<td>Human</td>
<td>Estrone-3-sulfate</td>
<td>40.2 ± 10.0</td>
<td>55.4 ± 8.4</td>
</tr>
<tr>
<td></td>
<td>Taurocholate</td>
<td>161.3 ± 6.0</td>
<td>39.4 ± 7.8</td>
</tr>
</tbody>
</table>

E-3-S, estrone-3-sulfate; \( K_m \), Michaelis constant; OATP, organic anion transporting polypeptide.
The kinetic properties of boatp1a2 within a 1-minute period. The Eadie-Hofstee plot analysis showed that the boatp1a2-mediated uptake of E-3-S presented biphasic kinetics (Fig. 3A), suggesting the presence of two kinetically distinct functional sites on boatp1a2. The kinetic parameters for the high- and low-affinity components of E-3-S uptake were 0.25 ± 0.08 and 46.6 ± 18.5 μM [Michaelis constant Km (Km)] and 24.5 ± 4.4 and 375 ± 61.2 pmol/mg protein/min (Vmax), respectively (Table 1). The transport kinetics of Tau was also investigated (Fig. 3B), and the Km and Vmax of this prototypic substrate were 40.2 ± 10.0 μM and 55.4 ± 8.4 pmol/mg protein/min, respectively (Table 1). We also analyzed the kinetics for these two substrates in HEK293 cells expressing human OATP1A2 and found out that there was only one binding component for E-3-S, with a Km value of 16.1 ± 3.4 μM and Vmax of 39.4 ± 7.8 pmol/mg protein/min. The Km and Vmax for Tau were 15.9 ± 6.0 μM and 24.1 ± 4.1 pmol/mg protein/min, respectively (Fig. 3, C and D; Table 1).

**Difference on pH Dependence for Multiple E-3-S Uptake Sites.**

It has been demonstrated previously that the transport activity of OATP family members are pH dependent (Nozawa et al., 2004; Leuthold et al., 2009; Tamai, 2012). To investigate whether functional sites of boatp1a2 are pH dependent, we performed the uptake experiments at two different pH. Our results showed that the high affinity component of E-3-S and Tau transport responded to the change of proton concentration, with lower pH demonstrating the higher uptake function (Fig. 4A). The low affinity component of E-3-S, on the other hand, was not sensitive to the change of pH (Fig. 4B).

**Inhibition Effect for Uptake of E-3-S.**

Because transport of E-3-S shown biphasic kinetics, we next examined which of the binding site for this substrate were shared by other substrates of boatp1a2. As shown in Fig. 5A, Tau inhibited the uptake of low concentration of E-3-S (50 nM) at a concentration-dependent manner but had no effect on the transport of high concentration of E-3-S (50 μM). In addition, BSP, which is a known substrate of OATPs, and rifampicin, an inhibitor of OATPs, also showed inhibitory effect on the high affinity component of E-3-S uptake. On the other hand, antitumor drug MTX only suppressed the uptake of high concentration of E-3-S (Fig. 5B).

**Discussion**

Because of the extent of its substrate specificity and expression in organs important for drug disposition and response, OATP1A2 is believed to be a key determinant for bioavailability of various drugs (Lee et al., 2005). Several Oatps were cloned from domestic animals,
such as cattle and dog, that showed high homology with their human ortholog (Geyer et al., 2004; Gui and Hagenbuch, 2010). Bovine Oatp1a2 was cloned in 2004 and shares high homology of amino acid sequence with human OATP1A2. Studies on oocytes expressing bOatp1a2 indicated that this transporter protein is functionally similar with that of human OATP1A2. In our present study, we generated a stable cell line expressing bOatp1a2 and found that, in such a system, the protein showed biphasic saturation kinetics for prototypic substrate E-3-S. The kinetic parameters for E-3-S are $0.25 \pm 0.08$ and $46.6 \pm 18.5 \mu M$ ($K_m$) and $24.5 \pm 4.4$ and $375 \pm 142 \text{ pmol/mg protein/min}$ ($V_{max}$), respectively, implicating both a high and low affinity site for E-3-S in the structure of bOatp1a2. This result is inconsistent with that of the study with the oocyte system, in which there was only one $K_m$ for E-3-S (9.6 $\mu M$) (Geyer et al., 2004). The difference may be attributable to the different systems used. A recently published report demonstrated that OATP2B1-mediated uptake of E-3-S exhibited biphasic saturation kinetics in Xenopus oocytes, whereas in previous studies, OATP2B1-expressing cells showed single saturation kinetics (Shirasaka et al., 2012). The presence of multiple binding sites of bOatp1a2 for E-3-S did not seem to be an artifact. We also investigated OATP1A2 in our HEK293 system and found that this human transporter protein showed similar saturation kinetics as in previous reports ($K_m = 16.1 \pm 3.4 \mu M$ for E-3-S in our current study) (Badagnani et al., 2006). On the other hand, the $K_m$ of another prototypic substrate Tau is $40.2 \pm 10.0 \mu M$ for bovine Oatp1a2 in our cellular system, which is comparable to that observed in Xenopus oocytes. Moreover, Tau showed inhibitory effect on the uptake of E-3-S in a concentration-dependent manner. However, such an inhibition only presented for the high affinity component of E-3-S transport. Of interest, the change of pH in the uptake solution seemed to only alter transport function for the high affinity site but not the low affinity component for E-3-S. Similar with the response of E-3-S high affinity binding site, transport function of Tau was higher at pH of 6.0, compared with that at pH of 7.4, further confirming that Tau shared

![Fig. 6. Secondary structure of bOapt1a2 (A) and OATP1A2 (B). Goldman Engelman Steitz hydrophobicity scale was used to generate topology of the transporter proteins. The extra transmembrane domain of human OATP1A2 was labeled as black diamonds.](image-url)
the same binding site of the high affinity component of E-3-S. When other substrates or inhibitor of Oatp1a2 was tested, both BSP and rifampicin showed suppressive effect on the high affinity functional site, whereas MTX inhibited the low affinity functional site. These results suggest that the high affinity component for E-3-S may be the location for most of the substrates transported by bOatp1a2.

The presence of multiple binding sites for E-3-S has been observed in other OATP family members, such as OATP1B1 (Tamai et al., 2001) and OATP2B1 (Shirasaka et al., 2012). However, studies on OATP1A2 showed that this transporter protein only has single saturation kinetics for E-3-S. Uptake studies on cells expressing OATP1A2 in our laboratory also indicated a single binding site for E-3-S. Although there is a high homology of amino acid sequence between bOatp1a2 and OATP1A2 (81.7% identity), there are four amino acid deletions in bOatp1a2, compared with OATP1A2. Such a difference may lead to a different secondary structure of the protein. With use of the Goldman Engelman Steitz hydrophobicity scale, the distribution of transmembrane regions of bOatp1a2 and OATP1A2 was analyzed (Fig. 6). The deletions of four amino acids in bOatp1a2 resulted in an 11 transmembrane domain (TM) structure, whereas OATP1A2 showed 12 transmembrane domains with both the amino and carboxyl termini located intracellularly. The presence of multiple binding sites for E-3-S in bOatp1a2 may be attributable to its different topology in comparison with that of OATP1A2. Of interest, use of the same hydrophobicity scale, another OATP member, OATP2B1 that had been proposed to contain multiple binding sites (Shirasaka et al., 1998), also showed an odd number of transmembrane domains (13 transmembrane domains, unpublished data). However, OATP1B1, which has long been known to have two E-3-S binding sites (Tamai et al., 2001), was predicted to share a 12 transmembrane domain topology with that of human OATP1A2. Therefore, more experimental analysis will be needed for clarifying the hypothesis.

In conclusion, our study demonstrated two binding sites of bOatp1a2 for E-3-S in HEK293 cell system. The high affinity binding site for E-3-S seemed to be responsible for most of the substrates tested and is sensitive to proton concentration change. The presence of such a biphasic saturation kinetics of bOatp1a2 differs from that of human OATP1A2. As an OATP member that shows high sequence homology with OATP1A2 and, thus, considered to be an ortholog to the human transporter protein, we may need to be cautious on data interpretation, depending on the systems used for the investigation.

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Authorship Contributions

Participated in research design: Hong, Sun.
Conducted experiments: Liu, Huang, Zhan, Zhang.
Contributed new reagents or analytic tools: Sun.
Performed data analysis: Hong, Huang.
Wrote or contributed to the writing of the manuscript: Hong.

References


Address correspondence to: Mei Hong, College of Life Science, Tianhe, Wuhan, Guangzhou, China. E-mail: mhf2788@scu.edu.cn

Bovine Oatp1a2 Contains Multiple Substrate Binding Sites

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