Intestinal absorption of bile acids: paradoxical behaviour of the 14 kDa ileal lipid-binding protein in differential photoaffinity labelling

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Photoaffinity labelling of brush border membrane vesicles from rabbit ileum with radiolabelled 3,3-azo and 7,7-azo derivatives of taurocholate identified integral membrane proteins of molecular masses 93 and 46 kDa, as well as a 14 kDa peripheral membrane protein, as components of the ileal Na^+/bile acid transport system [Kramer, Girbig, Gutjahr, Kowalewski, Jouvenal, Müller, Tripier and Wess (1993) J. Biol. Chem. **268**, 18035–18046]. Differential photoaffinity labelling in the presence of non-radiolabelled bile acid derivatives led, as expected, to a concentration-dependent decrease in the extent of labelling of the 93 and 46 kDa transmembrane proteins, which are the monomeric and dimeric forms of the ileal bile acid transporter protein. The extent of labelling of the 14 kDa ileal lipid-binding protein (ILBP), however, increased on the addition of unlabelled bile acids, the increase being dependent on the structure of the bile acid added. The possibility of artifacts was excluded by photoaffinity labelling experiments in the frozen state as well as by model calculations. The experimental results suggest that the binding of bile acids to ILBP can increase the affinity of ILBP for bile acids. These results would be in accordance with a substrate-load modification of transport activity and a positive-feedback regulation mechanism for active uptake of bile acid in the ileum.

INTRODUCTION

The enterohepatic circulation of bile acids is an efficient biological recycling system established by active Na^+-dependent bile acid transport systems in the terminal ileum and the liver, as well as in the proximal tubule of the kidney [1,2]. The bile acids are reabsorbed in the terminal ileum by a Na^+-dependent bile acid transport system and recirculate with portal blood to the liver, where they are taken up by the hepatocytes and resecreted into bile. This flux of bile acids through the hepatocyte regulates cholesterol 7α-hydroxylase, the rate-limiting enzyme for the conversion of cholesterol into bile acids [3]. By this mechanism the enterohepatic circulation of bile acids is a major regulator of serum cholesterol homeostasis. The physiology and specificity of intestinal bile acid absorption were intensively investigated *in vivo* and *in vitro* by Lack [4]. We have characterized, at a molecular level, putative protein components of the ileal Na^+/bile acid co-transport system from rabbit and rat by photoaffinity labelling with photoreactive bile acid analogues [5–9] leading to the identification of integral membrane proteins of molecular masses 93 and 99 kDa respectively. A soluble 14 kDa protein, ileal lipid-binding protein (ILBP), originally identified by Wilson and co-workers as a bile acid binder in the cytosol of ileocytes [10–13], is also closely associated with the brush border membrane of ileocytes, suggesting a direct function of ILBP in ileal bile acid uptake [7,8]. By expression cloning, Dawson and colleagues have characterized integral membrane proteins of 348 amino acid residues with an apparent molecular mass of 46 kDa as Na^+/bile acid co-transporters in hamster [14], man [15] and rat [16], whereas the rabbit transporter cloned by us has 347 residues (S. Stengelin, W. Becker, M. Maier, J. Rosenberger, A. Enhsen, P. Sauer, G. Wess and W. Kramer, unpublished work). The differences between the molecular masses of putative Na^+/bile acid co-transporters dependent on the methodology used were recently taken to indicate that the photolabelled 93 kDa protein in the rabbit is a non-covalently bound dimer of the Na^+/bile acid co-transporter protein [9]. Whereas the protein components of the ileal Na^+/bile acid co-transport system are well characterized today, the regulatory mechanisms of ileal bile acid transport are still poorly understood.

Intestinal transport processes can be influenced by the availability of substrate; a positive feedback mechanism in response to an increased substrate load was shown for the absorption of nutrients such as D-glucose [18] or amino acids [19], whereas the intestinal absorption of calcium or iron is up-regulated during a bodily deficiency of these minerals [20,21]. For the regulation of ileal bile acid absorption, conflicting results have been reported describing negative feedback mechanisms [22] as well as a down-regulation of ileal taurocholate transport in biliary-diverted rats [23–25]. In rabbit ileal brush border membrane vesicles we demonstated a functional relationship between the membrane-associated ILBP and the Na^+/bile acid co-transporting protein [7,8], putatively indicating a regulatory function of ILBP on the transporter activity. Here we describe a paradoxical behaviour of ILBP during photoaffinity labelling: the incorporation of photolabile bile acids into ILBP is stimulated by the presence of bile acids instead of being inhibited.

EXPERIMENTAL

Materials

Photoaffinity labelling was performed with the photolabile bile acid derivatives (7,7-azo-3α,12α-dihydroxy-5β[3β-^{3}H]cholan-24-oyl)-2-aminoethanesulphonic acid (specific radioactivity 20.25 Ci/mmol) and (3,3-azo-7α,12α-dihydroxy-5β[7β,12β-^{3}H]-cholan-24-oyl)-2-aminoethanesulphonic acid (specific radioactivity 5.9 Ci/mmol), synthesized as described [26–28]. [G-^{3}H]-Taurocholate (specific radioactivity 258.5 mCi/mmol) was obtained from DuPont–New England Nuclear (Dreieich, Germany) and recombinant rabbit ILBP was prepared as described elsewhere [11]. Male New Zealand White rabbits (weighing 4–5 kg) were used as a tissue source. They were purchased from Harlan Winkelmann (Borchen, Germany) and maintained on a normal

Abbreviation used: ILBP, ileal lipid-binding protein.
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chow diet. Marker proteins and other materials for protein electrophoresis were from Serva. Scintillators (Quickszint 501 and 361) and the tissue solubilizer Biolute were from Zinsser Analytic (Frankfurt, Germany). Protein was determined with a Bradford assay [29] kit from Bio-Rad (München, Germany).

Preparation of ileal brush border membrane vesicles and cytosol

Brush border membrane vesicles from the ileum of rabbits were prepared by the Mg^{2+} precipitation method and characterized as described previously [5–9]. For the preparation of ileal cytosol, the mucosa of a freshly excised rabbit ileum was scraped off, added to 10 mM Tris/Hepes (pH 7.4)/300 mM mannitol and treated with an Ultra-Turrax homogenizer to disrupt suspended cells. After centrifugation at 100000 ***g*** for 60 min the clear supernatant was used immediately as ileal cytosol for experiments.

Transport measurements

Uptake of radiolabelled bile acids by ileal brush border membrane vesicles was determined by the membrane filtration method as described previously [5–9]. Typically, the transport reaction was initiated by adding 10 μl of the vesicle suspension (50–100 μg of protein) equilibrated with 10 mM Tris/Hepes buffer (pH 7.4)/300 mM mannitol with 90 μl of incubation medium containing the substrates kept at 30 °C. The composition of the incubation medium for measurements in the presence of an inwardly directed Na^+ gradient was usually 10 mM Tris/Hepes (pH 7.4)/100 mM NaCl/100 mM mannitol, and in the absence of a Na^+ gradient it was 10 mM Tris/Hepes (pH 7.4)/100 mM KCl/100 mM mannitol. For measurement of bile acid uptake, these media contained [^{3}H]taurocholate at a concentration of 50 μM (0.75 μCi). At desired time points the transport reaction was terminated by the addition of 1 ml of ice-cold stop solution [10 mM Tris/Hepes (pH 7.4)/150 mM KCl]. The entire content was pipetted on the middle of a prewashed, prechilled filter kept under suction with the aid of a vacuum controller. The filters were rinsed immediately with 5 ml of ice-cold stop solution. The amount of radioactively labelled substrates taken up by the vesicles was measured after dissolving the filters in 4 ml of scintillator (Quickszint 361) by liquid-scintillation counting in a Packard Tri-Carb 2200 β-counter (Packard Instrument Co.).

Photoaffinity labelling and SDS/PAGE

Photoaffinity labelling with photoreactive bile acid analogues was performed as described previously [5–9]. Brush border membrane vesicles were incubated in 10 mM Tris/Hepes (pH 7.4)/100 mM NaCl/100 mM mannitol with the photolabile compounds. Freshly isolated cytosol from rabbit ileum (150 μg of protein) or recombinant ILBP (20 μg of protein) was adjusted with 10 mM Tris/Hepes (pH 7.4)/100 mM NaCl/100 mM mannitol to a protein concentration of 0.5 mg/ml and incubated for 5 min at 20 °C in the dark with 0.5–1 μCi (0.24–1 μM) of the bile acid photoprobes. For differential photoaffinity labelling, non-radiolabelled bile acid derivatives were added and adjusted to the concentrations indicated in the respective figures. Cross-linking was achieved by irradiation at 350 nm for 10 min. For photoaffinity labelling in the frozen state [9], the respective samples were incubated with photolabile radioactively labelled bile acid derivatives at 20 °C for the indicated periods and subsequently shock-frozen in liquid nitrogen, followed by irradiation for 10 min at 350 nm in the frozen state with a Rayonet Photochemical Reactor RPR-100 (The Southern Ultraviolet Co., Hamden, CT, U.S.A.). Protein was then collected by precipitation with chloroform/methanol [30] and analysed by SDS/PAGE on 12 % (w/v) gels [7,31]. The distribution of radioactivity was determined either by fluorography [32] or by slicing the gel tracks into 2 mm pieces, followed by digestion of their protein content with Biolute S and subsequent liquid-scintillation counting [7,31]. For Western blotting, proteins were transferred to nitrocellulose membranes (0.2 μm trans-blot transfer medium from Bio-Rad) from SDS/PAGE gels in a trans-blot cell (Bio-Rad) with 25 mM Tris/192 mM glycine (pH 8.3)/33 % (v/v) methanol. Blotting was performed at 300 mA for 3 h followed by 400 mA for 0.5 h. Immunodetection was performed with an anti-ILBP antibody generated against a fusion protein of the C-terminus of rabbit ILBP and *Escherichia coli* maltose binding protein [11] by using the Western light chemiluminescence detection system from Serva (Heidelberg, Germany).

RESULTS

Effect of bile acids on photoaffinity labelling of rabbit ileal brush border membrane vesicles

Photoaffinity labelling of rabbit ileal brush border membrane vesicles with radioactively labelled 3,3-azo or 7,7-azo derivatives of taurocholate in the presence of increasing concentrations of unlabelled bile acid derivatives revealed different behaviours of the bile acid-binding proteins of molecular masses 93, 87, 46 and 14 kDa. The extent of labelling of the 93, 87 and 46 kDa bands decreased as expected with increasing concentrations of unlabelled natural bile acids such as taurocholate, because these bile acids compete with the photoreactive bile acid analogues for identical binding sites (Figures 1a to 1d). However, the extent of labelling of the 14 kDa bile acid-binding protein [7,11] increased with increasing bile acid concentrations, reaching a characteristic maximum for each applied bile acid derivative. The decrease in the extent of labelling of the 93, 46 and 87 kDa bile acid-binding proteins, which were probably protein components of the active Na^+-dependent bile acid transport system (93 and 46 kDa) and a putative passive bile acid transport system (87 kDa) in the enterocyte brush border membrane, in the presence of increasing concentrations of non-radioactively labelled bile acids, is in accordance with transport measurements. The uptake of 50 μM [^{3}H]taurocholate by ileal brush border membrane vesicles was inhibited in a concentration-dependent manner by the presence of increasing concentrations of unlabelled bile acids such as taurocholate, cholate, ursodeoxycholate or taurochenodeoxycholate, indicating that all these compounds compete for binding and transport by the ileal Na^+/bile acid co-transport system (Figure 2). The unusual behaviour of the 14 kDa ILBP in the differential photoaffinity labelling experiments, with an increase in the extent of labelling of ILBP instead of a decrease, was dependent on the bile acid analogue used. With taurocholate as competing ligand, a maximum of labelling of the 14 kDa protein was observed reproducibly at taurocholate concentrations between 50 and 100 μM with a 3–4-fold stimulation of labelling compared with the controls (Figure 1a). With taurochenodeoxycholate, only a small increase in labelling (less than 2-fold) was observed with a maximum at about 25 μM, whereas the unconjugated bile acids cholate or ursodeoxycholate led to a profound stimulation of labelling of the 14 kDa protein with a maximum at 400–600 μM (Figures 1c and 1d).

To determine whether the increase in the extent of labelling of the 14 kDa ILBP in the presence of bile acids was caused by different amounts of ILBP in the brush border membranes after addition of the unlabelled bile acids, membrane vesicles were incubated with increasing concentrations of bile acids, as in the

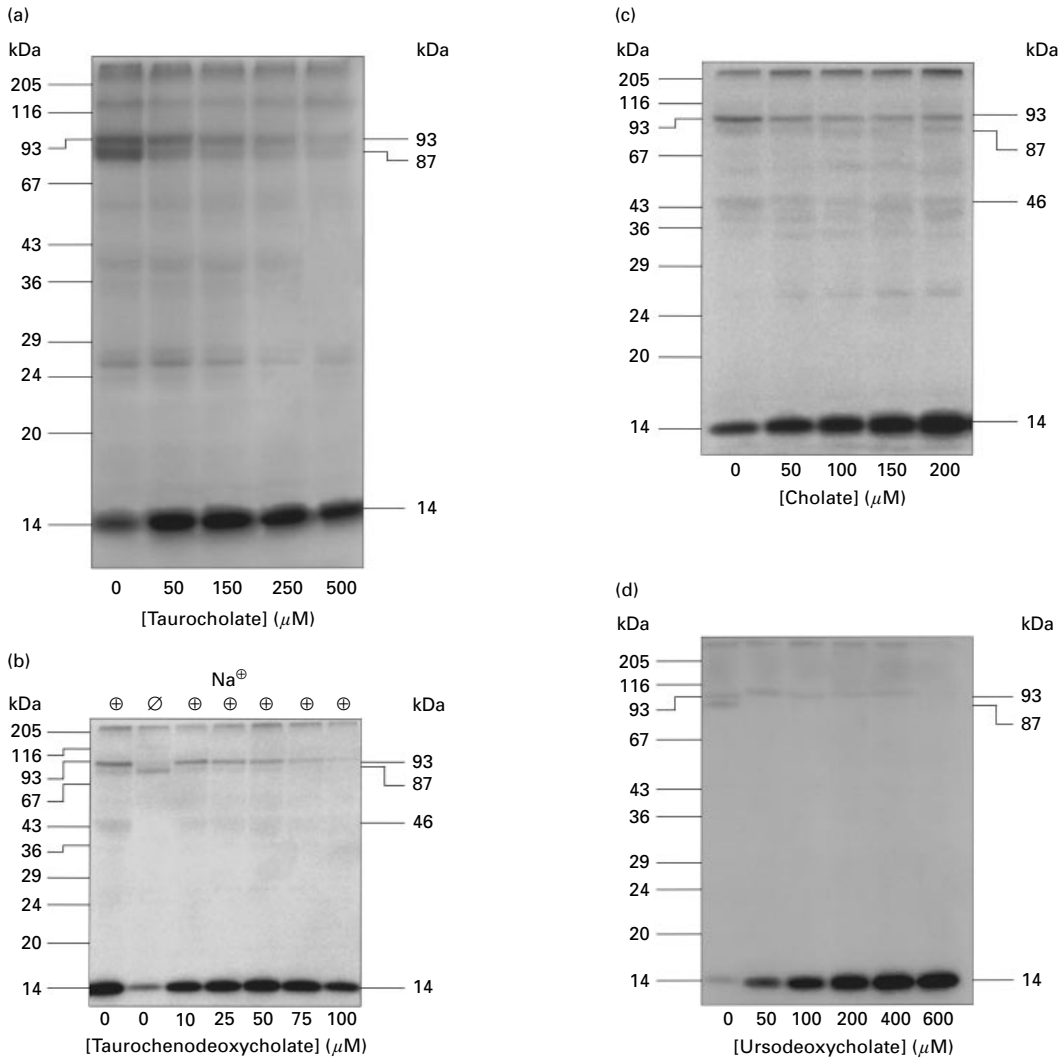


Figure 1 Effect of the bile acids taurocholate (a), taurochenodeoxycholate (b), cholate (c) and ursodeoxycholate (d) on photoaffinity labelling of bile acid-binding polypeptides in rabbit ileal brush border membrane vesicles by (7,7-azo-3α,12α-dihydroxy-5β[3β-^{3}H]cholan-24-oyl)-2-amino-ethanesulphonic acid

Rabbit ileal brush border membrane vesicles (150 μg of protein) equilibrated with 10 mM Tris/Hepes (pH 7.4)/300 mM mannitol were incubated for 5 min at 20 °C with 0.36 μM (1.5 μCi) (**a**) or 0.29 μM (1.5 μCi) (**b**–**d**) (7,7-azo-3α,12α-dihydroxy-5β[3β-^{3}H]cholan-24-oyl)-2-aminoethanesulphonic acid in 10 mM Tris/Hepes (pH 7.4)/100 mM NaCl/100 mM mannitol in the absence or presence of the indicated concentrations of bile acids. After irradiation for 10 min at 350 nm, vesicles were washed and membrane polypeptides were separated by SDS/PAGE followed by the detection of radioactively labelled polypeptides by fluorography.

differential photoaffinity labelling experiments, followed by SDS/PAGE and Western blotting with anti-ILBP antibodies. The amount of ILBP in the brush border membrane vesicles remained unchanged, which excluded the possibility that different amounts of ILBP resided in the brush border membranes (results not shown). The photoaffinity labelling experiments were usually performed at 20 °C with irradiation at 350 nm for 10 min. Under these conditions an exchange of the photoprobe and the competing ligands with the binding protein can occur. To study whether the increase in the extent of labelling of the 14 kDa protein in the presence of unlabelled bile acids might be caused by different association and dissociation kinetics of the photoprobe and the competing bile acids, we have performed differential photoaffinity labelling experiments of brush border membrane vesicles in the frozen state [9]. By shock-freezing of the probes in liquid nitrogen all molecular movements are fixed and subsequent photoaffinity labelling allows a snapshot to be taken of the molecular interactions between the photoprobe and respective binding proteins. An increased labelling of the 14 kDa protein under these conditions in the presence of excess concentrations of unlabelled bile acids would demonstrate unequivocally an increased binding of the photoprobe to the respective binding protein. Figure 3 (upper panel) shows that also in the frozen state the labelling of the 14 kDa ILBP by (3,3-azo-7α,12α-dihydroxy-5β[7β,12β-^{3}H]cholan-24-oyl)-2-aminoethanesulphonic acid was significantly increased by the presence of 300 μM ursodeoxycholate. In a further experiment, rabbit ileal brush border membrane vesicles were incubated with the photolabile bile acid analogue in the absence and in the presence of 300 μM ursodeoxycholate, and uptake photoaffinity labelling was performed in the frozen state [9,33]. After 5, 10, 20, 30 or 300 s of incubation all molecular movements were fixed by freezing in liquid nitrogen followed by cross-linking by light at 350 nm in the solid state. Figure 3 (lower panel) shows that the labelling of the 14 kDa

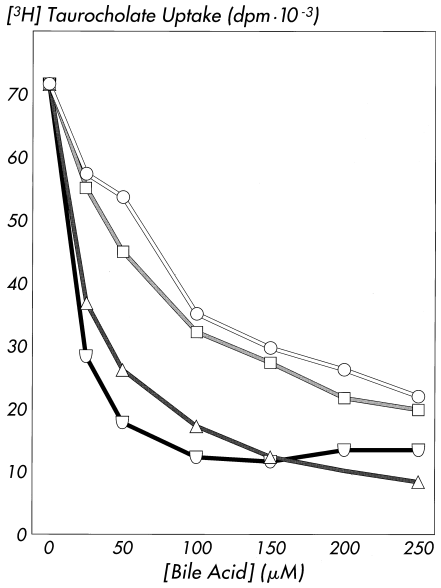


Figure 2 Effect of the bile acids ursodeoxycholate (○), taurocholate (□), deoxycholate (△) and taurochenodeoxycholate (▽) on Na^+-dependent [^{3}H]taurocholate uptake by rabbit ileal brush border membrane vesicles

Rabbit ileal brush border membrane vesicles (50 μg of protein) equilibrated with 10 mM Tris/Hepes (pH 7.4)/300 mM mannitol were incubated at 20 °C with 50 μM (0.75 μCi) [^{3}H]taurocholate in 10 mM Tris/Hepes (pH 7.4)/100 mM NaCl/100 mM mannitol in the absence or presence of the indicated concentrations of bile acids and uptake was measured after 60 s.

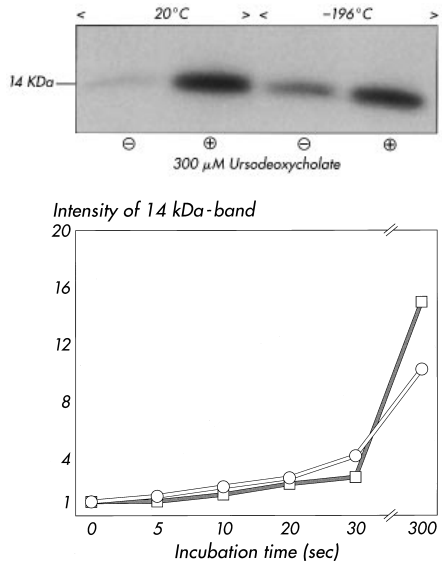


Figure 3 Photoaffinity labelling of rabbit ileal brush border membrane vesicles in the frozen state with (3,3-azo-7α,12α-dihydroxy-5β[7β,12β-^{3}H]cholan-24-oyl)-2-aminoethanesulphonic acid in the absence and in the presence of ursodeoxycholate

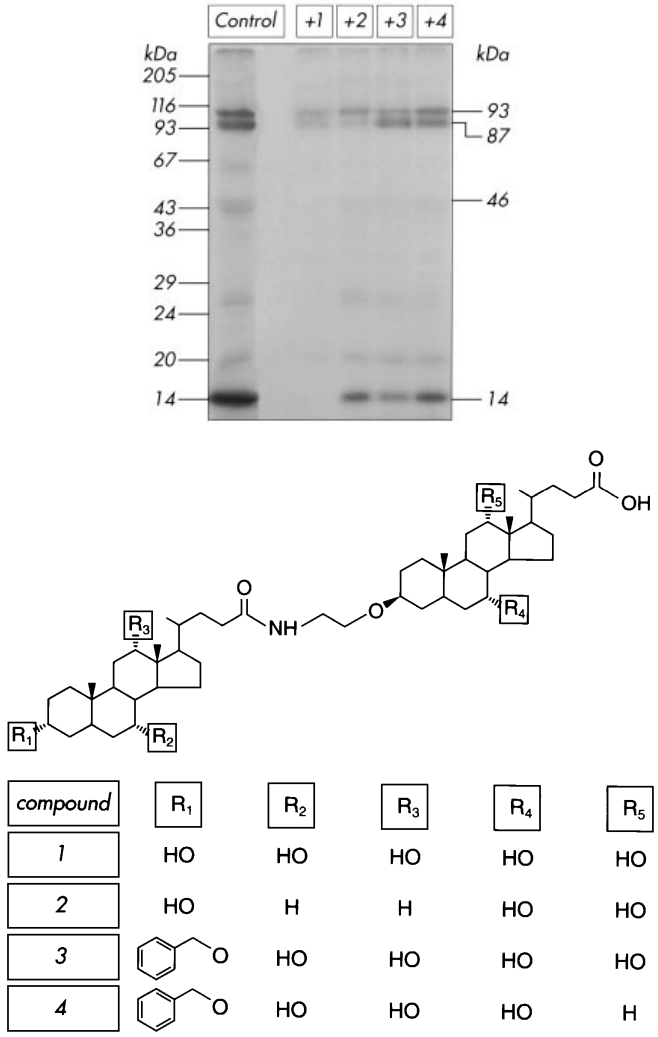
Upper panel: rabbit ileal brush border membrane vesicles (150 μg of protein) equilibrated with 10 mM Tris/Hepes (pH 7.4)/300 mM mannitol were incubated at 20 °C for 5 min with 0.84 μM (1 μCi) (3,3-azo-7α,12α-dihydroxy-5β[7β,12β-^{3}H]cholan-24-oyl)-2-aminoethanesulphonic acid in the absence or presence of 300 μM ursodeoxycholate. Subsequently the probes were irradiated for 10 min at 350 nm either at 20 °C or after being frozen in liquid nitrogen (−196 °C). After the vesicles had been washed, membrane proteins were separated by SDS/PAGE and radioactivity was detected by fluorography. Lower panel: rabbit ileal brush border membrane vesicles (150 μg of protein) were incubated at 20 °C with 0.84 μM (1 μCi) (3,3-azo-7α,12α-dihydroxy-5β[7β,12β-^{3}H]cholan-24-oyl)-2-aminoethanesulphonic acid in the absence or presence of 300 μM ursodeoxycholate. After the indicated durations the samples were frozen in liquid nitrogen and irradiated at 350 nm for 10 min in the frozen state. After SDS/PAGE, radiolabelled proteins were detected by fluorography and the intensity of labelling of the 14 kDa band was measured by densitometry of the fluorograms.

ILBP was inhibited by the presence of ursodeoxycholate up to an incubation period of 30 s owing to the lower intravesicular concentration of the photolabel in the presence of ursodeoxycholate compared with that in controls. After 5 min of Na^+-driven uptake, when equilibrium had nearly been achieved, the 14 kDa protein was significantly more highly labelled in the presence of ursodeoxycholate. It could be argued that the increased labelling of ILBP in brush border membrane vesicles was caused by an increased intravesicular concentration of the photoprobe at the beginning of the photolysis, either by trans-stimulation effects or by a non-specific increase in membrane permeability by a detergent-like effect of bile acids. Transport measurements with 1–50 μM (7,7-azo-3α,12α-dihydroxy-5β-cholan-24-oyl)-2-aminoethanesulphonic acid in the absence or presence of 300 μM cholate or ursodeoxycholate revealed that the uptake of the photoprobe was strongly inhibited by the presence of unlabelled bile acids. Consequently the intravesicular concentration of the photoprobe was lower at the beginning of photolysis, making a trans-stimulation effect unlikely. To exclude the possible non-specific detergent effect of bile acids, photoaffinity labelling experiments of rabbit ileal brush border membrane vesicles were also performed in the presence of the detergents Triton X-100, n-octyl glucoside or CHAPS, each at 300 μM. Whereas the labelling of ILBP was stimulated by the presence of cholate, it was significantly decreased by the detergents. Furthermore the labelling of recombinant ILBP or ILBP in ileal cytosol was also stimulated by bile acids (see Figures 5 and 6), which excludes the possibility of different accessibilities of the photoprobe to ILBP. The unexpected stimulation of ILBP photolabelling by bile acids can thus not be ascribed to a non-specific detergent effect but might be indicative of a regulatory function for the ileal Na^+/bile acid co-transport system.

Dimeric bile acid analogues are potent non-absorbable inhibitors of the ileal Na^+/bile acid co-transport system *in vitro* and *in vivo* [34,35]. Photoaffinity labelling of rabbit ileal brush border membrane vesicle with 3,3-azo or 7,7-azo derivatives of taurocholate in the presence of these inhibitors led, as expected, to a decrease in the extent of labelling of the 93 and 46 kDa forms of the bile acid transporter protein [9]. However, in contrast with monomeric transportable bile acids, these non-absorbable inhibitors also inhibited the labelling of the 14 kDa ILBP in brush border membrane vesicles (Figure 4). This inhibition of labelling of the membrane-bound ILBP by bile acid transport inhibitors was caused by a lower intravesicular concentration of the photoprobe. The ranking in the inhibition of labelling of the 93 and 14 kDa proteins by the bile acid transport inhibitors is correlated with their inhibitory potency on [^{3}H]taurocholate uptake by rabbit ileal brush border membrane vesicles (Table 1).

Photoaffinity labelling of recombinant ILBP and ileal cytosol

Similar differential labelling experiments were performed with recombinant ILBP and freshly isolated ileal cytosol. With the use of rabbit ileal cytosol and the 7,7-azo or 3,3-azo derivative of taurocholate as photoprobe, taurocholate led to only a small increase in the extent of labelling of the ILBP. In contrast, under identical experimental conditions, recombinant ILBP or ILBP in



compound	R_1	R_2	R_3	R_4	R_5
1	HO	HO	HO	HO	HO
2	HO	H	H	HO	HO
3		HO	HO	HO	HO
4		HO	HO	HO	H

Figure 4 Effect of bile acid transport inhibitors on photoaffinity labelling of rabbit ileal brush border membrane vesicles by (7,7-azo-3α,12α-dihydroxy-5β[3β-^{3}H]cholan-24-oyl)-2-aminoethanesulphonic acid

Rabbit ileal brush border membrane vesicles (150 μg of protein) were incubated at 20 °C for 5 min with 0.24 μM (1 μCi) (7,7-azo-3α,12α-dihydroxy-5β[3β-^{3}H]cholan-24-oyl)-2-aminoethanesulphonic acid in the absence or presence of the indicated bile acid transport inhibitors, each at 200 μM. After 10 min of irradiation at 350 nm, vesicles were washed and membrane proteins were separated by SDS/PAGE followed by detection of radioactivity by fluorography.

Table 1 Inhibition of [^{3}H]taurocholate uptake into rabbit ileal brush border membrane vesicles by bile acid reabsorption inhibitors

Rabbit ileal brush border membrane vesicles (50 μg of protein) equilibrated with 10 mM Tris/Hepes (pH 7.4)/300 mM mannitol were incubated for 1 min with 50 μM (0.75 μCi) [^{3}H]taurocholate in 10 mM Tris/Hepes (pH 7.4)/100 mM NaCl/100 mM mannitol in the absence and in the presence of increasing concentrations of the bile acid reabsorption inhibitors shown in Figure 4. The IC_x values were calculated after the subtraction of uptake rates in the absence of [Na^+]. (IC_x is that concentration of inhibitor leading to an x% inhibition of Na^+-dependent [^{3}H]taurocholate uptake.)

Compound	IC_{25}	IC_{50}	IC_{75}	IC_{100}
1	15	33	58	102
2	14	72	> 250	> 500
3	13	28	58	170
4	32	103	> 250	> 500

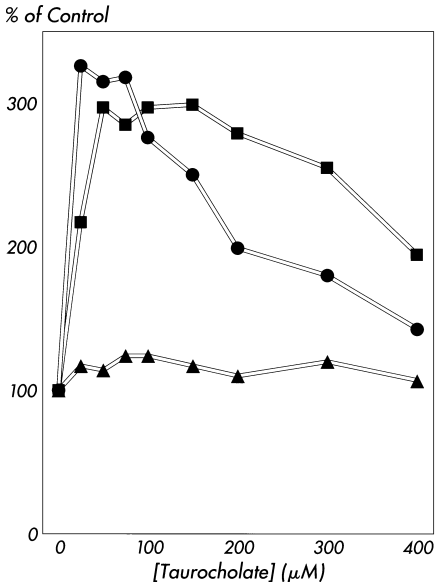


Figure 5 Differential photoaffinity labelling of rabbit ileal brush border membrane vesicles (●), rabbit ileal cytosol (▲) and recombinant rabbit ILBP (■) in the presence of taurocholate

Rabbit ileal brush border membrane vesicles or cytosol (150 μg of protein) or 25 μg of recombinant rabbit ILBP were incubated at 20 °C for 5 min with 0.42 μM (0.5 μCi) (cytosol and recombinant ILBP) or 0.84 μM (1 μCi) (brush border membrane vesicles) (3,3-azo-7α,12α-dihydroxy-5β[7β,12β-^{3}H]cholan-24-oyl)-2-aminoethane-sulphonic acid in the absence or presence of the indicated concentrations of taurocholate. After irradiation at 350 nm for 10 min, proteins were separated by SDS/PAGE followed by fluorography and densitometry of the 14 kDa-band.

ileal brush border membrane vesicles showed a 3–3.5-fold stimulation in the extent of photoaffinity labelling by taurocholate in the concentration range 25–100 μM (Figure 5). Next we investigated the effect of other bile acid derivatives on photoaffinity labelling of the ILBP in ileal cytosol. Figure 6 shows the marked effects of some natural bile acids. Whereas chenodeoxycholate and its taurine conjugate showed only an up to 2-fold stimulation, with a maximum between 25 and 50 μM, ursodeoxycholate and cholate strongly induced labelling of the ILBP, as with ileal brush border membrane vesicles. With ursodeoxycholate a 4–6-fold stimulation occurred with a maximum between 100 and 300 μM, whereas cholate produced a 7–10-fold increase with a maximum beyond 400 μM.

A crucial experiment for demonstrating the specificity of photoaffinity labelling is that performed in the presence of increasing concentrations of the non-radiolabelled photoprobe and substrate analogues that compete with the photoprobe for identical binding sites. It is generally accepted that the specific labelling of a particular protein should be inhibited in a concentration-dependent manner by increasing concentrations of unlabelled substrate and substrate analogues [36,37]. The paradoxical behaviour of the 14 kDa ILBP during photoaffinity labelling of rabbit ileal brush border membrane vesicles in the presence of increasing concentrations of unlabelled bile acids can therefore not be explained by the existence of only one ligand-binding site; either further binding sites or co-operative interactions of the ILBP in homo-oligomeric or hetero-oligomeric protein complexes have to be considered. With the assumption that ILBP has, as the other members of the fatty acid-binding protein superfamily, only one ligand-binding site, the interactions

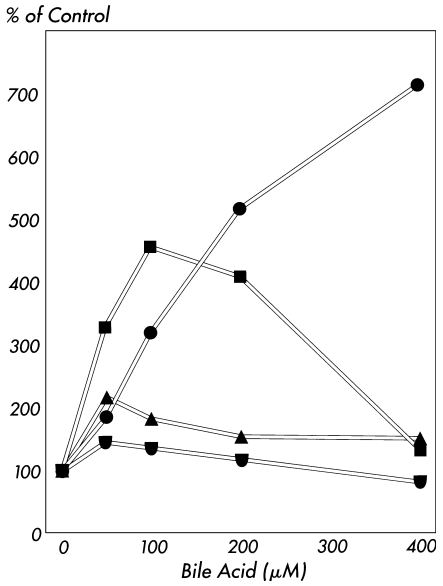


Figure 6 Differential photoaffinity labelling of rabbit ileal cytosol in the presence of cholate (●), ursodeoxycholate (■), chenodeoxycholate (▲) and taurochenodeoxycholate (▼)

Rabbit ileal cytosol (150 μg of protein) was incubated at 20 °C for 5 min with 0.12 μM (1 μCi) (7,7-azo-3α,12α-dihydroxy-5β[3β-^{3}H]cholan-24-oyl)-2-aminoethanesulphonic acid in the absence or presence of the indicated concentrations of bile acids. After irradiation at 350 nm for 10 min, proteins were separated by SDS/PAGE and the incorporation of the photoprobe into the 14 kDa ILBP was measured by densitometry of the fluorograms.

of the photoprobe L and the competing inhibitor I with ILBP can be described as follows:

$$P + L \rightleftharpoons P \cdot L \quad (1)$$

with $K_D = [P][L]/[P \cdot L]$,

$$P + I \rightleftharpoons P \cdot I \quad (2)$$

with $K_i = [P][I]/[P \cdot I]$,

$$[L]_t = [L] + [P \cdot L] \quad (3)$$

where $[L]_t$ is the total ligand concentration,

$$[I]_t = [I] + [P \cdot I] \quad (4)$$

where $[I]_t$ is the total inhibitor concentration, and

$$[P]_t = [P] + [P \cdot L] + [P \cdot I] \quad (5)$$

where $[P]_t$ is the total binding protein concentration.

The intensity of the photolabelled 14 kDa ILBP band corresponds to that fraction of P·L that is transformed to a covalent product P-L on cross-linking with light. With the assumption of an identical cross-linking rate of L in the P·L complex under otherwise identical experimental conditions such as preincubation time, temperature, irradiation time and wavelength, the amount of P-L measured as radioactively labelled 14 kDa protein is proportional to [P·L]. From the assumptions in eqns. (1–5), [P·L] is derived as follows:

$$[P \cdot L] = \frac{1}{2}\left([L]_t + [P]_t + K_D\left(1 + \frac{[I]_t}{K_i}\right) \pm \sqrt{[L]_t + [P]_t + K_D\left(1 + \frac{[I]_t}{K_i}\right)^2 - 4[P]_t[L]_t}\right) \quad (6)$$

According to the above relationship, [P·L] always decreases with increasing $[I]_t$, never exhibiting a peak maximum at a certain $[I]_t$ as we have shown in model calculations by varying $[L]_t$, K_D, K_i and $[I]_t$ over a wide range. The specific radioactivity is decreased by the addition of unlabelled photolabel to the radioactively labelled photoprobe. It could therefore be argued that the experimentally observed curves with maxima are the superimposition of two effects: a decrease caused by the decreased specific radioactivity of the photoprobe and an increase caused by the increase in [P·L] on the addition of unlabelled photoprobe. Even if these changes in the specific radioactivity of the photoprobe are considered, the model calculations never showed a maximum for [P·L] but resulted in saturation curves. The increase in the extent of photolabelling of the 14 kDa ILBP by photolabile bile acid derivatives in the presence of increasing concentrations of bile acids can therefore not be explained by the assumption of only one bile acid-binding site on the ILBP protein. The existence of a second binding site for bile acids on the ILBP protein, or the co-operative interaction of several ILBP molecules or of ILBP with further proteins, could account for the observed paradoxical behaviour of ILBP in differential photoaffinity labelling experiments.

DISCUSSION

The enterohepatic circulation of bile acids is a very efficient biological recycling system. In healthy human beings the bile acid pool of 1.5–4 g is cycled 6–15 times a day; overall 17–40 g of bile acids are reabsorbed in the terminal ileum each day. Only 250–500 mg of bile acids are lost daily with faeces, indicating an absorption efficiency of 97–99 % per cycle [1] during passage of the intestinal content along the terminal ileum. In animal species with a gall bladder, the majority of bile acids is stored in the gall bladder during fasting. After a meal the gall bladder content is secreted into the duodenum and the bile acids can exert their physiological function as biological detergents and lipase cofactors for the digestion and absorption of lipids and lipid-soluble vitamins. The terminal ileum is thus exposed to intestinal contents of various bile acid concentrations ranging from nearly no bile acids to the majority of the bile acid pool with concentrations up to 10 mM. A rapid adaptation of the transport system to these changing bile acid loads is therefore necessary to achieve an efficiency for bile acid reabsorption of more than 97 % per cycle. Transport processes in the small intestine can be influenced by the substrate load, by either an increase (positive feedback) or a decrease (negative feedback) in transport activity. Whereas dietary sugars or amino acids regulate their transport across the enterocyte by positive feedback mechanisms [18,19], controversial results for ileal bile acid reabsorption have been reported. In the rat and the guinea pig a negative feedback inhibition of ileal bile acid transport after feeding of taurocholate has been described [22], whereas other studies in the rat, with fasting, biliary diversion and extrahepatic cholestasis, led to the conclusion of a positive feedback mechanism for ileal bile acid reabsorption [23–25].

Our findings with the paradoxical behaviour of the 14 kDa ILBP protein on photoaffinity labelling in the presence of bile acids would be in accordance with positive feedback regulation of ileal bile acid reabsorption. The ileal bile acid transport protein uses an inwardly directed Na^+ gradient to transport bile acids across the brush border membrane into the ileocyte cytosol. ILBP is the only physiologically involved ileal cytosolic bile acid-binding protein, as we have shown by photoaffinity labelling of intact ileal tissue [33]. A bile acid-stimulated increase in the affinity/capacity of the ILBP for bile acids as observed in the

differential photoaffinity labelling experiments further increases the driving force for uptake. Such a modulation of the activity of the ileal bile acid reabsorption system by an intracellular increase of bile acid-binding affinity/capacity by ILBP would ensure a nearly quantitative extraction of bile acids from the intestinal contents and would also allow a maximal adaptation of transport activity to changing substrate loads. Our results show that such an adaptation can be achieved by the direct action of the bile acids on the protein components of the bile acid transport system. It remains to be clarified in further studies whether other mechanisms at transcriptional and post-translational levels are also involved. Preliminary experiments with cell lines co-transfected with the ileal bile acid transporter and ILBP genes support the above hypothesis, and further experiments involving the elucidation of the bile acid-binding site of the ILBP protein and knock-out approaches are necessary to obtain conclusive answers.

We thank Mrs. Susanne Winkler for excellent secretarial assistance and Dr. Dietmar Schwab for his valuable discussions.

REFERENCES

1 Carey, M. C. and Duane, W. C. (1994) in The Liver: Biology and Pathobiology (Arias, J. M., Boyer, J. L., Fausto, N., Jakoby, W. B., Schachter, D. and Shafritz, D. A., eds.), pp. 719–767, Raven Press, New York
2 Vlahcevic, Z. R., Heuman, D. M. and Hylemon, P. B. (1990) in Hepatology (Zakim, D. and Boyer, T. D., eds.), pp. 341–377, W. B. Saunders, Philadelphia
3 Einarrson, K. and Angelin, B. (1991) Curr. Opin. Lipidol. **2**, 190–196
4 Lack, L. (1979) Environ. Health Perspect. **53**, 79–90
5 Kramer, W., Burckhardt, G., Wilson, F. A. and Kurz, G. (1982) J. Biol. Chem. **258**, 3632–3627
6 Kramer, W., Nicol, S.-B., Girbig, F., Gutjahr, U., Kowalewski, S. and Fasold, H. (1992) Biochim. Biophys. Acta **1111**, 93–102
7 Kramer, W., Girbig, F., Gutjahr, W., Kowalewski, S., Jouvenal, K., Müller, G., Tripier, D. and Wess, G. (1993) J. Biol. Chem. **268**, 18035–18046
8 Kramer, W., Girbig, F., Gutjahr, U. and Kowalewski, S. (1995) Biochem. J. **306**, 241–246
9 Kramer, W., Wess, G., Bewersdorf, U., Corsiero, D., Girbig, F., Weyland, C., Stengelin, S., Enhsen, A., Bock, K., Kleine, H. et al. (1997) Eur. J. Biochem. **249**, 456–464
10 Gong, Y.-Z., Everett, E. T., Schwartz, D. A., Norris, J. S. and Wilson, F. A. (1994) Proc. Natl. Acad. Sci. U.S.A. **91**, 4741–4745

Received 18 December 1997/18 March 1998; accepted 8 April 1998

11 Stengelin, S., Apel, S., Becker, W., Maier, M., Rosenberger, J., Bewersdorf, U., Girbig, F., Weyland, C., Wess, G. and Kramer, W. (1996) Eur. J. Biochem. **239**, 887–896
12 Oelkers, P. and Dawson, P. A. (1995) Biochim. Biophys. Acta **1257**, 199–202
13 Lin, M. C., Kramer, W. and Wilson, F. A. (1990) J. Biol. Chem. **265**, 14986–14995
14 Wong, M. H., Oelkers, P., Craddock, A. L. and Dawson, P. A. (1994) J. Biol. Chem. **269**, 1340–1347
15 Wong, M. H., Oelkers, P. and Dawson, P. A. (1995) J. Biol. Chem. **270**, 27228–27234
16 Shneider, B. L., Dawson, P. A., Christie, D.-M., Hardikar, W., Wong, M. H. and Suchy, F. J. (1995) J. Clin. Invest. **95**, 745–754
17 Reference deleted
18 Solberg, D. H. and Diamond, J. M. (1987) Am J. Physiol. **252**, G574–G584
19 Karasov, W. H., Solberg, D. H. and Diamond, J. M. (1987) Am. J. Physiol. **252**, G614–G625
20 Nellans, H. N. and Kimberg, D. V. (1978) Am J. Physiol. **235**, E726–E737
21 Wheby, M. S., Jones, L. G. and Crosby, W. H. (1964) J. Clin. Invest. **43**, 1433–1442
22 Lillienau, J., Crombie, D. L., Munoz, J., Longmire-Cook, S. J., Hagey, L. R. and Hofmann, A. F. (1993) Gastroenterology **104**, 38–46
23 Higgins, J. V., Paul, J. M., Dumaswala, R. and Heubi, J. E. (1994) Am. J. Physiol. **267**, G501–G507
24 Dumaswala, R., Berkowitz, P. and Heubi, J. E. (1996) Hepatology **23**, 623–629
25 Sauer, P., Fitscher, B. A., Kloeters-Plachky, P., Stremmel, W., Stengelin, S., Kramer, W. and Stiehl, A. (1997) in Bile Acids in Hepatobiliary Diseases. Basic Research and Clinical Application (Paumgartner, G., Stiehl, A. and Gerok, W., eds.), pp. 188–190, Kluwer, Dordrecht
26 Kramer, W. and Kurz, G. (1983) J. Lipid Res. **24**, 910–923
27 Kramer, W. and Schneider, S. (1989) J. Lipid Res. **30**, 1281–1288
28 Kramer, W. (1981) Dissertation, Universität Freiburg
29 Bradford, M. M. (1976) Anal. Biochem. **72**, 248–254
30 Wessel, D. and Flügge, U. J. (1984) Anal. Biochem. **138**, 141–143
31 Kramer, W., Girbig, F., Gutjahr, U. and Leipe, I. (1990) J. Chromatogr. **521**, 199–210
32 Bonner, W. M. and Laskey, R. A. (1974) Eur. J. Biochem. **46**, 83–88
33 Kramer, W., Stengelin, S. and Wess, G. (1997) in Bile Acids in Hepatobiliary Diseases. Basic Research and Clinical Application (Paumgartner, G., Stiehl, A. and Gerok, W., eds.), pp. 161–178, Kluwer, Dordrecht
34 Wess, G., Kramer, W., Enhsen, A., Glombik, H., Baringhaus, K.-H., Böger, G., Urmann, M., Bock, K., Kleine, H., Neckermann, G. et al. (1994) J. Med. Chem. **37**, 873–875
35 Kramer, W., Wess, G., Baringhaus, K.-H., Böger, G., Enhsen, A., Falk, E., Friedrich, M., Glombik, H., Hoffmann, A., Neckermann, G. et al. (1995) in Bile Acids in Gastroenterology. Basic and Clinical Advances (Hofmann, A. F., Paumgartner, G. and Stiehl, A., eds.), pp. 205–220, Kluwer, Dordrecht
36 Bayley, H. and Knowles, J. R. (1977) Methods Enzymol. **46**, 69–114
37 Chowdhry, V. and Westheimer, F. H. (1979) Annu. Rev. Biochem. **48**, 293–325