Radiation-inactivation analysis of the Na^+/bile acid co-transport system from rabbit ileum

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The functional-unit molecular size of the Na^+/bile acid co-transport system and the apparent target size of the bile-acid-binding proteins in brush-border membrane vesicles from rabbit ileum were determined by radiation inactivation with high-energy electrons. The size of the functional transporting unit for Na^+-dependent taurocholate uptake was determined to 451 ± 35 kDa, whereas an apparent molecular mass of 434 ± 39 kDa was measured for the Na^+-dependent D-glucose transport system. Proteins of 93 kDa and 14 kDa were identified as putative protein components of the ileal Na^+/bile acid co-transporter in the rabbit ileum, whereas a protein of 87 kDa may be involved in passive intestinal bile acid uptake. Photoaffinity labelling with 3- and 7-azi-derivatives of taurocholate revealed a target size of 229 ± 10 kDa for the 93 kDa protein, and 132 ± 23 kDa for the 14 kDa protein. These findings indicate that the ileal Na^+/bile acid co-transport system is in its functional state a protein complex composed of several subunits. The functional molecular sizes for Na^+-dependent transport activity and the bile-acid-binding proteins suggest that the Na^+/bile acid co-transporter from rabbit ileum is a homotetramer $(AB)_4$ composed of four AB subunits, where A represents the integral 93 kDa and B the peripheral 14 kDa brush-border membrane protein.

INTRODUCTION

Bile acids synthesized from cholesterol in the liver are secreted into bile to aid in the digestion and absorption of fats and fat-soluble vitamins in the small intestine. The bulk of bile acids is absorbed in the terminal ileum by an active Na^+-dependent transport system, recirculated back to the liver with portal blood and secreted again into bile [1–4]. This enterohepatic circulation of bile acids with a strict organotropism for the small intestine and the liver under normal physiological conditions is established by specific Na^+-dependent bile acid transport systems in the brush-border membrane of ileocytes [1,2,5–7] and the sinusoidal membrane of hepatocytes [8,9]. The putative protein components of the bile acid carrier systems in the small intestine [6,7,10–14], the liver [15–21], blood [22] and the kidney [23] have been identified with photolabile azi- and azido-derivatives of conjugated and unconjugated bile acids [7,24–26]. The anatomical localization of the Na^+/bile acid co-transport system along the duodenum–ileum axis in the rabbit correlates with the co-expression of an integral 93 kDa bile-acid-binding membrane protein and a peripheral membrane-associated 14 kDa protein [7,27]. However, information about functional molecular size and structure of the native co-transporter is lacking. Radiation-inactivation analysis with high-energy electron radiation is a method permitting the measurement of the size of functional units *in situ* without the need for isolating the protein components of the transporter, which might alter its subunit assembly or functional properties [28–30]. This methodology has been successfully applied to various transport systems such as the Na^+/D-glucose co-transporter [31–35], the multispecific hepatic bile acid transporters [36–38], the Na^+/phosphate transporter from kidney [34], or the *p*-aminohippurate transporter in the basolateral membrane of renal proximal tubules [39]. In the present study we have applied this technology to determine the functional-unit molecular size and subunit composition of the Na^+-dependent taurocholate transport system in rabbit ileal brush-border membrane vesicles.

EXPERIMENTAL

Materials

Photoaffinity labelling was carried out with the sodium salts of (7,7-azo-3α,12α-dihydroxy-5β[3β-^{3}H]cholan-24-oyl)-2-aminoethanesulphonic acid (sp. radioactivity 20.25 Ci/mmol) or (3,3-azo-7α,12α-dihydroxy-5β[7β,12β-^{3}H]cholan-24-oyl)-2-aminoethanesulphonic acid (sp. radioactivity 5.9 Ci/mmol) synthesized as described previously [24–26]. [G-^{3}H]Taurocholic acid (sp. radioactivity 2.1 Ci/mmol) and D-[U-^{14}C]glucose (sp. radioactivity 285.5 Ci/mmol) were from Du Pont–New England Nuclear (NEN Division, Dreieich, Germany). Acrylamide and *NN*-methylenebisacrylamide were from Serva (Heidelberg, Germany), and the marker proteins for determination of molecular masses were from Sigma (München, Germany). Cellulose nitrate filters for transport studies (ME 25; 0.45 μm pore size; 25 mm diameter) were from Schleicher & Schuell (Dassel, Germany), and scintillators Quickszint 501, 361 and Unisolve I were from Zinsser (Zinsser Analytic G.m.b.H, Frankfurt, Germany). The kits (Merckotest) for determination of the enzymic activities of aminopeptidase N and γ-glutamyltransferase were from Merck (Darmstadt, Germany). Protein was determined by the method of Bradford [40] with the Bio-Rad kit (München, Germany). The dosimeter and radiation-dose-sensitive films were from Far West Technology (Goleta, CA, U.S.A.).

Preparation of ileal brush-border membrane vesicles

Brush-border membrane vesicles from the ileum of male New Zealand White rabbits (weighing 4–5 kg) were prepared by the Mg^{2+} precipitation method [41] as described previously [5,7,42–44]. The entire small intestine was removed, rinsed with 0.9% NaCl and divided into ten segments of equal length, numbered 1–10, proximal to distal. Segments 8–10 were used for the preparation of ileal vesicles. The final pellet was resuspended in cryoprotectant buffer (10 mM Tris/Hepes, pH 7.4, 300 mM

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mannitol, 10 mM mercaptoethanol) by using a 27-gauge needle at a protein concentration of 5 mg/ml. The ileal brush-border membrane vesicles were divided into batches under a stream of N_2 in 2 ml Nunc cryotubes (Kühn & Bayer, Nidderau, Germany) and immediately stored in liquid N_2 at −196 °C. During transport to the radiation laboratories, the radiation treatment and afterwards until analysis, the temperature was kept below −80 °C. The enrichment of brush-border membrane vesicles in the final preparation was assessed by enzymic and immunological methods as described previously [7]. Vesicles were enriched 19±4-fold in aminopeptidase N (EC 3.4.11.2) and 14.5±4-fold in γ-glutamyltransferase (EC 2.3.2.2), whereas basolateral membranes, endoplasmic reticulum, mitochondria, lysosomes and cytosol were not enriched [7]. Sucrase activity was determined as described by Dahlqvist [45]. The intactness of the vesicles was determined by measuring Na^+-dependent D-glucose uptake after 15 s of incubation, usually showing an overshoot uptake greater than 20-fold at 15 s.

Radiation inactivation

Irradiation was performed with high-energy electrons from a 10 MeV linear accelerator at the Strahlenzentrum of the Justus-Liebig-Universität, Giessen, Germany. The cryotubes with the ileal brush-border membrane vesicles were placed in the irradiation chamber in front of the linear accelerator. Chamber and samples were cooled with a stream of liquid N_2 flowing through the chamber. The temperature was kept constant in the range of −80 to 130 °C, and the actual temperature was measured with an electrode placed within the chamber. The dosimeter and the radiation-sensitive films were placed on each side of the samples in the irradiation chamber during irradiation. After irradiation and die-down of induced radioactivity, the samples were stored in liquid N_2 and transported back to the Hoechst laboratories. The following day, the irradiated frozen samples were opened and thawed at 37 °C. The vesicles were resuspended by passage through a 27-gauge syringe needle and subsequently placed on ice. Samples of these vesicles were then analysed for Na^+-dependent [^{3}H]taurocholate uptake, Na^+-dependent D-[^{14}C]glucose uptake, enzymic activity of aminopeptidase N and sucrase, as well as photoaffinity labelling of bile-acid-binding proteins as described below. The functional molecular masses were calculated from the empirical equation [34]:

$$\log M_r = 5.89 - \log D_{37,T} - 0.0028T$$

where D_{37} is the radiation dosage in Mrad generating 37% of residual biological activity compared with controls and T the temperature (in °C). In total 10 different irradiation experiments were performed, using 10 different membrane vesicle preparations.

Transport measurements

Uptake of radiolabelled substrates by brush-border membrane vesicles was determined by the membrane-filtration method [41] as described previously [6,7,42–44]. Typically, the transport reaction was initiated by adding 20 μl of the vesicle suspension (100 μg of protein) to 80 μl of incubation medium containing the radioactivity labelled substrate kept at 30 °C. The composition of the incubation medium for measurements in the presence of a 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 taurocholate uptake, these media contained 50 μM (0.75 μCi) [^{3}H]taurocholate and for glucose uptake 19 μM (1 μCi) D-[U-^{14}C]glucose. The transport reaction was terminated after 30 s for [^{3}H]taurocholate uptake, and after 15 s for D-[^{14}C]glucose uptake, by addition of 1 ml of ice-cold stop solution [10 mM Tris/Hepes (pH 7.4)/150 mM KCl]. The entire contents were pipetted on to the middle of a pre-washed pre-chilled filter kept under suction with the aid of a vacuum controller. The filter was rinsed immediately with 5 ml of ice-cold stop solution and then solubilized in scintillator Quickszint 361. The radioactivity remaining on the filter was counted by standard liquid-scintillation techniques. After correction for radioactivity from the medium bound to the filter in the absence of membrane vesicles and eventual chemilumininescence, absolute solute uptake was calculated and expressed as nmol/mg of protein. All experiments were performed in triplicate, and uptake values are given as means±S.D.

Photoaffinity labelling

Photoaffinity labelling with photoreactive bile acids was performed as described previously [6,7,42–44]. Typically, 30 μl of brush-border membrane vesicles (150 μg of protein) were added in the dark to 170 μl of 10 mM Tris/Hepes buffer (pH 7.4)/100 mM NaCl/100 mM mannitol containing the radiolabelled photoreactive 3,3- or 7,7-azo-derivatives of taurocholic acid. After 5 min of preincubation, the suspensions were irradiated for 10 min at 350 nm in a Rayonet Photochemical Reactor RPR-100 (The Southern Ultraviolet Co., Hamden, CT, U.S.A.) equipped with 16 RPR 3500 Å lamps. Afterwards, the suspensions were diluted with 1 ml of ice-cold buffer [10 mM Tris/Hepes (pH 7.4)/300 mM mannitol] and centrifuged for 30 min at 48000 *g*. The supernatants were carefully removed, and, after resuspension of the membranes in 100 μl of water, proteins were precipitated [46].

SDS/PAGE

SDS/PAGE was carried out in vertical slab gels (20 cm × 17 cm × 0.15 cm) in an electrophoresis system LE 2/4 (Pharmacia LKB Biotechnology, Freiburg, Germany) as described previously [7,10,47]. After staining with Serva Blue R 250, the gels were scanned with a densitometer CD 50 (DESAGA, Heidelberg, Germany), and radioactivity was determined either by slicing of the gels into 2 mm pieces and digestion of protein with Biolute S or by fluorography [48] as described previously [7,47].

RESULTS

Functional molecular mass of the Na^+-dependent [^{3}H]taurocholate and [^{14}C]glucose uptake systems

In order to establish the validity of the radiation-inactivation method for the Na^+/bile acid co-transport system from rabbit ileum, control experiments for the determination of the molecular masses of brush-border marker enzymes were performed. Aminopeptidase N (EC 3.4.11.2) is a typical brush-border enzyme of apparent molecular mass 105–120 kDa [49–52], and sucrase (EC 3.2.1.48) is a brush-border ectoenzyme of 122 kDa [52,53]. The molecular masses of these enzymes, measured when using intestinal brush-border membranes in the frozen state, were determined as 59 kDa for aminopeptidase N and 63 kDa for sucrase [52]. These values clearly underestimate the real molecular size of the polypeptide chains of the respective enzymes. Radiation analysis of kidney microvillar peptidases of freeze-dried proteins, however, revealed target-size molecular masses very similar to the accurate molecular masses of the purified enzymes [54]. In our hands, the molecular mass of aminopeptidase N was determined as 101±29 kDa and that of sucrase as

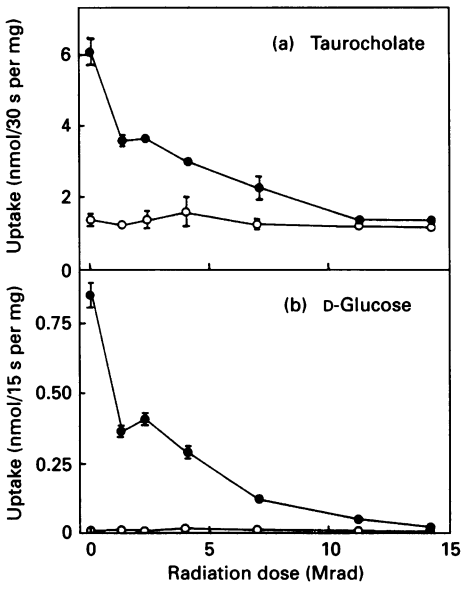

Figure 1 Dose-dependent radiation inactivation of Na^+/bile acid co-transport (a) and Na^+/D-glucose co-transport (b)

Ileal brush-border membrane vesicles were irradiated with the indicated doses of high-energy (10 MeV) electrons. Subsequently, 20 μl of membrane vesicles (100 μg of protein) equilibrated with cryoprotectant buffer was mixed at 30 °C with 80 μl of 10 mM Tris/Hepes buffer (pH 7.4)/100 mM mannitol, with 100 mM NaCl (●) or 100 mM KCl (○), containing either 50 μM (0.75 μCi) [^{3}H]taurocholate (**a**) or 19 μM (1 μCi) D-[U-^{14}C]glucose (**b**). Uptake was stopped after 30 s (taurocholate) or 15 s (D-glucose).

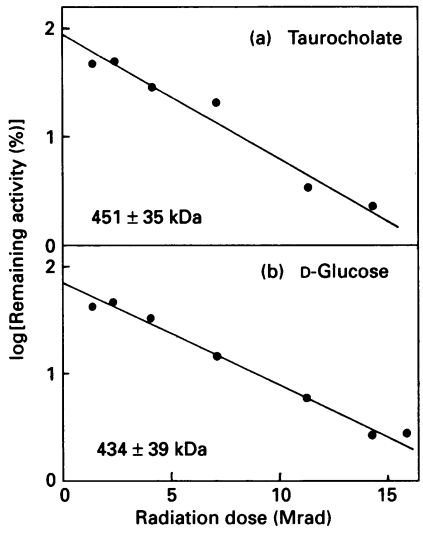

Figure 2 Determination of the functional molecular target size of the Na^+/bile acid co-transport system (a) and the Na^+/D-glucose co-transporter (b)

After irradiation of ileal brush-border membrane vesicles with the indicated doses of high-energy electrons, uptake of [^{3}H]taurocholate and D-[U-^{14}C]glucose was measured as described in Figure 1. The logarithm of residual Na^+-dependent transport activity [100 × (uptake after irradiation with dose x/control uptake) %] was plotted against the radiation dose.

105 ± 15 kDa, values very close to the actual molecular masses. These experiments revealed that our experimental approach should allow the determination of the accurate functional molecular mass of the Na^+/bile acid co-transport system. Initial irradiation experiments revealed a biphasic inactivation of D-glucose and taurocholate transport, with a very strong inactivation at low dosages, indicating very high molecular masses. Susceptibility of thiol groups in proteins to attack by radiolytic species, generated during the radiation procedure, may lead to an over-estimation of the actual molecular mass, as was shown for the molecular-mass determination of alcohol dehydrogenase in the absence and presence of thiol reagents [55]. After addition of scavenging agents such as dithiothreitol or 2-mercaptoethanol to the buffers, no such non-specific inactivation of Na^+-dependent glucose transport occurred. These findings are in accordance with the susceptibility of both the Na^+/D-glucose transporter and the Na^+/bile acid transporter to thiol-modifying or oxidizing agents [6,56]. After irradiation of ileal brush-border membrane vesicles with high-energy electrons in the dosage range of 0–25 Mrad, the uptake of [^{3}H]taurocholate and D-[^{14}C]glucose into the membrane vesicles was measured in both the absence and the presence of an inwardly directed Na^+ gradient ($[Na^+]_{out}$ = 100 mM, $[Na^+]_{in}$ = 0 mM). In all radiation-inactivation experiments, D-glucose measurements were performed as an internal standard to obtain information about the functional size of the ileal Na^+/bile acid co-transporter in direct comparison with the Na^+/D-glucose co-transporter. This is particularly important because the functional molecular mass of the D-glucose transporter determined in several investigations varied greatly, ranging from 110 to 1000 kDa [31–35,57].

Figure 1(a) shows that initial Na^+-dependent taurocholate uptake activity (30 s uptake) decreased with increasing radiation dosage asymptotically to the value of Na^+-independent taurocholate uptake equivalent to 15–20% uptake of control in the presence of Na^+. Figure 1(b) shows the dose-dependent inactivation of Na^+/D-glucose transport of the same vesicle preparation approaching uptake in the absence of Na^+ equivalent to less than 2% of uptake in the presence of a Na^+ gradient. It is evident that both transporters show a similar dose-dependence of radiation inactivation, indicating comparable functional molecular masses of the respective transporter complexes. The Na^+-independent uptake of both taurocholate and D-glucose was not affected by the radiation dose, demonstrating that the vesicles were not leaky and were not damaged by the irradiation procedure. This was also confirmed by equilibrium uptake measurements. Equilibrium uptake at 60 min and 30 °C remained constant and showed no dose-dependence on irradiation, indicating that the permeability of the membrane towards taurocholate or D-glucose was unaffected (results not shown). Furthermore, electron-microscopic investigations with kidney brush-border membrane vesicles [34] or rat liver basolateral membranes [36] revealed no alteration of the structural integrity of the vesicles upon irradiation. For the determination of the molecular mass of the Na^+-dependent transport systems for taurocholate and D-glucose, the logarithm of residual Na^+-dependent transport activity (uptake at dosage x/uptake of controls) was plotted against radiation dose, whereby the Na^+-dependent transport activity was uptake in the presence of a Na^+ gradient minus uptake in the absence of a Na^+ gradient. Figure 2(a) shows the determination of the molecular size of the Na^+/bile acid co-transport system, and Figure 2(b) that of the Na^+/D-glucose transporter, using the same vesicle preparation for both Na^+/bile acid and Na^+/D-glucose transport measurements. From seven independent experiments an average target size of 451 ± 35 kDa was calculated for the Na^+-dependent bile acid transport system, and 434 ± 39 kDa for the Na^+-dependent D-glucose transport

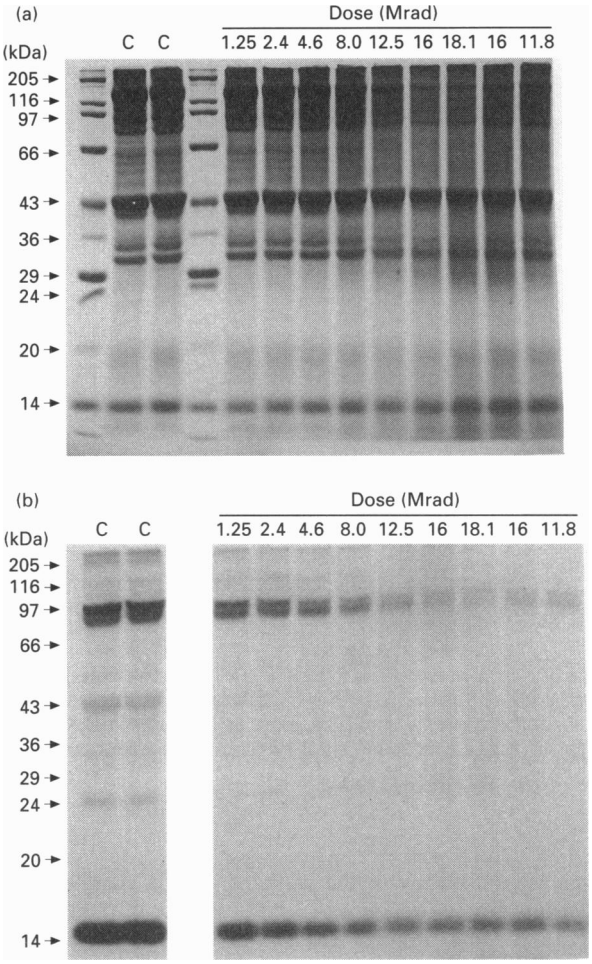

Figure 3 Effect of radiation inactivation on photoaffinity labelling of rabbit ileal brush-border membrane bile-acid-binding proteins by (7,7-azo-3α,12α-dihydroxy-5β[3β-^{3}H]cholan-24-oyl)-2-aminoethanesulphonic acid

Ileal brush-border membrane vesicles equilibrated with cryoprotectant buffer were irradiated with the indicated doses of high-energy electrons. Subsequently, 150 μg of membrane protein (30 μl) was mixed for 5 min in the dark at 30 °C with 170 μl of 10 mM Tris/Hepes buffer (pH 7.4)/100 mM mannitol/100 mM NaCl containing 0.49 μM (2 μCi) (7,7-azo-3α,12α-dihydroxy-5β[3β-^{3}H]-cholan-24-oyl)-2-aminoethanesulphonic acid and then photolabelled by irradiation at 350 nm for 10 min. After washing, membrane proteins were separated by SDS/PAGE on 12% gels. Proteins were fixed and stained with Serva Blue R 250 (**a**), and radioactivity was detected by fluorography of the dried gels for 8 weeks at −80 °C (**b**). The numbers at the top of each gel indicate the radiation dose in Mrad; C indicates controls not exposed to radiation inactivation. The 'kDa' values at the left indicate positions of standard marker proteins.

system. In every experiment performed, the Na^+/bile acid transporter showed a slightly higher molecular mass than the D-glucose transporter under conditions where the molecular masses of aminopeptidase N and sucrase were found to be very close to the actual molecular masses of the respective isolated proteins.

Functional molecular masses of the bile-acid-binding proteins of rabbit ileal brush-border membrane vesicles by photoaffinity labelling

To determine the functional molecular masses of the bile-acid-binding proteins of 93, 87 and 14 kDa, photoaffinity labelling using 3- and 7-azi-derivatives of taurocholate was performed with the radiation-inactivated brush-border membrane vesicles. Figure 3(a) shows that increasing radiation dose led to an increase in protein degradation. Proteins with molecular masses greater than 67 kDa were progressively destroyed with increasing radiation dose, whereas no significant loss of proteins with molecular masses less than 30 kDa could be detected. The corresponding fluorogram shows that the labelling of the 93 kDa protein strongly decreased with increasing radiation dose (Figure 3b); the labelling of the 87 kDa protein did not show such a strong dose-dependence. The labelling of the 14 kDa protein also decreased strongly with increasing radiation dose. Plotting the logarithm of residual labelling (% of labelling compared with control) of the respective bile-acid-binding proteins versus the radiation dose revealed that the labelling of the 93 kDa and 14 kDa proteins occurs biphasically, whereas labelling of the 87 kDa protein showed a monoexponential behaviour resulting in an apparent functional molecular mass of 114±35 kDa from three independent experiments (Figure 4). At low doses up to about 5 Mrad a very steep decrease in labelling of the 93 kDa and 14 kDa proteins occurred, followed by a much slighter decrease at radiation doses above 5 Mrad. A rough estimation of the molecular mass during the steep initial decrease yielded a molecular mass of 500–600 kDa for the 93 kDa and also for the 14 kDa protein. In the linear range at higher doses a functional molecular mass of 229±10 kDa for the 93 kDa protein was calculated, and 132±23 kDa for the 14 kDa protein.

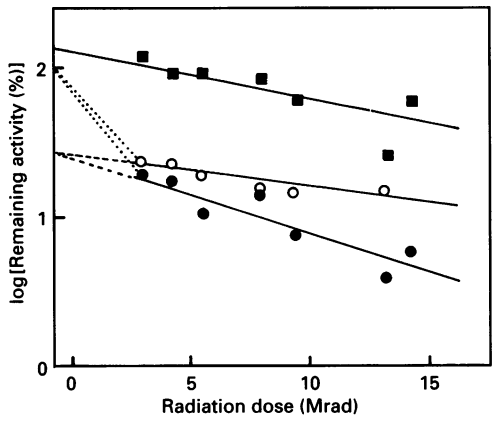

Figure 4 Determination of the functional molecular target sizes of bile-acid-binding proteins in rabbit ileal brush-border membrane vesicles after photoaffinity labelling with (7,7-azo-3α,12α-dihydroxy-5β[3β-^{3}H]cholan-24-oyl)-2-aminoethanesulphonic acid

Ileal brush-border membrane vesicles equilibrated with cryoprotectant buffer were irradiated with the indicated doses of high-energy electrons. After photoaffinity labelling with 0.49 μM (2 μCi) (7,7-azo-3α,12α-dihydroxy-5β[3β-^{3}H]cholan-24-oyl)-2-aminoethanesulphonic acid as described in Figure 3 and fluorography of the dried gels for 4 weeks, the fluorograms were scanned with a CD 50 densitometer. The logarithm of residual labelling [100 × (intensity of labelling after irradiation with dose x/intensity of labelling of controls) %] of the bile-acid-binding proteins of 93 kDa (●), 14 kDa (○) and 87 kDa (■) was plotted against radiation dose.

DISCUSSION

With photolabile azi- and azido-derivatives of conjugated and unconjugated bile acids [24–26] a 99 kDa protein was identified in the rat ileum as a putative Na^+/bile acid co-transporter, whereas a 94 kDa protein identified in rat jejunum is thought to be a component of a passive bile-acid re-absorption system [10,11,13,58]. In rabbit small intestine, an integral 93 kDa membrane protein and a peripheral 14 kDa bile-acid-binding protein are exclusively found in the terminal ileum concomitantly

with Na^+-dependent bile-acid transport activity, whereas a 87 kDa membrane protein is also found in the jejunum [7]. In the hamster ileum recently a Na^+-dependent bile acid transporter with a molecular mass of 38 kDa, as deduced from the cDNA sequence, has been cloned and characterized [59]. The relation of this Na^+/bile acid co-transporter from hamster ileum to the 93 and 14 kDa proteins from rabbit ileum remains to be determined.

The investigation of the molecular structure of transport systems is particularly difficult, because the protein components of transporters are embedded within the lipid bilayer membrane, and therefore structural information as well as function are lost upon solubilization and purification. The interaction of transporter components with each other may change during the exercise of function. One of the most powerful approaches to investigate protein–protein interaction of membrane-bound protein complexes is target inactivation analysis, if a signal indicating the function of the complex can easily be measured. A target size of 451 ± 35 kDa was found for the Na^+-dependent bile acid transport system from rabbit ileum, whereas in the same vesicles the radiation target size of Na^+-dependent D-glucose transport was 434 ± 39 kDa. In all experiments performed, the target size of the D-glucose transporter was slightly but significantly smaller than that of the Na^+/bile acid co-transport system. The target size for the D-glucose transporter in our hands was always greater than 290 ± 50 kDa, suggesting a homotetrameric structure [35]. The identification and cloning of a further subunit of the Na^+/glucose co-transport system [60], as well as the identification of a very strong and specific association of the 73 kDa transporter subunit with a 60 kDa protein building up an immunoreactive protein band of 120 kDa on Western blots [61], may indicate that different forms of the D-glucose transporter can exist in the brush border membrane.

A target size of 451 ± 35 kDa indicates that a least four 93 kDa subunits may build up the functional bile-acid transporter complex. Photoaffinity labelling of radiation-inactivated vesicles with azi-derivatives of taurocholate revealed a biphasic inactivation of labelling of the 93 kDa and the 14 kDa protein, whereas the 87 kDa bile-acid binder showed a monoexponential inactivation, determining its molecular mass as 114 ± 35 kDa. Up to 5 Mrad a rough target size of 500–600 kDa could be estimated for the 93 kDa and 14 kDa proteins, whereas above 5 Mrad target sizes of 229 ± 10.3 kDa for the 93 kDa and 132 ± 22.8 kDa for the 14 kDa protein were calculated. These findings suggest that neither the 93 kDa nor the 14 kDa bile-acid-binding protein binds bile acids in their monomeric form. The calculated functional molecular mass of 229 ± 10.3 kDa suggests that at least two 93 kDa subunits are necessary for bile acid binding. Bile acid binding to rat liver plasma membranes also occurs to dimers of the respective binding proteins of 48 and 54 kDa [38]. A functional target size of 132 ± 22.8 kDa for the 14 kDa bile-acid-binding protein suggests either a complex of 7–10 monomers or the association of the 14 kDa subunit with a further protein of about 100 kDa. Since different experimental approaches indicate that the 14 kDa and the 93 kDa bile-acid-binding protein interact with each other [7], it is tempting to speculate that the 14 kDa protein must be associated with the 93 kDa protein for binding of bile acids. From the experimental findings, the following structural model for the Na^+/bile acid co-transporter from rabbit ileum can be hypothesized.

(1) Binding of the bile acids to the 14 kDa protein occurs after association of the 14 kDa protein to the 93 kDa protein resulting in a functional molecular mass of 132 ± 22.8 kDa for the 14 kDa protein during photoaffinity labelling (1×93 kDa $+ 1 \times 14$ kDa).

The functional transporting complex A_4B_4 (451 ± 35 kDa) is built up of A_2B_2 subunits, yielding a functional molecular mass of 229 ± 10.3 kDa for the 93 kDa protein in photoaffinity-labelling studies (2×93 kDa $+ 2 \times 14$ kDa).

Preliminary evidence for this hypothesis has been found from chemical cross-linking studies; in addition to the 93, 87 and 14 kDa bands, faint bands of 200 and 400 kDa could be detected after cross-linking of photolabelled ileal brush-border membrane vesicles with bifunctional succinimide esters. Purification and sequencing of the 93 kDa and 14 kDa bile-acid-binding proteins is under way to study the molecular mechanism of ileal bile acid transport.

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