Biochimica et Biophysica Acta, 1103 (1992) 229–232

BBAMEM 75496

Functional molecular mass of the ^{14}C-azidobenzamidotaurocholic acid binding proteins in hepatocellular bile acid transport systems

Kornelia Ziegler and Reiner Heinrich Elsner

Institute of Pharmacology and Toxicology, Justus-Liebig University, Giessen (Germany)

(Received 1 July 1991)
(Revised manuscript received 30 September 1991)

Key words: Photoaffinity labeling; Taurocholate transport; Radiation inactivation; (Rat liver)

The apparent target size of ^{14}C-azidobenzamidotaurocholate binding proteins in basolateral rat liver plasma membranes (blPm) was determined by analysis of the radiation induced decrease of the binding of this photoreactive taurocholate analog to blPm. Radiation causes a dose-dependent mono-exponential reduction of binding of ABATC to the protein subunits with molecular masses of 48–50 and 52–54 kDa in SDS-PAGE. The minimal functional molecular mass of the 48–50 and 52–54 kDa ABATC binding proteins was determined to be 99 ± 8.2 and 93.2 ± 7 kDa, respectively.

Introduction

Recently the functional molecular mass (fmM) of the Na^+-dependent taurocholate transporter was determined by radiation inactivation of the transport function in sinusoidal rat liver plasma membrane vesicles (blPmv) [1]. Using the overshooting taurocholate uptake into blPmv as criterium of transport activity, a fmM of 170 kDa was calculated [1,2]. In the study presented in this paper, the fmM of the binding proteins of a photoreactive taurocholate analog, ^{14}C-azidobenzamidotaurocholic acid (ABATC), was determined by analyzing the decrease of its binding to blPmv after irradiation of the membranes with high energy electrons. ABATC specifically binds to proteins with molecular masses of 48–50, 52–54, 60, 54, 37 and 67 kDa [3,4]. The binding to those proteins can be prevented by preincubation with an excess of conjugated as well as unconjugated bile acids. Photolabeling with ABATC is sodium-independent, whereas the transport of ABATC in the dark is sodium-dependent [4]. According to expression cloning studies in *Xenopus leavis* oocytes [5] insertion of a 48–50 kDa protein in the oocyte membrane is sufficient to catalyze sodium-dependent taurocholate uptake. The 52–54 kDa protein, which is also labeled by chemical reactive [6–9] and photoreactive [10,11,4] taurocholate and cholate analogs is probably responsible for the uptake of unconjugated bile acids such as cholate or represents the sodium-independent bile acid transporter [12,13]. Since ABATC binds to both proteins, we determined the functional molecular masses for both. The proteins with molecular masses of 43 and 67 kDa are identical with actin [14] and albumin [10]. It is suggested that they are not carrier proteins for bile acids and we therefore did not investigate them. The same is true for the 60 and 37 kDa proteins.

Materials and Methods

Materials

^{14}C-Azidobenzamidotaurocholic acid was a gift of Prof. Dr. H. Fasold, Frankfurt, FRG. All other chemicals were purchased in best quality from the usual commercial sources.

Methods

Isolation of basolateral plasma membrane vesicles from rat liver

BlPmv from rat liver were prepared according to the method of Blitzer and Donovan (15) with some modifications [1]. Plasma membranes were suspended in 10 mM Hepes-KOH (pH 7.5), 300 mM sucrose at a final

Correspondence: K. Ziegler, Institute of Pharmacology and Toxicology, Justus-Liebig University, Frankfurterstrasse 107, 63 Giessen, Germany.

oncentration of 3.2 mg/ml. No decrease in enzyme nd transport activities could be found during storage f plasma membranes in liquid nitrogen (−196 °C) for months.

Na^+/K^+-ATPase in isolated blPmv, determined according to the method of Scharschmidt et al. [16] was nriched 22–26-fold, whereas 5'-nucleotidase [16] and Mg^{2+}-ATPase [17], marker enzymes for rat liver analicular plasma membranes were increased only 2-fold. The marker enzyme for microsomal membranes, glucose-6-phosphatase [18] was enhanced 2-old.

'adiation procedure

BlPmv stored in polypropylene vessels were kept nder liquid nitrogen (−196 °C) prior to irradiation. rradiation was performed at the Strahlenzentrum in iessen as described earlier [1].

Before irradiation of the samples, the dose distribution along the sample holder was determined after hort time irradiation by ferrous sulfate dosimetry.)uring irradiation dosimetry was performed with radition dose sensitive filmes (Far West Technology Inc., :A, USA), that were placed on the right and the left osition of each sample in the sample holder.

'alculation of the functional molecular masses

The fmM (molecular mass in daltons) can be calcuated by the empirical equation of Kepner and Macey 19] with a temperature corrected factor according to Kempner and Haigler [20]. Surviving activity of one rradiation experiment was plotted as a logarithmic unction of the radiation dose. Data were analyzed vith a linear regression program (least-squares nethod). D_{37}-values were determined from four radiaion runs. Alternatively, the internal standard enzyme nethod was used as published previously [1]. The logaithm of the experimentally determined D_{37}-values vere plotted against the logarithm of known molecular nasses of four standard enzymes (Na^+/K^+-ATPase, 200 kDa [19]; alkaline phosphatase, 72 kDa [21]; β-galactosidase, 464 kDa [22]; alcohol dehydrogenase, 78 kDa [23]). Target molecular weight for any D_{37}-value could be determined by this calibration plot with maximum deviations of 3.8% from the results, obtained by the equation of Kepner and Macey.

Photoaffinity labeling

After irradiation, blPmv (500 μg/ml) resuspended in phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 0.5 mM $MgCl_2 \cdot 6\ H_2O$, 4.5 mM $NaH_2PO_4 \cdot 2\ H_2O$, 1.5 mM KH_2PO_4, pH 7.4) were preincubated for 3 min at 37 °C. In the dark, 2.7 μCi of [^{14}C]ABATC was added to irradiated and non-irradiated plasma membranes and incubated for 3 min. The suspension was then taken up into quartz glas tubes and exposed to one high energy ultraviolet light flash (capacity 8000 μF (630 V, 952 Ws)) [3]. After photolysis, plasma membrane were washed twice with 30 mM Tris, 1 mM iodoacetic acid, 1 mM benzamidohydrochloride, 1 mM PMSF, 5 mM EDTA, 0.01 mM PCMB (pH 7.4) to remove unbound label.

Photoaffinity-labeled plasma membranes were separated by SDS gel electrophoresis under reducing conditions. Protein bands were stained with Coomassie blue. Radioactive proteins were visualized by fluorography. Quantitation of radioactivity was performed by slicing SDS-polyacrylamide rod gels, and counting the radioactivity in Lipoluma/Lumasolve/water (10:1:0.2, v/v).

Results

As shown in Fig. 1, increasing the radiation dose leads to an increase of protein degradation. Especially, proteins in higher molecular weight ranges are degraded at low radiation dose in accordance with target theory. Degradation products are seen in the lower molecular weight ranges.

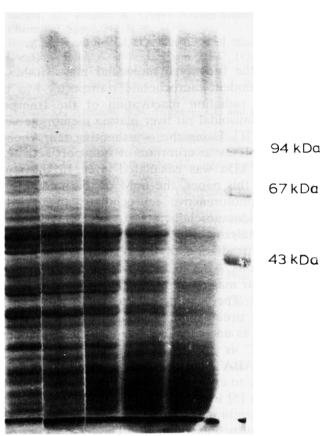

Fig. 1. SDS-PAGE of Coomassie blue-stained rat liver plasma membrane proteins before and after irradiation. Plasma membrane proteins of unirradiated (A) and irradiated (B, 10.5 Mrad; C, 15.3 Mrad; D, 22 Mrad) rat liver plasma membranes were separated on a 10% SDS-PAGE. Protein bands were stained with Coomassie blue.

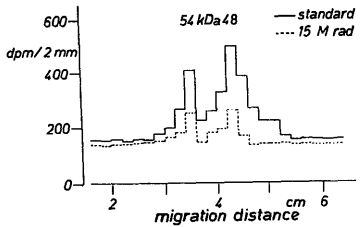

Fig. 2. Distribution of [^{14}C]ABATC-labeled proteins in unirradiated and irradiated rat liver plasma membranes. The plasma membrane proteins were separated in SDS-polyacrylamide-rod-gels. Radioactivity of the labeled proteins was determined by cutting the gel into discs of 2 mm and counting the radioactivity in a Packard liquid scintillation system (Tri Carb 2660).

In Fig. 2, the decrease of the [^{14}C]ABATC-labeling of plasma membranes irradiated in increasing doses of radiation is shown. Plotting the logarithm of the residual label bound to the proteins in the molecular mass range of 48–50 and 52–54 kDa versus the radiation dose, a monoexponential graph could be constructed for both proteins (fig. 3). For the 48–50 kDa protein a fmM of 99 ± 8,2 kDa and for the 52–54 kDa protein a fmM of 93.2 ± 7 kDa could be calculated using the approach of Kepner and Macey. With the internal standard enzyme method the fmM were calculated to be 101,2 and 95,07 kDa.

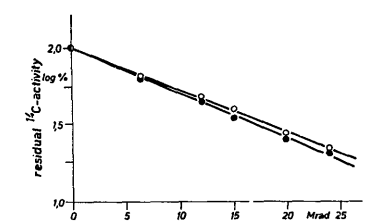

Fig. 3. Radiation inactivation of the [^{14}C]ABATC-labeling activity of unirradiated and irradiated rat liver plasma membranes. The logarithm of the percemual residual radioactivity of the 48–50 kDa and 52–54 kDa bands in SDS-polyacrylamide-rod-gel-electrophoresis was plotted versus the radiation dose. Shown is one typical experiment. All data were fitted to a straight line by the least-squares method. ●——●, 48–50 kDa; ○——○, 52–kDa.

Discussion

The molecular structure of the sodium-dependent taurocholate transport system is not precisely known. The same is true for the transport system for unconjugated bile acids such as cholate, which is sodium independent to an extent of about 50% [3]. In (photo)affinity labeling studies [4,6–11] proteins with molecular masses of 48–50 and 52–54 kDa were always labeled with taurocholate analogs but also with cholate derivatives. Since expression-cloning studies [5] made it evident that the 48–50 kDa protein is sufficient to catalyze the sodium-dependent uptake of taurocholate, the labeled 52–54 kDa protein does not appear to be a transport protein for taurocholate. On the other hand, as was shown in radiation inactivation studies, the fmM for the taurocholate transport protein is 170 kDa. These data suggest that sodium-dependent taurocholate transport is mediated by the cooperation of four protein subunits of molecular mass 48 kDa (fmM of the transport protein, 170 kDa [1]). In contrast, the cooperation of only two protein subunits is needed for the sodium-independent binding of ABATC (fmM of the binding protein, 99 kDa). Whether the higher fmM for the sodium-dependent taurocholate transport protein is due to the sodium-dependency of the transport remains unknown.

The other ABATC-labeled protein with a molecular mass of 52–54 kDa is thought to be a component of the transport system for cholate or the sodium-independent bile acid transporter [24]. In this case, binding of ABATC again needs the cooperation of two protein subunits (fmM 93.2 kDa). As will be shown in a forthcoming paper, two protein subunits cooperate to transport cholate into bl Pmv (fmM of the cholate transport protein, 100 kDa). In these cases, 50% of the transport of cholate as well as the binding of ABATC are sodium-independent. It would seem that for the sodium-substrate cotransport (taurocholate) the cooperation of a higher number of protein subunits is needed. Further studies, especially in reconstituted systems, will be required to solve this problem.

Acknowledgements

We thank Prof. M. Frimmer for valuable discussion, Dr. B. Boschek for critically reading the manuscript and P. Kronich for skillful technical assistance. Irradiation was kindly performed at the Strahlenzentrum of the University of Giessen. This work was supported by the Deutsche Forschungsgemeinschaft SFB 249.

References

1 Elsner, R.H. and Ziegler, K. (1989) Biochem. Biophys. Acta 983, 113–117.

2 Elsner, R.H. and Ziegler, K. (1989) Naunyn-Schmiedeberg's Arch. Pharmacol. Suppl. 340, R43.
3 Frimmer, M. and Ziegler, K. (1986) Biochim. Biophys. Acta 855, 143–146.
4 Ziegler, K., Frimmer, M., Müllner, S. and Fasold, H. (1989) Biochim. Biophys. Acta 930, 161–168.
5 Hagenbuch, B, Lübbert, H., Stieger, B. and Meier, P.J. (1990) J. Biol. Chem. 265, 5357–5360.
6 Ziegler, K., Frimmer, M., Möller, W. and Fasold, H. (1982) Naunyn-Schmiedeberg's Arch. Pharmacol. 319, 249–253.
7 Ziegler, K., Frimmer, M., Möller, W. and Fasold, H. (1982) Naunyn-Schmiedeberg's Arch. Pharmacol. 319, 254–261.
8 Ziegler, K., Frimmer, M., Müllner, S. and Fasold, H. (1984) Biochim. Biophys. Acta 773, 11–22.
9 Ziegler, K. (1985) Biochim. Biophys. Acta 815, 37–44.
10 Kramer, W., Bickel, U., Buscher, H.P., Gerok, W. and Kurz, G. (1982) Eur. J. Biochem. 129, 13–24.
11 Von Dippe, and Levy, D. (1983) J. Biol. Chem. 25, 8896–8901.
12 Stremmel, W. and Berk, P.D. (1986) J. Clin. Invest. 78, 822–826.
13 Berk, P.D. Potter, B.J. and Stremmel, W. (1987) Hepatology 7, 165–176.
14 Ziegler, K., Frimmer, M. and Fasold, H. (1984) Biochim. Biophys. Acta 769, 117–129.
15 Blitzer, B.L. and Donovan, C.B. (1984) J. Biol. Chem. 259, 9259–9301.
16 Scharschmidt, B.F., Keefe, E.B., Blankenship, N.M. and Ockner, R.K. (1979) J. Lab. Clin. Med. 93, 790–799.
17 Michell, R.H. and Hawthorne, J.N. (1965) Biochem. Biophys. Res. Commun. 21, 333–338.
18 Harper, A.E. (1967) in Methoden der enzymatischen Analyse, pp. 788–792, Verlag Chemie, Weinheim.
19 Kepner, G.R. and Macey, R.I. (1968) Biochim. Biophys. Acta 163, 188–203.
20 Kempner, E.S. and Haigler, H.T. (1982) J. Biol. Chem. 257, 9295–9301.
21 Pollet, R.J., Kempner, E.S. Standaert, M.L. and Haase, B.A. (1982) J. Biol. Chem. 257, 894–899.
22 Lo, M.M.S., Barnard, E.A. and Dolly, J.O. (1982) Biochem. 21, 2210–2217.
23 Nielsen, M. and Braestrup, C. (1988) J. Biol. Chem. 263, 11900–11906.
24 Fricker, G., Hugentobler, G., Meier, P.J., Kurz, G. and Boyer, J.L. (1987) Am. J. Physiol. 253, G816–G822.