Biochimica et Biophysica Acta, 983 (1989) 113–117
Elsevier

BBAMEM 70616

BBA Report

Determination of the apparent functional molecular mass of the hepatocellular sodium-dependent taurocholate transporter by radiation inactivation

Reiner Elsner and Kornelia Ziegler

Institute of Pharmacology, Justus Liebig University, Giessen (F.R.G.)

(Received 28 February 1989)

Key words: Taurocholate transporter; Radiation inactivation; Plasma membrane; (Rat liver)

The apparent target size of the sodium-dependent taurocholate transporter in basolateral rat liver plasma membrane vesicles, showing overshooting taurocholate uptake in the presence of sodium was estimated by radiation inactivation. Radiation at −105 to −120 °C and 2.5 Mrad/min causes a dose-dependent monoexponential reduction of the overshoot of taurocholate uptake in the presence of sodium. In contrast, taurocholate transport in the absence of sodium and taurocholate permeation at 4 °C remained totally unaffected by the radiation dose, indicating that the passive permeability of the membrane towards taurocholate remained unaffected. Radiation inactivation by high-energy electrons provides information about the size of the functional unit of the transporter in situ. The target size determined represents the size of the radiation-sensitive mass which is compact enough for significant energy transfer to occur within all parts of the transport system. The minimal functional molecular mass was determined to be 170 kDa for the sodium-dependent taurocholate transporter. To prove the validity of radiation inactivation data four internal standard enzymes were tested under identical conditions.

The hepatocellular uptake of bile acids is driven by a sodium gradient and the transmembranal potential [1,2]. In the presence of sodium, uptake of taurocholate exhibits overshoot phenomena. In contrast, replacing sodium by lithium, choline or potassium results in no overshoot. Apart from kinetic studies, photoaffinity labeling was used to characterize the taurocholate transport system in isolated hepatocytes and in basolateral plasma membranes [3,4]. Membrane proteins with molecular masses of 67, 54, 50, 43 and 37 kDa were identified as possible components of the transporter. Up to now, no other attempts have been made to determine the apparent molecular weight of the transporter. Therefore, radiation inactivation and target-size analysis were used for determination of the molecular mass of the sodium-dependent taurocholate transporter in intact membranes.

Rat liver basolateral plasma membranes were prepared according to the method of Blitzer and Donovan [5] with some modifications [6]. The enrichment of the specific activity of the basolateral marker enzyme Na^+/K^+-ATPase was 22–26 fold, that of the microsomal membrane marker glucose-6-phosphatase 2–2.2-fold. The morphology of the vesicles was determined by electron microscopy, which was kindly performed by Dr. Wahn, Institute of Virology, University of Giessen.

Uptake of taurocholate into basolateral plasma membrane vesicles was measured by a rapid membrane-filtration technique [5]. A mixture of taurocholate and [^{3}H]taurocholate was used at a final concentration of 1 μmol/l each. Uptake studies were performed at 37 °C in the presence and absence of sodium (100 mM NaCl was replaced by 100 mM KCl). Uptake was stopped after various times by adding 1 ml of ice-cold stop solution (100 mM NaCl or KCl, 100 mM sucrose, 10 mM Hepes-KOH (pH 7.5)). Sodium-dependent uptake was calculated by subtraction of the values for the uptake in potassium medium from those in sodium buffer.

For irradiation, 10 MeV electrons from a linear accelerator were used. Vessels with plasma membrane suspensions were stored before and after irradiation under liquid nitrogen at −196 °C. Samples were placed in the irradiation chamber in front of the linear accelerator. Chamber and samples were cooled with a stream of liquid nitrogen flowing through the chamber. The

Correspondence: K. Ziegler, Institute of Pharmacology, Justus Liebig University, Frankfurterstrasse 107, D-6300 Giessen, F.R.G.

temperature remained constant between −105 and −120 °C during the procedure and was measured with an electrode placed within the chamber. The dosimeter and radiation dose-sensitive films (Far West Technology Inc. CA. U.S.A.), were placed on each side of the samples in the radiation chamber during irradiation.

Radiation inactivation of four internal standard enzymes was performed to evaluate the validity of the radiation-inactivation method for the determination of the molecular weight of the taurocholate transporter. All enzyme activities were determined by recording spectrophotometric methods. The endogenous enzymes Na^+/K^+-ATPase and alkaline phosphatase were measured according to Scharschmidt et al. [7] and Keefe et al. [8] (at 37 °C and 405 nm using *p*-nitrophenyl phosphate as substrate), respectively. Alcohol dehydrogenase, added externally, was determined as described by Oppenheimer et al. [9] at 366 nm and a final concentration of 0.4 mg/ml; β-galactosidase according to Craven et al. [10] at 25 °C, 405 nm and 2-nitrophenyl-β-D-galactosidase as substrate.

Two approaches were used to calculate the molecular weight of the taurocholate transporter.

Approach one: The empirical equation of Kepner and Macey [11] was employed

$$\text{Molecular weight} = 6.4 \cdot 10^{11} / D_{37}(\text{rad})$$

D_{37} is the radiation dosage in rad that is necessary to obtain 37% residual biological activity. This value was calculated from a plot of the logarithm of the residual taurocholate transport activity versus the radiation dose. Because the experiments were performed at −105 to −120 °C, the empirical temperature correction factors of Kempner and Haigler [12] ($f = 10^{0.85 - 0.0028 \times C}$ where $C = -105$ to -120 °C) were used to correct inactivation data to 25 °C at which temperature the dosimetry with radiochrome dye films was done. The temperature correction factor for −105 °C used was $f = 2.4$; for −120 °C $f = 2.651$. The formula used for molecular weight determination therefore was:

$$\text{Molecular weight} = 6.4 \cdot 10^{11} \cdot f / D_{37}(\text{rad})$$

D_{37} values were determined for each of nine different radiation runs. The data of the inactivation experiments were analyzed by linear regression analysis (least-squares method). Plots of the logarithm of surviving activity versus radiation dose were linear over two orders of magnitude. Biological activity of the taurocholate transporter was expressed as the ratio of the sodium-dependent taurocholate uptake at 5–10 s to the uptake at equilibrium (3 min).

Approach two: Four internal standard enzymes namely Na^+/K^+-ATPase, alkaline phosphatase (both endogenous enzymes), β-galactosidase and alcohol dehydrogenase were irradiated. Nine different radiation runs were performed. The logarithm of the experimentally observed D_{37} values for these four enzymes was then plotted versus the logarithm of the known molecular weights of each enzyme to produce a calibration curve [11] that applies specifically to the conditions of these experiments. The D_{37} value observed for the taurocholate transport system is then converted to a molecular weight by use of this calibration curve.

In order to evaluate damage to the morphological

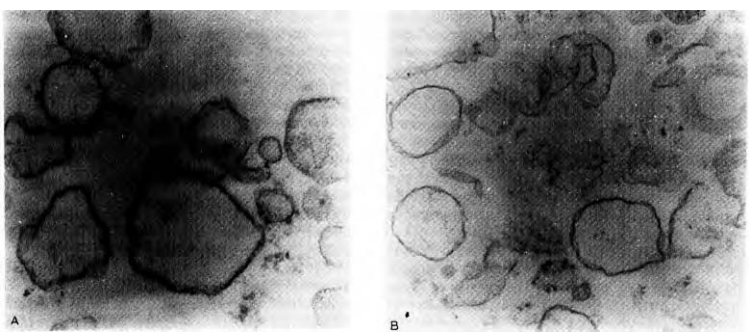

Fig. 1. Plasma membrane vesicles were fixed overnight in 0.1% sodium cacodylate buffer (pH 7.5) containing 2% glutaraldehyde and 2% paraformaldehyde. The solution was drawn off and the pellet was washed to remove glutar- and paraformaldehyde. Then the pellet was postfixed with a solution of 1% osmium tetroxide in ethanol. (A) Unirradiated vesicles, (B) irradiated (17 Mrad) vesicles.

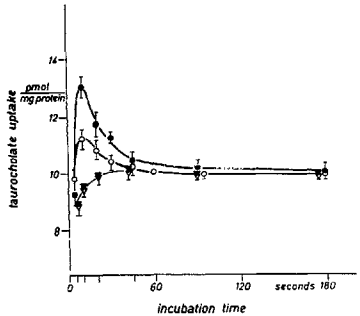

Fig. 2. Uptake of taurocholate in the presence (●, ○) and absence (▼, ▽) of sodium by untreated controls (●, ▼) and irradiated (○, ▽) plasma membrane vesicles. Plasma membrane vesicles, untreated (●, ▼) or irradiated (○, ▽) (50–80 μg protein in 10 mM Hepes-KOH, 300 mM sucrose (pH 7.4)) were preincubated for 2 min at 37° C before addition of 100 mM NaCl (●, ○) or KCl (▼, ▽), 100 mM sucrose, 10 mM Hepes-KOH (pH 7.5) containing 1 μmol/l [^{3}H]taurocholate or 1 μmol/l unlabeled taurocholate. Uptake was stopped after the times indicated by adding of 1 ml stop solution (100 mM NaCl or KCl, 100 mM sucrose, 10 mM Hepes-KOH (pH 7.5)). Vesicles were separated from buffer solution by vacuum filtration through 0.22 μm (Millipore) filters. Another 5 ml of stop solution were used to wash the filters. [^{3}H]Taurocholate uptake was measured by scintillation counting in Rotiszint 2220 (Roth AG, Karlsruhe, F.R.G.). Each point represents the mean of four determinations.

structure of vesicles by irradiation, vesicles were prepared for electron microscopy before and after irradiation. Electron microscopy of controls (Fig. 1A) and of irradiated (Fig. 1B) plasma membrane vesicles did not demonstrate any differences in membrane integrity. No increased membrane damage after irradiation with 17 Mrad was seen. We therefore assume no effects of radiation on the uptake of taurocholate into plasma membrane vesicles as a result of increasing leakiness of vesicles. This was further demonstrated by showing overshooting taurocholate uptake in irradiated membrane vesicles in the presence of sodium.

Comparison of the overshoot of taurocholate uptake between unirradiated and irradiated plasma membrane vesicles suggests that the relative impermeability of the vesicles is constant after irradiation (Fig. 2). Overshoot at 5–10 s of taurocholate uptake into plasma membrane vesicles in the presence of sodium occurs within a range of 1.4–1.5 of that at equilibrium. Equilibrium was reached in 2–3 min. As a function of radiation dosage, the height of the overshoot relative to the uptake at equilibrium decreased exponentially. In contrast, uptake in the absence of sodium (replacement by potassium) was virtually unaltered (Fig. 2). Equilibrium uptake remained constant within standard deviation, indicating no increase in leakiness of the vesicle membrane. Furthermore, uptake at 4° C remained totally unaffected by the radiation dosage, indicating that the passive permeability of the membrane towards taurocholate was unaffected (data not shown). D_{37} values for the sodium-dependent taurocholate transport activity were determined from inactivation plots as shown in Fig. 3. The result of a typical inactivation experiment is shown. The minimal functional molecular size of the taurocholate transporter calculated according to approach one (Kepner and Macey [11]) with a temperature correction factor (Kempner and Haigler [12]) was 173 ± 13 kDa using the D_{37} value of 9.5 ± 0.7. It is the first time that radiation-induced inactivation of the overshoot during initial uptake into vesicles has been used for calculation of the size of a carrier.

The reliability of the present investigation was tested by determination of the D_{37} values of four internal standard enzymes, two endogenous liver plasma membrane enzymes and two externally added proteins. The results of a typical experiment is shown in Fig. 4. Using

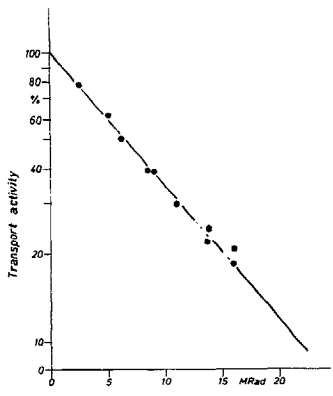

Fig. 3. Radiation inactivation of the taurocholate transporter as a function of radiation dosage. The residual sodium-dependent taurocholate transport activity (for details see Methods) was determined from uptake studies as shown in Fig. 2. The logarithm of the percentual surviving activity was plotted versus radiation dosage in Mrad. The straight line represents the least-squares line of the inactivation data of one typical inactivation experiment. Each point represents the mean of four determinations. Nine such radiation runs were performed to calculate the molecular weight of the transporter.

approach one and the D_{37} values shown for estimation of the molecular weights of the enzymes similar values to those determined and published by other authors could be calculated for the internal standard enzymes [13–15]: alcohol dehydrogenase 82 kDa; alkaline phosphatase 73 kDa; β-galactosidase 478 kDa; Na^+/K^+-ATPase 194 kDa. This demonstrates that the above method yields reliable results for determining the molecular weight of a molecule such as the sodium-dependent taurocholate transporter.

In addition, in a second approacn, the logarithm of the mean of the D_{37} values from nine different radiation runs and the logarithm of the molecular weights of the enzymes published in the literature were used to produce a calibration curve (Fig. 5) from which a molecular mass of 170 kDa was extrapolated for the taurocholate transporter.

In conclusion, the functional molecular mass of the overshooting sodium-taurocholate transporter of 170 kDa suggests that the carrier is composed of several subunits. Bile acids seem not to be translocated by a single component of the membrane proteins (molecular mass 50 and 54 kDa) which could be identified by various different (photo)affinity labels derived from bile acids. Our experiments suggest that combinations of the above proteins or combinations with unknown subunits form the functionally active transport system.

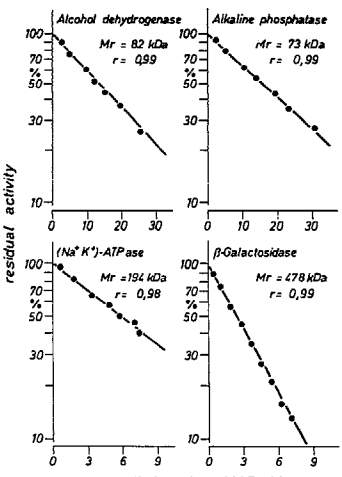

Fig. 4. Radiation inactivation of four internal standard enzymes as a function of radiation dosage. The result of a typical experiment is shown. The logarithm of percentual surviving enzyme activity was fitted to a straight line by linear regression analysis. D_{37} values were obtained from the plot to calculate the molecular weights of the enzymes by approach one (for details see Methods). r = regression coefficient. Irradiation of Na^+/K^+-ATPase was performed at −115 °C (f = 2.57), that of the other enzymes at −105 °C (f = 2.4).

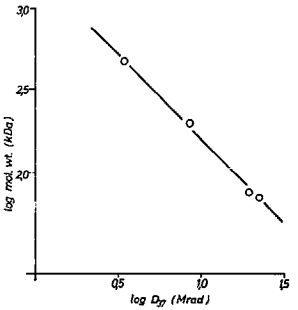

Fig. 5. Calibration curve for the determination of the molecular weight of the taurocholate transporter. The means of the D_{37} values for four internal standard enzymes were determined from nine different radiation runs. The logarithm of the known molecular weights of those enzymes was plotted versus the logarithm of the D_{37} values (as was done in Ref. 11) (D_{37} for Na^+/K^+-ATPase 8.56±1.07; for alkaline phosphatase 22.2±3.15; for β-galactosidase 3.42±0.24; for alcohol dehydrogenase 19.53±2.31). A linear function was observed which could be used to calculate the molecular weight of the taurocholate transporter. The molecular mass used for β-galactosidase was 464 kDa as described by Lo et al. in 1982 [13]; for Na^+/K^+-ATPase 200 kDa as described by Kepner and Macey in 1968 [11]; for alcohol dehydrogenase 78 kDa as described by Nielsen and Braestrup in 1988 [14]; for alkaline phosphatase 72 kDa as described by Pollet et al. in 1982 [15]. The molecular weight of the taurocholate transporter is seen to be 170 kDa (D_{37} 9.5 Mrad).

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 169.

References

1 Anwer, M.S., Kroker, R. and Hegner, D. (1976) Biochem. Biophys. Res. Commun. 73, 63–71.
2 Petzinger, E. and Frimmer, M. (1984) Biochim. Biophys. Acta 778, 539–548.
3 Ziegler, K., Frimmer, M., Möller, W. and Fasold, H. (1982) Naunyn-Schmiedeberg's Arch. Pharmacol. 319, 245–261.

4 Ziegler, K., Frimmer, M., Möller, W. and Fasold, H. (1982) Naunyn-Schmiedeberg's Arch. Pharmacol. 319, 249–253.
5 Blitzer, B.L. and Donovan, C.B. (1984) J. Biol. Chem. 259, 9259–9301.
6 Täfler, M., Ziegler, K. and Frimmer, M. (1988) Naunyn-Schmiedeberg's Arch. Pharmacol. 337, 231–237.
7 Scharschmidt, B.F., Keefe, E.B., Blankenship, N.M. and Ockner, R.K. (1979) J. Lab. Clin. Med. 93, 790–799.
8 Keefe, E.B., Scharschmidt, B.F., Blankenship, N.M. and Ockner, R.K. (1979) J. Clin. Invest. 64, 1590–1598.
9 Oppenheimer, H.L., Green, R.W. and McKay, R.H. (1967) Arch. Biochem. Biophys. 119, 552–559.
10 Craven, G.R., Steers, E. and Anfinsen, C.B. (1965) J. Biol. Chem. 240, 2468–2477.
11 Kepner, G.R. and Macey, R.I. (1968) Biochim. Biophys. Acta 163, 188–203.
12 Kempner, E.S. and Haigler, H.T. (1982) J. Biol. Chem. 257, 9295–9301.
13 Lo, M.M.S., Barnard, E.A. and Dolly, J.O. (1982) Biochemistry 21, 2210–2217.
14 Nielsen, M. and Braestrup, C. (1988) J. Biol. Chem. 263, 11900–11906.
15 Pollet, R.J., Kempner, E.S., Standaert, M.L. and Haase, B.A. (1982) J. Biol. Chem. 257, 894–989.