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Biochimica et Biophysica Acta 1190 (1994) 304–308

BBA Biochimica et Biophysica Acta

Effect of mitochondrial protein concentration on the efficiency of outer membrane removal by the cholesterol-selective detergent digitonin

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(Received 25 October 1993)

Abstract

The effects of different mitochondrial protein concentrations on the efficiency of digitonin titration of the outer mitochondrial membrane were investigated in isolated rat liver mitochondria. Isolated mitochondria were subjected to treatment with digitonin concentrations ranging from 0 to 0.40 mg digitonin per mg protein. This digitonin concentration range was used in incubations containing 5 to 50 mg mitochondrial protein per ml. Significant differences in the efficiency of outer membrane removal by digitonin titration were noted at protein concentrations of less than 20 mg per ml. Estimation of the effective concentration of digitonin required to remove 50% of the outer membrane indicated that in general, as the protein concentration decreases, the amount of digitonin required to remove the outer membrane increases. Significant differences were also noted in the amount of digitonin needed for removal of 95% of the outer membrane between 5, 10 and 20 mg/ml with the effect of protein concentration disappearing above 20 mg/ml. No effect of protein concentration was found on the disruption of the inner membrane by digitonin as judged by leakage of matrix marker enzyme activity and by release of inner membrane marker enzyme activity. The conclusions of these studies indicate that at relatively low mitochondrial protein concentrations (< 20 mg/ml), the efficiency of digitonin in removing the outer membrane is substantially reduced.

Key words: Mitochondrion; Membrane; Digitonin; Fractionation

1. Introduction

The mitochondrion of higher organisms consists of a dual membrane system; a relatively permeable outer membrane and an essentially impermeable inner membrane. Separation of the two membranes or removal of the outer membrane is frequently necessary when studying certain aspects of mitochondrial function. The removal of the outer membrane of mitochondria is achieved by a variety of means including osmotic shock [1], the use of pressure cells that effectively strip off the outer membrane [2] and by the use of the detergent digitonin [3]. Digitonin is a naturally occuring steroid glycoside derived from *Digitalis purpurea* and has been utilized in many cellular and subcellular systems as a cholesterol-specific detergent. Disruption and/or removal of relatively cholesterol-rich membranes is thus accomplished by the judicious use of digitonin. In cell systems the most common use for digitonin is the disruption of the plasma membrane for rapid isolation of subcellular organelles [4].

In mitochondria, digitonin has been used for two basic goals: the first of which is the treatment of isolated mitochondria with relatively low concentrations of digitonin (approx. 0.0125 mg digitonin/mg mitochondrial protein) to remove lysosomal contamination [5,6]. This procedure is effective in removing lysosomal contamination in mitochondrial preparations as determined by marker enzyme measurement, while preserving the respiratory function of the mitochondria. The second, and perhaps more common, use of digitonin in isolated mitochondria is the complete removal of the outer membrane [3]. Selective removal of the outer membrane of mitochondria by digitonin is due to the greater cholesterol content of the outer membrane when compared to that of the inner membrane [7]. In the original description of this precedure

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SSDI 0005-2736(93)E0322-2

by Schnaitman and Greenawalt [3] the mitochondrial protein concentration was kept relatively high (approx. 50 mg/ml). Recently several studies have appeared using protein concentrations which are substantially lower; ranging from 3 to 10 mg/ml [8–11]. From our own experience, the use of lower protein concentrations results in a greater ease in sample handling and marker enzyme assay and requires much less mitochondrial protein. During recent studies using a low protein concentration digitonin fractionation technique to localize a mitochondrial enzyme activity of interest [12], it became apparent that at low concentrations of protein the efficiency of digitonin removal of the outer membrane is considerably lower than that reported in the original descriptions of this procedure, however. This, and previous studies that have observed similar effects [9,10,13] have prompted the systematic characterization of this phenomenon using rat liver mitochondria reported here.

2. Materials and methods

2.1. Materials

Male Wistar rats, 200–250 g, were obtained from ALAB AB, Sollentuna, Sweden. Rats were allowed food and water ad libitum. Digitonin (> 99%) was obtained from Merck, Darmstadt, Gemany. Recrystallization of digtonin was performed according to the methods of Kun et al. [6] and the recrystallized digitonin was used within 15 days of its preparation. It was found that recrytallization of all commercially available digitonin is necessary in order to assure complete solubilization in aqueous solutions. Benzylamine-HCl and oxalacetic acid were obtained from Sigma. Fatty acid-free bovine serum albumin (BSA), Hepes and NADH were supplied from Boehringer-Mannheim, Germany.

2.2. Mitochondrial isolation

The rat was killed under ether ether anesthesia, the liver was perfused with phosphate-buffered saline, the liver was then removed and the mitochondria were prepared as described by Pedersen et al. [14] with the following modifications: 1.0 mM EDTA was included in the isolation medium (H-medium) through the first wash step and the concentration of Hepes in the isolation medium was 5 mM. The final mitochondrial pellet was resuspended in H-medium to a protein concentration of approx. 50–100 mg/ml and kept at 0–4°C until its use. The protein content of mitochondrial and submitochondrial suspensions was determined by the biuret reaction [15]. Mitochondria were used within 2 h of their preparation.

2.3. Digitonin titration of mitochondria and marker activity

All steps of the digitonin titration were carried out at 0–4°C. The final mitochondrial suspension was diluted to protein concentrations ranging from 10 to 100 mg/ml in H-medium. Solutions of varying digitonin concentrations were prepared in H-medium lacking BSA. The treatment of mitochondria with digitonin was by the addition of the mitochondrial suspension to the digitonin-containing solution in a ratio of 1:1 to a final volume of 250 μl. The resulting mixture was then submerged in ice-water and gently agitated on a rocking table for 15 min. The suspension was centrifuged at $20000 \times g$ for 2 min and a portion of the resulting supernatant was removed and used for the determination of marker enzyme activities. Monoamine oxidase (MAO; EC 1.4.3.4) was used as the outer membrane marker and malate dehydrogenase (MDH; EC 1.1.1.37) was used as the matrix marker. The activity of both MAO and MDH were determined by the spectrophotometric methods described by Schnaitman and Greenawalt [3]. Succinate dehydrogenase (SDH; EC 1.3.99.1) was used as the inner mitochondrial membrane marker and was determined according to the methods of Brdiczka et al. [16]. The total mitochondrial activity of MAO, MDH and SDH was determined in isolated mitochondrial preparations treated with 0.1% Triton X-100.

2.4. Nonlinear curve fitting and statistical analysis

Digitonin titration curves were fitted to a statistical sigmoid using the UltraFit software package (Biosoft, Cambridge, UK) which uses a nonlinear iterative Levenberg Least-Squares regression analysis technique. Data from individual titration curves were analyzed by curve fitting and estimates of the digitonin concentrations giving 50% and 95% monoamine oxidase release (EC_{50} and EC_{95}) were derived directly from the curve. One-way analysis of variance followed by a post hoc Student's t-test was used to determine differences between the calculated EC_{50} values and EC_{95} values.

3. Results and discussion

Digitonin has been used to selectively solubilize biological membranes in many different systems. Because of its relatively rich cholesterol content, the outer mitochondrial membrane was found to be removed by digitonin treatment in a rather selective manner [3]. This property of the dual membrane system of the mitochondrion allows the outer membrane to be 'titrated' away by progressively higher digitonin concentrations and has been utilized primarily in stud-

ies investigating the submitochondrial location of enzymes such as the glutathione transferase [8], the porin-hexokinase complex [9,10], carnitine palmitoyltransferase [13], creatinine kinase [17] and the NAD^+ glycohydrolase [12]. Recently, a thorough characterization of lipid populations in the outer and inner membranes was also reported [11] and it was found that for this purpose, digitonin was a preferable method to the osmotic swell-shrink-sonicate procedure. The original characterization of the removal of the outer mitochondrial membrane by digitonin described by Schnaitman and Greenawalt [3] used approx. 0.20 mg digitonin per mg protein in an incubation that contained greater than 50 mg per ml mitochondrial protein. This combination of digitonin and protein concentrations was reported to effectively remove 100% of the outer membrane as determined by marker enzyme activity. More recent reports have suggested that in order to remove the outer membrane, lower digitonin concentrations could be used; in the range of 0.065 to 0.12 mg digitonin per mg protein at protein concentrations comparable to those used in by Schnaitman and Greenawalt [6,14,18].

Recently, studies have appeared using much lower protein concentrations. At lower protein concentrations, however, a decrease in the originally reported efficiency of digitonin removal of the outer mitochondrial membrane has been observed [13] and partially characterized [9]. During recent studies in our laboratory which utilized the digitonin titration technique it was noted that the efficiency of digitonin removal of the outer membrane decreased as the protein concentration in the incubation decreased. A more thorough consideration of this phenomenon is reported here.

The results of digitonin titrations carried out at 5, 10, 20, 30 and 50 mg mitochondrial protein per ml are presented in Fig. 1. From these data an effect of protein concentration on the removal of outer membrane as assessed by release of monoamine oxidase activity into the supernatant is apparent. The lowest protein concentration (5 mg/ml) shows a substantially lower efficiency than the higher concentrations (30–50 mg/ml) with 10 and 20 mg/ml giving intermediate results. Measurement of the absolute amount of monoamine oxidase activity in the supernatant at each protein concentration indicated that at 0.40 mg digitonin per ml, equivalent amounts of the outer membrane were released (after adjusting for relative amounts of protein). One recent study in which a digitonin titration of rat liver mitochondria was conducted at 10 mg/ml protein indicated that removal of the outer membrane (as judged by monoamine oxidase activity) by digitonin was not complete, reaching a plateau at approx. 85% of the total mitochondrial activity [11]. In the present study, greater than 97% of the activity found in whole mitochondria is found in the supernatant, suggesting that most of the outer membrane is removed. Additionally, mitoplasts resulting from treatment of mitochondria with higher digitonin concentrations (> 0.30 mg/mg protein) were essentially devoid (< 1% of total) of monoamine oxidase activity.

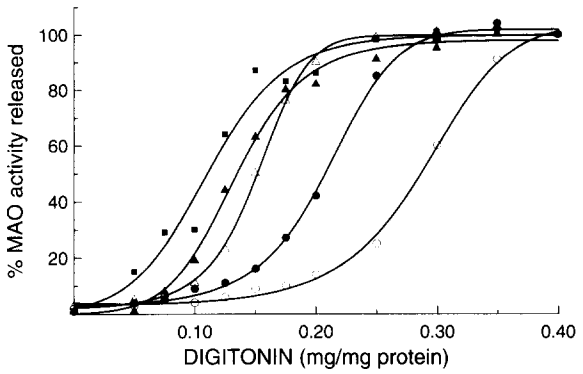

Fig. 1. Digitonin titration of the outer membrane marker monoamine oxidase (MAO) activity from isolated rat liver mitochondria. The data represent the percent of maximal acitivity released from mitochondria by digitonin with 100% representing the MAO activity present in the untreated mitochondrial preparation. Mitochondria at 5 (open circles), 10 (filled circles), 20 (open triangles), 30 (filled triangles) or 50 (filled squares) mg/ml were incubated with the indicated concentration of digitonin, centrifuged and the supernatant assayed for MAO activity as outlined in Materials and methods. The statistical analysis of these data is presented in Table 1.

Statistical analysis of the data presented in Fig. 1 is given in Table 1. Nonlinear regression analysis of titration curves derived from individual mitochondrial preparations allows the estimation of the 'effective concentrations' required to remove 50% (EC_{50}) and 95% (EC_{95}) of the outer membrane. Statistical comparison of these values indicates that there is a significant effect on the efficiency of digitonin removal of the outer membrane when the protein concentration in the titration incubation is lowered. Specifically, at protein concentrations lower than 20 mg/ml, a dramatic increase in the amount of digitonin required to remove 95% of the outer membrane is seen. No significant

Table 1
Effective digitonin concentrations required to remove 50% (EC_{50}) and 95% (EC_{95}) of the outer membrane of rat liver mitochondria [a]

Protein concentration (mg/ml)	EC_{50}	EC_{95}
5	0.287 ± 0.017 [b]	0.358 ± 0.020 [b]
10	0.207 ± 0.016 [b]	0.279 ± 0.019 [b]
20	0.151 ± 0.014 [c]	0.207 ± 0.028 [c]
30	0.135 ± 0.016 [c]	0.209 ± 0.027 [c]
50	0.105 ± 0.027 [b]	0.195 ± 0.014 [c]

[a] Values are derived from individual titration curves as described in Materials and methods and presented as the mean ± S.D., $n = 3$–8.
[b] Significantly different from all other groups in the column, $P < 0.05$.
[c] Significantly different from the 5 mg/ml and 10 mg/ml values in that column, $P < 0.01$.

changes in EC_{95} values are evident above 20 mg/ml indicating that the efficiency of digitonin is relatively constant if the protein concentrations are kept above 20 mg/ml. From the EC_{50} values, an apparent increase in the efficiency of digitonin continues up to 50 mg/ml. As can be seen from Fig. 1, this decrease in the EC_{50} values is a result of changes in the slope of the exponential phase of the sigmoidal curve as the protein concentration increases. The trends in EC_{50} and EC_{95} values at the different protein concentrations are presented in Fig. 2. Estimates of EC_{50} value from previous studies using rat liver mitochondria carried out at 3–3.5 mg/ml ($EC_{50} = \sim 0.25$) [8], 5 mg/ml ($EC_{50} = 0.39$) [9], 10 mg/ml ($EC_{50} = \sim 0.175$) [11], and 50 mg/ml ($EC_{50} = \sim 0.10$) [9] correspond reasonably well with those observed in the present study. Results of digitonin titration of rat brain mitochondria at 5 mg/ml indicate a much lower efficiency ($EC_{50} = \sim 0.50$) [10] and may represent a difference in the lipid composition of the outer mitochondrial membrane of the two organs.

As indicated by Jancsik et al. [9], the interaction between digitonin and membrane cholesterol is a second order process and thus as the protein concentration changes, the apparent requirement for digitonin will change. Hence from the data presented in Table 1 and Fig. 2, it appears from the EC_{95} values that the interaction between digitonin and cholesterol in rat liver mitochondria is a true second order process at protein concentrations less than 20 mg/ml and that it assumes a pseudo-first order character at higher protein concentrations; depending only on the concentration of digitonin (see Fig. 2). Thus at very low protein concentrations, very high digitonin concentrations will be required for complete removal of the outer membrane. Higher digitonin concentrations result in the disruption of the inner membrane with subsequent leakage of matrix components and, as the digitonin concentration is increased further, solubilization of the inner membrane [8,13]. Therefore it is reasonable to postulate that if the protein concentration is kept high, less disruption of the inner membrane will be seen at digitonin concentrations that are adequate to remove the outer membrane. However, the measurement of malate dehydrogenase (MDH) and succinate dehydrogenase (SDH) activities in the supernatants derived from mitochondria treated with the ED_{95} concentration of digitonin at each protein concentration indicates that leakage of MDH and release of SDH is equivalent among the different protein concentrations showing slight, but not significant increases at protein concentrations of 50 mg/ml (Table 2). This result indicates that incubation of rat liver mitochondria at low protein concentrations and relatively high digitonin concentrations does not affect the inner membrane to a greater extent than in high protein/low digitonin incubation. It remains to be seen whether this finding is applicable to mitochondrial from other tissues and species.

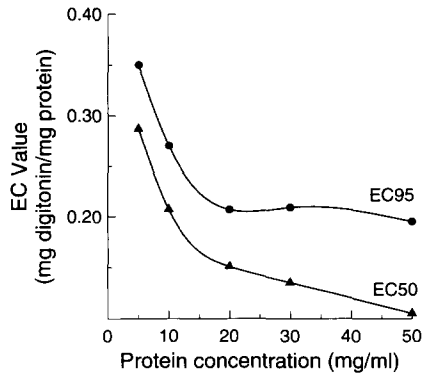

Fig. 2. Effective concentrations of digitonin required to remove 50% (EC_{50}) or 95% (EC_{95}) of the outer membrane of rat liver mitochondria *versus* the protein concentration of the incubation. Incubation conditions are outlined in Materials and methods. EC values are given in Table 1.

Table 2
Percent total SDH and MDH activity released from rat liver mitochondria at EC_{95} digitonin concentrations [a]

Protein concentration (mg/ml)	SDH	MDH
5	15±5	17±5
10	15±4	19±2
20	14±5	17±4
30	14±6	21±3
50	23±5	24±4

[a] EC_{95} digitonin values for each protein concentration are given in Table 1. Values are given as the percent of the total mitochondrial activity that was found in the post-digitonin supernatant as outlined in Materials and methods and represent the mean±S.D., $n = 4$. No significant differences were observed among the SDH or MDH values by ANOVA.

The use of digitonin to remove the outer mitochondrial membrane has been used both to isolate outer membrane free mitochondria (mitoplasts) and in 'titrations' of the outer membrane in studies concerned with the submitochondrial location of mitochondrial components. The isolation of outer membrane-free mitoplasts is particularly important when isolation of relatively pure inner membrane or matrix fractions are needed. Previous descriptions of the digitonin treatment of mitochondria for the purposes of subsequent use or fractionation of the resulting mitoplasts have recommended digitonin concentrations of 0.12 mg/mg protein [14,18] or 0.065 mg/mg protein [6]. The results of the present investigation would indicate that these concentrations are not adequate to assure complete removal of the outer membrane of rat liver mitochondria. Rather, as originally described by Schnaitman and Greenawalt, a digitonin concentration of approx. 0.20 mg/mg protein or greater is recommended

in the presence of relatively high (30–50 mg/ml) protein concentrations when isolating mitoplasts. Due to the dramatic decrease in the efficiency of digitonin removal of the outer membrane at relatively low (< 20 mg/ml) protein concentrations, this study also highlights the necessity for careful characterization of the digitonin effects on mitochondrial outer membrane removal when low protein concentrations are used. Finally, disruption of the mitochondrial inner membrane by digitonin does not appear to be affected by the protein concentration in the incubation.

4. References

[1] Sottocasa, G.L., Kuylenstierna, B., Ernster, L. and Bergstrand, A. (1967) Methods Enzymol. 10, 448–463.
[2] Decker, G.L. and Greenawalt, J.W. (1977) J. Ultrastruct. Res. 59, 44–56.
[3] Schnaitman, C. and Greenawalt, J.W. (1968) J. Cell Biol. 38, 158–175.
[4] Brocks, D.G., Siess, E.A. and Weiland, O.H. (1980) Biochem. J. 188, 207–212.
[5] Lowenstein, J., Scholte, H.R. and Wit-Peeters, E.M. (1970) Biochim. Biophys. Acta 223, 432–438.
[6] Kun, E., Kirsten, E. and Piper, W.N. (1979) Methods Enzymol. 55, 115–118.
[7] Akiyama, T., Takagi, S., Sankawa, U., Inari, S. and Saito, H. (1980) Biochemistry 19, 1904–1911.
[8] Ryle, C.M. and Mantle, T.J. (1984) Biochem. J. 222, 553–556.
[9] Jancsik, V., Lindén, M., Dorbani, L., Rendon, A. and Nelson, B.D. (1988) Arch. Biochem. Biophys. 264, 295–301.
[10] Dorbani, L., Jancsik, V., Lindén, M., Leterrier, J.F., Nelson, B.D. and Rendon, A. (1987) Arch. Biochem. Biophys. 252, 188–196.
[11] Hovius, R., Lambrechts, H., Nicolay, K. and De Kruijff, B. (1990) Biochim. Biophys. Acta 1021, 217–226.
[12] Boyer, C.S., Moore, G.A. and Moldéus, P. (1993) J. Biol. Chem. 268, 4016–4020.
[13] Zammit, V.A. and Corstorphine, C.G. (1985) Biochem. J. 230, 389–394.
[14] Pedersen, P.L., Greenawalt, J.W., Reynafarje, B., Hullihen, J., Decker, G.L., Soper, J.W. and Bustamente, E. (1977) Methods Cell Biol. 20, 411–480.
[15] Gornall, A.G., Bardawill, C.J. and David, M.M. (1949) J. Biol. Chem. 177, 751–766.
[16] Brdiczka, D., Dölken, G., Krebs, W., and Hofman, D. (1974) Hoppe-Seyler's Physiol. Chem. 355, 731–743.
[17] Kottke, M., Adams, V., Wallimann, T., Nalam, V.K. and Brdiczka, D. (1991) Biochim. Biophys. Acta 1061, 215–225.
[18] Greenawalt, J.W. (1974) Methods Enzymol. 31, 638–643.