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A robust method to screen detergents for membrane protein stabilization, revisited

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ABSTRACT

This report is a follow up of our previous paper (Lund, Orlowski, de Foresta, Champeil, le Maire and Møller (1989), J Biol Chem 264:4907–4915) showing that solubilization in detergent of a membrane protein may interfere with its long-term stability, and proposing a protocol to reveal the kinetics of such irreversible inactivation. We here clarify the fact that when various detergents are tested for their effects, special attention has of course to be paid to their critical micelle concentration. We also investigate the effects of a few more detergents, some of which have been recently advertised in the literature, and emphasize the role of lipids together with detergents. Among these detergents, lauryl maltose neopentyl glycol (LMNG) exerts a remarkable ability, even higher than that of β-dodecylmaltoside (DDM), to protect our test enzyme, the paradigmatic P-type ATPase SERCA1a from sarcoplasmic reticulum. Performing such experiments for one's favourite protein probably remains useful in pre-screening assays testing various detergents.

1. Introduction

Membrane proteins are generally solubilized in detergent for purification and further study, and many different detergents or amphiphilic scaffolds have been used for this purpose, including in the context of membrane protein crystallization for which long-term protein stability is especially essential [1–3]. In the case of SERCA1a, the paradigmatic P-type Ca^{2+}-ATPase from sarcoplasmic reticulum membranes, we previously showed [4] that the particular type of detergent used and the residual lipids associated with the detergent-solubilized protein were primary (although not exclusive) determinants for stabilization of this protein, and we proposed a general method for screening the ability of detergents to protect such an enzyme from time-dependent irreversible inactivation. This method (Fig. 1A) consists in first removing lipids from the membrane protein as fully as possible, by solubilizing it in a "fairly good" detergent, *i.e.* a detergent in which irreversible inactivation is slow compared with most other detergents ($C_{12}E_8$ was used in Ref. [4]; DDM was used in the present report) and then running the solubilized protein on a size exclusion column in the presence of the same detergent (and in the absence of any added lipid). In a second step the delipidated protein is diluted in the presence of a large w/w excess of the detergent to be tested (at a typical concentration of 1 mg/mL of this second detergent, a concentration significantly higher than the cmc of most used detergents) and incubated for various periods, under conditions where the residual amount of the initial detergent is expected to hardly interfere with the stabilizing or inactivating effect of the second detergent. At the end of this pre-incubation period of various duration, the protein is finally diluted, to assay its residual activity, into an assay medium containing a large concentration of a third detergent, known to be optimal for assaying this activity ($C_{12}E_8$ again was in fact used in Ref. [4], as well as in the present report), so that the second detergent in the preincubation medium (the one to be tested) gets highly diluted in the $C_{12}E_8$ micelles present during the ATPase assay and hardly interferes with the activity measurement. In the 1989 paper, this procedure allowed comparison between various detergents for their possible inactivating effects: more than thirty different detergents were tested for

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their irreversible inactivation of SERCA1a activity after storage (in various states of the enzyme: *"k_{E1}", "k_{E2}", or "k_{TO}"* in Table I of [4] stand for inactivation rates in the so-called Ca^{2+}-bound E1 or Ca^{2+}-free E2 states of SERCA1a, or during its turn-over, respectively). In the present report, in a general perspective applicable to any membrane protein, we wish to comment further on the data obtained by this procedure (which can play important roles in the functional exploration and the final crystallization of such protein), and to add to the list a few more detergents which have appeared in the meantime.

2. Materials and methods

SERCA1a-containing sarcoplasmic reticulum membranes were prepared from rabbit skeletal muscle, as described [4,5]. Delipidated SERCA1a was also prepared, essentially as described [4]: for this purpose, SERCA1a -containing membranes were first solubilized with DDM (instead of $C_{12}E_8$ as in Ref. [4]) and then run on a size-exclusion chromatography column (SuperDex 200 10/300 GL, GE Healthcare) with a mobile phase consisting of 25 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 50 mM Mops-Tris at pH 7.0 and 0.4 mg/mL DDM), allowing the elution, at a concentration of 0.32 mg protein/ml, of essentially delipidated SERCA1a (Fig 7A in Ref. [1]) together with its accessory modulator sarcolipin [6].

Time-dependent irreversible inactivation of SERCA1a was tested by its preincubation for various periods in the presence of various detergents (and in its Ca^{2+} bound "E1" form), in a standard buffer containing 100 mM KCl, 50 mM Tes-Tris, 1 mM $MgCl_2$ and 0.1 mM free Ca^{2+} (adjusted with EGTA if required), at pH 7.5 and 20 °C, using one or the other of two different protocols, as described below. Residual ATPase activities after preincubation were assayed using a classical coupled enzyme assay [4] using a similar medium, here with 0.05 mM free $CaCl_2$ and supplemented with 5 mM MgATP as well as pyruvate kinase, lactate dehydrogenase, phosphoenolpyruvate and NADH; this regenerating system kept working efficiently in the presence of the detergents tested, as systematically controlled by addition of a fixed amount of ADP. A cut-off filter (MTO J310A) was used to minimize photobleaching of NADH in our HP-Agilent 8453 diode array spectrophotometer. In Figs. 1A and B and 2A and B, after short incubation with DDM, the enzyme's activity measured in the presence of $C_{12}E_8$ and the small amount of DDM resulting from dilution of the preincubation medium was dubbed 100%. As small amounts of various detergents have different effects on activity in the presence of excess $C_{12}E_8$, not all curves start from exactly 100%. The experiments illustrated are representative of several experiments.

$C_{12}E_8$ (octa (ethylene glycol) docedyl monoether), DDM (β-dodecylmaltoside), LMNG (lauryl maltose neopentyl glycol) and DPC (dodecylphosphocholine, also known as F12-phoscholine or FC-12) were from Anatrace; DHPC (diC_7-phosphatidylcholine, with H for hepta), LPC (lysophosphatidylcholine, here from plant) and POPS (palmitoyl oleyl phosphatidylserine) were from Avanti; CyMal-5 (5-cyclo-maltoside) was from Fluka; CHAPS (3-[(3-cholamidopropyl)diméthylammonio]-1-propanesulfonate) was from Boehringer; OG (β-octyl glucoside) was from Sigma; cholate was recrystallized before use; other chemicals were of high quality. For these various detergents, their critical micelle concentration (cmc) was measured in a medium identical to the pH 7.5 buffer used here, according to the methyl orange method [7].

3. Results and discussion

1. Inactivating effects of detergents, as tested with delipidated SERCA1a.

To ensure that residual lipid bound to SERCA1a did not interfere with the ability of the tested detergents to stabilize or inactivate the solubilized protein, nearly complete delipidation was first achieved by running a concentrated batch of $C_{12}E_8$-solubilized SERCA1a through a SEC column in the presence of the same "first" detergent ($C_{12}E_8$ was used in Ref. [4]; in the experiments to be described below, DDM was used as an equally appropriate detergent). The resulting delipidated SERCA1a (Step 1 in Fig. 1 cartoon) with the accompanying "first" detergent was then diluted (typically down to 0.03–0.05 mg protein per mL) into a preincubation medium containing the detergent to be tested (the "second" detergent) at a concentration of 1 mg/mL (Step 2 in Fig. 1 cartoon), *i.e.* a concentration much higher than the residual concentration of the "first" detergent resulting from dilution of the delipidated protein (0.040–0.066 mg/mL). This residual concentration of $C_{12}E_8$, however, is close to the cmc for $C_{12}E_8$ (0.04–0.05 mg/mL [8]).

If the second detergent (the one to be tested) has a cmc much lower than 1 mg/mL, most of the residual first detergent will get diluted into the micelles of that second detergent, and the detergent belt around the delipidated protein will mainly contain the second detergent (Step 2A in Fig. 1 cartoon); as a result, the residual activity found for the protein after its incubation for various periods (Step 3 in Fig. 1 cartoon) will indeed mainly reveal the time-dependent inactivating or stabilizing effect of this second detergent (and the corresponding results in Table I of [4] are fully valid).

If, at variance, the detergent to be tested has a cmc higher than 1 mg/mL, it will be present only as monomers if it is only present at 1 mg/mL during the incubation, and in this case the protein, diluted into a water phase containing the first detergent at a concentration close to its cmc (e.g. $C_{12}E_8$ in Ref. [4] or DDM in the present study), will remain surrounded by a detergent belt mainly consisting of $C_{12}E_8$ (instead of the detergent to be tested (Step 2B in Fig. 1 cartoon), and subsequent incubation will only reveal the time-dependent inactivating or stabilizing effect of $C_{12}E_8$, the "first" detergent used for initial delipidation, nevertheless in the additional presence of free monomers of the second detergent. Table I in Ref. [4] includes a few such detergents with a high cmc, *e.g.* OG, CHAPS or cholate (cmc ~ 7–8 mg/mL for OG, and ~ 3–4 mg/mL for both CHAPS and cholate), with CHAPS and cholate being reported as fairly good at stabilizing SERCA1a (their inactivation rate constants are suggested to be only 4- or 3-fold faster than the "best" detergent, DDM (k_{E1} of 0.055 or 0.042 min^{-1}, respectively, versus 0.016 min^{-1}), unfortunately with no mention of whether special attention had been given to the high cmcs of those detergents.

We therefore here first repeated similar experiments with those high cmc detergents, testing their effects with delipidated SERCA1a (Panels A&B in Fig. 1). In the present experiments, SERCA1a had been first delipidated with DDM and eluted from the SEC column at 0.32 mg protein/mL (with 0.4 mg DDM/mL in the running buffer), and for incubation with the detergent to be tested it was diluted 5-fold (*i.e.* down to 0.064 mg protein/mL), hence with a final residual concentration of non-bound DDM of 0.08 mg DDM/mL, again a concentration close to the cmc of DDM [9]. But for testing the effect of OG, CHAPS or cholate, the detergent concentration used was now significantly higher (10, 5 or 5 mg/mL, respectively) than the cmc of these detergents. Samples were incubated for various periods, and their residual activity was measured after 20-fold dilution into the ATPase assay medium, containing excess $C_{12}E_8$ as previously (here at 2.5 mg/mL). Controls with either 2 or 1 mg/mL of those high cmc detergents (*i.e.* concentrations lower than the cmc of those detergents) were included for comparison with the results reported in Table I of [4], as well as controls with or without DDM.

In these experiments, when measurements were performed after diluting delipidated SERCA1a into a medium containing detergent at a concentration lower than its cmc, i.e. OG at 2 mg/mL only, CHAPS at