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Hydrogenated and fluorinated surfactants derived from Tris (hydroxymethyl)-acrylamidomethane allow the purification of a highly active yeast F1-F0 ATP-synthase with an enhanced stability

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Abstract Loss of stability and integrity of large membrane protein complexes as well as their aggregation in a non-lipidic environment are the major bottlenecks to their structural studies. We have tested $C_{12}H_{25}$-S-poly-*Tris*-(hydroxymethyl) acrylamidomethane (H_{12}-TAC) among many other detergents for extracting the yeast F_1F_0 ATP-synthase. H_{12}-TAC was found to be a very efficient detergent for removing the enzyme from mitochondrial membranes without altering its sensitivity towards specific ATP-synthase inhibitors. This extracted enzyme was then solubilized by either dodecyl maltoside (DDM), H_{12}-TAC or fluorinated surfactants such as C_2H_5-C_6F_{12}-C_2H_4-S-poly-*Tris*-(hydroxymethyl)acrylamidomethane (H_2F_6-TAC) or C_6F_{13}-C_2H_4-S-poly-*Tris*-(hydroxymethyl)acrylamidomethane (F_6-TAC), two surfactants exhibiting a comparable polar head to H_{12}-TAC but bearing a fluorinated hydrophobic tail. Preparations from enzymes purified in the presence of H_{12}-TAC were found to be more adapted for AFM imaging than ATP-synthase purified with DDM. Keeping H_{12}-TAC during the Ni-NTA IMAC purification step or replacing it by DDM at low concentrations did not however allow preserving enzyme activity, while fluorinated surfactants H_2F_6-TAC and F_6-TAC were found to enhance enzyme stability and integrity as indicated by sensitivity towards inhibitors. ATPase specific activity was higher with F_6-TAC than with H_2F_6-TAC. When enzymes were mixed with egg phosphatidylcholine, ATP-synthases purified in the presence of H_2F_6-TAC or F_6-TAC were more stable upon time than the DDM purified enzyme. Furthermore, in the presence of lipids, an activation of ATP-synthases was observed that was transitory for enzymes purified with DDM, but lasted for weeks for ATP-synthases isolated in the presence of molecules with Tris polyalcoholic moieties. Relipidated enzymes prepared with fluorinated surfactants remained highly sensitive towards inhibitors, even after 6 weeks.

Keywords Membrane protein complexes · F_1F_0 ATP-synthase · Detergent · Fluorinated surfactants

Abbreviations

Tris	Tris(hydroxymethyl)aminomethane

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H_{12}-TAC	$C_{12}H_{25}$-S-poly-Tris(hydroxymethyl) acrylamidomethane
Chol-TAC	Cholesteryl-COC_2H_5-S-poly-Tris-(hydroxymethyl)acrylamidomethane
H_2F_6-TAC	C_2H_5-C_6F_{12}-C_2H_4-S-poly-Tris-(hydroxymethyl)acrylamidomethane
F_6-TAC	C_6F_{13}-C_2H_4-S-poly-Tris-(hydroxymethyl) acrylamidomethane
DDM	dodecyl maltoside
$C_{12}E_8$	octaethyleneglycol mono-n-dodecylether
CHAPS	3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate
bigCHAP	N,N-bis[3-(D-gluconamido) propyl]cholamide
Dig	digitonin
PC	egg phosphatidylcholine
AFM	atomic force microscopy
BN-PAGE	blue-native poly-acrylamide gel electrophoresis
cmc	critical micellar concentration
SMP	sub-mitochondrial particles
NTA	nitrilo-triacetic acid
IMAC	immobilized metal affinity chromatography
PK	pyruvate kinase
LDH	L-lactate dehydrogenase
PEP	phosphoenolpyruvate
PMSF	phenylmethanesulfonyl fluoride
ε-ACA	ε-aminocaproic acid
DCCD	dicyclohexyl carbodiimide
DPR	detergent/protein (W/W) ratio
LPR	lipid/protein molar ratio

Introduction

Structural studies of large membrane complexes have been hampered by the difficulty to handle them upon extraction, solubilization, purification and crystallization steps. Many reviews and textbooks describe in details detergents and methods to manipulate such complexes (Bowie 2001; Garavito and Ferguson-Miller 2001; Hunte et al. 2003; Privé 2007). Loss of stability and integrity of oligomeric proteins as well as their aggregation in a non-lipidic environment are not easy to overcome and are the major brakes to structural studies.

The yeast F_1F_0 ATP-synthase is a complex of 590 kDa consisting of 17 distinct subunits. This enzyme is embedded in the inner mitochondrial membrane, where it uses the proton electrochemical gradient generated by the respiratory chain to synthesize ATP from ADP and inorganic phosphate. This enzyme represents 10% of total yeast mitochondrial proteins. It comprises a catalytic head F_1 (α_3,β_3,γ,δ,ε), a channel region (9_{10}, 6), a peripheral stalk (4, 8, d, OSCP, i, f, h) (Velours and Arselin 2000) and non-essential subunits (e, g and k) that were found to specifically be associated with dimeric and oligomeric ATP-synthases (Arnold et al. 1998; Arselin et al. 2003). F_1 catalyzes ATP hydrolysis but ATP synthesis requires the association of F_1 to the channel region and the peripheral stalk. High sensitivities to specific ATP-synthase inhibitors like dicyclohexyl carbodiimide (DCCD) or oligomycin indicate a good physical and functional coupling between F_1 and the rest of the enzyme. Crystallographic data have been obtained on the catalytic head F_1 (Kabaleeswaran et al. 2006, 2009), on a sub-complex F_1-9_{10} (Stock et al. 1999) and on a soluble part of the peripheral stalk (Dickson et al. 2006) but the structure of the whole enzyme remains to be elucidated to understand its energetic coupling mechanism.

Although the complex extracted and isolated in the presence of dodecyl maltoside (DDM) contained most subunits, subunit 6 involved in the proton channel and all the subunits of the peripheral stalk were further lost during crystallization (Stock et al. 1999). In similar experiments carried out in our group with lower DDM concentrations upon the purification and crystallization steps, the purified complex contained 14 distinct subunits and like in Stock et al. (Stock et al. 1999) only six of them ($\alpha_3\beta_3\gamma\delta\varepsilon 9_{10}$) were found in electron density maps (manuscript in preparation). This indicates that DDM may not be the most appropriate detergent for structural studies of ATP-synthase and pointed to the necessity to find alternative detergents/surfactants to preserve the enzyme integrity and activity upon all the steps required for its isolation. New non-ionic synthetic detergents like $C_{12}H_{25}$-S-poly-*Tris*(hydroxymethyl)acrylamidomethane (H_{12}-TAC) have been engineered to extract and solubilize membrane proteins (Pucci et al. 1991, 1993). H_{12}-TAC has a thio dodecanoyl chain combined with a polar group made of Tris polyalcoholic moieties. Other molecules, like C_2H_5-C_6F_{12}-C_2H_4-S-poly-*Tris*(hydroxymethyl)acrylamidomethane (H_2F_6-TAC) or C_6F_{13}-C_2H_4-S-poly-*Tris*(hydroxymethyl)acrylamidomethane (F_6-TAC) have a polar head chemically similar to H_{12}-TAC but their aliphatic chains are totally or partially fluorinated (see Fig. 1 for chemical structures) (Barthélémy et al. 1999; Chabaud et al. 1998; Myrtil et al. 1995; Pavia et al. 1991). F_6-TAC has an aliphatic chain with eight carbons in which the six terminal carbons are perfluorinated, while the fluorinated chain of H_2F_6-TAC is end-capped with an ethyl group leading to a hemifluorinated hydrophobic chain with ten carbon atoms. The grafting of such an ethyl group was designed to improve the H_2F_6-TAC affinity towards protein hydrophobic surface. Fluorocarbon surfactants are more

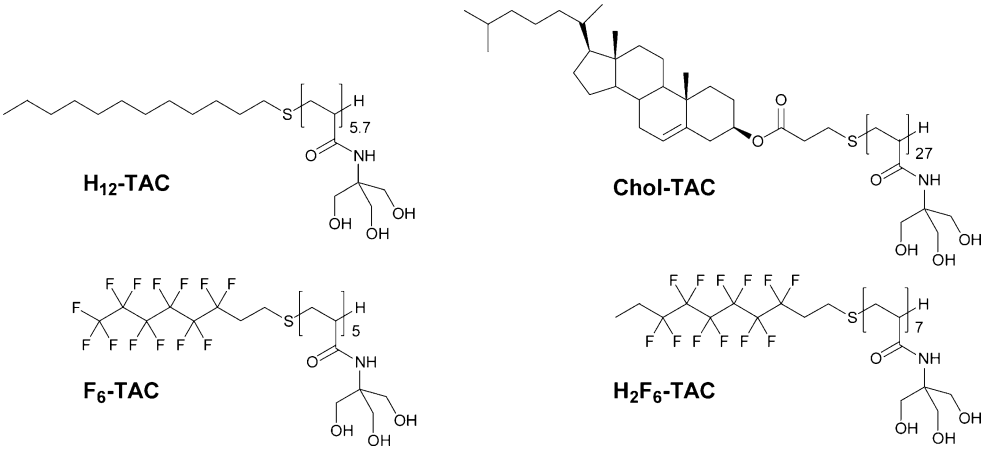

Fig. 1 Chemical structures of poly-Tris derivatives surfactants H_{12}-TAC, H_2F_6-TAC, F_6-TAC and Chol-TAC

hydrophobic than hydrocarbon surfactants and their critical micellar concentration (cmc) is much lower than the cmc of hydrocarbon analogues. Moreover they are lipophobic (Krafft 2001) and consequently unable to extract membrane proteins from their native lipidic environment (Pucci et al. 1993) whereas they are very effective agents to maintain them in solution after their extraction from the membrane by classical detergents (Chabaud et al. 1998). These fluorinated compounds have been shown to preserve activity and to prevent aggregation of membrane proteins such as bacteriorhodopsin, cytochrome b_6f complex or bacterial outer membrane protein OmpA (Breyton et al. 2004). Their role as molecular chaperones for insertion of diphtheria toxin in lipid bilayers has also been reported (Posokhov et al. 2008; Rodnin et al. 2008) and they were successfully used to maintain in solution a mecanosensitive channel during its expression in a cell-free system (Park et al. 2007).

Here we compare ATP-synthases extracted with DDM or H_{12}-TAC. We found that H_{12}-TAC was very efficient for extracting the yeast ATP-synthase from the mitochondrial membrane. Furthermore, H_{12}-TAC purified ATP-synthase molecules adsorbed on mica plates were still active and suitable for atomic force microscopy (AFM) imaging. We also show results on ATP-synthases extracted with H_{12}-TAC and kept in solution with DDM, H_{12}-TAC, H_2F_6-TAC or F_6-TAC. The presented data demonstrate the efficiency of F_6-TAC to stabilize this large complex after its extraction from membranes, with or without additional lipids.

Material and methods

Chemicals

H_{12}-TAC is a non-ionic detergent with a low cmc (~0.15 mM) synthesized as previously described (Pucci et al. 1991). The batch used in the present work had an average number of 5.7 Tris motifs (<MW> ≈ 1,200 g/mol). H_2F_6-TAC (<MW> ≈ 1,615 g/mol, cmc ~0.45 mM), F_6-TAC (<MW> ≈ 1,378 g/mol, cmc ~0.5 mM), Chol-TAC (<MW> ≈ 5,200 g/mol, cmc ~0.38 mM) were synthesized as described in (Pavia et al. 1991) (Fig. 1). DDM (Anagrade, cmc ~0.16 mM), octyl glucoside (cmc ~21 mM), Anzergent 3–12 (cmc ~2.8 mM) were purchased from Anatrace. CHAPS (cmc ~5 mM) and bigCHAP (cmc ~3.4 mM) were from Calbiochem. $C_{12}E_8$ was a gift from Pr M. le Maire. PK, LDH from rabbit muscle and inhibitor cocktail were purchased from Roche. Digitonin, ATP, NADH, DCCD, HEPES, EDTA and oligomycin were from Sigma-Aldrich; PEP was from Fluka.

Detergent extraction

Mitochondria were obtained with the zymolyase method (Guérin et al. 1979) from a genetically modified D273-10/B/A strain (*met6*, *his3*, *ura3*, *i-his*$_6$) in which a $(His)_6$-tag had been added at the C-terminus of subunit *i*, a component of the peripheral stalk. Preparations were adjusted to a mitochondrial protein concentration of 10 mg mL^{-1} and incubated in extraction buffer "A" (150 mM potassium acetate, 10% (V/V) glycerol, 2 mM PMSF, 2 mM ε-ACA, 30 mM HEPES pH 7.4 and EDTA-free protease inhibitor cocktail) supplemented with appropriate concentrations of H_{12}-TAC, DDM or other detergents. After a 30 min incubation at 4 °C, samples were centrifuged (30 min, 4 °C, 25,000 ***g***) and extracted proteins (0.4 mg) were analyzed under native conditions (BN-PAGE) on a 3–13% linear gradient polyacrylamide gel (Schägger et al. 1994). ATPase activity was revealed directly on gel (Grandier-Vazeille and Guérin 1996) before Coomassie brilliant blue staining. ATPase activities of extracts were measured as previously described (Somlo 1968). Enzyme purity was assessed by silver staining after Tricine SDS-PAGE.

Purification of ATP-synthase from yeast mitochondria

Submitochondrial particles (SMP) were prepared according to (MacLennan et al. 1968) and centrifuged at 48,000***g*** for

45 min at 4 °C. Pellets were suspended at 10 mg of starting mitochondrial proteins per mL of buffer "A" containing 0.75% H_{12}-TAC (W/V) (6.65 mM). After 30 min of incubation at 4 °C, the extract was clarified by centrifugation (30 min, 4 °C, 25,000 ***g***). The supernatant was diluted with two volumes of washing buffer "W" (50 mM NaCl, 10% (V/V) glycerol, 10 mM imidazole, 20 mM sodium phosphate pH 7.9) and mixed with Ni-NTA-agarose beads (Qiagen) (0.25 mL of slurry per 10 mg of starting mitochondrial proteins). After an overnight incubation at 4 °C, beads were washed with 25 volumes of buffer "W" containing either DDM (0.32 mM), H_{12}-TAC (0.85 mM), H_2F_6-TAC (1.15 mM) or F_6-TAC (1.2 mM). ATP-synthase was eluted with buffer "E" (50 mM NaCl, 10% (V/V) glycerol, 250 mM imidazole, 20 mM sodium phosphate pH 7.9) containing either DDM (0.64 mM) or TAC derivatives at the above-mentioned concentrations.

The ATPase activity of extracted F_1F_0 ATP-synthase was evaluated as described in (Somlo 1968) or for the Ni-NTA purified enzyme by monitoring NADH oxidation at 340 nm through a coupled reaction with pyruvate kinase (PK) and lactate dehydrogenase (LDH) (Tietz and Ochoa 1958). An absorbance decrease at 340 nm reflects ATPase activity. Activity assays were performed at 28 °C under magnetic stirring in 1.5 mL of activity buffer (20 units mL^{-1} of PK, 30 units mL^{-1} of LDH, 5 mM ATP, 2 mM PEP, 0.2 mM NADH, 5 mM $MgCl_2$, 50 mM HEPES pH 7.5). The molar extinction coefficient of NADH at 340 nm was 6,220 M^{-1} cm^{-1} and the pathlength was 1 cm.

Protein concentration was estimated by the Lowry method (Lowry et al. 1951) after a trichloroacetic acid precipitation step and solubilization of pellets in 5% SDS (W/V).

Incubation with egg phosphatidylcholine

Egg phosphatidylcholine (PC) purified according to (Singleton et al. 1965) was suspended in 5 mM $MgCl_2$, 50 mM HEPES pH 7.5 to a concentration of 10 mg mL^{-1} and then sonicated three times, for 5 min at 4 °C (75TS Annemasse sonicator, 120 V). Purified enzymes were mixed just after their elution from the IMAC resin with PC to a lipid/protein molar ratio of ~250 and stored at 13 °C. ATPase activity was determined at 28 °C using the PK/LDH coupling reaction.

Preparation of specimens for AFM imaging

Freshly cleaved mica plates (15×8 mm^2 for enzymatic activity control and 5×5 mm^2 for AFM) were incubated for 1 min in 1 mM $NiCl_2$. Mica plates were washed with water and incubated with the enzyme (0.1 mg mL^{-1}) in buffer "P" (100 mM NaCl, 25 mM D(+)trehalose, 2 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM PMSF, 20 mM Tris-HCl pH 8.0) containing either 0.85 mM H_{12}-TAC or 0.64 mM DDM. After 10 min of incubation, plates were rinsed with buffer "P" gradually diluted in observation buffer (50 mM KCl, 2 mM $MgCl_2$, 10 mM Tris-HCl pH 8.0) to decrease detergent concentration step by step and to remove unadsorbed materials (Schabert and Engel 1994).

The ATPase activity of F_1F_0 ATP-synthase bound on mica was controlled by monitoring NADH oxidation at 340 nm (Tietz and Ochoa 1958). In typical experiments plates incubated or not with ATP-synthase were plunged out of the light beam in stirred reaction mixture, removed, and plunged again.

AFM experiments were performed at room temperature in a liquid cell with a Nanoscope IIIa Multimode scanning probe microscope (Digital Instruments, Santa Barbara, CA) equipped with an "E" scanner (maximum X and Y scan of 14 μm). Mica plates were glued on a Teflon disc and mounted in the liquid cell. Silicon nitride cantilevers with 0.06 N m^{-1} and 0.35 N m^{-1} nominal spring constants and tips with a semi-angle of 35° were used. Force curves (or approach-retract curves) were obtained in the force calibration mode and collected by measuring the cantilever deflection (or the amplitude of cantilever oscillations) as the sample was moved towards the tip. The experimental force (or approach-retract) curves were obtained with a Z scan velocity between 2 μm s^{-1} and 8 μm s^{-1} and drive amplitude and set point were optimized for each image. Images were recorded in tapping mode with a scan angle of 90°. For each experiment, three images were recorded: height, phase and amplitude. The amplitude (signal error) was also recorded in order to avoid artefact during measurement. For clarity, only height images are shown.

Preparation of specimens for electron microscopy

F_1F_0 ATP-synthases purified in the presence of H_{12}-TAC were diluted 20–40 fold in water and immediately applied for 1 min to formvar/carbon-coated copper grids, previously glow discharged, washed with distilled water and stained with 2% uranyl acetate for 1 min. Specimens were examined on a Hitachi 7650 transmission electron microscope operating at 80 kV.

Results

The enzyme extracted by H_{12}-TAC is highly active and sensitive to specific inhibitors

When mitochondria were incubated with increasing concentrations of DDM, the maximum of ATPase specific activity that was detected in the detergent extract was about 4 μmol min^{-1} mg^{-1} for a detergent/protein ratio (DPR) of

0.25 g/g (Fig. 2a). The lower activity observed at a DPR of 0.75 (g/g) was reproducible in several experiments. For higher concentrations of DDM, a small increase in activity was observed as well as a decrease in DCCD and oligomycin (not shown) sensitivity, indicating a slight functional and/or a physical uncoupling of ATP-synthase. When DDM-extracted proteins were analyzed by BN-PAGE, only monomeric forms were obtained with a DPR from 0.25 to 2.00 (Fig. 2b and c).

As an alternative to DDM for ATP-synthase extraction, we looked for detergents that could extract ATP-synthase without affecting enzyme sensitivity toward its inhibitors and that could preferably preserve oligomeric species. Many detergents usually used to extract membrane proteins were tested. Some of them like $C_{12}E_8$, octyl glucoside or Anzergent 3–12 disrupted the interactions between F_1 and F_0, even when low concentrations (around their cmc) were used (not shown). Monomeric forms of ATP-synthase were obtained with the zwitterionic detergent CHAPS (4 mM to 64 mM) (not shown). Dimeric and oligomeric forms of ATP-synthase were observed with non ionic detergents like bigCHAP (up to 22 mM) and digitonin (below 6 mM), a detergent commonly used to study oligomeric forms of ATP-synthase (Schägger and Pfeiffer 2000) but that cannot be used for structural studies because of its impurity. When H_{12}-TAC was used to extract mitochondrial proteins, the highest ATPase specific activity (6 µmol min^{-1} mg^{-1}) was detected for a DPR of 0.75 (g/g) and sensitivity towards DCCD was maintained (Fig. 2a). The same inhibition trend was observed with oligomycin (not shown). For higher

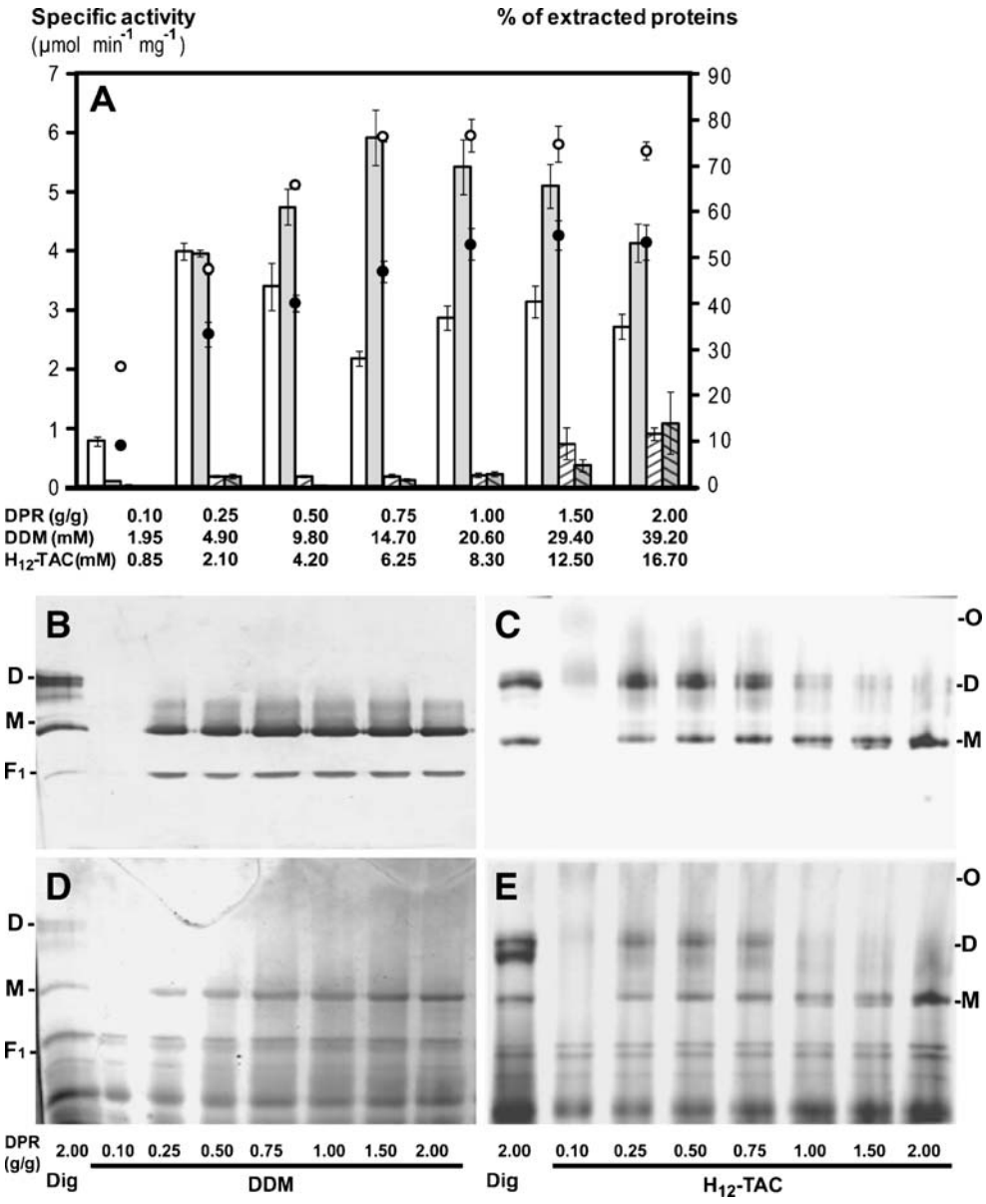

Fig. 2 Analyses of DDM and H_{12}-TAC extracts. **a** ATPase specific activities (µmol min^{-1} mg^{-1}) were measured in DDM (white bars) or H_{12}-TAC (*grey bars*) extracts obtained at various DPR without or with ATPase inhibitor (DCCD (5 µg mL^{-1}), *hatched bars*). Protein concentrations were measured in triplicate for each extract. Extracted protein quantities (DDM extracts (*open circles*) and H_{12}-TAC extracts (*plain circles*)) are given in percentage of starting mitochondrial proteins (*right axis*). Values are the average of three independent experiments. For clarity, detergent concentrations (g/g of mitochondrial proteins) used for extraction are also given in mM. 40 µl of DDM (**b**) or H_{12}-TAC (**d**) extracts were analyzed by BN-PAGE and ATPase activity was revealed in gel. Gels were then stained with Coomassie brilliant blue (**c** DDM extracts, **e** H_{12}-TAC extracts). O: oligomers; D: dimers; M: monomer; F_1: free catalytic sector of ATP-synthase. Dig: Digitonin

H_{12}-TAC ratios, above 1 g/g, ATPase activity in the extracts decreased, with a lower sensitivity towards ATPase inhibitors. Although Chol-TAC has a cholesterol moiety like digitonin and a polar head chemically similar to H_{12}-TAC, it had a very poor extraction power even at a concentration around 20 × cmc (not shown).

A BN-PAGE analysis of mitochondrial extracts obtained with increasing concentrations of H_{12}-TAC (DPR from 0.10 to 2.00) is shown in Fig. 2d and e. With DPR between 0.1 and 0.25, mainly dimeric species were extracted. A faint band, corresponding to a higher oligomeric (O) species was also detected when ATPase activity was revealed in gel (Fig. 2d). When DPR were comprised between 0.50 and 1.00, dimeric (D) and monomeric (M) species were present in extracts and above a DPR of 1.00, monomeric species became predominant. Only a very faint active band of free F_1 was present with 2% H_{12}-TAC and no corresponding band was discernible on the Coomassie blue stained gel (Fig. 2e).

Activity due to free F_1 sectors was observed on BN-PAGE for digitonin- and DDM-extracted samples (Fig. 2b), but no free F_1 activity was detected in solution in DDM extracts as indicated by a high sensitivity towards DCCD (Fig. 2a). This F_1 band with a strong ATPase activity on BN-PAGE may indicate a higher weakness of the DDM extracted enzyme upon electrophoresis compared to H_{12}-TAC extracted complexes.

As there were less total proteins extracted with H_{12}-TAC than with DDM, H_{12}-TAC seems more useful to specifically extract the ATP-synthase from yeast mitochondrial membranes than DDM. As ATP-synthase oligomeric structures were better preserved with H_{12}-TAC than with DDM, H_{12}-TAC seems to be a milder detergent compared to DDM. For these reasons, H_{12}-TAC was preferred for the extraction step of the purification protocol of the yeast ATP-synthase.

To minimize contamination by matricial soluble proteins, H_{12}-TAC extraction was performed on sub-mitochondrial particles (SMP). Figure 3 shows specific activities that were measured in extracts obtained from SMP at various H_{12}-TAC concentrations. Starting from SMP, the highest specific activity (20 µmol min^{-1} mg^{-1}) was obtained for a detergent/mitochondrial protein ratio of 0.75 (W/W). Performing H_{12}-TAC extraction on mitochondria or on SMP did not alter oligomer/monomer ratios within the same range of H_{12}-TAC concentrations (data not shown).

Enzymes purified in the presence of H_{12}-TAC or DDM are monomeric

We first attempted to purify the dimeric entities starting from SMP with H_{12}-TAC using a DPR of 0.25 for the extraction step, a concentration that gave small enzyme quantities but for which a large proportion of dimeric species was found in the extract. Enzymes were bound on Ni-NTA agarose beads and eluted with 0.85 mM H_{12}-TAC (0.1% W/V). BN-PAGE analyses of eluates indicated that dimeric species were lost upon this unique purification step. Decreasing detergent concentrations upon washing steps did not prevent the loss of dimers.

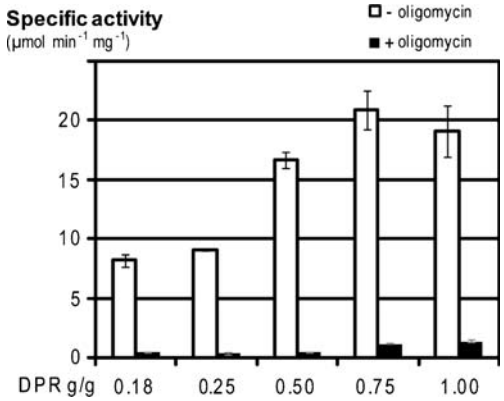

Fig. 3 Analyses of ATPase specific activity in H_{12}-TAC extracts from SMP. ATPase activities in H_{12}-TAC extracts were measured in triplicate in the absence or in the presence of oligomycin (6 µg mL^{-1}). DPR values were calculated using starting mitochondrial protein quantities

Thus we performed ATP-synthase purification using 6.25 mM H_{12}-TAC (*i.e.* a DPR of 0.75) for the SMP extraction step, a concentration that gave a mix of monomeric and dimeric enzymes in the extract and corresponded to the highest specific activity (Fig. 3). However, after the Ni-NTA IMAC step, ATPase specific activity dropped to ~4 µmol $min^{-1}mg^{-1}$ and sensitivity towards oligomycin was decreased. When H_{12}-TAC was replaced by 0.64 mM DDM upon washing and elution steps, the specific activity was ~2 µmol min^{-1} mg^{-1} and sensitivity towards inhibitors was often lost or dramatically decreased. This drop could vary from 50% to almost 100% depending on preparations or ATPase activity measurement conditions (different pH and enzyme concentrations).

Analyses by BN-PAGE of enzymes purified after the Ni-NTA IMAC step in the presence of DDM or H_{12}-TAC (Fig. 4a) indicated that the purified ATP-synthase was a homogeneous monomer containing all ATP-synthase subunits except e and g. These two components required for enzyme dimerization were not revealed by Western blot (not shown). Western blot analyses did not also reveal the presence of the Pi or ADP/ATP carriers in enriched ATP-synthase fractions, as was the case with the ATP synthasome from rat mitochondria (Chen et al. 2004; Ko et al. 2003). Figure 4b shows the high degree of purity of ATP-synthase prepared in the presence of H_{12}-TAC.

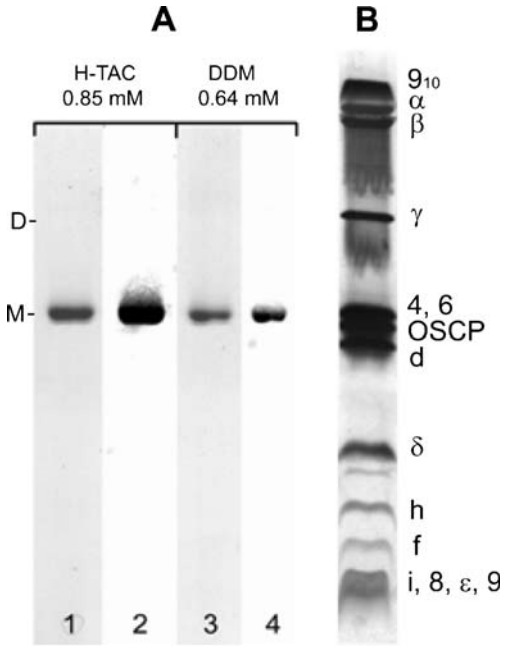

Fig. 4 Native and denaturing electrophoresis analyses of Ni-NTA IMAC-purified samples. **a** BN-PAGE showing monomers purified in the presence of H_{12}-TAC (lanes *1* and *2*) or DDM (lanes *3* and *4*) revealed by ATPase activity (lanes *2* and *4*) or Coomassie brilliant blue (lanes *1* and *3*). M: monomer. D: migration zone of dimers. **b** Silver stained Tricine SDS-PAGE (12.5% (W/V) polyacrylamide slab gel) of ATP-synthase purified with H_{12}-TAC. ATP-synthase subunits are indicated

AFM imaging of the yeast ATP-synthase

Atomic Force Microscopy imaging on membrane protein samples is extremely dependent on enzyme preparations. To determine if H_{12}-TAC purified enzymes could be imaged with this technique, isolated enzymes were adsorbed on mica plates. Imaging in liquid by contact mode AFM of ATP-synthase deposits destroyed the observed objects. Thus all images were obtained in tapping mode (Fig. 5). Control experiments performed without protein showed no deposit on the surface and looked like regular mica surfaces (insert A of Fig. 6).

AFM images of Fig. 5a and b as well as the sample observed after the activity assay (insert B of Fig. 6) showed that the whole mica surfaces were covered by a relatively dense monolayer. A significant number of very large aggregates (Fig. 5b, *black arrows*) remained on mica in spite of extensive rinses. Such aggregates were, however, more numerous with DDM (results not shown) indicating that DDM purified enzymes were less suitable for informative AFM observations. As shown in Fig. 5b smaller particles were clearly visible between aggregates. At the experimental resolution, particles appeared as roughly spherical objects. When the surface shown in Fig. 5c was scanned with higher magnifications some isolated spherical particles were observed (see Fig. 5d–e). Consecutive scans in the same area did not alter the deposits because the images were recorded with a vertical force adjusted to a few hundreds of piconewtons (almost 300 pN) to avoid displacement of isolated and weakly bound proteins. At forces above this value, proteins were flattened and reproducible images could not be recorded. Images recorded with higher magnification than in Fig. 5e in order to enhance the resolution did not bring more detailed information than the one shown in Fig. 5e and did not allow identifying F_1 and F_0 sectors. Statistical analyses on isolated particles like those indicated by arrows on Fig. 5d, gave a mean width of 25 nm. Both shape and size were in good agreement with those measured on chloroplast F_1F_0 ATP-synthase by AFM in contact mode (Neff et al. 1997). However the measured mean height of isolated particles was around 5 nm (±1 nm) instead of 9 nm obtained for the chloroplast enzyme in constant force mode.

Some objects appeared spherical (Fig. 5d, arrows) and some looked like adjacent balls with slightly different radii and height (Fig. 5d, asterisks). This lack of homogeneity could reflect different orientations of the enzyme on mica plate and the two adjacent balls might correspond to the two sectors of the enzyme, seen on its long axis. Figure 5f shows an electron micrograph obtained after negative staining of a deposit of the H_{12}-TAC purified sample, on which the two sectors of the enzyme can clearly be recognized (Fig. 5f, insert). Particles seem larger when imaged by AFM but the observed size is a convolution of the actual size of the molecule with that of the tip. Although the experimental resolution obtained with the AFM tip is low, these data clearly indicate that H_{12}-TAC is a promising surfactant for AFM observations of membrane protein complexes.

ATPase activity of mica-adsorbed enzymes

To ensure that bound enzymes were still active, we used a sensitive activity assay in which ADP produced by adsorbed ATP-synthase is used by coupled enzymes (PK/LDH) to lead to NADH oxidation. A typical experiment is shown in Fig. 6. When mica plates treated with buffer "P" containing only 0.85 mM H_{12}-TAC (without enzyme) were immersed in the activity buffer, no decrease in the absorbance was detected (step 1). By AFM, no object was observed (Fig. 6, insert A). NADH oxidation was detected only when the plate, pre-incubated with buffer "P" containing 0.85 mM H_{12}-TAC and 0.1 mg mL^{-1} of ATP-synthase and then extensively rinsed, was immersed in the activity buffer (step 3). When the plate was removed, NADH oxidation was reduced but the rate of oxidation was higher than the one detected prior to plate addition (step 4). This is probably due to some ATP-synthases (or free F_1 sectors) that were released after 10 min of reaction in the stirred medium. Once again, after addition of the plate, an increase in NADH oxidation was

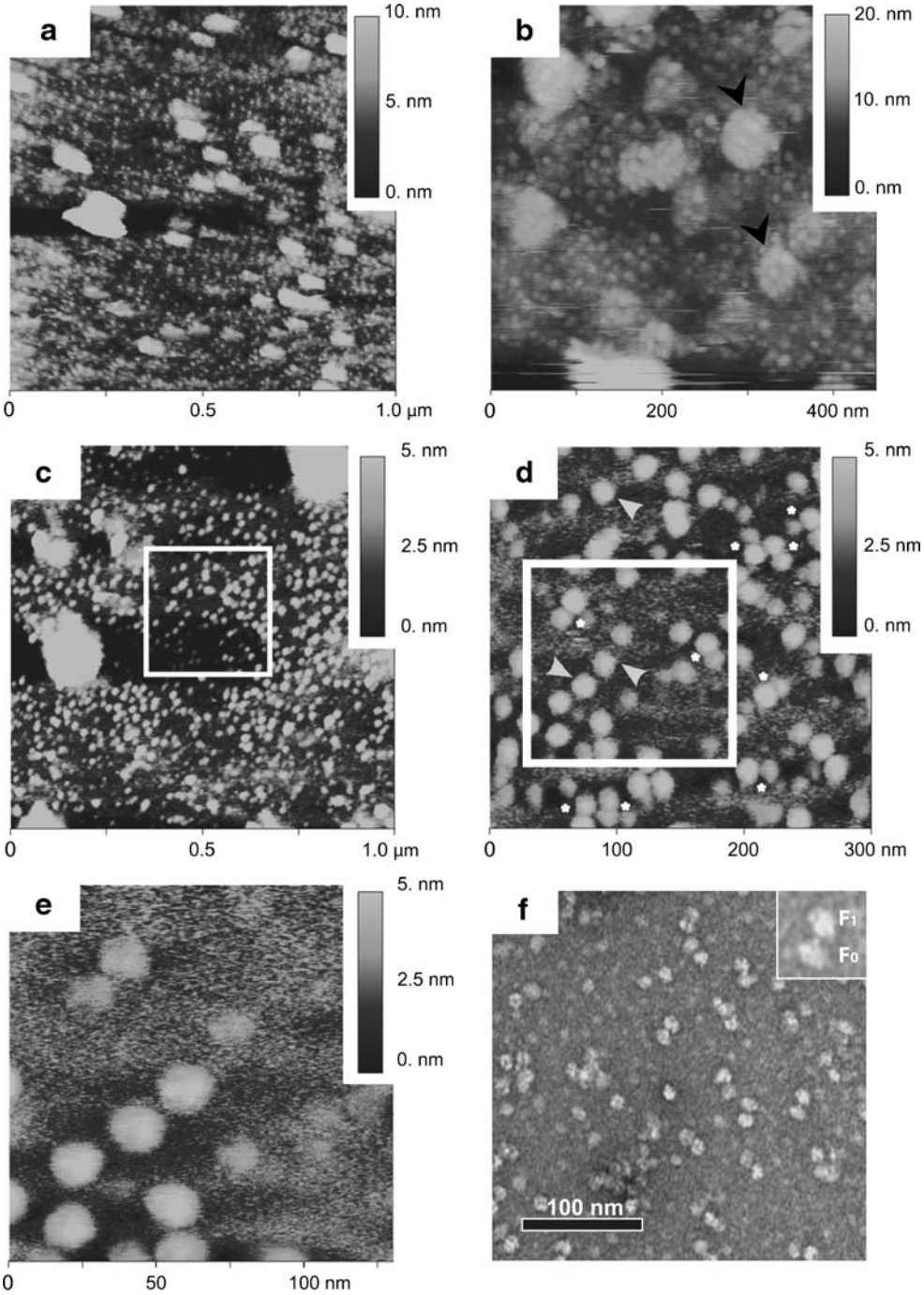

Fig. 5 Imaging of H_{12}-TAC purified ATP-synthases with AFM and electron microscopy. AFM images of two different samples deposited in the presence of 0.85 mM H_{12}-TAC show dense monolayers of ATP-synthase: **a** Sample 1 (drive amplitude 150 mV, set-point voltage 1.7 V), **b** Sample 2 (drive amplitude 340 mV, set-point voltage 1.7 V); *Black arrows* indicate aggregates. **c**–**e**) AFM serial images of another sample deposited in the presence of 0.15 mM H_{12}-TAC show isolated particles (drive amplitude 350 mV, set-point voltage 0.56 V). Images **d** and **e** correspond to new scans of the boxed area shown on the previous image. *Arrows* indicate spherical objects and asterisks two adjacent spherical objects of different diameters **f** Electron micrograph of negatively stained H_{12}-TAC purified ATP-synthases. The insert shows a zoom of a F_1F_0 ATP-synthase

observed (step 5). After the plate was removed, a decrease in NADH oxidation was recorded (step 6). Finally this plate was imaged by AFM (Fig. 6, insert B). These results clearly show that enzymes were adsorbed on mica and were still active.

H_2F_6-TAC and F_6-TAC preserve sensitivity towards inhibitors

In order to avoid ATPase activity loss upon the Ni-NTA IMAC purification step, we tested whether fluorinated surfactants such as H_2F_6-TAC and F_6-TAC could advantageously replace H_{12}-TAC or DDM if they were used at the elution step from the IMAC resin. It can be seen from Fig. 7a that F_6-TAC was the most effective compound to maintain a high activity of the purified enzyme. Two days after the day of extraction from mitochondrial membranes (day referred as day 0), activities in the presence of F_6-TAC were three and six times higher than activities detected at day 2 for enzymes purified with H_{12}-TAC and DDM, respectively (Fig. 7a). Samples purified in the presence of H_2F_6-TAC displayed a higher ATPase activity than with DDM and H_{12}-TAC a few

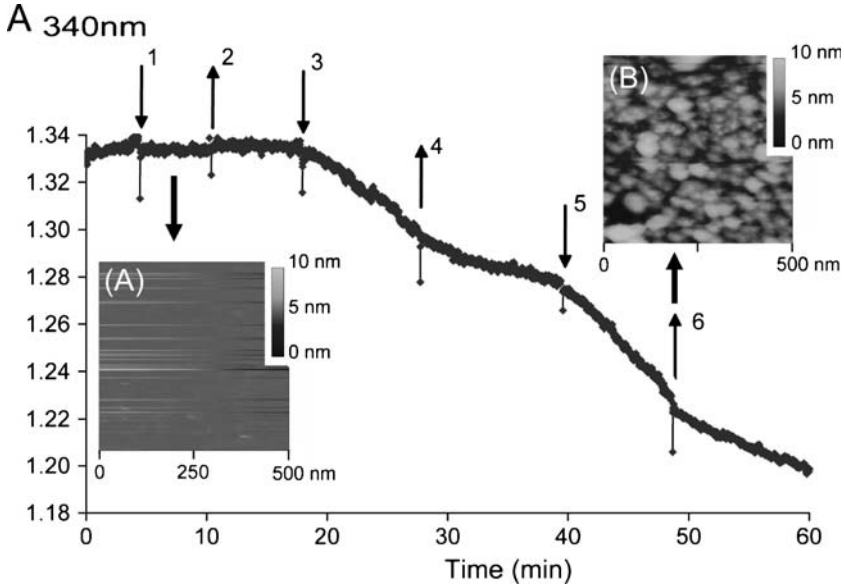

Fig. 6 ATPase activity of mica-adsorbed yeast ATP-synthase. Step 1: addition of the mica plate pre-incubated with buffer "P" alone; Step 2: removal of the mica plate; Step 3: addition of the plate pre-incubated with ATP-synthase (0.1 mg mL^{-1} in buffer "P"); Step 4: removal of the plate; Step 5: addition of the same plate as in step 3; Step 6: removal of the plate. In insert, AFM images in tapping mode of a mica surface **a** pre-incubated with buffer "P" with 0.85 mM H_{12}-TAC (5.5 cmc) or **b** pre-incubated with 0.1 mg mL^{-1} of ATP-synthase in the same buffer

days after enzyme extraction, but this activity quickly dropped upon storage at 4 °C to lower values than the ones observed with H_{12}-TAC and DDM after long storage times. A small reactivation of the enzyme purified in the presence of DDM was observed and for all surfactants but H_2F_6-TAC, all activity values converged to ~4 µmol min^{-1} mg^{-1}.

It has to be noted that specific activities were dependent on the concentration of enzymes added in the cuvette of measurement. Endogenous lipids or detergents/surfactants or even small subunits may dissociate upon dilution in the reactive medium and induce this protein concentration dependence. Hence, whatever the sample (DDM, H_{12}-TAC, H_2F_6-TAC or F_6-TAC) around 7 µg mL^{-1} of enzyme were always added in the cuvette. This dependence was not observed when additional lipids were added to the purified enzymes just after the Ni-NTA IMAC step.

When mixed with phosphatidylcholine, DDM, H_{12}-TAC, H_2F_6-TAC and F_6-TAC samples displayed, just after lipid addition, a strong increase in specific activity (Fig. 7b).

Kinetics of enzyme reactivation with lipids were slower with H_2F_6-TAC than with other detergents/surfactants, but this activity after 2 weeks to 3 weeks reached values close to the ones detected with H_{12}-TAC and F_6-TAC. Note that for H_{12}-TAC samples values were dispersed. This may reflect various endogenous lipid contents from one preparation to another. With the DDM purified sample the reactivation was only transitory and activity was divided by two after 6 weeks of storage.

We also followed the ATPase sensitivity towards DCCD and oligomycin for 39 days after enzyme extraction. For non relipidated samples (Fig. 7c), a decrease in enzyme sensitivity was observed for the H_{12}-TAC and H_2F_6-TAC purified samples, whereas in the presence of F_6-TAC sensitivities were always above 90%, even after 6 weeks. Sensitivity towards inhibitors was lost for the DDM purified enzyme. For all samples, the same inhibition tendency was observed with DCCD (not shown) and oligomycin. For relipidated samples (Fig. 7d), enzymes purified in the presence of fluorinated surfactants and H_{12}-TAC were still extremely sensitive to inhibitors, even after 6 weeks of storage at 13 °C. In contrast, relipidation of the DDM sample restored sensitivity towards inhibitors but this sensitivity decreased slowly with long storage.

Discussion

Solubilizing large membrane protein complexes is usually achieved by the use of classical detergents which often destabilize these large assemblies. Here we showed that using H_{12}-TAC, a surfactant bearing an oligomeric non ionic polar head derived from Tris, instead of DDM for the extraction step, better preserved the enzyme oligomeric forms. Using H_{12}-TAC instead of DDM for the unique chromatographic step also led to an enzyme activity that was twice higher with H_{12}-TAC than with DDM. As the two molecules have a dodecyl moiety this may be explained by the bulkier polar head of H_{12}-TAC that decreases the curvature radius of the surfactant torus (Abla et al. 2008) around the membrane domain and could influence the enzyme activity. However, with both detergents an activity drop of the enzyme was observed between the extraction and the Ni-NTA IMAC purification steps. To alleviate this problem, we have used fluorinated compounds, such as H_2F_6-TAC and F_6-TAC. These surfactants could minimize activity drop and F_6-TAC was more stabilizing than H_2F_6-TAC. The higher ATPase activity detected with these fluorinated compounds was probably

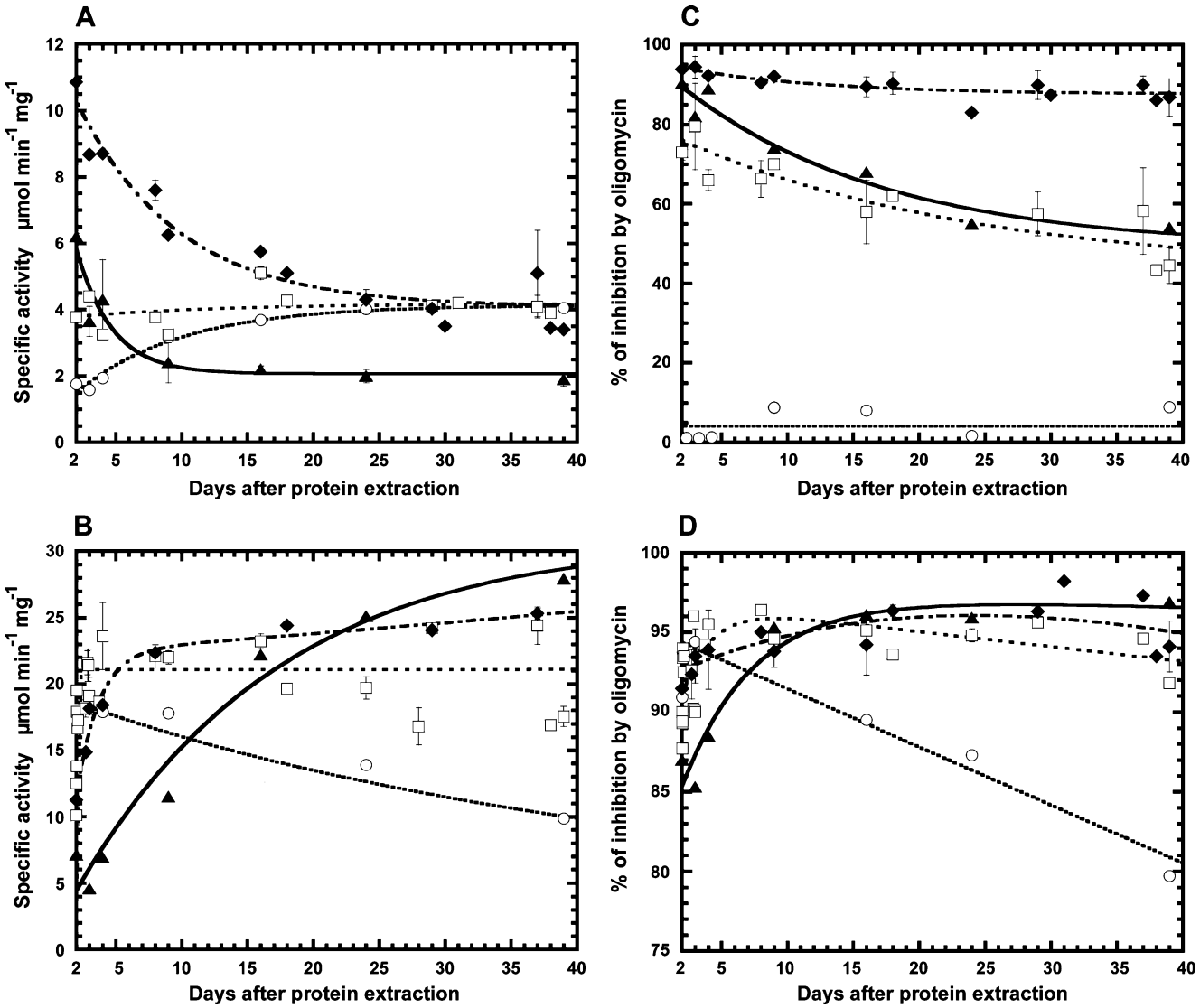

Fig. 7 Stability of ATPase activity and oligomycin sensitivity of ATP-synthase purified with different surfactants and incubated without or with PC. Specific ATPase activity (*left*) and inhibition by 6 µg mL^{-1} of oligomycin (*right*) of non relipidated ATP-synthase (**a** and **c**) or relipidated with PC at a LPR of 250 (**b** and **d**). Inhibitions (**c** and **d**) are expressed in percentage of the remaining activity determined at indicated days after extraction from membranes. Enzymes isolated in the presence of DDM (▪▪▪○▪▪▪), H_{12}-TAC (– –□– –), H_2F_6-TAC (—▲—) and F_6-TAC (– ·◆– ·). Measures in triplicate were performed from 2 days to 39 days after the extraction from membranes. Values are the averages of two or three independent purifications except for H_2F_6-TAC where only one experiment could be carried out

due to their more rigid hydrophobic part. Their rigid chains could have a lesser denaturing effect than hydrogenated alkyl chains by less removing peripheral subunits or by keeping some important endogenous lipids (Lebaupain et al. 2006).

Our experiments, performed in the presence of PC argue in favour of a loss of lipids during the single purification step with hydrogenated surfactants. Whereas the ATPase specific activity of the F_6-TAC purified sample almost doubled after relipidation (from 12 µmol min^{-1} mg^{-1} without PC to above 20 µmol min^{-1} mg^{-1} with PC), the ATPase activity was increased ten-fold when the DDM purified enzyme was mixed with PC, to reach the same values as those obtained with enzymes purified in the presence of fluorinated surfactants and relipidated. This indicates that the drop in activity of the DDM or H_{12}-TAC purified ATP-synthases did not arise from the loss of important subunits or the dissociation of the two ATP-synthase sectors since this could be reversed by a simple addition of lipids. While enzymes purified in the presence of H_{12}-TAC or DDM were reactivated in less than 1 h with lipids, this reactivation was slower with samples isolated in the presence of fluorinated surfactants. This may reflect different protein/surfactants dissociation rates or a low

association rate of lipids with protein-fluorinated surfactant complexes. This effect is more pronounced with H_2F_6-TAC indicating that the ethyl group designed to facilitate the adsorption of the hydrophobic chain onto trans-membrane domains of proteins has a strong impact on surfactant/lipid exchange. With F_6-TAC, it took 2 days to 3 days of incubation with PC before reaching a high activity value. It has to be noted that after 10 months of storage with lipids at 13 °C an ATPase activity of 14 µmol min^{-1} mg^{-1} was still measured for the F_6-TAC purified sample and the remaining activity was fully sensitive towards inhibitors. Hence F_6-TAC combined or not with lipids, seems to be the appropriate surfactant to maintain a high activity level of ATP-synthase for periods of time compatible with 3D crystallization experiments. Although the polar head is paucidisperse, we have started 3D crystallisation screens but no hit has yet been obtained. Since F_6-TAC has been shown not to alter lipid layers (Rodnin et al. 2008), this is also a promising molecule for 2D crystallization experiments either for reconstituting ATP-synthase in liposomes or for the monolayer crystallization method (Dauvergne et al. 2008; Lévy et al. 1999). We have tried to image with AFM F_6-TAC purified enzymes but have not yet obtained interesting results. Images obtained with H_{12}-TAC enzyme preparations have shown that this detergent is suitable for AFM experiments and allowed us to clearly distinguish spherical objects. A specific activity of ~4 µmol min^{-1} mg^{-1} was measured for the H_{12}-TAC purified ATP-synthase. The activity derived from the slope in Fig. 6 was around 0.9 nmol of ATP hydrolyzed per min. Thus if each enzyme on mica occupies at most a surface of ~220 nm^2 (enzyme lain along its longitudinal axis), and assuming all bound enzymes are as active as the free purified enzyme, this would indicate that nearly 40% of the plate would be covered by the enzyme. This estimation is in agreement with the surface coverage seen in Fig. 5d and e, but since there was some heterogeneity in deposits, as seen in the insert B of Fig. 6, it cannot be ascertain that all bound enzymes were as active as the enzyme in solution.

Electron microscopy (Rubinstein et al. 2003) and X-ray structures (Stock et al. 1999) gave 11×11 nm^2 and 20×11 nm^2 for the minimum and maximum projection sizes of mitochondrial ATP-synthase, if the object is seen from a top view or a lateral view respectively. Whatever the orientation adopted by the adsorbed enzymes, there is clearly an underestimation of the actual height. The apparent flattening is due to the AFM signal that combines various mechanical informations such as wear processes, elastic or visco-elastic response as well as topography, the size and the shape of the cantilever. For typical commercial cantilevers with a length ranging from 100 µm to 200 µm, a width of about 20 µm and an oscillation amplitude of about 10 nm, hydrodynamic damping induces forces that can reach values as high as several nanonewtons close to the surface (Dubourg et al. 2003). When imaging biological samples, one seeks forces in the piconewton range; therefore hydrodynamic forces produce a large background that may mask the more specific interactions between the tip apex and the biological sample. This viscous damping effect reduces the AFM sensitivity when working in dynamic mode to measure the phase and the amplitude. In order to minimize these effects, the width and the length of the cantilever must be reduced or the tip height must be increased. It was shown that the hydrodynamic forces due to the drag fluid could be decreased by almost one order of magnitude when reducing the cantilever width (Maali et al. 2005). Hence the current experimental device requires an adaptation for the use of such cantilevers in order to enhance the ATP-synthase image resolution. However, even if tips and AFM electronics still need to be improved to reach resolution as the one obtained with electron microscopy, an important point is the fact that binding on mica did not abolish ATPase activity. This shows potential for future dynamic experiments under the AFM tip.

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