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Biochimica et Biophysica Acta 1196 (1994) 145–153

BBA Biochimica et Biophysica Acta

Solubilization of the overexpressed integral membrane protein alkane monooxygenase of the recombinant *Escherichia coli* W3110[pGEc47]

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Received 13 April 1994

Abstract

The integral membrane-bound alkane monooxygenase (AlkB) from *Pseudomonas oleovorans* has been overexpressed in the recombinant *Escherichia coli* strain W3110[pGEc47] and expression levels of 10 to 15% relative to the total cell protein were reached. The amount of phospholipids in induced cells is about 3-fold higher compared to the wild-type and AlkB has been shown to be located in small membrane vesicles. We present here a study on the solubilization of these AlkB containing membrane vesicles by different detergents with special emphasis on structural requirements for a surfactant preserving the activity of AlkB. Moreover, the effects of the detergents used on the complete alkane hydroxylase system was studied.

Keywords: Detergent; Solubilization; Recombinant *E. coli*; Alkane monooxygenase; Overexpression

1. Introduction

The *Pseudomonas oleovorans* alkane hydroxylase system consists of three components: alkane hydroxylase (AlkB), rubredoxin (AlkG) and rubredoxin–NAD^+ reductase (AlkT). The molar ratio of these components in vivo is about 50:10:1 [1,2]. AlkB is an integral cytoplasmic membrane non-heme monooxygenase which is expressed and active both in *Escherichia coli* W3110[pGEc47] and *Pseudomonas oleovorans*. In *E. coli* W3110[pGEc47] AlkB is overexpressed after induction with dicyclopropylketone to about 10–15% of the total cell protein [3]. This was the first report on the significant overexpression of a foreign bacterial cytoplasmic membrane protein in *E. coli*. Using density gradient centrifugation it was shown that AlkB is highly enriched in low-density membrane vesicles. The topology of this enzyme has been studied in detail [4] and a model of AlkB has been developed. According to this model, AlkB contains six membrane spanning domains. A short part of the amino-terminus and a large carboxy-terminal domain are located in the cytoplasm.

Detergents have the ability to disrupt structures like membranes that are held together by hydrophobic forces [5]. For this reason detergents have been widely used to liberate membrane-bound proteins. The critical feature is the proper choice of detergent, since it is the detergent that disrupts lipid bilayers and replaces the lipids surrounding a particular protein [5], and thus has a substantial effect on protein stability. Hjelmeland and Chrambach [6] stated that the nature of the membrane being solubilized also plays an important role in the consideration of an appropriate detergent. Therefore, the detergent of choice should solubilize the membrane without denaturing the protein of interest.

We report here studies on the solubilization of the overexpressed integral membrane protein AlkB of the recombinant *E. coli* W3110[pGEc47] using a number of

Abbreviations: n-Octylglycoside, 1-*O*-n-octyl-β-D-glucopyranoside; n-dodecylglycoside, 1-*O*-n-dodecyl-β-D-glycopyranosyl(1-4)-α-D-glucopyranoside; MEGA-8, *N*-D-gluco-2,3,4,5,6-pentahydroxyhexyl-*N*-methyloctanamide; MEGA-10, *N*-D-gluco-2,3,4,5,6-pentahydroxyhexyl-*N*-methyldecanamide; Triton X-100, octylphenolpoly(ethyleneglycolether)$_{10}$; Triton X-100$_{hydr}$, octylcyclohexanepoly(ethyleneglycolether)$_{10}$; Thesit, dodecylpoly(ethyleneglycolether)$_9$; GENAPOL X-080, isotridecylpoly(ethyleneglycolether)$_8$; CHAPS, 3-((3-cholamidopropyl)dimethylammonio)-1-propane sulfonate; CHAPSO, 3-((3-cholamidopropyl)dimethylammonio)-2-hydroxy-1-propane sulfonate; *N*-dodecylsulfobetaine, *N*-dodecyl-*N*,*N*-dimethyl-3-ammonio-1-propane sulfonate; SDS, sodium dodecyl sulfate; Zwittergent 3-08, 3-(octyldimethylammonio)propane-1-sulfonate; Zwittergent 3-10, 3-(decyldimethylammonio)propane-1-sulfonate; Zwittergent 3-12, 3-(dodecyldimethylammonio)propane-1-sulfonate; Zwittergent 3-14, 3-(tetradecyldimethylammonio)propane-1-sulfonate; Zwittergent 3-16, 3-(Hexadecyldimethylammonio)propane-1-sulfonate.

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SSDI 0005-2736(94)00216-9

different detergents. Ionic, zwitterionic and nonionic detergents have been involved in this study and tested for their ability to solubilize AlkB under maintenance of its activity. Structural requirements for the effective solubilization with preservation of the activity of AlkB are discussed in detail. Furthermore, the effect of several detergents on the assay system has been investigated.

2. Materials and methods

2.1. Materials

NADPH, tetracycline, bovine serum albumin and detergents were purchased from Boehringer-Mannheim at the highest purity available. The Zwittergent detergents were purchased from Calbiochem. Salts, Pefabloc (serine proteinase inhibitor) and medium components were from Merck. Buffers and octane were products from Fluka. Bio-Beads SM-2 were purchased from Bio-Rad. Coomassie Brilliant Blue G-250 was a product of Serva. Spinach ferredoxin-$NADP^+$ reductase was purchased from Sigma. Dicyclopropylketone (DCPK) was a product of Aldrich. Purified rubredoxin (AlkG) was a generous gift of Jaap Kingma, University of Groningen (Netherlands).

2.2. Strain

Throughout the experiments, wild-type *E. coli* W3110((F⁻1⁻IN(rrnD-rrnE)1); laboratory collection) was used. Plasmid pGEc47 which contains the whole *alk* system (*alkB-L* as well as *alkST*) cloned in the broad host range vector pLAFR1 was constructed according to [1].

2.3. Cultivation and media

Recombinant *E. coli* were pre-cultured on LB-medium (250 ml) containing 10 g tryptone, 5 g NaCl and 5 g of yeast extract (pH 7). Minimal medium contained E2 salts supplemented with 2 ml 1 M $MgSO_4$ and 1 ml of MT microelement stock per l [7] and 1.0% (w/v) glucose as carbon source. Tetracycline was added to all cultures to a final concentration of 12.5 $\mu g\ ml^{-1}$. Cells were grown in a 42 l fermenter with a working volume of 25 l. The pO_2 was adjusted to 80% and the cells were stirred at 600 rpm. As anti-foam agent propylene glycol was added to a final concentration of 0.08 ml/l. When induction of the *alk* system was required, DCPK was added 4 h after inoculation to a final concentration of 0.05% (v/v). After cells started to become stationary, new medium components were added and the culture was allowed to grow until the second stationary phase was reached. Cell densities were determined from the absorbance at 450 nm and expressed in mg cell dry weight per ml, as described before [8]. The final cell dry weight reached was 1 mg/ml.

2.4. Cell disruption

Cells were harvested at 5000 rpm (4°C, 12 min) using a Heraeus cryofuge (Heraeus, Osterode). The pellet (90 g cell wet weight) was resuspended in disruption buffer consisting of 100 mM triethanolamine (TEA)-NaOH buffer (pH 7.4), supplemented with 50 μM Pefabloc and 2 mM sodium dithionite to a final density of 12% (w/v). Cells were disrupted twice in a Dyno-Mill (Bachofen, Basel) using a 600 ml-flow through container, 0.1–0.2 mm glass beads (Bachofen) and 2000 rpm at a flow rate of 5 l/h. Temperature was maintained at 0°C using a cryostat type D-M-K 30 (Bachofen, Basel). The glass beads were washed with disruption buffer. Subsequently, the cell debris was removed by centrifugation (12000 rpm, 10 min, 4°C, Sorvall 5534). The resulting crude extract (850 ml) contained 4.2 mg/ml of protein and 320 units of AlkB. Aliquots of 40 ml were stored at −20°C until further use.

2.5. Standard assay procedure for AlkB

The alkane hydroxylase assay was carried out in 1 ml reaction volume, containing 50 mM TEA-NaOH buffer (pH 7.4), 300 μg rubredoxin, 0.4 unit spinach ferredoxin–$NADP^+$ reductase, 0.2 mM NADPH and variable amounts of AlkB containing sample. For 2 min the background reaction was monitored at 340 nm (22°C) using a molar absorption coefficient of 6.22 $mM^{-1}\ cm^{-1}$ in a Cary IE spectrophotometer (Varian, Basel). Subsequently, the reaction was started by the addition of 10 μl octane (50 mM in acetone) and followed for further 2 min. Controls using acetone instead of octane showed no activity. Activity measurements were carried out in duplicate with less than 4% relative standard deviation. One unit of alkane hydroxylase (AlkB) activity is that amount of enzyme which oxidizes 1 μmol NADPH/min under the conditions specified.

2.6. Solubilization experiments

Initial solubilization experiments were carried out at a protein concentration of 2 mg/ml. Stock detergent solution (10%, w/v) was added to aliquots of crude extracts to yield detergent concentrations of 0, 0.11, 0.32, 1.0 and 3.0%. The absorbance at 375 nm (initial absorbance 0.8) of the mixture was then recorded for 1 h. The temperature was 22°C. Absorbance was corrected for dilution by the detergent solution and given as percent, normalized to 1 (A_{375}: 0.8) in the absence of detergent. The absorbance of the detergent solutions alone was negligible at all concentrations tested.

A second solubilization experiment using detergents selected in the initial detergent mapping was carried out at a protein concentration of 3 mg/ml at different detergent to protein ratios. Special tubes with a nominal volume of 5.0 ml were used (Beckman). After incubation of AlkB-

containing crude extract with detergent for 1 h, the mixture was centrifuged at 105 000 × *g* for 1 h at 4°C using a Beckman ultracentrifuge (Model LB-70M) and a SW65 Ti rotor. As control AlkB-containing crude extract without the addition of detergent was run. The resulting supernatant was assayed for AlkB activity as described above.

2.7. Protein determination

Protein contents were determined as described by Lowry et al. [9] using the Bio-Rad Lowry detergent compatible assay system in the presence of sodium dodecylsulfate. Bovine serum albumin (Boehringer) was used as a standard. The absorption at 750 nm was recorded in a Uvikon 810 spectrophotometer (Kontron).

2.8. Determination of critical micellar concentration (CMC)

The CMC value of hydrogenated Triton X-100 has not been reported yet and was determined by the method described by Samsonoff and co-workers [10].

3. Results

3.1. Fed batch fermentation of recombinant E. coli W3110[pGEc47]

The recombinant *E. coli* W3110[pGEc47] was grown on minimal medium on the 30-l scale in a fed batch mode of operation. The growth curve of this fermentation is shown in Fig. 1. After three cell doublings, the *alk* genes were induced with the gratuitous inducer dicyclopropylketone (DCPK). As described earlier [3] using small flasks cultures, the induction of the *alk* genes has an effect upon the final cell density reached when compared to the non-induced strain. The final cell density reached in the presence of inducer was about 70% of the density reached in its absence. The final cell density of *E. coli* W3110[pGEc47] on the first step of the 25-l fermentation was exactly the same as described by Nieboer and co-workers [3] for the small scale. After the first stationary phase was reached, new medium components were added and the strain was allowed to grow for an additional 24 h. The final cell density reached was 30% higher compared to the density of the first step. After harvesting and disrupting the cells a total activity of 320 units of AlkB was obtained (11 U/l fermentation broth).

3.2. Initial solubilization of AlkB

Fig. 2 presents the data of the initial detergent screening. The highly turbid crude extract (A_{375}: 1.6) obtained after cell disruption was incubated with detergent solutions of the concentrations indicated in the figure and the absorbance at 375 nm was measured. After 1 h of incubation at room temperature, aliquots were withdrawn. Subsequently, the remaining activity of AlkB was measured spectrophotometrically. Activities are given as percent, normalized to 1 (0.3 U/ml) in the absence of detergent.

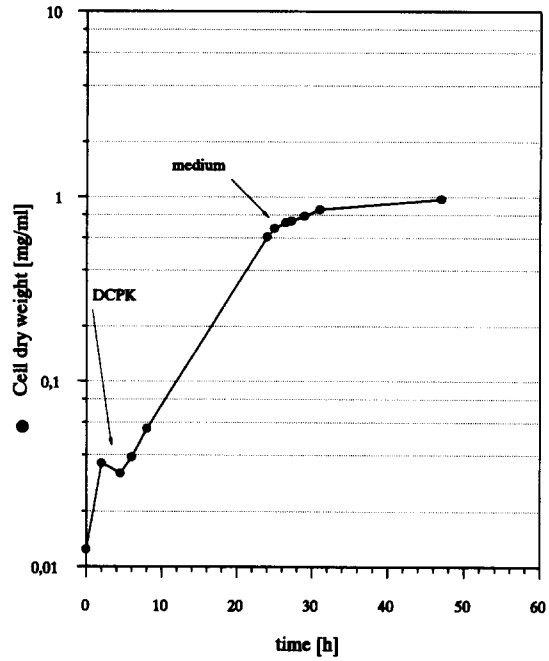

Fig. 1. Growth curve of *E. coli* W3110[pGEc47] in fed batch mode of operation. *E. coli* W3110[pGEc47] was precultured on LB-medium (250 ml) to a final density of 7.4 (21 h). The 30 l fermenter was inoculated with this preculture (1%) to a density of 0.012 g/l cell dry weight. The medium was E2+MT with 1% glucose as carbon source (see Materials and methods). After 4 h dicyclopropylketone (DCPK) was added to a final concentration of 0.05%. After cells started to become stationary, new medium components were added and the culture was allowed to grow for additional 20 h to reach the final cell density of 1 g/l cell dry weight.

The detergents vary significantly in their effects on AlkB activity and their ability to influence the turbidity of the crude extract containing membrane vesicles. Some detergents like MEGA-8 and GENAPOL X-080 were ineffective in solubilizing the lipid vesicles. The increase in turbidity on raising the detergent concentration seen for some surfactants (CHAPSO, GENAPOL X-080, deoxycholate) can be explained either by the formation of mixed micelles, or by fusion of small vesicles forming larger vesicles, or by the formation of enlarged structures such as cylindrical micelles [11]. Octylglycoside was only able to partially solubilize the membrane vesicles well above its CMC value. Other detergents, such as the Zwittergent series, sodium deoxycholate, Triton X-100, hydrogenated Triton X-100 and dodecylmaltoside, effectively solubilized the membrane vesicles containing AlkB. Depending on the characteristics of the detergent itself, the general rule can be given that the detergent concentration has to be above the CMC value of the particular detergent to solubilize the AlkB containing vesicles effectively. There are also some

detergents like GENAPOL X-080 which do not behave according to this general rule. The ionic detergents such as sodium deoxycholate and sodium dodecylsulfate solubilize AlkB containing membranes even well below their CMC values. However, most of the non-ionic detergents can be regarded as 'well-behaved'. This rule has also been observed using liposomes as membrane models [12].

Only five detergents out of 18 tested had no or little effect on the remaining activity of AlkB at concentrations up to 3% (detergent/protein ratio of 15), and simultaneously were able to reduce the turbidity of the crude extract to a low level. These detergents were CHAPS, Zwittergent 3-16, Triton X-100 and hydrogenated Triton X-100 and dodecylmaltoside. With the exception of CHAPS, which has a relatively high CMC, all of the detergents mentioned in the previous sentence have CMC values below 0.1%.

3.3. Determination of the CMC value of Triton X-100 (hydrogenated)

Fig. 3 presents the graphical determination of the CMC value of hydrogenated Triton X-100, which has not been

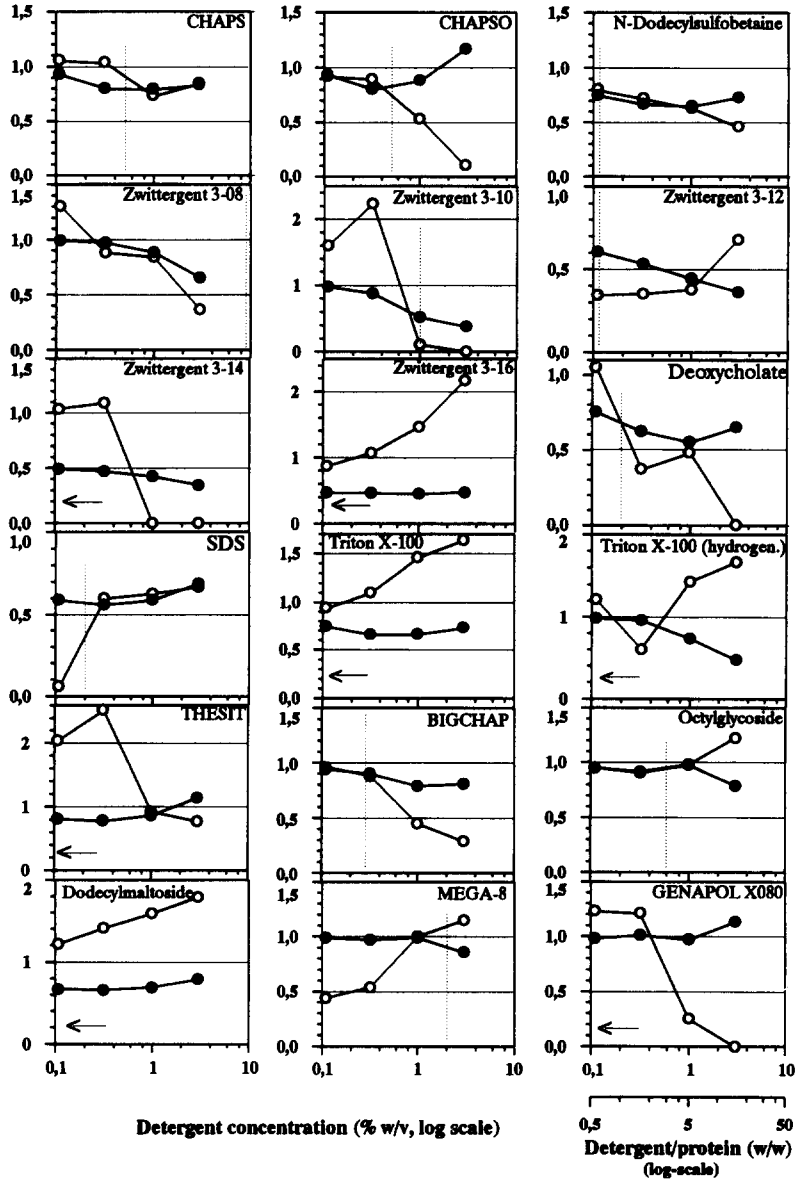

Fig. 2. Solubilization of AlkB containing lipid vesicles from *E. coli* W3110[pGEc47] and effects on the remaining activity of AlkB. The turbidity of crude extract in detergent solution of the indicated concentration is represented by closed circles (●), normalized to the original absorbance at 375 nm which was 0.8. The critical micellar concentration (CMC) is given by the dashed vertical line, or if off scale, by the horizontal arrow. AlkB was incubated with the indicated concentrations of detergent and then diluted 10-fold for assay of remaining octane hydroxylation activity. Activity is expressed normalized to the activity measured in the absence of detergent using the same incubation conditions (○). The protein content was set to 2 mg/ml in all experiments. Different detergent concentrations were used (0%, 0.11%, 0.32%, 1%, 3%) to obtain detergent-to-protein ratios between 0 and 15 (see additional axis). Absorbances at 375 nm were corrected for dilution by the detergent solution (10% stocks).

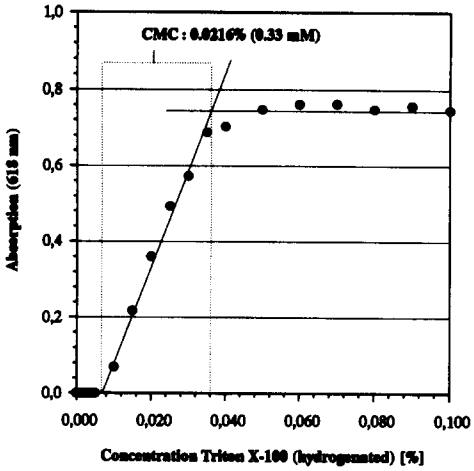

Fig. 3. Determination of the critical micellar concentration (CMC) of hydrogenated Triton X-100. Different concentrations of Triton X-100 (reduced) were mixed with Coomassie Brilliant Blue G-250 (0.05 mg/ml) and incubated for 10 min. The change of absorbance was measured at 618 nm and plotted versus the final detergent concentration. The CMC value is given by the concentration reached at one half of the linear increase.

reported yet. The change of absorption at 618 nm is plotted versus the concentration of Triton X-100 (hydrogenated) and the CMC value is determined as described by Samsonoff and co-workers [10] to 0.0216% or 0.33 mM. Hence, the CMC value of hydrogenated Triton X-100 is within the range of CMC values reported for Triton X-100 (0.2–0.9 mM).

3.4. Optimization of the protein / detergent ratio of selected detergents

In a second set of experiments several detergents selected from the initial screening were tested using a different solubilization criterion than turbidity measurements. The most often used criterion, sedimentation at 105 000 × *g* for 1 h, had been chosen and the retention of AlkB activity in the supernatant after solubilization was measured. Nevertheless, some very small AlkB-containing membrane vesicles still stay in the supernatant on prolonged centrifugation at 105 000 × *g* (see Fig. 4). A range of detergent-to-protein ratios covering the range between 0.1 and 25 have been tested in this set of experiments. The results of these studies are presented in Fig. 4.

Solubilization of AlkB containing vesicles with Triton X-100 resulted in an optimum detergent-to-protein ratio of 15 with 100% recovery of AlkB in the supernatant, whereas solubilization using CHAPS resulted in a lower optimum ratio of 10 with 90% recovery of activity. In contrast, solubilization using octylglycoside was only effective at relatively high detergent-to-protein ratios of more than 20 with maximum recovery of AlkB activity of about 60%.

3.5. Influence of detergents on the AlkB assay system

The assay system for the determination of AlkB is presented in Fig. 5. Since this assay system for measuring the activity of AlkB contains several proteins, and detergent is brought into the assay mixture for measuring the

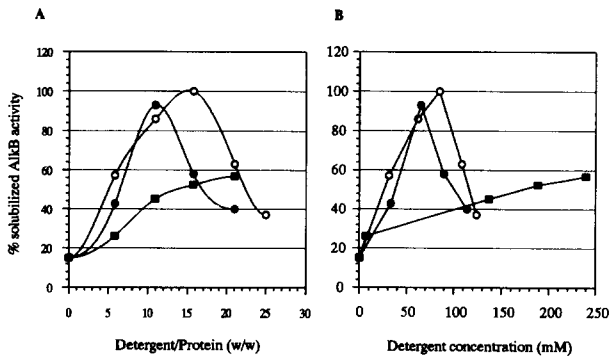

Fig. 4. Optimization of the detergent-to-protein ratio for three different detergents selected from the initial solubilization experiment. The experiment was carried out at a protein concentration of 3 mg/ml at various detergent-to-protein ratios. Three different detergents were tested: Triton X-100 (○), CHAPS (●) and octylglycoside (■). In part A the percentage of solubilized AlkB is plotted against the detergent-to-protein ratio and in part B against the final detergent concentrations. After incubation of AlkB-containing crude extract with detergent for 1 h, the mixture was centrifuged at 105000× *g* for 1 h at 4°C. As control AlkB-containing crude extract without the addition of detergent was run and the AlkB activity seen in the supernatant is due to very small AlkB-containing membrane vesicles.

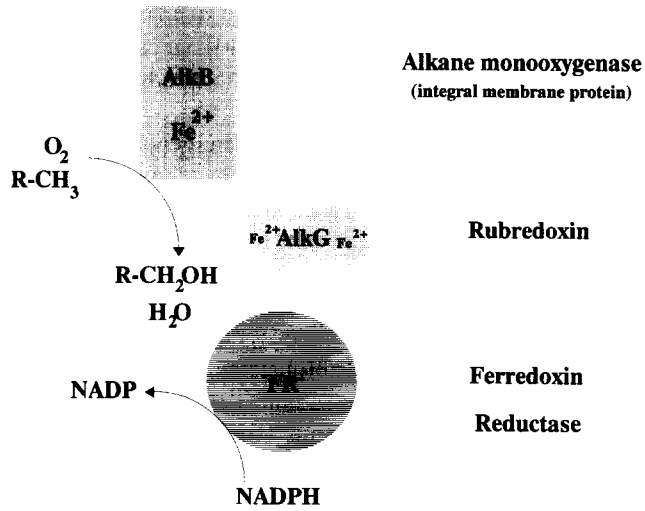

Fig. 5. Assay system for the determination of the activity of the alkane monooxygenase (AlkB). Electrons are transferred from NADPH via ferredoxin reductase (spinach) to rubredoxin (AlkG) and further transferred to the integral membrane component AlkB, which contains the catalytic centre to bind different alkanes or alkenes and oxidizes these compounds at the expense of molecular oxygen and the electrons derived from NADPH. The native system contains rubredoxin reductase instead of ferredoxin reductase.

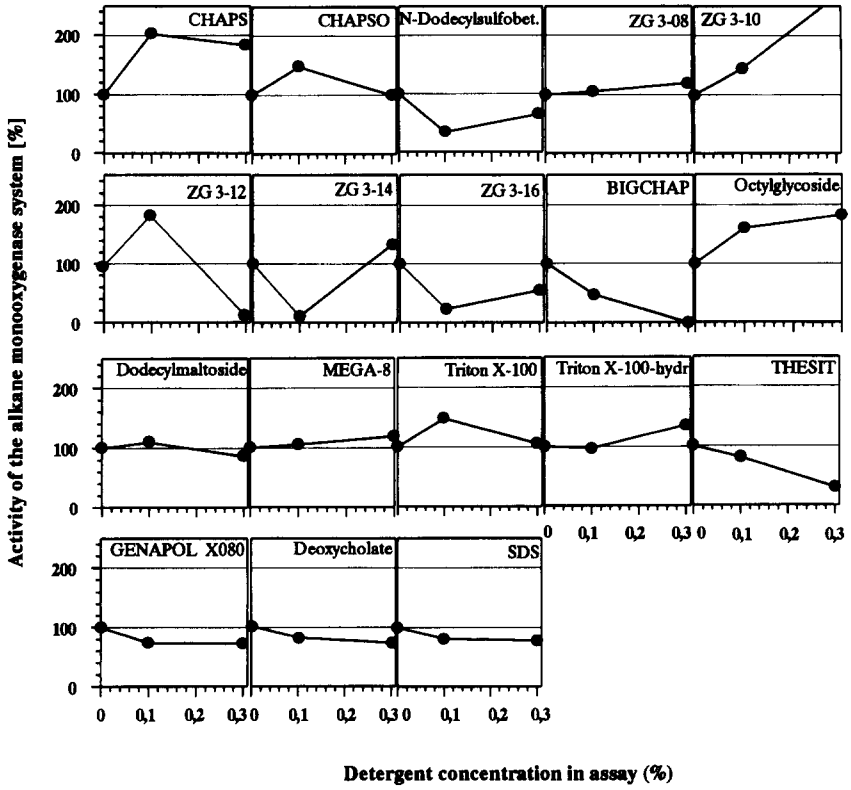

Fig. 6. Influence of different detergents on the alkane monooxygenase complex. The activity of the alkane monooxygenase system was measured as described in Materials and methods at different final concentrations of detergent given in the figure. The activity of the alkane monooxygenase system is given relative to the activity measured in the absence of detergent (100%, 86 mU/mg).

remaining activity of AlkB, the influence of different detergents on the complete alkane hydroxylase system was investigated in separate experiments. The results of these experiments are shown in Fig. 6. Unfortunately, the detergents can not be removed using the standard procedure described by Horigome and Sugano [13], since AlkB binds strongly to the highly hydrophobic Bio-Beads SM-2 (data not shown).

As shown in Fig. 6, most of the detergents tested more or less influence the AlkB assay system at final concentrations up to 0.3%. Eight out of 18 detergents had no significant effect in average on the assay system (CHAPSO, Zwittergent 3-08, Zwittergent 3-12, Zwittergent 3-14, Triton X-100, hydrogenated Triton X-100, dodecylmaltoside and MEGA-8). Further six detergents had slightly inhibitory effect (*N*-dodecylsulfobetaine, Zwittergent 3-16, Thesit, GENAPOL X-080, sodium deoxycholate, sodium dodecylsulfate), three detergents (CHAPS, Zwittergent 3-10, octylglycoside) had activating influence and only one detergent (BIGCHAP) strongly inhibited the assay components.

4. Discussion

The alkane hydroxylase has been isolated several times from *Pseudomonas oleovorans* [14,15] using 0.1% sodium deoxycholate, but a thorough detergent screening has not been published yet. Nieboer et al. [3] showed that AlkB expression in recombinant *E. coli* W3110[pGEc47] changes the composition of the phospholipids, as well as the fatty acid composition of the membranes. Since the lipid composition of membranes influences the sensitivity to detergents [16,6], previous procedures are hardly transferable to the solubilization of AlkB from the recombinant *E. coli*.

4.1. Initial solubilization experiments

If the ratio of normalized AlkB activity to normalized absorbance for incubations at 1% is taken for purpose of comparison, the values of the five detergents are 1 (CHAPS), 2.9 (Zwittergent 3-16), 2.3 (Triton X-100), 2.1 (Triton X-100 hydrogenated) and 2.3 (dodecylmaltoside). Hence, in contrast to the liposome system described by Womack and co-workers [12], there are only little differences in the lowest concentration of detergent that is able to solubilize the lipids.

It is noteworthy that all detergents used in the solubilizing experiments are especially purified compounds for applications in membrane protein biochemistry. However, the differences between Zwittergent 3-12 (CalBiochem, 99% by GC) and *N*-dodecylsulfobetaine (Boehringer, ho-

mogeneous by TLC) may be explained by differences in their purity.

Focusing on the influence of the five detergents mentioned above on the activity of AlkB, it turns out that, with the exception of CHAPS, these detergents increase greatly the remaining activity compared to the untreated control. This might be due to the high degree of packing of AlkB in the membrane vesicles [3] leading to inaccessibility of AlkB to the other proteins forming the active alkane monooxygenase complex. On solubilization, these formerly unaccessible AlkB molecules become accessible resulting in higher activity.

Another interesting observation was the influence of sodium dodecylsulfate (SDS) on the solubilization and activity of AlkB. Below the CMC value of SDS AlkB is completely inactivated. Increasing the concentration of SDS above the CMC value (0.2%), AlkB activity is maintained to about 60% compared to the initial activity without the addition of SDS. Hence, AlkB is one exception to the behaviour of most enzymes which completely loose their activity upon addition of SDS [5].

Sodium deoxycholate was used by Katopodis [15] at a final concentration of 0.1% to solubilize AlkB isolated from *Pseudomonas oleovorans*. Unfortunately, Katopodis did not show additional data of the solubilization experiment. As shown in Fig. 2, increasing the concentration of sodium deoxycholate above 0.1% leads to increasing inactivation of AlkB. Hence, this detergent is not suitable for the solubilization of active AlkB.

Womack and co-workers [12] gave the general recommendation derived from experiments with liposomes that, for effective solubilization with minimum damage to proteins, detergents should be used at concentrations at or, preferably, slightly below their CMC values. This would lead to the formation of mixed micelles containing protein, lipid and detergent molecules [5]. These mixed micelles are known to interfere with separation methods, especially with gel permeation chromatography. In contrast, in our study of the solubilization of AlkB, the enzyme can be solubilized by certain detergents at concentrations that lead to mostly complete delipidation of AlkB. However, there are also cases, such as GENAPOL X-080, where increasing the concentration of the detergent into the delipidation region leads to complete loss of activity of AlkB. Therefore, care has to be taken concerning the generalization of the behaviour of some enzymes in model systems and applying this behaviour to other integral membrane proteins. There still remains the requirement for empirical screening [17].

4.2. Structural requirements of detergents for the solubilization of active AlkB

From Fig. 2 several structural requirements for a detergent can be derived that both solubilizes AlkB containing membrane vesicles and preserves the activity of the alkane monooxygenase. For instance, Zwittergent 3-08, CHAPS and CHAPSO solubilize AlkB containing vesicles relatively ineffectively compared to longer chain zwitterionic detergents such as Zwittergent 3-14 and 3-16. The latter detergents cause the highest decrease in turbidity among all of the surfactants tested.

The influence of the hydrophilic head group can be seen comparing certain detergents with each other. Zwittergent 3-12, dodecylmaltoside and sodium dodecylsulfate (SDS) have the same C_{12} alkyl chain, but differ in their head groups. Zwittergent 3-12 with a zwitterionic group solubilizes AlkB containing membranes more effectively than dodecylmaltoside and SDS. BIGCHAP, CHAPS, CHAPSO and deoxycholate have also nearly the same hydrophobic group and differ only in the hydrophilic regions. Among these detergents, deoxycholate having a small ionic head group solubilizes AlkB containing membranes most effectively. The structures of CHAPS and CHAPSO differ only very slightly in one hydroxyl group, demonstrating a strong structural requirement for the zwitterionic group $-N^+(CH_3)_2-(CH_2)_3-SO_3^-$ to solubilize AlkB containing vesicles.

Regarding the maintenance of the AlkB activity, another set of requirements for a suitable detergent can be derived. The length of the alkyl side chain should be between C_8 to C_{16} and the hydrophilic part at best is a glycosyl or polyethoxy group. The alkyl chain should not be branched at its distal part (GENAPOL X-080), but it might be branched in the proximal region (Triton series). *N*-Alkylsulfobetaines are known to be strongly denaturing [18]. Unexpectedly, Zwittergent 3-16 did not denature AlkB while solubilizing the membrane vesicles very effectively.

From these observations the following picture of an ideal detergent for the solubilization of functional AlkB can be drawn. The detergent should contain either a zwitterionic or polyethoxy head group and the alkyl chain should be between C_{12} and C_{16} without terminal branches (proximal branches are allowed). In contrast to previous studies [19,20], the alkylglycosides were not very efficient in solubilization of AlkB containing membranes. On the other hand, these compounds did not denature AlkB and they might be used to preserve AlkB activity at further stages of the purification procedure.

Nieboer and co-workers [3] presented a detailed description on the effects of the overexpression of AlkB (10–15% of the total protein) on the lipid composition of *E. coli* W3110[pGEc47]. Interestingly, they found a strong increase of 16:1 and 18:1 unsaturated fatty acids, while the saturated 16:0 stayed constant and 12:0, 14:0, 17:0cyc, 18:0 and 19:0cyc decreased on induction of AlkB. The decrease seen for 17:0cyc and 19:0cyc has been interpreted [3] by binding of the precursors 16:1 and 18:1 to AlkB, leading to inaccessibility to the cyclopropane fatty acyl synthetase [21,22]. Regarding the phospholipids in induced W3110[pGEc47], cardiolipin was present at a significant

higher level compared to *E. coli* W3110 and the concentration of phosphatidylglycerol, the precursor of cardiolipin, decreased. The amount of phosphatidylethanolamine (PE) remained approximately constant (80% of total lipid) and they did not observe significant differences between the lipid compositions of cytoplasmic membrane and AlkB containing membrane vesicles. Hence, the major lipid components of induced *E. coli* W3110[pGEc47] are 16:1 and 18:1 phosphatidylethanolamines. Since the conformation of the double bonds in the unsaturated fatty acids is *cis* rather than *trans* (unpublished data), the links in the fatty acids lead to a disruption of the ordered packing of phospholipids in the bilayer resulting in space for the incorporation of integral membrane proteins. The shift of the fatty acid composition from saturated to unsaturated fatty acids is consistent with the incorporation of AlkB into the membrane vesicles.

At pH 7.4, the pH used in the solubilization experiments, the terminal amino group of PE is positively charged, resulting in the zwitterionic structure -O-PO_2^--O-CH_2-CH_2-NH_3^+. The complementary zwitterionic structures are present in the zwitterionic detergents (-$N^+(CH_3)_2$-CH_2-CH_2-CH_2-SO_3^-), which solubilized AlkB containing membranes most effectively. An alkyl chain of C_{12} to C_{16} leads to preservation of the AlkB structure which is consistent with the fatty acid composition of membrane lipids in AlkB containing vesicles.

4.3. Optimization of the detergent-to-protein ratio

As described by Hjelmeland and Chrambach [6,23], two cases can be observed: (i) solubilization without progressive inactivation of enzyme activity at high detergent concentrations and (ii) solubilization with an apparent optimum detergent concentration. As can be seen from Fig. 4, in the case of AlkB containing vesicles, octylglycoside behaves as described under (i) while Triton X-100 and CHAPS behave as described under (ii) showing an optimum concentration of detergent.

Comparison of the results obtained in the optimization experiment with the initial solubilization experiment based on turbidity measurements, demonstrates the general applicability of the latter method to screen the effectivity of detergents. For example, solubilization of AlkB containing crude extract with Triton X-100 (see Fig. 2) and the analysis by the turbidity measurement demonstrates optimum activity recovery and reduction of turbidity at a detergent-to-protein ratio of 15 (3% Triton X-100). The same conformity can be seen for CHAPS and octylglycoside as well.

4.4. Influence of detergents on the AlkB assay system

Positive effects of the detergents on the assay system may be attributed to better accessibility of AlkB entrapped in membrane vesicles to the other assay components like rubredoxin and ferredoxin–$NADP^+$ reductase. Negative influences are in some cases due to inactivation of AlkB (compare with remaining activities shown in Fig. 2), in other cases attributed to the other proteins in the assay mixture. For example, *N*-dodecylsulfobetaine shows negative influence on the stability of AlkB (see Fig. 2) at final concentrations up to 0.3% (about 70–80% remaining activity), and also exhibited negative effect on the complete alkane hydroxylase system (about 80% of the maximum activity without surfactant). That means, *N*-dodecylsulfobetaine most probably only affects AlkB, but not the other proteins in the assay. On the other hand, Thesit does not affect the stability of AlkB negatively, but shows negative influence on the assay components.

In conclusion, there are considerable differences between the detergents tested concerning the ability to solubilize AlkB and, simultaneously, maintaining its enzymatic activity. Solubilization of the dense packed overexpressed integral membrane-bound enzyme alkane monooxygenase leads to an increase in activity due to better accessibility by the other proteins forming the active multi-component complex. The detergent of choice should contain either a zwitterionic or polyethoxy head group and the alkyl chain should be between C_{12} and C_{16} without terminal branches, while proximal branches are allowed. In a recent paper [3] evidences were presented that $C_{16:1}$ and $C_{18:1}$ phosphatidylethanolamines are the major lipids bound to AlkB during overexpression in *E. coli* W3110[pGEc47]. The structure of the zwitterionic detergent leading to the most effective solubilization while preserving the activity is similar to the lipid components surrounding AlkB.

Acknowledgements

We thank Dr. Bruno Winter, Dr. Jan van Beilen and Maarten Nieboer for stimulating discussions.

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