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Purification of a phosphatase which hydrolyzes phosphatidic acid, a key intermediate in glucolipid synthesis in *Acholeplasma laidlawii* A membranes

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Abstract

A phosphatidic acid phosphatase (PAP; EC 3.1.3.4.), dephosphorylating phosphatidic acid (PA) to diacylglycerol (DAG), was identified and purified from the plasma membrane of *Acholeplasma laidlawii* A. After four purification steps, including membrane preparation, Tween 20 solubilization, preparative gel electrophoresis and electro-elution, PAP was purified about 400 times to near homogeneity. The molecular weight of PAP was according to SDS–polyacrylamide gel electrophoresis ≈ 25 kDa and the enzyme was a stable and integral membrane protein. It is proposed to catalyze the first enzymatic step in the important glucolipid pathway of *A. laidlawii*. No essential cofactors or activator lipids were found. However, some divalent cations and phosphate analogues were potent inhibitors. Beside the in vivo substrate (PA), PAP was found to dephosphorylate *p*-nitrophenylphosphate. This less stringent specificity makes alternative in vivo functions for PAP plausible, the importance which is discussed. © 1997 Elsevier Science B.V.

Keywords: Enzyme; Phosphatidic acid phosphatase; Phosphatidic acid; Diacylglycerol; Glucolipid; Membrane protein; (*Acholeplasma laidlawii*)

1. Introduction

In the single membrane of the small and cell wall-less prokaryote *Acholeplasma laidlawii* A (i) a constant surface charge density, given by the anionic polar membrane lipids [1,2], (ii) similar phase equilibria, close to a potential bilayer to nonbilayer phase transition [3], and (iii) an almost constant spontaneous curvature [4], are metabolically maintained for the bilayer lipid mixture under a variety of conditions in vivo, see Rilfors et al. [5]. This involves extensive changes in the relative amounts of the individual lipids as well as between the phospholipid, phosphoglucolipid and glucolipid classes (e.g., [2]). Key lipids

Abbreviations: PAP, phosphatidic acid phosphatase; PA, phosphatidic acid; *p*NPP, *p*-nitrophenylphosphate; DOPG, dioleic phosphatidylglycerol; DAG, diacylglycerol; PGP, phosphatidylglycerophosphate; MGlcDAG, monoglucosyldiacylglycerol; DGlcDAG, diglucosyldiacylglycerol; MABGPDGlcDAG, monoacylbisglycerophosphoryl–DGlcDAG; GPDGlcDAG, glycerophosphoryl–DGlcDAG; MAMGlcDAG, monoacyl–MGlcDAG; MADGlcDAG, monoacyl-DGlcDAG; 16:0, palmitic acid; 18:1*c*, oleic acid; TLC, thin-layer chromatography; BSA, bovine serum albumin; BCA, bicinchoninic acid; SDS, sodium dodecyl sulphate; PAGE, polyacrylamide gel electrophoresis; NOGA, *N*-octanoyl-β-D-glucosylamine; CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio)]-1-propane sulfonate; EDTA, ethylenediaminetetraacetic acid

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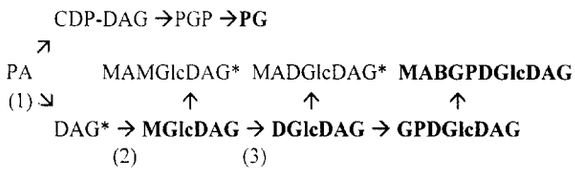
in this regulation are the monoglucosyldiacylglycerol (MGlcDAG) and diglucosyldiacylglycerol (DGlcDAG), forming mainly nonlamellar and lamellar phases with unsaturated acyl chains, respectively. Under certain conditions variants of these two with an extra acyl chain (i.e., monoacyl; MA) attached to the glucose head can also be made [6]; lipids of this latter type also occur in certain Gram-positive bacteria [7]. Most likely, the different lipid-synthesizing enzymes in *A. laidlawii* sense and set the different physical properties of the bilayer in a coordinate manner. The biosynthetic pathways have not been formally established but are considered to be as follows:



Lipids in bold are always made, albeit in varying amounts see [2,5]. Lipids with an asterisk may accumulate under certain conditions [2,6], especially when the phase equilibria of MGlcDAG are shifted from nonlamellar towards lamellar phases by more saturated acyl chains. These latter three (i.e., *) all form nonbilayer/nonlamellar aggregates, but with varying propensities. PA (phosphatidic acid), made by the enzymatic addition of two acyl chains to *sn*-glycerol-3-phosphate, CDP–DAG (cytidine-diphosphate–DAG) and PGP (phosphatidylglycerol-phosphate) are minor precursors. PA, CDP–DAG and DAG have all been detected in *A. laidlawii* membranes [8].

The important glucolipid pathway (above) is probably initiated by enzyme 1, a hypothetical PA-phosphatase producing DAG; enzyme 2 is the recently purified MGlcDAG synthase [9], and enzyme 3 is the following DGlcDAG synthase [1]. However, in certain Gram-positive bacteria phosphatidylglycerol (PG) is the donator of the *sn*-glycerol-1-phosphate (GP) polar head of the phosphoglucolipids; this also yields a free DAG moiety [7]. For *A. laidlawii* this would mean:

DGlcDAG + PG → GP–DGlcDAG + DAG

GPDGlcDAG can in turn probably be modified to a monoacyl-bis-glycerophosphoryl variant (i.e., MABGP–DGlcDAG), see Haukson et al. [10] for structures. The released DAG from these reactions is a potential substrate for the MGlcDAG synthase (see above). However, under different conditions in vivo the membrane amounts of GPDGlcDAG and MABGPDGlcDAG are substantially less than the glucolipid amounts [2,6]. Hence, DAG released from PG during phosphoglucolipid synthesis is most likely not sufficient for the glucolipid synthesis.

Consequently, an enzymatic step producing DAG from PA (phosphatidic acid phosphatase; PAP; EC 3.1.3.4), as indicated here and as found in sugar-lipid containing photosynthetic organisms, is likely to be present in *A. laidlawii*. The present work describes the identification, characterization and purification of such an enzyme, and supports the metabolic pathways suggested (above).

2. Materials and methods

2.1. Growth conditions and protein determination

Strain A-EF22 of *A. laidlawii* was grown at 28°C in a tryptose–bovine serum albumin (BSA) medium supplemented with 0.12 mM oleic acid (18:1*c*). Cells were harvested by centrifugation 20 min at 20 000 × *g*, washed twice and stored at −80°C in β-buffer (0.05 M Tris–HCl pH 7.4, 0.15 M NaCl and 0.01 M 2-mercaptoethanol). The protein content was determined by the BCA or the Micro-BCA kit (Pierce) in micro-titre plates at 540 nm by a Multiskan MCC/340 (Labsystem). BSA was used as reference, and an average of three analyzes were performed for each sample.

2.2. Cell lysis and membrane preparation

Intact cells, dissolved in β-buffer to ≈ 22 mg protein per ml, were centrifuged at 20 000 × g for 20 min and the pellet was resuspended in β-buffer diluted to osmolytic concentration (1:20 v/v). The solution was stirred for 30 min at 37°C for cell lysis and then centrifuged at 2500 × *g* for 20 min to remove intact cells. Membranes were collected at 39 000 × *g* for 40 min. About 3% of the total PAP activity was found in the supernatant (data not shown). The membranes were washed once with 1:20 β-

[1] Vikström et al., manuscript in preparation.

buffer. All centrifugation steps during the enzyme preparation were performed at 4°C.

2.3. Partial solubilization of membranes with Tween 20

Membrane pellets were suspended in 1:20 β-buffer containing 2.5% (w/v) of the detergent Tween 20. After smooth stirring for 2 h at 20°C the solution was centrifuged at $37\,000 \times g$ for 2 h. This procedure solubilizes ca. 40% of the membrane proteins [11], but less than 2% of the total PAP activity was found in the micellar supernatant. The extracted pellet was suspended in 1:20 β-buffer.

2.4. Preparative SDS–polyacrylamide gel electrophoresis

A 9 cm 12% acrylamide separating gel (ratio acrylamide to bis-acrylamide 30/0.8 w/w), and a 3 cm 4% stacking gel, was polymerized at 6°C in a 37 mm wide tube of a preparative gel apparatus (BioRad model 491 Prep Cell). The discontinuous buffer system of Laemmli [12] was used. The electrophoresis chambers and the elution reservoir were filled with running buffer (0.025 M Tris, 0.192 M glycine, and 0.1% SDS (w/v)). Tween 20-extracted membranes (7 mg protein/ml) were mixed (1:2, v/v) with loading buffer containing 0.06 M Tris–HCl pH 6.8, 2% SDS (w/v), 5% 2-mercaptoethanol, 10% glycerol (v/v) and 0.025% bromphenol blue (w/v) and incubated for 10 h at 6°C with intermittent stirring. A 9 ml sample was loaded upon the gel 12 h after the stacking gel was casted. The electrophoresis was run in the cold (6°C) for 22 h at an average power of 8.5 W (200 V constant voltage), with circulation (100 ml min^{-1}) of the lower chamber buffer through a 2 m tube submerged in a cold water bath. The elution buffer flow rate was 1.0 ml min^{-1}; fractions of 10 ml were collected from the gel bottom after the dye front, starting at ≈ 10 h of run time, and analyzed for protein concentration and PAP activity. Highest specific activity was detected 19 h from the start. 8 Fractions containing PAP activity were saved.

2.5. Separation in SDS slab gels

Fractions from the preparative SDS–PAGE column containing PAP activity were concentrated in 10 K Microsep™ micro-concentrators (Filtron) by centrifugation at $5000 \times g$ for 30 min. A $2X$ volume of SDS–gel cocktail [13] was mixed with the concentrate and incubated for 2 h at 20°C (without boiling). Proteins were then separated by the discontinuous SDS–PAGE system of Neville as described by Nyström et al. [13]. The preparative slab gel (1 mm thickness/15% (w/v) acrylamide) was run for 1300 V h at 20°C. Gels were stained for 30 min in 0.25% (w/v) Coomassie Brilliant Blue dissolved in methanol/acetic acid/H_2O (50:7:43), and destained for 2 h in methanol/acetic acid/H_2O (20:5:75).

2.6. Electroelution

4 slices, each corresponding to one Coomassie-stained protein band, were cut out from the gel slab and electro-dialyzed (BioRad electro-eluter model 422) for 3 h at 50 mA constant current in 50 mM NH_4HCO_3 and 0.1% (w/v) SDS. The volume above the dialysis membrane (12–15 kDa cut-off) was then collected, concentrated as above and analyzed for protein composition and concentration, and PAP enzyme activity (see below).

2.7. Determination of protein purity and molecular mass of PAP

The extent of protein purification during the procedures was analyzed by SDS–PAGE in thin slab gels (15% acrylamide), essentially run and processed as the thicker slabs (above). Electrophoresis calibration kit from Pharmacia LKB Biotechnology were used as molecular mass markers.

2.8. Enzyme assays

The general procedure for the enzymatic hydrolysis of PA to diacylglycerol (DAG) and phosphate was performed in protein/lipid/detergent mixed-micelles. Dioleoylphosphatidic acid (DOPA) (Avanti Polar Lipids, USA), dissolved in 1,1,1-trichloroethane, was mixed with [^{14}C]dipalmitoylphosphatidic acid (DPPA) (DuPont) labeled in the glycerol backbone. After evaporation under a stream of N_2 for 30 min, 50 μl assay buffer was added (0.2 M Tris–Maleate pH 8.0 and 40 mM Triton X-100 (Riedel-de-Haën)). This mixture was incubated for 3 h at 4°C and agitated by vortexing at least 3 times. 50 μl of

protein solution from the purification procedure (above) was added to the lipid/detergent emulsion to yield a final concentration of 3 mM PA (with 22 mCi/mol lipid of [^{14}C]PA) and 20 mM Triton X-100, and mixed by vortexing (10 s) before incubation for 30 min at 37°C. The enzymatic hydrolysis was stopped by 375 μl methanol/chloroform 2:1 (v/v). Lipids were then extracted with a modified Bligh and Dyer method [14], and separated by thin-layer chromatography (TLC) on silica gel 60 plates (Merck) developed in chloroform/aceton (96:4 v/v). Proper lipid references were included on the TLC plates. The conversion of PA to DAG (see below) was quantified using electronic autoradiography (Packard Instant Imager). The specific activity of the PA phosphatase was expressed as nmol DAG synthesized per h and mg of protein.

During the course, we found that *p*-nitrophenylphosphate (*p*NPP) could serve as an enzyme substrate for monitoring PAP activity. Because of the simple detection of the released colored product, this was used for routine screening of the phosphatase activity during the purification procedures. 50 μl of protein solution was mixed with 40 μl of assay buffer (above) in a micro-titre plate. After addition of 10 μl 50 mM *p*NPP (Sigma Diagnostics) the plate was smoothly shaken and incubated for 30 min at 37°C. The reaction was stopped by 100 μl of 0.5 M NaOH, and the intensity of the yellow color (released *p*-nitrophenol; *p*NP) was determined at 405 nm. The background absorbance was subtracted from the average values. The enzymatic formation of *p*-nitrophenol was expressed as nmol per h and mg of protein, taking the molar extinction coefficient at 405 nm as 18800 M^{-1} cm^{-1}. In some experiments, the phosphate enzymatically released from the PA, lyso-PA or *p*NPP substrates was quantified using the method of Baginski et al. [15].

2.9. Identification of DAG

It is well known that diacylglycerol can undergo isomerization under certain circumstances [16]. Unlabeled *rac*-1,2-dioleoyldiacylglycerol and *sn*-1,3-dioleoyldiacylglycerol were used as TLC references, stained with Rhodamin 6G after development, and visualized by UV light. Spots corresponding to 1,2-DAG and 1,3-DAG were revealed, the latter with a higher R_f-value in the developer used. However, quantification of DAG after the PAP assays showed that less than 15% of the total [^{14}C]DAG enzymatically released was the 1,3-DAG species.

3. Results and discussion

3.1. Phosphatidic acid phosphatase

A. laidlawii strain A contained an enzymatic activity that could hydrolyze PA, radioactively labeled in the glycerol backbone, to yield phosphate and 1,2-DAG. The latter species was identified by its unique migration in thin-layer chromatography. When solubilized cells were assayed with rising concentration of PA, an increasing synthesis of DAG was recorded up to a saturating state (Fig. 1). Due to the water-insoluble character of the PA substrate and the DAG product the assays were performed in mixed micelles of the detergent Triton X-100 plus PA substrate, sometimes also with other lipids or amphiphiles present, see materials and methods. Essentially all enzyme activity was confined to the purified cytoplasmic membranes. This PAP activity could not be detached from the membranes by salt washings (e.g., NaCl) usually performed to release peripheral membrane proteins, nor by chaotropic agents like 0.8 M NaBr and 0.8 M KSCN (data not shown). Hence, the enzymatic dephosphorylation of PA, visualized in Fig. 1, seems to be localized to the plasma membrane of the bacterium.

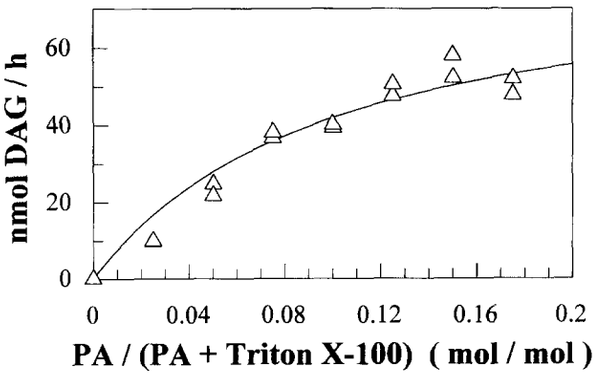


Fig. 1. PA phosphatase in *A. laidlawii*. Intact cells were solubilized with TX-100 and the activity analyses were performed in PA/TX-100 mixed micelles (see Section 2).

3.2. Solubilization of membranes

Total solubilization of the membranes by the anionic detergent SDS, followed by enzyme assay in the same detergent, completely abolished the PAP activity. Several mild (nonionic) detergents, like Tween 20 [11] or NOGA [17], yielded a partial solubilization of the membrane proteins where most (≈ 90%) of the PAP activity remained in the insoluble fraction after ultracentrifugation (≈ 30% of the protein), see materials and methods. Such membrane fractions are enriched in hydrophobic proteins compared to native membranes [11]; i.e., studies by Johansson et al. showed that *A. laidlawii* proteins not soluble in Tween 20, contain more hydrophobic amino acid residues than the Tween-soluble proteins [18]. A selective detergent solubilization, or retainment in the membrane matrix, has previously been the basis for the initial separation and purification of several *A. laidlawii* membrane proteins [11], including a *p*-nitrophenylphosphatase with a molecular mass determined to ≈ 25 kDa [19]. Like PAP, the latter enzyme was not solubilized by Tween 20 and it was therefore used as a marker during the preparation procedures here. For PAP a ten fold enrichment in the Tween 20 insoluble fraction was obtained, compared to intact cells. In conclusion, the behavior of PAP towards different salts and detergent indicates that it is an intrinsic, probably very hydrophobic membrane protein, or a protein with one or two transmembrane anchoring domains but where the rest could be hydrophilic.

3.3. Enzyme purification

PAP remained active in membranes after Tween extraction, and activity lost in SDS-solubilized samples could be restored by the addition of surplus nonionic detergent (Tween 20 or Triton X-100, see Table 1). A high thermal stability was also noted in neutral detergents, e.g., CHAPS; no PAP activity was lost after 3 days at 20°C. Purification of the enzyme protein based on charge interactions failed, i.e., ion exchange or hydroxyapatite column chromatography in the presence of Triton X-100 detergent (data not shown), perhaps due to a potentially dominating hydrophobic character of the enzyme (cf. above).

A strong enrichment of the PAP enzyme activity was obtained by preparative SDS gel electrophoresis (cf. Section 2). The activity was regained after diluting the SDS (to 2 mM) in the obtained column fractions and supplementing with Triton X-100 (to 20 mM), see Fig. 2. Elution profiles for activity towards the two substrates PA and *p*NPP followed each other closely. The extent of enrichment for the PAP activity in the best fractions were hundred fold, yielding 6 bands on a SDS slab gel (Fig. 3).

The protein bands in the high-activity fractions were electro-eluted, following a preparative SDS slab gel. The fractions were then assayed for enzyme activity, and analyzed in thin SDS slab gels. Results from a silver-stained gel indicated a purification to near homogeneity (≈ 400-fold enrichment). The molecular mass of the protein band corresponding to the PAP activity was determined to ≈ 25 kDa (see

Table 1
Purification scheme for the phosphatidic acid phosphatase from *A. laidlawii*

Purification step	Total activity (nmol h^{-1})	Protein (mg)	Specific activity (nmol $h^{-1} mg^{-1}$)	Yield (%)	Enrichment
1. Intact cells	1 920 000	1100	1745	100	1
2. Membranes	1 200 000	156	7700	63	4
3. Tween 20-solubilized membrane remains	462 000	51	18 100	24	10
4. Preparative SDS gel electrophoresis					
One fraction:	59 000	0.31	190 000	3	109
Pool of 4 fractions:	184 800	1.39	133 100	10	76
5. MicroSep-conc., SDS slab gel and electroelution	4600	0.006	767 000	0.2	440

The purification steps were performed as described in Section 2.

Fig. 3). The observation that PAP is an intrinsic membrane protein together with the findings that the enzyme is possible to reactivate after denaturation in a SDS-solution, makes the comparison in mobility with water-soluble proteins in SDS–PAGE less trustable. If the structure of PAP is differently denaturated compared to the reference proteins then the determined size of PAP might be incorrect. However, there were no difference in mobility of the proteins if the samples studied were boiled or not.

In the last two steps of the purification procedure PAP is kept soluble by SDS, probably in a nonactive configuration. The total activity recovered after those steps are dependent on the reactivation of the enzyme. Reactivation of the *p*-nitrophenylphosphatase by Hjertén [19] regained 100% of the lost activity, but after 2 weeks of dialysis. Therefore, the activity of PAP detected here are probably to low compared to the amount of PAP (Table 1). However, the activity regained after this reproducible purification procedure is most likely catalyzed by the single protein purified.

3.4. Influence of ions and PH

The consecutively acting MGlcDAG and DGlcDAG synthases both have Mg^{2+} as an essential cofactor [9,20]. 10 mM of the metal chelator EDTA did not change the PAP-activity in fractions eluted from the preparatory column significantly, compared to the reference samples, indicating an independence of divalent ion cofactors (Table 2). None of the ions tested yielded any stimulation, but Cd^{2+}, Hg^{2+} and Zn^{2+} caused increasing inhibition. Low concentrations of molybdate and vanadate, potent inhibitors for phosphatases, abolished nearly all the PAP-activity. Phosphate, on the other hand, was not a strong inhibitor.

The dephosphorylation of both PA and *p*NPP occurred in a pH range between 6 and 10, with an optimum of about pH 8. This is fairly similar to the pH range and maximum growth for *A. laidlawii* cells, respectively.

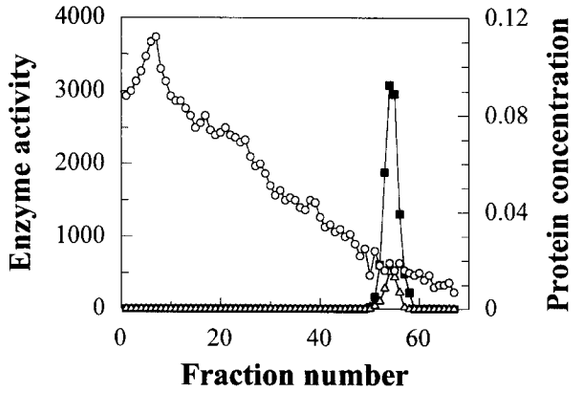


Fig. 2. Preparative column SDS–polyacrylamide gel electrophoresis. Elution profiles of protein (○), μg ml^{-1}; PA phosphatase (■), nmol DAG h^{-1} ml^{-1}; and *p*-nitrophenyl phosphatase (△), 10× nmol *p*NP h^{-1} ml^{-1}, from a standard run of preparative SDS–PAGE (see Section 2).

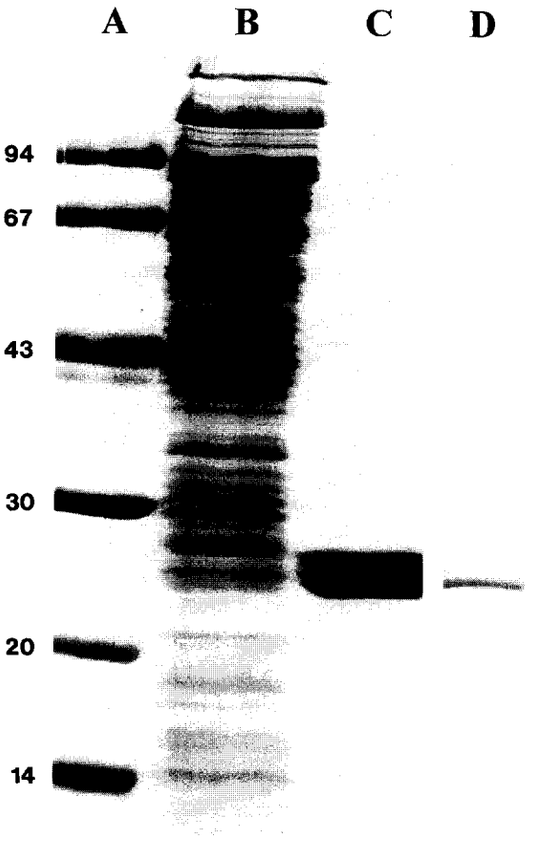


Fig. 3. Analytical SDS–polyacrylamide gel electrophoresis. The gel, stained with Coomassie Brilliant Blue, shows the protein yield from different steps in the purification procedure. Lane A: Molecular mass markers with the size (kDa) indicated at the side of the figure; Lane B: *A. laidlawii* membranes; Lane C: High activity fraction from a preparative SDS–PAGE run; Lane D: Homogenous fraction after electro-elution.

Table 2
Ion and lipid influence on PAP-activity

Sample [a]	% activity
Reference	100
10mM EDTA	104
1 mM $CdCl_2$	71
1 mM $HgCl_2$	53
1 mM $ZnCl_2$	49
1 mM Na_2MoO_4	10
1 mM $NaVO_3 \times H_2O$	1
20 mM pNPP	41
15% DOPG [b]	99
30% DOPG [b]	99

The enzyme was partially purified by preparative SDS–polyacrylamide gel electrophoresis, diluted 10 times with assay buffer (see Section 2) and incubated for 12 h at 4°C. The assays were performed in a PA/Triton-X 100 mixed micelle solution supplemented with the specific ion or lipid. The synthesis of DAG or *p*NP was detected as described in Section 2. The values are averages of two experiments.

[a] The samples contain about 0.02% SDS (w/v).

[b] The concentration of DOPG is defined as % of total amphiphiles.

3.5. Substrate specificity

Activity studies on the 440-fold purified enzyme (Figs. 2 and 3) indicated that PAP may dephosphorylate both the PA and *p*NPP substrates. Hence, these two activities presumably reside in a single (purified) protein enzyme unit. This was supported from the inhibiting effect of *p*-nitrophenylphosphate on the PAP activity (Table 2). A concentration of 20 mM *p*NPP clearly interfered with the enzymatic hydrolysis of PA, yielding DAG. This inhibition seems to be competitive since a release of *p*-nitrophenol was observed at the same time. A *p*-nitrophenylphosphatase activity has previously been found firmly associated with the membranes of several mycoplasma species including *A. laidlawii* [21,22]. In the latter species, the activity seems not to be dependent upon the physical state of the membrane lipids, in contrast to e.g. the ATP phosphatase [21]. The lipid independency seems to be valid even for the PAP-activity. No significant difference in activity were detected together with the anionic lipid DOPG (Table 2). The specificity in chain length of the substrate were not analyzed but PAP was active against both the di-16:0 and di-18:1*c* species of PA. Interestingly, a fraction of partially purified PAP did also dephosphorylate lysophosphatidic acid.

3.6. Functional role of PAP

The findings that PAP seems to be a rather unspecific enzyme suggests that other substrates, like lipids with a free phosphate group bound to the polar head, can be hydrolyzed by PAP. The *pgp* B gene in *E. coli* [23] coding for a phosphatidylglycerophosphate (PGP) phosphatase was shown to dephosphorylate not only PGP, but also phosphatidic acid and lysophosphatidic acid. Since PGP is an intermediate in the phospholipid pathway of *A. laidlawii* as well (see above), PAP might be a multi-functional phosphatase with two functions in the lipid metabolism, i.e., the phospholipid and the glucolipid pathways. Interestingly, the gene product from *pgp* B is an intrinsic membrane protein with a molecular mass of 28 kDa, similar to the size determined here for the PAP. An eukaryotic phosphatidate phosphohydrolase (PAP-2) was purified from the plasma membrane of rat liver [24,25]. PAP-2 was identified as two forms with the masses of 51 and 53 kDa in a SDS-gel, but treatment with *N*-glycanase decreased the apparent molecular mass to 28 kDa for both.

The very limited number of genes in *Mycoplasma genitalium* [26], the smallest species found so far, has raised the question whether enzymes with more than one function may exist in such cells. A potential function of PAP in both the glucolipid and phospholipid pathways may be a mechanism to join two house-keeping activities in the same enzyme in *A. laidlawii*, another small genome mycoplasma.

The turnover number here determined for PAP (Table 1) is in the same range or even higher as the consecutive synthesis of MGlcDAG [9]. The specific activity in the latter was determined to 79 nmol MGlcDAG per h and mg of protein. Therefore, it is concluded that PAP has the ability to produce enough DAG for the glucolipid synthesis in the membrane of the cell. However, this does not exclude the possibility that a phosphatidyl transferase, producing DAG from PG, is expressed in *A. laidlawii*.

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