Purification and properties of a phosphate-irrepressible membrane-bound alkaline phosphatase from *Zymomonas mobilis*

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The phosphate-irrepressible alkaline phosphatase of the ethanol-producing bacterium *Zymomonas mobilis* was solubilized from membranes by the cationic detergent *N*-cetyl-*N,N,N*-trimethylammonium bromide (CTAB) and purified by fast protein liquid chromatography. The purified enzyme was a monomeric protein of molecular mass 54 kDa, highly resistant to heat and to ionic strength. The alkaline phosphatase of growing *Z. mobilis* cells (ZAPase), remained active in the presence of an ethanol concentration as high as 100 g l⁻¹. However, *in vitro*, the stability of the purified ZAPase was severely affected at low ethanol concentration (7.8 g l⁻¹), showing the importance of the membrane environment *in vivo*. ZAPase differed from other bacterial alkaline phosphatases by having a higher affinity for the substrate 4-nitrophenylphosphate, a higher K_i for phosphate, only a partial reactivation by Zn²⁺ after EDTA inhibition, and a higher specific activity.

Introduction

Zymomonas mobilis, an obligately fermentative micro-organism which converts glucose, fructose and sucrose to ethanol via the Entner–Doudoroff pathway (Swings & De Ley, 1977), has been previously reported as containing a membrane-bound and phosphate-irrepressible alkaline phosphatase (Michel & Baratti, 1989). Constitutive and phosphate-irrepressible alkaline phosphatases (APases) have been described in *Capnocytophaga* (Poirier & Holt, 1983), *Bacteroides ruminicola* (Cheng & Costerton, 1973) and *Bacillus licheniformis* (Hulett, 1987) but these enzymes were not found associated with membranes. Although not exceptional (Von Tigerstrom & Stelmashuk, 1986), the membrane location of bacterial APases is rather rare since these enzymes are generally detected in the periplasmic space of most of the Gram-negative bacteria studied (Bhatti *et al.*, 1976; Cheng & Costerton, 1973; Filloux *et al.*, 1988; Garen & Levinthal, 1960; Kobori *et al.*, 1984; Poirier & Holt, 1983; Roy *et al.*, 1982). Thus, with respect to its mode of expression

(absence of phosphate repression) and its location (membrane-bound), the enzyme of *Z. mobilis* (ZAPase) can be considered as atypical. A characteristic such as membrane location might reflect specific requirements in relation to the alcoholic environment of the bacterium. Little is known about phosphate metabolism in *Z. mobilis*. However, alkaline and acid phosphatase activities have been described in *Z. mobilis* CP4 (Pond *et al.*, 1989) and an APase has been purified from *Z. mobilis* ZM4 by chromatography on multifunctional adsorbents (Reyes & Scopes, 1991). This paper deals with the purification by fast protein liquid chromatography (FPLC) of a highly active ZAPase from detergent-solubilized membranes of the ZM1 and ZM4 strains of *Z. mobilis*.

Methods

Bacterial strains, and growth conditions. *Zymomonas mobilis* ZM1 (ATCC 10988) and ZM4 (ATCC 31821) were grown statically at 30 °C, in complex medium as previously described (Michel & Baratti, 1989). Growth was monitored by measurement of optical density at 450 nm.

Phosphatase assay. APase was assayed at 30 °C in a reaction mixture containing 0.1 ml enzyme extract and 0.9 ml 0.1% (w/v) 4-nitrophenyl phosphate (pNPP) in 0.1 M-Tris/HCl pH 8.0. The amount of substrate hydrolysed per unit time was calculated from the absorbance of 4-nitrophenol liberated using a molar absorption coefficient of $1.62 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ at 410 nm. One unit was defined as the amount of enzyme which liberates 1 μmol nitrophenol min⁻¹. The use of HEPES buffer instead of Tris buffer in the assay decreased activity by only 30%,

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Abbreviations: APase, alkaline phosphatase; ZAPase, *Zymomonas mobilis* alkaline phosphatase; p-NPP, 4-nitrophenyl phosphate; CTAB, cetyltrimethylammonium bromide; DOC, sodium deoxycholate; OTAC, octadecyltrimethylammonium chloride; SLS, sodium lauryl-sarcosinate.

suggesting that transphosphorylation on Tris (Roy *et al.*, 1982) was limited. Therefore, Tris buffer was used for the assay. APase activities at different pH values were determined according to Von Tigerstrom (1984).

Preparation of cell extracts. Cells grown in complex medium were centrifuged (7000 g, 10 min, 4 °C) washed in 10 mM-Tris/HCl pH 7.4 and resuspended in the same buffer after centrifugation. Cells were disrupted after passage through a French pressure cell (two passages at 294 MPa, one passage at 392 MPa) or ultrasonically disintegrated using a Branson Sonifier (three periods of 15 s at 45 W, with 45 s intervals between periods). Membranes were recovered in the pellet after ultracentrifugation of the cell extract (170 000 g, 90 min, 4 °C).

Protein assay. Proteins were assayed as described by Schaeferle & Pollach (1975) with BSA as a standard. Detergent-treated samples were assayed after removal of the detergent according to Wessel & Flugge (1984).

Solubilization of APase from *Z. mobilis* membranes. Membranes resuspended in 10 mM-Tris/HCl pH 7.5 were incubated with detergents (final concentration 0.5%, w/v) for 30 min at 20 °C under mild agitation. Treatment was carried out with anionic detergents (sodium lauryl sarcosinate and sodium deoxycholate), non-ionic detergents (Triton X-100, Triton X-114, Tween 80, Brij 35 and Nonidet P40), cationic detergents (*N*-cetyl-*N,N,N*-trimethylammonium bromide, octadecyltrimethylammonium chloride) and a zwitterionic detergent (*N*-dodecyl-*N,N*-dimethylammonio-3-propanesulphonate). Detergent-treated membranes were centrifuged (100 000 g, 90 min, 4 °C) and the solubilized membrane components recovered in the supernatant.

Purification of membrane-bound APase. *Z. mobilis* cells were grown in a 20 litre fermenter containing 19 litres of complex medium (100 g glucose l⁻¹). The membranes were obtained as described above after disruption in a French pressure cell, resuspended in 10 mM-Tris/HCl pH 7.5 and treated for 1 h at 20 °C with 1% (w/v) *N*-cetyl-*N,N,N*-trimethylammonium bromide (CTAB). The insoluble fraction was removed by ultracentrifugation (100 000 g, 90 min, 4 °C) and the supernatant containing APase dialysed for 24 h at 4 °C against 10 mM-Tris/HCl, 0.25% CTAB, pH 8.5. The dialysed fraction was loaded on a Q-Sepharose column (2.5 cm × 40 cm, filled with 160 ml gel, Pharmacia) equilibrated with 10 mM-Tris/HCl, 0.25% CTAB, pH 8.5 and the adsorbed proteins eluted with a two-step KCl gradient, using an FPLC system (Pharmacia). The first gradient step (0–0.5 M-KCl) was carried out within 30 min and the second step (0.5–1 M-KCl) within 1 h (flow rate 1 ml min⁻¹). Fractions (1.4 ml) were collected. Fractions containing APase were pooled, dialysed for 24 h at 4 °C against 10 mM-Tris/HCl, 0.25% CTAB, pH 7.5 and applied to a Mono Q column (0.5 cm × 5 cm, filled with 1 ml gel, Pharmacia) equilibrated with 10 mM-Tris/HCl, 0.15% CTAB, pH 7.5. The adsorbed proteins were eluted in 10 mM-Tris/HCl, 0.25% CTAB, pH 7.5 within 1 h using a linear KCl gradient (0 to 0.1 M-KCl in 10 mM-Tris/HCl, 0.25% CTAB, pH 7.5). Fractions of 0.6 ml were collected. Fractions containing APase activity were pooled, concentrated on ultrafree-PF membranes (UFP1-LGC14, Millipore) and applied to a Superose 12 column (1 cm × 30 cm, filled with 24 ml gel, Pharmacia). Proteins were eluted with 100 mM-Tris/HCl, 0.15% CTAB, pH 7.5. Elution of the proteins was monitored as absorbance at 280 nm; 0.6 ml fractions were collected and assayed for APase activity. Fractions containing APase activity were pooled and concentrated on ultrafree-PF membranes.

Substrate specificity. Determination of substrate specificity was carried out at 1 mM concentration with the following substrates: 4-nitrophenyl phosphate (*p*NPP), AMP, ATP, glucose 6-phosphate and fructose 6-phosphate. The amount of inorganic phosphate liberated by APase activity was determined according to Saheki *et al.* (1985).

Determination of kinetic parameters. The K_m was determined from Hanes plots (substrate concentration/velocity against substrate concentration) which were shown to give more precise results than Lineweaver–Burk plots. Substrate was added to the reaction mixture at the following concentrations: 0.67, 1.34, 2.0, 2.7, 6.7, 13.4, 20.1, 26.8, 33.5 µM. The K_i for phosphate was determined from Dixon plots (1/velocity against inhibitor concentration). KH_2PO_4 was added to the reaction mixture at the following concentrations: 2.5, 1.0, 0.5, 0.25, 0.1 and 0.05 µM. Assays were carried out using three different substrate concentrations close to or above the K_m value (2.7 µM-, 6.7 µM-, and 13.4 µM-*p*NPP).

Polyacrylamide gel electrophoresis. One dimensional SDS-PAGE (10.5%, w/v, acrylamide) and two-dimensional PAGE were performed according to Laemmli (1970) and O'Farrell (1975), respectively. APase staining on gels was carried out as described previously (Michel & Baratti, 1989).

Results

Enzyme solubilization

Detergents of four different classes (anionic, neutral, cationic and zwitterionic) were tested for their ability to release APase from a *Z. mobilis* membrane preparation without affecting activity. In our experimental conditions, the enzyme was not solubilized by Triton X-100 and only low levels of unsedimentable APase were obtained after treatment with Tween 80, Nonidet P40 and *N*-dodecyl-*N,N*-dimethyl-ammonio-3-propanesulphonate (3 to about 8% of the total activity) (Table 1). Sodium deoxycholate (DOC), Triton X-114 and Brij 35 were more efficient solubilizers since 34 to 47% of the total activity was released from membranes treated with these detergents (Table 1). By contrast, more than 80% of the APase activity was released by sodium lauryl-sarcosinate (SLS), *N*-cetyl-*N,N,N*-trimethylammonium

Table 1. Effectiveness of detergents in the solubilization of the APase of *Z. mobilis*

Detergent	Charge	Soluble APase (%)*
None	0	ND
Sodium laurylsarcosinate (SLS)	—	81.2
Sodium deoxycholate (DOC)	—	34.4
Triton X-100	0	ND
Triton X-114	0	46.9
Tween 80	0	3.0
Brij 35	0	34.4
Nonidet P40	0	7.9
Cetyltrimethylammonium bromide (CTAB)	+	87.5
Octadecyltrimethylammonium chloride (OTAC)	+	82.3
Dodecyltrimethylammonio-3-propanesulphonate	±	4.8

* Results are expressed as percentages of the activity recovered in the soluble fraction after treatment of membranes with detergents as described in Methods. ND, not detected.

bromide (CTAB) and octadecyltrimethylammonium chloride (OTAC). Removal of the ionic detergent from the enzyme preparation by dialysis or replacement with Triton X-100 led to complete enzyme inactivation. The requirement of ionic detergents for enzyme solubilization suggested that in *Z. mobilis*, APase is bound through strong interactions to some components of the cell membrane. CTAB, the most efficient solubilizing agent, did not affect enzyme stability and therefore, it was selected for the purification procedure.

Purification procedure

APase from *Z. mobilis* ZM1 (ZAPase) was purified by FPLC from a membrane fraction solubilized with CTAB, using a procedure involving three chromatographic steps (Table 2). Treatment of a membrane fraction with 1% (w/v) CTAB resulted in a 7.6-fold purification, with an activity yield of 87%. The soluble fraction was loaded on a Q-Sepharose column and the adsorbed proteins eluted with a two-step KCl gradient. ZAPase eluted at 0.75 M-KCl with a 100.4-fold purification (Fig. 1*a*, Table 2). The enzyme was further purified on a Mono Q column and eluted with a linear KCl gradient (0 to 0.1 M) at 0.05 M-KCl (Fig. 1*b*). The specific activity was increased 12-fold compared to the previous step, with a yield of 54%. The active fractions were pooled, concentrated by ultra-filtration and loaded onto a Superose 12 column. Proteins were separated into four major peaks and the activity recovered into the first eluted peak (Fig. 1*c*). The purification was achieved with an overall yield of 36.3% and the final purification factor was 8693-fold (Table 2). The specific activity of the enzyme (89×10^4 IU mg^{-1}) differed considerably from that reported by Reyes & Scopes (1991) (37 IU mg^{-1}). Such a difference could be explained by the procedure used for enzyme solubilization. The ZAPase purified by Reyes & Scopes (1991) was extracted from membranes by Triton X-100 treatment. In our hands, this neutral detergent was inefficient, and release of ZAPase from membranes with a good

Fig. 1. Elution profiles of APase from Q Sepharose (*a*), Mono Q (*b*) and Superose 12 (*c*) columns. The samples loaded were as described in Methods. ●, A_{280} ; ○, APase activity; —, KCl gradient.

efficiency (80% of the total activity) was only achieved by using ionic detergents. On the other hand, the specific activity of the ZAPase was considerably higher than that described for the enzymes of *Lysobacter enzymogenes* (438 IU mg^{-1}) (Von Tigerstrom & Stelmashuk, 1986), *Escherichia coli* (1500 IU mg^{-1}) (Torriani, 1966) and *Micrococcus sodonensis* (82 IU mg^{-1}) (Glew & Heath, 1971). The high specific activity of ZAPase could also be explained by the presence of CTAB simulating the native environment of the enzyme in the membrane. However, ZAPase was shown to be a minor protein in *Z. mobilis*, since it accounted for only 0.004% of total proteins (0.008% of membrane proteins). *Z. mobilis* APase was also purified from strain ZM4 with almost identical results (not shown). Thus, although present in very low

Table 2. Purification of the APase from *Z. mobilis* ZM1

Source of fraction	Total amount of:		Sp. act (units mg^{-1})	Yield %	Purification (-fold)
	Enzyme ($10^{-3} \times$ units)	Protein (mg)			
Crude extract	978	9310	105	100	1
Membranes	850	4600	185	87	1.8
CTAB-soluble extract	851	1070	795	87	7.6
Q-Sepharose	686	65.0	10550	70	100.4
Mono Q	529	4.30	123000	54	1190
Superose 12	355	0.40	887500	36	8690

Fig. 2. Purity of the enzyme preparation from strain ZM1. Proteins were analysed by SDS-PAGE at different steps of the purification procedure. Lane 1, molecular mass standards; lane 2, crude extract; lane 3, membranes; lane 4, CTAB-soluble fraction; lane 5, Q Sepharose eluate; lane 6, Mono Q eluate; lane 7, Superose 12 eluate. ZAPase is indicated by an arrow.

amounts in cells, *Z. mobilis* APase displayed a high catalytic activity.

Homogeneity and molecular mass

Protein fractions were analysed by SDS-PAGE at different steps of the purification procedure. Active fractions eluted from the Superose 12 column contained only one protein of 60 kDa as indicated by SDS-PAGE (Fig. 2). This molecular mass is slightly different from that previously reported after activity staining (56 kDa) (Michel & Baratti, 1989). However, it should be pointed out that protein denaturation was only partial when proteins were stained for phosphatase activity on gels. Indeed, heat treatment and reducing agent were omitted. Moreover, it cannot be excluded that trace amounts of the cationic detergent CTAB still remained associated with the protein, even after dialysis, and affected electrophoretic mobility in the SDS system. The molecular mass of the native ZAPase was determined from the elution pattern after calibration of the Superose 12 column with standard proteins. It was estimated to be about 54 kDa. This result is in agreement with the molecular mass (55 kDa) of the APase purified by Reyes & Scopes (1991). However, as emphasized by Tanford & Reynolds (1976), concern is sometimes expressed when the molecular mass of a native protein is determined in the presence of an ionic detergent. Indeed, although ZAPase purified in the presence of CTAB remained in a native state as shown by activity assay, a dissociation into subunits cannot be excluded. Nevertheless, retention of activity in the presence of detergent is consistent with the absence of disruption of interpeptide bonds important for primary function (Tanford & Reynolds, 1976). Thus, by comparison with the molecular mass on

SDS-PAGE, and with the results obtained by Reyes & Scopes (1991), it could be concluded that the APase of *Z. mobilis* was most probably a monomeric protein. Two-dimensional PAGE analysis of the purified enzyme (data not shown), showed several isoforms which are consistent with the results previously reported (Michel & Baratti, 1989). Very similar results were obtained with the APase purified from strain ZM4: the same molecular mass on SDS-PAGE and Superose 12 column, and presence of isoforms revealed by two-dimensional PAGE.

Effect of pH, temperature and ionic strength on activity

The effect of pH on ZAPase activity was studied according to Von Tigerstrom (1984). Activity on *p*NPP was observed between pH 6 and pH 11 and was maximal at pH 8. These results are in agreement with the pH range reported for membrane-bound or periplasm-located APases in other bacteria (Garen & Levinthal, 1960; Vinter *et al.*, 1987; Von Tigerstrom, 1984). However, the optimum pH for APase activity found in the present work (pH 8), differed from that reported by Reyes & Scopes (1991) (pH 9).

ZAPase activity was assayed in a temperature range from 4 °C to 60 °C. Activity increased with temperature up to 40 °C and decreased upon further heating.

The APase of *Z. mobilis* was shown to be particularly resistant to ionic strength and remained fully active when the assay mixture contained up to 2.5 M-KCl (the highest KCl concentration tested).

Stability

Incubation of the enzyme for 1 h (30 °C) at different pH values before the assay showed that ZAPase was fully stable in the pH range from 6 to 9. The purified enzyme was stable at -20 °C and around +20 °C for 4 months, but its activity decreased by 40 % after 2 months and by 55 % after 4 months at 4 °C. Enzyme inactivation at 4 °C was most probably due to the gradual crystallization of the solubilizing agent CTAB at this temperature. Furthermore, incubation of the enzyme at high temperatures showed that ZAPase remained stable for at least 1 h at 60 °C, but further increase of temperature caused inactivation. Above 70 °C, the enzyme was completely inactivated. This result was consistent with the heat resistance of other APases, particularly those of *Lysobacter enzymogenes* (Von Tigerstrom, 1984; Von Tigerstrom & Stelmashuk, 1986), *Micrococcus sodonensis* (Glew & Heath, 1971) and *E. coli* (Garen & Levinthal, 1960).

The effect of pH and temperature on both activity and stability of APase from strain ZM4 was identical to that observed for the enzyme from strain ZM1.

Table 3 *Effect of inhibitors on APase activity*

APase was incubated for 15 min at 30 °C in the presence of inhibitors at indicated concentrations and assayed. Results are expressed as percentages relative to the control (100 % = 3.5×10^5 IU ml⁻¹).

Inhibitor	Activity (%) inhibitor concn of:		
	0.1 mM	1 mM	5 mM
Sodium arsenate	33	10.9	0
Potassium phosphate	100	71.8	38.7
Potassium cyanide	77.8	39.6	0
EDTA	62.5	0	0
β -Hydroxyquinoline	85.6	65.1	13.5
Iodoacetate	85.5	79.1	73.5
β -Hydroxymercuribenzoate	85.9	83	79.4
L-Cysteine	100	93.2	60.4
β -Mercaptoethanol	100	100	100

Sensitivity to inhibitors

The stability of ZAPase activity was tested in the presence of various inhibitors of enzyme activity. Potassium phosphate, potassium cyanide, sodium arsenate and especially EDTA (in increasing order) were the most powerful inhibitors studied, and their effect was concentration-dependent (Table 3). Activity was not greatly affected by reducing agents such as β -mercaptoethanol and L-cysteine. This result was quite surprising since in a previous study, ZAPase activity was shown to be detected on gels, after SDS-PAGE, only when the usual treatment with reducing agents was omitted (Michel & Baratti, 1989). These contradictory results can be explained by the action of SDS on APase, exposing masked disulphide bonds to inhibitors. Furthermore, the enzyme activity was not significantly affected by thiol-group inhibitors such as *p*HMB and iodoacetate, suggesting that thiol groups might not be essential for activity.

On the other hand, ethanol (31.6 g l⁻¹) suppressed ZAPase activity. Incubation of the enzyme in the presence of 7.8 g ethanol l⁻¹ for 1 h resulted in a 50 % inhibition of activity (data not shown). These results were particularly surprising since cellular APase was shown to retain activity in cultures producing much higher ethanol concentrations (50–100 g l⁻¹), *in vivo* (data not shown). The highest resistance of ZAPase to ethanol in growing cells could be related to the membrane location of the enzyme.

Effect of metal ions on activity

ZAPase was inactivated by 1 mM-EDTA and its reactivation studied by adding various metal ions (5 mM concn). Addition of MnCl₂, CuSO₄, CoCl₂, BaCl₂, NiCl₂, NaCl and KCl did not cause any reactivation of the

enzyme. In the presence of MgCl₂ and CaCl₂ 4 % of the initial activity was recovered. ZnCl₂ was the most potent activator but only 25 % of the initial ZAPase activity could be recovered (data not shown). By comparison, 66 % of the APase activity of *Lysobacter enzymogenes* (Von Tigerstrom & Stelmaschuk, 1986) and 82 % of that of a *Pseudomonas* sp. (Kobori & Taga, 1980) was recovered after addition of 0.05 mM and 0.5 mM-ZnCl₂ respectively, to an EDTA-treated enzyme preparation. Similarly, EDTA-inactivated APase from *Micrococcus sodonensis* recovered 90 % of its initial activity when further treated with 1 mM-Ca²⁺ (Glew & Heath, 1971).

Substrate specificity

The activity of the purified ZAPase was assayed in the presence of different phosphate esters. ZAPase exhibited a broad substrate specificity. Results (expressed as percentages relative to *p*NPP) showed that the highest activities were obtained with *p*NPP (100), ATP (95.8 ± 13.2) and AMP (82.8 ± 15.7), while glucose 6-phosphate (19.3 ± 8.0) and fructose 6-phosphate (23.6 ± 6.9) were hydrolysed to a lesser extent.

Kinetic constants

ZAPase exhibited a very high catalytic activity since the k_{cat} was estimated to be about 50×10^6 min⁻¹. Kinetic study of the purified enzyme showed a K_m for *p*NPP hydrolysis of $5 \pm 2 \times 10^{-6}$ M. This K_m value was lower than those found in *E. coli* (1.2×10^{-5} M) (Garen & Levinthal, 1960), *L. enzymogenes* (0.56 – 3.4×10^{-4} M) (Von Tigerstrom & Stelmaschuk, 1986) or *Vibrio cholerae* (2.2×10^{-4} M) (Roy *et al.*, 1982), showing a greater affinity for this substrate. Competitive inhibition by phosphate was observed, with the inhibition constant ($K_i = 2 \pm 0.2 \times 10^{-4}$ M) higher than for the enzyme from *E. coli* ($K_i = 3.8 \times 10^{-6}$ M) (Garen & Levinthal, 1960) or *Vibrio cholerae* ($K_i = 3.3 \times 10^{-5}$ M) (Roy *et al.*, 1982).

Discussion

The alkaline phosphatases from *Z. mobilis* ZM1 and ZM4 (ZAPases) were found to be identical on the basis of molecular mass, pH optimum, and the effects of temperature, inhibitors, metal ions and ionic strength.

Under the conditions used, the *phoA* gene was not highly expressed in *Z. mobilis*, since ZAPase only accounts for 0.004 % of the cellular proteins of this bacterium. Like most of the APases described, the APase of *Z. mobilis* was fully active at pH 8 and was inhibited by the cation chelator EDTA. However, in comparison with the APases of other micro-organisms, the purified enzyme exhibits a higher specific activity, only a partial

renaturation by ZnCl_2 after EDTA treatment and a lower sensitivity to inhibition by phosphate. Furthermore, during growth of *Z. mobilis*, ZAPase remained active in spite of the high ethanol concentrations produced. The relative ineffectiveness of this alcohol in causing inhibition of activity *in vivo* contrasts markedly with the sensitivity of the purified enzyme to ethanol *in vitro*. These results suggest that ZAPase shows some specificities (e.g. membrane location) which could be required for the adaptation of the micro-organism to an alcoholic environment.

Our results also indicated that in both ZM1 and ZM4 strains, the purified ZAPase was a monomeric protein exhibiting high stability in the presence of CTAB. It accounted for the main phosphate-irrepressible APase activity present in these strains. Indeed, except for isoenzymes, no other APase could be further detected or purified. It has been reported that two different structural genes encoding, respectively, a dimeric and a monomeric APase were present in *Bacillus subtilis* (Hulett *et al.*, 1990). On the other hand, studies on *Z. mobilis* CP4 (Pond *et al.*, 1989) revealed four APase activities including a major phosphate-repressible APase. Recently, Reyes & Scopes (1991) reported the purification of an APase from ZM4 using multifunctional adsorbents. These authors found two forms (ALP-1 and ALP-2) of APase which may differ in the amount of attached neutral lipids or the detergent Triton X-100 originating from the extraction. The ALP-2 form was purified and some of its properties reported. However, ALP-2 was not shown to be identical to the ZAPase obtained using our purification procedure. Indeed, although the two enzymes exhibited a similar molecular mass (55 and 54 kDa, respectively), their pH optimum, their substrate specificity and their specific activity were not identical.

On the other hand, the chemical composition of the detergent used for ZAPase solubilization seemed to be more important than the ionic charge, since OTAC and CTAB (cationic) were as active as SLS (anionic). Moreover, DOC (anionic) and dodecyltrimethylammonio-3-propanesulphonate (zwitterionic) were not shown to be efficient solubilizing agents (especially the latter). The importance of the chemical composition of the detergent was also observed using uncharged compounds. Indeed, some of the neutral detergents used solubilized up to 47% of the total ZAPase (Triton X-114), while others showed a poor effectiveness (Nonidet P40, Tween 80 and Triton X-100). The result obtained with Triton X-100 was particularly surprising since it contrasted with the effectiveness of this detergent on APase solubilization from *Z. mobilis* membranes (Reyes & Scopes, 1991). However, neutral detergents are known to be less efficient than ionic detergents in the ability to break protein interactions. It cannot be excluded that in

our experimental conditions, disruption of interactions between ZAPase and other membrane components required more specific solubilizing agents than Triton X-100.

Thus, the differences observed between our results and those obtained by Pond *et al.* (1989) on the one hand, and Reyes & Scopes (1991) on the other, could be related either to strain specificities or to the experimental procedures used. Although we did not detect another form of APase in addition to the enzyme described in this work, it cannot be excluded that several forms of APase exist in *Z. mobilis*.

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