Biochimica et Biophysica Acta, 1068 (1991) 174–178

ADONIS 000527369100344B

BBAMEM 75366

Membrane-associated ATPase from *Zymomonas mobilis*; purification and characterization

Leticia Reyes and Robert K. Scopes

Centre for Protein and Enzyme Technology, La Trobe University, Bundoora, VIC (Australia)

(Received 27 December 1990)
(Revised manuscript received 6 May 1991)

Key words: ATPase, F_0F_1-; ATP; Membrane enzyme; Purification; (*Z. mobilis*)

The major ATPase (adenosinetriphosphatase, EC 3.6.1.3) activity present in the membrane of *Zymomonas mobilis* has been isolated, using a novel combination of multifunctional hydrophobic adsorbents. On subjecting the preparation to gel filtration, activity was lost, but could be restored by reconstituting fractions from the column. Subunit composition of the fractions indicated that the *Zymomonas mobilis* ATPase is of the F_0F_1 type, and so is probably involved in proton pumping. The contributon of this ATPase to the overall ATP turnover in the cells has been calculated to be approximately 20% and so it may be partly responsible for the phenomenon of 'uncoupled growth' observed with *Zymomonas mobilis*.

Introduction

The use of the anaerobic bacterium *Zymomonas mobilis* for industrial alcohol production has some advantages compared with yeast, the chief one being that the rate of fermentation per g biomass is 3–4 times faster [1]. This is because (a) the fermentation process, via the Entner-Doudoroff pathway, produces only one ATP molecule per glucose fermented, whereas yeast produces two; and (b) the occurrence of 'uncoupled' growth [2,3] in which the ATP turnover is independent of cellular growth, results in a greater demand for ATP in *Zymomonas mobilis* [4].

An ATPase (adenosinetriphosphatase, EC 3.6.1.3) has been partially purified from *Zymomonas mobilis* [4]; its K_m was determined to be 1 mM. Measurement of ATP levels in growing cells suggested that they were controlled through this ATPase, which, because of its relatively high K_m, increased in activity as the ATP level rose.

ATPases are expected to have a physiological function, either related to energy-consuming processes such as ion pumps, or as ATP synthases linked to proton gradients. As *Zymomonas mobilis* is normally grown in anaerobic conditions, oxidative phosphorylation is absent. However, some ATP synthesis may occur in aerobic conditions as a result of the membrane-linked glucose dehydrogenase and cytochrome systems present in the organism [5,6]. Thus the major ATPases in *Zymomonas mobilis* are expected to be associated with membrane transport systems. Disruption of the cells may compromise the activity measurements compared with their activity in intact cells; nevertheless, ATPase activities related to these transport systems are normally detected in subcellular preparations.

The present report describes the isolation of the major ATPase activity associated with the membrane fraction isolated from *Zymomonas mobilis*, and compares its properties with those of other bacterial ATPases, in particular the F_0F_1-ATP synthases from *Escherichia coli* [7,8], and from anaerobes such as *Clostridium pasteurianum* [9]. The isolation procedure involved the use of multifunctional hydrophobic adsorbents which we have previously used for the purification of both membrane-associated and cytoplasmic enzymes [10,11].

Materials and Methods

Materials

Divinylsulphone, 3,5-dinitrotyrosine, benzylmercaptan and other organic ligands were purchased from

Correspondence: R.K. Scopes, Centre for Protein and Enzyme Technology, La Trobe University, Bundoora, VIC 3083, Australia.

Aldrich Chemicals. ATP, UTP, CTP, GTP, ITP, AMP, and NADH were from Sigma. Sepharose CL-4B, and materials for electrophoresis were from Pharmacia.

Preparation of multifunctional adsorbents

Adsorbents were prepared by methods described elsewhere [10,11]. Sepharose CL-4B was activated either with divinylsulphone or with epichlorhydrin, and amino- or thiol-containing ligands, which included hydrophobic, polar and charged substituents, were coupled at alkaline pH.

Growth of organism

Zymomonas mobilis strain ZM4 (ATCC 31821) was grown as described previously [12] except that the pH was maintained at 5.6 with NH_4OH. The medium contained 15% (w/v) glucose, 0.5% yeast extract, 0.05% KH_2PO_4, 0.02% $MgSO_4$, 20 mg/l ferrous ammonium sulphate and 2 mg/l Ca pantothenate. Between 80 and 100 g (wet weight) of cell were harvested from a 4 litre culture.

Preparation of Zymomonas membrane fractions

Cells were harvested and washed once with a 10 mM K-Mes buffer (pH 6), containing 2 mM $MgCl_2$ and 30 mM KCl (buffer A). Washed cells (150 g) were suspended in 500 ml of 50 mM K-Mes, pH 6 (buffer B) and lysozyme was added at 0.2 mg/ml. After slow stirring overnight, the mixture was divided into about 100-ml portions,which were sonicated (Rapidis 600) at power 5 for 30 min using a 9 mm probe. The resulting mixture was centrifuged at $35000 \times g$ for 15 min at 4°C. After washing the collected pellet with buffer B, it was then solubilized with 1% Triton X-100 in the same buffer overnight. The supernatant was collected after centrifugation at $100000 \times g$ at 4°C for 30 min.

ATPase activity, protein determination and analysis

ATPase activity was measured in a continuous assay following the production of ADP using pyruvate kinase and lactate dehydrogenase [7]. The assay was carried out in 20 mM K-Mes buffer (pH 6.5), containing 2 mM $MgCl_2$ and 50 mM NaCl, using 0.5 mM ATP, 0.15 mM NADH, 0.5 mM phospho*enol*pyruvate and 5 units/ml each of pyruvate kinase and lactate dehydrogenase. One unit is defined as the amount of enzyme producing 1 μmol of ADP per min at 25°C.

For characterizing the pH profile and metal ion requirements, the assay procedure involved measuring the released P_i by the method of Saheki et al. [13], using 1 mM ATP.

The protein concentration was determined using Coomassie Brilliant Blue G250 as described by Sedmak and Grossberg [14] with bovine serum albumin as a standard.

Polyacrylamide gel electrophoresis was carried out using a Pharmacia Phast system. SDS-gels were stained with Coomassie Blue R250.

Results

Screening of multifunctional adsorbents

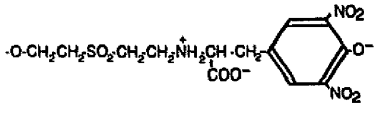
Screening of the prepared multifunctional adsorbents was carried out as described elsewhere [10]. Screening was carried out both with and without the presence of 0.5 M Na_2SO_4, in 10 mM K-Mes buffer (pH 6) containing 2 mM $MgCl_2$, 30 mM KCl, 1 mM dithiothreitol and 0.1% Tween-80. A suitable adsorbent for ATPase should meet the following requirements: (i) a high recovery in enzyme activity with the lowest amount of contaminating proteins; (ii) a separation of ATPase from alkaline phosphatase, a membrane-bound enzyme which can also utilize ATP as a substrate; and, (iii) ATPase should be able to be desorbed with a mild eluent.

About 20 multifunctional adsorbents were screened; agarose-DVS-3,5-dinitrotyrosine (as a negative adsorbent) and agarose-DVS-benzylmercaptan (as a positive adsorbent) used in the presence of sodium sulphate (Fig. 1) proved to be the most efficient.

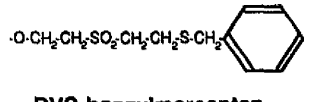
Purification of ATPase from Zymomonas membrane fractions

About 40 ml of solubilized membrane fraction prepared as described in Materials and Methods was applied to a 12 ml DVS-3,5-dinitrotyrosine column (70×15 mm) equilibrated with the pH 6 K-Mes buffer and 0.5 M Na_2SO_4. The column was washed with the starting buffer. A virtually complete recovery of ATPase (free of alkaline phosphatase), with a 2-fold purification, was obtained in the flow-through.

The unadsorbed fraction was applied to the DVS-benzylmercaptan column (75×25 mm) equilibrated with the same buffer. (The two columns could be coupled in tandem, and disconnected after washing



DVS-3,5,-dinitrotyrosine



DVS-benzylmercaptan

Fig. 1. The multifunctional adsorbents used in the purification of ATPase from *Zymomonas mobilis*.

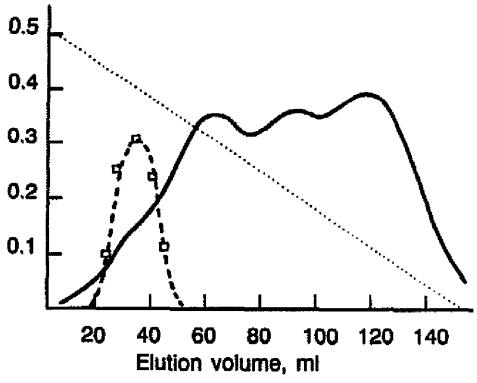


Fig. 2. Elution profile of *Z.mobilis* ATPase on DVS-benzylmercaptan column. The unabsorbed material from the dinitrotyrosine column was applied to the column in the presence of 0.5 M Na_2SO_4, and activity eluted by application of a decreasing gradient from 0.5 M to zero Na_2SO_4. ———, A_{280}; □ --- □, ATPase activity (U/ml); ·····, Na_2SO_4 concentration (M). The vertical scale is appropriate for each variable.

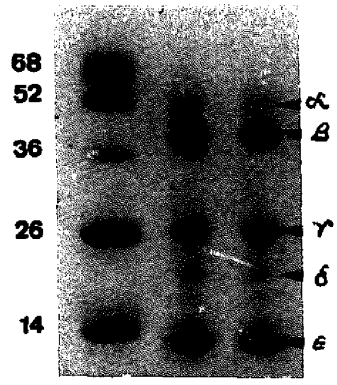


Fig. 4. SDS-PAGE of *Z. mobilis* ATPase obtained after DVS-benzylmercaptan column. The ATPase subunits are shown: α (50 kDa), β (43 kDa), γ (25 kDa), δ (20 kDa), and ε (13–15 kDa). The molecular mass marker sizes are indicated on the left.

through with the starting buffer.) After washing the benzylmercaptan column with the starting buffer, elution was achieved with a gradual decrease in the concentration of Na_2SO_4 in the same buffer. The elution profile is given in Fig. 2. ATPase was purified 25-fold, with 88% recovery.

Further purification of this fraction was attempted by gel filtration. An 8 × 50 mm (100 ml) column of GCL-2000 (superfine grade, Amicon Corp.) was used, equilibrated in a buffer consisting of 20 mM K-Mes, (pH 6), 1 mM dithiothreitol, 0.05% v/v Tween-80 and 0.25 mM phenylmethylsulphonyl fluoride. The elution diagram is shown in Fig. 3. Several peaks were observed; ATPase, amounting to only 5% of the applied activity, was found in the area labelled *b* in Fig. 3, corresponding to a molecular mass of 80–90 kDa.

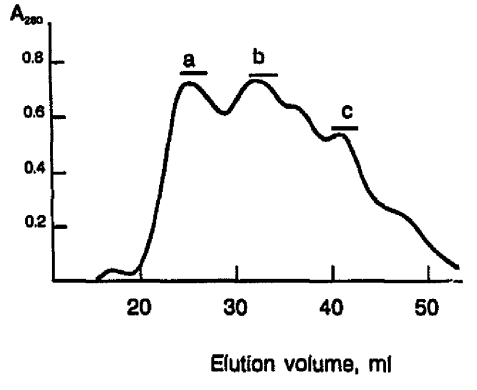


Fig. 3. Gel filtration of *Z.mobilis*. Fractions eluted from the DVS-benzylmercaptan column between 20 and 40 ml (see Fig. 2) were combined and concentrated by ultrafiltration to 1 ml and applied to a 100 ml gel filtration column(GCL 2000). Peak *a*, 400 kDa fraction; peak *b*, 80–90 kDa fraction; peak *c*, 25 kDa fraction. (For details, see text.) Weak ATPase activity was found around peak *b*.

Reconstitution of ATPase

Although most of the ATPase activity was lost during gel filtration, incubation of a sample of the ATPase in the gel filtration buffer without passing through the column did not result in any significant loss in activity. Thus it was possible that the loss of activity on the column was due to separation of subunits, rendering the enzyme inactive. The fractions from the gel filtration column were investigated by recombining them to see if the original activity could be restored.

The tubes comprising fraction *b* with low ATPase activity were combined. On adding samples from the other tubes to samples of this active fraction it was found that an increase of activity occurred only on addition of samples from the area labelled *a* in Fig. 3, corresponding to a molecular mass of about 400 kDa.

To a mixture of both fractions *a* and *b*, further additions were made. Small amounts of protein from tubes in the area labelled *c* in Fig. 3, corresponding to a molecular mass of 25 kDa, caused a further increase in the ATPase activity. The maximum activity achieved was approximately 30% of the activity applied to the column; nevertheless the specific activity of this reconstitued sample was higher than before application to the column. It represented a 6-fold increase over fraction *b* alone, and this occurred at protein ratios of approximately 3:2:1 for *a:b:c*. Addition of samples from other tubes to this mixture failed to restore any more activity.

The summary of the purification of ATPase from *Z. mobilis* is given in Table I.

TABLE I

Purification of ATPase from 40 ml solubilized membrane fraction from Z. mobilis

ATPase activity and mg protein were determined as described under Materials and Methods

	Total protein (mg)	Total units (U)	Specific activity (U/mg)	Purification -fold	%Recovery
Crude extract	448	121	0.27	(1)	(100)
Not adsorbed on DVS-3,5-dinitrotyrosine	264	120	0.45	2	99
Eluted from DVS-benzylmercaptan	16	106	6.62	25	88
Active fraction from GCL-2000	2.5	6	2.4	9	5
GCL-2000 fraction reconstituted	2.5	37	12.72	47	30

Gel electrophoresis of ATPase fractions

Analysis on SDS-PAGE of the ATPase fraction obtained from the benzylmercaptan column showed major peptides of sizes 50 kDa, 43 kDa, 25 kDa, 20 kDa and smaller subunits of 13–15 kDa (Fig.4). These sizes are in good agreement with the sizes of the five subunits (α, β, γ, δ,and ϵ) of F_1-ATPase from bacteria [7,15–18], chloroplast [19] and mitochondria [20]. However impurity proteins could be contributing some of these bands.

Analysis of the fractions from the gel filtration column showed one major subunit (43 kDa) in the 80–90 kDa fraction *b*; this is the approximate size of the catalytic subunit in F_1- ATPases [8]. The fractions conferring additional activity on reconstitution showed subunits at 50 kDa (fraction *a*) and 25 kDa (fraction *c*). Thus it is concluded that the complete ATPase activity required subunits of 50 kDa, 43 kDa, and 25 kDa.

Kinetic characterization of purified ATPase

Substrate specificity. The relative activities of the enzyme when ATP, ITP, UTP, CTP and GTP at 1 mM each were used as substrates were 100, 97, 163, 82 and 80, respectively. The enzyme did not hydrolyze AMP or *p*-nitrophenyl phosphate, indicating that it was free of both 5'-nucleotidase [21] and phosphatases [11].

A Lineweaver-Burk plot using ATP as the substrate yielded a linear pattern with an apparent K_m value of 0.1 mM, which is ten times smaller than the reported K_m found in earlier studies [4].

Cation requirement. The cation requirement of ATPase was investigated. Either Mg^{2+} or Ca^{2+} was able to act as complexing ion to the ATP equally well. No activity was observed without a divalent cation, and the presence or absence of Na^+ or K^+ had little effect on the activity.

pH optimum. The pH optimum of ATPase was determined by assaying the enzyme at pH 4.0–5.5 (Na-acetate buffer), pH 5.5–7.0 (K-Mes), pH 7.0–9.0 (Tricine-KOH), and pH 8.0–10.0 (glycine-NaOH). The enzyme showed the highest activity at pH 9 with either Tricine-KOH or glycine-NaOH as buffers.

Discussion

Uncoupled growth in *Zymomonas mobilis* is a result of ATPase activity in excess of that required for the normal growth and maintainance of the cells. The rapid production of ADP stimulates glycolysis to regenerate ATP, and so accelerates ethanol production. Thus the rate of ethanol production is largely dependent on the action of the ATPases. Among the enzymes capable of hydrolysing ATP found in *Zymomonas mobilis*, alkaline phosphatase (membrane-bound [11]), 5'-nucleotidase (periplasmic [21]) and a true ATPase (membrane-bound) have been described.

We have isolated acid and alkaline phosphatases [11], and now the ATPase from *Zymomonas mobilis* membrane preparations. The isolation procedure, which was effectively one step, involved the use of novel hydrophobic adsorbents which selectively bound the ATPase and other components in the detergent-solubilized membrane fraction. Some impurities were undoubtedly still present at this stage; nevertheless the polyacrylamide-SDS pattern gave major bands all of which are typical of F_0F_1-ATPases, and the specific activity of the preparation was comparable to that of purified enzyme from other sources [7,8]. Gel filtration resulted in the loss of most activity, which could be partly regained by recombining fractions from the column.

It is of interest that the best substrate was UTP, which has been reported to be one of the main nucleotide triphosphates in *Zymomonas mobilis* [22]. The enzyme was not stimulated by monovalent cations, indicating that it was not involved in the active transport of Na^+ or K^+ across the membranes. Its resem-

blance to ATPases that are responsible for ATP synthesis by electron transport in aerobic bacteria calls into question its real role. *Zymomonas mobilis* does have an electron transport chain which can operate for direct oxidation of glucose using the PQQ-linked glucose dehydrogenase and acceptors leading to cytochrome *o* [5,6]. One may speculate that the ATPase described synthesizes ATP in aerobic conditions (in which *Zymomonas mobilis* will grow, but poorly), but acts as a proton pump ATPase in anaerobic conditions, especially towards the end of fermentations that produce high amounts of ethanol, causing the cell membrane to become leaky.

We cannot directly relate our ATPase to that of Lazdunski and Belaich [4]. However, it is probably the same enzyme; their K_m determination could have been in error due to under-estimation of rates at low ATP concentration because of substrate depletion. Using the couple enzymic assay, ATP is recycled; consequently our K_m estimate of 0.1 mM is likely to be accurate. In which case fluctuations in ATP levels in the cell around 0.5 to 1.5 mM would not result, through this ATPase, in significant differences in ATP turnover, as they had suggested.

It can be calculated that *Zymomonas mobilis* cells fermenting in optimum conditions turn over ATP at a rate of 200–250 μmol per min per g wet weight at 30°C [23,24]. The contributions to this turnover by the acid phosphatase, the alkaline phosphatase, the 5'-nucleotidase and the present ATPase are calculated to be approximately 4%, 7%, 2% and 20%, respectively. However, the 5'-nucleotidase would not be expected to have access to intracellular ATP, nor may the other phosphatases. In intact cells the specific orientation of the enzyme, and the physiological concentrations of ions may result in either lower or higher values for ATP degradation by this ATPase, but the calculation of 20% turnover serves to demonstrate that it is significantly involved in affecting the rate of glycolysis, and so is at least partly responsible for the rapid fermentation rates in *Zymomonas mobilis*.

References

1 Rogers, P.L., Lee, K.J., Skotnicki, M.L. and Tribe, D.E. (1982) Adv. Biochem. Eng. 23, 37–84.
2 Cole, H., Wimpenny, J.W. and Hughes, D.E. (1966) Biochem. J. 100, 81P.
3 Gunsalus, I.C. and Shuster, C.W. (1961) in The Bacteria: A Treatise in Structure and Function, Vol. 2 (Gunsalus, I.C. and Stanier, R.Y., eds.), pp. 1–51, Academic Press, New York.
4 Lazdunski, A. and Belaich, J.P. (1972) J. Gen. Microbiol. 70, 187–197.
5 Strohdeicher, M., Schmidz, B., Bringer-Meyer, S. and Sahm, H. (1988) Appl. Microbiol. Biotechnol. 27, 378–382.
6 Strohdeicher, M., Neuss, B., Bringer-Meyer, S. and Sahm, H. (1990) Arch. Microbiol. 154, 536–543.
7 Vogel, G. and Steinhart, R. (1976) Biochemistry 15, 208–216.
8 Satre, M., Lunardi, J., Pougeois, R. and Vignais, P.V. (1979) Biochemistry 18, 3134–3139.
9 Clarke, D.J. and Morris, J.G. (1976) Biochem. J. 154, 725–729.
10 Scopes, R.K. and Porath, J. (1990) Bioseparation 1, 5–9.
11 Reyes, L.B. and Scopes, R.K. (1991) Bioseparation, in press.
12 Scopes, R.K., Testolin, V., Stoter, A., Griffiths-Smith, K. and Algar, E. (1985) Biochem. J. 228, 627–634.
13 Saheki, S., Takeda, A. and Shimazu, T. (1985) Anal. Biochem. 148, 277–281.
14 Sedmak, J.J. and Grossberg, S.E. (1977) Anal. Biochem. 79, 544–552.
15 Mileykovskaya, Y.I., Abuladze, A.N. and Ostrovsky, D.N. (1987) Eur. J. Biochem. 168, 703–708.
16 Biketov, S.F., Kasho, V.N., Kozlov, I.A., Mileykovskaya, Y.I., Ostrovsky, D.N. and Skulachev, V.P.T. (1972) Eur. J. Biochem. 120, 241–250.
17 Sone, N., Yoshida, M., Hirata, H. and Kagawa, Y. (1975) J. Biol. Chem. 250, 7917–7923.
18 Kagawa, Y., Sone, N., Yoshida, M., Hirata, H. and Okamoto, H. (1976) J. Biochem. 80, 141–151.
19 Nelson, N., Deters, D.W., Nelson, H. and Racker, E. (1973) J. Biol. Chem. 248, 2049–2055.
20 Knowles, A.F. and Penefsky, H.S. (1972) J. Biol. Chem. 247, 6617–6623.
21 Neu, H.C. (1967) J. Biol. Chem. 242, 3896–3904.
22 Barrow, K.D., Collins, J.G., Leigh, D.A., Rogers, P.L. and Warr, R.G. (1984) Appl. Microbiol. Biotechnol. 20, 225–232.
23 Lee, K.J., Skotnicki, M.L., Tribe, D.E. and Rogers, P.L. (1980) Biotechnol. Lett. 2, 339–344.
24 Algar, E.M. and Scopes, R.K. (1985) J. Biotechnol. 2, 275–289.