

Defined subcomplexes of the A₁ ATPase from the archaeon *Methanosarcina mazei* Gö1: biochemical properties and redox regulation

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Received 25 March 2003; revised 29 April 2003; accepted 30 April 2003

First published online 13 May 2003

Edited by Stuart Ferguson

Abstract The potential A₁ ATPase genes *ahaA*, *ahaB*, *ahaC*, *ahaD*, *ahaE*, *ahaF*, and *ahaG* from the anaerobic archaeon *Methanosarcina mazei* Gö1 were overexpressed in *Escherichia coli* DK8 (pTL2). An A₁ complex was purified to apparent homogeneity and shown by Western blot and N-terminal sequence analyses to contain subunits A, B, C, D, and F but to be devoid of subunits E and G. Further removal of subunit C was without effect on ATPase activity. The enzyme was most active at pH 5.2 and required bisulfite and acetate for maximal activity. Kinetic studies confirmed three new inhibitors for A₁ ATPases (diethylstilbestrol and its derivatives hexestrol and dienestrol) and identified redox modulation as a new type of regulation of archaeal A₁ ATPases.

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Key words: Archaeon; A₁ ATPase; Subcomplex; Kinetic property; Regulation; *Methanosarcina mazei* Gö1

1. Introduction

Methanogenic, halophilic and thermoacidophilic archaea synthesize ATP by means of ion gradient-driven phosphorylation. The key enzyme is A₁A₀ ATP synthase which converts the electrochemical ion potential across the membrane into ATP [1]. Like the F₁F₀ ATPase, A₁A₀ ATPase catalyzes ATP synthesis in vivo, but according to its subunit composition and the primary sequence of its major subunits, it is more related to eukaryal V₁V₀ ATPases [2]. Therefore, the enzyme can be regarded as a chimera of F₁F₀ and V₁V₀ ATPases. Unfortunately, biochemical and structural data of the A₁A₀ ATPases are scarce and even their subunit composition is far from being settled.

To obtain insights into the structure–function relationship of A₁A₀ ATPases using defined systems we started a molecular approach and succeeded in the construction of plasmid pSÖ1 containing the ATPase genes *ahaE*, *ahaC*, *ahaF*, *ahaA*, *ahaB*, *ahaD* and *ahaG* from *Methanosarcina mazei* Gö1. The F₁F₀ ATPase-negative strain *Escherichia coli* DK8 was used as a host to express the A₁A₀ ATPase genes. This strain grows aerobically by oxidation of glucose through glycolysis that is coupled to ATP formation; NADH oxidation is carried out by the electron transport chain with concomitant reduction of

oxygen. *E. coli* DK8 (pSÖ1) did grow on glucose, and cell free extracts did indeed hydrolyze ATP [3]. However, in vivo rates of ATP hydrolysis (that are assumed to be low at neutral pH) do apparently not inhibit growth of *E. coli* DK8 (pSÖ1). Here, we report the purification of the A₁ complex produced heterologously and will show that it is composed of subunits A, B, C, D, and F. Additional experiments narrowed down the minimal functional core unit of A₁ ATPases to an A₃B₃DF subcomplex. Furthermore, kinetic studies revealed three new inhibitors for A₁ ATPases (diethylstilbestrol and its derivatives hexestrol and dienestrol) and identified redox modulation as a new type of regulation of archaeal A₁ ATPases.

2. Materials and methods

2.1. Organisms and plasmids

E. coli strain DK8 (1100Δ[*uncB-uncC*] *ilv::Tn10*) [4] was grown on LB at 37°C. Plasmids used were pGEM-4Z (Promega, Mannheim, Germany), pSÖ1, pRT103 [3], and pTL2 (this study).

2.2. Purification of the A₁ ATPase

Cells were grown in a volume of 8 l in a fermenter. The expression of the A₁ ATPase genes was induced during growth on LB by addition of 0.25% (w/v) lactose. Five hours after addition of lactose cells were harvested and broken up by three passages through a French pressure cell. 1 and 0.1 mM phenylmethylsulfonyl fluoride was added to the crude extract and to the buffers used for purification, respectively. Membranes were removed by centrifugation (100 000×g, 90 min, 4°C). The ATPase was precipitated by addition of ammonium sulfate-saturated TMDG buffer (50 mM Tris–HCl, 5 mM MgCl₂, 1 mM dithioerythritol, 10% (v/v) glycerol, pH 7.5) to 65% saturation. After 30 min incubation on ice the precipitated proteins were pelleted by centrifugation (12 000×g, 30 min, 4°C) and dissolved in 20 ml TMDG buffer. The A₁ ATPase was precipitated again at 55–65% saturation and dissolved in 2 ml of TMDG buffer. This protein solution was applied to a gel filtration column (BioPrepSE1000/17, Bio-Rad, Munich, Germany) at a flow rate of 60 cm/h in buffer A (50 mM Tris–HCl, 150 mM NaCl, pH 6.9). The fractions containing ATPase activity were pooled and applied to an ion exchange column (BioScale DEAE5, Bio-Rad), equilibrated with buffer A. Elution was performed with a salt gradient (0.15–0.45 M NaCl) and fractions with ATPase activity were pooled. After concentration by Centrex UF-2 ultrafiltration columns (Schleicher and Schuell, Dassel, Germany) the sample was applied to a gel filtration column (Sephacryl S-300HR(16/60), Amersham Pharmacia Biotech, Freiburg, Germany) at a flow rate of 7.5 cm/h in buffer A at 7°C.

2.3. ATPase activity

At first, ATPase activity was measured in MES buffer at 37°C according to [5]. The assay was started by addition of Na₂-ATP to a final concentration of 4 mM. Samples were taken at 0, 0.5, 1, 1.5, and 2 min, and phosphate released was determined as described [6]. In the course of this study, the assay buffer was optimized and, finally, ATP-

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ase activity was measured in 100 mM MES, 8 mM MgCl₂, 30 mM NaHSO₃, 40 mM Na-acetate, pH 5.2. One unit of activity is defined as 1 μmol ATP hydrolyzed per min.

2.4. Molecular biology

All procedures were performed according to standard techniques [7]. The construction of plasmid pTL2 was performed in two steps. First, the upstream region of *ahaE* (starts 46 bp downstream of *ahaI*) was amplified by PCR introducing a *SalI* restriction site at the 5' terminus (5'-CTTAAATTCGTCGACCATGCTTT-3') and a *XbaI* restriction site at the 3' terminus (5'-CGGTATCTAGAAGTT-3'). The 416 bp fragment was cloned into pGEM-4Z (pTL0). Then, the 7.8 kb *XbaI/KpnI* fragment of pRT103 was inserted into the *XbaI* and *KpnI* sites of pTL0. The resulting plasmid (pTL2) contained 162 bp upstream of *ahaE*, the A₁ ATPase genes *ahaE-ahaG*, and the 5' terminus of *hypF*.

2.5. Other methods

Western blot analyses were performed as described [8,9]. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and native PAGE were carried out as described [10,11]. The gels were stained either with Coomassie brilliant blue G-250 [12] or with silver [13]. Protein was determined by the methods of Lowry et al. [14] or Bradford [15]. For N-terminal sequencing, the proteins were blotted on a BioTrace[®] polyvinylidene difluoride membrane (pore size 0.45 μm, PALGelma-Sciences, Rosdorf, Germany) according to [16]. The protein bands were excised from the membrane and sequenced with a model 473A sequencer from Applied Biosystems using a faster version of the standard cycle.

3. Results

3.1. Purification of a heterologously produced A₁ ATPase complex

We have shown previously that the A₁ ATPase can be produced in *E. coli* DK8 that is devoid of the F₁F₀ ATPase but contains the potential A₁ ATPase *ahaA*, *ahaB*, *ahaC*, *ahaD*, *ahaE*, *ahaF*, and *ahaG* on an expression plasmid [3]. To determine the subunit composition of the complex, the ATPase was purified. The cell free extract of *E. coli* DK8 (pSÖ1) had a specific ATPase activity of 0.35 U/mg. The A₁ ATPase was enriched 30-fold by chromatography (see Section 2) with a yield of 10% and a specific activity of 9.6 U/mg protein (Table 1). The apparent molecular mass of the complex was determined by gel filtration to be 355 kDa.

3.2. Subunit composition of the A₁ complex

The A₁ ATPase complex was resolved into five different polypeptides with molecular masses of 65, 55, 41, 28, and 9 kDa (Fig. 1). These were identified by Western blot analyses with antisera against subunits A, B, C, and F as well as by N-terminal sequence analyses as subunits A (MEVKGE), B (VKEYKTITQIAGP), C (MRLLENLWGKKPSR), D (AQ-QDVKPTRSEL), and F (MELAVIGKSEF). The experimentally determined start codons match the ones deduced from the DNA sequences [5]. Indeed, the gene *ahaA* starts with the

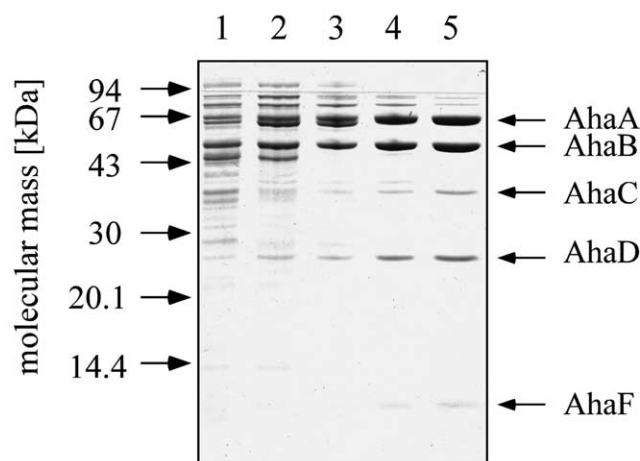


Fig. 1. Subunit composition of the A₁ ATPase. 10 μg of protein was applied to a 12.5% SDS–polyacrylamide gel. Lane 1, cytoplasm of *E. coli* DK8 (pTL2); lane 2, (NH₄)₂SO₄ precipitate; lane 3, pooled BioPrepSE1000/17 fractions; lane 4, pooled DEAE eluate; lane 5, pooled Sephacryl S-300 HR fractions.

unusual codon GTG. The N-terminal methionine was removed from subunits B and D.

Although plasmid pSÖ1 encodes the ATPase genes *ahaE* and *ahaG*, the products were not found in the purified ATPase. *ahaE* is preceded by an apparently untranslated region of 208 bp which is not present on plasmid pSÖ1. To analyze whether this DNA fragment enhances the expression of *ahaE*, a new clone (pTL2) was generated which carries in front of the A₁ ATPase-encoding genes (*ahaE*, *ahaC*, *ahaF*, *ahaA*, *ahaB*, *ahaD*, and *ahaG*) 162 bp of the intergenic region present between *ahaE* and *ahaI*. pTL2 was expressed in *E. coli* DK8, but AhaE could not be detected using the anti-AhaE antiserum. The enzyme purified from *E. coli* DK8 (pTL2) had the same subunit composition and the same specific activity as the enzyme produced in *E. coli* DK8 (pSÖ1). From these experiments it can be concluded that subunits E and G are dispensable for A₁ ATPase assembly and function.

3.3. Kinetic properties of the A₃B₃CDF complex

The A₃B₃CDF complex had a requirement for Mg²⁺ but other divalent cations could substitute for Mg²⁺ in the order Fe²⁺ (55%) > Zn²⁺ (41%) > Mn²⁺ (30%) > Ni²⁺ (27%) > Ca²⁺ (10%) > Co²⁺ (1%). At an optimal Mg²⁺:ATP ratio, the enzyme was most active at pH 5.2 (Fig. 2). The ATPase showed a high specificity for ATP. GTP was hydrolyzed with 48% activity whereas the pyrimidine nucleotides were hydrolyzed with 20 (CTP) and 33% (UTP). Other compounds such as pyrophosphate, ADP or AMP were not hydrolyzed. Enzymatic activity was stimulated three-fold by max. 30 mM bi-

Table 1
Purification of the A₁ ATPase from *M. mazei* Gö1

Purification step ^a	Protein (mg/ml)	ATPase activity (U)	Specific activity ^b (U/mg)	Enrichment (fold)	Yield (%)
Cytoplasm	35	244	0.35	1	100
(NH ₄) ₂ SO ₄	42	90	1	2.7	37
BioPrepSE1000/17	2.3	52	1.9	5.4	22
BioScaleDEAE	1.6	28.6	4.6	13.1	11.7
Sephacryl S-300HR	0.9	25	9.6	27.4	10.3

^aATPase was purified and enzyme activity was determined as described in Section 2.

^bActivity was determined in the optimized buffer.

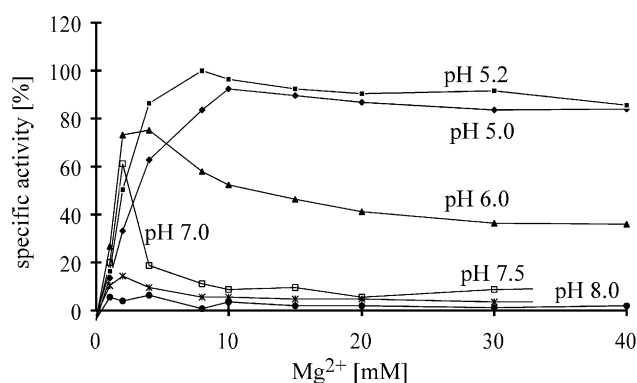


Fig. 2. Mg^{2+} dependence of ATPase activity at different pH values. The A_1 ATPase activity was determined in the optimized buffer containing 100 mM MES, 30 mM $NaHSO_3$, 40 mM Na-acetate, 8 mM $MgCl_2$, 4 mM ATP, and increasing $[Mg^{2+}]$, at pH 5.0 (◆), 5.2 (■), 6.0 (▲), 7.0 (□), 7.5 (*), and 8.0 (●). 100% activity corresponds to 9.6 U/mg protein.

sulfite. To determine the effect of organic acids on activity, acetate, propionate, succinate, or maleinate was added to the assay in increasing amounts to a final concentration of 80 mM. However, only acetate had a pronounced stimulatory effect: starting at 5 mM the activity increased linearly with increasing acetate concentration until an optimum was reached at 40 mM. Maximal stimulation was three-fold.

From these results, the optimal assay buffer was deduced to be 100 mM MES-HCl, pH 5.2, 40 mM acetate, 30 mM $NaHSO_3$, and Mg^{2+} :ATP in a ratio of 2:1. Using this buffer, an apparent K_m for Mg-ATP of 1.3 ± 0.3 mM and a maximal velocity of the reaction of 13 ± 3 U/mg protein was determined.

3.4. Inhibitor sensitivity of the A_3B_3CDF complex

Previously, we had shown that ATP hydrolysis as catalyzed by the cell-free extract of *E. coli* DK8 (pSÖ1) was inhibited by diethylstilbestrol (DES) and its derivatives hexestrol and dienestrol [3]. This was verified with the purified enzyme. The activity of the A_1 ATPase was inhibited by DES ($I_{50} = 3$

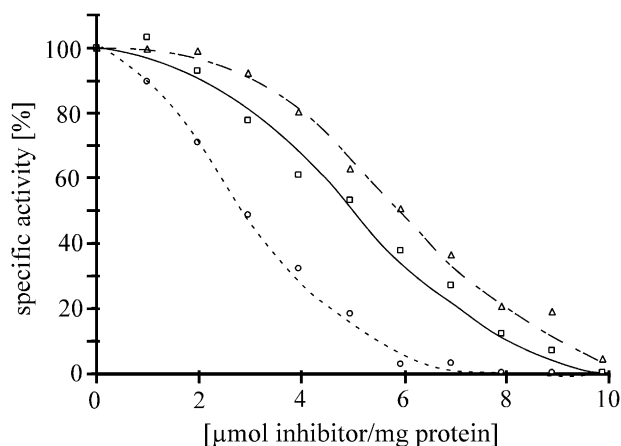


Fig. 3. Inhibition of the A_1 ATPase by DES and its derivatives. The ATPase activity was determined in optimized buffer containing increasing amounts of DES (dashed line), hexestrol (solid line), and dienestrol (dot-dashed line). The enzyme was preincubated with the inhibitors for 30 min at 25°C. The control received the solvent only. 100% activity corresponds to 9.6 U/mg protein.

$\mu\text{mol/mg protein}$), by dienestrol ($I_{50} = 6 \mu\text{mol/mg protein}$) and by hexestrol ($I_{50} = 5 \mu\text{mol/mg protein}$) (Fig. 3). Attempts to restore enzymatic activity by diluting out the inhibitor were unsuccessful, indicating a tight binding of the inhibitor to A_1 or an irreversible inhibition.

3.5. Isolation and redox modulation of the A_3B_3DF subcomplex

The isolated A_1 ATPase was applied to a Sephacryl[®] S-400 HR column (10/30, Pharmacia) equilibrated with 50 mM Tris-HCl (pH 8.1) and 150 mM NaCl. The protein eluted as an A_1 subcomplex without subunit C as shown by SDS-PAGE (Fig. 4A, lane 1). This preparation had the same ATPase activity as the A_3B_3CDF complex indicating that subunit C is dispensable for activity.

Redox modulation of the closely related V-ATPase leads to changes in structure and activity of this enzyme [17–19]. To examine whether this also applies to the A_1 ATPase, we determined the effect of dithiothreitol (DTT) on enzyme function. Under non-reducing conditions, subunit A migrates as a diffuse band (A_1) and two new polypeptides (I, II) with apparent molecular masses of 100 and > 150 kDa were obtained

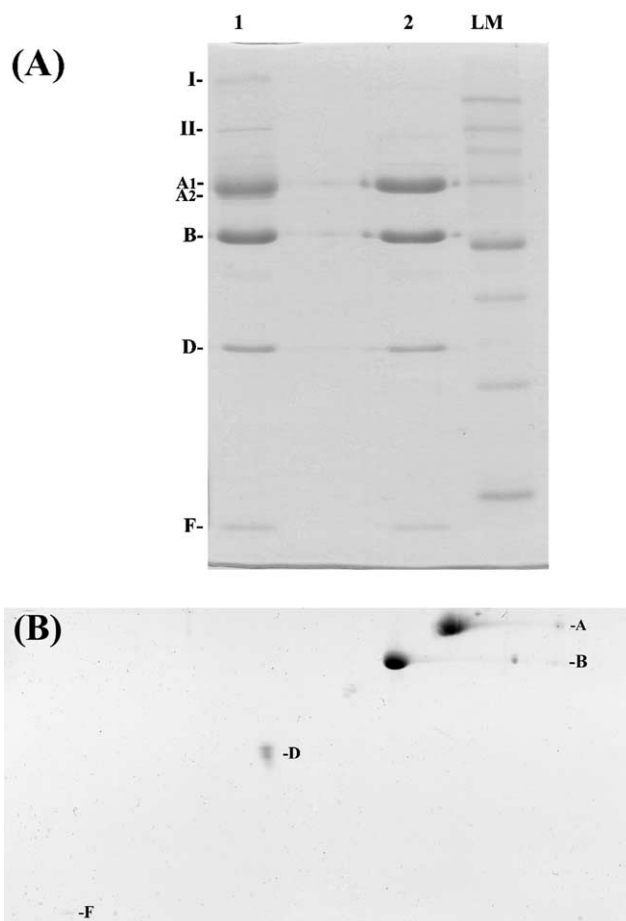


Fig. 4. Analysis of the mobility of the reduced and non-reduced A_3B_3DF subcomplexes. A: The enzyme (8 μg) was incubated with (lane 2) or without (lane 1) 10 mM of DTT for 10 min before being applied to a 17.5% total and 0.4% cross-linked acrylamide gel. LM, molecular mass marker. B: Two-dimensional SDS-PAGE to analyze the bands I and II. Lane 1 of the gel in A were cut out, destained, soaked in buffer consisting of 20 mM Tris-HCl (pH 8.1) and 150 mM NaCl, 50 mM DTT, and 0.1% SDS, and placed on a 17.5% total and 0.4% cross-linked acrylamide gel.

(Fig. 4A, lane 1). DTT (10 mM) prevented formation of polypeptides I and II and subunit A migrated as a more defined band. ATPase activity was increased by 20% under reducing conditions. To identify the polypeptides present in I and II, they were cut out of the gel, destained, and subjected to SDS-PAGE in the presence of DTT. As shown in Fig. 4B, the polypeptides I and II represent A-B and B-B adducts, respectively. These data clearly demonstrate redox modulation of the A_1 ATPase.

4. Discussion

We recently overproduced an A_1 ATPase complex from the mesophilic anaerobic archaeon *M. mazei* Gö1 in *E. coli* [3]. Here, the enzyme was purified to apparent homogeneity and its subunit composition was determined. N-terminal sequence analyses and immunological data revealed subunits A, B, C, D and F but subunits E and G were not detected, although the encoding genes are present on the expression plasmid and expressed in *E. coli* DK6 (pSÖ1) [3]. An explanation for this apparent discrepancy might be that subunits G and E are apparently unstable in the absence of subunits H, I, and/or K and, therefore, not assembled into the complex.

It is obvious from the data presented here that subunits C and E are dispensable for ATPase activity, and tryptic digestion of an A_3B_3CDF subcomplex removed subunits C and F but retained 65–70% of ATPase activity [20]. Taken together, these data indicate that an A_3B_3D complex would be the minimal core unit of an A_1 ATPase. The same holds true for the F_1F_0 ATPase [21,22], in which an $\alpha_3\beta_3\gamma$ subcomplex was described as the core unit. Subunits A, B, and D are indeed similar to subunits β , α , and γ of F_1F_0 ATPases [1].

A mechanism of regulating ATPase activity has been described for the closely related V-ATPase in which cysteine residues, located at the catalytic A subunit, are able to form disulfide bonds, leading to reversible inhibition of V-ATPase activity [23]. In the present report, we demonstrate that in the oxidized state A-B and B-B products occur in the archaeal A_3B_3DF subcomplex, which are abolished in the reduced enzyme, resulting in an increase of hydrolytic activity. There is a striking similarity with recent data of the A_1 complex in which A-B and B-B disulfide formations were generated after incubation with the nucleotide analogue 5'-adenylyl-imidophosphate (*closed* conformation), induced by Cu^{2+} [24]. It is of interest that subunit A in the oxidized A_1 subcomplex, containing six Cys residues (Cys 28, 64, 173, 255, 372) [5], appears in two forms (A_1 , A_2). The oxidized form of the vacuolar A subunit, which is 50% identical to the A ATPase A subunit, migrates as a diffuse (A_1) and a more defined second band (A_2) [25]. Many disulfide bond containing proteins migrate faster in SDS-PAGE than do their reduced counterparts, presumably because the disulfide form is more compact [26]. The fact that subunit A in the oxidized subcomplex appears in two forms (A_1 and A_2) implies that not all three subunits are in a *closed* conformation. Like the most reactive Cys residue, Cys 254 of the vacuolar A subunit [23], the archaeal A subunit has a conserved Cys 255 in close proximity to the P-loop [5],

which might play a role in disulfide bond formation in the archaeal subcomplex. Further experiments will be required to elucidate the importance of this redox modulation for the archaeal ATPase *in vivo* and whether significant structural alterations occur in the stalk subunit(s) as described for the V_1 complex [25].

Acknowledgements: This study was supported by grants from the Deutsche Forschungsgemeinschaft (MU 801/10 and GR 1475/9-1).

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