Defined subcomplexes of the A_1 ATPase from the archaeon *Methanosarcina mazei* Gö1: biochemical properties and redox regulation

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Abstract The potential A_1 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 A_1 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 A_1 ATPases (diethylstilbestrol and its derivatives hexestrol and dienestrol) and identified redox modulation as a new type of regulation of archaeal A_1 ATPases.

Key words: Archaeon; A_1 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 A_1A_o ATP synthase which converts the electrochemical ion potential across the membrane into ATP [1]. Like the F_1F_o ATPase, A_1A_o 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 V_1V_o ATPases [2]. Therefore, the enzyme can be regarded as a chimera of F_1F_o and V_1V_o ATPases. Unfortunately, biochemical and structural data of the A_1A_o ATPases are scarce and even their subunit composition is far from being settled.

To obtain insights into the structure–function relationship of A_1A_o 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 F_1F_o ATPase-negative strain *Escherichia coli* DK8 was used as a host to express the A_1A_o 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 A_1 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 A_1 ATPases to an A_3B_3DF subcomplex. Furthermore, kinetic studies revealed three new inhibitors for A_1 ATPases (diethylstilbestrol and its derivatives hexestrol and dienestrol) and identified redox modulation as a new type of regulation of archaeal A_1 ATPases.

2. Materials and methods

2.1. Organisms and plasmids

E. coli strain DK8 (1100Δ[*uncB-uncC*] *ilv*::Tn*10*) [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 A_1 ATPase

Cells were grown in a volume of 8 l in a fermenter. The expression of the A_1 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 $MgCl_2$, 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 A_1 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 Na_2-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 $MgCl_2$, 30 mM $NaHSO_3$, 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 *Sal*I restriction site at the 5′ terminus (5′-CTTAAATTTCGTCGACCATTGCTTT-3′) and a *Xba*I restriction site at the 3′ terminus (5′-CGGTATCTAGAAGTT-3′). The 416 bp fragment was cloned into pGEM-4Z (pTL0). Then, the 7.8 kb *Xba*I/*Kpn*I fragment of pRT103 was inserted into the *Xba*I and *Kpn*I sites of pTL0. The resulting plasmid (pTL2) contained 162 bp upstream of *ahaE*, the A_1 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, PALLGelman-Sciences, Rossdorf, 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 A_1 ATPase complex

We have shown previously that the A_1 ATPase can be produced in *E. coli* DK8 that is devoid of the F_1F_o ATPase but contains the potential A_1 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 A_1 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 A_1 complex

The A_1 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 (AQQDVKPTRSEL), and F (MELAVIGKSEF). The experimentally determined start codons match the ones deduced from the DNA sequences [5]. Indeed, the gene *ahaA* starts with the unusual codon GTG. The N-terminal methionine was removed from subunits B and D.

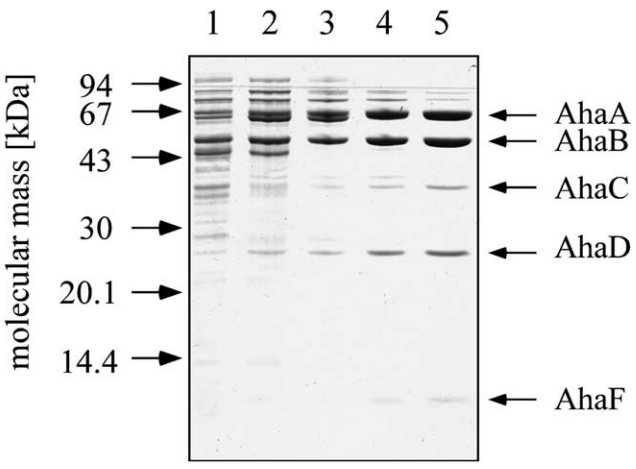

Fig. 1. Subunit composition of the A_1 ATPase. 10 μg of protein was applied to a 12.5% SDS–polyacrylamide gel. Lane 1, cytoplasm of *E. coli* DK8 (pTL2); lane 2, $NH_2(SO_4)_2$ precipitate; lane 3, pooled BioPrepSE1000/17 fractions; lane 4, pooled DEAE eluate; lane 5, pooled Sephacryl S-300 HR fractions.

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 A_1 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 A_1 ATPase assembly and function.

3.3. Kinetic properties of the A_3B_3CDF complex

The A_3B_3CDF complex had a requirement for Mg^{2+} but other divalent cations could substitute for Mg^{2+} in the order Fe^{2+} (55%) > Zn^{2+} (41%) > Mn^{2+} (30%) > Ni^{2+} (27%) > Ca^{2+} (10%) > Co^{2+} (1%). At an optimal Mg^{2+}: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 A_1 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
$(NH_4)_2SO_4$	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

[a]ATPase was purified and enzyme activity was determined as described in Section 2.
[b]Activity was determined in the optimized buffer.

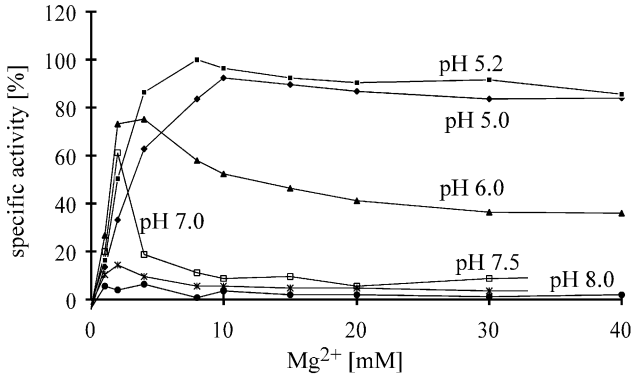

Fig. 2. Mg^{2+} dependence of ATPase activity at different pH values. The A_1 ATPase activity was determined in the optimized buffer containing 100 mM MES, 30 mM $NaHSO_3$, 40 mM Na-acetate, 8 mM $MgCl_2$, 4 mM ATP, and increasing [Mg^{2+}], at pH 5.0 (♦), 5.2 (■), 6.0 (▲), 7.0 (□), 7.5 (*), and 8.0 (●). 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 µmol/mg protein), by dienestrol (I_{50} = 6 µmol/mg protein) and by hexestrol (I_{50} = 5 µmol/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.

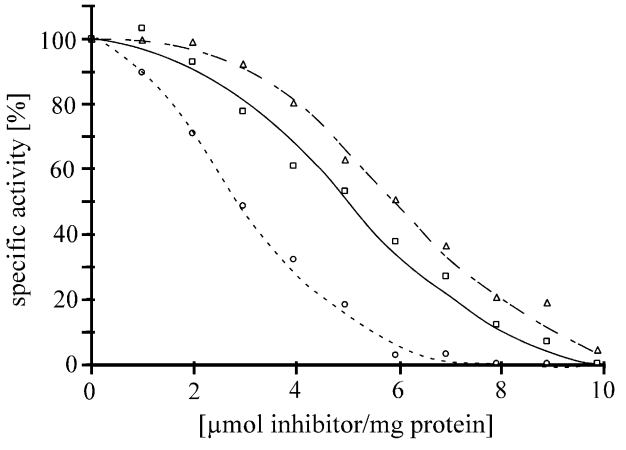

Fig. 3. Inhibition of the A_1 ATPase by DES and its derivatives. The ATPase activity was determined in optimized buffer containing increasing amounts of DES (dashed line), hexestrol (solid line), and dienestrol (dot-dashed line). The enzyme was preincubated with the inhibitors for 30 min at 25°C. The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

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

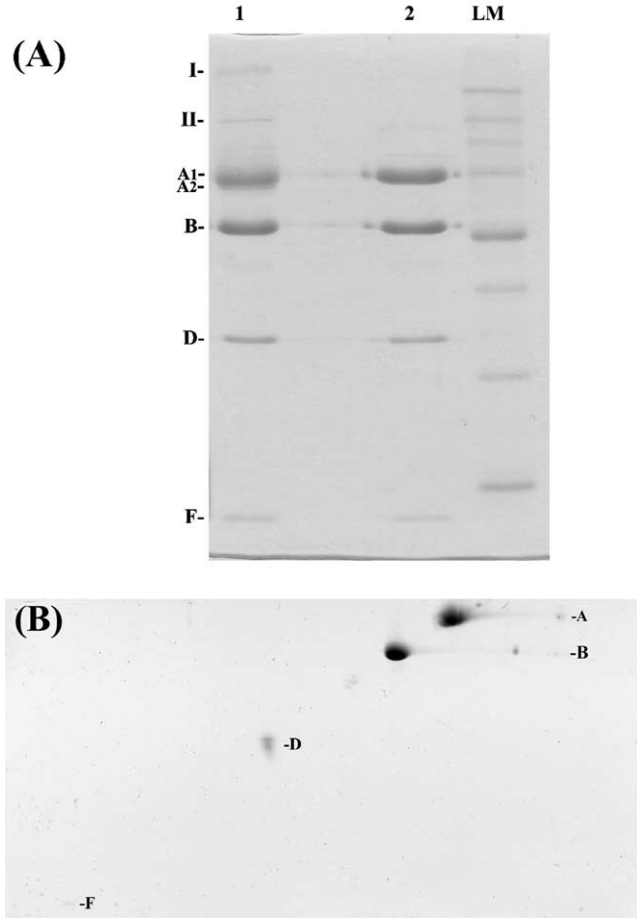

Fig. 4. Analysis of the mobility of the reduced and non-reduced A_3B_3DF subcomplexes. A: The enzyme (8 µg) was incubated with (lane 2) or without (lane 1) 10 mM of DTT for 10 min before being applied to a 17.5% total and 0.4% cross-linked acrylamide gel. LM, molecular mass marker. B: Two-dimensional 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_o 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_o 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].

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