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Published in:
Archives of Microbiology

DOI:
10.1007/s00203-006-0165-x

Publication date:
2006

Arch Microbiol (2006) 186:513–517
DOI 10.1007/s00203-006-0165-x

SHORT COMMUNICATION

The *ntp* operon encoding the Na^+ V-ATPase of the thermophile *Caloramator fervidus*

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Received: 4 May 2006 / Revised: 14 July 2006 / Accepted: 21 August 2006 / Published online: 12 September 2006

Abstract The V-type ATPase of the thermophile *Caloramator fervidus* is an ATP-driven Na^+ pump. The nucleotide sequence of the *ntpFIKECGABD* operon containing the structural genes coding for the nine subunits of the enzyme complex was determined. The identity of the proteins in two pairs of subunits (D, E and F, G) that have very similar mobilities on SDS-PAGE of the purified complex (24.3 and 22.7 kDa, and 12.3 and 11.6 kDa) was established by tryptic digestion of the protein bands followed by mass spectrometric analysis of the peptides.

Keywords ntp Operon · V-ATPase · Thermophile · *Caloramator fervidus* · Molecular motor · Central stalk

Introduction

The V-type ATPase of *Caloramator fervidus* pumps Na^+ ions out of the cell against the sodium ion motive force at the expense of the hydrolysis of ATP (Speelmans et al. 1994). The enzyme complex consists of an integral membrane part V_0 that is responsible for the translocation of the Na^+ ions and a cytoplasmic, membrane associated part V_1 that hydrolyzes ATP. V_0 is built of subunits K and I, V_1 consists of seven subunits, termed A, B, C, D, E, F, and G (Höner zu Bentrup et al. 1997). Like its eukaryotic counterparts and the well-studied F-type ATPases, the enzyme functions as two coupled molecular motors (for an overview, see Junge and Nelson 2005). ATP hydrolysis in V_1 drives the physical rotation of a stalk that is contained in the central cavity of a hexagonal arrangement of alternating A and B subunits. The central stalk is connected to a multimeric assembly of K subunits in V_0 (the 'rotor') that rotates in the plane of the membrane against the static part of V_0 formed by the I subunit. The rotation free energy is used to pump Na^+ ions across the membrane at the interface of the rotor and the I subunit.

Our studies of the V-type ATPase of *C. fervidus* have focused on the structure of the stalk region in the complex. Electron microscopy of the purified enzyme complex (Höner zu Bentrup et al. 1997; Ubbink-Kok et al. 2000) followed by single particle averaging, visualized a peripheral stalk in the complex for the first time for any ATPase complex (Boekema et al. 1997). Further studies demonstrated that the stator structure, in fact, consisted of two peripheral stalks that are positioned at an angle of 120° around the complex (Ubbink-Kok et al. 2000; Boekema et al. 1999). Subsequently, similar observations were made with V-type and A-type ATPases from other sources by others (Coskun et al. 2004; Zhang et al. 2003; Bernal and Stock 2004; Venzke et al. 2005). A major challenge remains the localization of the different subunits in the stalk regions. Electron microscopy studies of a series of subcomplexes of the V-type ATPase of *C. fervidus* demonstrated that the central stalk was composed of

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two subunits. Subunit E forms an elongated structure sitting in the cavity of the V_1 headpiece and subunit C a spherical structure that provides the connection with the rotor in V_0 (Chaban et al. 2002). The assignment of subunit E to the central stalk is a matter of debate (e.g., Yokohama et al. 2003; Chaban et al. 2004; Arata et al. 2002). Building subcomplexes from isolated subunits is likely to be the best way to resolve this issue. Here, we describe the first step in this approach: the sequencing of the *ntp* operon of *C. fervidus* that contains the structural genes for the V-type ATPase subunits.

Materials and methods

Growth conditions

Caloramator fervidus ATCC 43024 was grown anaerobically at 68°C in trypton-yeast extract-glucose medium (Patel et al. 1987) as described (Ubbink-Kok et al. 2000). *Escherichia coli* DH5α was grown in Luria Bertani Broth (LB) medium at 37°C under continuous shaking at 150 rpm. When appropriate, ampicillin was added to the medium at a final concentration of 50 μg/ml.

Sequencing of the *ntp* operon

Chromosomal DNA was extracted from a culture of *C. fervidus* following standard procedures. A course sequence of the region containing the *ntp* operon was obtained using a variety of strategies all based on the PCR technique. A first fragment was obtained using degenerated primers based on two highly conserved regions in the D subunits of V-type ATPases found in a number of low GC Gram positives (Lolkema et al. 2003). Assuming the same gene order as observed on the chromosome of these organisms, upstream sequences were obtained using degenerated forward primers targeted at conserved regions in the A and B subunits in combination with specific backward primers based on the already known sequence. Also, degenerated forward primers were designed based on the N-terminal sequence of subunits A, B, and C that were determined before using the isolated subunits of the purified enzyme complex (Höner zu Bentrup et al. 1997). PCR products were ligated directly into the pGEM-T Easy vector (System I; Promega, Madison, USA) and after propagation in *E. coli* DH5α, one strand of the inserts was sequenced using the M13 primer set.

The missing ends of the operon at the 5′ and 3′ end of the sequence obtained with the above strategies were determined using an inverted PCR approach as follows. A 6-base cutting restriction site A was selected close to the end of the known sequence. Two specific primers were designed, one against each strand, at sites just in front of and following site A. The primers extend in opposite directions and away from site A. A 4-base cutting restriction enzyme was selected that does not cut in between site A and the end of the known sequence. Genomic DNA (1 μg) was digested to completion with this enzyme in the appropriate buffer. Following heat-killing of the enzyme, the sample was diluted tenfold with ligase buffer containing T4 DNA ligase and incubated at room temperature for 2 h. Circularized DNA fragments were ethanol-precipitated, resuspended in 8 μl of water and restricted at site A in the appropriate buffer. Successful ligation of the DNA fragment containing the known sequence plus a stretch of unknown sequence follows from the production of a distinct band in a PCR reaction using the two specific primers. The PCR fragments were ligated into the pGEM-T Easy vector and sequenced as above.

The final base sequence was determined by designing specific forward and backward primers producing DNA fragments of ~500 base pair lengths that were overlapping by ~100 base pairs. The PCR products were sequenced on both strands and the final sequence was determined by matching the results obtained from the different fragments and from the complementary strands. Sequencing was done at ServiceXS, Leiden, The Netherlands. Open reading frames were determined using CloneManager (Sci Ed Central) and by aligning with the known sequences of related organisms. Putative promoters and terminators were identified at http://www.softberry.com/berry.phtml. The sequence was deposited in GenBank and is available under accession number DQ369724.

Mass spectrometry analysis

The V-type Na^+-ATPase of *C. fervidus* was purified to homogeneity as described (Ubbink-Kok et al. 2000). The subunits were separated by SDS-PAGE using a 12 or 15% gel as indicated and visualized by Coomassie Brilliant Blue staining. One gel was used to cut out the protein bands corresponding to the molecular masses of 12 and 10 kDa (the putative F and G subunits). A second gel was run for a longer period of time to obtain a better separation of the protein bands running at 26 and 25.5 kDa (the putative E and D subunits) allowing the two bands to be cut out of the gel without significant cross-contamination. The pieces of gel were fragmented in smaller pieces, destained in 50 mM ammonium bicarbonate in 40% ethanol, dehydrated

by a three times repeated treatment with 100 µl acetonitril, and dried completely using a SpeedVac centrifuge. The pieces of gel were reswollen by adding 20 µl of a 10 ng/µl trypsin or Arg-C solution and the samples were incubated overnight at 37°C. The peptides were extracted from the fluid by shaking for 20 min with 30 µl of a mixture of 60% acetonitril and 1% trifluoroacetic acid (TFA) in water. The extracted peptides were dried in a SpeedVac centrifuge and dissolved in 10 µl of 0.1% TFA in water. Aliquots of 0.75 µl of the peptide suspension were spotted on the MALDI target and mixed on the target in a 1:1 ratio with the matrix solution consisting of 10 mg/ml α-cyano-4-hydroxycinnamic acid (dissolved in 70% acetonitril and 0.1% TFA). The spots were allowed to dry completely before the MALDI-TOF experiment was performed on the Applied Biosystems 4700 Proteomics Analyzer.

Materials

Sequence grade modified porcine trypsine was obtained from Promega. Arg-C was obtained from Sigma. All other were reagents were reagent grade and obtained from commercial sources.

Results and discussion

The *ntp* operon of *C. fervidus*

The nucleotide sequence of a stretch of 9554 base pairs on the chromosome of *C. fervidus* was determined. The stretch that has a CG content of 32.6% contains the *ntp* operon consisting of the nine structural genes coding for the subunits of the V-type Na^+-ATPase as depicted in Fig. 1. The order of the genes *ntpFIKECGABD* was as observed in a number of other Gram-positives with low CG content that contain a V-type ATPase (Lolkema et al. 2003) among which the well studied enzyme from *Enterococcus hirae* (Takase et al. 1994). The genes are very densely packed with only 0.8% base pairs in the intergenic regions between the start codons of the *ntpF* and *ntpD* genes. The *ntpF*, *ntpC*, *ntpA*, and *ntpB* genes extend into the coding regions of the downstream genes. Upstream of the *ntpF* gene, two putative promoter sequences were identified with initiation of transcription sites at positions 520 and 889. Immediately downstream of the stop codon of the *ntpD* gene with only three base pairs in between, a putative terminator sequence capable of forming a stem-loop structure (free energy of −13.4 kcal/mol) was found starting at position 9508 (Fig. 1).

The translated amino acid sequences of the 9 genes in the *ntp* operon are most similar to the corresponding subunits of the V-type ATPases of *Clostridium tetani* and *Clostridium perfringens* (Table 1). Sequence identities with the *C. tetani* subunits range from 36% for the NtpF subunit which is part of the stalk region to 85% for the NtpB subunit which is the catalytic subunit in the headpiece. It may be noted that *C. fervidus* originally was named *Clostridium fervidus* (Collins et al. 1994).

The start codon of the F subunit

The start codons of all the genes in the *ntp* operon could be unambiguously assigned based on the sequence except for the *ntpF* gene. The 5′ region of the coding sequence was TTGGCAAAGGAAGTT GTAAAG (the two potential start codons at positions 1043 and 1055 are underlined) which translates into the amino acid sequence LAKEVVK. The F subunit is the least conserved subunit and alignment with the corresponding subunits from other sources did not yield a conclusive result (not shown). The protein band corresponding to the F subunit was extracted from the gel following SDS-PAGE of the complete

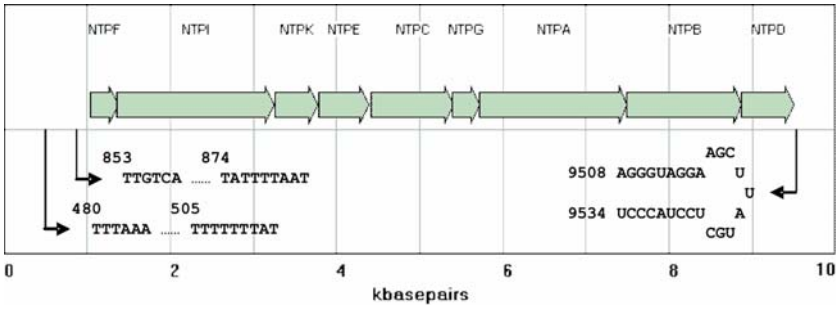

Fig. 1 Genetic organization of the *ntp* operon of *C. fervidus*. *Arrows* represent the nine genes coding for the subunits of the V-type ATPase of *C. fervidus*. Two putative promoter sites were indicated on the *left* and a terminator sequence forming a stem-loop structure was indicated on the *right*. All positions are relative to the first base of the sequenced fragment

Table 1 Subunits of the V-type ATPase of *C. fervidus*

Subunit	*Caloramator fervidus*			*Clostridium tetani*		
	Base pairs[a]	Amino acids	MW (kDa)	gi number[b]	Amino acids	Identity (%)
NtpF	1043–1369	108	12.3	28210699	108	36
NtpI	1356–3248	630	72.3	28210700	656	41
NtpK	3262–3771	169	17.6	–[c]	157	50
NtpE	3788–4384	198	22.7	2821-702	201	38
NtpC	4417–5403	328	38.4	28210703	333	39
NtpG	5396–5719	107	11.6	28210704	105	59
NtpA	5725–7497	590	66.0	28210705	592	75
NtpB	7490–8875	461	51.2	28210706	461	85
NtpD	8875–9504	209	24.3	28210707	212	74

[a] Relative to the first base pair of the sequenced fragment

[b] NCBI protein database identifier

[c] NtpK incorrectly annotated in the genome sequence

enzyme complex isolated from *C. fervidus* (Fig. 2; Ubbink-Kok et al. 2000). The sample was treated with Arg-C, a proteolytic enzyme that cuts at the C-terminal side of arginine residues unless the next residue is a proline. The digest was analyzed by MALDI-TOF mass spectrometry. A peptide was obtained with an observed *M*/*Z* of 2338.27, which corresponds to the N-terminal peptide AKEVVKQVIDVERQAEEIVR of the F subunit when the start codon is TGG at position 1043 and the first residue is removed. The identity of the peptide was confirmed by tandem MS. No peptide indicating translation at the second initiation site was detected.

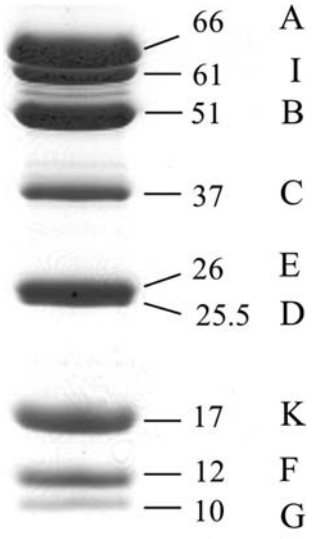

Fig. 2 The subunits on SDS-PAGE of the purified V-type ATPase of *C. fervidus*. The enzyme complex was purified as described (Ubbink-Kok et al. 2000) and 20 µg was loaded on a 12% SDS-PAGE. Indicated on the *right* were the apparent molecular masses of the A, I, B, C, E, D, K, F, and G subunits that were inferred from a set of marker proteins (not shown)

Identification of the subunits after SDS-PAGE

The calculated molecular masses of the D and E subunits (24.3 and 22.7 kDa) and of the F and G subunits (12.3 and 11.6 kDa) are very similar (Table 1). During SDS-PAGE of the complete V-ATPase enzyme complex isolated from *C. fervidus* (Ubbink-kok et al. 2000), especially the D and E subunits run with almost the same mobility, corresponding to molecular masses of 25.5 and 26 kDa (Fig. 2). The assignment of the two bands, D and E, was based on differences in the intensities after Coomassie staining that were observed for the corresponding bands of the *E. hirae* complex (Murata et al. 1997). Subunits E and D of the *E. hirae* complex are well separated on SDS-PAGE with apparent molecular masses of 24 and 27 kDa, respectively. The assignment resulted in a different order of mobility for the two subunits of the two organisms and has been a matter of debate in studies of the subunit composition of the central stalk of the complex. Electron microscopy and single particle analysis of a subcomplex of the *C. fervidus* complex showed subunit E as an elongated density, partly sitting in the central cavity of the AB complex in very much the same way as the γ subunit in F-type ATPases (Chaban et al. 2002). The finding was a surprise because it was generally believed that in V-type ATPases the D subunit would be the central stalk. To confirm the assignment of the subunits, the protein bands of the *C. fervidus* enzyme were extracted from the gel and digested with trypsin followed by analysis of the peptides by MALDI-TOF mass spectrometry. Table 2 lists three peptides for each of the four protein bands analyzed that unambiguously identify the subunits. It follows that the original annotation of the subunits was correct. In spite of the higher molecular mass, subunit D of *C. fervidus* has a higher mobility than

Table 2 Assignment of subunits D, E, F, and G after SDS-PAGE of the holoenzyme

Protein band (kDa)	Observed *M*/*Z*	Subunit	Peptide[a]	From	To	Calculated MW (Da)
26	1021.6	E	IVEEFKEK	62	69	1020.6
	1430.7		FNQSFLYEINR	149	159	1429.7
	1663.9		LSSETLNLNSGFILR	172	186	1662.9
25.5	2136.1	D	AVMGAEFLQEAISMPAETIK	88	107	2135.1
	1592.8		NIMSVSVPVMNFVR	114	127	1591.8
	1517.8		VNALEYVMIPQLK	185	197	1516.8
12	841.4	F	QVIDVER	3	9	840.4
	1021.5		EIIENFEK	50	57	1020.5
	1405.7		EAQPIYDEANKK	62	73	1404.7
10	1101.5	G	GVDTPEEAQR	25	34	1100.5
	1540.8		EGYGLIFVTETIAK	42	55	1539.8
	1004.5		EIDETIER	56	63	1003.5

[a] Peptide identified by MALDI-TOF mass spectrometry following tryptic digestion of the protein band

subunit E on SDS-PAGE. Two factors may contribute to the different relative electrophoretic behavior of the D and E subunits of *E. hirae* and *C. fervidus*. First, the calculated molecular masses of the D subunits are 27.1 and 24.3 kDa for the *E. hirae* and *C. fervidus* subunits, respectively. Second, the E subunit of *E. hirae* is significantly more hydrophobic than the E subunit of *C. fervidus*. The higher hydrophobicity of the former may results in the binding of a higher amount of SDS during SDS-PAGE resulting in a higher mobility.

Acknowledgment We appreciate the use of the mass spectrometry facility at the Dutch Proteomics Center in the Membrane Enzymology group at the University of Groningen headed by Prof. B. Poolman.

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