Volume 316, number 1, 89–92 FEBS 12020 January 1993

N-terminal amino acid sequences of the subunits of the Na^+-translocating F_1F_0 ATPase from *Propionigenium modestum*

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Received 1 December 1992

We report here the N-terminal protein sequences of the subunits of the ATPase from *Propionigenium modestum*. Subunits c, b, δ, α and β start with an N-terminal methionine residue, the γ and ε subunits have an alanine N-terminus, from which *N*-formylmethionine was hydrolyzed by posttranslational modification, and subunit a contains a blocked N-terminus. Each of the N-terminal sequences exactly matches a portion of the DNA sequence in the gene encoding the respective subunit protein on the *unc* operon. Thus, the exact translational start for each subunit protein can be identified and the primary structures of the protein transcripts can be clearly defined. Based on these data the putative size of the open reading frame that was envisaged from the DNA sequence had to be revised for the α and δ subunits.

F_1F_0 ATPase; *Propionigenium modestum*; Initiation of translation; Start of coding sequence

1. INTRODUCTION

The strictly anaerobic eubacterium *Propionigenium modestum* grows from fermentation of succinate to propionate and CO_2 at the remarkable low free energy span of $\Delta G^{\circ\prime} \approx -20$ kJ/mol [1]. Part of this free energy is converted into an electrochemical gradient of Na^+ ions by membrane-bound methylmalonyl-CoA decarboxylase [2]. The $\Delta\tilde{\mu}Na^+$ thus established provides the direct driving force for ATP synthesis by a Na^+-translocating F_1F_0 ATPase [3,4]. The unique property of the *P. modestum* ATPase to use Na^+ instead of H^+ as coupling ion for ATP synthesis has attracted special interest in the structural peculiarities associated with this switch in ion specificity.

Protein structural analyses have indicated a composition of the *P. modestum* ATPase from eight different subunits; the five denoted $\alpha, \beta, \gamma, \delta, \varepsilon$ form the F_1 sector and the three denoted a, b, c are constituents of the more firmly membrane-bound F_0 moiety [4]. The homology between these subunits and those of the *E. coli* ATPase has been most strikingly demonstrated by in vitro and in vivo complementation studies. ATPase hybrids consisting of F_0 from *P. modestum* and F_1 from *E. coli* were found to be functional and to catalyze Na^+ translocation like the *P. modestum* enzyme [5,6].

The genes encoding the ATPase subunits have been cloned and sequenced [6–10]. The genes are clustered on the genome (*unc* operon) and occur in the same order as in *E. coli*. In order to clearly identify the reading frames and thus the primary structure of the ATPase subunits encoded by these genes, the N-terminal sequences of the individual subunits must be known. So far, protein sequencing has only been performed with subunit c [8]. We report here the N-terminal sequences of the other ATPase subunits except subunit a which apparently contains a blocked N-terminus.

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2. EXPERIMENTAL

2.1. *Purification of F_1F_0-ATPase*

The ATPase of *Propionigenium modestum* was isolated from membranes by extraction with Triton X-100 and fractionated by precipitation with poly(ethylene glycol) as described [4].

2.2. *N-terminal sequencing*

ATPase subunits were separated by SDS-PAGE according to Schägger et al. [11] and electroblotted onto a hydrophobic poly(vinylidene difluoride) (PVDF) membrane (Millipore) [12]. Blot staining was performed with 0.1% Coomassie G250.

Filter pieces with the respective subunits were used directly for N-terminal sequence analysis using a protein sequencer (model 470/A; Applied Biosystems) with on-line PTH-(phenylthiohydantoin) amino acid detection on a HPLC (model 120; Applied Biosystems). For all subunits the first seven amino acids were sequenced.

2.3. *Immunization and immunoblot analysis*

The synthetic peptide RWSSKNLEVVLERKQAFFET which comprizes a hydrophilic sequence within the N-terminal region of the a subunit of the *P. modestum* ATPase was custom-synthesized by Cambridge Research Biochemicals (Cambridge, UK) and conjugated to keyhole limpet hemocyanin.

A rabbit (species: New Zealand White) was first immunized with 0.1 mg conjugated peptide emulsified with complete Freund's adjuvant. Following immunizations were carried out with 0.1 mg peptide and incomplete Freund's adjuvant. Blood was withdrawn from an ear vein approximately ten days after immunization. It was then allowed to clot overnight at 4°C. After centrifugation the serum was collected and stored at −20°C.

For immunoblotting, the ATPase subunits were separated by SDS-PAGE as described above and electroblotted onto a nitrocellulose membrane. Unspecific binding sites were blocked with PBS (phosphate-buffered saline)/0.5% Tween for one hour. 100 μl of polyclonal antibody were incubated with 2 ml *E. coli* lysate overnight at 4°C. This pretreated antibody was diluted with 10 ml PBS/0.05% Tween/0.05 g BSA (bovine serum albumin) and incubated with the blot for 5 h. After washing with PBS/0.05% Tween and labeling with goat anti-rabbit IgG (H+L) conjugated to alkaline phosphatase (Bio-Rad) (diluted 1:1500 with PBS/0.05% Tween/0.5% BSA) the color development reaction was performed with BCIP (5-bromo-4-chloro-3-indoyl phosphate *p*-toluidine salt) and NBT (*p*-nitro blue tetrazolium chloride).

2.4. *Computer analyses*

Sequences used for computer analyses were taken from the EMBL database (Accession No. X58461 for *P. modestum*ATPase and Accession No. J01594 for *E. coli* ATPase). Sequence comparison was done with the program BESTFIT from the GCG software package version 7.1 using the default values. Molecular masses of the subunits of *P. modestum* ATPase were calculated with the program PEPTIDESORT.

3. RESULTS AND DISCUSSION

To define the primary structure of all ATPase subunits encoded by the *unc* operon of *P. modestum*, N-terminal protein sequencing is required in addition to the DNA sequences that have already been published [6–10]. The individual ATPase subunits were therefore separated by SDS-PAGE, blotted onto a PVDF membrane and subjected to seven cycles of automated N-terminal sequencing. Seven different sequences were obtained and are shown in Table I. It is also shown that each of these sequences exactly matches a stretch of DNA sequence in the vicinity of the putative N-terminus of subunits α, β, γ, δ, ε, b and c. These data thus clearly define the open reading frames encoding these ATPase subunits and the N-terminal amino acid in each mature polypeptide chain. These N-terminal protein sequences also provide insight into the initiation of translation which requires an initiation codon (ATG, GTG, or more rarely TTG) and a Shine–Dalgarno sequence (consensus AGGAGG) in appropriate distance upstream the initiation codon [3]. These initiation signals for translocation are marked in Table I and are described below for each of the ATPase subunits. All following DNA sequence positions are referred to EMBL database (Accession No. X58461).

Subunit c

The N-terminal sequence (Table I) confirms previous protein sequencing data of this subunit [8]. The N-terminal methionine is translated from an ATG start codon (position 2053 of the DNA sequence) located 10 base pairs downstream to an AGGAGG Shine–Dalgarno consensus sequence.

Subunit b

Our protein sequencing data show that initiation of translation of this subunit uses the rare TTG start codon (position 2430). ATG, 15 base pairs downstream the TTG start codon is not used as translational start of the b subunit. Utilization of this TTG start codon is reasonable, because an AGGAGG Shine–Dalgarno sequence is located 8 base pairs upstream, while the ATG codon at position 2445 is not flanked by an appropriate ribosome binding site.

Subunit δ

Ambiguity about the start of this subunit could be solved by N-terminal protein sequencing. It is now clear that ATG at position 2933 of the DNA sequence is the initiation codon rather than an ATG 102 nucleotides downstream that was previously assumed to initiate translation of this polypeptide [6]. A putative ribosome binding sequence (AGG) is located 9 base pairs upstream of the actual start codon.

Subunit α

Interestingly, the rare TTG start codon (position 3493) is used again to initiate translation of the α-subunit. A Shine–Dalgarno consensus AGGAGG is located 9 base pairs upstream of this initiation codon. The protein sequence data exclude start of the α-subunit with an ATG start codon (position 3676), 183 nucleotides further downstream, which was proposed to initiate translation of this protein based on the DNA sequence data (EMBL database, Accession No. X58461).

Subunit γ

Synthesis of this protein is initiated by an ATG start codon (position 5008), 7 base pairs downstream a putative ribosome binding site. The N-terminal *N*-formylmethionine has been cleaved off in the pure protein which therefore starts with the following amino acid alanine.

Subunit β

Translation of the β-subunit is initiated by a GTG start codon (position 5906) located 7 base pairs downstream a AGGAGG Shine–Dalgarno consensus sequence.

Subunit ε

ATG (position 7198) is used as start codon for the ε-subunit. A putative ribosome binding sequence is located 10 base pairs upstream of the start codon. The ε-subunit is processed like the γ-subunit and starts with alanine that follows the cleaved *N*-formylmethionine.

Subunit a

No PTH amino acids were released during Edman degradation from the a subunit, suggesting that this polypeptide is substituted at its N-terminal amino acid residue. A protein band corresponding to the a subunit could be clearly identified on the PVDF blot used for sequencing by immunological techniques. For this, a

polyclonal antibody was raised in a rabbit against a synthetic polypeptide of 20 amino acid residues within the N-terminal region of the a subunit. This antibody reacted specifically with a band on the blot that migrated as expected for subunit a (Fig. 1). Based on the DNA sequence the translation of the a subunit is predicted to start with ATG (position 1120) that is located 4 base pairs downstream a putative ribosome binding site (GGAG).

Also shown in Fig. 1 are the locations of the other subunit bands on the PVDF blot, as revealed from N-terminal protein sequencing. As noted previously, subunit c forms strong aggregates that move slowly on SDS-PAGE [4]. This protein was therefore identified by sequencing in the vicinity of the α-and β-subunits. The sizes of all ATPase subunits from *P. modestum* deduced from the primary structures are summarized in Table II and are compared to the corresponding data from the *E. coli* enzyme [14]. The α- and β-subunits of the *P. modestum* enzyme are shorter by 42 and 31 residues, respectively, than those of the *E. coli*ATPase. The γ, δ and ε subunits are of very similar size and the a, b and c subunits of the *P. modestum* ATPase are larger than the corresponding *E. coli* subunits by 18, 12 and 10 residues, respectively. The highest degree of identity exists between the β- and α-subunits (70 and 57%, respectively), while the identity among the other corresponding subunits from these two ATPases though evident is clearly less pronounced, ranging from 25 to 38%.

The *P. modestum* ATPase has deformylated N-terminal methionine on subunits c, b, δ, α and β. The *E. coli* ATPase has *N*-formylmethionine on subunit c and N-terminal methionine residues on the b, δ and α subunits. In both ATPases the N-terminus of the α subunit is blocked. Assuming the translational start of the a subunit in *P. modestum* at ATG (position 1120), the N-terminal *N*-formylmethionine residue would be followed by two lysine residues, which makes removal of the *N*-formylmethionine rather unlikely [15]. Interestingly, the N-terminal *N*-formylmethionine has been removed from the γ- and ε-subunits from both ATPases and from the β-subunit of the *E. coli* ATPase. In all these cases the cleavage occurred to an adjacent alanine residue, which is known to favour cleavage of *N*-formylmethionine residues [15]. The methionine was not removed from the *P. modestum* β-subunit which contains glutamate instead of alanine as the second residue. All subunits from which *N*-formylmethionine is hydrolyzed, in addition to alanine at position 2, also

Table I.

Amino terminal sequences determined by protein sequence analysis of isolated *P. modestum* ATPase subunits and alignment with the corresponding DNA sequence. For each subunit the first seven amino acids were determined by N-terminal sequencing. Start codons are highlighted in bold and putative ribosome binding sites are underlined. DNA sequences are numbered according to the sequence deposited in the EMBL database (Accession No. X58461). The downstream ATG codons shown in the δ-subunit (position 3035) and in the α-subunit (position 3676) have been assumed on the basis of the DNA sequence to function as start codons for these proteins.

Subunit		Sequences
c	protein	M D M V L A K , etc. to C-terminus
	DNA	AAGGAGGGAATCAAGAT**ATG**GATATGGTATTAGCTAAA
		2053
b	protein	M A P Q N M P , etc.
	DNA	GGAAGGAGGTAGACAAC**TTG**GCACCACAAAATATGCCT
		2430
δ	protein	M I E A Q V G RRYAEAIYEIAESNDNVKELYE T L N G V M , etc.
	DNA	GAGGTAGGGGAAGAGAA**ATG**ATAGAAGCACAAGTTGGT.....GAAACTTTAAATGGAGTAATG
		2933 3035
α	protein	M K I R P E E ISGIIKTEIENYKKSLDVKTSGSVVQVGDGIARIYGLSNAKAGELLEFP N G I T G M
	DNA	GTAGGAGGTGTAATCAC**TTG**AAGATCAGACCGGAAGAG.....CCTAACGGGATTACAGGGATG
		3493 3676
γ	protein	A A G K E I K , etc.
	DNA	TAAAGAGAGGTGAGACC**ATG**GCTGCGGGAAAAGAGATAAAG
		5008
β	protein	M E N K G V I , etc.
	DNA	GAAAAGGAGGCAATTTG**GTG**GAAAATAAGGGAGTTATT
		5906
ε	protein	A T F K L E V , etc.
	DNA	AAGGGAGCTGAATAATC**ATG**GCGACTTTCAAATTAGAAGTG
		7198

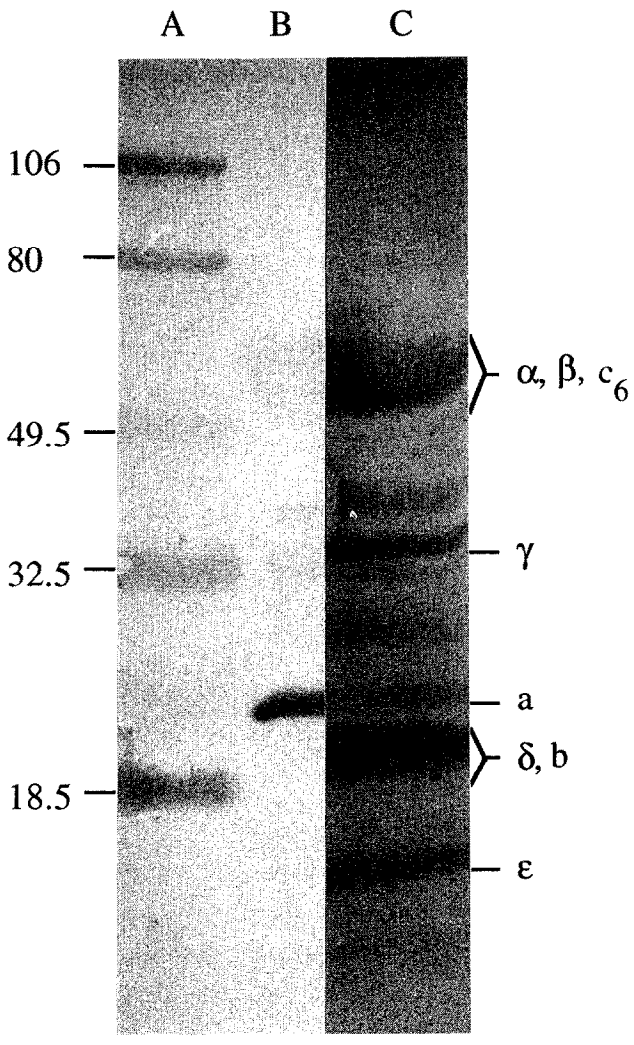

Fig. 1. (A) Molecular weight markers in kDa. (B) Immunoblot of the separated subunits developed with antiserum against a 20-mer synthetic peptide of the N-terminal region of the a subunit. (C) Position of the *P. modestum* ATPase subunits separated by SDS-PAGE and blotted onto a PVDF membrane as revealed from N-terminal protein sequencing.

contain lysine at position 5, except for the *E. coli* ε-subunit which has a tyrosine residue at the 5th position.

Another point that deserves mention is that in all cases where multiple copies of subunits are translated from the mRNA, the ribosome binding site has the consensus AGGAGG, whereas variations in this sequence are found in all cases where only one subunit copy is translated.

Table II

Comparison of the size and amino acid sequence identity of the ATPase subunits from *Propionigenium modestum* and *E. coli*. Posttranslational processing of N-terminal methionine residues were not taken into account.

Subunit	Number of amino acids		Molecular mass [kDa]		Percent identity
	P. modestum	*E. coli*	*P. modestum*	*E. coli*	
α	471	513	51.1	55.3	57.2
β	429	460	46.6	50.3	69.6
γ	282	287	31.8	31.6	38.4
δ	174	177	19.9	19.3	28.3
ε	137	139	15.4	15.1	27.4
a	289	271	32.2	30.3	25.2
b	168	156	19.2	17.2	30.1
c	89	79	8.7	8.3	25.0

Acknowledgements: Protein sequencing was performed at the Proteinchemie Service Labor of the ETH by Rocco Falchetto and Peter James.

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