Biochimica et Biophysica Acta 1777 (2008) 583–591

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbabio

Probing the functional tolerance of the *b* subunit of *Escherichia coli* ATP synthase for sequence manipulation through a chimera approach ☆

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ARTICLE INFO

Article history:
Received 1 February 2008
Received in revised form 5 March 2008
Accepted 7 March 2008
Available online 16 March 2008

Keywords:
ATP synthase
b subunit
Chimeric protein
Coiled coil
Right-handed coiled coil
Hendecad pattern

ABSTRACT

A dimer of 156-residue *b* subunits forms the peripheral stator stalk of eubacterial ATP synthase. Dimerization is mediated by a sequence with an unusual 11-residue (hendecad) repeat pattern, implying a right-handed coiled coil structure. We investigated the potential for producing functional chimeras in the *b* subunit of *Escherichia coli* ATP synthase by replacing parts of its sequence with corresponding regions of the *b* subunits from other eubacteria, sequences from other polypeptides having similar hendecad patterns, and sequences forming left-handed coiled coils. Replacement of positions 55–110 with corresponding sequences from *Bacillus subtilis* and *Thermotoga maritima b* subunits resulted in fully functional chimeras, judged by support of growth on nonfermentable carbon sources. Extension of the *T. maritima* sequence N-terminally to position 37 or C-terminally to position 124 resulted in slower but significant growth, indicating retention of some capacity for oxidative phosphorylation. Portions of the dimerization domain between 55 and 95 could be functionally replaced by segments from two other proteins having a hendecad pattern, the distantly related E subunit of the *Chlamydia pneumoniae* V-type ATPase and the unrelated Ag84 protein of *Mycobacterium tuberculosis*. Extension of such sequences to position 110 resulted in loss of function. None of the chimeras that incorporated the leucine zipper of yeast GCN4, or other left-handed coiled coils, supported oxidative phosphorylation, but substantial ATP-dependent proton pumping was observed in membrane vesicles prepared from cells expressing such chimeras. Characterization of chimeric soluble *b* polypeptides *in vitro* showed their retention of a predominantly helical structure. The *T. maritima b* subunit chimera melted cooperatively with a midpoint more than 20 °C higher than the normal *E. coli* sequence. The GCN4 construct melted at a similarly high temperature, but with much reduced cooperativity, suggesting a degree of structural disruption. These studies provide insight into the structural and sequential requirements for stator stalk function.

1. Introduction

Ion-translating ATP synthase/ATPases of the F-, A-, or V-types utilize a rotational mechanism for coupling ion movement through the membrane-bound sector, F_0, A_0, or V_0, to the synthesis or hydrolysis of ATP by the peripheral catalytic sector, F_1, A_1, or V_1. In the prototypical F-ATP synthase of *Escherichia coli*, the central rotor subcomplex is composed of the $\gamma\varepsilon c_{10}$ while the stator is composed of $\alpha_3\beta_3\delta ab_2$. The *b* subunit dimer forms the peripheral stator stalk, linking the *a* subunit of F_0 with $\alpha_3\beta_3\delta$ of F_1. The stator stalk must resist the torque imposed by the rotor so that the γ subunit turns inside $\alpha_3\beta_3$, generating conformational changes associated with ATP synthesis. The ATP synthases of most eubacteria have homodimeric stator stalks, but photosynthetic species, amongst others, contain heterodimers of two *b*-type subunits, *b* and *b′*. The stator stalks of chloroplast ATP synthase are also heterodimeric, with subunits denoted I and II. The stator stalk of mitochondrial ATP synthase has a different architecture; one of its subunits is called *b* but bears little sequence similarity to the eubacterial and chloroplast *b* family. See recent reviews of ATP synthase [1–4].

The soluble domain of *E. coli b*, expressed without the N-terminal transmembrane domain, has been characterized as a highly extended, helical dimer with substantial coiled coil character [5–8]. Deletion analysis identified a central dimerization domain bounded approximately by positions 53 and 122 [8]. The C-terminal region is essential for binding $\alpha_3\beta_3\delta$ [9,10], and may be called the δ-binding or F_1-binding domain, while the region between the membrane and the dimerization domains has been termed the tether domain [11].

The sequence of eubacterial *b* is not well conserved, but multiple sequence alignments reveal an unusual 11-residue (hendecad) pattern

Abbreviations: ACMA, 9-amino-6-chloro-2-methoxyacridine; Ag84, antigen-84; CD, circular dichroism; DSC, differential scanning calorimetry; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone; VATE, E subunit of V-ATPase

☆ This work was supported by a grant from the Canadian Institutes of Health Research (MT-10237).

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doi:10.1016/j.bbabio.2008.03.004

in the dimerization domain [11–13]. Hendecad patterns are thought to be typical of right-handed coiled coils [14,15]. Hendecad positions are denoted *a* through *k*; the *a* and *h* positions in the *b* family are most often occupied by small residues, usually alanine, while the *d* and *e* positions are often occupied by larger nonpolar residues. These positions form a hydrophobic, right-handed strip on the helix $b_{61–122}$ crystal structure [12]. Protein chemical evidence has shown this strip to be the dimerization interface, so it seems likely that dimerization will form a novel two-stranded, right-handed coiled coil [13]. However, modeling studies and analysis of inter-residue distances by ESR have led to the proposal that a left-handed coiled coil is also possible [16,17], so the nature of the structure remains controversial.

In the current work, we sought to ask what modifications to the sequence of *b* will support its function as the stator stalk. Since few point mutations of *b* affect function [18,19], we adopted the approach of constructing chimeras in which substantial sections of the *E. coli b* polypeptide are replaced by exogenous sequences. We began by substituting corresponding regions from other eubacterial *b* subunits, then extended the approach to sequences from other proteins with hendecad repeats that should be compatible with RHCC, and finally to left-handed coiled coils. The effects of these substitutions provide insight into the parts of *b* that tolerate changes, and the types of change they tolerate without loss of function.

2. Materials and methods

2.1. Materials

Genomic DNA from *Thermotoga maritima* was purchased from the American Type Culture Collection. Genomic DNA from *Chlamydia pneumoniae* AR3 was a kind gift from Dr. Robert Brunham of the University of British Columbia Centre for Disease Control, Vancouver British Columbia, Canada. Plasmid pPH5253 [20] carrying the gene encoding Ag84 from *Mycobacterium tuberculosis* was kindly provided by Dr. Peter W. Hermans, Laboratory of Pediatric Infectious Diseases, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands. *Saccharomyces cerevisiae* DNA was kindly provided by Dr. Chris Brandl, Department of Biochemistry, University of Western Ontario. *Bacillus subtilis* DNA was prepared from bacterial cells. *E. coli* strain KM2 [21], carrying a chromosomal deletion of *uncF*, pDM8 [6], carrying a synthetic *uncF* gene, and pBAD24 [22], an expression vector utilizing the arabinose control system, have been described.

Synthetic oligonucleotides were obtained from Sigma. Monoclonal antibody α-II was the generous gift of Drs. Rod Capaldi and Robert Aggeler of the University of Oregon, Eugene, Oregon. Polyclonal antibodies to the soluble domain of *E. coli b* subunit were raised in a rabbit and purified by affinity chromatography on a column of b_{MERC} coupled to Sulfo-link resin (Pierce), prepared as described [9].

Table 1
Complementation of *uncF* strain KM2 by chimeric *b* subunits

Strain/plasmid/*b* type	Exogenous residues inserted[a]	*E. coli* residues replaced	Growth on acetate[b] [IPTG] (μM)				
			0	1	3	10	30
1100/pSD80/WT	na[c]	na	+++	+++	+++	+++	+++
KM2/pSD80/none[d]	na	na	–	–	–	–	–
KM2/pDM8/WT[d]	None	None	+++	+++	++	+	tr[e]
KM2/pSD200/Tm-1	E58-Q111	D55-E108	+++	++	+	–	–
KM2/pSD246/Tm-2	E58-A127	D55-V124	++	++	tr	–	–
KM2/pJW3/Tm-3	M1-Ser164	M1-L156	–	–	–	–	–
KM2/pJW4/Tm-4	R40-Ser164	Q37-L156	–	–	–	–	–
KM2/pJW5/Tm-5	M1-Ala127	M1-V124	–	–	–	–	–
KM2/pJW6/Tm-6	R40-Ala127	Q37-V124	tr	–	–	–	–
KM2/pSD245/Tm-7	R40-Q111	Q37-E108	tr	tr	–	–	–
KM2/pPK2/Bs-1	Q65-V120	D55-E110	+++	+++	+	–	–
KM2/pSD203/GCN4-1	Q252-V278	K67-A94	tr	tr	–	–	–

[a] Inserted sequences are from *T. maritima* except for pPK2 which was from *B. subtilis*.
[b] Growth was tested on minimal medium with 0.2% sodium acetate as described under Materials and methods. Growth was scored based on colony diameter after incubation at 37 °C for 2 days as follows: +++, >0.4 mm; ++, 0.25–0.4 mm; +, 0.1–0.25 mm; tr, <0.1 mm. All strains grew well on glucose.
[c] na, not applicable.
[d] pSD80 is the negative control plasmid while pDM8 is the positive control with *E. coli b* sequence.
[e] tr, trace.

Table 2
Growth on acetate medium of KM2 with *b* subunit chimeras in pBAD24

Sequence source	Designation	Exogenous residues inserted	*E. coli* residues replaced	Plasmid	Growth[a]
E. coli b subunit					
	WT	none	none	pSD205	+++
	Null	na[b]	na[b]	pBAD24	–
T. maritima b subunit					
	Tm-1	E58-Q111	D55-E108	pSD248	+++
	Tm-2	E58-A127	D55-V124	pSD252	+
	Tm-6	R40-A127	Q37-V124	pSD247	+
	Tm-7	R40-Q111	Q37-E108	pSD251	++
C. pneumoniae V-ATPase E subunit					
	VATE-1	K23-K78	D55-E110	pSD250	–
	VATE-2	K23-H63	D55-E95	pSD255	+
	VATE-3	E26-K78	K58-E110	pSD256	+
	VATE-4	N35-K78	K67-E110	pSD257	+
	VATE-5	E26-K67	K69-E110	pSD258	–
	VATE-6	E26-K78	K58-E110	pSD259	–
	VATE-7	N35-H63	K67-E95	pSD260	+++
	VATE-8	E26-H63	K58-E95	pSD264	+++
M. tuberculosis Ag84					
	Ag84-1	S108-A163	D55-E110	pSD204	–
	Ag84-2	S108-D148	D55-E95	pSD277	+++
	Ag84-3	S125-A163	A72-E110	pSD278	–
	Ag84-4	S125-D148	A72-E95	pSD279	+++
Left-handed coiled coils					
	GCN4-1	Q252-V278	K67-A94	pSD249	tr[c]
	GCN4-1C[d]	Q252-V278	A68-A94	pSD280	–
	GCN4-2	Q252-V278	E71-R98	pSD253	–
	GCN4-2C	Q252-V278	A72-R98	pSD254	–
	GCN4-4	V257-K275	A61-N80	pSD261	tr
	GCN4-5	V257-K275	A72-K91	pSD262	tr
	GCN4-5C	V257-K275	Q73-K91	pSD275	tr
	GCN4-7	V257-K275	R83-V102	pSD270	–
	GCN4-8	V257-K275	A61-N80, R83A[e]	pSD271	tr
	GCN4-8C	V257-K275	T62-N80, R83A	pSD276	tr
	Eea1-1[f]	L1320-Q1338	A72-K91	pSD272	–
	Tropo-1[g]	V246-K264	A72-K91	pSD273	–
	Tropo-2	L50-Q68	A72-K91	pSD274	–

[a] Growth was scored as described in Table 1 after two days incubation on acetate plates containing 30 μM arabinose at 37 °C.
[b] na, not applicable.
[c] tr, trace.
[d] Designations ending in "C" indicate replacement of the removed *b* segment with an equal number of leucine zipper residues, resulting in a discontinuity in the hydrophobic surface.
[e] These chimeras also contained the R83A point mutation that stabilizes the coiled coil structure of b_2.
[f] Eea1, human Eea1 (early endosome antigen 1).
[g] tropo, rabbit tropomyosin A.

2.2. Plasmid construction

Recombinant DNA procedures were carried out by standard methods. To construct pJW3, the entire *uncF* gene (encoding *b*) of *T. maritima* was amplified by PCR using 5′-CGGCGGTACCATAGAGGCATTGTGCTGTGGGCTTTCTGGAG-3′ as forward primer and 5′-GACGGCAGCTTGAGACCTTATGACTTTTCTATCTCCT-3′ as reverse primer. The forward primer contains a synthetic KpnI site, a Shine–Dalgarno sequence, and changes the start codon from TTG to GTG, in order to match *E. coli b*. The reverse primer contains a BsaI site that leaves a 5′ overhang compatible with HindIII; this strategy was used since *T. maritima uncF* contains an internal HindIII site. The PCR product was cut with KpnI and BsaI and then ligated into the corresponding KpnI and HindIII sites in pDM8 in order to make pJW3. Sequences encoding natural or chimeric *b* subunits were transferred from pDM8-type plasmids, with the *lac* gene expression control system into pBAD24 [22] with the arabinose gene expression control system using the EcoRI and HindIII sites. The plasmid carrying the synthetic *b* sequence in pBAD24 was called pSD205.

For chimera construction, primers included restriction enzyme sites suitable for cloning products in-frame into the synthetic *uncF* sequence in pDM8 or pSD205. DNA for preparing chimeras incorporating sequences from *b* subunits of *T. maritima* or *B. subtilis*, the E subunit of the V-ATPase of *C. pneumoniae*, the Ag84 protein of *M. tuberculosis* Ag84, or yeast GCN4, were obtained by PCR amplification from either genomic DNA or plasmids containing the appropriate cloned gene. DNA for preparing