Journal of Structural Biology 180 (2012) 509–518

Contents lists available at SciVerse ScienceDirect

Journal of Structural Biology

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

Relevance of the conserved histidine and asparagine residues in the phosphate-binding loop of the nucleotide binding subunit B of A_1A_O ATP synthases

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ARTICLE INFO

Article history:
Received 17 July 2012
Received in revised form 28 September 2012
Accepted 1 October 2012
Available online 11 October 2012

Keywords:
P-loop
Subunit B
A-ATP synthases
Mutants
Methanosarcina mazei Gö1

ABSTRACT

The nucleotide binding sites in A-ATP synthases are located at the interfaces of subunit A and B, which is proposed to play a regulatory role. Differential binding of MgATP and -ADP to subunit B has been described, which does not exist in the related α and B subunits of F-ATP synthases and V-ATPases, respectively. The conserved phosphate loop residues, histidine and asparagine, of the A-ATP synthase subunit B have been proposed to be essential for γ-phosphate interaction. To investigate the role of these conserved P-loop residues in nucleotide-binding, subunit B residues H156 and N157 of the *Methanosarcina mazei* Gö1 A-ATP synthase were separately substituted with alanine. In addition, N157 was mutated to threonine, because it is the corresponding amino acid in the P-loop of F-ATP synthase subunit α. The structures of the subunit B mutants H156A, N157A/T were solved up to a resolution of 1.75 and 1.7 Å. The binding constants for MgATP and -ADP were determined, demonstrating that the H156A and N157A mutants have a preference to the nucleotide over the wild type and N157T proteins. Importantly, the ability to distinguish MgATP or -ADP was lost, demonstrating that the histidine and asparagine residues are crucial for nucleotide differentiation in subunit B. The structures reveal that the enhanced binding of the alanine mutants is attributed to the increased accessibility of the nucleotide binding cavity, explaining that the structural arrangement of the conserved H156 and N157 define the nucleotide-binding characteristics of the regulatory subunit B of A-ATP synthases.

1. Introduction

Adenosine 5′-triphosphate (ATP) is an essential component in energy transfer in all living creatures from archaea to eukaryotes. The synthesis of ATP in eukaryotic and prokaryotic cells is brought about by F_1F_O ATP synthases (Junge et al., 1997; Ballmoos et al., 2009), while in archaea the A_1A_O ATP synthases (A-ATP synthase) are responsible for the production of ATP (Deppenmeier and Müller, 2008). The A-ATP synthase is composed of nine subunits in a proposed stoichiometry of A_3:B_3:C:D:E_2:F:H_2:*a*:c_x (Grüber and Marshansky, 2008). As depicted for the bacterial F_1F_O ATP synthase (α_3:β_3:γ:δ:ε:*a*:b_2:c_x) and the eukaryotic V_1V_O ATPase (A_3:B_3:C:D:E:F:G_2:H_x:*a*:*d*:*c*:*c*′:*c*″), the enzyme consists of a soluble A_1 sector, containing the catalytic sites, and a membrane embedded A_O sector, which is involved in ion-translocation (Boekema et al., 1999; Böttcher and Gräber, 2000; Müller et al., 2005). The origin of the energy transducer, V-ATPase, is related to A-ATP synthase (Nelson, 1992). The nucleotide binding subunits A and B of the A_1/V_1 part and the corresponding β and α subunits of the F_1 part display the highest degree of sequence similarity within the enzymes (Iwabe et al., 1989; Nelson, 1992).

Subunit A of the A-ATP synthases contain the catalytic nucleotide-binding sites, based on covalent modification and on sequence homology with the catalytic subunits A and β of the related V-ATPases and F-ATP synthases, respectively (Iwabe et al., 1989; Oldendzenski et al., 1998). They possess the consensus sequence GXXXXGKT(S), commonly referred to as the phosphate binding loop (Walker et al., 1982; Saraste et al., 1990). Such P-loop sequence is not conserved at the equivalent position in subunit B of both A-ATP synthases and V-ATPases (Schäfer et al., 2006; Oldendzenski et al., 1998). In contrast, the functional motif is conserved at nucleotide binding sites of the related α subunits ($_{169}$GDRQTGKT$_{176}$) of F-ATP synthases (Walker et al., 1982). The P-loop sequence of B subunits in both A-ATP synthases and V-ATPases comprises of peptide SASGLPHN (Schäfer et al., 2006; Inatomi et al., 1989; Puopolo et al., 1992). Previously, it was shown that subunit B (WT-B) of the *Methanosarcina mazei* Gö1 A-ATP synthase binds MgATP and -ADP (Schäfer et al., 2006; Kumar et al., 2009). The trail of the nucleotide to the actual binding pocket has been highlighted by trapping the nucleotides in transition states in crystal structures of the subunit B mutant R416W (Kumar

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http://dx.doi.org/10.1016/j.jsb.2012.10.001

et al., 2008; Manimekalai et al., 2009). Lately, the nucleotide binding to WT-B was also demonstrated in the presence of reported inhibitors such as NBD-Cl (Hunke et al., 2010), providing information for nucleotide binding ability of subunit B of A-ATP synthases.

Irrespective of possessing a similar glycine rich sequence motif, the conserved lysine and threonine residues in the P-loop of α and β subunits of F-ATP synthases are reported to have different roles (Jounouchi et al., 1993). In β these residues are located near the γ-phosphate of the ATP molecule and stabilize the negative charge (Abrahams et al., 1994), whereby the lysine residue is involved in transition state stabilization and threonine co-ordinates with the essential magnesium ion (Menz et al., 2001). In comparison, in subunit α the corresponding lysine and threonine have been described to be not absolutely necessary for catalysis. In contrast, the mutation of the equivalent H180 and N181 residues of the P-loop ($_{174}$SASGLPHN$_{181}$) of the yeast V-ATPase subunit B resulted in a drop of 30–50% enzymatic activity (Liu et al., 1996). Although the nucleotide-binding subunits α and B of F-ATP synthases and V-ATPases, respectively, have similar binding constants for MgATP and MgADP, subunit B of A-ATP synthases binds MgATP with a higher preference (K_D = 22 ± 3 μM) than MgADP (K_D = 50 ± 3 μM), as demonstrated for the *M. mazei* Gö1 A-ATP synthase subunit B (Kumar et al., 2009). Based on these and its inhibitory traits (Hunke et al., 2010) subunit B has been proposed to function as a regulatory subunit.

In order to understand the role of the conserved H156 and N157 residues in the P-loop of subunit B of A-ATP synthases and to recognize the differential nucleotide binding ability, alanine mutants were generated in subunit B of A-ATP synthases from *M. mazei* Gö1. Additionally, the residue N157 of subunit B was mutated separately to threonine, as it is the corresponding residue in the P-loop sequences of the related α subunits of F-ATP synthases from *Escherichia coli* and its equivalent residue (T241) in subunit A of A-ATP synthase showed a nucleotide bound conformational variation (Kumar et al., 2010). The crystal structure of mutant proteins H156A, N157A and N157T were determined at a resolution of 1.75 and 1.7 Å. The ability of nucleotide analogues, MgATP ATTO-647N and MgADP ATTO-647N, to bind to these mutant proteins were evaluated by fluorescence correlation spectroscopy (FCS). Although the mutant proteins had significant preference for the nucleotide over the WT-B, they lost the ability to distinguish MgATP and MgADP, indicating the importance of histidine and asparagine residues in nucleotide-binding and regulation.

2. Material and methods

2.1. Construction and purification of single mutants H156A, N157A and N157T of subunit B

Three different P-loop mutant constructs (H156A, N157A, N157T) of subunit B of the A-ATP synthase from *M. mazei* Gö1 were generated by overlap extension PCR method (Ho et al., 1989) using subunit B insert in pET9d1 (Grüber et al., 2002) as template. In two PCR reactions one flanking primer 5′ -TTTCTCCATGGTCAAAGAGTACAAGACGATCACTCAGATTGCAGGGCC -3′ (forward primer *a*) and 5′ -TTTGAGCTCTCACTTAGCCTTTCTGTGTGCCGGGTGATATTTCTGG -3′ (reverse primer *d*) incorporating *Nco*1 and *Sac*1 (underlined) restriction enzymes sites, respectively were used that hybridized at each end of the subunit B sequence. In addition, the internal primer 5′ -TTTCTCAGCATCAGGCCTGCCAGCGAATGAAATCGCCCTGCAGATTG -3′ (forward primer *b*) and 5′ -CAATCTGCAGGGCGATTTCATTCGCTGGCAGGCCTGATGCTGAGAAA -3′ (reverse primer *c*) was used that hybridized at the site of the mutation and contains the mismatched bases (underlined) in case of H156A mutant. In separate PCRs two fragments of the subunit B gene were amplified by using primer pairs *a*/*b* and *c*/*d*, respectively. The overlap allowed one strand from each fragment to act as a primer on the other, and extension of this overlap by flanking primers (*a* and *d*) resulted in the mutant product. Similar strategy was employed to generate mutant constructs N157A and N157T via overlap extension PCR method wherein same flanking primers (*a* and *d*) were used. The internal primers for constructing N157A mutant construct comprised of 5′ -CTCAGCCATCAGGCCTGCCACACGCGGAAAATCGCCCTGCAGATTGCA- 3′ (forward primer *b*) and 5′ -TGCAATCTGCAGGGCGATTTCCGCGTGTGGCAGGCCTGATGCTGAG- 3′ (reverse primer *c*), whereas the internal primers for N157T mutant construct comprised of 5′ -CTCAGCATGAGGCCTGCCACACACCGAAATCGCCCTGCAGATTGCA- 3′ (forward primer *b*) and 5′ -TGCAATCTGCAGGGCGATTTCGGTGTGTGGCAGGCCTGATGCTGAG- 3′ (reverse primer *c*). Locations of the mismatch in internal primers (*b* and *c*) are underlined. Following digestion with *Nco*1 and *Sac*1, the PCR product was ligated separately into the pET9d1-His$_6$ vector. The mutations were verified by DNA sequencing. The verified plasmids were finally transformed into *E. coli* BL21 (DE3) cells (Stratagene, CA, USA) for protein expression using electroporation method. To produce subunit B mutant proteins, liquid cultures were shaken in LB medium containing 30 μg/ml kanamycin. At an OD$_{600}$ of 0.6, the cells were induced by the addition of 1 mM isopropyl-β-D-thiogalactoside and growth was further allowed at 30 °C for 4 h. The mutant proteins were isolated according to Kumar et al. (2008), and the purity of the protein sample was monitored by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE; Laemmli, 1970). The SDS-gels were stained with Coomassie Brilliant Blue G250. Peak fractions were pooled and concentrated on Centricon 30 kDa concentrators (Millipore). Protein concentrations were estimated by the bicinchoninic acid assay (BCA; Pierce, Rockford, IL, USA).

2.2. Crystallization of subunit B P-loop mutant proteins

The mutants H156A, N157A and N157T were crystallized by hanging drop vapour diffusion method at 18 °C. The protein drops were set for crystallization by mixing equal volumes (2 μl) of the protein and reservoir solution. The reservoir solution comprised of 15% (v/v) glycerol, 20% (v/v) PEG 400, 0.1 M NaCl, 0.1 M sodium citrate (pH 5.0) and 2 mM TCEP (Tris(2-carboxyethyl) phosphine). Good quality crystals were obtained over a period of 3 weeks which were cryo-protected by bathing them in mother-liquor solution incorporating 30% glycerol prior to flash cooling in liquid nitrogen. For co-crystallization with nucleotides, the purified subunit B mutants were divided into three batches and incubated separately with 2 mM MgATP, 2 mM MgADP and 2 mM MgAMP-PNP at 4 °C for 30 min on a sample rotator. The protein-ligand complexes were then set up for crystallization in the same optimized conditions as described above. Diffraction quality crystals in case of H156A could be obtained in empty and in the presence of MgATP and MgADP, whereas the N157A mutant generated crystals in empty and in the presence of MgATP. For the N157T mutant only empty form yielded diffraction quality crystals. The crystals were cryo-protected using mother liquor supplemented with 30% glycerol and frozen in liquid nitrogen.

2.3. Data collection and structure determination

Diffraction data for the subunit B mutant protein H156A was collected at 100 K in Taiwan beamline 12B2 and RIKEN BL26B2 (Spring-8, Japan) using the Q4R ADSC and Mar CCD 225 detectors. For N157A and N157T mutant proteins, X-ray diffraction data were collected at 140K on beamline 13B1 at the National Synchrotron Radiation Research Center (NSRRC, Hsinchu, Taiwan) using the ADSC Quantum 315 CCD detector. The nucleotide free form of