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NMR structures of α -proteobacterial ATPase-regulating ζ -subunits

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Abstract

NMR structures of ζ -subunits, which are recently discovered α -proteobacterial F_1F_0 -ATPase regulatory proteins representing a Pfam protein family of 246 sequences from 219 species (PF07345), exhibit a four-helix bundle, which is different from all other known F_1F_0 -ATPase inhibitors. Chemical shift mapping reveals a conserved ADP/ATP-binding site in ζ -subunit, which mediates long-range conformational changes related to function, as revealed by the structure of the *Paracoccus denitrificans* ζ -subunit in complex with ADP. These structural data suggest a new mechanism of F_1F_0 -ATPase regulation in α -proteobacteria.

Introduction

F_1F_0 -ATPases are molecular nanorotors found in bacteria, mitochondria and chloroplasts, and consist of a transmembrane domain (F_0 or rotor) and an ATP synthesizing/hydrolyzing domain (F_1 or stator) [1]. ATPase regulation has long attracted keen interest for its fundamental role in regulating in-cell energy reservoirs and cell membrane potentials [1-4]. F_1F_0 -ATPases operate either as ATPases or ATP synthases, depending on the sense of the F_0 domain rotation [5]. A flux of H^+ , Na^+ or K^+ ions through the F_1F_0 -ATPase, induced by electrochemical potentials across the membrane, results in a clockwise rotation when viewed from the outer side, which generates ATP from ADP and P_i . A counter-clockwise rotation induced by ATP hydrolysis reestablishes the membrane potential under anaerobic

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The chemical shifts of Js- ζ , Pd- ζ and Pd- ζ -ADP have been deposited in the Biological Magnetic Resonance Bank (<http://www.bmrb.wisc.edu/>) with the accession codes 17002, 18018, and 19510, respectively. The atomic coordinates of the bundles of 20 conformers used to represent the solution structures of Js- ζ , Pd- ζ and Pd- ζ -ADP have been deposited in the Protein Data Bank (PDB; <http://www.rcsb.org/pdb/>), with the accession codes 2KZC, 2LL0 and 2MDZ.

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conditions, by pumping H^+ or Na^+ ions from the inner to the outer membrane space. Two ATPase-regulatory proteins have been structurally characterized: the bacterial ϵ -subunit [6] and the mitochondrial IF_1 protein [1]. The ϵ -subunits [1,6,7] block the ATPase rotation by interacting with the central rotor and the stator [1,8], and the mitochondrial IF_1 protein functions primarily by obstructing the gyration of the stator [2]. The more recently discovered α -proteobacterial ζ -subunit has also been suggested to interact with the stator, complementing the activity of the ϵ -subunit [9,10]. Here, we describe the NMR structures of two ζ -subunits, investigate their ATP binding, and present evidence that these structural data are applicable to the ζ -subunit protein family of 246 sequences from 216 species (PF07345).

NMR structure of the ζ -subunit reveals a new ATPase-regulating architecture

The NMR structure determination of the ζ -subunits from *Jannaschia* sp. *CCSI* (*Js- ζ*) and *P. denitrificans* (*Pd- ζ*) with the automated J-UNIO protocol [11-14], in combination with the torsion angle dynamics algorithm CYANA-3.0 [15] yielded well-defined structures, as indicated by the statistics of bundles of 20 CYANA conformers (Table S1). Both proteins form a down-up-down-up four-helix bundle, with $\alpha 1$ and $\alpha 3$ arranged in antiparallel fashion, and $\alpha 2$ and $\alpha 4$ oriented at angles of about 45° relative to the other two helices (Fig. 1, a and b). The four helices of *Js- ζ* and *Pd- ζ* can be superimposed with a backbone RMSD of 1.3 Å (Fig. 1c). Larger structural differences prevail in the loop between $\alpha 2$ and $\alpha 3$, which is less well-defined in both structures (Fig. 1, a and b). Broader lines in the 2D [^{15}N , 1H]-HSQC spectra for residues in this loop further indicate conformational dynamics on the millisecond timescale [16,17], as frequently observed at or near protein active sites [18]. In *Pd- ζ* , the N-terminal extension appears to be poorly defined when judged from the bundle of conformers obtained by superposition for best fit of $\alpha 1$ to $\alpha 4$. However, the presence of a well-defined $\alpha 1'$ helix (residues 7 to 18), for which the bundle of 20 NMR conformers can be superimposed with a local backbone RMSD of 0.69 Å (Table S1), indicates that the segment 1–19 forms an N-terminal subdomain which undergoes hinge motions with respect to the protein core.

Alignment of *Js- ζ* with other α -proteobacterial ζ -subunits (Fig. S1) reveals high sequence homology for $\alpha 1$, $\alpha 2$ and $\alpha 3$, and more diversity for $\alpha 4$. High conservation is also observed for the N-terminal 19-residue segment and the loop linking $\alpha 2$ and $\alpha 3$. Combined with sequence-based prediction of regular secondary structures, these data indicate high conservation of the three-dimensional structures for the 246 members of the ζ -subunit family.

ATP- and ADP-binding increases flexibility in the ζ -subunit

The ζ -subunit architecture does not resemble other known F_1F_0 -ATPase inhibitors, which suggests a novel mechanism of action. Since ATP regulates the activity of the ϵ -subunit in bacteria [8], we investigated ATP addition to the N-terminally truncated *Js- ζ* and to *Pd- ζ* . For both proteins, superposition of the 2D [^{15}N , 1H]-correlation spectra in the absence and presence of 10 equivalents of ATP showed that ATP-binding caused large chemical shifts of residues located near the N-terminal end of $\alpha 1$ and in the loop between $\alpha 2$ and $\alpha 3$ (Fig. 2, a–

g), thus identifying the ATP binding site. The sequence segments affected by ATP-binding to J_s - ζ are among the most highly conserved ones in the ζ -subunit family (Fig. S1), so that the observations in Fig. 2, a and d, can be expected to prevail for most or all of its 246 members.

In Pd- ζ , chemical shift changes were also observed for residues in $\alpha 1'$, which has been described as the “inhibitory subdomain” in the ζ -subunit [10], and $\alpha 4$ (Fig. 2, e), indicating that ATP-binding induces global changes of the protein structure. This motivated the determination of a ligand-bound structure of Pd- ζ . Since the ATP complex precipitated within a few hours, and the data of Fig. 2, e and f, showed that ATP and ADP induce chemical shifts in binding-site residues, e.g. T54 and T57, as well as peripheral residues located at the N-termini, e.g. F15 and H17, with similar magnitude and orientation, we determined an NMR structure of Pd- ζ in phosphate buffer containing 10 mM ADP (Pd- ζ -ADP).

The NMR assignments of free Pd- ζ were used as a starting platform and validated with three 3D heteronuclear-resolved [^1H , ^1H]-NOESY experiments. Superposition for best fit of residues 2–102 in the NMR bundle representing the resulting Pd- ζ -ADP structure (Fig. 3a) showed a similar global architecture to free Pd- ζ (Fig 1b), but the global backbone RMSD indicated apparent lower precision of the structure determination (Table S1). This decreased precision is not due to selective loss of NOE constraints as a result of conformational mobility about the hinge located at residues R28 and R29. For example, in the Pd- ζ -ADP complex we detected long-range NOEs between residues V56 and N30, which are located on the same side of the hinge, but not between V56 and residue A27, which are located on different sides of the hinge. In free Pd- ζ both sets of NOE were observed. Chemical shift of the ligand in the nucleotide-binding site can be excluded as the source of the imprecise line broadening by observation of increased plasticity for residues near the N-terminal chain end, which are located far from the ADP binding site and would, therefore, not be susceptible to chemical exchange effects, unless these initiate conformational changes far from the binding site.

Local superpositions for best fit of discrete polypeptide segments then showed that the Pd- ζ -ADP core of residues 22–102 consists of two well-defined subdomains (Fig 3, c and d) which undergo hinge motions relative to each other, enabled by increased structural disorder and concomitant increased flexibility of the central parts of $\alpha 1$ and $\alpha 4$ under the influence of ADP binding. As indicated in Fig. 3b, $\alpha 1$ and $\alpha 4$ are thus broken up into two pairs of shorter helices, $\alpha 1a$ and $\alpha 1b$, and $\alpha 4a$ and $\alpha 4b$, respectively. Furthermore, $\alpha 1'$ is locally less well-defined in Pd- ζ -ADP than in Pd- ζ (Table S1).

Discussion

NMR studies with the α -proteobacterial ζ -subunit family reveal a novel ATPase-regulatory architecture. Although its structure clearly differs from the ε -subunit, some tentative inferences on structure–function correlations can be drawn from dynamic features related to ATP-binding in the two proteins. For the bacterial ε -subunit, increased flexibility is required for F_1F_0 -ATPase inhibition, where two helices block the rotation of the central stalk at low

ATP concentrations [1,8]. NMR studies revealed that at higher concentrations, ATP reduces the conformational space accessible to the two helices by constraining them close to the protein core, which prevents interaction of the ϵ -subunit with the F_1F_0 -ATPase [19]. For the ζ -subunit, we now observe increased flexibility of the helices $\alpha 1'$, $\alpha 1$ and $\alpha 4$ at high ATP concentrations, suggesting, in analogy to the ϵ -subunit, that both chain termini are involved in ζ -subunit activity by participating in the interactions with the F_1F_0 -ATPase central stalk. These conclusions from the present structural data are independently supported by biochemical studies: Binding of Pd- ζ to ATP was characterized using isothermal titration calorimetry [10,20], Pd- ζ interactions with the ATPase central stalk were evidenced using chemical cross-linking experiments²⁰, and a functional role of the N-terminal segment with $\alpha 1'$, was implicated by the observation that the N-terminally truncated construct of Pd- ζ , with residues 23–102, appeared to be inactive in inhibitory assays [10,20]. The notion appears to emerge that structural plasticity related to ATP-binding is important for the activity of both α -proteobacterial subunits ϵ and ζ , and that the two subunits may play complementary roles in F_1F_0 -ATPase regulation. While the ϵ -subunit functions at low ATP-concentrations by inhibiting F_1F_0 -ATPase activity and thus preventing depletion of cellular ATP, the ζ -subunit may block ATP-synthase activity at higher ATP concentrations, reducing excessive H^+ and Na^+ intake, and thus preventing cell damage.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Research Highlights

The NMR structure of the α -proteobacterial ζ -subunit reveals a novel ATPase-regulating architecture

The ζ -subunit exhibits an ATP/ADP binding site

Nucleotide binding to the ζ -subunit induces long-range structural changes related to function

Fig. 1.

NMR structures of the ζ -subunits from *Jannaschia sp* and *P. denitrificans*. (a) Stereo view of a bundle of 20 energy-refined CYANA conformers of the N-terminally truncated Js- ζ with residues 20–102 superimposed for best fit of residues 22–55 and 67–102 (Table S1). The residue positions bounding the segments used for the superposition are indicated in italics. (b) same as (a) for full-length Pd- ζ with residues 1–102. (c) Superposition of the conformers closest to the mean coordinates of the bundles in panels (a) and (b). The flexibly disordered N-terminal 19-residue segment of Pd- ζ is not shown.

Fig. 2.

NMR studies of ATP- and ADP-binding to N-terminally truncated *Js-ζ* and to *Pd-ζ*. (a) and (b) Superposition of the 2D ^{15}N , ^1H -HSQC spectra of *Js-ζ* in the absence (red) and presence (green) of 10 mM ATP. The dotted square in (a) outlines the spectral region shown in (b). Signals of those residues which showed chemical shifts $\delta = [(0.2\delta_{\text{N}})^2 + (\delta_{\text{H}})^2]^{1/2} \geq 0.035$ ppm upon addition of ATP are identified. (c) Stereo view of a ribbon presentation of *Js-ζ*. Positions of residues with chemical shifts $\delta \geq 0.035$ ppm are colored orange. The chain ends and selected residues (see text) are identified. (d) Superposition of the 2D

[^{15}N , ^1H]-HSQC spectra of Pd- ζ without addition of a ligand (red), with 10 mM ATP (green), and with 10 mM ADP (blue). The dotted frame outlines the spectral region shown in (e) and (f). (e) Superposition of the spectra of Pd- ζ without (red) and with (green) addition of 10 mM ATP. (f) Same presentation as in e for the addition of 10 mM ADP (blue). In (b), (e) and (f), corresponding NMR signals of the protein without and with ligand bound are connected by black lines. (g) Ribbon diagram of Pd- ζ , same presentation as in (c).

Fig. 3.

NMR structure of Pd- ζ in complex with ADP. (a) Stereo view of a bundle of 20 Pd- ζ conformers in complex with ADP superimposed for best fit of the backbone atoms of residues 22–55 and 67–102 (Table S1). (b) Ribbon presentation of the conformer closest to the mean coordinates of the bundle in (a). The dotted line represents the poorly defined polypeptide segment 1–19 (c). Bundle of the 20 Pd- ζ conformers in (a) superimposed for best fit of residues 30–55 and 67–94 (Table S1). (d) Same as (c), with superposition for best fit of residues 22–29 and 95–102.