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Minireview

The role of the stalk in the coupling mechanism of F_1F_0-ATPases

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Abstract

The extrinsic and intrinsic membrane sectors of F_1F_0-ATPases are linked by a slender stalk 40–50 Å in length. The stalk transmits the energy produced by oxidative or photosynthetic phosphorylation from the intrinsic sector, F_0, to the catalytic sites in the extrinsic F_1 sector. How this is achieved is unknown, but long-range conformational changes linked to transmembrane proton transport may be involved. In bacterial and chloroplast F_1F_0-ATPases, the stalk is probably a composite of subunits δ and ε, part of the γ-subunit, and the extrinsic membrane domains of 2 subunits (identical or non-identical according to the species) that are bound to the membrane by their N-terminal regions. The stalk in the bovine mitochondrial enzyme appears to be more complex, and the γ, δ, ε, OSCP, F_6, b and d subunits all contribute to it. A bovine stalk complex has been assembled in vitro from bacterially expressed OSCP, F_6, b and d, both in the presence and in the absence of F_1-ATPase. One molecule of each of these subunits is present in the assembled complexes, as there is also in each native F_1F_0-ATPase assembly. Providing that suitable crystals can be obtained, the stalk complex and the F_1·stalk complex may permit the high resolution structure of bovine F_1-ATPase to be extended into the stalk domain.

Key words: F_1F_0-ATPase; Coupling mechanism; Stalk domain

1. Introduction

The proton translocating F_1F_0-ATPases in the energy transducing membranes of eubacteria, chloroplasts and mitochondria have many common features. For example, their gross structures observed by electron microscopy are similar and consist of three domains, namely the extrinsic and intrinsic membrane domains, F_1 and F_0, respectively, and the slender stalk 40–50 Å long that links them together [1,2]. The existence of the stalk has been questioned (see references cited in [2]), and it has been suggested that it may be an artefact of negative staining. However, the visualization of the stalk by electron microscopy of unstained specimens of the *Escherichia coli* enzyme [2] has rebutted this criticism effectively. The 90 Å particle attached to this stalk is F_1-ATPase, the extrinsic membrane domain of the ATP synthase, which contains the catalytic sites of the enzyme (for reviews see [3,4]). If the stalk is ruptured, the F_1-ATPase particle is released intact as a soluble globular complex. The intrinsic membrane domain, F_0, has a transmembrane proton transporting mechanism involved in harnessing energy stored in the proton potential gradient across the energy transducing membrane [5,6]. A central question for understanding ATP formation is, how is this energy channelled into the F_1 domain to drive ATP synthesis? There is considerable evidence that the energy transmission from F_0 to F_1 is indirect and is mediated by long-range conformational changes that are induced by proton transport [7,8]. These changes must pass through the stalk. In alternative directly coupled mechanisms, the protons are proposed to be conducted from F_0 via the stalk to the catalytic sites in F_1, where they participate directly in ATP formation [9,10]. Therefore, irrespective of whether the coupling mechanism is direct or indirect, the stalk plays a central role in this mechanism, and knowledge of its structure is crucial for understanding the process of energy transduction. Our current knowledge about the structure of the stalk is reviewed below.

2. Stalk subunit compositions in bacteria and chloroplasts

The F_1F_0-ATPases are multisubunit enzymes, ranging from the simplest bacterial complexes, exemplified by the *E. coli* enzyme, which has 8 different polypeptides, to the F_1F_0-ATPases from photosynthetic bacteria and chloroplasts, which have 9 subunits, to the F_1F_0-ATPase from bovine heart mitochondria, in which 16 constituent polypeptides have been characterized. Nonetheless, as the sequences of the subunits of F_1F_0-ATPases have revealed, there are many extensive similarities between bacterial, chloroplast and mitochondrial enzymes. For example, homologues of all five of the subunits of the F_1 domain of the *E. coli* enzyme (α, β, γ, δ and ε; stoichiometries 3:3:1:1:1) are also found in the chloroplast and mitochondrial enzymes [11].

In the F_0 sectors of F_1F_0-ATPases, homologues of

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subunits a and c, two of the three components of *E. coli* F_0, are also present in the chloroplast and mitochondrial assemblies. They are both hydrophobic proteins and are essential components of the transmembrane proton transporting mechanism [5,6]. Neither of them is likely to form part of the stalk, but subunit b, the third constituent of *E. coli* F_0, is almost certainly an important component of this domain. It has a hydrophobic N-terminal region, which is thought to make a single transmembrane α-helix, followed by an extensive highly charged region, that lies outside the lipid bilayer, where it can interact with the F_1 subunits [12–15]. There are two molecules of subunit b in each *E. coli* F_1F_0-ATPase complex [16]. These conclusions are supported by studies of subunit b lacking its N-terminal hydrophobic region. It forms a stable dimer that competes with F_0 in binding with *E. coli* F_1-ATPase [15].

In contrast to subunits a and c, the F_1F_0-ATPases in chloroplasts and photosynthetic bacteria do not contain subunits that are significantly related in sequence to the *E. coli* subunit b, but they each have two different subunits with a general distribution of hydrophobic and charged residues related to those of *E. coli* b (see [17]). They are known as subunits b and b′ in photosynthetic bacteria, and as subunits II and IV in chloroplasts. It has been assumed that there is one of each of these subunits per photosynthetic bacterial or chloroplast complex, and that they play a similar role to the two identical b subunits in the *E. coli* F_1F_0-ATPase.

In the structure of bovine F_1-ATPase determined by X-ray crystallography at 6.5 Å resolution [18], a stem protrudes about 40 Å from the approximately spherical body of the particle, and, next to it, a pit penetrates 35 Å into the particle. It is probable that the stem is derived from the stalk in the intact mitochondrial F_1F_0-ATPase, and that it is composed of the δ and ε subunits and part of the γ subunit. The pit may be occupied by part of the OSCP subunit, which forms a stable 1:1 complex with F_1-ATPase [19], and is required for re-association of F_1 with membranes from which F_1 has been removed [20,21]. The stem in the bacterial and chloroplast F_1F_0-ATPases is likely to have a similar composition to the mitochondrial one. Therefore, they probably contain part of their γ-subunits, together with the δ and ε subunits (the homologues of mitochondrial OSCP and δ, respectively, in bacteria and chloroplasts) and the extrinsic membrane domains of the two subunits b (or one each of b and b′ in photosynthetic bacteria and chloroplasts). Two recent observations are consistent with this proposal. In chloroplasts, the γ, δ and ε subunits interact with the membrane domain [22], and in the *E. coli* enzyme, the γ and ε subunits interact with each other in the middle of the F_1 particle (viewed end-on). They are mobile during the catalytic cycle, which is consistent with their role in energy transmission by conformational changes [23].

3. Which subunits are in the stalk in the mitochondrial enzyme?

Amongst the 16 different proteins in the bovine F_1F_0-ATPase complex, other subunits, in addition to the OSCP and the components of the F_1 stem (γ, δ and ε), contribute to the stalk. For example, if the membranes are stripped more stringently than is needed for OSCP removal, a subunit known as F_6 is removed, and must be added back together with OSCP for the reassembly of F_1 with stripped membranes [24,25]. Subunit F_6 has no extensive hydrophobic regions in its sequence [26]. It is attached to the matrix surface of the inner mitochondrial

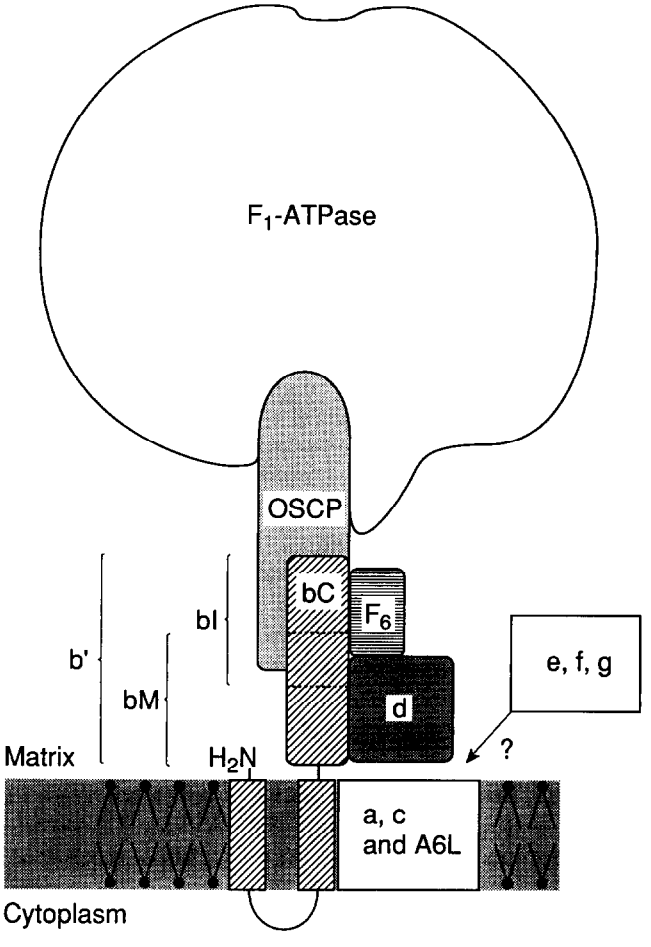

Fig. 1. Schematic representation of possible arrangements of some subunits of bovine F_1F_0-ATPase. The various subunits are not drawn to scale. The OSCP is shown interacting with the 'pit' and 'stem' regions of F_1-ATPase [18]. The diagonally hatched subunit b, has two transmembrane α-helical spans near its N-terminus (amino acids 30–53 and 58–79) [28]. The positions of the fragments b′, bI, bM and bC (amino acids 79–214, 121–214, 79–164 and 165–214) used in the reconstitution experiments are indicated. Subunits F_6 and d interact primarily with subunit b, and to a lesser extent with each other. The hydrophobic subunits a, c and A6L are placed within the lipid bilayer. It is not known how they interact with each other or with subunit b. The locations of subunits e, f and g are unknown, as the question mark indicates, but subunit f has an extensive hydrophobic region [39], and is therefore likely to be at least partially within the lipid bilayer.

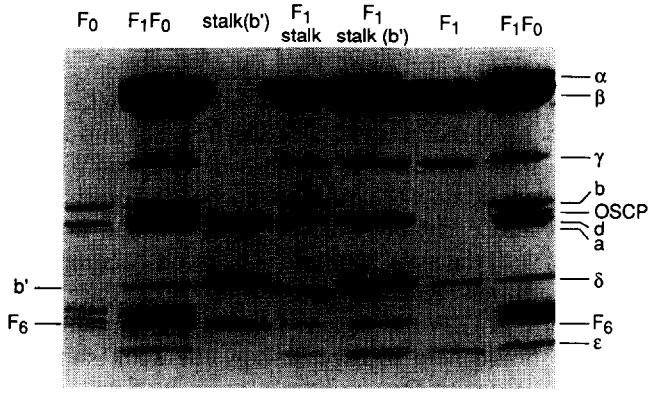

Fig. 2. The subunit compositions of the stalk and F_1·stalk complexes derived by in vitro assembly of subunits of bovine F_1F_0-ATPase in the absence and presence of bovine F_1-ATPase. The subunits were separated by SDS-PAGE and stained with Page blue 83 dye. The bovine F_0, F_1 and F_1F_0-ATPase complexes are shown for reference. The positions of the subunits of F_1F_0-ATPase are indicated on the right, and the positions of b′ and F_6 are shown on the left. The F_1·stalk complex contains intact subunit b and was made by adding b, OSCP, d and F_6 to F_1. The stalk (b′) and F_1·stalk (b′) complex contains fragment b′, which migrates on the gel at approximately the same position as the δ-subunit. The latter complex was made by association of bovine F_1-ATPase with a pre-formed stalk complex containing OSCP, b′, F_6 and d.

membrane, as demonstrated by proteolysis of stripped inner mitochondrial membranes (see below), where it can form part of the stalk. Homologues of F_6 have been found in other eukaryotic F_1F_0-ATPases [27].

In vitro assembly experiments described below have helped to confirm that subunits b and d are also stalk components. The hydrophobic profile of subunit b [28] has two consecutive hydrophobic stretches from amino acids 30–53 and 58–79 that could form the arms of an α-helical hair-pin spanning the inner membrane twice. The remainder of its sequence is highly charged and is capable of making important interactions either with other subunits in the stalk or, as in the analogous subunit b in the bacterial F_1F_0-ATPases, with F_1 subunits. Homologues of bovine b are present in the rat [29] and yeast [30] mitochondrial enzymes, and a human homologue has also been sequenced [31]. Subunit d has no extensive hydrophobic regions in its sequence [28], and, like F_6, it is probably attached to the matrix surface of the inner mitochondrial membrane. Homologues of bovine d have been identified in the yeast and rat enzymes [32,33].

Several studies have emphasized the role of bovine b in energy transduction [34–37]. In inner mitochondrial membranes that have been treated with chaotropes, the C-terminal part of bovine subunit b is exposed to proteolysis on the matrix side [36,38]. In sub-mitochondrial particles that have been striped of F_1-ATPase and OSCP with guanidine hydrochloride [37], the main sites of trypsin cleavage in F_0 are in F_6, which is completely digested away, and the bonds Arg^{166}-Gln^{167}, and Lys^{120}-Arg^{121} and Arg^{121}-His^{122}, at distances of 48 and 93–94 amino acids from the C-terminus of subunit b, respectively. The same subunits are similarly exposed to digestion by chymotrypsin. Trypsin also removes amino acids 1–4 from subunit d; the other subunits in F_0 appear to be relatively resistant to proteolysis [37].

The bovine F_0 domain is now thought to be an assembly of 9 different polypeptides [39]. They are subunits a, b, c, d and F_6, which have been discussed already, and A6L, e, f, and g. Homologues of A6L and e have been found in the rat mitochondrial F_1F_0-ATPase [40,41]. Rat subunit A6L is also known as chargerin II, and is said to have an essential role in energy transduction [40]. The C-terminus of bovine A6L may be exposed to the matrix [38], and interaction of this region with the stalk cannot be excluded. Subunits f and g were discovered in the bovine enzyme recently [42], and it is not yet known whether there are homologues elsewhere. Subunit f may have a single α-helical transmembrane span, but nothing is known about either the functions of subunits e, f or g, or about their locations. Therefore, it is possible that any of them could interact with the stalk domain in the bovine enzyme.

4. Assembly of the mitochondrial stalk complex in vitro

Interactions between bovine F_1-ATPase and proteins that probably form the stalk in the F_1F_0-ATPase, have been investigated by in vitro assembly, using the OSCP, F_6 and subunits b and d, and fragments of subunits b and d (see fragments b′, bI, bC and d′ in Fig. 1) produced by over-expression in *E. coli*. Each of these proteins was tested by gel-filtration chromatography for its ability to form a stable binary complex with F_1-ATPase. Only the OSCP could do so. Ternary complexes, F_1·OSCP·(b′, bI or bC), were obtained by mixing F_1-ATPase together with OSCP and either b′, bI or bC, but b′, bI and bC seemed to be bound in sub-stoichiometric quantities. However, when F_6 was added to F_1-ATPase together with OSCP and any one of b′, bI or bC, the stoichiometric quaternary complexes F_1·OSCP·(b′, bI or bC)·F_6 were observed. Therefore, the presence of F_6 enhances the binding of b′, bI and bC to F_1·OSCP, although F_6 does not appear to form a strong direct interaction with the binary complex. By adding either d or d′ to F_1 together with OSCP, b′ (or bI) and F_6, the pentameric complexes F_1·OSCP·b′(or bI)·F_6·d(or d′) were formed. These latter experiments suggest that the N-terminal region of subunit d is unimportant in forming the complex, and that its main interactions with subunit b and the other subunits involve amino acids 121–214 in the latter protein. Subunit d did not form a stable complex with F_1 in the presence of OSCP and b′, and the F_1·OSCP·b′ complex, lacking d, was recovered. Therefore, the binding of subunit d in the complex depends on the additional

presence of F_6. Finally, a pentameric complex was formed between F_1, OSCP, b, F_6 and d (see Fig. 2). The binary complexes b′·OSCP and b′·d were observed by mixing b′ and OSCP and b′ and d, respectively, a ternary complex formed from b′, d and F_6, and a stoichiometric quaternary complex between OSCP, b′, F_6 and d was also isolated (see Fig. 2). This quaternary complex containing b′ is referred to as the 'stalk (b′)' complex. The pre- formed stalk (b′) complex can make a 1:1 complex with F_1-ATPase, as shown in Fig. 2.

5. The stoichiometry of mitochondrial stalk subunits

By S-carboxymethylation with iodo-2-[^{14}C]acetic acid of the subunits of F_1F_0-ATPase that contain cysteine residues (subunits α, γ, ε, OSCP, b, d, f and c), and by measurement of the radioactivity incorporated into α, γ, OSCP, b, d and f, the stoichiometry $\alpha_3\gamma_1b_1OSCP_1d_1f_1$ was obtained. By similar means, the stoichiometry $\alpha_3\gamma_1b_1OSCP_1d_1$ was found in the F_1·stalk complex. The ratio of b:d in F_0 was 1:1, and the ratio of b′:d:OSCP in the stalk was 1:1:1. The molar ratio of each subunit in the complex was also estimated by measuring the PTH amino acids released by N-terminal sequencing. The same ratios were obtained as in the radioactive labelling experiments, and the observed values additionally suggested that there is 1 mol of F_6 per mol of the various complexes. Therefore, the mol ratio of b:OSCP:d:F_6 in bovine F_1F_0-ATPases appears to be 1:1:1:1 and not 2:1:1:2, as proposed by Hekman et al. [38]. Lippe et al. [35] have also proposed 2 mol of subunit b/mol of F_1, and Penin et al. [43] have suggested that there are 2 mol of OSCP/mol of F_1 in the pig enzyme. The stoichiometry of 1 mol of b/mol of F_1 implies that either the structure of the stalk in the mitochondrial enzyme differs significantly from the bacterial and chloroplast enzymes, or that other subunits or combinations of other subunits of the mitochondrial enzyme (such as d and F_6) fulfil the role of the extrinsic membrane domain of the second bacterial b subunit.

6. Conclusions

An understanding of the coupling mechanism of F_1F_0-ATPases depends on knowledge of the stalk between the F_1 and F_0 domains of the enzyme, and of how the stalk communicates with the proton transporting mechanism in F_0 and with the catalytic sites in the β-subunits of F_1. Different arrangements of subunits are found in the stalk regions of F_1F_0-ATPases from various sources. The stalks in bacterial and chloroplast enzymes are probably similar to each other, and 4 or 5 different subunits contribute to their structure. In bovine heart mitochondria as many as 7 proteins help to form the stalk. Some of the interactions between subunits in the bovine stalk have been identified by in vitro assembly experiments, and an extended bovine F_1-ATPase complex containing stalk subunits has been obtained. Therefore, a future task will be to obtain suitable crystals of the F_1·stalk and of the separate stalk complex. It will also be important to try and understand how the proton transporting mechanism in the a and c subunits of the F_0 membrane domain interacts with the stalk subunits. The recently completed high resolution structure of bovine F_1-ATPase (J.P. Abrahams, A.G.W. Leslie and J.E. Walker, unpublished results) should help to explain how the stalk subunits communicate with the catalytic sites in the β-subunits.

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