MAMMALIAN ATP SYNTHASE: NOVEL INSIGHTS INTO THE ROLES OF ITS SUPERNUMERARY SUBUNITS

by

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Abstract

ATP synthase (F_OF_1) in mammals is an important and well recognized complex located on the inner mitochondrial membrane. It synthesizes ATP from ADP and P_i in the presence of Mg^{2+} by utilizing an electro-chemical gradient of protons across this membrane generated by an electron transport chain. The overall process is referred to as "oxidative phosphorylation". More recent studies reported within or near the past decade have shown that the ATP synthase complex exists also on the plasma membrane of several mammalian tissues and have referred to it as the "ectopic" ATP synthase. At this location the ATP synthase may have important functions other than a role in ATP synthesis, or in addition to a role in ATP synthesis. Also, of some interest have been the findings that some of the soluble components of the ATP synthase such as subunit F_6 and the regulator IF_1 are present in human serum and interact with and affect the activity of the ectopic ATP synthase. For these reasons six soluble non catalytic subunits (d, OSCP, Factor B, F_6 and IF_1) of mitochondrial ATP synthase were investigated in some detail to determine to what extent they independently interact with the catalytic F_1 component of the ATP synthase and influence its activity. Of particular interest were subunits d, OSCP, and F_6, as these are known as "supernumerary subunits", i.e., non-catalytic or extra subunits whose roles are not well defined.

Maltose-Binding Protein (MBP) pull-down assays done in in a phosphate saline (PBS) buffer containing Triton X-100 showed that subunits d, F_6, Factor B and OSCP can individually interact with the catalytic F_1 subcomplex. These interactions were not affected appreciably by either conditions containing more salt (NaCl) or the addition of ATP, ADP and Mg^{2+}, properties that may be common among mammalian species and also allowed within the mitochondrial structure. We also found that each subunit tested could independently affect the catalytic ATP hydrolytic activity by measuring the free phosphate released from ATP

catalyzed by the isolated F_1-ATPase in the presence of each tested subunit. Finally, we carried out an MBP pull-down assay without the detergent (Triton X-100) being present and reconfirmed these interactions. These data indicate that each of the soluble non-catalytic subunit components of ATP synthase in mammals can bind to the catalytic F_1 subcomplex and affect its activity. Therefore, we suggests that within many animal and human cells such regulation of ATP synthase is likely to occur both at its well established mitochondrial location, and at its more recently established cell membrane location.

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Introduction:

ATP Synthase

ATP: Biological Energy

Adenosine-5'-triphosphate (ATP) is a common energy source for most chemical reactions in all living organism. In humans and animals ATP is made in sub-cellular organelles called "mitochondria", or the so-called "power houses" of the cell. Most of the energy derived from food or stored in a human, animal, or other organism is extracted through oxidation predominantly to produce the reduced high-energy electron carriers NADH (Nicotinamide adenine dinucleotide) and $FADH_2$ (Flavin adenine dinucleotide). In subcellular organelles called "mitochondria", these electrons are transferred via an electron transport chain (ETC) consisting of NADH dehydrogenase (complex I), succinate dehydrogenase (complex II), ubiquinone, bc1-complex (complex III), cytochrome c (Cyt c), and cytochrome c oxidase (complex IV) where the final electron acceptor oxygen is reduced to water. Significantly, electron transport in the ETC is coupled to the proton pumping activity of inner membrane complexes I, III, and IV, which generate across this membrane an electrochemical gradient called the proton motive force Δp. This serves as the driving force for synthesizing ATP from adenosine-5'-diphosphate (ADP) and phosphate (P_i) by ATP synthase (complex V) via the backflow of protons across the membrane. ATP synthase is referred to also as F_OF_1 or F_OF_1-ATP synthase as it is comprised of two major functional units; F_O, a proton translocating unit and F_1, a catalytic unit on which ATP is made from ADP and phosphate (P_i) in the presence of Mg^{2+}.

ATP synthases are responsible for producing most cellular ATP in aerobic organisms. To make ATP, this highly asymmetric enzyme uses the electrochemical gradient inherent in H^+ or Na^+ (in some anaerobic bacteria) generated across energy-coupling membranes, such as the inner mitochondrial membrane (noted above), the thylakoid membrane of plants, or the plasma membrane of bacteria (1). Specifically, these ATP synthases convert electrochemical

ion gradient energy into mechanical energy via rotation of the c-ring of the F_O domain. This mechanical energy is transmitted from the F_O through the central stalk to the F_1 domain containing bound ADP and P_i converting them at this catalytic center into ATP where useful chemical energy is stored. Because of its central role in biological energy production the ATP synthase has been regarded as one of the most important enzyme complexes in the human body. Significantly, greater than 90% of cellular ATP is generated by the continuous activity of ATP synthases to provide most of the energy necessary for human life. The human body stores and maintains a small amount of ATP (~50 grams), but has to generate a large amount of ATP equal approximately to its body weight to sustain its biological activities every day (2).

Structure of ATP Synthase in Bacteria

ATP synthases of bacteria such as *Escherichia coli* are associated with the plasma membrane. This simplest ATP synthase form is comprised of eight different subunits types in a stoichiometric relationship of $\alpha_3\beta_3\gamma\delta\varepsilon ab_2c_{10}$. This includes both the basic and core subunit compositions of the catalytic and rotary components of the enzyme (Table 1 and Figure 1). ATP synthase can be isolated also as two separate functional protein complexes, the membrane-embedded F_O and the water-soluble F_1 subcomplex. Isolation procedures may vary widely depending on the laboratory involved (3). The F_1 subcomplex derives its name from both the term "Fraction 1", the first ATP synthase/ATPase related factor isolated from bovine heart. The F_O subcomplex (written with a subscript letter "o", not "zero") derives this "O" from the first letter of the word "oligomycin" a natural antibiotic isolated from *streptomyces diastatochromogenes*. Oligomycin upon binding to the F_O moiety of the complete ATP synthase (F_OF_1) from many sources is a potent inhibitor of its catalytic activities, ATPase and ATP synthesis. Thus, oligomycin by binding to F_O confers inhibitory sensitivity to bound F_1 thereby inhibiting its catalytic forward and reverse functions, i.e., ATP synthesis and ATP hydrolysis.

In contrast to mitochondria, ATP synthases from chloroplasts and most bacteria including *E. coli* have a low sensitivity to oligomycin.

The F_1 subcomplex (holoenzyme) is a water soluble fraction consisting of five different subunits in the stoichiometry of $\alpha_3\beta_3\gamma\delta\varepsilon$ and can catalyze ATP synthesis or hydrolysis. The isolated F_1 can display only ATPase activity and hence is also called F_1-ATPase. The subunits, α and β, have a similar amino acid sequence and tertiary structure. The $(\alpha\beta)_3$ unit is alternately arranged to form the catalytic hexameric complex with 3 catalytic nucleotide binding sites, one at each α/β interface, and 3 non-catalytic nucleotide binding sites, one each at the other α/β interfaces. The catalytic nucleotide binding sites are located mainly on the β-subunits while the non-catalytic binding sites are located mostly on the α-subunits. The function of the three non-catalytic sites is not yet fully understood but it has been suggested that they may have regulatory roles. The small subunit pair γε is located within the central cavity of the catalytic center. The γ-subunit resides within the center of the $(\alpha\beta)_3$ hexamer and rotates at the expense of an electrochemical proton gradient set up across the mitochondrial inner membrane by the electron transport chain. This induces upon contact sequential conformational changes in the three catalytic sites containing ADP, P_i, and Mg^{2+} resulting in ATP synthesis and its release. In bacteria, the ε-subunit is an inhibitor of the ATPase activity, thus preventing wasteful ATP hydrolysis. It also contributes to the central stalk together with the γ-subunit in connecting F_O to F_1. The δ-subunit is located on top of F_1 and together with the b-subunit acts as a peripheral stalk that also helps connect F_O and F_1.

The F_O subcomplex in bacteria is comprised of three subunits, "a", "b", and "c", in the stoichiometry ab_2c_n (n=10 in *E. coli*) and acts as a membrane proton channel. The a-subunit contains two separated half channels for protons that end on both sides of the membrane. A proton that enters from one half channel of the a-subunit is transferred to the adjacent c-

subunit in the middle of the membrane. The c-subunits form an oligomeric ring within the membrane with 10 subunits in *E. coli*. However, 8-15 subunits have been reported to comprise the c ring in various species. Proton transfer to a c-subunit from the a-subunit induces a stepped rotation of the whole c ring. At the same time the release of the proton from an adjacent c-subunit to the other half channel of the a-subunit occurs. Two identical b-subunits form an unusual right-handed coiled coil structure that encompasses the whole protein from the membrane to the top of the enzyme to help connect F_O to F_1. This coiled coil structure plays an essential role in keeping the catalytic unit from co-rotating with the γ-subunit.

In terms of a functional unit, the ATP synthase can be divided into 4 subdomains. In *E. coli*, the catalytic head, $(\alpha\beta)_3$, the first subdomain, catalyzes either ATP synthesis or ATP hydrolysis depending on whether or not an electrochemical gradient exists across the membrane. The proton channel, the second domain, consists of an ac_{10} unit that provides a pathway for proton translocation. The third domain, a central stalk, is comprised of the subunits γ and ε which connect F_O and F_1 at the center of the catalytic center and couple between mechanical force and chemical energy during catalysis. The fourth and final domain is the peripheral stalk ($b_2\delta$) that links F_O and F_1 together on one side of the enzyme and endures the rotary strain during ATP synthesis. During the reactions of ATP synthesis/hydrolysis the $\gamma\varepsilon c_{10}$ rotor complex rotates within the center of the $\alpha_3\beta_3$ catalytic head, while the peripheral $b_2\delta$ stalk stabilizes the head against enduring strain.

Mechanism of ATP Synthase

The ATP synthase is currently considered a nanomachine composed of two rotary motors, F_O (c_{10} ring) and F_1 ($\gamma\varepsilon$) responsible for two opposing rotations. The two motors are physically connected by two stalks, a central one and a peripheral one. With protons flowing through the proton channel powered by the electrochemical potential of the protons across

the membrane, the c-ring rotates against the ab_2-subunits in a clockwise direction as viewed from the membrane. This rotation then drives the rotation of the γε-subunit pair of the F_1 motor. During this rotation the asymmetry of the γ-subunit induces a series of conformational changes (one at each catalytic site) that facilitate ATP synthesis. In contrast, when the electrochemical potential is small or decreases, ATP hydrolysis at the catalytic site becomes favorable and drives the rotation of the F_1 motor and then the F_O motor in the reverse direction. If this is the mitochondrial ATP synthase located on the inner mitochondrial membrane facing the matrix, and ATP is present, then in the absence of an electrochemical proton gradient ATP hydrolysis would be expected to occur with protons being pumped out of the matrix and hydroxyl groups (OH^-) left therein.

The catalytic mechanism of the ATP synthase has been explained by the "binding change mechanism" proposed by Boyer (4) through numerous biochemical approaches. According to this model, the catalytic F_1 domain contains three sites, one at each β-subunit. At any given moment in the catalytic reaction cycle, each of the three catalytic sites is considered to be in a different conformation and each site's binding affinity differs for ATP or ADP and P_i. One is in the tight conformation which binds ATP tightly. A second is in the loose-binding conformation which binds ADP and P_i but not ATP. Finally, a third is in the open conformation after releasing newly synthesized ATP. The conformation of the β-subunit changes in the direction of open, loose, tight and open again for the next reaction during ATP synthesis or in reverse direction during ATP hydrolysis. This model assumes an asymmetric conformation for the three identical αβ pairs in F_1. The asymmetric arrangement of homologous catalytic-subunits is induced by the rotation of a single eccentrically coiled coil γ-subunit in the center of the catalytic complex. The conformation of each β-subunit is changed sequentially as it interacts with the rotating γ-subunit. In one complete rotation, the

γ-subunit drives conformational changes in all three β-subunits that induce/drive the synthesis of ATP at each of the three catalytic sites. Thus, three ATPs are synthesized per each 360° turn of the γ-subunit.

During ATP synthesis the electrochemical potential gradient of protons is required for the release of formed ATP but not for the formation of the covalent bond of ATP from ADP and P_i. Oxygen isotope exchange studies confirmed that there is a rapid inter-conversion via several reversible hydrolysis and synthesis steps of ATP from bound ADP and P_i at the catalytic site even on un-energized F_OF_1 complexes on the membrane in the absence of a proton gradient and also on isolated F_1 when the incorporation of ^{18}O into the P_i during ATP hydrolysis was analyzed (4,5). Also, the purified F_1-ATPase could synthesize ATP in the presence of organic solvents such as dimethyl sulfoxide (DMSO) that increased the affinity of the enzyme for P_i. The ATP remains tightly bound to the enzyme and is not released to the medium (6). These findings demonstrated that F_1 alone can synthesize enzyme-bound ATP from medium ADP and P_i without free energy input. Instead, input of energy provides a driving force primarily for the release of newly synthesized ATP thus allowing more ADP and P_i to bind to the catalytic site and ATP to be synthesized and released.

The first high-resolution X-ray structure of the F_1-ATPase from bovine heart mitochondria clearly confirmed that the three β-subunits in F_1 have different catalytic site conformations (7). In the crystal structure of bovine mitochondrial F_1, one of the three catalytic sites is filled with the non-hydrolysable ATP analog, AMP-PNP (5'-adenylyl imidodiphosphate) (assumed to be the tight conformation). The second site is filled with ADP (the loose conformation). The third site has no bound ligand (the open conformation). Hence, each β-subunit is referred to as β_{TP}, β_{DP}, and β_E. The occupied β-subunits, β_{TP} and β_{DP}, show similar structures in a closed conformation, and the empty β_E-subunit is in an open

conformation. The main difference between a closed and an open conformation is found in the C-terminal domain of the β-subunit in what is referred to as the DELSEED-loop, a helix-turn-helix structure containing the conserved DELSEED motif. The DELSEED-loop is in an up position when the catalytic site on the respective β-subunit is filled with nucleotide but is extended downward in the β_E-subunit in the absence of bound nucleotide. The DELSEED-loop of each of the three β-subunits makes contact with the γ-subunit suggesting an essential role for this motif in coupling between catalysis and rotation of the γ-subunit.

Whether the ATP synthase (F_OF_1) is making ATP or hydrolyzing ATP in biological systems, e.g., mitochondria, bacteria, and chloroplasts, proton translocation is involved. The subunits a and c in the F_OF_1 of *E. coli* are directly involved in proton movement through the membrane. Each c-subunit has a hairpin structure, which is composed of two long α-helices and a short hydrophilic loop at the cytoplasmic side of the membrane. In the c_{10} ring, the loop region forms an extensive area contacting both the foot of the central stalk and the subunits γ and ε. It couples the rotation of the c ring with the rotation of the F_1 motor. The a-subunit has five transmembrane helices and resides outside of the c-ring. According to the "half channel mechanism" the a-subunit includes two half-channels that allow protons to enter and pass partway to deliver protons to the c ring. During the rotation of the c ring, the a-subunit also acts in part as a stator via its interaction with subunit b, a homodimer within the membrane-spanning region (Fig. 1). The b-subunit homodimer extends from the membrane to the top of F_1 (Fig 1). Together with subunits a and δ, the b-subunit functions as a peripheral stalk to hold in place a stator part of F_O (δ-subunit) and a stator part of F_1 (subunit a) to avoid their co-rotation with the motor parts.

Extensive biochemical studies on the F_O subunits have been carried out to identify amino acid residues involved in proton translocation. Several charged residues important in

this process were found. A highly conserved residue, Arg210 in *E. coli*, directly interacts with subunit c and is thought to be involved in proton transfer (8). In the c-subunit, Asp61 (in *E. coli*, Glu in other species) located within the phospholipid bilayer in the middle of the C-terminal helix is viewed as playing an essential role in proton binding and release through inter-conversion of protonated and de-protonated forms of this conserved carboxylate residue.

In a "two half channel model" for proton translocation through F_O the a-subunit is viewed as providing two aqueous channels to access the a/c interface on the membrane. Each half channel is independent, disconnected from the other, and exposed on opposite sides. Also, each half channel interacts with a different neighboring c-subunit. During proton translocation Arg210 of subunit a is believed to be interacting with Asp61 of subunit c. In the ATP synthesis mode a proton enters one half channel exposed to the side with a high proton motive force and then binds to the carboxyl residue, Asp61, of the c-subunit at the interface between a and c-subunits. The acidic group now neutralized is allowed to rotate toward the hydrophobic lipid layer. This c-subunit continues to rotate until it encounters subunit a and then releases the proton via the other half channel of this subunit to the other side of the membrane. The number of c-subunits in the c ring determines the number of protons released during one c ring rotation. Therefore, in *E. coli* ATP synthase, the c ring has ten c-subunits and hence requires ten protons for one full rotation while the catalytic F_1 makes 3 ATPs. In the ATP-driven proton-pumping mode, the reaction occurs in the reverse direction.

Single Molecule Assay

In 1997, Noji *et al.* (9) first demonstrated the direct observation of the rotational mechanism of the F_1 moiety of ATP synthase by video microscopy. This single molecule assay provided verification of a previous model based on biochemical assays and other remarkable advances to understand the mechanical dynamics in the study of ATP synthases. In the

experiment conducted by Noji *et al.* (9) they attached the $\alpha_3\beta_3\gamma$ subcomplex derived from the thermophilic Bacillus strain PS3 to a Ni-nitrilotriacetic acid (Ni-NTA) covered glass coverslip via a His tag linked to the N terminus of each β-subunit. To magnify the small position changes of the γ-subunit, a fluorescently labelled, biotinylated actin filament was attached to the γ-subunit through streptavidin. After the addition of ATP they visualized the unidirectional rotation of the γ-subunit in the F_1-ATPase and the anti-clockwise rotation as viewed from the membrane-facing side of F_1. In addition, they clearly confirmed the minimal catalytic unit, $\alpha_3\beta_3\gamma$ in F_1-ATPase. The fluorescent actin filament attached to the γ-subunit showed more than 100 turns/second and the torque generated from the ATP-driven F_1 motor was determined to be more than 40 pN·nm at saturating ATP concentrations. At low ATP concentrations, the torque averaged over all steps was 44 pN·nm irrespective of the frictional load (10). Considering that 3 ATPs were produced in one rotation, the mechanical work done in each 120° step was calculated to be 80-90 pN·nm (the torque of 40-44 pN·nm multiplied by $2\pi/3$ radian), which is approximately equal to the free energy released during the hydrolysis of one ATP molecule (~ 90 pN·nm /molecule) and, therefore, the efficiency of energy conversion by the F_1 motor was extremely high implying nearly 100% efficiency and a tight mechanochemical coupling of rotation to ATP synthesis from ADP and P_i within the F_1-ATPase (10-12).

Subsequent single molecule experiments identified the distinct 120° rotation step, which is consistent with the threefold symmetry of the $\alpha_3\beta_3$ catalytic sites as seen in the crystal structure of bovine F_1-ATPase (7). The 120° step was further divided into 80° and 40° sub-steps using high speed imaging by lowering the ATP concentration when binding of an ATP molecule was slowed down (13). The pause before the 80° step was dependent on ATP concentration and triggered by ATP binding and thus defined the "ATP-waiting" or "ATP-

binding" dwell. The duration of the pause before the 40° sub-step was independent of the ATP concentration, but significantly increased when the reaction was slowed by either a mutant F_1 with a very low rate of ATPase activity or by ATPγS (adenosine 5'-O-(3-thiotriphosphate), a slowly hydrolyzing ATP analog as the substrate. In order to resolve the rotational process into discrete rotational steps (14,15), the reaction scheme of F_1 during one complete rotation of the γ-subunit was studied extensively by simultaneous observations of the rotation and binding/unbinding events of fluorescently labeled ATP. These experiments showed distinct sub-steps regarding substrate binding/release involved in the reaction scheme. Through one revolution of the γ-subunit, each β-subunit accomplishes a single reaction of ATP hydrolysis. Based on degrees (°) of rotation, ATP binds the F_1 motor at 0°, is hydrolyzed between 0° and 200° and is released as the products ADP and P_i at 240° and 320°, respectively. The second β-subunit sequentially begins the reaction at an 120° interval (Figure 2).

At the time these experiments were conducted, the question of whether or not the chemo-mechanical coupling within the F_1 motor is completely reversible was very important. Attaching magnetic beads to the γ-subunit was carried out to manipulate solely the rotary direction of this subunit with electrical magnets. The minimal catalytic subcomplex, $\alpha_3\beta_3\gamma$, was attached to a glass surface through a His-tag modified at the termini of the β-subunits and a magnetic bead coated with streptavidin was attached to the biotinlyated γ-subunit. After the forced rotation of the γ-subunit in a clockwise direction using external magnets was accomplished, the investigators involved clearly confirmed that ATP was synthesized by using the luciferin/luciferase luminescence assay. However, the mechano-chemical coupling efficiency was very low [a coupling ratio of 0.5 ± 0.4 (mean ± s.d.) ATPs produced per turn]. However, in the presence of the ε-subunit, the efficiency was dramatically increased to ~77% [an average value of 2.3 ± 1.6 (mean ± s.d.) ATPs produced per turn]. This finding implied

that although the ε-subunit is not a component of the minimal catalytic unit for ATP hydrolysis (16), it has an essential function during ATP synthesis.

A different experimental approach using the ATP synthase (F_OF_1) incorporated into liposomes was developed to monitor γ-subunit rotation within the holoenzyme during proton-driven ATP synthesis. Single-molecule fluorescence resonance energy transfer (FRET) was used to monitor a change in the distances between the b-subunit of the peripheral stalk and the γ-subunit in the central rotor. *E. coli* ATP synthase (F_OF_1) molecules were selectively labelled at the b-subunit and the γ-subunit with two different fluorophores, a FRET donor and acceptor. The double-labeled F_OF_1-ATP synthases molecules were then incorporated into liposomes and single-molecule fluorescence resonance energy transfer (FRET) was measured during ATP hydrolysis in the presence of ATP. Also, ATP synthesis was measured in the presence of a transmembrane pH difference (ΔpH) plus an additional electric potential difference ($\Delta\psi$) across the liposomal membrane. As in the earlier experiment with isolated F_1, the rotation of the γ-subunit was stepwise during proton-driven ATP synthesis, showing three distinct distances to the b-subunits. Also, the direction of rotation during ATP synthesis was shown to be opposite to that of ATP hydrolysis (17,18). Finally, the rotation of the ε-subunit relative to the b-subunit was confirmed using a similar approach that involved labeling the ε-subunit rather than the γ-subunit (19). The direct verification of rotation of the c-subunit ring in *E. coli* ATP synthase was revealed with a similar FRET experiment. *E. coli* ATP synthases containing the a-subunit fused with EGFP (enhanced green fluorescent protein) as the FRET donor and the c-subunit covalently attached with Alexa568-maleimide as the FRET acceptor were reconstituted singly into liposomes. The intermolecular distance between the stator and the labeled c-subunit was monitored during ATP synthesis in the energized liposomes together with the pH difference and electrical potential. Significantly, the investigators detected multiple

stepping rotations interpreted as subsequent 36° steps as expected from the 10-fold symmetry of the c-ring from *E. coli* (20).

Direct demonstration of single F_1-ATPase and F_OF_1-ATP synthase molecules became the new essential tool to improve our understanding about the energy-conversion mechanism and the conformational changes of this enzyme during catalysis. A number of experiments have been performed with the recombinant or purified enzyme from several species sources. Usually the enzyme that originated from either the *thermophilic Bacillus* strain PS3 or *E. coli* was used for single molecule experiments because of easy handling and high stability. Also, direct observation of a rotary operation within a Na^+-translocating ATP synthase (21) and chloroplast ATP synthase during both ATP hydrolysis (22) and ATP synthesis (23) have been reported. Therefore, the rotatory mechanism demonstrated for both the F_1-ATPase and F_OF_1-ATP synthase is likely a ubiquitous phenomenon in all ATP synthases present in prokaryotes as well as in eukaryotes. However, with the exception of yeast ATP synthase (24), the single molecule study with ATP synthases from other sources remains to be carried out. Regarding the more complicated structure and different subunit composition in mitochondrial ATP synthase, a single molecule study may not only provide critical information for understanding the mechanism and regulation of the enzyme but also the function of the additional supernumerary subunits during the catalysis.

Mitochondrial ATP Synthase and Its Supernumerary Subunits

The overall structure of ATP synthases and their basic catalytic mechanisms for ATP synthesis and hydrolysis have been highly conserved among species. Nevertheless, there are some differences in terms of subunit composition. Both the ATP synthase of bacteria and that of chloroplasts have similar subunit components except for the nature of the b dimer and the number of the c-subunits in the c rings. In most bacteria such as *E. coli*, the b-subunit

forms the peripheral stalk consisting of a homodimer of two identical b-subunits generated from a single gene but subunits I and II as named in chloroplasts or b and b' in photosynthetic bacteria make a heterodimer with two different gene products but homologous b-like polypeptides. The number of c-subunits in the c-ring naturally varies depending on the species. The smallest number that has been identified so far is 8 in the mammalian mitochondrial ATP synthase (25) while alkaliphilic cyanobacterium *Spirulina platensis* has the largest number, i.e., 15 c-subunits in the F_O rotor ring which was verified by high-resolution atomic force microscopy topographs (26) and the crystal structure of the purified c rings (27). The mitochondrial ATP synthase of the yeast and bacterial ATP synthase from *E. coli* and *Bacillus* PS3 have 10 c-subunits (28-30). The c ring of the Na^+-dependent ATP synthases isolated from *Ilyobacter tartaricus* and *Propionigenium modestum* contained 11 c-subunits (31) and the heterologous overexpression of the c-subunit gene from either organism in *E. coli* could fully assemble into the c rings with the identical stoichiometry of 11 c-subunits (32). Therefore, intrinsic sequence information of the c-subunits seems to be critical to determine the formation of the species-specific c ring structure with any particular quantity of c-subunit. In addition, subunit III oligomers of the chloroplast ATP synthase, equivalent to the c ring in mitochondria and bacteria, were isolated from spinach and shown by atomic force microscopy to be cylindrical structures with 14 symmetrically distributed subunit IIIs (33).

Mitochondrial ATP synthase has not only all core components of the enzyme but also several supernumerary subunits without a counterpart in bacteria and chloroplasts. It is generally accepted that mitochondrial ATP synthase has 15 different subunit types because most purified F_OF_1-ATP synthases contain these subunits. Of these, 10 form the F_O domain (a, A6L, b, c, d, e, F_6, f, g and OSCP), while the F_1 domain is composed of only 5 subunit types (α, β, γ, δ, ε) (34,35). Among them the ε-subunit of the F_1 complex and the subunits d, F_6, e,

f, g and A6L of the F_O complex have been found exclusively in the mitochondrial ATP synthase and are thus considered as supernumerary subunits. In addition, the inhibitory Factor 1 (IF_1) occasionally regarded as the 16th subunit (36) and Factor B as the 17th subunit (37,38), despite significant loss of both proteins during purification of the ATP synthase complex, also have no homologous proteins in bacteria and chloroplasts (Table 1).

F_1 in Mitochondrial ATP Synthase Relative to F_1 in Bacteria and Chloroplasts

Because the F_1 domain plays a directly essential role in catalytic reactions, all basic component are highly conserved throughout diverse species from *E. coli* to mammals, especially the α and β-subunits that form catalytic hexamers (39,40). The primary sequence of the γ-subunit is rather poorly conserved but shows locally well-conserved regions that are important in binding to neighboring subunits. The mitochondrial δ-subunit is homologous to the subunit ε in bacteria and chloroplasts and is composed of an N-terminal β-sandwich with 10 β-strands and a C-terminal α-helical hairpin with 2 α-helices. The overall structure of the mitochondrial δ-subunit in the crystal structure of bovine mitochondrial F_1 is very similar to that of the purified bacterial subunit ε (41). However, the structural studies of the isolated γε-complex and F_1-ATPase from *E. coli* have shown that the C-terminal region of ε can adopt another conformation which extends along subunit γ toward the β-subunit (42,43). This subunit in bacteria and chloroplasts is well known as an inhibitor of ATPase activity that prevents wasteful ATP hydrolysis. Later it was shown that the electrostatic interaction between the positive charges in the C-terminal α-helical domain of the ε-subunit and the negative charges in the DELSEED motif in the β-subunit are important for the inhibitory effect of the ε-subunit (44). In contrast, there is no evidence so far that the mitochondrial δ-subunit has a similar inhibitory effect on the ATP hydrolysis reaction or forms a similar interaction with the β-subunit. Indeed, the C-terminal region of the mitochondrial δ-subunit is not as basic as

the counterpart of the C-terminal α-helix of the bacterial and chloroplast ε-subunit (45). Its C-terminal α-helices form a hairpin. The contracted conformation is located on the surface of c-subunits and might be stabilized with the mitochondrial ε-subunit (41). It was also proposed that in mitochondria a specialized protein, known as IF_1 may adapt a similar inhibitory function as that of the bacterial and chloroplast ε-subunit. This is because the α-helical N-terminal region of IF_1 is rich in basic amino acid residues and can penetrate into the interface between subunits α_{ADP}-β_{ADP} within the catalytic $\alpha_3\beta_3$ hexamer. As a result, IF_1's N-terminal region can be in close proximity to the side-chain carboxyl groups of the β-DELSEED sequence within the crystal structure of the F_1-ATPase-IF_1 complex to play an inhibitory role by preventing the hydrolysis activity of mitochondrial ATP synthase (46,47).

The only unique mitochondrial subunit in F_1 is the ε-subunit, which has no counterpart in either bacterial or chloroplast ATP synthases (34). A "null mutant" study of the ε-subunit in yeast grown on non-fermentable substrates showed that it is not an essential component of ATP synthase (48). However, another study recently reported that the ε-subunit is an essential component for oxidative phosphorylation using the ε-subunit-deletion mutants transformed with a plasmid for doxycycline-regulated expression of the wild-type ε-subunit. Only 25% down-regulation in the expression of the ε-subunit induced with doxycycline in yeast was enough to result in complete rapid growth arrest in non-fermentable media because of F_O-mediated proton leaks (49). It was suggested that the loss of the ε-subunit affects the stability of the δ-subunit within the central stalk and this leads to uncoupling via a high proton leakage through F_O. This report is consistent with previous structural studies in which the ε-subunit was shown to form a stable heterodimer with the δ-subunit (50) that within the central stalk provides most of surface to contact the upper surface of the membrane rotor, i.e., c_{10} rings (51). Therefore, the mitochondrial specific ε-subunit might be important for the stability of

the ATP synthase within the central stalk through tight interactions with subunits γ and δ. Other studies showed that the silencing of the ε-subunit of mitochondrial ATP synthase in mammalian HEK293 cells induced a decrease in both the activity and content of the complex and an unexpected accumulation of the c-subunit (52). Interestingly, analysis of human fibroblasts obtained from a patient with a homozygous missense mutation,Tyr12Cys, that corresponds to Tyr11 of the mature ε-subunit and is a highly conserved amino acid in eukaryotes, showed a similar biochemical phenotype (53). Also, in this regard, an essential role of the ε-subunit during the biogenesis and assembly of ATP synthase was suggested.

F_O in Mitochondrial ATP Synthase Relative to F_O in Bacteria and Chloroplasts

Most of the supernumerary subunits of mitochondrial ATP synthase have been found in its F_O subcomplex. Among its F_O subunit composition of a, A6L, b, c, d, e, F_6, f, *g* and OSCP, the subunits d and F_6 are supernumerary components in the peripheral stalk and e, f, g, A6L are supernumerary subunits and integral membrane proteins of the F_O complex. Factor B and IF_1 are also additional supernumerary subunits with specific regulatory functions. All peripheral stalks across species apparently play the same essential function by preventing the co-rotation of the catalytic hexamer with a rotary motor thus making the catalytic unit responsive to the rotating γ-subunit during ATP synthesis. The existence of these supernumerary subunits in the ATP synthase of mitochondria are absent in ATP synthases from *E. coli* and chloroplast thus raising a very interesting question about whether these subunits have additional functions specific to mitochondria.

In bacteria and chloroplasts, the peripheral stalk (or stator stalk) of the ATP synthase consists of only three proteins, the δ-subunit and either a b_2 dimer formed from two identical b-subunits expressed from a single gene in some eubacteria or a bb' dimer (or b_Ib_{II} dimer) formed from two different proteins of the homologous genes in photosynthetic bacteria and

chloroplasts respectively. Each b-subunit forms an α-helix extending from the membrane to the top of F_1. The functional unit, the b_2 dimer, serves as the primary external scaffold in the peripheral stalk and connects F_1 and F_O through protein interactions involving the a-subunit of F_O in the membrane and the δ-subunit at the top of F_1. In contrast to the *E. coli* ATP synthase, the mitochondrial ATP synthase has a peripheral stalk composed of four different subunits designated as subunit b, OSCP, subunit d, and F_6 (or H in *S. cerevisiae*). A single subunit b forms a continuous, slightly curved α-helix extending from the membrane with two transmembrane helices to the top of F_1 as the primary stem. OSCP is a connector between subunit b and the top of F_1. Additional supernumerary subunits, subunits d and, F_6 surround and probably strengthen the upper part of subunit b.

The OSCP "Stalk" Subunit within F_O in Mitochondrial ATP Synthase

OSCP (oligomycin sensitivity-conferring protein) helps mediate in part the sensitivity of the inhibition of the intact mitochondrial F_OF_1-ATP synthase to oligomycin, the natural antibiotic first isolated from the Gram-positive bacterium *Streptomyces diastatochromogenes*. Oligomycin does not directly interact with the OSCP but instead binds the proton channel and specifically blocks proton translocation through F_O thus inhibiting both ATP synthesis and ATP hydrolysis (54). As a component of the peripheral stalk, the OSCP connects and stabilizes the two subcomplexes F_1 and F_O of ATP synthase and is essential for coupling the proton flux to ATP synthesis. Thus, the oligomycin-sensitive ATPase activity conferred by the OSCP has been regarded as an indicator of the functional integrity of the enzyme on the membrane. Dissociation or uncoupling of F_1 from F_O results in a loss in the capacity of its ATPase activity to be inhibited by oligomycin. Interestingly, the chloroplast and *E. coli* ATPases have no or little sensitivity to oligomycin. Recently the high-resolution crystal structure of the c_{10} ring of the yeast mitochondrial ATP synthase containing oligomycin showed that this

inhibitor binds to the surface of the c_{10} ring that includes the carboxyl side chain of the Glu59 residue. This critical residue allows proton translocation across the membrane. The difference in sensitivity between the mitochondrial and *E. coli* ATP synthases to oligomycin is most likely due to differences in the amino acid compositions and sequences of subunit c in that region that bind this inhibitor of the mitochondrial enzyme (54).

OSCP is the functional counterpart of the bacterial chloroplast δ-subunit but has its own distinct characteristics. Historically OSCP has been considered as a subunit of the F_O moiety because it is absent from preparations of F_1-ATPase. In contrast, the bacterial δ-subunit remains associated with the purified bacterial F_1-ATPase and is regarded as a component of the F_1 moiety. The primary amino acid sequence of bovine OSCP (190 residues long in its mature form) has ~27% sequence identity to the δ-subunit of *E. coli* (177 residues long). Surprisingly, OSCP also contains some residues homologous with both the b-subunit of *E. coli* ATP synthase and the mitochondrial ADP/ATP carrier (55,56) and has been suggested often as a hybrid protein between the δ and b-subunits of *E. coli* (56,57). Moreover, the δ-subunit of both the *E. coli* and spinach chloroplast ATP synthases are acidic proteins (Theoretical pI= 4.94 and 4.68 respectively) whereas OSCP is a basic protein (pI=9.83). In spite of these different properties, both OSCP of the mitochondrial ATP synthase and the δ-subunit of the *E. coli* and chloroplast ATP synthases are functionally essential proteins to connect F_O to F_1 over the species. OSCP was the only candidate to interact directly with F_1-ATPase among subunits forming the peripheral stalks in bovine heart mitochondria when the assay was performed by gel-filtration chromatography (58). In addition, in yeast and *E. coli* the OSCP and δ-subunit respectively are the last subunits assembled into the ATP synthase (F_OF_1) and are key players connecting the subunit a-stator component with the F_1-c ring structure preassembled during the biogenesis of the ATP synthase (59,60). Despite the difference in the

primary sequence of the OSCP and the δ-subunit, their overall structures appear to exhibit a similar architecture based on overall similarities in predicted secondary structure. Solution NMR studies indicate that the N-terminal regions of both the bovine OSCP and *E. coli* δ-subunit consist of a bundle of six α-helices with extremely similar folds. In addition, the C-terminal regions of both proteins appeared to be largely unfolded in solution but were proposed as similar structures based on sequence homologies (61,62). Circular dichroism spectroscopy studies with the δ-subunit from spinach chloroplasts and with the OSCP from porcine mitochondrial ATP synthase also showed almost identical recordings and predictions of their secondary structure (63).

Based on the above studies, OSCP is placed at the very top of the F_1 catalytic unit interacting with both the N termini of the α-subunit and the C terminal regions of the b-subunit within the peripheral stalk. The latter physically connects the catalytic F_O domain to the F_1 subcomplex. A previous study with deletion mutants of OSCP from bovine mitochondrial ATP synthase showed that the N-terminal end of OSCP is involved in its interaction with F_1 while the C-terminal end of OSCP is important for the interaction with F_O (64). Mild trypsin treatment of soluble mitochondrial F_1 which removes small peptides from the N-terminal regions of α (residues 1-15) and β-subunits (residues 1-7) without a significant effect on the hydrolytic activity, resulted in the loss of the binding to the OSCP (65,66). An NMR study with the N-terminal domain of bovine OSCP (residues 1-120) and a peptide corresponding to residues 1-25 of the α-subunit revealed their interaction with 1:1 stoichiometry. Neighboring hydrophobic surfaces of helices 1 and 5 in the N-terminal domain of bovine OSCP were found to be involved in the interaction with hydrophobic side-chains of the α-subunit peptide. Residues 1-8 of the α-subunit are more essential for the interaction. Interactions between the N-terminal regions of the β-subunit peptide (residues 1-15) and

OSCP were not identified. (62,67). Interestingly, the complex of bovine mitochondrial OSCP with the α-subunit peptide is quite similar to the corresponding complex between the N-terminal region of the *E. coli* δ-subunit (residues 3-105) and the N-terminal region of the α-subunit (residue 1-22). This is despite the difference in surface charge between bovine OSCP and the *E. coli* δ-subunit (61).

Both bovine OSCP and the *E. coli* δ-subunit suffer from torque energy which is generated by the rotor during catalysis and is estimated to be equivalent to about 50 kJ/mol. They have to resist this rotary strain to hold the catalytic domain (68). Therefore, the interaction between either OSCP or the δ-subunit and F_1 must be sufficiently strong to withstand the rotary strain. About 50 kJ/mol corresponds to a binding affinity (Kd) of about 1 nM. The binding affinity between bacterial δ and F_1 was about 1 nM, roughly corresponding to a free energy of binding of about 50 kJ/mol and was enhanced to ≤3 pm in the presence of the soluble cytoplasmic domain of the b-subunit (69). A similar binding affinity for the chloroplast δ-subunit binding to chloroplast F_1 was reported (70). These results indicate that the δ-subunit in *E. coli* and chloroplast binds to F_1 tightly enough to tolerate the elastic strain and prevent the stator sector from co-rotation with the rotor. In contrast, bovine OSCP binds to the isolated F_1 with somewhat lower affinity, Kd = 80 nM for one high affinity site and Kd = 6-8 μM for two lower-affinity sites. These values are not enough to hold the catalytic domain and explain why the OSCP is not retained in the F_1 preparation in mitochondrial ATP synthase unlike the δ-subunit in *E. coli* and chloroplasts (71). However, the affinity of OSCP for F_1 was increased to 1.7-5 nM when the affinity between OSCP and F_1 in the form incorporated with F_O on the membrane was measured (72). It was suggested that OSCP binds more tightly to F_1 via a conformational change in the holoenzyme, i.e., F_OF_1-ATP synthase. The other components in the peripheral stalk in the mitochondrial enzyme may contribute to the

increased affinity of OSCP for F_1 directly by providing another interaction with F_1 and/or by indirectly supporting the interaction between OSCP and F_1. However, the other subunits in the peripheral stalks did not form a stable binary structure with the bovine F_1 subcomplex that could be retained after gel-filtration chromatography. Only OSCP was able to do so (58).

The b-Subunit in Mitochondrial ATP Synthase

The C- terminal region of the OSCP of mitochondrial ATP synthase and the equivalent subunit, the δ-subunit in bacteria and chloroplasts, interact with the C-terminal regions of the b-subunit. In most bacteria including *E. coli* the peripheral stalks have the b_2 dimer genetically expressed from a single copy of the gene. Each *E. coli* b-subunit is a 156 residue polypeptide with four distinct functional domains. The N-terminal 24 amino acid sequence is a considerably hydrophobic domain that is buried in the membrane as one transmembrane α-helix. This region is required to form the F_O subcomplex via a strong interaction with subunit a in the membrane. The tether domain (b25-52) is not essential for dimer formation but links the membrane domain to the following sequence essential for dimerization. This domain seems to be quite flexible because it showed a high tolerance for deletions of up to 11 amino acids, insertions up to 14 amino acids, and for facilitating formation of dimers of b-subunits differing in the length of this region without affecting the activity or assembly of the *E. coli* ATP synthase. Therefore, it was proposed that the tether domain controls the elasticity of the stator and functions to store the transient elastic energy necessary to efficiently couple two motors at different stepping angles with ten 36-degree rotations within F_O (containing 10 c-subunits in *E. coli*) coupled to three 120-degree rotations occurring during the stepwise translocation of protons during ATP synthesis (73).

The dimerization domain (b53-122) extends along the F_1 part of the ATP synthase forming much of the peripheral stalk region. Proper interactions in the dimerization domain

are critical for the function of ATP synthase and an elongated, parallel, highly α-helical coiled-coil structure of a b_2 dimer is generally accepted. However, the correct structure of the b_2 dimer whether in a staggered or in an in-register conformation, and whether in an atypical right-handed coiled coil or in a left-handed coiled coil, is still under debate (74). The C-terminal δ-binding domain (b123-156) is essential for the binding of F_1 or the δ-subunit. A direct interaction with the δ-subunit has not only been well established but several lines of evidence also suggest a direct interaction between the b_2 dimer and the α and/or β-subunit of F_1 (75). Interestingly, the monomeric b-subunit displayed no affinity for F_1-ATPase. In contrast, formation of the b_2 dimer is indispensable for its interaction with F_1 (76).

Despite two identical b-subunits in the *E. coli* peripheral stalk, each b-subunit appears to have a district function in terms of its interactions with the other subunits. A recent report indicated that in the *E. coli* ATP synthase one of the two b-subunits is involved in the interactions necessary to connect F_O and F_1, and that the other might stabilize the stator as do the mitochondrial subunits F_6 and d (77). Another study reported that one b-subunit of the b_2 dimer is necessary for interacting with the δ-subunit while the other is responsible for interacting with the membrane part of F_O (78).

A major stem of the peripheral stalk of mitochondrial ATP synthase is composed of one b-subunit which extends from the inner membrane with two transmembrane helices to the top of the F_1 subcomplex. Its C-terminal region interacts with the C- terminal region of OSCP with the help of F_6 and the opposite part, its N-terminal region, interacts with subunit a on the membrane. Based on a recent x-ray crystallographic study of the bovine peripheral stalk, it was suggested that the stalk is rather rigid, different from that reported for the *E. coli* ATP synthase (79) (Figure 3A). In this study several interactions such as b-d, b-F_6 and d-F_6 in the bovine peripheral stalk were also confirmed. The overall structure of bovine F_1-ATPase

reconstituted with the peripheral stalk in the x-ray crystals provided important supportive information about protein-protein interactions (80) (Figure 3B). The elongated stator was positioned along the non-catalytic $\alpha_{DP}\beta_{TP}$ interface of the F_1 subcomplex. Helices 1 and 5 among the 6 α-helical bundles of the N-terminal domain of OSCP (residues 1-113) provided the binding site for residues 6-17 of subunit α_E, largely via hydrophobic interactions that probably contribute to the high-affinity binding site. The putative interactions of the OSCP with the N-terminal regions of subunit α_{DP} or α_{TP} were proposed as the two low-affinity sites already reported (71). The interpreted structure of the C-terminal domain of OSCP consisted of a β-hairpin followed by 2 α-helices, which formed an extended 5-helix bundle with the N-terminal α-helix of the F_6 and the C-terminal region of the long α-helix of the b-subunit (80).

The F_6 and d Subunit in Mitochondrial ATP Synthase

In bovine mitochondria, coupling factor 6 (F_6), a component present in the peripheral stalk, is a small protein composed of 76 amino acids in its mature form, i.e., after enzymatic deletion of the signal peptide from an immature form with 108 amino acids. This subunit was first purified crudely about 40 years ago and was shown to be necessary for oxidative phosphorylation and the capacity to restore ATP-P_i exchange and oligomycin-sensitive ATPase activity in reconstitution studies with F_6-depleted mitochondria. The solution structure of F_6 determined by NMR spectroscopy is highly flexible. In the mature sequence F_6 consists of two α -helical regions from residues 7-22 and 34-51 that cross over each other at a ~120° angle. F_6 also consists of a largely unstructured linker that connects the α-helices (81). However, in yeast ATP synthase, subunit h, weakly homologous to F_6, showed a more linear arrangement along the axis of the peripheral stalk, and the C terminus of the subunit was located close to the membrane (82). In contrast, the structure of the bovine peripheral stalk indicated that the C-terminal region of F_6 was positioned on the upper part of the stalk,

a distance of about 70 Å away from the membrane despite a similar extended structure to that of yeast subunit h. The yeast subunit h has 16 more amino acids, which has been suggested in part as the explanation for its position (79). In *in vitro* reconstitution experiments involving several combinations of recombinant subunits, F_6 formed stable tertiary complexes with either OSCP and b or b and d and hence may have a function in stabilizing these interactions (58). In addition and interestingly, F_6 was identified in the plasma of both rats and humans and suggested to be a novel vasoconstrictor acting on the ectopic ATP synthase on the plasma membrane (83).

Subunit d is composed of five α -helical regions (residues 11-19, 24-44, 52-59, 64-78 and 85-122), which align along the long α-helix of subunit b. Here, they interact with subunit b via one parallel and two antiparallel coiled-coil interactions. Interestingly, the δPKC isozyme, which previously was proposed to play important roles in ischemic injury and cell death, binds to subunit d in cardiac myocytes during prolonged hypoxia. Here it inhibits the activity of ATP synthase implicating subunit d as a modulator of the heart myocyte enzyme.

In a recent x-ray crystal structure of the F_1-ATPase in complex with the peripheral stalk (Figure 3B), a segment before residue 146 of subunit b has moved inward more closely (~9°) to the F_1 domain relative to the structure containing only the b-d-F_6 subcomplex of the peripheral stalk (79,80). This segment's movement has generated two unanticipated contacts between the d and the β_{TP}-subunit and between F_6 and the β_{TP}-subunit. These two unanticipated contacts were considered as artifacts produced during the crystal generation and ignored because they had not detected such interactions in earlier reconstitution experiments (58,80). However, results of other studies had already reported these interactions based on the chemical cross-linking approach. (84,85). Also, the direct interaction between F_6 and the β-subunit *in vitro* has been identified by immunoprecipitation analysis using an F_6 antibody in a

mixture of solubilized human endothelial cells and purified F_6 (86).

Membrane Embedded Subunits in Mitochondrial ATP Synthase

The membrane embedded subunits in mitochondrial ATP synthase include subunits a, b, c, e, f, g and A6L. The c-subunit forms an oligomeric assembly as a c ring with specific copy numbers among species. The c ring in the structure of the bovine F_1-c-ring complex contains eight c-subunits. All vertebrates, and probably all or most invertebrates, have such a copy number based on the highly conserved sequences of c-subunits. The mammalian ATP synthase is a highly efficient enzyme based on its H^+/ATP ratio, i.e., the number of protons translocated for one ATP synthesized by the enzyme. Yeast, *E. coli,* and thermophilic *Bacillus* PS3 have 10 c-subunits in their c rings. The spinach chloroplast and the cyanobacterial *Spirulina platensis* have 14 and 15 copies of c-subunits respectively. The species specific copy number of the c-subunit might be able to be changed in response to the surrounding environment in bacteria (87). The c ring together with a-subunit provides a transmembrane path for protons through the membrane for which detailed structural information is not available. However, it likely has five transmembrane helices. In *E. coli* the b_2 dimer of the stator interacts with subunit a on the membrane and one b_2 dimer seems to be close to the c ring (78). However, in the membrane embedded region of ATP synthase determined by electron cryo-microscopy subunit b is localized away from the c ring and subunit a is between subunit b and the c ring thus maintaining interactions in both directions (88). In addition to the subunits a, b, and c found in the membrane-bound region of the bacterial ATP synthase, mitochondrial ATP synthases contain several small accessory membrane proteins, e, f, g and A6L referred to as "supernumerary" subunits. Each of the subunits has a single transmembrane span (89) and is not involved directly in the activity of ATP synthase. It has been suggested that they may play various roles, e.g., in stabilizing the ATP synthase core structure, regulating the assembly

process, and determining the oligomeric state of the enzyme.

Factor B and IF_1 in Mitochondrial ATP Synthase

Coupling factor B (or "Factor B" as it is also named) can restore oxidative phosphorylation and partial energy-driven reactions in either non-phosphorylating or factor B depleted submitochondrial particles prepared from heavy bovine heart mitochondria by sonication in the presence of ammonia and ethylenediaminetetraacetic acid (EDTA). Factor B is thought to block a proton leak through a second latent (still unidentified) proton channel across the membrane sector of F_O and consequently maintains a proton motive force high enough to facilitate ATP synthesis. It is a highly conserved protein throughout animal species but has not been identified in yeasts or prokaryotes (90). X-ray crystallography studies of Factor B showed an oblong, oval-shaped structure composed of a globular N-terminal domain with the putative anchoring motif in the first ten amino acids and the C-terminal domain containing four leucine-rich repeats, LRR (Figure 4). Cross-linking studies using the recombinant Factor B containing an unnatural photoreactive amino acid analog substituted for the Trp2 position identified its association with subunits e and g of F_O as well as the ADP/ATP carrier. Based on a study with a deletion mutant of Factor B involving residues 2-4 at the N-termini, it was suggested that the proximal N-terminal amino acids are important for its function and protein-protein interactions (91).

The inhibitory factor 1 (or simply IF_1) is the most well-known regulatory protein of ATP synthase. As the F_OF_1-ATP synthase has a reversible activity, it can hydrolyze ATP coupled to the proton pumping from the matrix into the intermembrane space to restore the mitochondrial membrane potential in the absence of a respiratory driven proton gradient, e.g., under ischemic conditions. However, this leads to the rapid depletion of cellular ATP, and it is here where IF_1 plays an important role in order to prevent the futile hydrolysis of ATP.

Bovine IF_1 is a heat-stable, basic protein with 84 amino acids and is highly conserved throughout the animal species. Yeasts also have a homologous IF_1 protein, of which the activity can be stabilized by the accessory proteins STF1, STF2 and STF3 specifically identified in yeast (92).

The structure of the mature IF_1 protein is α-helical along most of its length. Assays for inhibition of ATP synthase on IF_1-depleted submitochondrial particles with several truncated forms of bovine IF_1 identified the minimal inhibitory sequence as consisting of residues 14-47 (93). In addition, the dimerization of IF_1 depends on its C-terminal region consisting of residues 37-84, while residues 32-44 in the N-terminal region of IF_1 are required for its oligomerization. At low pH (<6.7), IF_1 exists as a homo-dimer by complimentary hydrophobic interactions of the C-terminal region of each monomer forming an antiparallel double-stranded coiled-coil. Under this condition the two minimal inhibitory sequences are at opposite ends and can inhibit two ATP synthases simultaneously. Conversely, when the pH rises above neutrality, IF_1 homo-dimers can form tetramers or higher oligomers by forming antiparallel coiled-coils in their N-terminal regions. This hides the inhibitory sequences of the dimers. Consequently, IF_1 now cannot inhibit the ATPase activity of ATP synthase. In bovine IF_1, this pH-dependent mechanism has been shown to be mediated by a histidine-rich region (residues 48-70) (36).

The molecular crystal structure of bovine F_1-ATPase with a monomeric fragment of IF_1 consisting of residues 1-60 which removed most of the dimerization domain was characterized at 2.1-Å resolution (94,95) (Figure 5). In this structure residues 1-46 of the monomeric IF_1 lie within F_1-ATPase. The longer helix (residues 21-46) occupies a deep groove at the interface between the β_{DP} and α_{DP}-subunits and extends from the external surface of F_1 to the central cavity surrounding the γ-subunit. It binds four subunits of F_1-ATPase, the C-

terminal end of the β_{DP}, α_{DP}, and β_{TP} subunits and the N-terminal helix of the γ-subunit. Residues 14-18 form a short helix that turns suddenly at almost 90° by the short linker (residues 19-20) to make additional contacts with the N-terminal helix of the γ-subunit. The unstructured region (residues 8-13) crosses the central cavity and interacts with the nucleotide binding domain of the α_E-subunit. It is reasonably proposed that the initial binding of IF_1 takes place at the interface of the α_E and β_E-subunits forming an inhibited complex between bovine F_1-ATPase and the monomeric IF_1. This complex seems to be formed from the conversion of this initial interface to the β_{DP} and α_{DP} interface after two 120° rotations during ATP hydrolysis. Consequently, in the fully inhibited structure, the extensive interactions between IF_1 and subunits of F_1-ATPase stabilize the structure enough to block the next 120° rotation of the γ -subunit such that it can no longer consume the ATP for ATP hydrolysis. When the proton motive force is recovered, ATP synthase is reactivated and changes the direction of rotation of the central stalk to that required for ATP synthesis. The reversal of the direction of rotation of the rotor evidently destabilizes the interactions of IF_1 with F_1-ATPase and leads to it release from the inhibited catalytic interface (94,96). The extreme N-terminal region of IF_1 is not involved in its initial binding to F_1-ATPase but contributes to the stability of the inhibited complex as determined by a kinetic approach with yeast mutants. Instead, an interaction of the middle part of yeast IF_1, in the vicinity of Leu40 (the homologue of bovine IF_1- Leu45) with the extreme C-terminal region of the β-subunit is presumably involved in the initial or recognition step (95,97).

Proteins in Addition to Factor B and IF_1 that Interact with Mitochondrial ATP Synthase

It was widely believed that the activity of ATP synthesis *in vivo* was governed passively by either the concentration of substrates for the ATP synthase such as ADP, P_i or the

electrochemical potential of H^+. However, the relatively constant levels of these parameters during periods of ATP demand could not completely account for the regulation of the enzyme implicating additional regulatory mechanisms, e.g., protein-protein interactions with regulatory proteins or post-translational modifications of the enzyme. In this regard, and in order to identify a binding partner or partners to ATP synthase, it seemed important to better understand its regulation.

Usually the subunit composition and nomenclature that have been ascribed to F_OF_1-ATP synthase have been based on the composition of the complex when isolated using standard chromatography. Therefore, it is not surprising that additional proteins have been identified since the earlier studies. Thus, in bovine heart mitochondria, two additional proteins MLQ, a 6.8-kDa proteolipid with the N-terminal sequence Met-Leu-Gln containing a single membrane domain, and AGP (or DAPIT, a diabetes-associated protein in insulin-sensitive tissue) were identified (98,99). It was first suggested that the two proteins affect the dimeric or oligomeric state of ATP synthase. Recently, however, it appears that the MLQ and AGP also play an important role in the assembly and stabilization of ATP synthase. This is because the expression levels of MLQ and AGP also show a significant correlation with the ATP synthase population in mitochondria. In addition, also isolated from mitochondria is "CaBI", a calcium-binding ATPase inhibitor protein, highly expressed in skeletal and cardiac muscle tissues and released from the enzyme in the presence of elevated Ca^{2+}. This protein may regulate the activity of ATP synthase in response to a need for ATP in these muscle tissues but the molecular structure is still unknown (100,101). In yeast there are several additional ATP synthase subunits such as i/j and k (products of yeast genes ATP18, ATP19, respectively). They are involved in the stepwise assembly of yeast ATP synthase dimers and oligomers (102). Finally, there are the proteins STF1 and STF2 (stabilizing factors 1 and 2) that associate with

yeast IF_1 (103).

In addition to the above proteins that appear to have a direct relationship with ATP synthase, there are several other proteins identified as binding partners with already well-known functions. For example, the αβ-subunits of F_1 were reported to interact with the 14-3-3 protein, which may induce a multitude of functionally diverse signaling proteins to regulate the activity of the enzyme. In addition, the non-catalytic-subunits, e.g., OSCP, d, b, and F_6 of mitochondrial ATP synthase have been found to provide binding sites for other proteins such as δPKC and CYPD (Cyclophilin D) (104). This accumulating evidence involving "ATP synthase interacting proteins" expands current knowledge not only about the modulation of ATP synthase *per se* but to this enzymes' "family' of subunits participating in other cellular functions, i.e., playing "moonlighting" roles.

In addition, numerous studies have provided strong evidence that ATP synthase can form a super protein complex. ATP synthase can be successfully isolated as a functional monomer from various species but an oligomeric organization developing from a homo-dimer in yeast, mammalian and plant mitochondria was identified by either size exclusion chromatography or by blue-native polyacrylamide gel electrophoresis (BN-PAGE) (105). The homo-dimer was suggested to be involved in shaping the cristae of mitochondria depending on an angular association of the dimer with two ATP synthases. Involvement of several subunits including e, g, k, i, IF_1, Factor B, the peripheral stalk, MLQ, and AGP was suggested as having a role in generation of ATP synthase dimers. However, this suggestion promoted considerable debate due to the different results obtained among species.

ATP synthase may form super-complexes with components of the electron transport chain, especially cytochrome c oxidase (complex IV). This so-called respirasome was also identified in detergent-resistant lipid rafts from several mammalian tissues. Another example

is the ATP synthasome consisting of an ATP synthase monomer in complex formation with the adenine nucleotide carrier (ANT) and the phosphate carrier (PIC) that was suggested to have a beneficial effect in supplying the ATP synthase with its substrates ADP and P_i in order to make ATP (106).

Ectopic ATP Synthase on the Plasma Membrane

It was generally believed that because the ATP synthase generates ATP from ADP and P_i using an electrochemical proton gradient across the inner mitochondrial membrane, it was localized exclusively on the inner membrane of mitochondria. However, a number of groups have recently identified the enzyme at the plasma membrane of a wide range of cell types and suggested novel physiological roles. At this location the enzyme is frequently referred to as "Ectopic ATP synthase" where ectopic signifies an "abnormal place".

On the plasma membrane the catalytic F_1 sector of the ectopic ATP synthase protrudes into the extracellular space contrary to that of bacterial ATP synthase and was found to serve as a receptor for multiple ligands. Also, the ectopic ATP synthase showed its own enzymatic activity of ATP synthesis and ATP hydrolysis as that on the inner membrane of mitochondria. ATP and/or ADP generated by the enzyme in the extracellular space may participate in several cellular responses through purinergic signaling for extracellular nucleotides. P2X receptors are ATP-gated ion channels that mediate rapid, non-selective passage of cations (Na^+, K^+, Ca^{2+}) leading to depolarization of the cell membrane. Membrane depolarization subsequently activates voltage-gated calcium channels, thus resulting in an increase in intracellular Ca^{2+}, which is responsible for activating numerous signaling molecules. This is especially the case in impulse propagation in the nervous system and in muscle contractility. P2Y receptors are G-protein-coupled receptors (GPCRs) that are activated by ATP or ADP. They exert various biological functions based on their G-protein coupling

involved in proliferation, differentiation, apoptosis, metabolism, secretion, and cell migration (107). Moreover, ATP synthase may under certain conditions, i.e., where it operates in the direction of ATP hydrolysis, pump protons via the channel in its F_O component into the extracellular space thus regulating pH. This is especially important for tumor and endothelial cells within a low pH environment (108,109).

Additionally, it should be noted that the ectopic ATP synthase was first discovered in tumor cell lines. In 1994 Das *et al.* identified and reported the presence of the β-subunit of the ATP synthase on the surface of tumor cell lines (110). To find tumor surface proteins that interact with viable human peripheral blood lymphocytes (HPBLs), plasma membrane proteins of the K562 tumor cell line were labeled with biotin and were then reacted with freshly isolated viable HPBL. A major protein with a 51.5kD molecular mass among the biotinylated tumor plasma membrane proteins was further purified, and using NH_2-terminal amino acid sequence analysis unexpectedly identified as the β-subunit of ATP synthase (F_OF_1). From these data they suggested that the ATP synthase expressed on the surface of tumor cells is an important ligand in a natural killer (NK) cell-mediated cytolytic pathway. Later apolipoprotein A-I (apoA-I), the major protein component in high density lipoproteins (HDL) was identified as an enhancer required for optimal activation of a subset of non-conventional T lymphocytes, Vγ9Vδ2 T cells. Both T cell receptor (TCR) of Vγ9Vδ2 T cells and ATP synthase bind to a de-lipidated form of apoA-I and mediate the activation of the T cells (111).

Also, ectopic ATP synthase was identified as the receptor for angiostatin, a naturally occurring inhibitor of angiogenesis (112). ATP synthase was found also to be present on various human tumor cell surfaces and more active at low extracellular pH, a unique characteristic of the tumor microenvironment. Angiostatin inhibited the activity of the ATP synthase and induced tumor cell death (108). This cytotoxicity of angiostatin to tumor cells

was also found to be pH dependent with a higher cytotoxic effect occurring at low pH (~6.5). Therefore, it was suggested that ATP synthase plays a role in maintaining the intracellular pH of tumor cells under acidic conditions through proton pumping to the extracelluar space coupled with ATP synthesis, of which inhibition by angiostatin causes the intracellular acidification and results in tumor cell death. However, it was reported that the intracellular acidification itself inhibited only cell proliferation but not apoptosis. This suggested that simultaneous inhibition of extracellular ATP generation resulting from the inhibition of the cell surface ATP synthase, could also play a critical role in tumor cell death induced by angiostatin (113) In this regard, the cell surface ATP synthase has been considered as an important potential target for cancer therapy (114).

Ectopic ATP synthase has been shown also to be expressed in normal cells or tissues and hence has been suggested to have a novel biological function depending on cell or tissue types. In hepatocytes, ectopic ATP synthase was identified as the receptor for apoA-I and regulator of HDL endocytosis (115). The binding of apoA-I to ectopic ATP-synthase stimulated its hydrolytic activity and induced endocytosis of HDL protein particles dependent on the generation of ADP by the ATP synthase. This was mediated by the purinergic P2Y13 receptor because small interfering RNA to the P2Y13 receptor abolished endocytosis of HDL (116). In endothelial cells, ectopic ATP synthase is the receptor of angiostatin (112), which inhibits endothelial cell migration and proliferation by suppressing ATP production by ectopic ATP synthase. This suggested that ATP synthase on the normal endothelial surface plays an important role in cell proliferation and migration. As that in hepatocytes, the ATP hydrolytic activity of ectopic ATP synthase on the surface of endothelial cells can be activated by interaction with apoA-I, promoting proliferation and inhibiting apoptosis (117). The resulting ADP, the product of ATP hydrolysis could bind and activate the P2Y receptor and the

downstream signaling pathway but the exact mechanism is still unknown. However, this is a good example that ectopic ATP synthase in spite of its interaction with the same ligand can play a different role depending on cell type. In neural cells, amyloid precursor protein (APP) and amyloid beta-peptide (Aβ) bind to ectopic ATP synthase and partially inhibit both its ATP synthetic and hydrolytic activity (118). The accumulation of the ATP synthase α-chain was found in amyloid plaques in an Alzheimer disease animal model. Therefore, it has become of particular interest to understand the malfunctioning of the cell surface ATP synthase in neuronal degenerative diseases (119,120).

The initial finding of ectopic ATP synthase on the cell surface, especially the α and β-subunits of the F_1 sector, was unexpectedly obtained during the characterization of a binding partner for a specific ligand. More recently the combination of both biochemical and proteomic approaches have revealed the existence of several other mitochondrial proteins associated with the plasma membrane. Thus, in addition to the ATP synthase, other complexes involved in oxidative phosphorylation were determined to localize to the plasma membrane in several kinds of cells and tissues (121). Interestingly, these complexes were found to be enriched in lipid rafts, detergent-resistant plasma-membrane microdomains that contain high concentrations of cholesterol and glycosphingolipids. Significantly, lipid rafts are known to serve as signaling centers for biological processes including endocytosis, assembly of signaling molecules, and trafficking of membrane proteins. In this regard, it is should be noted that Caveolins are structural proteins that induce conformational changes in lipid rafts, i.e., from a flat membrane to an invaginated structure, so-called "caveolae" that serve as a marker for lipid rafts. The ATP synthase β-subunit can associate directly with caveolins, confirmed both by co-immunoprecipitation and a GST-caveolin-1 fusion protein pull-down assay (122). This is consistent with the observation of a patchy and punctate staining of ATP synthase α and β-

subunits on the plasma membrane as revealed by confocal microscopy (123). Despite these finding, the mechanism by which the ATP synthase is translocated to the plasma membrane is not totally understood. One possibility involves random fusion events between the mitochondria and the plasma membrane (124).

The α and β-subunits of mitochondrial ATP synthase are not the only subunits of this complex that have been identified on the plasma membrane. For example, in HELA cells, 10 subunits of ATP synthase (α, β, γ, b, d, e, f, g, F_6, and OSCP) were detected in the detergent-resistant fraction using an unbiased quantitative proteomic approach (125). In C2C12 myotubes (126), 11 components of ATP synthase, α, β, δ, γ, b, d, e, g, F_6, IF_1, and OSCP were identified in the isolated lipid rafts. Ectopic localization of subunits d and OSCP of the F_O sector on the plasma membrane of an osteosarcoma cell line was determined by an immunocytochemical study (127). More recently active ectopic F_OF_1-ATP synthases were identified by in-gel ATPase activity staining in extracts of plasma membranes prepared from rat liver and had a similar molecular weight to the monomeric ATP synthase present in the mitochondrial membrane. The ATPase activity of the complex was inhibited by the specific mitochondrial ATP synthesis/ATPase inhibitor oligomycin. Interestingly, the complex contained all F_1 subunits, (α, β, δ, γ and ε) and the F_O subunits a, b, d, OSCP and A6L encoded by the mitochondrial genome) (124).

Coupling factor 6 (CF6 or F_6) is well known to be a component of the peripheral stalk of mitochondrial ATP synthase. However, CF6 was first introduced as a novel inhibitor of the synthesis of prostacyclin, a potent vasodilator and inhibitor of platelet aggregation. CF6 was purified from the heart of spontaneously hypertensive rats and interestingly showed an effect from the outside of the cells (128). A few years later it was confirmed that CF6 is present on the surface of rat and human vascular endothelial cells and released into the plasma by

tumor necrosis factor (TNF)-α and shear stress through activation of the NF-kB signaling pathway (83,129,130). CF6 on the surface of human endothelial cells interacts directly with the β-subunit of ATP synthase, different from its role as part of the peripheral stalk in mitochondrial ATP synthase. CF6 binding to the cell surface ATP synthase stimulates ATP hydrolytic activity and causes intracellular acidification. This is because ATP hydrolysis is coupled to inward proton translocation into the cell (86). Clinically, higher plasma levels of CF6 were reported in human patients with hypertension, acute myocardial infarction, end-stage renal disease, and diabetes (131).

More recently, IF_1 has also been identified in human serum mostly as an active dimeric form. Its level in serum was positively correlated with HDL-cholesterol and negatively with triglycerides. It suggested that the circulating IF_1 may inhibit the hydrolytic activity of ectopic ATP synthase and impair endocytosis of HDL protein particles in hepatocytes (132). Like the F_6 and IF_1, the other soluble subunits may function at the cell surface to regulate the ectopic ATP synthase. However, OSCP and IF_1 are the only known soluble proteins directly interacting with the F_1 subcomplex in mitochondria.

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Table 1

Subunit Nomenclature, Composition and Stoichiometry of F_OF_1-ATP Synthase in Bacteria, Chloroplast and Mitochondria

Subunits are aligned horizontally at each row based on sequence or functional homology. *****; The subscripted number next to the subunits α, β, b and c indicates the stoichiometric number of each subunit within the F_OF_1-ATP synthase.

Sector	Bacteria	Chloroplast	Mitochondria	
	E. coli	**Spinarch**	**Yeast**	**Mammalian**
F_1	α_3*	α_3*	α_3*	α_3*
	β_3*	β_3*	β_3*	β_3*
	γ	γ	γ	γ
	δ	(OSCP)	(OSCP)	(OSCP)
	ε	ε	δ	δ
			ε	ε
F_O	a	a (or IV)	Su6 (or 6)	a
	b_2*	b and b' (or I and II)	B (or 4)	b
	c_{10}*	c_{14}* (or III)	c_{10}* (or Su9)	c_8*
			Su8	$A6L_m$
			OSCP	OSCP
			D	d
			E	e
			F	f
			G	g
			H	F_6
			i (or j)	
			K	
			INH	IF_1
			STF1	
			STF2	
			STF3	
				Factor B

Figure 1

Structural Model of *E. coli* F_OF_1-ATP Synthase

The model was created from available structural data and modeling approaches. The overall architecture of the enzyme comprises two subcomplexes, one being the membrane-embedded F_O complex and the other the water-soluble F_1 complex. The water-soluble F_1 portion is made up of $\alpha_3\beta_3\gamma\delta\varepsilon$. F_O in bacteria is comprised of three subunits in a stoichiometry ab_2c_n (n=10 in *E. coli*). With the proton flow through the proton channel powered by the electrochemical potential of protons across the membrane, the c-ring rotates against the ab_2-subunits in a clockwise direction along with the central rotor, $\gamma\delta\varepsilon$ as viewed from the membrane, and ATP is produced from ADP and P_i in the catalytic hexamer, $\alpha_3\beta_3$. This figure was adapted from Figure 1, Weber (2006), in *Biochimica et Biophysica Acta* (75) with permission (© copyright Elsevier, 2006, All Rights Reserved)

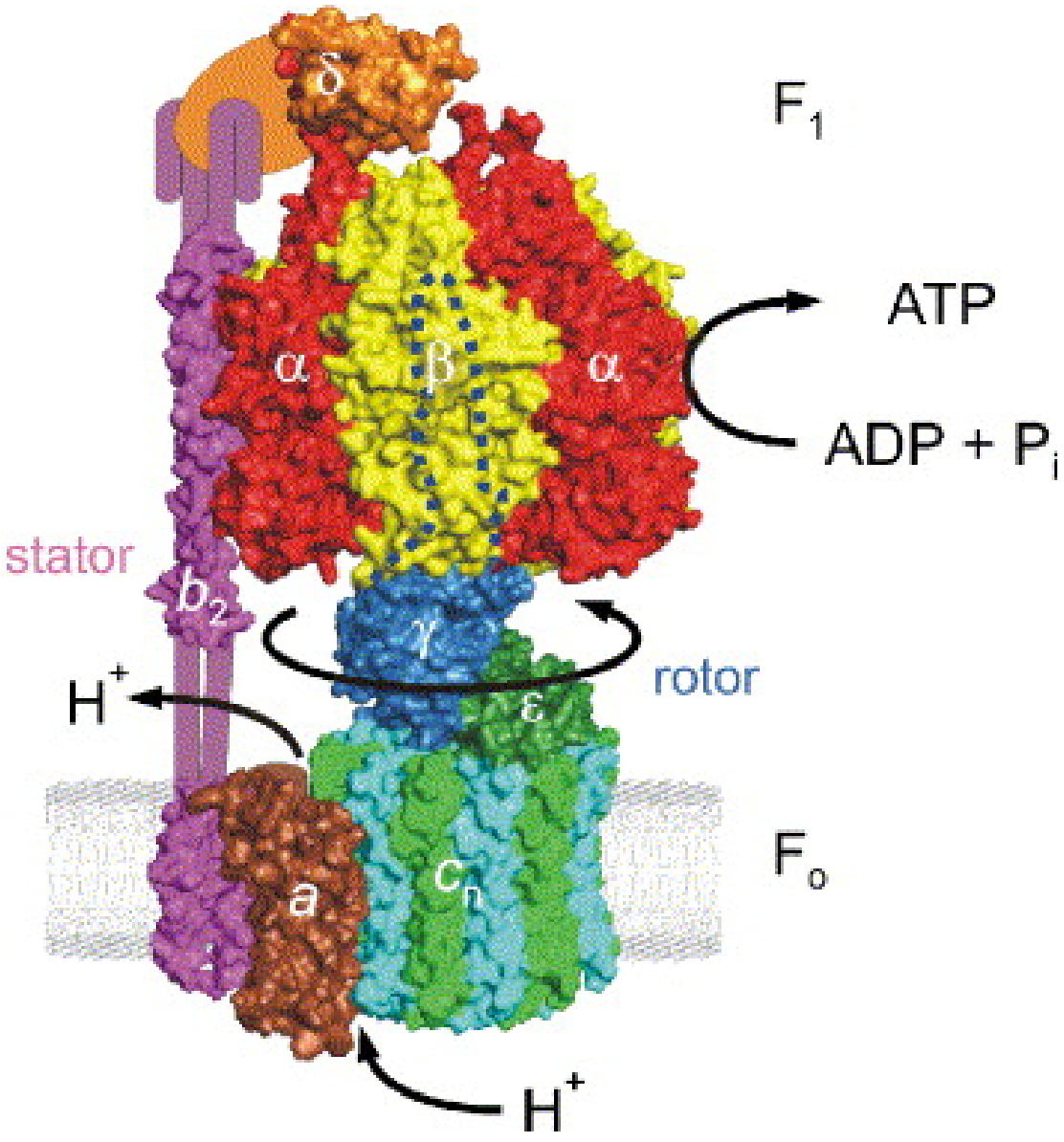

δ
F1
ATP
α
β
α
ADP + Pi
stator
b2
γ
rotor
H+
ε
Fo
a
cn
H+

Figure 2

Mechanochemical Coupling Scheme of F_1-ATPase under ATP Hydrolysis Conditions

The scheme depicts the binding and catalytic events on one catalytic site shown as a grey circle during one rotation of the γ-subunit (red arrow). The other two catalytic sites shown as green circles experience the same events simultaneously with an offset by each of 120°. The blue catalytic site retains the bound ATP until the γ-subunit rotates 200° from the ATP binding angle (0°). At 200°, the catalytic site hydrolyzes ATP into ADP and P_i, each of which is released, the ADP at 240° and the P_i at 320°. This figure was reproduced from Figure 3, Watanabe and Noji (2013), in *FEBS Letters* (133) with permission (© copyright Elsevier, 2013, All Rights Reserved)

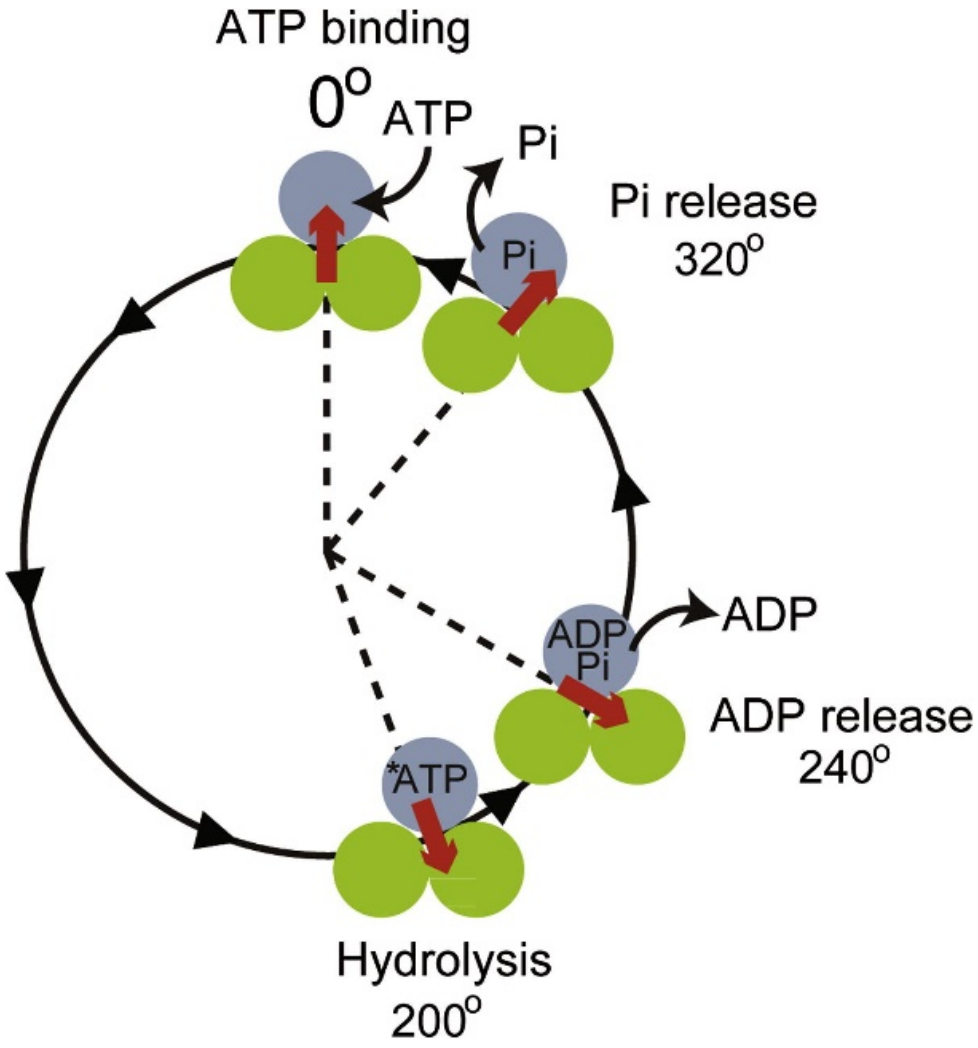

ATP binding
0°
ATP
Pi
Pi release
320°
Pi
ADP
ADP
Pi
ADP release
240°
*ATP
Hydrolysis
200°

Figure 3

Structure of the Peripheral Stalk and F_1-ATPase-Peripheral Stalk Complex from Bovine Heart Mitochondria

(**A**), Subcomplex consisting of subunit d residues 1-124, the entire F_6 (76 residues), and the soluble regions for subunits b (residues 79-184) of bovine ATP synthase. These were overexpressed from a single operon of an expression plasmid in *E.coli* and purified. Crystals were obtained for the complex and subjected to X-ray crystallography. A final model of this structure was generated. The resolved model includes residues 79-183 of subunit b (magenta), residues 3-123 of subunit d (orange) and residues 5-70 of F_6 (green). The structure is available in the Protein Data Bank (PDB code 2CLY). This figure was adapted from Figure 1, Dickson *et al.* (2006), in *The EMBO Journal* (79) with permission (Copyright © 2006 European Molecular Biology Organization, All Rights Reserved) (**B**), Structure of bovine F_1-ATPase-peripheral stalk complex. This was obtained by reconstitution of F_1-ATPase, the OSCP and the above subcomplex of subunits b, d, and F_6. The final model of the complex contains the complete F_1 domain, 3α (red), 3β (yellow), γ (blue), δ (magenta), and ε (green) and the peripheral stalk, residues 1-146 and 169-189 of the OSCP (teal); residues 122-207 of subunit b (pink); residues 5-25 and 35-57 of F_6 (pea green); and residues 30-40, 65-74, and 85-91 of subunit d (orange). The structure is available in the Protein Data Bank (PDB ID code 2WSS). This figure was adapted from Figure 1, Rees *et al.* (2006), in *Proceedings of the National Academy of Sciences* (80) with permission.

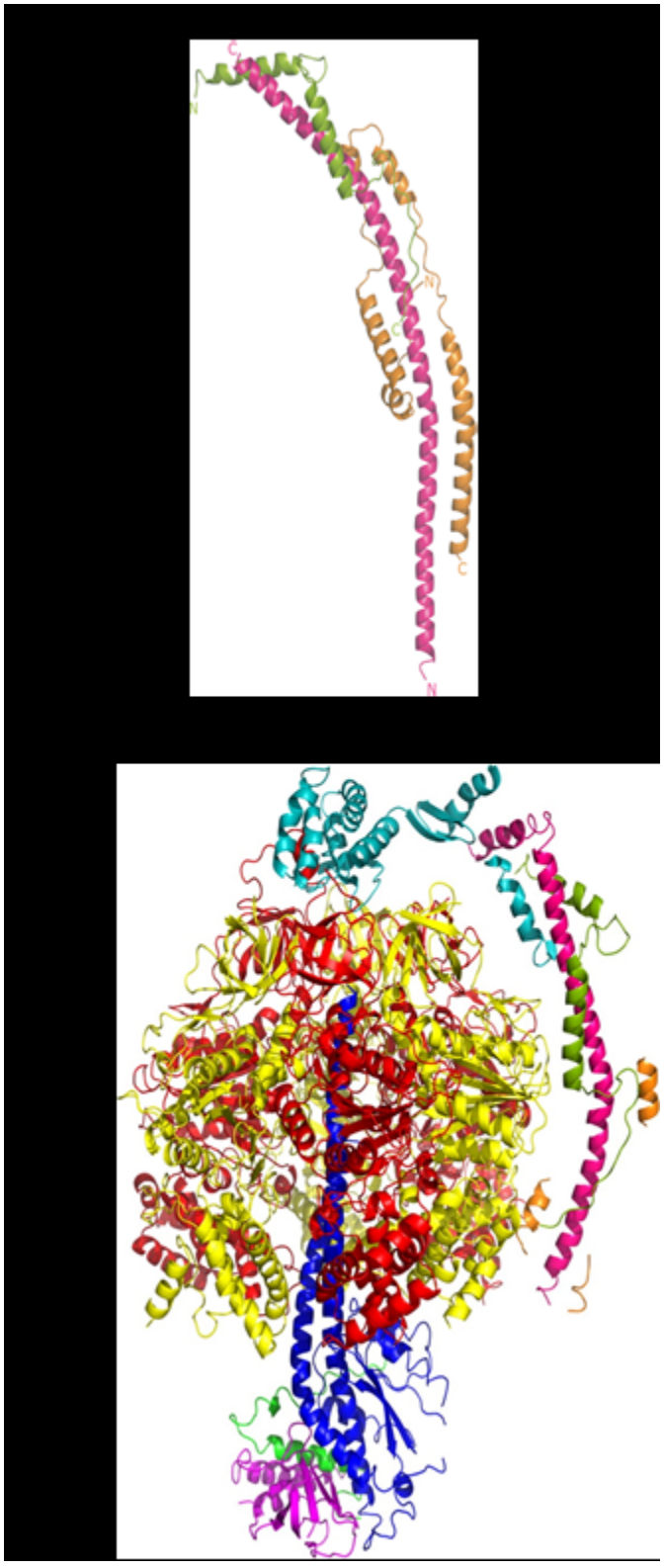
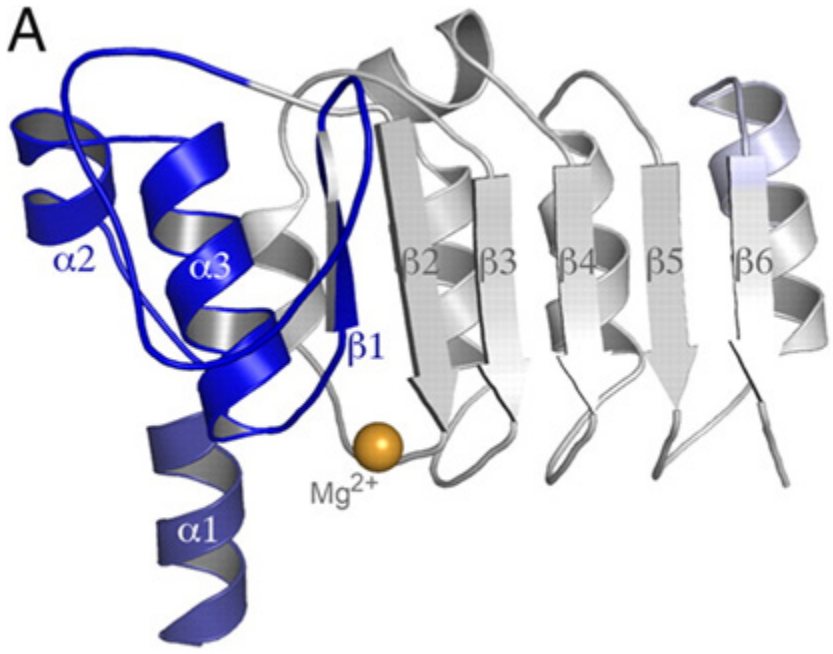


Figure 4

Structure of Bovine Mitochondrial Factor B

(**A**), Overall structure of bovine mitochondrial Factor B containing a substitution of residue 3, glycine to glutamate. Oval-shaped molecule folds into two domains, the N-terminal domain (residues 1-61, blue) and the C-terminal LRR (four leucine-rich repeats)-containing domain (residues 62-175, gray). The N-terminal domain is composed of three helices (α1-3) and one β-strand. The first N-terminal α-helix (residues 1-10) containing four aromatic residues and four hydrophobic residues is suggested to anchor factor B to the matrix side of the mitochondrial inner membrane. The C-terminal LRR domain contains four LRRs, which was previously suggested to mediate protein-protein interactions. There is a metal ion binding site, the Mg^{2+} at the lower end of the β1-β2 strand pair. (**B**), View from the matrix side to the membrane. The structure is available in the Protein Data Bank (PDB ID code 3E2J, and 3E4G). This figure was adapted from Figure 1, Lee *et al.* (2008), in *Proceedings of the National Academy of Sciences* (134) with permission. Copyright (2008) National Academy of Sciences, U.S.A.


A
α2
α3
β1
β2
β3
β4
β5
β6
Mg2+
α1

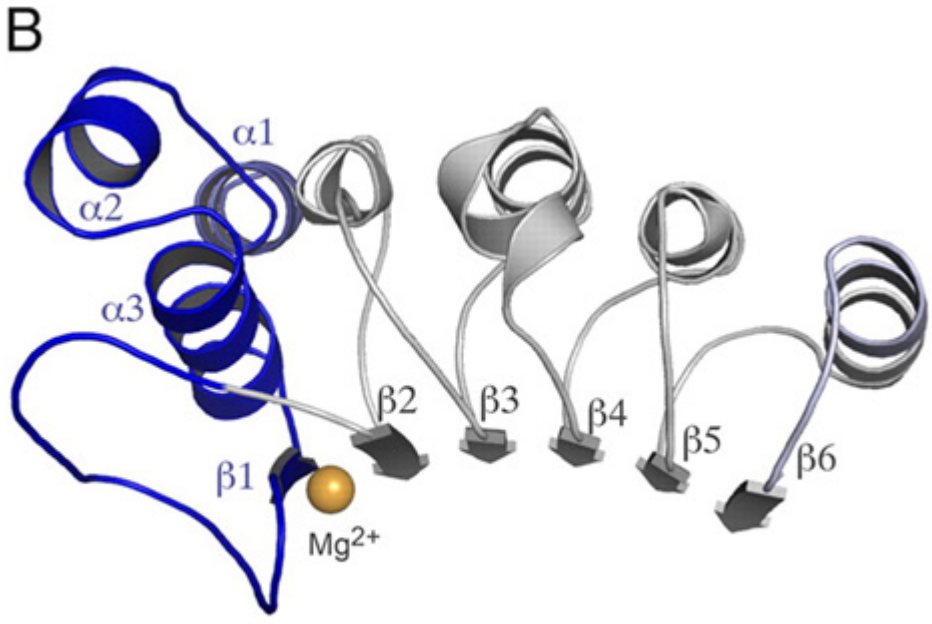
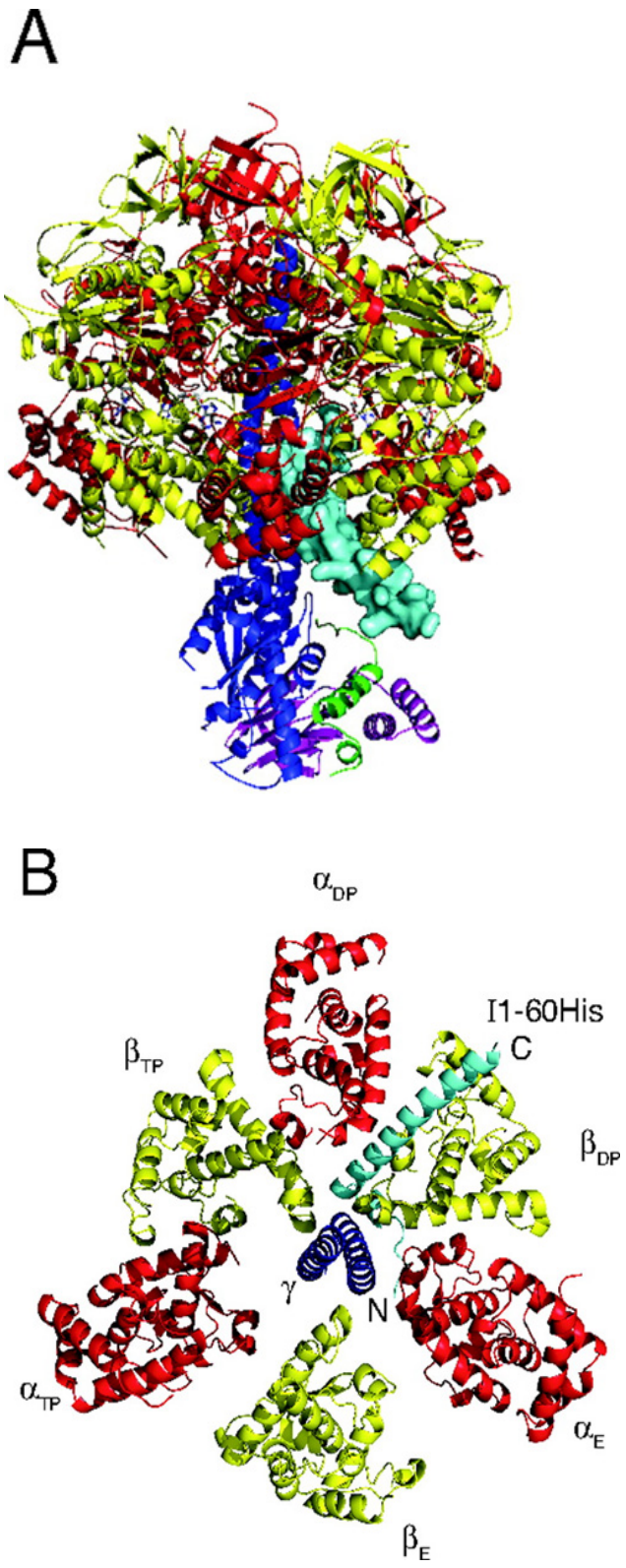

B
α1
α2
α3
β2
β3
β4
β5
β6
β1
Mg2+

Figure 5

Structure of the Bovine F_1-ATPase Inhibited by Monomeric IF_1 (I1-60His)

(**A**), Side view. The overall structure of the F_1-ATPase-IF_1complex contains the 3α (red), 3β (yellow), 1 γ (dark blue), 1δ (magenta), and 1ε (green) subunits shown in ribbon form and residues 8-50 of I1-60His (light blue) shown in solid form. (**B**), Bottom view. Residues 8-46 of I1-60His lie within the F_1 domain showing interactions of the inhibitor protein with the subunits of F_1-ATPase. The structure is available in the Protein Data Bank ((PDB ID code 2V7Q). This figure was adapted from Figure 1, Gledhill *et al.* (2007), in *Proceedings of the National Academy of Sciences* (94) with permission. Copyright (2007) National Academy of sciences, U.S.A.


A
B
α_{DP}
I1-60His
C
β_{TP}
β_{DP}
γ
N
α_{TP}
α_{E}
β_{E}

Chapter 2

Expression, Purification and Characterization of Recombinant Supernumerary Subunits and Interactions with Purified Rat F_1-ATPase

Summary

ATP synthase (F_OF_1) in mammals is an important protein complex that catalyzes the final step in the mitochondrial process referred to as "oxidative phosphorylation" that in the presence of Mg^{2+} is responsible for making ATP from ADP and Pi. Although the ATP synthase has been known for many years to reside on the inner membrane of the mitochondria facing the matrix, a number of more recent studies have demonstrated that this complex exists also on the plasma membrane of several different mammalian tissues. This led to the suggestion that the ATP synthase on the cell surface may be involved in functions other than ATP synthesis *per se*. In addition, some of the known subunits of the mitochondrial ATP synthase e.g., the supernumerary subunit F_6 and the ATPase inhibitor protein IF_1 were found to play a role as circulating ligands in human serum that interact with the cell surface ("ectopic") ATP synthase (F_OF_1) regulating its activity. For these reasons, we investigated whether, in addition to F_6 and IF_1, other soluble subunits of mitochondrial ATP synthase, e.g., d, OSCP, and Factor B can also interact independently with ATP synthase and influence its catalytic activity.

We purified each of the human and rat ATP synthases' soluble supernumerary subunits following their overexpression in *E. coli* as MBP fusion proteins. Then, using a MBP pull-down assay, we showed that the subunits d, F_6, Factor B and OSCP can individually interact with the catalytic F_1 subcomplex in a phosphate saline (PBS) buffer condition. These interactions were not noticeably affected by either NaCl or the addition of ATP, ADP and Mg^{2+}. In addition, as shown by others such interactions seem to be common among mammalian species and also allowed within mitochondria.

Based on this work it is concluded that each known supernumerary subunit of the ATP synthase can independently bind to the catalytic F_1 moiety and modulate its activity, and

that this occurs whether or not the F_1 moiety is associated with its F_O component residing within the mitochondrial inner membrane or within the cell membrane.

Introduction

The mammalian mitochondrial ATP synthase (F_OF_1) is the enzyme complex required for the production of most cellular ATP. It is located on the mitochondrial inner membrane, frequently on invaginations of this membrane called "cristae" that face the soluble space known as the "matrix". Here, the ATP synthase uses the potential energy stored in the electrochemical gradient of protons (H^+s) generated by the electron transport chain to synthesize useful biological energy, ATP, from ADP and inorganic phosphate (P_i). The ATP synthase is also located in association with the plasma membrane of bacteria such as *E. coli* and the thylakoid membrane of plant chloroplast. The overall structure and the basic catalytic mechanism of the ATP synthase are evolutionarily conserved in all organisms.

It is generally accepted that mitochondrial ATP synthase has two functional protein subcomplexes, known as F_1 and F_O, with 15 different subunit types, some in multiple copies. The water-soluble F_1 subcomplex contains the catalytic site involved in ATP synthesis or its hydrolysis. It is comprised of five different subunit types in the stoichiometry of $\alpha_3\beta_3\gamma\delta\varepsilon$. The membrane embedded F_O subcomplex functions as a proton channel and contains 10 different subunit types that include a, A6L, b, c_8, d, e, F_6, f, g and OSCP. The two subcomplexes, F_1 and F_O, are physically connected together by a central stalk and a peripheral stalk. The central stalk is composed of the F_1 subunits γ, δ, and ε that interact with a ring (c_8 ring) composed of 8 copies of subunit c. The c_8 ring is buried in the inner membrane of mitochondria and induces an asymmetric conformation of the catalytic hexamer $\alpha_3\beta_3$ through mainly the γ-subunit during catalysis. The peripheral stalk consists of single copies of the subunits b, d, F_6 (coupling factor 6) and OSCP (oligomycin sensitivity conferral protein). It extends from subunit a of the F_O domain within the inner membrane of mitochondria to the top of the catalytic F_1 domain and serves as a stator to prevent the co-rotation of the catalytic hexamer with the central stalk,

thus making the catalytic hexamer responsive to the rotating γ-subunit during ATP synthesis.

Despite the similarity of the overall structure and the basic catalytic mechanism for ATP synthases among species, the mitochondrial ATP synthase contains some regulatory or supernumerary subunits, most of which are within the F_O subcomplex. The only mitochondrial specific-subunit in F_1 is the ε-subunit, which has no counterpart in ATP synthases isolated from either bacteria or chloroplasts (1). Among the subunit components of the mitochondrial F_O subcomplex, the subunits d and F_6 which are found in the peripheral stalk and e, f, g and A6L which are found as the integral membrane proteins of the F_O complex are the mitochondrial specific-subunits. In addition, the inhibitory Factor 1 (IF_1) (2) and Factor B (3,4) are occasionally regarded as additional subunits of the mitochondrial ATP synthase and also have no homologous counterpart in bacteria and chloroplasts. The existences of these supernumerary subunits in the ATP synthase of mitochondria that are absent from the ATP synthases of *E. coli* and chloroplasts raises a very interesting question. That is, are the supernumerary subunits specific to the mitochondria involved in functions specific to the mitochondria, i.e., functions that are not performed by either bacteria or chloroplasts?

The striking structural difference of the mitochondrial ATP synthase from that of bacteria (*E. coli*) and chloroplasts is found in the peripheral stalk. In bacteria and chloroplasts, the peripheral stalk (or "stator stalk") of the ATP synthase consists of only three proteins, i.e., the δ-subunit and either a b_2 dimer formed with two identical b-subunits expressed from a single gene in some eubacteria or bb' dimer (or $b_I b_{II}$ dimer) formed with two different proteins of the homologous genes in photosynthetic bacteria and chloroplasts respectively. The functional unit, the b_2 dimer serves as the primary external scaffold in the peripheral stalk and connects F_1 and F_O through protein interactions with the a-subunit of F_O on the membrane and with the δ-subunit at the top of the catalytic domain. On the other hand, in the peripheral

stalk of the mitochondrial ATP synthase the single b-subunit forms a continuous, slightly curved α-helix extension from the membrane with two transmembrane helices to the top of F_1 as the primary stem (5,6). OSCP is a connector between the b-subunit and the top of the catalytic F_1 domain. Additional supernumerary subunits, i.e., subunits d and F_6 surround and probably strengthen the upper part of subunit b according to a recent structural study of the bovine complex. Therefore, whether one b-subunit of the mitochondrial peripheral stalk could fulfill the function of two b-subunits of the bacterial peripheral stalk has been an interesting question. A study about the interaction of each b-subunit in *E. coli* suggested that only one b-subunit is involved in helping connect F_1 and F_O. The same may be the case for the single b-subunit in mitochondrial ATP synthase. In *E. coli* the other b-subunit may serve the role of subunits F_6 and d in mitochondria, i.e., to stabilize the F_1 catalytic unit. (7).

In reconstitution experiments *In vitro* using several combinations of recombinant subunits known to be components of the bovine peripheral stalk, OSCP was the only protein found to bind directly to isolated bovine F_1-ATPase. However, in the recent x-ray crystal structure of the complex formed with bovine F_1-ATPase and the peripheral stalk, the segment before residue 146 of subunit b has moved inward 9° closer to the F_1 domain relative to that found for the structure containing only the b-d-F_6 subcomplex of the peripheral stalk (8). Two unanticipated contacts were generated between the d and β_{TP}-subunit and between the F_6 subunit and the β_{TP}-subunit. They considered these findings as artifacts produced during the crystal generation and ignored them because they had not detected such interactions in earlier reconstitution experiments (6,9). However, some studies had reported these interactions already via a chemical cross-linking approach (10,11). Also, the direct interaction between F_6 and the β-subunit *in vitro* had been identified by immuno-precipitation analysis using an antibody to F_6 in a mixture containing solubilized human endothelial cells and the purified F_6

protein (12).

Factor B is thought to block a proton leak through a second latent (still unidentified) proton channel across the membrane sector of F_O and consequently maintains the proton motive force high to stimulate ATP synthesis (13). Using a cross-linking assay, Factor B was found to be associated with subunits e and g of the F_O subcomplex as well as with the ADP/ATP carrier. However, the investigators could not detect co-sedimentation of factor B with the F_OF_1-ATP synthase in an assay using sucrose density gradient centrifugation (14). The bovine IF_1 is a heat-stable, basic protein with 84 amino acids and the most well-known regulatory protein of ATP synthase. IF_1 plays an important role in preventing the futile hydrolysis of ATP in the absence of the respiratory generated proton gradient such as ischemic conditions. In the structure of bovine F_1-ATPase inhibited by a monomeric fragment of IF_1, the N-terminal region (residues 1-46) of IF_1 with a long α helical structure was inserted into a deep groove at the interface between the β_{DP} and α_{DP} subunits (15).

These soluble supernumerary subunits of ATP synthase may also function at the plasma membrane along with the enzyme. For many years, it was generally believed that because the ATP synthase of animals and humans generates ATP from ADP and Pi using an electrochemical proton gradient across the mitochondrial inner membrane that this enzyme complex is localized exclusively on this membrane. However, recently a number of groups have identified the ATP synthase, later named the ectopic ATP synthase, on the plasma membrane of a wide range of cell types. Novel physiological roles have been suggested for this ectopic ATP synthase.

On the plasma membrane the catalytic F_1 sector of the ectopic ATP synthase is protruding into the extracellular space, in contrast to the inward orientation of the bacterial ATP synthase. In addition, the ectopic ATP synthase was shown to serve as a receptor for

various extracellular ligands. As the ATP synthase on the inner mitochondrial membrane, the ectopic ATP synthase was shown to catalyze also ATP synthesis and ATP hydrolysis. ATP and ADP generated by the enzyme in the extracellular space may participate in several cellular responses through a purinergic signaling mechanism involving extracellular nucleotides. Also, the ectopic ATP synthase may play an important role in maintaining intracellular pH homeostasis as its ATP hydrolytic activity on the cell surface would be coupled to proton translocation into the cell (16,17).

The initial finding of an ectopic ATP synthase, especially the α and β-subunits of the F_1 sector, on the cell surface was unexpectedly made during the characterization of a binding partner for a specific ligand. Since that discovery, not only have the α and β-subunits of the ATP synthase been found on the plasma membrane but also its other components. For example, more recently active ectopic ATP synthases (F_OF_1) were identified by in-gel ATPase activity staining in extracts of plasma membranes prepared from rat liver and showed a similar molecular weight to the monomeric ATP synthase present in the mitochondrial inner membrane. Also, the ATPase activity of the plasma membrane ATP synthase complex was inhibited by the specific mitochondrial ATP synthase inhibitor oligomycin. Interestingly, it is indicated that extracts from rat liver plasma membranes contain all F_1 subunits (α, β, δ, γ and ε) and F_O subunits (subunits a, b, d, OSCP, and A6L). The identification of subunits a and A6L encoded by the mitochondrial genome, and the others by the nuclear genome, suggested that the active ATP synthase found on the plasma membrane had been previously assembled in the mitochondria. Then, by some still unknown mechanism, this mitochondrial ATP synthase was transported into the plasma membrane to become the ectopic ATP synthase (18).

Coupling factor 6 (CF6 or F_6) is a known component of the peripheral stalk of mitochondrial ATP synthase. However, the novel function of F_6 was first introduced as the

inhibitor of the synthesis of prostacyclin which is a potent vasodilator and an inhibitor of platelet aggregation. CF6 is present on the surface of rat and human vascular endothelial cells and is released into the plasma by tumor necrosis factor (TNF)-α and shear stress through activation of the NF-kB signaling pathway (19-21). F_6 on the surface of human endothelial cells interacts directly with the β-subunit of ATP synthase, different from the case in mitochondrial ATP synthase. F_6 binding to the cell surface ATP synthase stimulates ATP hydrolytic activity and causes intracellular acidification because ATP hydrolysis is coupled to inward proton translocation, i.e., into the cell (12). Clinically, higher plasma levels of CF6 have been reported in human patients with hypertension, acute myocardial infarction, end-stage renal disease, and diabetes (22). More recently, IF_1 has been identified also in human serum, mostly as an active dimeric form. Its level in serum was positively correlated with HDL (High-density lipoprotein)-cholesterol and negatively with triglycerides. It suggested that the circulating IF_1 may inhibit the hydrolytic activity of ectopic ATP synthase and impair endocytosis of HDL protein particles in hepatocytes (23).

When all of the above noted findings, suggestions, and/or thoughts are considered together, it is tempting to speculate that several soluble subunits of the plasma membrane located ATP synthase may under certain conditions be released and play "moonlighting roles" as regulators of cellular activity. Specifically, the soluble subunits OSCP, subunit d, F_6, Factor B, and IF_1 could be considered as candidates for this kind of action. For this reason, this chapter focused on determining to what extent the supernumerary subunits OSCP, d, and F_6 as well as Factor B and IF_1 interact directly with F_1-ATPase.

Materials and Methods

Cloning of Subunits: Subunit d, F_6, Factor B, IF_1 and OSCP from Human and Rat Origin

All primers were specifically designed to generate MBP-fusion proteins containing only the mature sequence as found in the mitochondria. The templates used originated from human and rat genes. Each forward primer started with the first nucleotide sequence of each mature subunit and the reverse primer contained a stop codon and a following EcoRI sequence to be inserted into the expression vector. Table 1 shows the nucleotide sequences of each primer set and its source of templates used to amplify each subunit.

Conventional PCR (Polymerase chain reaction) was performed to generate a DNA fragment for each desired subunit using a specific EST clone purchased from Open Biosystems. For the OSCP subunit (rat) a pET-15b-Rat-OSCP clone (24) had already been prepared in our laboratory as the template. Also, a pMAL-c2-Rat-IF_1 clone for a Rat-IF_1 MBP fusion protein previously prepared in our lab (25) was available. PCRs were performed with Pfu polymerase to generate blunt ended PCR products. Each PCR product was treated with EcoRI to generate the restriction site and T4 Polynucleotide Kinase to add 5´-phosphates at each end for ligation. Each was then inserted using T4 DNA ligase into the XmnI and EcoRI site of pMAL-c2X or the SnaBI and EcoRI sites of pMAL-c2G which had been treated already with calf intestinal alkaline phosphatase (New England Labs, NEB) to remove the 5' phosphate and to block self-ligation. Because both human and rat OSCP have an endogenous Factor Xa cleavage site (^{14}IEGR$^{17}\downarrow$ (26) in both the mature human and rat OSCP protein sequences), these PCR products were incorporated into the pMAL-c2G vector. *E. coli* DH5α cells were transformed with each ligation mixture and then plated on LB agar containing ampicillin. To determine the presence of the correct insert, some ampicillin-resistant colonies

were selected and each purified DNA plasmid was analyzed with several combinations of restriction enzyme digestion. Finally, the correct sequences of all DNA constructs were confirmed by DNA sequencing. *E. coli* BL21 (DE3) was used for overexpression and subsequent purification and pull down assay.

Protein Purification of MBP Fusion Proteins

MBP fusion proteins were purified following the manufacturer's instruction using amylose resins (NEB). *E. coli* strain BL21 (DE3) cells containing each DNA construct were incubated to an A_{600} of ~0.5 at 30 ℃ with vigorous shaking in LB medium containing 0.2% glucose and 100 μg/ml ampicillin. Isopropyl-β-d-thiogalactopyranoside (IPTG) was added to a final concentration of 0.1-0.5 mM to induce protein overexpression. The cells were then grown for more than 4 hours. The cells were collected by centrifugation and resuspended in ice-cold column buffer (20 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA, pH 7.4) supplemented with 1 mM phenylmethyl-sulfonyl fluoride (PMSF). After overnight incubation at -80℃, the cells were thawed in cold water and broken with sonication on ice using a Branson Sonifier® cell disrupter at a power setting of 35 for a total of 5 min with 50% pulses. The supernatant was obtained by centrifugation at 10,000 x *g* for 30 min at 4 °C and diluted 5 times with column buffer. The diluted crude extract was loaded onto a column containing amylose resin that had been already equilibrated with 10 column volumes of the column buffer. After enough washing with the column buffer the MBP fusion proteins were eluted with the column buffer containing 10 mM maltose and pooled.

Cleavage of the Fusion Protein and Purification of the Subunits

Each purified MBP fusion protein was treated with Genenase I for OSCP or Factor Xa for the others to separate the full-length mature subunit from the MBP tag. To purify the protein of interest, the fusion protein cleavage mixture was dialyzed with 20 mM Tris-HCl, 25

mM NaCl, pH 8.0 overnight at 4 °C with several buffer changes and applied to a diethylaminoethanol (DEAE) Sepharose CL-6B column equilibrated with the same buffer.

A profile of fractionated proteins was obtained using DEAE-Sepharose ion exchange chromatography that included a series of steps with increasing concentrations of NaCl. The gradient started in with 25 mM NaCl and was increased to 500 mM NaCl (a 25 mM increase at each step). Gradient solutions were buffered with 20 mM Tris-HCl, pH 8.0. Fractions containing each subunit of interest were pooled. Most of the OSCP precipitated after cleavage with Genenase I. The precipitates were collected by centrifugation and then denatured with 6 M guanidine hydrochloride. Stepwise dialysis was performed at 4°C starting with a DEAE buffer containing 1 M guanidine hydrochloride, 0.5 M arginine and 1 M DTT. Then, the concentration of these additives was gradually reduced to 0. The refolded fraction was collected by centrifugation and loaded onto carboxymethyl (CM) Sepharose CL-6B. The fractions containing OSCP were pooled and reapplied to the amylose resin to remove the MBP protein.

Preparation of Mitochondria

Mitochondria from rabbit skeletal muscle (27) and rat liver (28,29) were prepared according to published methods with some modifications. All procedures were at 0-4 °C unless stated otherwise.

The skeletal muscle was excised from the hind legs of rabbits and washed in 150 mM KCl, 25 mM Tris-HCl (pH 7.4). The fat and connective tissues were removed, and the muscle was minced into small pieces with scissors and fine razor blades. The mince (~50 g) was transferred to a pre-chilled 1-L commercial Waring blender in 900 ml of homogenization buffer (5 mM $MgSO_4$, 1 mM EDTA, 50 mM Tris-HCl, 100 mM KCl, pH 7.4) and homogenized at maximum speed three times for 5 s with 30 s intervals. Homogenates were

centrifuged for 10 min at 800 × *g* in a GSA rotor. The pellets were re-suspended, homogenized, and re-centrifuged again as described above. Both supernatants were collected, filtered through eight layers of cheesecloth and then centrifuged at 10,000 x *g* for 10 min. The pellets were washed with 50 ml of homogenization buffer and re-centrifuged at 9,000 x *g* for 10 min two times. The final mitochondrial pellets were re-suspended in 0.25 M sucrose to give about 20 mg of protein/ml and stored at -80°C.

Livers were quickly removed from animals and minced with scissors. A 25% (w/v) suspension of liver mince was prepared in ice-cold H-medium (70 mM sucrose, 210 mM mannitol, 2 mM HEPES, 0.05% BSA (pH 7.5)) and homogenized in a glass Thomas homogenizer by applying four up and down cycle with a Teflon pestle rotating at about 1,500 rpm. The homogenates were centrifuged at 1,100 x *g* for 3 min. The resulting pellets were re-suspended in the original volume of H-medium, homogenized by applying two strokes rotating at about 1,500 rpm, and re-centrifuged again as described above. These steps were repeated two more times. All supernatant fractions obtained after each homogenization and centrifugation step were combined and centrifuged at 6,800 x *g* for 15 min. The resulting sediments were gently re-suspended with H-medium to half the volume of the supernatants in the previous step and centrifuged at 20,000 x *g* for 10 min. Again the resulting sediments were gently re-suspended in H-medium to half the volume of the supernatants in the previous step and centrifuged at 20,000 x *g* for 10 min. The final mitochondria were re-suspended in H-medium to give 20 mg of protein/ml and stored at -80°C.

Purification of Rat Liver F_1-ATPase

The enzyme was purified by the method developed by Pedersen *et al.* (30). The purified enzyme in 250 mM KPi and 5.0 mM EDTA pH 7.5 was divided into 100 µl aliquots, lyophilized and stored at -80 °C until use. The purified enzyme as lyophilized samples was re-

dissolved in distilled water and precipitated twice at room temperature with an ammonium sulfate solution containing 3.5 M ammonium sulfate, 1 mM EDTA, and 10 mM Tris-HCl, pH 7.5. Finally, the enzyme was dissolved in 50% glycerol, 50 mM HEPES, pH 7.4.

MBP Pull-Down Binding Assay

MBP pull-down assays were performed under several conditions as indicated in figure legends. In some cases, 100 μl of amylose resin was incubated with 100 μg of each purified MBP fusion protein from which maltose had been removed via a DEAE column and dialysis. However, in most cases, 100 μl of amylose resin was incubated with *E. coli* cell lysates from which the protein of interest had already been overexpressed with IPTG and disrupted with sonication. After incubation for 1 hour at 4°C and washing three times with the ice-cold column buffer and a buffer specifically indicated in figure legends.

Because of the cold lability of the isolated F_1-ATPase, the following procedure was performed at room temperature. MBP fusion proteins bound to amylose beads were incubated with 1 mg of the mitochondrial extracts solubilized with 1% Triton X-100 or 15 μg of F_1-ATPase purified from rat liver with gentle shaking at room temperature for more than 1 hour. During each incubation, the resin was washed three times with 1 ml of the same buffer noted above in order to remove unbound proteins. The resin was subjected to centrifugation at 500 x *g* for 2 min at each step. Sodium dodecyl sulfate (SDS) sample buffer was added to each sample, which subsequently was boiled for 5 min to release the bound proteins. Samples, 10~15 μl including beads, were analyzed by 13% SDS-polyacrylamide gel electrophoresis (PAGE) gels and visualized with Coomassie blue staining. In a separate experiment, the same volume of each sample was loaded onto SDS-PAGE and subjected to western blot analysis using an anti-β-subunit antibody.

For western-blotting, proteins on the gels after SDS-PAGE were transferred onto a

PVDF (polyvinylidene) membrane using a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad). This was followed by blocking overnight at 4°C with TBST (Tris-buffered saline: 50 mM Tris-Cl, pH 7.5. 150 mM NaCl, 0.1 Tween-20) containing 5% BSA. The PVDF membrane was incubated with gentle shaking overnight at 4°C with an anti-β-subunit antibody at a dilution of 1:10,000. Then, the PVDF membrane was washed extensively and incubated with peroxidase-labeled anti-rabbit IgG for 1 hour at room temperature with gentle shaking. The PVDF membrane was washed with TBST and then incubated with the enhanced chemiluminescence (ECL) Western blotting detection reagents (Amersham). Finally, the PVDF membrane was exposed to Amersham Hyperfilm ECL to detect chemiluminescent signals.

ATPase Activity Assay

To assess the inhibitory effect on the catalytic activity of F_1-ATPase resulting from the 1 mM AMP-PNP used in the reconstitution buffer (9), we used the malachite green ATPase assay (31,32) to measure the amount of Pi released in the presence of ATP and $MgCl_2$. For a 300 µl reaction, 0.6 µg of F_1-ATPase was incubated in 394 µl total volume of a reaction buffer containing 20% glycerol, 100 mM HEPES (pH 7.4), 1 mM AMP-PNP for 20 min. For a physiological salt condition, NaCl was added to give a concentration of 150 mM in the reaction buffer. A volume of 98 µl was removed from each reaction mixture during this incubation and therefore prior to adding a 50 mM ATP-Mg^{2+} solution. This served as a zero time standard for the malachite green reaction described below. The ATPase reaction was started by adding 4 µl of 50 mM ATP-Mg^{2+} solution to the remaining 196 µl volume of each reaction. At 2 min and 5 min, 100 µl of the reaction mixture was taken and immediately added to 800 µl of the malachite green reaction solution freshly prepared by mixing three volumes of 0.045% malachite green hydrochloride, one volume of 4.2% ammonium molybdate tetrahydrate in 4

N HCl and 1/200 volume of 4% Triton X-100. Then, 100 µl of 34% citric acid was added to stop color development. After about 20 min, the OD_{660} was measured using Varian Cary 50 Bio UV/ Visible Spectrophotometer. Samples were read against a zero time standard that lacked the ATP- Mg^{2+} solution. Also, the endogenous amount of free phosphate in the ATP-Mg^{2+} solution was measured at the same time. Protein concentration was measured with a colorimetric Bio-Rad Protein Assay kit based on the Bradford assay using BSA as a standard (33).

Results

Purification of Subunits

Constructs were designed to generate a MBP fusion protein for each of the following ATP synthase subunits: d, F_6, Factor B, IF_1 and OSCP (originating from human and rat). A MBP fusion system for overexpressing proteins in bacteria was used to obtain the mature form of each subunit after a specific cleavage with either the protease Factor Xa or Genease I followed by the identification of their interaction with F_1-ATPase via a pull down assay using the MBP as an affinity tag. All subunits were first purified as MBP fusion proteins using an amylose affinity column and then further separated from MBP and proteases using ion-exchange columns such as DEAE or CM Sepharose after specific protease cleavage. All of the proteins, each as an MBP fusion protein, were successfully overexpressed in soluble form in the bacterial cytoplasm.

F_6 and IF_1 were passed through a DEAE column and also found in the washing fractions (Figure 2, 4). The pooled fractions containing either F_6 or IF_1 were dialyzed to remove any maltose and then subjected to amylose affinity column chromatography to remove any residual MBPs. The fractions containing Factor B and subunit d overlapped with the fraction containing MBP and therefore after dialysis were loaded onto the amylose affinity column to remove the MBP (Figure 1, 3). Compared to a previous study (24), the MBP fusion protein system improved the solubility and overexpression of the MBP-OSCP fusion proteins. However, OSCP freed from MBP showed self-aggregation. Also, some undigested MBP-OSCP fusion proteins were present after treatment with the protease, Genease I (Figure (Figure 5). Therefore, OSCP was solubilized with 6 M guanidine hydrochloride and induced to refold by step-wise dialysis. Then, the refolded OSCP was further isolated using a CM Sepharose column (Figure 5). All of the purified proteins were subsequently loaded onto an

amylose column to further remove any residual MBP proteins.

The SDS-PAGE analysis presented in Figure 6 shows all of the purified supernumerary proteins, subunit d, F_6, Factor B, IF_1, and OSCP originated from rat and human, and also the purified F_1-ATPase derived from rat liver with five distinct subunit components α, β, γ, δ, and ε.

MBP Pull Down Assay

A previous study demonstrated that among the subunits comprising the peripheral stalks, OSCP is the only protein that strongly interacts and remains with the F_1-ATPase after gel filtration chromatography and also after *in vitro* reconstitution experiments (9). However, in an earlier study of x-ray crystals generated from a complex of the bovine mitochondrial F_1-ATPase with the peripheral stalk, the subunits F_6 and d were also found to be in contact with the β-subunit of the enzyme. However, this work with subunits F_6 and d gave little attention because of the above data (6) even though these interactions had been previously investigated by a chemical cross-linking approach and reported. (10,11). Also, in a study undertaken to find a receptor for the circulating F_6 subunit, a direct interaction between F_6 and the β-subunit *in vitro* was identified by immuno-precipitation analysis. Specifically, an F_6 antibody was used in a mixture containing solubilized human endothelial cells and the purified F_6 protein (12). Frequently, Factor B has been considered as a component of the ATP synthase complex (3). It was suggested that it binds to subunits e and g of the F_O complex based on a cross-linking study (14) and also to the ADP/ATP carrier based on a co-sedimentation study. However, because they used a photo-reactive unnatural amino acid for residue Trp2 in the cross-linking study, it may have limited an interaction with Factor B's extreme N-terminal region that was also suggested to be involved in the attachment of the protein to the membrane (34). The increasing number of recent reports revealing an "ectopic" ATP synthase, i.e., on the cell

surface that is almost identical to that found in mitochondria, as well as reports of known supernumerary subunits of this enzyme, e.g., F_6 and IF_1 in human serum encouraged us to carry out studies to better understand their significance. Specifically, using a MBP pull-down assay, the interaction of each soluble subunit, i.e., subunit d, F_6, Factor B, IF_1, and OSCP, with the ATP synthase's catalytic F_1 moiety was monitored in a phosphate buffered saline (PBS) solution.

To investigate the possible interaction of the soluble supernumerary subunits with the catalytic F_1 moiety, we performed MBP pull-down assays in a PBS medium. This is because we were interested in determining later to what extent the supernumerary subunits found for the mitochondrial ATP synthase also interact with the F_1 moiety of the ATP synthase found on the plasma membrane. To commence these studies, mitochondria were isolated from rat livers and then solubilized with 1% Triton X-100. Each rat supernumerary protein was expressed in fusion with MBP (i.e. not cleaved) and bound to amylase beads. Then, the solubilized mitochondrial extracts were incubated with each amylose bead-attached MBP fusion protein. As a control, MBP-lacZα (MBP2*-β-galactosidase α fragment fusion protein), was overexpressed from the original vector, pMAL-c2X without any insert. Significantly, the subunits d, F_6, Factor B and OSCP as MBP fusion proteins were found to successfully pull-down the ATP synthase β-subunit, as detected by immunoblotting with an antibody to the β-subunit. High NaCl concentration (100 mM more added in PBS) had no effect on either the amount of these subunits bound to the amylose beads or the amount of β-subunit caught by these subunits (Figure. 7).

Both MBP-lacZα, the negative control, and MBP-IF_1 did not pull down the β-subunit despite the considerable amount of MBP fusion protein attached to the amylose beads. The N-terminal region of IF_1 must be inserted into the interface between the α_{DP} and β_{DP} subunits

of F_1-ATPase in order to block this enzyme's hydrolytic activity based on the respective structures of F_1-ATPase and monomeric IF_1 (15,35,36). Therefore, a relatively larger MBP region combined to the N terminal region of IF_1 within the MBP-IF_1 fusion protein is unlikely to allow IF_1 to interact with the F_1-ATPase. Such could be used as another negative control following the pull-down assays.

Finally, as was found to be the case for IF_1, MBP-fusion proteins involving other ATP synthase subunits (d, F_6, Factor B, and OSCP) mentioned above were also incubated with the purified F_1-ATPase from rat liver. The same result as the above (Figure. 8) was obtained. These data indicate that subunits d, F_6, Factor B, and OSCP can interact with the F_1-ATPase moiety individually and that high concentrations of NaCl has little effect on these interactions.

Independence of Mammalian Species

The structure and the sequences of the common subunit components of ATP synthases are highly conserved among diverse organisms even across prokaryotes and eukaryotes. The amino acid sequence of the supernumerary specifically associated with mitochondrial ATP synthase also shows high sequence similarity/identity among various mammalian species (37-41). Therefore, it is reasonable to expect that the above interactions are common in mammals. To confirm whether these interactions are general among mammalian mitochondrial subunits, the mitochondrial extracts isolated from rabbit skeletal hind muscle solubilized with 1% Triton X-100 were incubated with MBP fusion proteins of each rat subunit (Figure 9). In another set of experiments, purified F_1-ATPase from rat liver was incubated with each human MBP fusion protein (Figure 10). In both cases, subunit d, F_6, Factor B and OSCP were shown to bind F_1-ATPase and a high concentration of NaCl failed to affect these interactions. In conclusion, these studies taken together with those already described above, would suggest that interactions of the supernumerary subunits with the F_1-

catalytic unit of the mitochondrial ATP synthase may be common among various mammalian ATP synthases.

Independence of ATP, ADP, and Mg^{2+}

Previously it was reported that F_6 bound to the β_{DP}-subunit of the ectopic ATP synthase because its interaction was suppressed by adenosine diphosphate (ADP) and a β-subunit antibody in a binding assay of ^{125}I-F_6 to the surface of human umbilical vein endothelial cells abbreviated "HUVEC" (12). The binding of IF_1 to the soluble F_1-ATPase required the presence of Mg^{2+} and ATP and subsequent ATP hydrolysis (15). However, these components were not present in the above binding experiments. To investigate whether ATP, ADP and Mg^{2+} could influence the interaction of MBP-F_6 or MBP-IF_1 with F_1-ATPase these agents were added to the pull-down assay with PBS. None were able to affect these interactions. In conclusion, F_6 can interact with F_1-ATPase independent of the presence of Mg^{2+}, ADP and ATP molecules (Figure 11). Moreover, it was reconfirmed that MBP-IF_1 could not interact with the F_1-ATPase not because of the absence of Mg^{2+} and ATP but because of the interruption by the MBP region of the fusion protein.

MBP Pull Down Assays in the Reconstitution Buffer

In experiments reported here, individual MBP-d, MBP-F_6 and MBP-OSCP fusion proteins interacted directly with rat F_1-ATPase in a PBS buffer. This is in contrast to a previous report (9) in which OSCP was the only one of these three subunit types to bind strongly to bovine F_1-ATPase. This earlier study had been carried out specifically to investigate the interaction of each subunit composing the peripheral stalks with the F_1-ATPase (9). Therefore, we decided to use a similar buffer condition in our MBP pull-down assays. First, we tried adding AMP-PNP, a non-hydrolysable analogue of ATP, to inhibit the catalytic activity and pause the rotation of F_1-ATPase under our conditions, i.e., PBS buffer. The inhibition of

purified rat F_1-ATPase by 1 mM AMP-PNP was determined by phosphate release from ATP using the malachite green assay (Figure 12). Each rat MBP fusion protein freshly attached to amylose beads was incubated with purified F_1-ATPase in PBS with or without 1 mM AMP-PNP, 20 μM ADP and 2 mM $MgCl_2$. Independent of these additives, MBP-d, -F_6, -Factor B and -OSCP could pull-down rat F_1-ATPase (Figure 13). Second, each MBP protein and purified F_1-ATPase were incubated in reconstitution buffer, which contained 20 mM Tris (pH 7.5), 50 mM sucrose, 100 mM NaCl, 2 mM $MgSO_4$, 1 mM EDTA, 0.001 % PMSF, 8 mM dithiothreitol (DTT), 0.02% NaN_3, 10% glycerol with or without 1 mM AMP-PNP, 20 μM ADP and 2 mM $MgCl_2$ (9) (Figure 13). Again, we found that not only MBP-OSCP but also MBP-d, MBP-F_6 and MBP-Factor B are able to associate with the F_1-ATPase.

The reconstitution buffer was based on that used previously to generate X-ray quality crystals for the peripheral stalk and F_1-ATPase (6). Therefore, these interactions are likely to contribute to the mitochondrial structure. However, the reason why the subunits d, F_6, and Factor B were not retained with the F_1-ATPase during the previous study employing gel filtration and centrifugation in a sucrose gradient remains unknown.

Discussion

OSCP in the peripheral stalk has been generally considered as the only protein of the stalk that interacts with the F_1-ATPase catalytic unit of the mitochondrial ATP synthase (9). However, there are some reasons to disregard this generalization. First, in the structure of F_1-ATPase with the peripheral stalk, there are also significant contacts between the enzyme and both subunits d and F_6 (6). The resolved helix 2 (residues 30-40) of subunit d has a contact with the β_{TP} -subunits, and the residues 43-50 of the C-terminal helix (residues 34-51) of F_6 bind residues 247 and 301 of the β_{TP} -subunit. The structure has a 9° kink around residue 146 in subunit b compared to the structure containing the peripheral stalk alone. This generated an inward movement of the lower region of the stalk toward the catalytic domain. Later the authors disregarded these findings because the structure without the kink from the subcomplex containing subunits b, d and F_6 fit well with the overall electron cryo-microscopy map and there had been observed no such interaction in previous reconstitution experiments (6). Second, cross-linking studies also reveal these interactions (10,11). Thus, F_6 and subunit d were able to cross link with subunit α and/or β-subunits using either the chemical cross-linker disuccinimidyl tartarate (DST) or 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC). The above candidate regions of F_6 (43 LFKLKQMY 50) and subunit d (30 LKSWNETLTS 40) from the structure determined by x-crystallography contain the amino acids Lys and Glu that react with these reagents. Third, F_6 was found to interact with the β-subunit *in vitro* under extracellular conditions, i.e., phosphate buffered saline (12). The biological active site on F_6 involved in increasing blood pressure after intravenous administration into rats resides in the C-terminal portion of the precursor of F_6 (residues 55-108). These residues correspond to residues 13-76 in the mature sequence (42) which also includes the candidate region of F_6. Fourth and finally, the interaction between OSCP and F_1-

ATPase is relatively weak compared to the interaction of the *E. coli* δ-subunit with F_1-ATPase. Therefore, mitochondrial OSCP is considered as a F_O component. In contrast, the *E. coli* δ-subunit is usually purified with the catalytic F_1 subcomplex. In terms of the binding affinity, OSCP did not show sufficient affinity for F_1 to sustain this catalytic domain from rotating with the central stalk during ATP synthesis (43). For these reasons, there are likely additional interactions involving other subunits of the peripheral stalk and F_1-ATPase.

Using sucrose density gradient centrifugation and cross-linking with the photo-reactive unnatural amino acid pbenzoyl-L-phenylalanine (pBpa) substituted for Trp2 of bovine Factor B, it was suggested that the protein interacted with subunits e and g of F_O as well as the ADP/ATP carrier (14). Because the N-terminal extreme regions (residues 1-10) were suggested to anchor factor B to the matrix side of the mitochondrial inner membrane (34), interactions with membrane embedded proteins would be possible via the N-terminal region. The remaining region of Factor B is exposed to the matrix region of mitochondria and is likely to provide a binding site for the catalytic F_1 moiety of the ATP synthase. Interestingly, Factor B contains a C-terminal LRR motif domain (four leucine-rich repeats) which was suggested to be involved in a variety of protein-protein interactions. However, if Factor B interacts with subunits e and g of F_O, it should overcome the obstruction of the structure of the peripheral stalk that prevents it from interacting with the catalytic F_1 headpiece of the ATP synthase (F_OF_1). According to the intact bovine mitochondrial ATP synthase resolved by electron determined cryo-microscopy of single particles, subunits e and g are positioned at distal regions from the center of the enzyme, confronting the peripheral stalk before the F_1 (5).

MBP-IF_1 did not pull down F_1-ATPase under any condition and acted as a negative control throughout this study. The N-terminal regions of IF_1 do contribute to the inhibition

of the hydrolytic activity of F_1-ATPase. In this regard, IF_1's end must be deeply inserted into one of the three catalytic interfaces between the α and β-subunits and reach beyond the central cavity of F_1, thus promoting high affinity and specific interactions involving five different subunits (15). Therefore, C-terminal affinity tags such as hexahistidine or glutathione-S-transferase (GST) on IF_1 not only allowed the protein to interact with F_1-ATPase but also provided the highly selective tools necessary to purify the active F_OF_1-ATP synthase complex from mitochondria (44). Interestingly, a small globular-like protein, PsaE with an 8kDa molecular weight attached to the N-terminal region of yeast IF_1 did not interrupt the binding of IF_1 to F_1-ATPase as determined by retention of IF_1's capacity to completely inhibit F_1's ATPase activity but showed decreased stability. This result suggested that the extreme N-terminal region of IF_1 preceding the minimal inhibitory sequence (residues 14-47 in bovine heart) may turn in the opposite direction while the inhibitory sequence interacts with the catalytic site's interface and may ultimately be involved in the stabilization of the IF_1 inhibited F_1-ATPase. However, we have used the MBP protein fused to the N-terminus of IF_1. Its molecular weight is 42.5 kDa, which is undoubtedly large enough to interrupt the penetration of the inhibitory sequence of IF_1 into the catalytic interface. However, no β-subunit pull-down by MBP-IF_1 was detected.

In summary, in work reported here we demonstrated that in phosphate buffered saline the mammalian mitochondrial ATP synthase subunits d, F_6, Factor B and OSCP can each bind alone to the isolated F_1-ATPase moiety. These interactions seem to be strong enough to sustain media containing 100 mM NaCl in PBS or 1% Triton X-100. F_6 and IF_1 are found also in human serum where the former has been proposed to function as an endogenous vasoconstrictor and the latter as a regulator for HDL-cholesterol metabolism. Both interact with the ectopic ATP synthase on the cell surface and affect its catalytic activity. Like F_6 and

IF_1, the subunits d, Factor B and OSCP will be new candidates to investigate as potential binding partners of the ectopic ATP synthase to determine whether they are required for inducing the enzyme to participate in novel functions or well established cellular functions. However, to suggest participation of any one of the 3 above noted subunits in a cellular function by their binding to the cell surface, that subunit must fulfill the following conditions. First it must be released from the ectopic ATP synthase. This could result from shear stress generated by blood flow because we already know that F_6 of the ectopic ATP synthase and ATP are released from vascular endothelial cells by shear stress (19,45). Second, it must be established whether they do appear in human serum or remain on the cell surface. Several studies have already reported that an almost complete set of the above noted subunits are at the plasma membrane of various cell types (46). However, for a supernumerary subunit of an ATP synthase/ATP complex located on the plasma membrane to regulate the activity of that complex, it must be released and therefore be detectable in the serum. At the extracellular location, the supernumerary subunit should be able to modulate the catalytic activity of the ectopic ATP synthase whether or not it is functioning as an ATPase or an ATP synthase.

This study also provides suggestive evidence to describe the structure of mitochondrial ATP synthase because of the same interaction in the reconstitution buffer which has been a basic condition to generate several x-ray crystals of mitochondrial ATP synthase or a subcomplex thereof. However, several questions arise. One question is why were these interactions not identified in previous binding assays such as the gel-filtration assay for components of the peripheral stalk (9) and the sucrose density gradient centrifugation assay for Factor B (14)? A second question is whether each subunit can discriminate among the catalytic states of the β-subunits? If not, there would be detected three binding sites on F_1 for each supernumerary subunit. However, in this study all supernumerary subunits exhibited a

1:1 stoichiometry with F_1-ATPase within the isolated ATP synthase. It was suggested also that excess subunits are degraded quickly if they are not assembled into the complex. However, in certain pathogenic conditions such as a cancer where cells overexpress IF_1 (47), additional interactions would be possible.

In conclusion, we purified all subunits of the rat liver ATP synthase/ATPase complex that have been referred to as "supernumerary subunits" and then performed experiments that demonstrated that each supernumerary subunit interacts with the catalytic F_1-ATPase moiety of the ATP synthase/ATPase complex. As this complex is known to be associated with both the mitochondrial inner membrane as well as the plasma membrane of mammalian cells (ectopic location), the results of experiments reported in this thesis provide new information that is likely to be relevant to better understanding the structure, function, and regulation of the ATP synthase complex at both locations.

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Table 1

Primer Set and Templates Used to Generate Each MBP-fusion Protein and Purify a Mature Form of Each Protein

Each forward Primer started with the first nucleotide sequence of each mature subunit, and the reverse primer contained a stop codon followed by an EcoRI sequence (5'GAATTC3') to be inserted into the expression vector for the MBP fusion protein. An IMAGE Clone ID indicates the EST clone used as the template in each PCR reaction. The last column indicates the region of a mature form in the sequence of the initially translated protein and thus the final fragment generated in the study. (**a**), The DNA clone for MBP-Rat IF_1 was previously prepared (25). (**b**), For MBP-Rat OSCP, the template was the Rat-OSCP clone incorporated into the pET vector (24)

DNA Constructs Information

Gene	Forward Primer	Reverse Primer	IMAGE Clone ID	Mature Amino Acids (length)
Human IF_1	5'GGCTCGGACTC GTCGGAGAG3'	5'AGCGAATTCTCAATGCT CACTATTCTTTAG3'	3877506	26 - 106 (81)
Rat IF_1[a]				26 - 107 (82)
Human F_6	5'AATAAGGAACTT GATCCTATAC3'	5'AGCGAATTCTCAGGCCT GGGGTTTTTCGATG3'	3357779	33 - 108 (76)
Rat F_6	5'AATAAGGAACTT GATCCTGTAC3'	5'AGCGAATTCTCAGGAC TGGGGTTTGTCGAGG3'	6919556	33 - 108 (76)
Human Factor B	5'TTCTGGGGCTG GTTGAATGCAG3'	5'AGCGAATTCTTACTTCA ATTGTAATTTTAGTTCC3'	6165658	41 - 215 (175)
Rat Factor B	5'TTCTGGGAGTG GTTGAATACAG3'	5'AGCGAATTCTTACTTCA GGTTTAATTTTAGTTCC3'	7135201	26 - 200 (175)
Human Subunit d	5'GCTGGGCGAAA ACTTGCTC3'	5'AGCGAATTCTTATAAAT TCTCAATTGGTTGG3'	6020835	2 - 161 (160)
Rat Subunit d	5'GCTGGGCGCAA ACTTGCTC3'	5'AGCGAATTCTCACAGG TTCTCGATGGGCTG3'	6921557	2 - 161 (160)
Human OSCP	5'TTTGCCAAGCTT GTGAGGC3'	5'AGCGAATTCTTAGACAA TCTCCCGCATAGCC3'	4126036	24 - 213 (190)
Rat OSCP[b]	5'TTTTCGAAGCTT GTAAGGCC3'	5'AGCGAATTCTCAGAGC AGATCCCGCATGG3'		24 - 213 (190)

a: Rat IF_1 : Lebowitz and Pedersen, Arch. Biochem. Biophys., 301 (1), 64-70 (1993)
b: Rat OSCP : Golden and Pedersen, Biochemistry, 37(39):13871-81 (1998)

Figure 1

SDS-PAGE/Coomassie Blue Staining Analysis of Subunit d of Mitochondrial ATP Synthase Following Its Over-expression in *E. coli* and Subsequent Purification

E. coli BL21 (DE3) cells (**lane 1**) containing pMAL-c2X incorporated with human or rat subunit d were grown in liquid culture medium and over-expression was induced by IPTG (**lane 2**). After purification of the MBP fusion protein (**lane 3**) using affinity chromatography with an amylose resin, elution with maltose, and subsequent cleavage with Factor Xa (**lane 4**), the mature form of each subunit d protein was purified by DEAE ion-exchange chromatography (**lane 5**). "**M**" is a size marker and arrowheads (◀) in the gel and on the right side of the gel indicate protein bands of human and rat subunit d respectively.

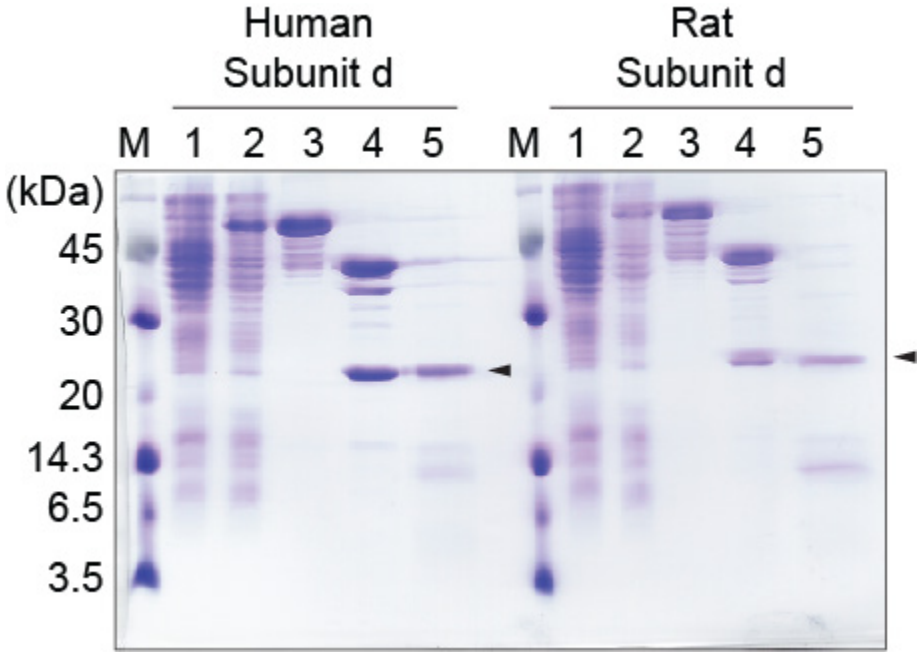

Human
Subunit d
Rat
Subunit d
M 1 2 3 4 5 M 1 2 3 4 5
(kDa)
45
30
20
14.3
6.5
3.5

Figure 2

SDS-PAGE/Coomassie Blue Staining Analysis of the F_6 subunit of Mitochondrial ATP Synthase Following Its Over-expression in *E. coli* and Subsequent Purification

E. coli BL21 (DE3) cells (**lane 1**) containing pMAL-c2X incorporated with human or rat F_6 were grown in liquid culture medium and over-expression was induced by IPTG (**lane 2**). After purification of the MBP fusion protein (**lane 3**) using affinity chromatography with an amylose resin, elution with maltose, and subsequent cleavage with Factor Xa (**lane 4**), the mature form of each F_6 protein was purified using DEAE ion-exchange chromatography (**lane 5**). "**M**" is a size marker and arrowheads (◀) in the gel and on the right side of the gel indicate protein bands of human and rat F_6 respectively.

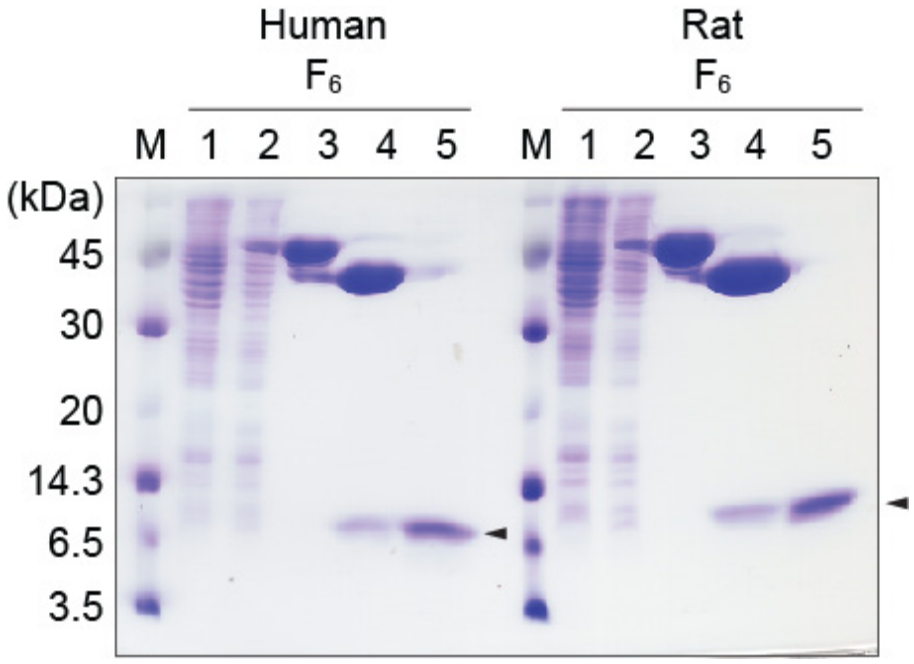

Human
F6
Rat
F6
M 1 2 3 4 5 M 1 2 3 4 5
(kDa)
45
30
20
14.3
6.5
3.5

Figure 3

SDS-PAGE/Coomassie Blue Staining Analysis of Factor B of Mitochondrial ATP Synthase Following Its Over-expression in *E. coli* and Subsequent Purification

E. coli BL21 (DE3) cells (**lane 1**) containing pMAL-c2X incorporated with human or rat Factor B were grown in liquid culture medium and over-expression was induced by IPTG (**lane 2**). After purification of the MBP fusion protein (**lane 3**) using affinity chromatography with an amylose resin, elution with maltose, and subsequent cleavage with Factor Xa (**lane 4**), the mature form of each Factor B protein was purified with DEAE ion-exchange chromatography (**lane 5**). "**M**" is a size marker and arrowheads (◀) in the gel and on the right side of the gel indicate protein bands of human and rat Factor B respectively.

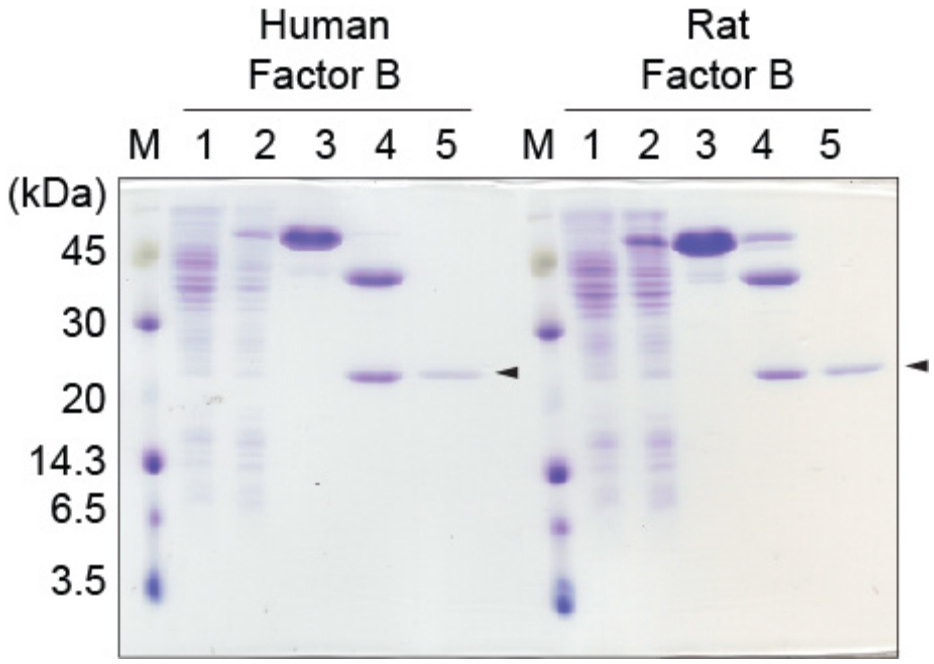

Human
Factor B
Rat
Factor B
M 1 2 3 4 5 M 1 2 3 4 5
(kDa)
45
30
20
14.3
6.5
3.5

Figure 4

SDS-PAGE/Coomassie Blue Staining Analysis of IF_1 of Mitochondrial ATP Synthase Following Its Over-expression in *E. coli* and Subsequent Purification

E. coli BL21 (DE3) cells (**lane 1**) containing pMAL-c2X incorporated with human or rat IF_1 were grown in liquid culture medium and over-expression was induced by IPTG (**lane 2**). After purification of the MBP fusion protein (**lane 3**) using affinity chromatography on an amylose resin and elution with maltose and subsequent cleavage with Factor Xa (**lane 4**), the mature form of each IF_1 protein was purified with DEAE ion-exchange chromatography (**lane 5**). "**M**" is a size marker and arrowheads (◀) in the gel and on the right side of the gel indicate protein bands of human and rat IF_1 respectively.

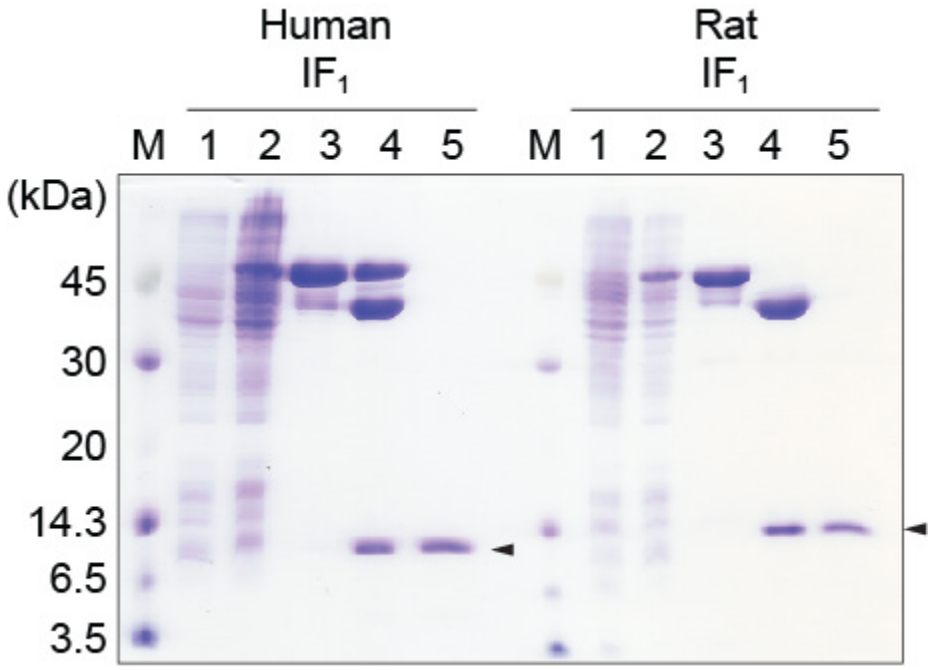

Human
IF1
Rat
IF1
M 1 2 3 4 5 M 1 2 3 4 5
(kDa)
45
30
20
14.3
6.5
3.5

Figure 5

SDS-PAGE/Coomassie Blue Staining Analysis of OSCP of Mitochondrial ATP Synthase Following Its Over-expression in *E. coli* and Subsequent Purification

E. coli BL21 (DE3) cells (**lane 1**) containing pMAL-c2G incorporated with human or rat OSCP were grown in liquid culture medium and over-expression was induced by IPTG (**lane 2**). Each MBP fusion protein (**lane 3**) was purified using affinity chromatography on an amylose resin and elution with maltose. After subsequent cleavage with Genenase I, the released OSCP was precipitated with undigested MBP-OSCP fusion proteins (**lane 5**) with MBP proteins remaining in the supernatant (**lane 4**). The pellet with OSCP was solubilized with 6 M guanidine hydrochloride and then refolded by step-wise dialysis. Finally, the mature form of each OSCP protein was separated with CM Sepharose CL-6B (**lane 6**). "**M**" is a size marker and arrowheads (◀) in the gel and on the right side of the gel indicate protein bands of human and rat OSCP respectively.

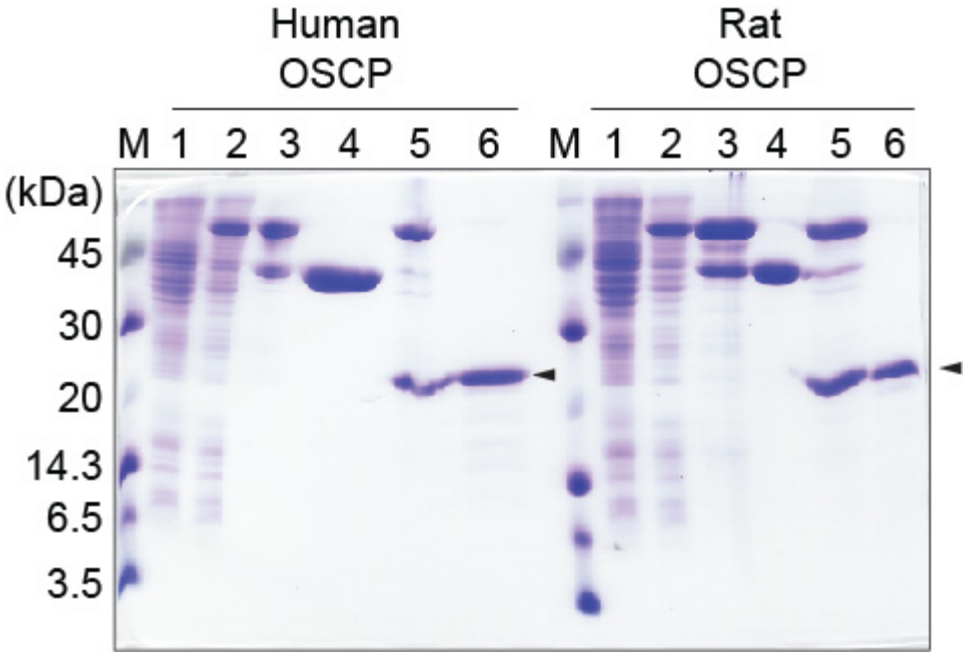

Human
OSCP
Rat
OSCP
M 1 2 3 4 5 6 M 1 2 3 4 5 6
(kDa)
45
30
20
14.3
6.5
3.5

Figure 6

SDS-PAGE Analysis with Colloidal Coomassie G-250 Staining of the Purified Subunits

The final purified subunits derived from rat and human were loaded into Tricine SDS-PAGE (48) and stained with colloidal Coomassie G-250 (49). F_1 refers to Rat F_1-ATPase isolated from rat livers. α, β, γ, δ, ε and accompanying arrows indicate each subunit of F_1-ATPase. **Lane 1**, purified subunit d (either human or rat); **lane 2**, purified F_6 subunit; **lane 3,**: Factor B; **lane 4**, IF_1; **lane 5**, OSCP.

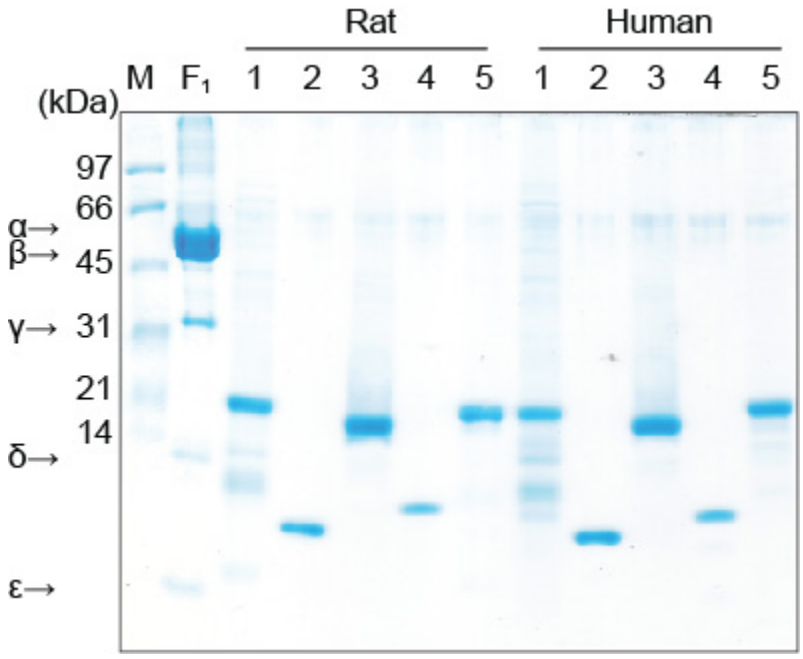
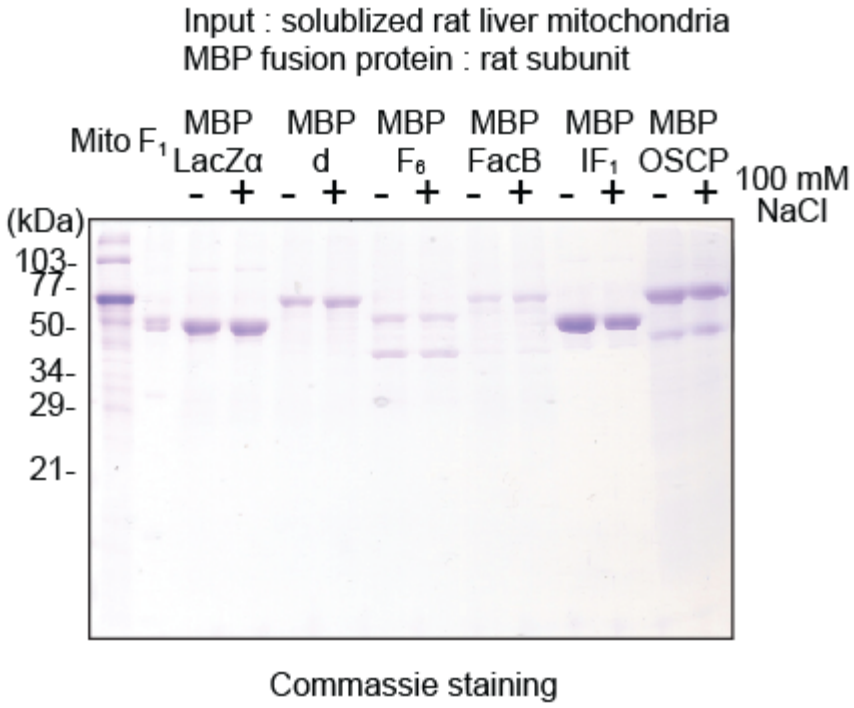

Rat
Human
M
F1
1
2
3
4
5
1
2
3
4
5
(kDa)
97
66
α→
β→
45
γ→
31
21
14
δ→
ε→

Figure 7

MBP Pull-Down Assay with Rat MBP Fusion Proteins and Extracts of Rat Liver Mitochondria

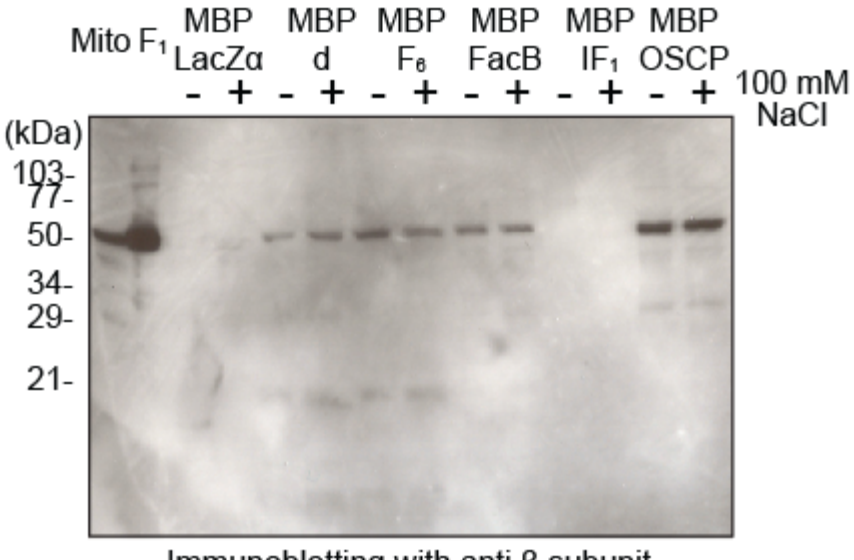
Each rat MBP fusion protein bound to amylose beads was incubated with extracts of rat liver mitochondria that had been prepared by solubilizing with 1% Triton X-100 in PBS (Phosphate Buffered Saline: 10 mM Na_2HPO_4, 2 mM KH_2PO_4, 137 mM NaCl, and 2.7 mM KCl) with (**+**) or without (**-**) additional 100 mM NaCl at room temperature. After incubation for one hour and washing three times with the same buffer, proteins attached to amylose resins were eluted with SDS sample buffer. The same amount of each sample was loaded for (**A**) Coomassie blue staining and (**B**) western blotting with an antibody to the β-subunit of F_1-ATPase.

A



Commassie staining

B



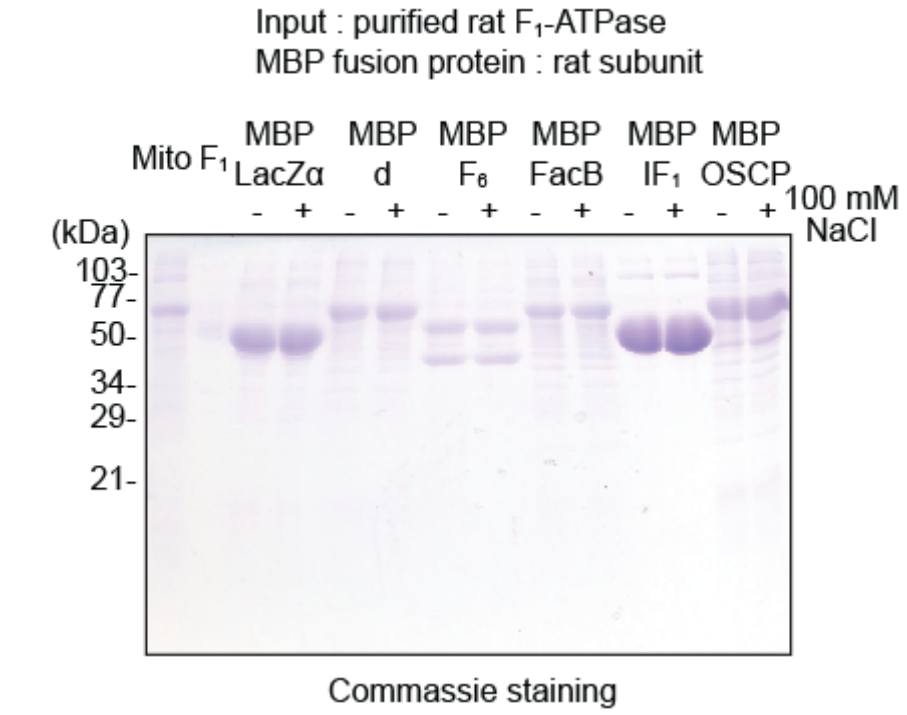
Immunoblotting with anti β subunit

Figure 8

MBP Pull-Down Assay with Rat MBP Fusion Proteins and Purified Rat F_1-ATPase

Each rat MBP fusion protein bound to amylose beads was incubated with 15 μg of isolated rat F_1-ATPase in PBS, 1 % Triton X-100 with (**+**) or without (**-**) additional 100 mM NaCl at room temperature. After incubation for one hour and washing three times with the same buffer, proteins attached to amylose resins were eluted with SDS PAGE sample buffer. The same amount of each sample was loaded for subsequent (after electrophoresis) (**A**) Coomassie blue staining and (**B**) western blotting with an antibody to the β-subunit of F_1-ATPase.

A


Input : purified rat F1-ATPase
MBP fusion protein : rat subunit
Mito F1
MBP LacZα
MBP d
MBP F6
MBP FacB
MBP IF1
MBP OSCP
- + - + - + - + - + - +
100 mM NaCl
(kDa)
103-
77-
50-
34-
29-
21-
Commassie staining

B

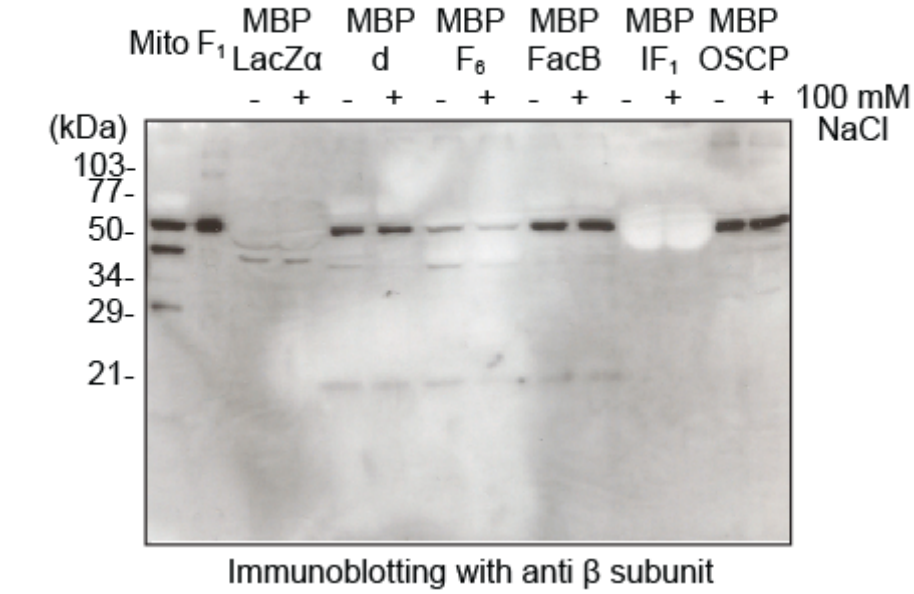

Mito F1
MBP LacZα
MBP d
MBP F6
MBP FacB
MBP IF1
MBP OSCP
- + - + - + - + - + - +
100 mM NaCl
(kDa)
103-
77-
50-
34-
29-
21-
Immunoblotting with anti β subunit

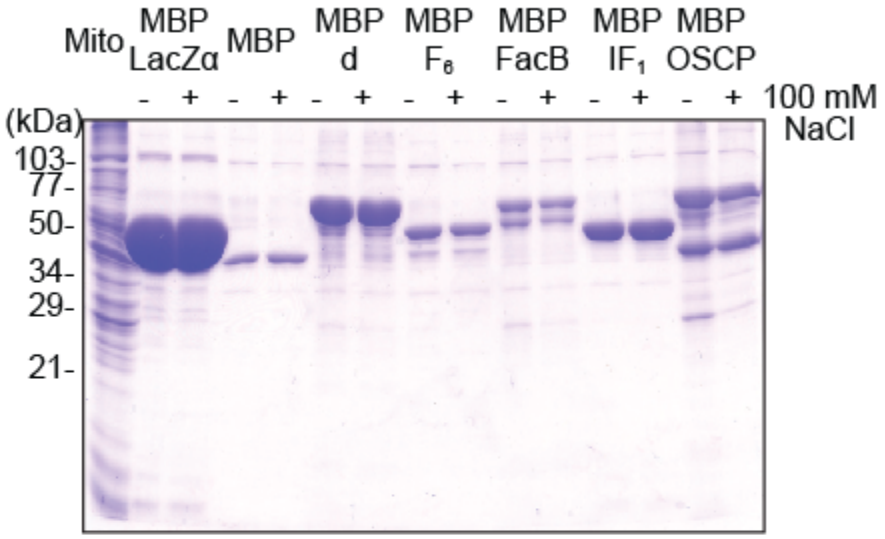
Figure 9

MBP Pull-Down Assay with Rat MBP Fusion Proteins and Extracts of Rabbit Skeletal Mitochondria

Each rat MBP fusion protein bound to amylose beads was incubated with extracts of rabbit skeletal mitochondria solubilized with 1% Triton X-100 in PBS at room temperature with (**+**) or without (**-**) additional 100 mM NaCl. After 1 hr incubation and 3 times washing with the same buffer, proteins attached to amylose resin were eluted with SDS sample buffer. The same amount of each sample was then loaded onto SDS PAGE gels for (**A**) Coomassie blue staining and (**B**) western blotting with an antibody to the β-subunit of F_1-ATPase.

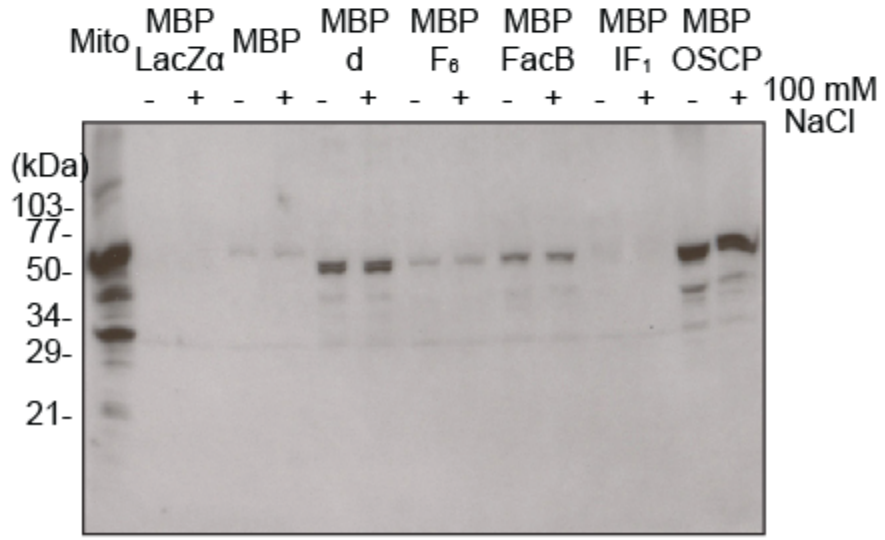
A

Input : solublized rabbit skeletal mitochondria
MBP fusion protein : rat subunit



Commassie staining

B



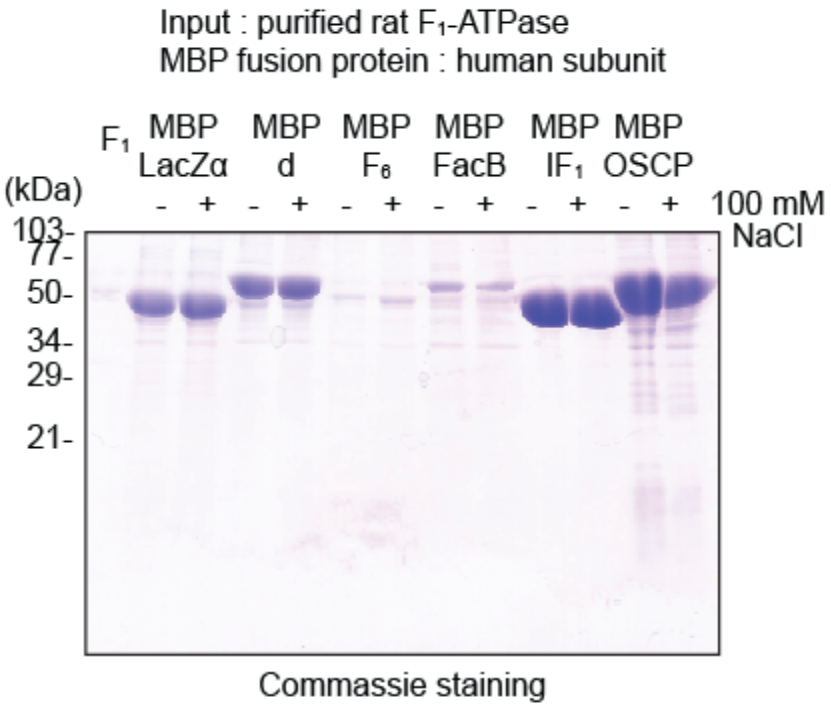
Immunoblotting with anti β subunit

Figure 10

MBP Pull-Down Assay with Human MBP Fusion Protein and Purified Rat F_1-ATPase

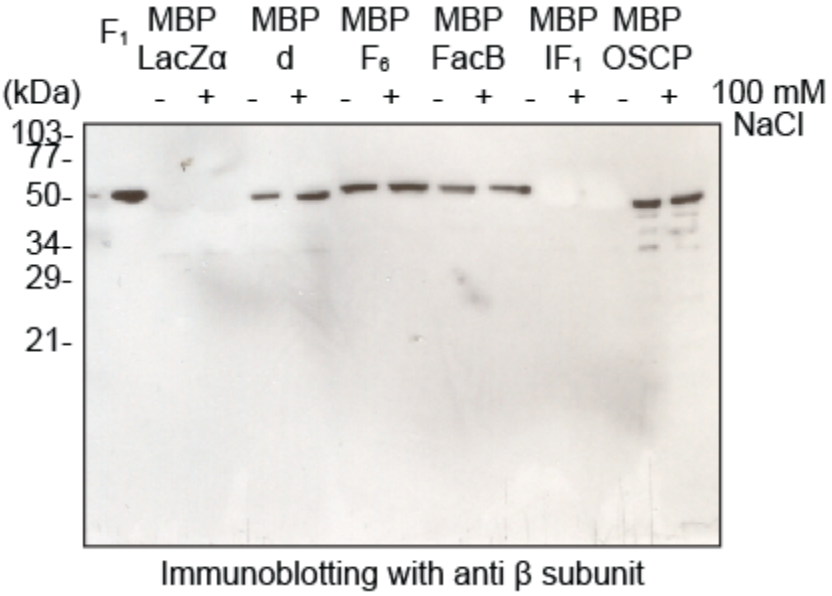
Each human MBP fusion protein bound to amylose beads was incubated with 15 μg of isolated rat F_1-ATPase in PBS, 1 % Triton X-100 with (**+**) or without (**-**) additional 100 mM NaCl at room temperature. After 1 hr incubation and 3 times washing with the same buffer, proteins attached to amylose resins were eluted with SDS sample buffer. The same amount of each sample was loaded for (**A**) Coomassie blue staining and (**B**) western blotting with an antibody to the β-subunit of F_1-ATPase.

A



Commassie staining

B



Immunoblotting with anti β subunit

Figure 11

MBP Pull-Down Assay with Rat MBP-F_6 and Rat MBP-IF_1 with Purified Rat F_1-ATPase under Conditions Containing ATP, ADP, or Mg^{2+}

MBP-F_6, MBP-IF_1 or MBP-lacZα fusion protein bound to amylose beads was incubated with 15 μg of isolated rat F_1-ATPase in PBS, 1 % Triton X-100 at room temperature (**lane 1**) or simultaneously under conditions containing also 2 mM $MgCl_2$ (**lane 2**), 2 mM $MgCl_2$ and 1 mM ADP (**lane 3**) or 2 mM $MgCl_2$, 1 mM ATP (**lane 4**). After 1 hr incubation and 3 times washing with the same buffer, proteins attached to amylose resins were eluted with SDS sample buffer. The same amount of each sample was loaded onto SDS PAGE gels for subsequent (after electrophoresis) (**A**) Coomassie blue staining and (**B**) western blot analysis, the latter with an antibody to the β-subunit of F_1-ATPase.

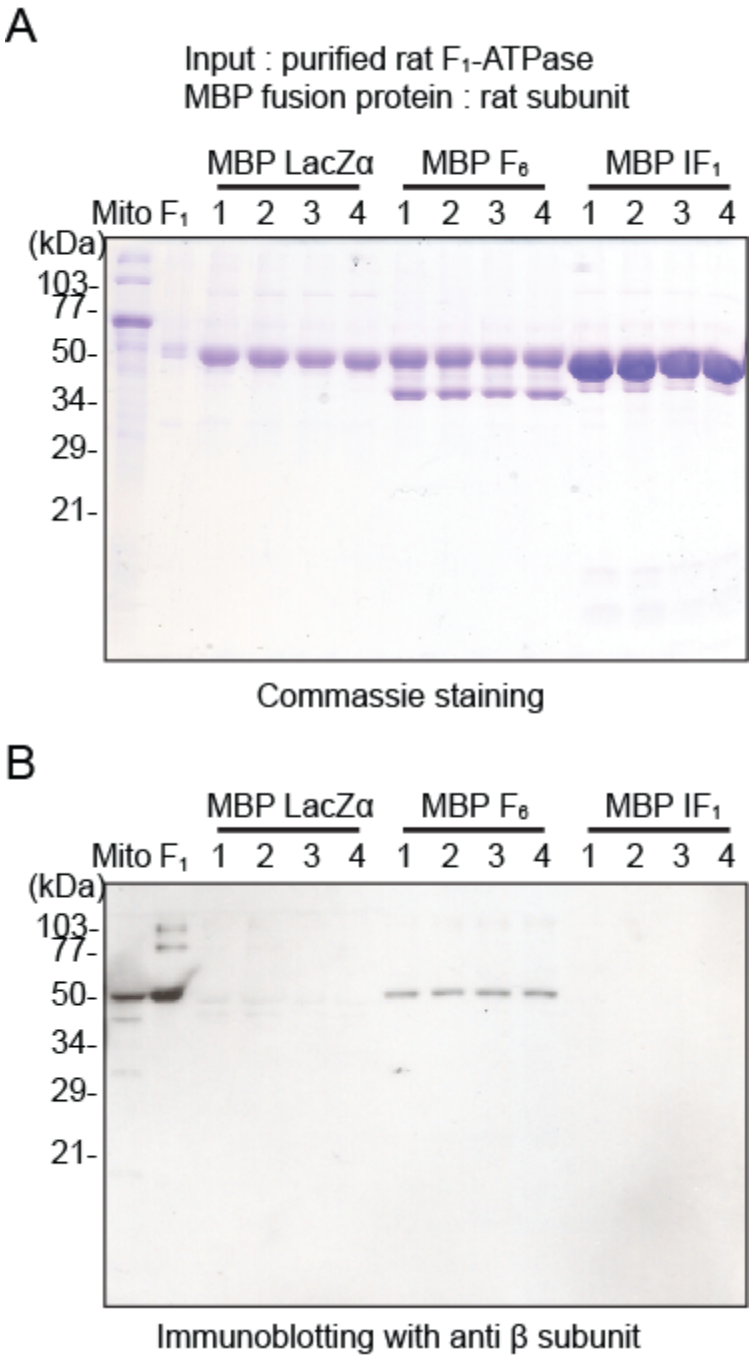

A
Input : purified rat F1-ATPase
MBP fusion protein : rat subunit
MBP LacZα
MBP Fε
MBP IF1
Mito F1
1 2 3 4 1 2 3 4 1 2 3 4
(kDa)
103-
77-
50-
34-
29-
21-
Commassie staining
B
MBP LacZα
MBP Fε
MBP IF1
Mito F1
1 2 3 4 1 2 3 4 1 2 3 4
(kDa)
103-
77-
50-
34-
29-
21-
Immunoblotting with anti β subunit

Figure 12

Inhibition of F_1-ATPase by AMP-PNP

The ATP hydrolytic activity of rat F_1-ATPase occurring for 2 minutes and 5 minutes was measured by the malachite green ATPase assay. Each sample was read against a zero time standard that had been removed previously from the reaction sample before adding the ATP-Mg^{2+} solution. The graph are expressed as percent of control ATPase activity (100%) for each reaction. Experiments were repeated 3 times. All results containing both F_1-ATPase and AMP-PNP were compared to the control reaction with F_1-ATPase alone by using Student's t-test. Data are presented as the mean± S.D (standard deviation). *** ($p < 0.001$) indicates a statistically significant effect of AMP-PNP on the ATPase hydrolytic activity of rat F_1-ATPase.

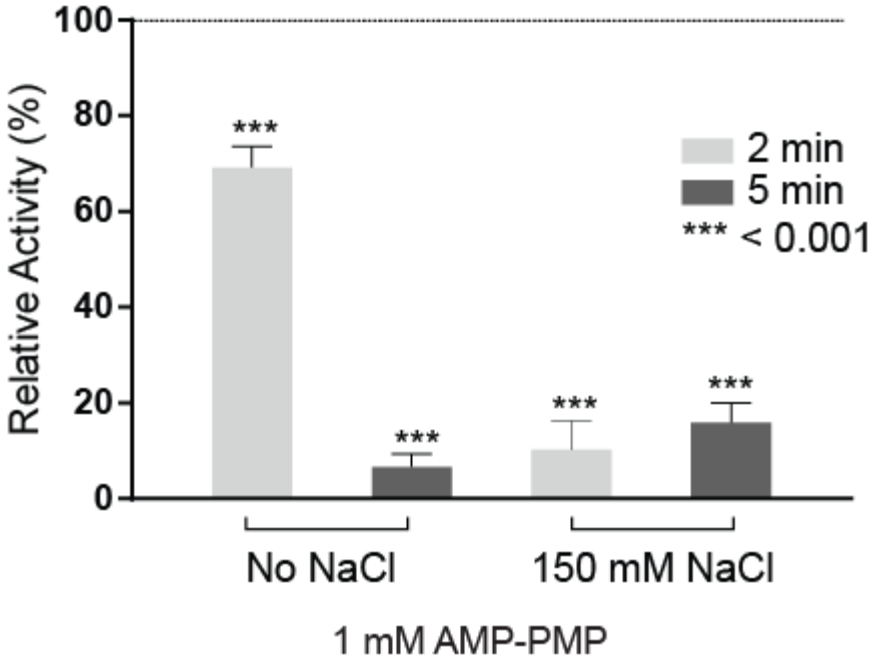
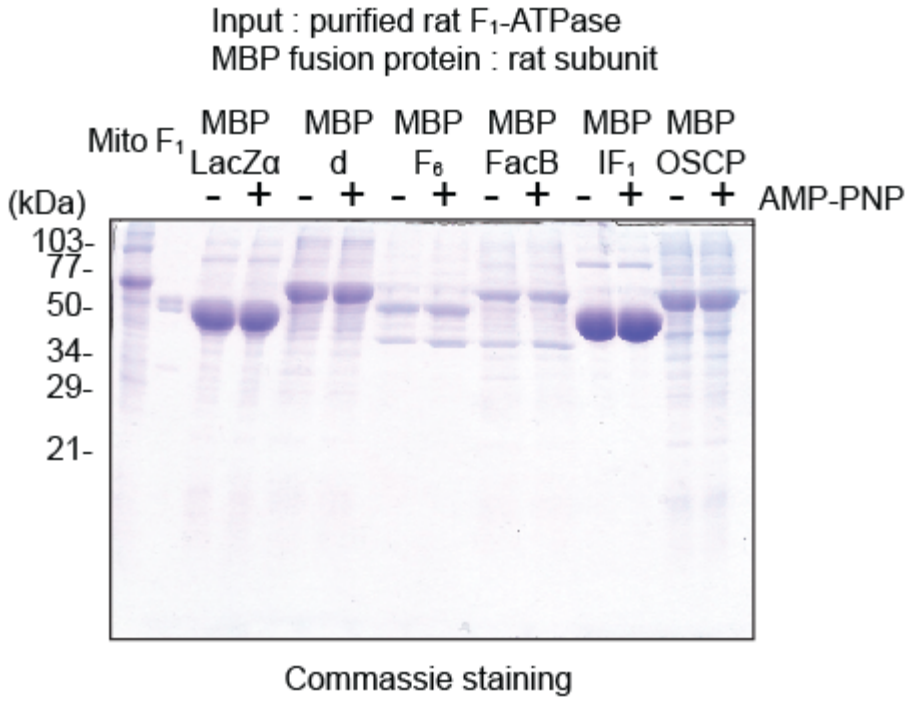

100
80
60
40
20
0
Relative Activity (%)
***
***
***
***
2 min
5 min
*** < 0.001
No NaCl
150 mM NaCl
1 mM AMP-PMP

Figure 13

MBP Pull-Down Assay of Purified Rat F_1-ATPase in the Condition Containing AMP-PNP

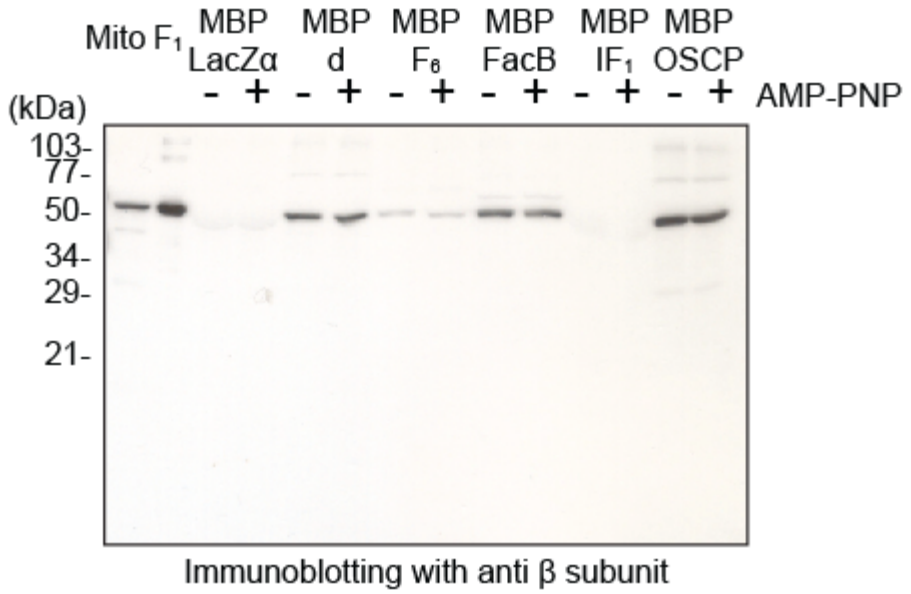
Each rat MBP fusion protein bound to amylose beads was incubated with 15 μg of isolated rat F_1-ATPase in PBS, 1 % Triton X-100 (**-**). The (**+**) lanes refer to conditions in which 1 mM AMP-PNP, 20 μM ADP, 2 mM $MgCl_2$ were present. After 1 hour incubation and 3 times washing with the same buffer, proteins attached to amylose resins were eluted with SDS sample buffer. The same amount of each sample was loaded for (**A**) Coomassie blue staining and (**B**) western blotting with an antibody to the β-subunit of F_1-ATPase.

A



Commassie staining

B

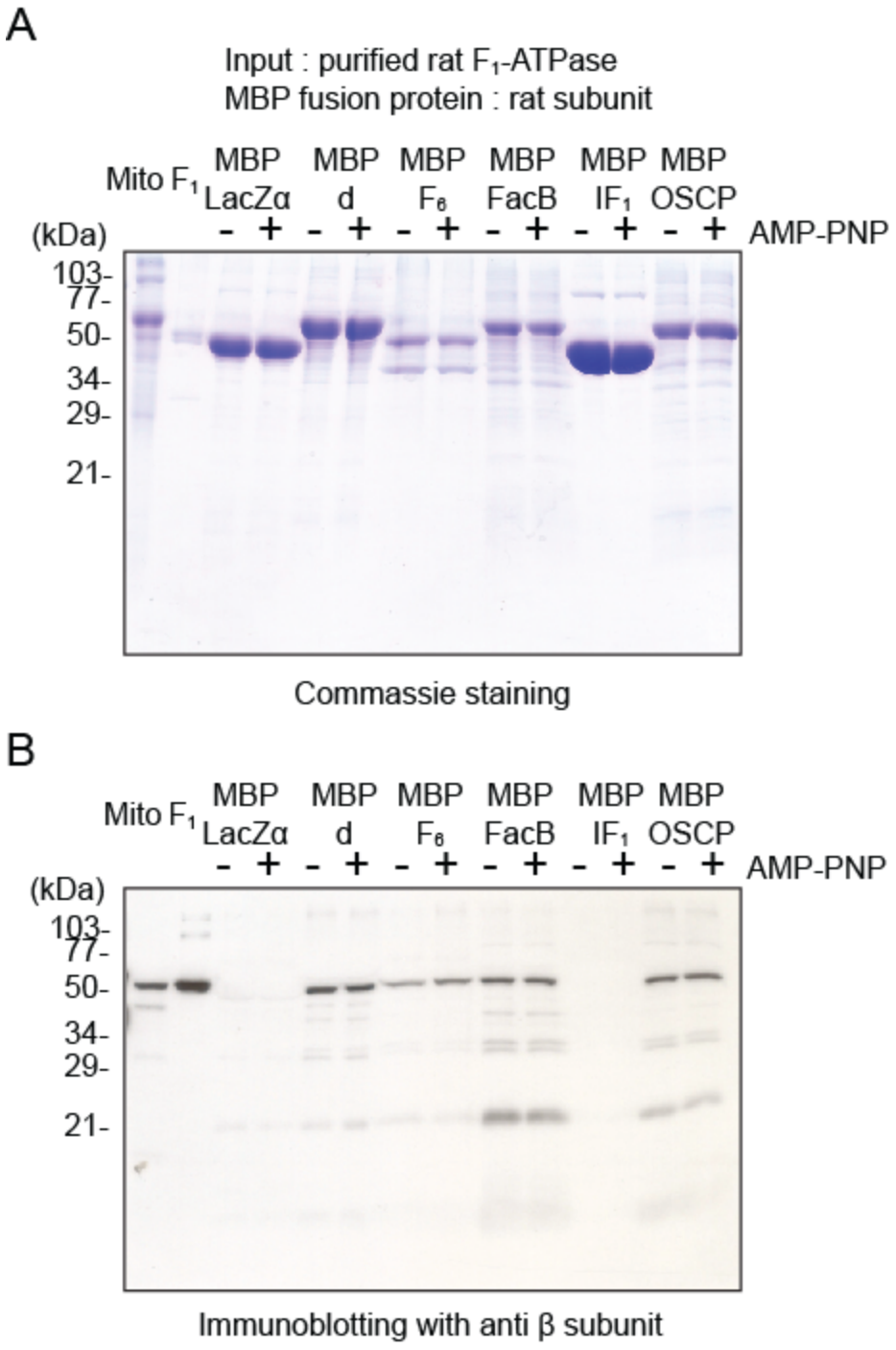


Immunoblotting with anti β subunit

Figure 14

MBP Pull-Down Assay of Purified Rat F_1-ATPase in a Reconstitution Buffer

Each rat MBP fusion protein bound to amylose beads was incubated with 15 μg of isolated rat F_1-ATPase in a reconstitution buffer (9) (20 mM Tris (pH 7.4), 50 mM sucrose, 100 mM NaCl, 2 mM $MgSO_4$, 1 mM EDTA, 0.001 % PMSF, 8 mM DTT, 0.02% NaN_3, and 10% glycerol) with (**+**) or without (**-**) 1 mM AMP-PNP, 20 μM ADP, and 2 mM $MgCl_2$ at room temperature. After 1 hr incubation and washing 3 times with the same buffer, proteins attached to amylose resins were eluted with SDS sample buffer. The same amount of each sample was loaded for (**A**) Coomassie blue staining and (**B**) western blot analysis with an antibody to the β-subunit of F_1-ATPase.


A
Input : purified rat F1-ATPase
MBP fusion protein : rat subunit
Mito F1
MBP LacZα
MBP d
MBP F6
MBP FacB
MBP IF1
MBP OSCP
- + - + - + - + - + - + AMP-PNP
(kDa)
103-
77-
50-
34-
29-
21-
Commassie staining
B
Mito F1
MBP LacZα
MBP d
MBP F6
MBP FacB
MBP IF1
MBP OSCP
- + - + - + - + - + - + AMP-PNP
(kDa)
103-
77-
50-
34-
29-
21-
Immunoblotting with anti β subunit

Chapter 3

Effect of Supernumerary Subunits on the ATPase Activity of F_1 Catalytic Component

Summary

Having shown that four of the rat liver mitochondrial ATP synthase's F_O subunits, d, F_6, Factor B and OSCP, can bind to its isolated catalytic F_1-ATPase component, we proceeded to determine whether each subunit alone could also affect its ATP hydrolytic activity. The free phosphate released from ATP following its hydrolysis by isolated F_1-ATPase in the presence of each subunit was measured under two different conditions, i.e., with and without NaCl (150 mM). The results showed under both conditions that subunits d, F_6 and Factor B, each alone, had a stimulatory effect on the ATPase activity of F_1. However, the subunit OSCP showed a biphasic effect, first displaying a stimulatory effect on the ATPase activity of F_1 at low concentrations and then a loss of this effect with a small inhibitory effect, at higher concentrations. In contrast, in the presence of NaCl, OSCP had a stimulatory effect on the ATP hydrolytic activity (ATPase activity) of F_1-ATPase in a concentration-dependent manner.

IF_1, a well-known inhibitory factor of the ATPase activity of F_1, also showed an inhibitory effect in experiments reported here in which NaCl was deleted but showed little effect when NaCl was present. Several combinations of the subunits d, F_6, Factor B, OSCP and IF_1 also confirmed the inhibitory effect of IF_1 and the stimulatory effects of subunits d, F_6, Factor B and OSCP in recovering the F_1-ATPase activity inhibited by IF_1. However, the data suggest that the subunits d, F_6, Factor B and OSCP do not share the same binding site as IF_1. Finally, an MBP pull-down assay was carried out without the detergent Triton X-100 and the same results were obtained thus eliminating the possibility that this detergent might have contributed to the results obtained.

Introduction

The mitochondrial ATP synthase's supernumerary subunits d, F_6 and OSCP are components of the peripheral stalk necessary during ATP synthesis to help retain the catalytic hexamer $(\alpha\beta)_3$ of the enzyme static against rotation of the central stalk (rotor) comprised of subunits γ, δ and ε. Also, supernumerary subunits d, F_6 and OSCP are known to be required for the F_1 subcomplex to reconstitute onto the F_O subcomplex and restore the fully active oligomycin sensitive holo-enzyme, i.e., the F_OF_1-ATPase/ATP synthase complex on the mitochondrial inner membrane. Therefore, the assays used to reveal the effects of the aforesaid supernumerary subunits, and other supernumerary subunits of the ATP synthase on the activities of the enzyme were focused on monitoring either the association of F_1 and F_O by a gain in oligomycin sensitive ATPase activity or the restoration of oxidative phosphorylation in a mitochondrial membrane preparation depleted of a certain subunit by a specific treatment. These experiments were necessary as reports related to rigorously determining whether there is a direct effect of each supernumerary subunit on the catalytic properties, i.e., ATP synthesis and ATP hydrolysis, of the mitochondrial ATP synthase are scarce. In fact, to our knowledge none have been reported for the rat liver enzyme.

During experiments to identify among the components of the peripheral stalk those that interact with the purified bovine F_1-ATPase, OSCP was found to stimulate the ATP hydrolytic activity and prevent cold inactivation of the complex, and subunits d and F_6 together with subunit b supported the effect of OSCP (1). In addition, the extracellular treatment of primary human umbilical vein endothelial cells (HUVECs) with F_6 increased the ATP hydrolytic activity of the ectopic ATP synthase inducing an intracellular acidification that supported proton transport into cells (2).

Factor B is suggested to block a proton leak, likely within the F_O part of the ATP

synthase. Factor B is lacking in ammonia, EDTA-treated submitochondrial particles (AE-SMP) prepared from well-coupled heavy bovine heart mitochondria subjected to ultrasound (sonication) at pH ~8.8 (adjusted with ammonium hydroxide). This creates a proton leak determined by a loss of both ATP-driven membrane potential formation and reverse electron transfer from succinate to NAD^+ (3). Therefore, a commonly used assay for Factor B has been to determine whether the suspected protein restores the membrane potential upon adding it to AE-SMPs that lack Factor B.

Significantly, IF_1 is a well-known and characterized inhibitor protein of the ATP synthase. It can inhibit the ATP hydrolytic activity but not the ATP synthetic activity of the complete ATP synthase (F_OF_1). IF_1 can inhibit also the ATPase activity of F_1 in the absence of F_O. Its capacity to bind to F_1-ATPase is highly dependent not only on pH but also on salt concentration (4). However, most reports have concentrated on the pH-dependent process because pH in the mitochondrial matrix decreases when the proton electrochemical gradient across the inner membrane is lost during hypoxic/ischemic conditions.

Also, IF_1 has been found in human serum where it is believed to be a regulator of the ectopic "cell surface" ATP synthase. In fact, IF_1 has been suggested to play an important role in the regulation of cholesterol HDL metabolism (5), not only because it is present in human serum but because it also positively correlates with HDL-cholesterol. For IF_1 to inhibit the ectopic ATP synthase, it must overcome the physiological salt concentration.

In this Chapter, we describe experiments in which interactions between each soluble supernumerary subunit and the catalytic F_1-ATPase moiety of the ATP synthase were monitored. The expectation was that such interactions would affect the catalytic ATPase activity of F_1. To avoid misinterpreting any of the results obtained that might have resulted from the detergent Triton X-100, we performed all MBP pull-down assays without detergent.

Materials and Methods

ATPase Activity

The malachite green ATPase assay was used to measure the amount of Pi resulting from the ATP hydrolytic activity of F_1-ATPase (6,7). For a 300 µl reaction, 0.6 µg of F_1-ATPase and varying amounts of each supernumerary subunit as described in figure legends were incubated in a 294 µl total volume of reaction buffer for 20 min. From this a 98 µl volume was taken as a zero time control before adding a 50 mM ATP-Mg^{2+} solution. The ATPase reaction was started by adding 4 µl of 50 mM ATP-Mg^{2+} solution to the remaining 196 µl volume of each reaction. At 2 min and 5 min, 100 µl of the reaction medium was taken and immediately added to 800 µl of the malachite green reaction solution. The reaction solution had been freshly prepared by mixing three volumes of 0.045% malachite green hydrochloride, one volume of 4.2% ammonium molybdate tetrahydrate in 4 N HCl and 1/200 volume of 4% Triton X-100. Then, 100 µl of 34% citric acid was added to stop color development. After about 20 min the OD_{660} was measured using Varian Cary 50 Bio UV/ Visible Spectrophotometer. Samples were read against a zero standard which had been removed before adding the ATP-Mg^{2+} solution. Also, the endogenous amount of free phosphate in the ATP-Mg^{2+} solution was measured at the same time. Protein concentration was measured with a colorimetric Bio-Rad Protein Assay kit based on the Bradford assay using BSA as a standard (8).

MBP Pull-Down Binding Assay with F_1-ATPase Pre-incubated with Purified Rat IF_1

To investigate whether IF_1 binding to F_1-ATPase inhibits the binding of other subunits, i.e., subunits d, F_6, Factor B and OSCP, an MBP pull-down assay was performed with each MBP fusion protein containing the purified IF_1 protein with some modifications. Amylose resins (100 µl), each with an attached MBP fusion protein, were freshly prepared from *E. coli*

cells overexpressing each protein and put in 450 µl of buffer containing 100 mM HEPES, 10% glycerol, 1 mM ATP, 2 mM $MgCl_2$, 1% Triton X-100 as necessary for assaying ATPase activity. Purified F_1-ATPase (15 ug) dissolved in 50% glycerol, 50 mM HEPES, pH 7.4, was incubated with purified IF_1 at 20-30 fold molar excess over F_1-ATPase in a 50 µl total volume of 100 mM HEPES, 10% glycerol, 1 mM ATP, 2 mM $MgCl_2$, 1% Triton X-100 at room temperature. After 20 min the solution was combined with the amylose beads, and then the same procedure as described in Chapter 2 was followed.

MBP Pull-Down Binding Assay without Triton X-100

MBP pull-down assays were performed using several buffer conditions and all without Triton X-100 as indicated in the figure legends. We followed the same procedure as described in Chapter 2.

Results

ATPase Activity

We found that the rat liver mitochondrial ATP synthase's supernumerary subunits d, F_6, Factor B and OSCP can interact directly with its F_1-ATPase catalytic component. To determine whether these interactions affect the catalytic activity of F_1-ATPase, the amount of Pi released from ATP was determined upon incubating in separate experiments the enzyme with increasing concentrations of each purified supernumerary subunit. The malachite green ATPase assay was used to measure the free phosphate released via a color change. This was measured in a spectrophotometer at OD_{660}. The purified subunits F_6, d, and Factor B showed a stimulatory effect on the hydrolytic activity of purified F_1-ATPase while as expected purified IF_1 showed an inhibitory effect (Figure 1). Moreover, these effects of IF_1 on ATPase activity of F_1 occurred in a concentration-dependent manner. In contrast to IF_1, F_6, d, and Factor B, the supernumerary subunit OSCP showed a biphasic effect. Initially, OSCP stimulated the ATPase activity as the concentration of this subunit increased to a maximum of 10 times more than the amount of F_1-ATPase. Then, OSCP slightly inhibited the activity at the higher concentration.

Under conditions where150 mM NaCl was present, F_1-ATPase alone showed a lower activity than under conditions without NaCl. The purified subunits F_6, d, and Factor B showed a stimulatory effect on the hydrolytic activity of purified F_1-ATPase (Figure 2). Remarkably, with NaCl present OSCP showed a highly stimulatory effect in a concentration-dependent manner in contrast to conditions without NaCl. Interestingly, the inhibitory effect of IF_1 was reduced significantly under conditions containing NaCl.

Combinations

Several supernumerary subunit combinations in the assays for ATPase activity showed

an effect similar to that of individual subunits (Figure 3). In HEPES buffer without NaCl, F_6, d, Factor B and OSCP, each added in an amount 10 times more than F_1-ATPase, showed a stimulatory effect on its ATPase activity while IF_1 showed an inhibitory effect. The most effective stimulatory combination was the mixture of d, F_6, Factor B and OSCP. In other experiments, F_6, d, Factor B and OSCP were able to mitigate the inhibitory effect of IF_1. This is of interest as IF_1 inserts its N-terminal region into the interface between subunits α and β whereas based on structural data subunits F_6, d, Factor B and OSCP are thought to bind to the surface of F_1-ATPase. In 150 mM NaCl, IF_1 did not show an inhibitory effect.

Sharing the IF_1 Binding Site

The subunits d, F_6, Factor B or OSCP were each able to recover the activity of F_1-ATPase inhibited by IF_1. This suggested that these subunits may be competitive with IF_1 for its binding site on F_1 (F_1-ATPase). To test this possibility, MBP pull-down assays were performed following incubation of each subunit with purified rat F_1 that had been pre-incubated with more than a 30 times molar excess of IF_1 relative to F_1 (Figure 4). If the subunits noted above are competitive with IF_1 for a binding site on F_1, it would contribute to a reduction or loss of isolated F_1 in the pull-down assay for each subunit. However, even in the presence of IF_1, the amount of pull-downed F_1-β-subunit by the subunits d, F_6, Factor B or OSCP did not change. Therefore, these findings indicate that the subunits d, F_6, Factor B and OSCP do not block the interaction of IF_1 with F_1.

IF_1 in NaCl

Another interesting finding was that IF_1's inhibitory effect was significantly reduced in reactions containing 150 mM NaCl. To date, a change in pH has been considered as the most important factor responsible for IF_1's capacity to inhibit the ATPase activity of F_1. However, IF_1 was found in human serum suggesting that it must be able to inhibit the atypical (cell

surface) ATP synthase at physiological salt concentration. To investigate the effect of NaCl on the IF_1 inhibitory activity at optimal pH (~6.5), we compared the ATPase activity of F_1 in the presence of IF_1 both at pH 6.5 (in 100 mM Bis-Tris and MES buffer) and at pH 7.4 (in 100 mM HEPES buffer) (Figure 5). At either pH, NaCl negatively affected the inhibitory action of IF_1 on the ATP hydrolytic activity of F_1 although at pH 6.5 IF_1 did function a little more actively than at pH 7.4.

MBP Pull Down Assays without Triton X-100

In order to rule out any additional effect of the detergent (Triton X-100), e.g., in denaturing a protein (subunit) or promoting a certain interaction, pull down assays were carried out without detergent and 10 % glycerol was added to improve the stability of F_1. As under the previously described conditions with detergent, all assays showed the same results. First, the MBP-fusion proteins constructed with ATP synthase subunit d, F_6, Factor B or OSCP successfully pulled down rat F_1 as determined by immunoblotting with an antibody against the β-subunit. In contrast, the negative controls, MBP-lacZα or MBP-rat-IF_1 in which the MBP protein was attached to the N-terminus of rat IF_1, did not capture any β-subunit. Second, we reconfirmed these interactions under the HEPES buffer condition used for the malachite green ATPase assay (Figure 6, Lanes 1 and 2), and under the PBS condition used extra-cellularly in the previous assays (Figure 6. Lane 3 and 4). In both cases, adding more NaCl did not affect the binding data. Third, MBP-rat subunit d, MBP-rat Factor B and MBP-rat OSCP, as well as MBP-rat F_6, could also pull down the F_1-ATPase independent of treatments with ATP, ADP, Mg^{2+} and EDTA (Figure 6. Lane 5 to 9). Forth, adding IF_1 to and pre-incubating it with F_1-ATPase did not reduce the amount of the captured β-subunit by subunit d, F_6, Factor B and OSCP, suggesting that these subunits bind to sites on F_1 independent of IF_1 (Figure 6. Lane 10). Taken together, these results demonstrate that the

supernumerary subunits d, F_6, Factor B and OSCP of the mitochondrial rat liver ATPase/ATP synthase complex can each associate with its F_1-ATPase catalytic unit and affect its activity.

Discussion

Effect of Rat Liver ATP Synthase's Supernumerary Subunits on the Activity of Its F_1-ATPase Catalytic Component

In agreement with a previous study to assess the effect of the supernumerary subunits forming the peripheral stalk of the bovine heart mitochondrial ATPase/ATP synthase on its ATPase activity (1), in work reported here on the rat liver ATPase/ATP synthase we showed that subunits d, F_6 and OSCP (each alone) also have a stimulatory effect on the ATPase activity of its isolated rat F_1-ATPase component. In addition, we demonstrated that rat liver Factor B alone can also stimulate the activity of rat F_1-ATPase but with a lesser effect than the other subunits tested. Considering that the primary role of Factor B is believed to be to block a putative proton leak through the F_O subcomplex, then its role as a direct regulator of the ATPase activity of F_1 may be secondary. Interestingly, it was reported that Bcl-x_L has a role similar to Factor B in the mitochondria. Thus, Bcl-x_L can block a proton leak within the ATP synthase complex and interact directly with the β-subunit of the ATP synthase as determined by co-immuno-precipitation experiments. Similar to Factor B as shown in this study, the recombinant Bcl-x_L protein alone can stimulate the ATP hydrolytic activity of purified ATP synthase (9).

Recently various studies have demonstrated that some of the above noted supernumerary subunits that actively participate in the regulation of the catalytic activity of F_OF_1-ATP synthase are involved also in other types of interactions, i.e., "moonlighting roles". Thus, subunit d interacted with the δPKC isozyme and inhibited ATP hydrolytic activity of the ATP synthase following prolonged hypoxia (10). This effect was independent of IF_1, a putative regulator of ATP hydrolysis during ischemia. Also, a peptide inhibitor made up of the binding sequence of ATP synthase's subunit d together with an inhibitor of the δPKC

interaction reduces cardiac troponin I release from ischemic rat hearts. This finding suggests that subunit d may play a significant role in helping prevent cardiac ischemic injury (11). Also, OSCP is known to be a direct binding partner with other proteins such as Sirtuin3 (12) and cyclophilin D (CyPD) (13) as well as with 1,4-benzodiazepine (Bz-423), an inhibitor of ATP synthase, (14). Interestingly, Sirtuin3 binding stimulates ATPase activity which is mediated by deacetylation of OSCP, and Bz-423 is an uncompetitive inhibitor that inhibits both ATP synthesis and hydrolysis. In addition, it is well known that CyPD is an activator of the mitochondrial permeability transition pore (PTP) involved in cell death and binds to the same binding site on OSCP as Bz-423.

Taken together, studies reported here provide support for the view that the supernumerary subunits indicated above can modulate the activity of ATP synthase alone or through other regulators. Thus, the experimental results provide rather compelling evidence that the indicated supernumerary subunits do regulate the catalytic activity of the F_1-ATPase subcomplex, and likely that Sirtuin3, CyPD, Bz-423, and other agents act through these subunits to further regulate the enzyme activity of ATP synthase. Supernumerary subunits d, F_6 and OSCP as components of the peripheral stalk not only connect with the catalytic $\alpha_3\beta_3$ hexamer of F_1 and the proton channel of F_O to facilitate structural and functional coupling required for ATP synthesis, but also modulate the activity of the enzyme complex either directly or via native regulators. Also, it is both interesting and important to note that the subunit "Factor B" modulates the activity of ATP synthase by not only blocking the proton leak through the F_O subcomplex, but also via its direct interaction with the enzyme.

Significantly, subunits d, F_6, Factor B and OSCP are able to rescue F_1 from inhibition induced by its natural inhibitor IF_1. Although the mechanism by which the aforesaid subunits do this remains unknown, it seems likely that each is able to induce via a conformational

change the release of IF_1 from F_1 rather than by competing for IF_1's binding site on F_1. This is based on MBP pull-down assays to test whether these proteins share the same binding site with IF_1 on F_1. The results support the view that IF_1 shares its binding site on F_1 with no other subunit. Thus, the subunits d, F_6, Factor B and OSCP were unable to block competitively the binding of IF_1 to F_1-ATPase.

Effect of ATP Synthase Supernumerary Subunits on the Ectopic ATP Synthase in the Extracellular Environment

As note earlier, the supernumerary subunit F_6 is present in human serum and was found to bind to the β-subunit of the ectopic ("cell surface") ATP synthase of endothelial cells and increase its ATP hydrolytic activity (2). This resulted in an increase of proton influx into the cells and a decrease of intracellular pH. Significantly, in work reported here, we also showed that purified F_6 can stimulate the ATP hydrolytic activity of the isolated rat F_1-ATPase. Although to date, subunits d, Factor B and OSCP have not been reported in human serum, we would predict that these subunits, as F_6, would also have a stimulatory effect on the ATPase activity of the cell surface ATP synthase. Also, considering the higher effects of the subunits d and OSCP than that of F_6, we might predict that they would have a higher stimulatory effect on the ATPase activity of ATP synthase in an extracellular environment. Nevertheless, we must wait until we know the extent to which these proteins appear in the serum and whether this occurs under normal physiological conditions or more so in certain pathological/disease states.

Considering physiological salt concentration, IF_1 in serum may have a problem in affecting the activity of the ectopic ATP synthase. In our analysis, IF_1 did not inhibit the purified F_1-ATPase at physiological pH (pH 7.4) and salt concentration (150 mM NaCl) in HEPES buffer. At pH 6.5, the optimal pH for IF_1, it also did not show statistically significant

inhibition of the F_1-ATPase. Significantly, it has been previously reported that at high salt concentration IF_1 easily dissociates from the ATP synthase resulting in its reactivation (15-17). Although IF_1 has been found in human serum and suggested to regulate HDL-cholesterol metabolism in hepatocytes and endothelial cells, it must be explained how IF_1 affects the catalytic activity of the ectopic ATP synthase at physiological pH and salt concentration.

MBP Pull Down Assay without Triton X-100

Triton X-100 used in the MBP pull-down assay is described in Chapter 2. It is a detergent that has been used in an early step in the purification of both the yeast and mammalian ATP synthase, i.e., to extract them from mitochondria (18,19). However, it has been reported that Triton X-100 can remove IF_1 from the ATP synthase at higher concentrations causing it to disassociate F_1 from F_O in yeast (20). Therefore, we designed another set of MBP pull-down assays to rule out any possibility that the detergent induces denaturation of F_1 or promotes non-specific interactions. Indeed, because these assays were performed together with negative controls, we can verify the results regardless of addition of the detergent. Through this set of MBP pull-down assays without the detergent, we also reconfirmed the interactions of the subunits d, F_6, Factor B and OSCP with F_1.

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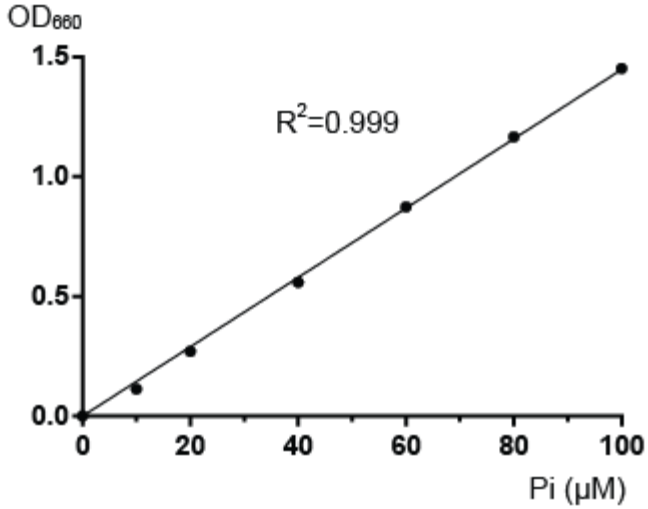
Figure 1

Effect of Supernumerary Subunits on the ATPase Activity of Isolated Rat F_1-ATPase Assayed in the Absence of NaCl

ATPase activity was assayed as described under Methods. The ATPase activity of F_1-ATPase alone obtained after 5 minutes in the presence of 20% Glycerol, 100 mM HEPES (pH 7.4) was used as a control; results are expressed as percent of control ATPase activity. Supernumerary subunits were present in molar excess as indicated in each graph. Experiments were repeated 3 times. Raw data were analyzed using ANOVA a subsequent Dunnett's post hoc test to analyze the statistical significance relative to the control. Data are presented as the mean± S.D (standard deviation). * ($p < 0.05$), ** ($p < 0.01$) and *** ($p < 0.001$) indicate a statistically significant effect on the hydrolytic activity of rat F_1-ATPase. (**A**), standard curve with KH_2PO_4; (**B**), reactions containing purified rat subunit d; (**C**), reactions containing purified rat F_6; (**D**), reactions containing purified rat Factor B; (**E**), reactions containing purified rat subunit IF_1; (**F**), reactions containing purified rat OSCP.

A



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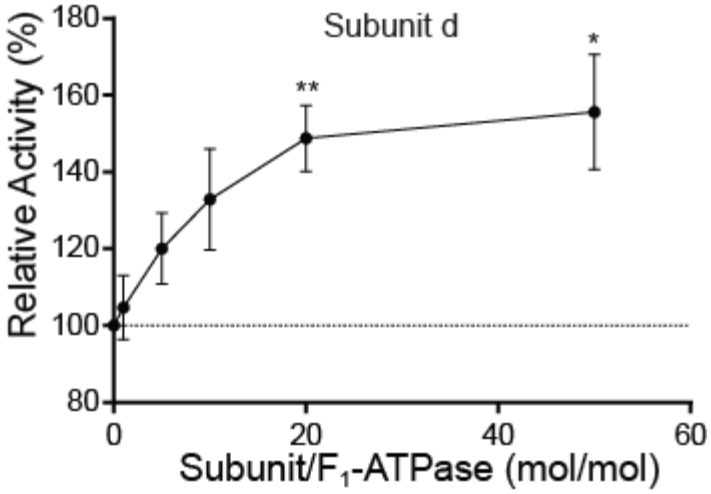

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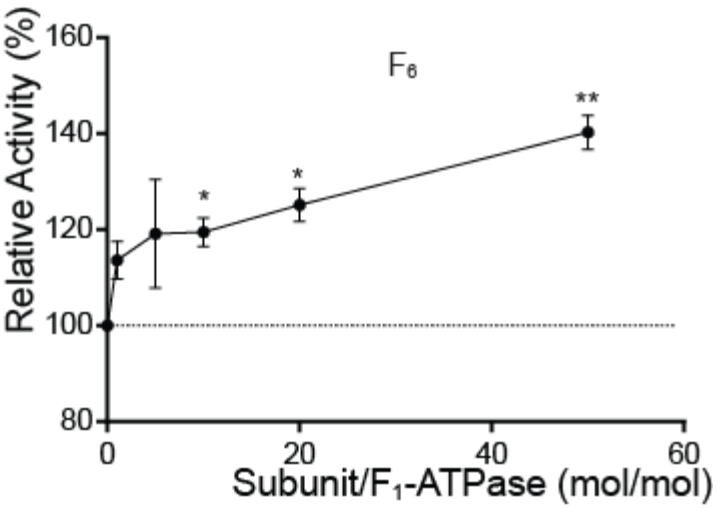

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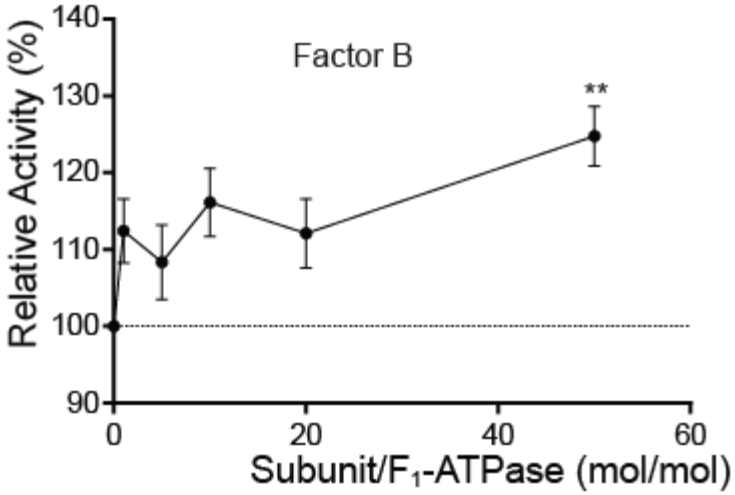
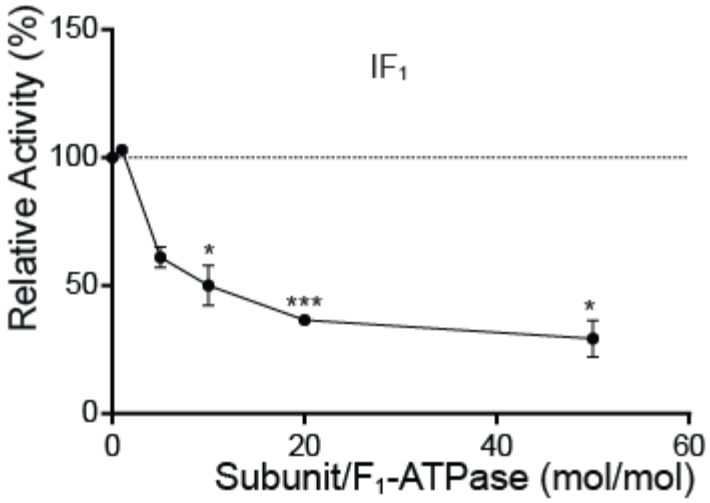

Factor B
Relative Activity (%)
140
130
120
110
100
90
0
20
40
60
Subunit/F1-ATPase (mol/mol)
**

E


IF1
Relative Activity (%)
150
100
50
0
0
20
40
60
Subunit/F1-ATPase (mol/mol)
*
***
*

F

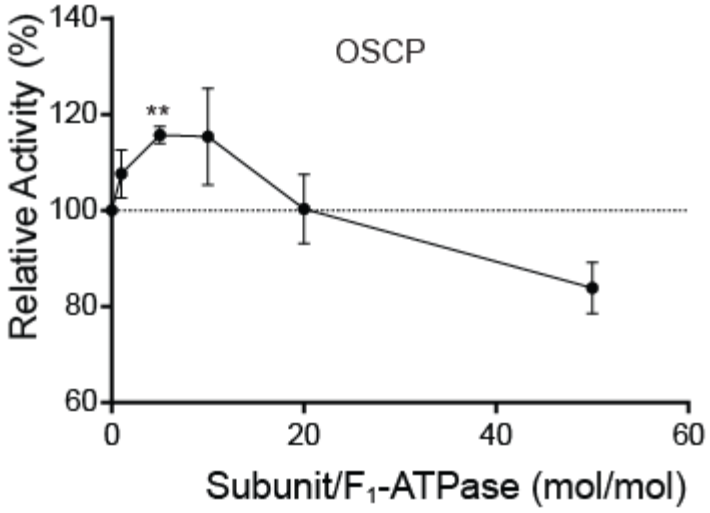
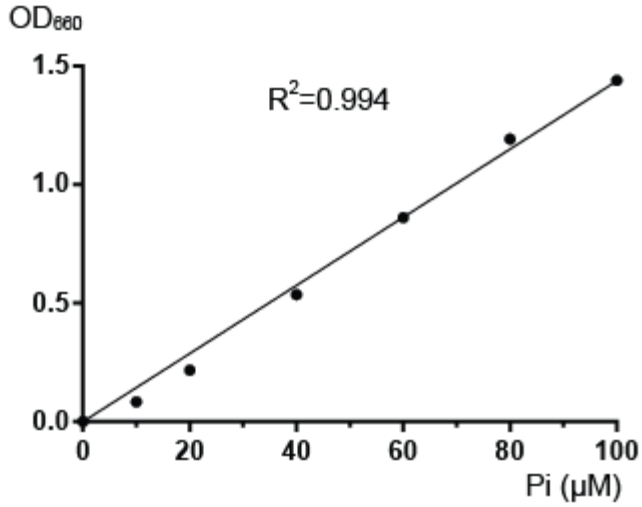

OSCP
Relative Activity (%)
140
120
100
80
60
0
20
40
60
Subunit/F1-ATPase (mol/mol)
**

Figure 2

Effect of Supernumerary Subunits on the ATPase Activity of Isolated Rat F_1-ATPase Assayed with 150 mM NaCl

ATPase activity was measured as described in the legend to Figure 1 except that 150 mM NaCl was included in all assays. Experiments were repeated 3 times. Raw data were analyzed using ANOVA a subsequent Dunnett's post hoc test to analyze the statistical significance relative to the control. Data are presented as the mean± S.D (standard deviation). * ($p < 0.05$), ** ($p < 0.01$) and *** ($p < 0.001$) indicate a statistically significant effect on the hydrolytic activity of rat F_1-ATPase. (**A**), standard curve with KH_2PO_4; (**B**), reactions containing purified rat subunit d; (**C**), reactions containing purified rat F_6; (**D**), reactions containing purified rat Factor B; (**E**), reactions containing purified rat subunit IF_1; (**F**), reactions containing purified rat OSCP.



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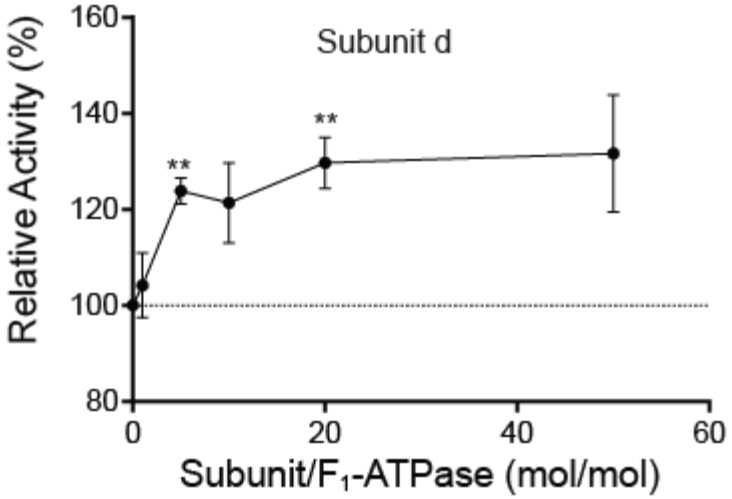

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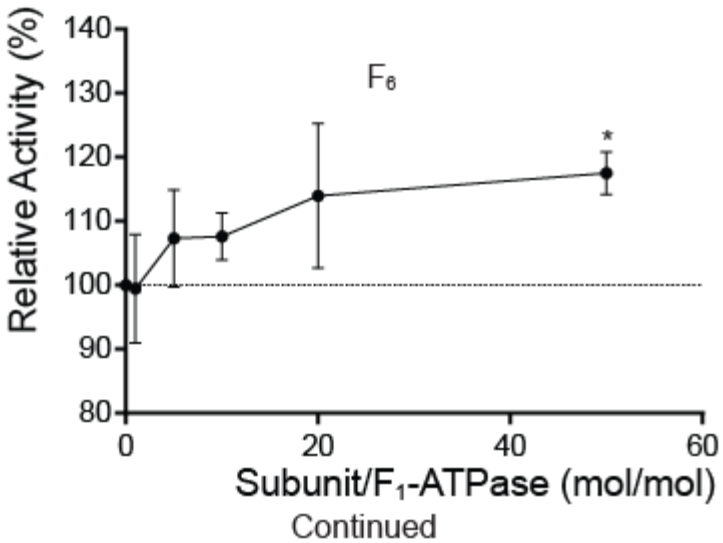

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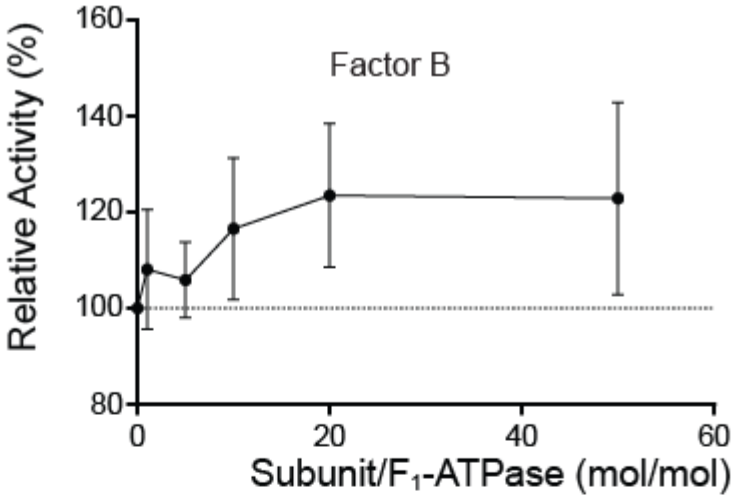
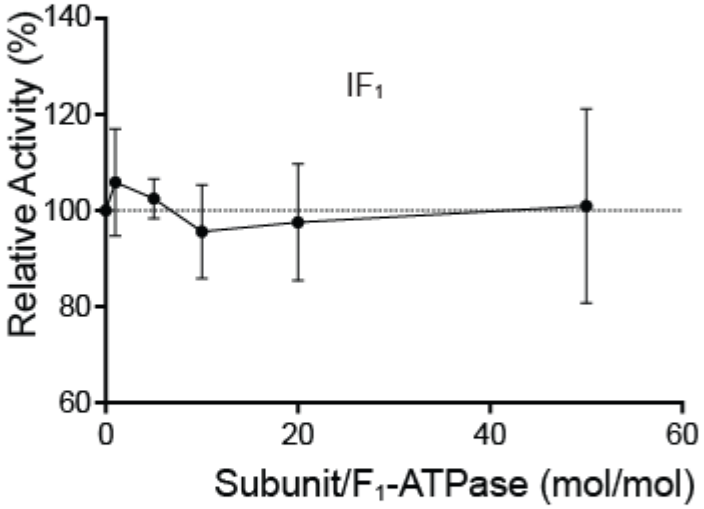

Factor B
Relative Activity (%)
160
140
120
100
80
0
20
40
60
Subunit/F1-ATPase (mol/mol)

E


IF1
Relative Activity (%)
140
120
100
80
60
0
20
40
60
Subunit/F1-ATPase (mol/mol)

F

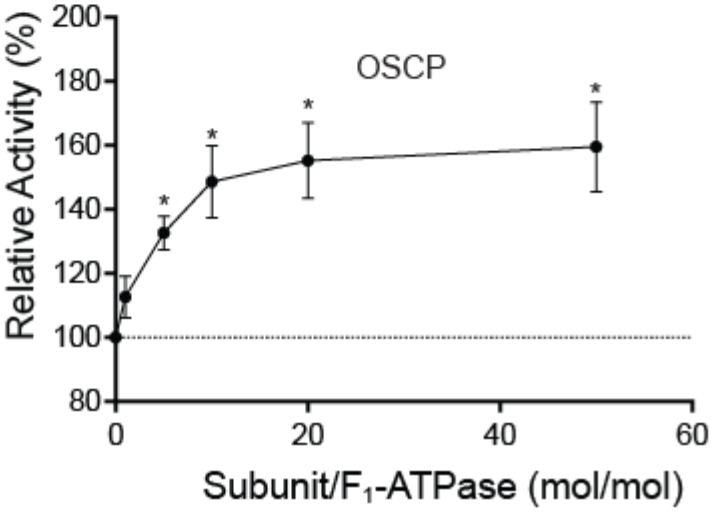
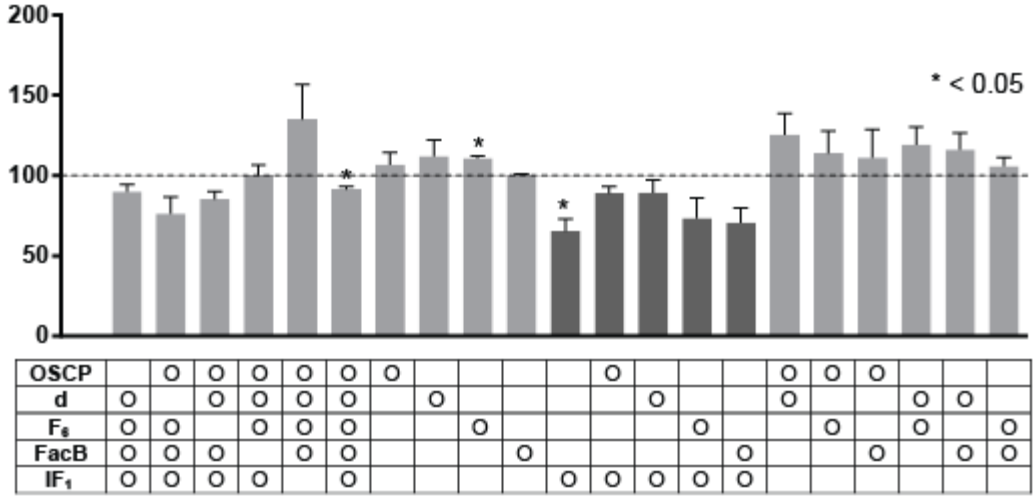

OSCP
Relative Activity (%)
200
180
160
140
120
100
80
0
20
40
60
Subunit/F1-ATPase (mol/mol)
*
*
*
*

Figure 3

ATPase Activity of Isolated Rat F_1-ATPase Incubated with Combinations of Supernumerary Subunits

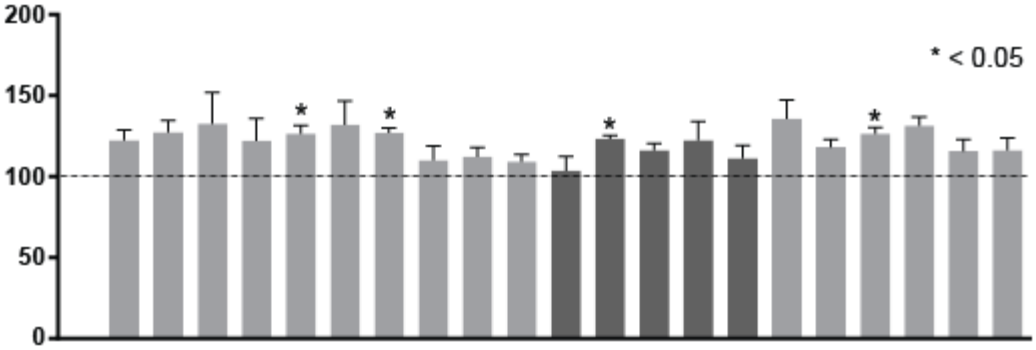
F_1-ATPase was incubated either alone or with a 10-fold molar excess of the indicated purified supernumerary subunits in the reaction buffer of (**A**) 20% glycerol and 100 mM HEPES (7.4) or (**B**) 20% glycerol, 150 mM NaCl, and 100 mM HEPES (pH 7.4) for 20 min and ATPase activity was measured after 5 min of incubation with ATP. The data are expressed in the graph as activity relative to the control, the reaction containing F_1-ATPase alone. Dark bars were used to emphasize the recovering effects of the subunits d, F_6, Factor B and OSCP on the hydrolytic activity of F_1-ATPase inhibited by IF_1. Experiments were repeated 3 times. Raw data were analyzed using ANOVA a subsequent Dunnett's post hoc test to analyze statistical significance relative to the control. Data are presented as the mean± S.D (standard deviation). * ($p < 0.05$) indicates a statistically significant effect on the hydrolytic activity of rat F_1-ATPase.

A



OSCP		O	O	O	O	O	O					O				O	O	O			
d	O		O	O	O	O		O					O			O			O	O	
F_6	O	O		O	O	O			O					O			O		O		O
FacB	O	O	O		O	O				O					O			O		O	O
IF_1	O	O	O	O		O					O	O	O	O	O						

B



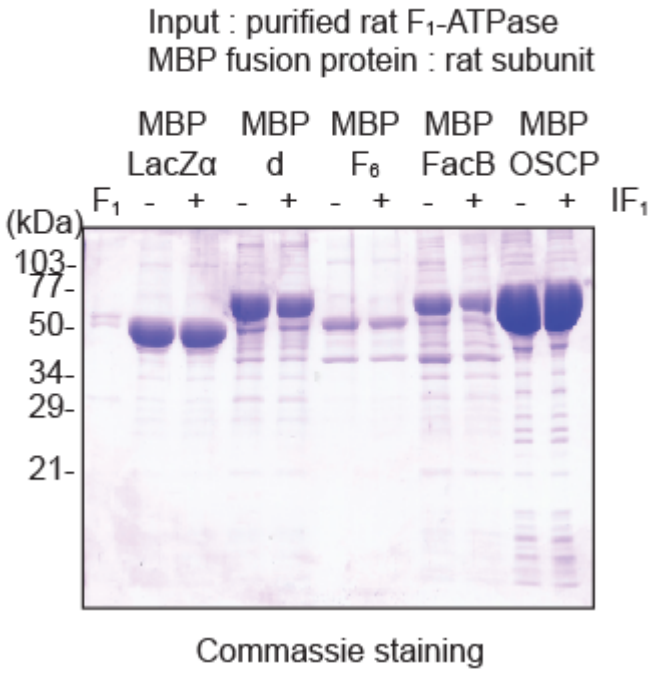
OSCP		O	O	O	O	O	O					O				O	O	O			
d	O		O	O	O	O		O					O			O			O	O	
F_6	O	O		O	O	O			O					O			O		O		O
FacB	O	O	O		O	O				O					O			O		O	O
IF_1	O	O	O	O		O					O	O	O	O	O						

Figure 4

MBP Pull-Down Binding Assay with F_1-ATPase Pre-incubated with Purified Rat IF_1

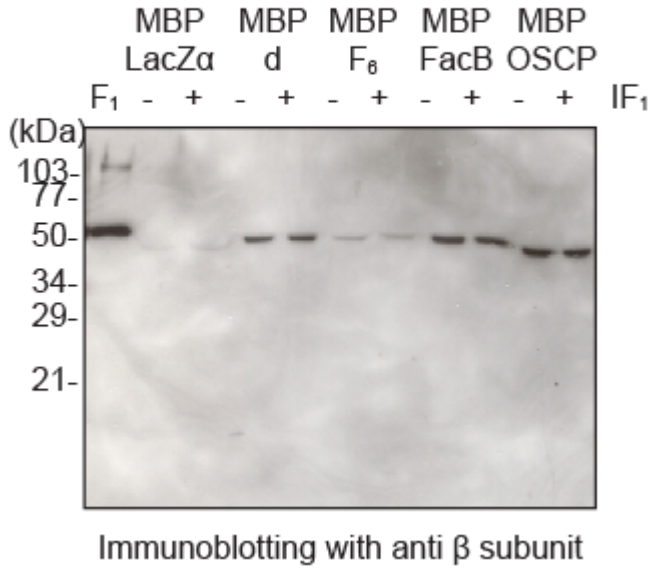
To identify whether the subunits d, F_6, Factor B or OSCP bind to the same site on F_1-ATPase as IF_1, 15 μg of F_1-ATPase was pre-incubated with a 30-fold molar excess of purified rat IF_1 for 20 min. Considering the requirement of ATP hydrolysis for IF_1 binding to F_1-ATPase and the previously noted condition to measure ATPase activity, the incubation buffer contained 100 mM HEPES (pH 7.4), 20% glycerol, 1 mM ATP, 2 mM $MgCl_2$, and 1% Triton X-100. Other steps are as indicated in Chapter 2.

A



Commassie staining

B



Immunoblotting with anti β subunit

Figure 5

ATPase Assay Activity of Isolated Rat F_1-ATPase and IF_1 at pH 6.5 vs. 7.4

The ATPase activity of isolated F_1-ATPase was assayed either in HEPES, pH 7.4, or in 100 mM Bis-Tris/100 mM MES, pH 6.5. Both buffers contained 20% glycerol. For physiological salt concentration, 150 mM NaCl was added. A 10 fold molar excess of IF_1 was incubated with F_1-ATPase for 20 min. Subsequent procedures were the same as described above for the malachite green reaction. Results are expressed as activity relative to the control, i.e., the reaction containing F_1-ATPase alone. Experiments were repeated 3 times. Raw data were analyzed using ANOVA a subsequent Sidak's post hoc test to identify statistical significance relative to the control. Data are presented as the mean± S.D (standard deviation). *** ($p < 0.001$) indicates a statistically significant effect on the hydrolytic activity of rat F_1-ATPase.

A

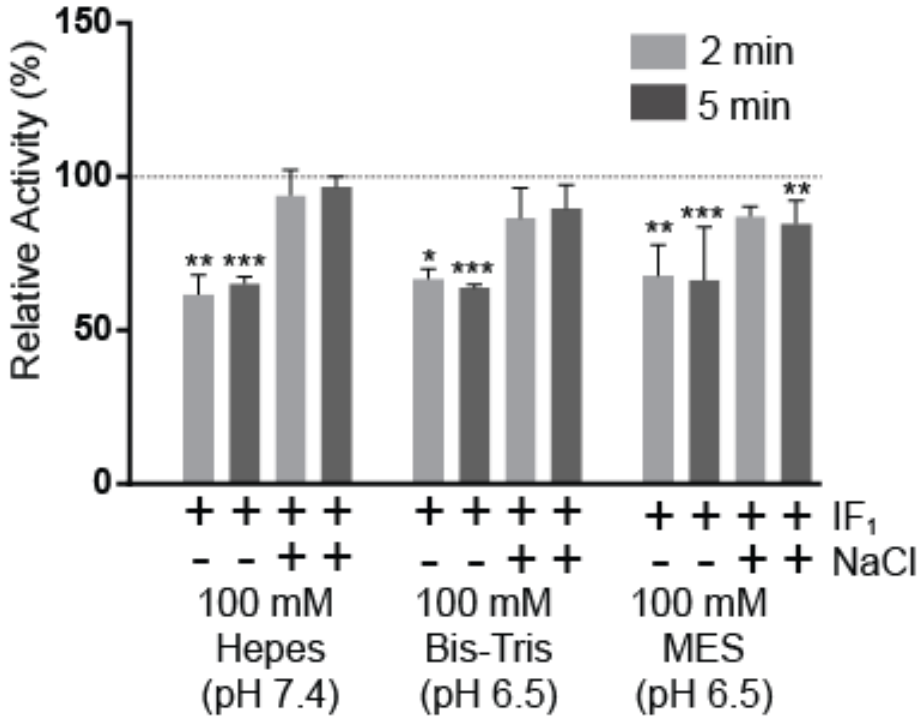
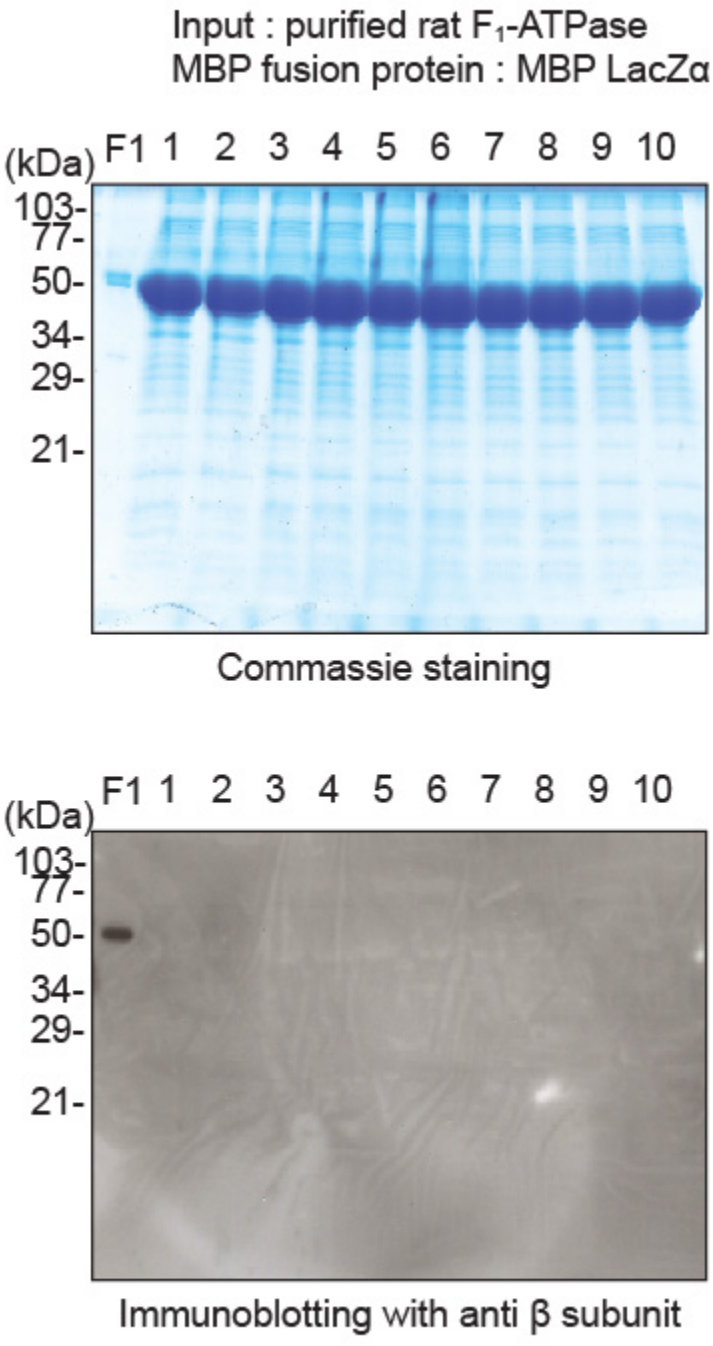

150
100
50
0
Relative Activity (%)
2 min
5 min
** ***
*
***
**
***
**
+ + + + + + + + + + + + IF1
- - + + - - + + - - + + NaCl
100 mM Hepes (pH 7.4)
100 mM Bis-Tris (pH 6.5)
100 mM MES (pH 6.5)

Figure 6

MBP Pull-Down Binding Assay without Triton X-100

Amylose resins (100 µl), each with a MBP fusion protein attached, were freshly prepared following each protein's overexpression in *E. coli* cells. Each was then added to 15 µg of F_1-ATPase solution. These MBP pull-down assays were performed in several buffer conditions without Triton X-100. The following steps were carried out as indicated in Chapter 2. (**A**), MBP pull-down assay with MBP-lacZα; (**B**), MBP pull-down assay with rat MBP-subunit d; (**C**), MBP pull-down assay with rat MBP-F_6; (**D**), MBP pull-down assay with rat MBP-Factor B; (**E**), MBP pull-down assay with rat MBP-IF_1; (**F**), MBP pull-down assay with rat MBP-OSCP. Each lane contains the following composition. **Lane 1**, 100 mM HEPES (pH 7.4), 10% glycerol; **lane 2**, 100 mM HEPES (pH 7.4), 10% glycerol, 150 mM NaCl; **lane 3**, PBS, 10% glycerol; **lane 4**, PBS, 10% glycerol, 100 mM NaCl; **lane 5**, PBS, 1 mM ATP, 2 mM $MgSO_4$, 10% glycerol; **lane 6**, PBS, 1 mM ADP, 2 mM $MgSO_4$, 10% glycerol; **lane 7**, PBS, 2 mM $MgSO_4$, 10% glycerol; **lane 8**, PBS, 1 mM ATP, 1 mM ADP, 2 mM $MgSO_4$, 10% glycerol; **lane 9**, PBS, 1 mM EDTA, 10% glycerol; **lane 10**, PBS, 1 mM ATP, 2 mM $MgSO_4$, 10% glycerol, and 20-fold molar excess of purified rat IF_1 to rat F_1-ATPase.

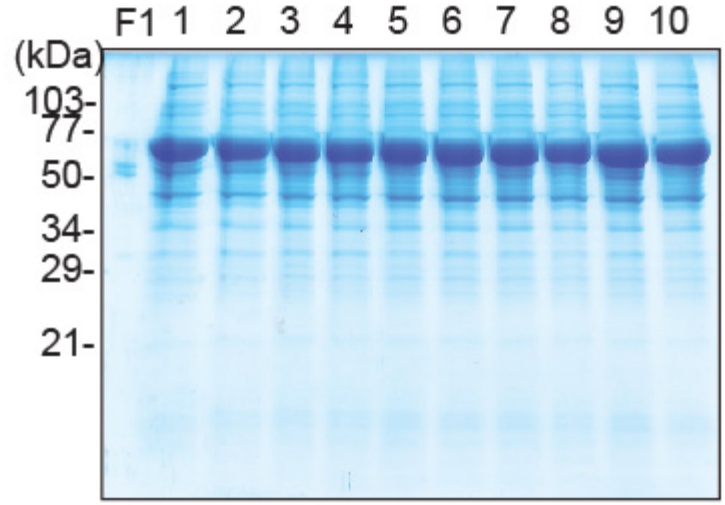
A


Input : purified rat F_1-ATPase
MBP fusion protein : MBP LacZα
(kDa)
F1 1 2 3 4 5 6 7 8 9 10
103-
77-
50-
34-
29-
21-
Commassie staining
(kDa)
F1 1 2 3 4 5 6 7 8 9 10
103-
77-
50-
34-
29-
21-
Immunoblotting with anti β subunit

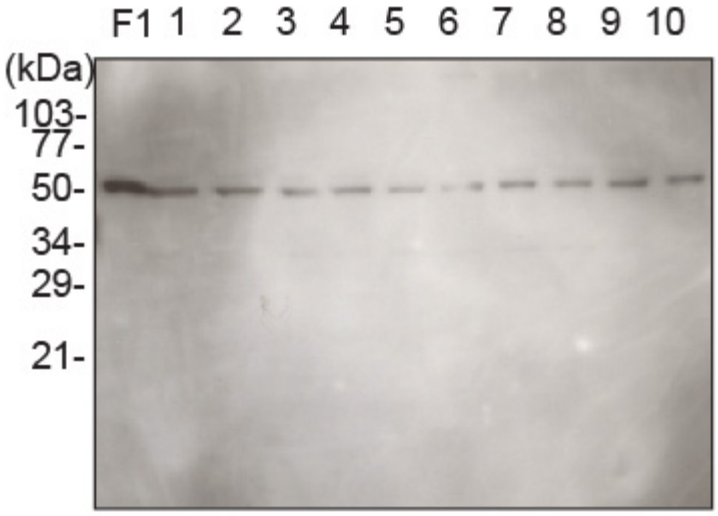
Continued

B

Input : purified rat F_1-ATPase
MBP fusion protein : MBP rat subunit d



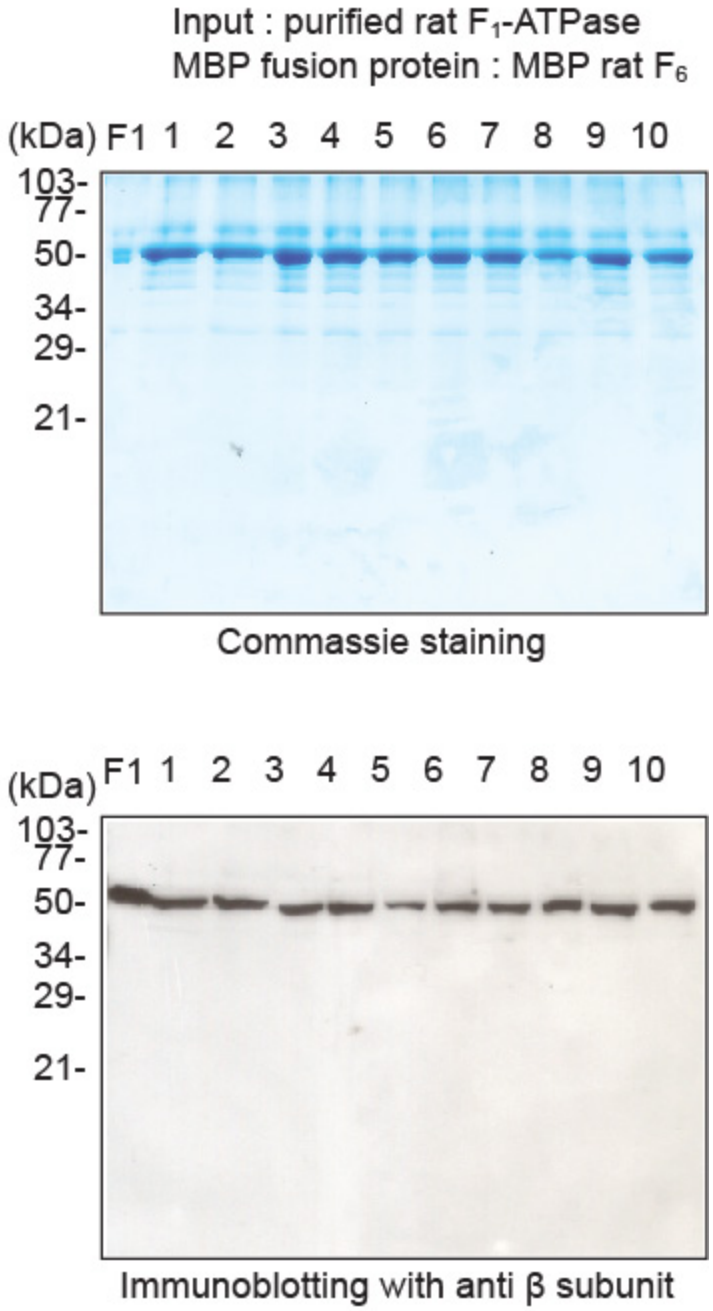
Commassie staining



Immunoblotting with anti β subunit

Continued

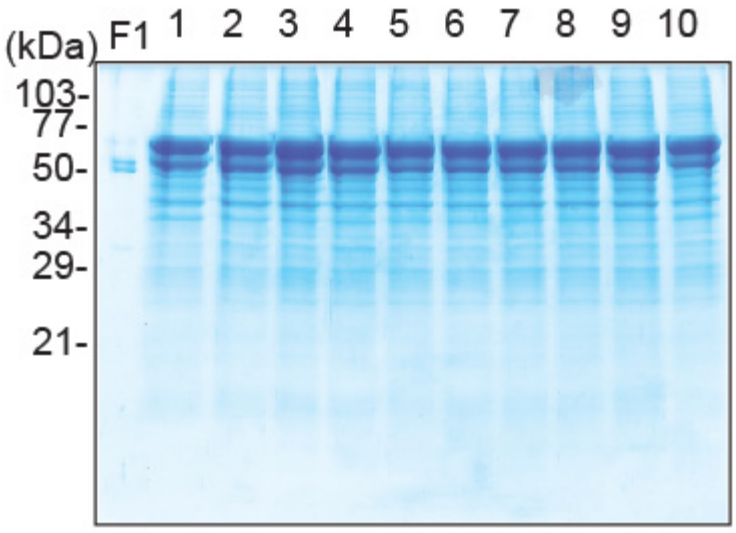
C


Input : purified rat F_1-ATPase
MBP fusion protein : MBP rat F_6
(kDa) F1 1 2 3 4 5 6 7 8 9 10
103-
77-
50-
34-
29-
21-
Commassie staining
(kDa) F1 1 2 3 4 5 6 7 8 9 10
103-
77-
50-
34-
29-
21-
Immunoblotting with anti β subunit

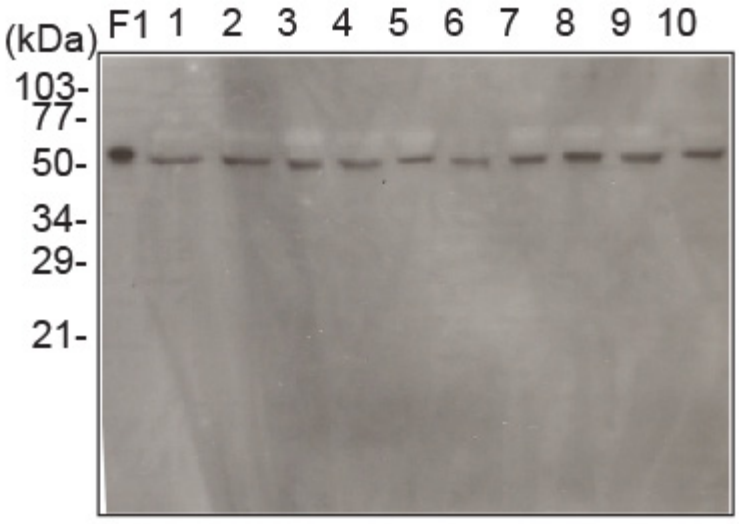
Continued

D

Input : purified rat F_1-ATPase
MBP fusion protein : MBP rat Factor B



Commassie staining

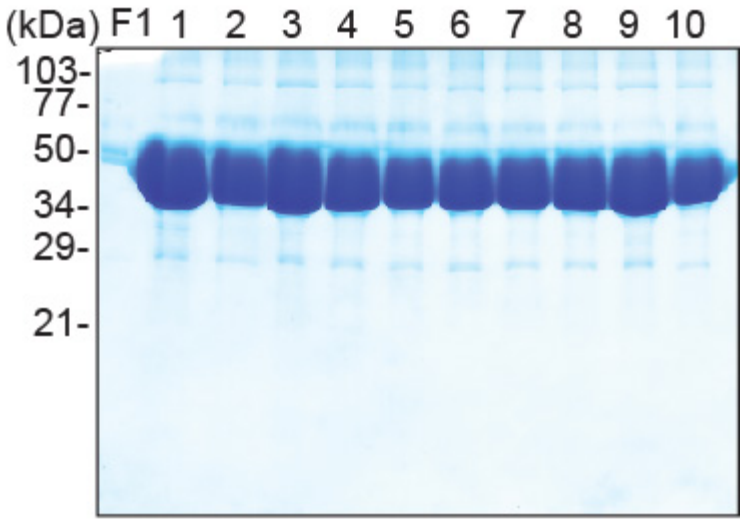


Immunoblotting with anti β subunit

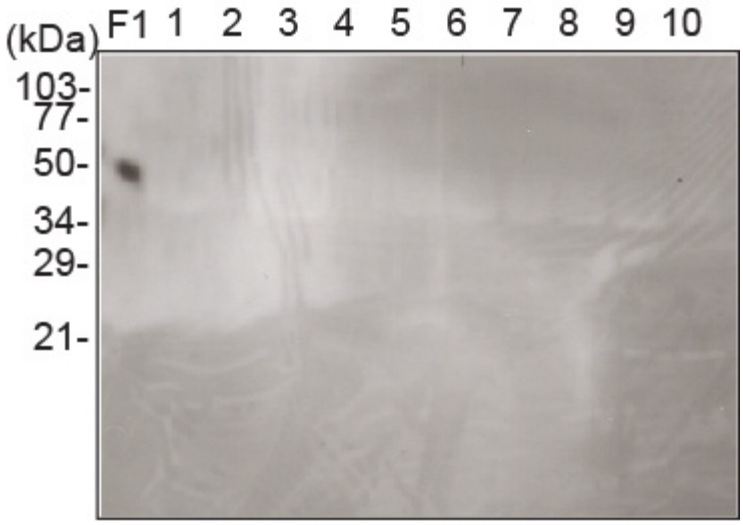
Continued

E

Input : purified rat F_1-ATPase
MBP fusion protein : MBP rat IF_1



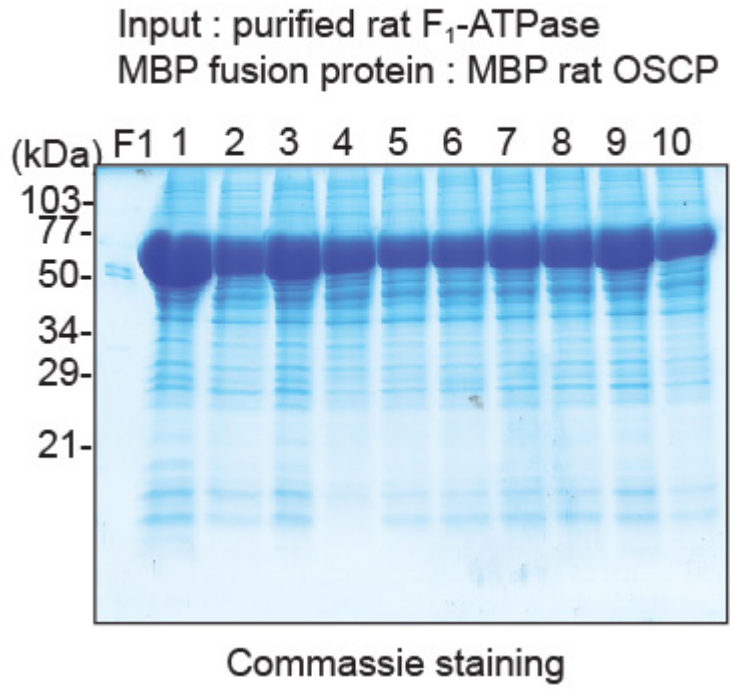
Commassie staining

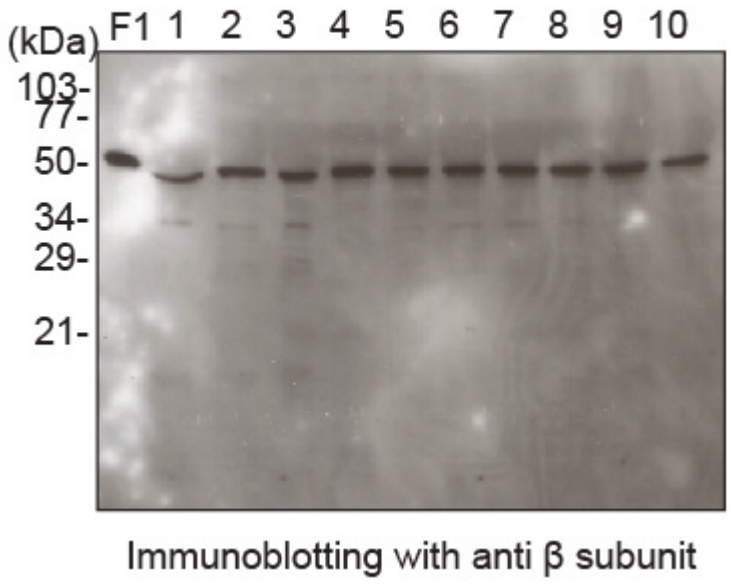


Immunoblotting with anti β subunit

Continued

F


Input : purified rat F_1-ATPase
MBP fusion protein : MBP rat OSCP
(kDa)
F1 1 2 3 4 5 6 7 8 9 10
103-
77-
50-
34-
29-
21-
Commassie staining


(kDa)
F1 1 2 3 4 5 6 7 8 9 10
103-
77-
50-
34-
29-
21-
Immunoblotting with anti β subunit

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Teaching : In an experimental class of pharmacology

THESIS

B.S.	Comparison between Hwacheongbyeo and Hwacheong ms-h Line on Enzymes Related to the Starch Biosynthesis by Northern Blotting.
M.S.	A Study on HRC (Histidine-rich Ca^{2+} Binding Protein) of the Sarcoplasmic Reticulum.
PhD.	Mammalian ATP synthase: Novel Insights into the Roles of Its Supernumerary Subunits.

PUBLICATIONS

Han Gil Lee, Hara Kang, Do Han Kim, and Woo Jin Park (2001) Interaction of HRC (Histidine-rich Ca^{2+}-Binding Protein) and Triadin in the Lumen of Sarcoplasmic Reticulum. *The Journal of Biological Chemistry*. 276(43): 39533-38.

Han Gil Lee, So Young Kim, Du Sik Kim, Su Ryeon Seo, Syng-Ill Lee, Dong Min Shin, Patrick De Smet, Jeong Taeg Seo (2009) 1*H*-[1,2,4]Oxadiazolo[4,3-*a*]Quinoxalin-1-One Inhibits Neurite Outgrowth and Causes Neurite Retraction in PC12 Cells Independently of Soluble Guanylyl Cyclase. *Journal of Neuroscience Research*. 87(1):269-77.

So Young Kim, Jeong Mi An, **Han Gil Lee**, Sik Kim Du, Chae Uk Cheong and Jeong Taeg Seo (2011) 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one induces cell cycle arrest and apoptosis in HeLa cells by preventing microtubule polymerization. *Biochemical and Biophysical Research Communications*. 408(2):287-92.

Du Sik Kim, Jeong Mi An, **Han Gil Lee**, Su Ryeon Seo, Seon Sook Kim, Ju Yeon Kim, Jeong Wan Kang, Yun Soo Bae and Jeong Taeg Seo (2013) Activation of Rac1-dependent redox

signaling is critically involved in staurosporine-induced neurite outgrowth in PC12 cells. *Free Radical Research*. 47(2):95-103.

Han Gil Lee, Du Sik Kim, Seong Ah Moon, Jeong Wan Kang, Jeong Taeg Seo (2015) Staurosporine induces ROS-mediated, microtubule-dependent formation of cellular processes in human gingival fibroblasts and rat cortical astrocytes. *International Journal of Oral Biology*. In preparation.

Han Gil Lee, Young H. Ko, and Peter L. Pedersen (2014) Mammalian ATP synthase: Novel Insights into the roles of its supernumerary subunits. In preparation.