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MINI-REVIEW

Evidence from Immunological Studies of Structure–Mechanism Relationship of F_1 and F_1F_0[1]

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Abstract

Monoclonal and polyclonal antibodies directed against peptides of F_1-ATPase or F_1F_0-ATPase synthase provide new and efficient tools to study structure–function relationships and mechanisms of such complex membrane enzymes. This review summarizes the main results obtained using this approach. Antibodies have permitted the determination of the nature of subunits involved in the complex, their stoichiometry, their organization, neighboring interactions, and vectorial distribution within or on either face of the membrane. Moreover, in a few cases, amino acid sequences exposed on a face of the membrane or buried inside the complex have been identified. Antibodies are very useful for detecting the role of each subunit, especially for those subunits which appear to have no direct involvement in the catalytic mechanism. Concerning the mechanisms, the availability of monoclonal antibodies which inhibit (or activate) ATP hydrolysis or ATP synthesis, which modify nucleotide binding or regulation of activities, which detect specific conformations, etc. brings many new ways of understanding the precise functions. The specific recognition by monoclonal antibodies on the β subunit of epitopes in the proximity of, or in the catalytic site, gives information on this site. The use of anti-α monoclonal antibodies has shown asymmetry of α in the complex as already shown for β. In addition, the involvement of α with respect to nucleotide site cooperativity has been detected. Finally, the formation of F_1F_0-antibody complexes of various masses, seems to exclude the functional rotation of F_1 around F_0 during catalysis.

Key Words: Monoclonal; polyclonal; antibodies; F_1-ATPase; F_1-F_0-ATP synthase; immunological approach; structure; assembly; mechanisms; regulation.

[1]Abbreviations: IF_1, natural protein inhibitor of the ATPase-ATP synthase; OSCP, oligomycin sensitivity-conferring protein; DCCD, dicyclohexylcarbodiimide; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoreses; F_1, F_1-ATPase, coupling factor F_1 of ATPase; F_1F_0, F_1F_0-ATP synthase, ATPase–ATP synthase complex.

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Introduction

The proton-translocating ATPase-ATP synthase of mitochondria, chloroplasts, and bacterial cell membrane has similar structure and catalyzes hydrolysis or synthesis of ATP. It consists of two main parts, the hydrophilic F_1 bearing the catalytic sites and the hydrophobic membrane sector F_0 involved in proton translocation. F_1 can be isolated as a water-soluble ATPase and thus provides a system to study the hydrolytic mechanism. Efficient ATP synthesis can be obtained only if F_1 is connected to the F_0 sector. The subunit composition of F_1 is very similar for all organisms: $\alpha_3\beta_3\gamma\delta\varepsilon$ (see reviews: Amzel and Pedersen, 1983; Senior, 1985; Vignais and Satre, 1984; Godinot and Di Pietro, 1986).

The inhibitory proteins associated to F_1 have similar functions but appear to belong to different protein classes whether in mitochondria, chloroplasts, or bacteria (Schwerzmann and Pedersen, 1986). In *Escherichia coli* and other bacterial systems, the membrane sector F_0 consists of three subunits *a*, *b*, and *c* with an approximate stoichiometry of 1:2:3–15 (Walker *et al.*, 1984; Foster and Fillingame, 1982). The binding of DCCD to the subunit *c* prevents the H^+ translocation. In mitochondria the nature of the peptides of the membrane sector is not as well understood as in *Escherichia coli.* However, an oligomycin sensitivity-conferring protein (OSCP) and factors F_6 and B have been described as involved in the connection of F_1 to F_0 (see reviews of Hatefi, 1985, Sanadi 1982) while a DCCD-binding protein, equivalent to the *E. coli* subunit *c*, appears as a main constituent of the F_0 sector (see review: Sebald and Hoppe, 1981). More recently a proteolipid (5,870 Da) has been characterized in yeast F_0 (Velours *et al.*, 1984) and another protein of apparent molecular mass of 24,000–25,000 Da has been purified from either yeast (Velours *et al.*, 1987) or beef heart (Walker *et al.*, 1987).

Monoclonal and polyclonal antibodies directed against peptides from F_1-ATPase or F_1F_0-ATP synthase have provided new and efficient tools to study structure–function relationships and mechanisms of such complex membrane enzymes. The present review summarizes the main progress obtained in this field.

General Structure and Organization of F_1F_0 Complex

The elucidation of the structure and arrangement of such a complex enzyme is a prerequisite for understanding its mechanisms.

Nature of Constitutive Subunits Detected with Antibodies and Coded by Either Mitochondrial or Nuclear Genome

As early as 1971, Tzagoloff and Meagher, using a polyclonal antibody directed against F_1, succeeded in immunoprecipitating the F_1F_0 complex from yeast mitochondrial Triton extracts (Tzagoloff and Meagher, 1971). The analysis of the complex by gel electrophoresis permitted the detection of at least nine peptide subunits including α, β, γ, δ, ε from F_1 and other peptides from F_0, some of which encoded in mitochondrial genome. Later, this approach was widely used to study the biogenesis of F_1F_0 complex and to identify the subunits coded by either nuclear or mitochondrial genome. It has been shown that two subunits among thirteen identified are mitochondrially encoded. They correspond to the proteins coded by the ATPase 6 gene (24,800 Da) and to the URF A6L gene (7,900 Da) of mt DNA of mammalians (Anderson *et al.*, 1981). With yeast the DCCD binding protein is also mitochondrially encoded (Macino and Tzagoloff, 1979) in addition to the two proteins equivalent to the mammalian ones (Macino and Tzagoloff, 1980; Esparza *et al.*, 1981). In bacteria the F_0 subunits *a*, *b*, and *c* have been studied mainly with mutation approaches rather than with antibodies (Futai and Kanazawa, 1983).

Purification of Subunits

When a protein has no directly titrable activity, antibodies are very useful in the development or improvement of the purification procedure. This approach has been successfully used to work out a new procedure of purification of OSCP that is simple, rapid, and efficient (Archinard *et al.*, 1986b). Monoclonal anti-OSCP antibodies permitted testing the efficiency of each purification step after separation of proteins with SDS-PAGE, transfer to nitrocellulose, and estimation of the amount of OSCP present by immunodecoration. Once the protocol has been established, antibodies are no longer required. This experimental approach can be of general use.

Stoichiometry of Subunits in the Complexes

The stoichiometry of F_1 subunits has for a long time been a matter of controversy between $\alpha_3\beta_3\gamma\delta\varepsilon$ or $\alpha_2\beta_2\gamma_2\delta_2\varepsilon_2$. The titration of α and β subunits in mitochondrial F_1 using monoclonal antibodies contributed to proving the $\alpha_3\beta_3$ stoichiometry (Moradi-Améli and Godinot, 1983). The α_3 stoichiometry has been directly visualized by immunoelectron microscopy which permitted the labeling of α in F_1 from *Escherichia coli* (Lünsdorf *et al.*, 1984) and F_1 from chloroplasts (Tiedge *et al.*, 1985) with monoclonal antibodies.

In the case of OSCP, monoclonal antibodies were used to demonstrate a stoichiometry of two OSCP per F_1F_0 complex by an immunoelectrotransfer blot technique (Penin *et al.*, 1985).

Joshi *et al.* (1981) reported that analysis of beef heart submitochondrial particles using a competitive binding ELISA indicated factor B stoichiometric with F_1.

Using HPLC and immunoelectron microscopy, Harris *et al.* (1986) were able to directly demonstrate the stoichiometry of one IF_1 per F_1.

Determination of Nearest Neighbors of Subunits

To study neighboring interactions between subunits, cross-linking experiments have been widely used. In many cases antibodies have served to identify the subunits present in the cross-linked products. For example, with isolated F_1, cross-linked products with OSCP have shown that both α and β subunits of beef heart interact with OSCP (Dupuis *et al.*, 1985). In the pig heart membranes the use of monoclonal antibodies has shown that OSCP was cross-linked with α and β subunits of F_1 and to a subunit of M_r 24,000 Da (Archinard *et al.*, 1986a). The latter result is in agreement with cross-linked products observed by Torok and Joshi (1985) between a protein of 24,000 Da and OSCP in beef heart mitochondria. In *Escherichia coli*, similar experiments have demonstrated that certain domains of F_0 subunits *a* and *b* extend from the membrane and are in close vicinity to one another and the F_1 catalytic subunits β (Aris and Simoni, 1983). Süss (1986), in the same way, has detected the following interactions inside the chloroplast complex CF_1–CF_0: α–ε, α–CF_0I, α–CF_0II, α–δ, β–γ, β–CF_0–II, β–ε, β–CF_0–III, γ–CF_0–II, γ–ε, γ–CF_0–III, CF_0–III–ε. In *E. coli*, Dunn (1982) has shown that ε binds to the γ subunit.

Topography and Vectorial Orientation of Subunits

As early as 1969 Racker and co-workers used anti-cytochrome *c* and anti-F_1 antibodies to localize the binding sites of cytochrome *c* on the cytosolic face of mitochondrial inner membrane and the F_1 ones on the inner face of the same membrane (Christiansen *et al.*, 1969). More recently, the vectorial orientation of F_1–F_0 subunits in the membrane has been studied in more detail. Antigenicity of protein domains is generally related to hydrophilicity and to surface accessibility. (Hopp and Woods, 1981; Kyte and Doolittle, 1982; Boger, *et al.*, 1986; Parker and Hodges, 1986). Therefore, domains embedded inside the membrane bilayers are poorly antigenic. This is why antibodies are useful for identifying domains directly accessible from the surface or after treatment of surfaces.

The natural protein inhibitor IF_1 has been known for a long time to be external to F_1 structure. Many laboratories have prepared anti-IF_1 antibodies and have attempted to use them to precisely localize IF_1 and determine its role (see below) and interactions (Dreyfus *et al.*, 1981; Tuena de Gomez-Puyou *et al.*, 1982, 1983; Beltran *et al.*, 1984; Husain *et al.*, 1985a,b; Audinet *et al.*, 1986; Jackson and Harris, 1986; our laboratory, unpublished work). Only one site per F_1 is available for binding IF_1 on one β subunit, despite the presence of three β subunits (Harris *et al.*, 1986).

According to Jackson and Harris (1986) an anti-IF_1 antibody conceals residues 54, 58, and 65 of IF_1 without affecting interaction between IF_1 and F_1. In contrast, Audinet *et al.* (1986) have reported that an antiserum preventing the ability of IF_1 to inhibit F_1-ATPase was directed against the sequence spanning His 48–Lys 58 of IF_1. In spite of the apparent discrepancies, Audinet *et al.* (1986) conclude that the sequence His 48–Lys 58 might not play a strategic role in the interaction between IF_1 and F_1. Finally, the combined results of both groups suggest an external position of the span 48–65 in IF_1 bound to F_1.

The ε subunit of *E. coli* appears to have an inhibitory function equivalent to that of IF_1 in mitochondrial F_1–F_0. Although ε and δ subunits are both required for the binding of F_1 to F_0 (see Review by Futai and Kanazawa, 1983), monoclonal antibodies raised against these subunits bind to the membrane-bound F_1–F_0 complex, indicating that ε and δ are substantially exposed in the complex (Dunn and Tozer, 1987). The same authors (Tozer and Dunn, 1987) have demonstrated that two monoclonal antibodies and ε subunit as well interact with the carboxyl-terminal region of β in *E. coli* isolated F_1.

With mitochondrial complexes both α and β subunits are accessible from the matrix side of the membrane to monoclonal antibodies as shown by immunoelectron-microscropy: gold-labeled protein A permits the visualization of anti-α, anti-β monoclonal antibodies complexed with membrane-bound F_1 (Archinard *et al.*, 1986a). The various monoclonal antibodies bind on isolated α or β, on active or denatured F_1, and on membrane-bound F_1, but with very different affinities (Moradi-Améli and Godinot, 1987). In this work, the comparison of affinities for the various conformations of subunits is a good tool for comprehending their topography and orientations. One anti-β antibody recognizes the glu 168–met 200 sequence of β (Clerc *et al.*, see below) which is partly shielded inside the β subunit. Other anti-β antibodies directed against the C-terminal fragment (Clerc *et al.*, 1986) bind more easily to isolated β than to isolated or membrane-bound F_1, indicating that binding sites are partially buried in purified or membrane-bound F_1. Two monoclonal anti-α antibodies appear very interesting since one recognizes a

membrane-buried domain while the other one binds very easily to membrane-bound F_1 and therefore recognizes a domain exposed at the surface of the complex.

Epitopes recognized by monoclonal anti-β antibodies and involved in catalytic events have been located for the first time on the C-terminal end of β in our laboratory by combining chemical and enzymatic cleavages of isolated β and binding of antibodies on the fragments separated by electrophoresis and immunoblotting (Clerc *et al.*, 1986). More recently, Tozer and Dunn (1987) have used a similar approach with *E. coli* F_1. It should be stressed that our monoclonal anti-β antibodies, raised against the pig heart F_1, which recognize the C-terminal end of β, have a wide cross-reactivity with all species tested (pig and beef heart, rat liver, *Saccharomyces cerevisiae, Schizosaccharomyces pombe, Aspergillus niger, Micrococcus luteus, E. coli*, spinach chloroplast, *Chlamidomonas renhardii, Anacystis nidulans*) (Archinard *et al.*, 1984, and unpublished results from our Laboratory). Similar results were obtained by Dunn *et al.* (1985) with monoclonal antibodies raised against β from *E. coli.*

The accessibility of OSCP to monoclonal antibodies has been qualitatively visualized by using the protein-A-gold electron microscopy immunocytochemistry and quantitatively estimated by immunotitration of OSCP in depolymerized or intact membranes. Since the amount of OSCP immunotitrated was the same in both cases, these experiments demonstrated the perfect accessibility of the OSCP domain recognized by the antibodies from the matrix side of the membrane (Archinard *et al.*, 1986a). More recent experiments have shown that the corresponding domain is located near the N-terminal end of OSCP (Colorio *et al.*, 1987). Contrary to monoclonal antibodies raised against β, our anti-OSCP antibodies appear mostly species specific, and react only with pig and beef heart. This might be related to the strong antigenicity of OSCP as demonstrated in beef heart (Robbins *et al.*, 1981).

The beef heart factor B component has been studied with both polyclonal (Lam and Yang, 1969) and monoclonal antibodies (Joshi *et al.*, 1985). It appears well shielded in the H^+-ATPase F_1F_0 complex. The vectorial and topographic organization of the F_0 membrane part of the *E. coli* complex has been studied by combining genetic and immunological approaches. By immunoprecipitation with anti-F_1 antibodies, of detergent-solubilized F_1F_0 complexes partially purified from cells bearing mutations in subunits *a* and *b*, Vik and Simoni (1987) and Aris and Simoni (1983) have shown that a mutation on subunit *b* (gly 9 $\rightarrow$ asp) reduced the binding of F_1 to F_0 and that F_1 interacts with both *c* and mutant *b* subunits. Antisera against *a* and *b* subunits which in wild type precipitate *a* and *b* in a ratio of about 1:2 no longer do it with the mutant *b* subunit or when the *a* subunit is ended at

residue 235. These experiments indicate that the C-terminal end of *a* and the N-terminal end of *b* are important in the interaction between these subunits. In addition, F_1-antiserum precipitates *b* and *c* from preparations missing *a*, which suggests that *b* and *c* interact with F_1 independently from *a*. Moreover, *b* interacts with β since a cross-linked product has been identified by immunoblotting (Aris and Simoni, 1983). Perlin and Senior (1985) have shown that the latter interactions occur at the C-terminal end of *b* which extends to the cytoplasmic side while the N-terminal end is embedded in the membrane. Other immunological studies with antibodies raised against subunit *a* indicate that the polypeptide chain is accessible from both sides of the membrane (Deckers-Hebestreit and Altendorf, 1986).

Antibodies directed against the *c* subunit prevent the binding of F_1 to F_0 (Loo and Bragg, 1982). As shown by solid-phase radioimmunoassay, this binding involved α and/or β subunits of F_1 and the arginine residues of the polar central region of *c* (DCCD-binding protein in *E. coli*). The results are consistent with a looped arrangement of *c* in the membrane in which the C-terminal and N-terminal regions of the molecule are at the periplasmic surface and the polar central region, interacting with F_1, is at the cytoplasmic surface of the cell membrane (Loo *et al.*, 1983). Other immunological studies have also shown that *c* is accessible from both sides of the cytoplasmic membrane (Deckers-Hebestreit and Altendorf, 1986; Deckers-Hebestreit *et al.*, 1986).

The conclusions obtained by immunological methods with *E. coli* F_1F_0 concerning the orientation of subunit *c* have been confirmed for the equivalent mitochondrial DCCD-binding protein with other techniques, mainly chemical modification of residues exposed at the surface of inverted membranes depleted from F_1 (see Review of Hatefi, 1985). In addition, with the mitochondrial F_1F_0 complex, Ludwig *et al.* (1980) have attempted to identify the peptides which are exposed in isolated F_1F_0 complex or at the surface of inverted submitochondrial particles (depleted or not from F_1). They have labeled the surface of these various particles with a nonpenetrating reagent [^{35}S]diazobenzene sulfonate (DABS), immunoprecipitated the F_1F_0 complex, and measured the amount of labeling in the various subunits. They have shown that all F_1 subunits were accessible to radiolabeling with [^{35}S]DABS although labeling indicates that α, γ, and ε are partially shielded by interaction with the membrane sector in F_1F_0 complex. This shielding was even more important in inverted submitochondrial particles. OSCP and a 24–25-kDA protein could also be labeled in inverted submitochondrial particles, indicating their external position in the membrane-bound complex.

Finally, antibodies labeled with fluorescent markers have permitted the determination of the distance between chloroplast CF_1 and the membranes. Dansyl-labeled monovalent antibodies reacted with the whole molecule of

CF_1 and the isolated α, β, γ, and ε subunits. The antigenic sites for monovalent anti-CF_1 were found to be located more than 35 Å from the surface of the lipid bilayer, indicating that the head of CF_1 extends away from the membrane (Baird *et al.*, 1979).

Functions of Subunits and Catalytic Mechanism of ATP Hydrolysis and Synthesis

Experiments concerning more precisely the role of each subunit will be considered first. Then, aspects related to catalytic mechanisms and regulations will receive attention.

Functions of Individual Subunits

α Subunit. The role of this subunit is not yet clearly understood although α isolated from *E coli* can bind adenine nucleotides (Dunn and Futai, 1980). Most laboratories have suggested the presence of nucleotide sites at the interface α/β (see review by Senior, 1985). Several groups have obtained monospecific polyclonal anti-α antibodies or monoclonal antibodies able to inhibit ATPase activity of soluble or membrane-bound F_1 of various sources: *E. coli* (Smith and Sternweis, 1982; Dunn *et al.*, 1985); *Micrococcus lysodeikticus* (Urban and Salton, 1983; Larraga *et al.*, 1981); chloroplasts (Kanner *et al.*, 1975); heart mitochondria (Moradi-Améli *et al.*, 1987; Moradi-Améli, 1987). A stimulation of ATPase activity was also observed by Dunn *et al.* (1985). Inhibitions of photophosphorylation and light-induced ATPase activity of chloroplasts were observed with polyclonal anti-α antibodies (Nelson *et al.*, 1973).

The mechanism of these inhibitors or activations has not been studied in detail. Moreover, it must be verified that inhibitions could not result from the dissociation of F_1 subunits. Indeed, Hadikusumo *et al.* (1986) have shown that some subunit-specific antibodies do not drive all the subunits of the complex during immunoprecipitation experiments, which means that binding of the antibody induces a dissociation of subunits.

Recently, in our laboratory (Moradi-Améli *et al.*, 1987, Moradi-Améli, 1987), a detailed study of inhibitions of both ATPase and ATP synthase activities of isolated or membrane-bound F_1 from pig heart by a monoclonal anti-α antibody (Godinot *et al.*, 1986) has produced more information. The inhibitions are correlated to the binding of one IgG or of two Fab fragments per mole of F_1. This suggests that the three α-subunits are not equivalent in F_1 and that the epitope recognized by this antibody is not equally accessible in the three α-subunits of active F_1. It is not a simple matter of steric

hindrance, since Tiedge *et al.* (1985) were able to bind three anti-α monoclonal IgG per molecule of chloroplast F_1 and Lünsdorf *et al.* (1984) also three anti-α monoclonal IgG per *E. coli* F_1. The functional asymmetry demonstrated with our specific monoclonal anti-α antibody is in agreement with the heterogeneity of α subunits demonstrated in our Laboratory (Falson *et al.*, 1986) with F_1 from *Schizosaccharomyces pombe* and in the case of chloroplast F_1 (Nalin *et al.*, 1985). In these studies, inhibitions of ATPase activity were correlated to chemical labeling. Single-crystal X-ray diffraction studies (Amzel and Pedersen, 1983) have shown that the three α–β couples in F_1 are not structurally equivalent.

The above inhibition induced by the binding of our anti-α-antibody (one IgG only per F_1) does not appear to directly involve the catalytic site(s). Indeed, the rate of ITP hydrolysis is not affected while that of ATP hydrolysis is inhibited by about 50%. The binding of the antibody is likely to maintain ADP produced by ATP hydrolysis in the regulatory site, strictly specific of adenine nucleotide (Di Pietro *et al.*, 1981; Baubichon *et al.*, 1981), and responsible for hysteretic inhibition of F_1 (Di Pietro *et al.*, 1980). Since the hysteretic inhibition is linked to the binding of ADP on only one β subunit (demonstrating the asymmetric function of β) (Fellous *et al.*, 1984) and since the above antibody is α-specific, it means that the binding of this antibody prevents normal interactions between α and β, by changing the conformation of α.

β Subunit. This subunit has been extensively studied, and it has been known for a long time that it contains substrate binding sites and that its role is essential for ATP hydroysis and ATP synthesis. Known amino acid sequences of β (about nine) from various species exhibit strong homologies of about 70% (Walker *et al.*, 1985). As mentioned above (topograpy and vectorial orientation), antigenicity of proteins is related to the hydrophilicity and surface exposure of domains. Therefore the sequence permits the prediction of the antigenicity. Homologous sequences should produce cross-reactivity. The use of antibodies raised against β from many various surfaces has widely demonstrated these homologies, much before the sequences were known. Cross-reactivity of polyclonal antibodies was observed between the following: rat liver and yeast mitochondria, Swiss chard chloroplast, and *E. coli* (Rott and Nelson (1981). *Rhodospirillum rubrum* polyclonal antibodies recognize lettuce chloroplasts (Philosoph and Gromet-Elhanan, 1981); Pig heart monoclonal anti-β antibodies react with pig heart, beef heart, rat liver, *Aspergillus niger, Saccharomyces cerevisiae, Schizosaccharomyces pombe* mitochondria, spinach chloroplasts, *E. coli, Micrococcus luteus, Chlamidomonas renhardii,* and *Anacystis nidulans* F_1 (Archinard *et al.*, 1984, and unpublished results from our laboratory); anti sweet potato F_1 antibodies cross-react with β subunits from guinea pig liver mitochondria and pea and

spinach chloroplasts (Iwasaki *et al.*, 1984); monoclonal anti *E. coli* β antibodies cross-react with *Azotobacter macrocytogenes, Bacillus subtilis, Micrococcus luteus, Nocardia rhodocrous, Lactobacillus casei, Streptococcus faecalis,* spinach chloroplasts, and rat liver mitochondria (Dunn *et al.*, 1985). Polyclonal antibodies against corn chloroplastic F_1 detect similar reactivities with mitochondrial forms of F_1 from the same species or from beef heart, or with spinach chloroplasts, or *E. coli* (Spitsberg *et al.*, 1985). Polyclonal antisera raised against the β-subunit of the thermoacidophilic archaebacterium *Sulfolobus acidocaldarius* give cross-reactivity with cellular or membrane extracts of a number of archaebacteria, eubacteria, and chloroplasts, *Methanobacterium, Methanosarcina, Methanolobus, Halobacterium,* and *Escherichia coli,* and with purified F_1 from beef heart (Lübben *et al.*, 1987). Antisera raised against *Escherichia coli* F_1 cross-react with the β subunit of the thermophilic bacterium PS_3 (Steffens *et al.*, 1987). All these studies stress the strong phylogenic conservation of most epitopes in β. This strict conservation shows the strong requirements of sequences involved in active conformations and catalytic mechanisms.

Polyclonal or monoclonal antibodies can be used to inhibit ATP hydrolysis in soluble or membrane-bound F_1, or to inhibit ATP synthesis in the membrane-bound complex. For example, monovalent anti-β antibodies inhibited photophosphorylation in chloroplasts (Gregory and Racker, 1973). Other polyclonal antibodies against *Rhodospirullum rubrum* β inhibited. ATP-dependent activity in the chromatophores of this organism (Philosoph and Gromet-Elhanan, 1981). Larraga *et al.* (1981) obtained a polyclonal anti-β antibody able to inhibit membrane-bound F_1 in *Micrococcus lysodeikticus* better than the isolated F_1. This indicates a different conformation of F_1 after isolation. Hadikusumo *et al.* (1984) prepared monoclonal anti-yeast β antibodies either inhibitory or stimulatory of ATPase activity in isolated F_1F_0. The stimulatory effect could be due to conformational changes but the inhibitions observed with two anti-β antibodies were due to a dissociation of one or more of the subunits from the complex (Hadikusumo *et al.*, 1986). Dunn *et al.* (1985) obtained four monoclonal antibodies against *E. coli* β that inhibit the ATPase activity of soluble F_1 up to 90% while the inhibition is only 40–60% with the membrane-bound enzyme. They also obtained antibodies that do not inhibit ATPase activity because they can only bind to isolated β-subunit. From their studies they concluded that all antibodies obtained which exhibited cross-reactivity were found to recognize sites which were not exposed in intact ATPase, implying that the surfaces which lie between subunits are more highly conserved. However, results obtained with our monoclonal antibodies raised against pig heart β subunits do not agree with this conclusion: at least one well-conserved epitope located on the C-terminal end of β binds two overlapping antibodies which produce at least a partial inhibition of both ATPase and ATP synthesis activities (Moradi-

Améli and Godinot, 1987; Clerc *et al.*, 1986). One of the antibodies decreases the rate of ATP synthesis without modifying the rate of ATP hydrolysis (Archinard *et al.*, 1984). In addition, this antibody also prevents the ADP-induced hysteretic inhibition of ATPase (Archinard *et al.*, 1984 and Di Pietro *et al.*, 1980). Another anti-β antibody inhibits ATPase activity of isolated F_1 at slightly acidic pH in the presence of inorganic phosphate while ATP hydrolysis and ATP synthesis in the membrane-bound complex are not affected (Gautheron *et al.*, 1984).

Minor Subunits: γ, δ, ε. Smith and Sternweis (1982), using monospecific rabbit antisera to each of the different subunits of *E. coli* F_1, have shown that, in addition to β, the γ subunit is required for ATP hydrolysis. This is in agreement with cross-linking experiments on pig heart F_1 showing that the reticulation of γ with α and β was directly correlated with the loss of ATPase activity (Godinot *et al.*, 1979). Moreover, chemical modifications of γ in *Schizosaccharomyces* F_1 resulted in ATPase inhibition (Falson *et al.*, 1986). Mollinedo *et al.* (1980) have also shown that, in addition to β, small portions of α and γ are required for the hydrolytic activity of F_1-ATPase, probably at the level of active conformations in *Micrococcus lysodeikticus*. Urban and Salton (1983) have shown that γ is essential for ATPase activity as well as α and β subunits in *Micrococcus lysodeikticus*, using polyclonal antibodies. The same studies have suggested that δ and ε are involved in the attachment of F_1 to the membrane.

With thermophilic bacterium PS_3, Yoshida *et al.* (1979) have shown that γ and ε were both protected against their corresponding antibodies when F_1 was attached to the membrane. Sternweis *et al.* were conclusive on the requirement of δ and ε for the binding of F_1 to F_0 in *E. coli* (Sternweis, 1978; Futai *et al.*, 1974; Smith and Sternweis, 1977). However, the ε subunit of *E. coli* is described by Dunn and Tozer (1987) as a tightly bound but dissociable inhibitor of the ATPase activity of soluble F_1. The ε subunit can be easily removed from soluble F_1 using anti-ε-antibodies coupled to Sepharose (Sternweis, 1978). The binding of anti-ε-antibody to F_1 can reverse the inhibition of F_1-ATPase by the ε-subunit without gross displacement of ε from its normal position (Dunn and Tozer, 1987). Other results from this team (Dunn *et al.*, 1987) suggest that the ε subunit slows a conformational change that is required to reduce the affinity at the active site and provokes a reduced rate of product release. In another study made on *Micrococcus lysodeikticus*, the ε subunits could be effectively released by an electrochemical gradient as shown by cross-immunoelectrophoresis (Urban and Salton, 1983).

IF_1: The function of ε in bacteria has often been compared to that of the eukaryotic IF_1 which also inhibits the F_1-ATPase activity and can be quite easily released. For example, Dreyfus *et al.* (1981) have shown that anti-IF_1-antibody reactive sites were unmasked upon generation of a protonmotive force by initiation of respiration, thus releasing the inhibition

of ATPase activity. Despite the presence of three β-subunits, only one site per F_1 molecule is available for binding IF_1 and producing the inhibition, as demonstrated by measuring the binding of [^{125}I]-labeled antibodies (Beltran *et al.*, 1984) or by immunoelectron microscopy (Harris *et al.*, 1986).

OSCP: Immunotitrations with monoclonal anti-OSCP antibodies have shown that two OSCP are present per molecule of F_1 inside mitochondria (see above). The same antibodies were used to follow the progressive depletion of membrane in OSCP by various treatments (Gautheron *et al.*, 1985). Then, the depleted membranes were reconstituted with purified OSCP and F_1 and the reassociation of OSCP was correlated to the recovery of oligomycin sensitivity of ATP hydrolysis, efficient proton fluxes, and ATP synthesis. Two OSCP per molecule of F_1 were required for full recovery of all these energy-transfer linked reactions. However, the binding of one OSCP could induce a large but incomplete oligomycin sensitivity of ATPase activity. These experiments have shown that OSCP is required to adjust the fitting of F_1 to F_0 for a correct channelling of protons efficient for ATP synthesis (Penin *et al.*, 1986).

Factor B: Joshi *et al.* (1985) described that two anti-factor B monoclonal antibodies inhibited factor B-stimulated ATP-dependent reverse electron flow activity in reconstitution experiments of factor B-deficient submitochondrial particles with purified factor B. Joshi *et al.* (1981) have also reported that a polyclonal anti-beef heart mitochondrial factor B antibody cross-reacted with *E. coli*, chloroplasts, *Paracoccus denitrifians*, and the thermophilic bacterium, PS_3. This suggests the presence of proteins similar to factor B in many systems, although the above antibody gave negative results with rat liver mitochondria and with purple membranes.

Bacterial Subunit c or DCCD-Binding Protein: Loo and Bragg (1982) have shown that an antiserum against *E. coli* DCCD binding polypeptide blocked the leakage of protons through F_0 in F_1-stripped everted membrane vesicles. Deckers-Hebestreit *et al.* (1986) have obtained an anti-subunit *c* antiserum which specifically recognizes the native conformation of subunit *c* present in its oligometric form or reconstituted into liposomes. The work of Vik and Simoni (1987) concerning the role of *a*, *b*, and *c* subunits in the binding of F_1 to F_0 has been discussed above (topography and vectorial orientation).

General Mechanisms

The use of specific antibodies appear especially interesting to approach the mechanisms of complex membrane enzymes such as F_1F_0 or even isolated F_1. As early as 1975, Monteil and Roussel obtained specific anti-F_1 antisera

which produced uncompetitive inhibition of isolated bacterial F_1 ATPase activity (Monteil and Roussel, 1975). Whiteside and Salton (1970), in contrast, described anti-ATPase antibodies which acted as noncompetitive inhibitors. The interpretation of these observations was difficult in terms of mechanisms since polyclonal antibodies may bind to several sites. As shown above, many polyclonal and monoclonal antibodies have been raised against specific subunits and used to inhibit ATPase activity and more seldom ATP synthesis. Hadikusumo *et al.* (1984) have isolated anti-α and anti-β monoclonal or polyclonal antibodies which either inhibited or stimulated yeast F_1 ATPase activity. They suggested that the binding of antibodies might induce conformational changes resulting in the partial dissociation of F_1F_0 subunits. Dunn *et al.* (1985), using *E. coli*, have found that all their antibodies against α and β which recognize the whole ATPase have effects (inhibition or stimulation) on ATP hydrolysis. They concluded that these results support the concept that conformational freedom is very important in the catalysis. In our case, a monoclonal anti-β antibody $19D_3$ raised against pig heart mitochondrial F_1 can inhibit ATPase activity only at slightly acidic pH and in the presence of P_i, when the monovalent form of inorganic phosphate is dominant (Gautheron *et al.*, 1984). However, this antibody does not affect ATP synthesis probably because it poorly binds to membrane-bound F_1 (Moradi-Améli and Godinot, 1987). Moreover, this $19D_3$ antibody has been shown to recognize the C-terminal end of the β subunit (Clerc *et al.*, 1986) and to bind to a strategic domain. Another anti-β monoclonal antibody 5G11 inhibits ATP synthesis without modifying ATP hydrolysis (Archinard *et al.*, 1984). Its binding overlaps with the previous one, $19D_3$, and is located between amino acid 373 and 393 in the terminal C span of the β sequence (Clerc *et al.*, 1986, Moradi-Améli and Godinot, 1987). These observations mean that the mechanisms of ATP synthesis and ATP hydrolysis are not symmetric and proceed via different conformations or different pathways or both. Shoshan and Shavit (1979) have also described differential inhibitions of ATP hydrolysis and ATP synthesis with polyclonal antibodies against lettuce chloroplast F_1. The monoclonal anti-α antibody $7B_3$, raised against heart mitochondrial F_1, inhibits both ATP hydrolysis and synthesis (our Laboratory, unpublished results, and Moradi-Améli, 1987), but does not inhibit ITP hydrolysis. In addition, it prevents hysteretic inhibition produced by ADP-binding at the regulatory site described by Di Pietro *et al.* (1980). Since IDP cannot bind to the regulatory site, these results mean that $7B_3$ slows or prevents the release of ADP issued from ATP hydrolysis, this latter ADP being retained at the regulatory site (Moradi-Améli *et al.*, 1987). Among polyclonal or monoclonal antibodies which inhibit ATPase activity, none appears to be competitive with ATP. This suggests that the catalytic nucleotide site is not exposed on the surface of the active complex. Dunn *et al.*

(1985) reported that all their anti-α and anti-β raised against *E. coli*, which exhibited a large cross-reactivity with other species, were found to recognize sites not exposed in intact ATPase. This indicates that well-conserved regions in F_1, essential for activity, appear buried inside the complex. Our monoclonal anti-β antibodies 5G11 and $19D_3$, which also exhibit a large cross-reactivity and recognize the C-terminal end of F_1 containing the binding sites of nucleotide analogues such as fluorosulfonylbenzoyl adenosine (FSBA), at the level of tyrosine 368 (Esch and Allison, 1978), are easily accessible on the surface of the isolated β subunit but much less to β assembled in the active complex. This proves that the nucleotide sites involved in catalysis are either at the interface between subunits or deeply buried in the active complex due to a conformational change occurring during the assembly of β inside the complex (Moradi-Améli and Godinot, 1987). Another monoclonal anti-β 14D5 only reacts with urea-denatured F_1. Its binding site is located between amino acids 168–200 of β. According to Duncan *et al.* (1986) this region is also strongly involved in the catalytic nucleotide binding. Homologous sequences have been described for several nucleotide-binding proteins (Walker *et al.*, 1982; Fry *et al.*, 1986) exhibiting similar tertiary folding topology. On these basis, Duncan *et al.* (1986) have proposed a working model for the tertiary structure of the nucleotide-binding domain of the β subunit of *E. coli* F_1 comprising the segment of residues 148–297. The model was derived from secondary structure prediction and from comparison of the amino acid sequences.

Although the catalytic site is deeply buried inside the complex and therefore not easily accessible to study with antibodies, antibodies are good probes to detect conformational changes linked to catalytic activities and to reveal conformations of proteins inside the membrane. For example, the binding of ATP to F_1 was shown to strongly decrease the apparent affinity of three anti-β monoclonal antibodies and of one anti-α; the same antibodies revealed that the conformation of F_1 inside the membrane was similar to that of soluble F_1 saturated with ATP (Moradi-Améli and Godinot, 1987).

Richter and McCarty (1987) have obtained an antiserum against the isolated ε subunit of chloroplast F_1 which removes ε from F_1. Interestingly this antiserum is only effective in removing ε from membrane-bound F_1 when a proton gradient has been induced across the membrane by illumination. These results support the existence of energy-dependent changes in the conformation of ε in the ATP synthase complex. These changes may be a part of the mechanism of energy-dependent activation of chloroplast ATP synthase.

The F_1F_0-ATP synthase of photosynthetic bacteria, unlike that of heterotrophic bacteria and of chloroplasts, is inhibited by oligomycin as the mitochondrial enzyme (Fillingame, 1981). Gromet-Elhanan *et al.* (1985)

have reconstituted a hybrid F_1F_0 using β-less *R. rubrum* chromatrophores with β subunits purified from *E. coli* or *R. rubrum* F_1. In each case photophosphorylation and ATPase activity were restored at different rates. However, the hybrid F_1F_0 containing *E. coli* β was not sensitive to oligomycin, contrary to the homologous reconstituted *R. rubrum* system. Both reconstituted systems were inhibited by DCCD, and by an antibody raised against *E. coli* F_1, although the hybrid system was much more sensitive to the antibody. The authors conclude that *R. rubrum* β plays a role that the *E. coli* β cannot fulfill in conferring oligomycin sensitivity to the *R. rubrum* F_1F_0 complex.

As mentioned above, the use of anti-α monoclonal antibodies have permitted demonstrating the functional as well as structural asymmetry of the mitochondrial complex F_1F_0. In addition, the effects of the anti-α $7B_3$ suggest that α subunits are involved in nucleotide site cooperativity (Moradi-Améli and Godinot, 1987, unpublished results).

The precise molecular mechanisms of catalysis and regulation of ATP synthesis and hydrolysis are still controversial. Many models have been proposed (see review by Vignais and Satre, 1984). The possibility of three equivalent alternating catalytic sites is difficult to reconcile with the asymmetric structure and behavior of F_1F_0. Gresser *et al.* (1982) have proposed a mechanism of rotational catalysis in which the α–β couples would rotate with respect to the minor subunits. If the rotation is an essential feature of the mechanism, the rate of ATP synthesis should be inhibited either completely or proportionally to the load carried by F_1. Bivalent immunoglobulins (IgG) or monovalent Fab fragments of an anti-α monoclonal antibody (7B3) were bound to F_1 present in electron transport particles (2 Fab or 2 IgG/F_1). This binding similarly inhibited the rate of ATP synthesis by a maximum of about 50%. When anti-mouse immunoglobulins were added to the F_1–$7B_3$ (IgG) complex, no change in the rate of inhibition was observed. In conclusion, the rate of ATP synthesis was the same when F_1 was loaded with 100 kDa (2Fab), 300 kDa (2 IgG, $7B_3$), or 900 kDa (2 IgG + 4 anti-mouse IgG). Therefore, the rotation of the α-subunits during ATP synthesis is extremely unlikely (Moradi-Améli and Godinot, 1988). These results do not completely exclude a possible rotation of some F_0 subunits inside the membrane during proton translocation as proposed by Cox *et al.*, 1984. However, such quick rotations of proteins in a membrane have to be demonstrated and, in any case, they would not explain how phosphate is transferred to ADP during ATP synthesis.

As a general conclusion, it is obvious that the use of antibodies has not solved all the problems related to energy-linked ATP synthesis. The characterization of more antibodies to F_1 and F_0 subunits should lead to a better understanding of the structure of F_1F_0 and the mechanism of ATP-synthesis.

These antibodies should be also very important for understanding membrane pathologies, mechanism of deficiencies, eventual tissue specificities, and changes during differentiation and mutations (Côté and Boulet, 1985; Di Mauro *et al.*, 1987; Vayssière *et al.*, 1987).

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