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YEARLY REVIEW

STRUCTURE AND FUNCTION OF THE COUPLING-FACTOR OF PHOTOPHOSPHORYLATION

This topic deals with the catalytic part of the ATP-synthase present in the thylakoids from higher plants, green algae, photosynthetic bacteria and cyanobacteria. We also refer to some specific results obtained with proton-ATPase from *Escherichia coli*, thermophilic bacterium PS_3 and mitochondria. The most important change in this field is the very fast development of our knowledge of the primary structure of the constituents of the ATP-synthase.

Molecular weight and subunit stoichiometry of the coupling-factor

In higher plants, ATP-synthase is mostly present in stroma lamellae (Faludi-Daniel *et al.*, 1983). The enzyme is formed of the coupling-factor, CF_1 or F_1, localized in the outer part of the thylakoids and of the CF_0 or F_0, hydrophobic part, buried in the membrane. The F_1 is composed of 5 subunits, α-β-γ-δ-ϵ, and F_0 of 3, I-II-III (Alt *et al.*, 1983). Chloroplast genes coding for the subunits α, β, ϵ and III are photogenes (de Heij *et al.*, 1984; Rodermel and Bogorad, 1985). A similar F_1 has been also extracted and purified from cyanobacteria, *Synechococcus* (Lubberdings *et al.*, 1983; Van Walraven *et al.*, 1984) and *Mastigocladus laminosus* (Frei *et al.*, 1984) and photosynthetic bacteria, *Rhodospirillum rubrum* (Khananshvili and Gromet-Elhanan, 1982) and *Rhodopseudomonas sphaeroides* (Müller *et al.*, 1983).

β Subunits from F_1 of different organisms present a high homology (Nelson and Cidon, 1984) and generally have a molecular weight lower than that of the corresponding subunit α (Merchant and Selman, 1983). However, in *Dunaliella* order of migration of subunits α and β in polyacrylamide gel electrophoresis, is inversed (Selman-Reimer *et al.*, 1984). The recent determinations give a molecular weight of 400 kD, instead of 325 kD, for CF_1 in spinach (Moroney *et al.*, 1983) and *Chlamydomonas reinhardii* (Merchant *et al.*, 1983). These results are consistent with a stoichiometry $\alpha_3\,\beta_3\,\gamma\,\delta\,\epsilon$ which is in accord with the analyses of uniformly labelled $[^{14}C]CF_1$ (Merchant *et al.*, 1983; Süss and Manteuffel, 1983). The subunit δ is easily lost during CF_1 purification (Moase and Green, 1981; Finel *et al.*, 1984) and Berzborn *et al.* (1984) propose that the true stoichiometry is 3 δ per CF_1. Electron microscopy of negative stained CF_1 exhibits a six-fold symmetry (Moase and Green, 1981; Akey *et al.*, 1983) or a three-fold symmetry when α subunits have bound a monoclonal specific antibody (Tiedge *et al.*, 1984), indicating a stoichiometry $\alpha_3\beta_3$.

Primary structure and organization of the coupling-factor

Determination of the primary structure of the ATP-synthase begins with the analysis of the DNA sequence of the genes coding for the different subunits in the following organisms. Subunits α, β and ϵ are sequenced in tobacco (Deno *et al.*, 1983; Deno and Sugiura, 1984; Shinozaki *et al.*, 1983), subunits β and ϵ in maize (Krebbers *et al.*, 1982), subunits β and ϵ in spinach (Zurawski *et al.*, 1982), subunits β and ϵ in barley (Zurawski and Clegg, 1984). Genes for the subunits α, β and ϵ are located in pea and will be soon sequenced (Huttly and Gray, 1984). Subunit α has been partially sequenced in *Chlamydomonas reinhardii* (Hallick, 1984) and genes coding for subunits α, β and ϵ have been localized (Woessner *et al.*, 1984). The five subunits of F_1 are sequenced in *Rhodopseudomonas blastica* (Tybulewicz *et al.*, 1984).

The secondary structure of each subunit of the ATP-synthase from *Escherichia coli* has been inferred from the amino acid sequence. Organization of the peptidic chains in the membrane has also been proposed from the calculated hydropathy of the amino acid sequences (Hoppe and Sebald, 1984, Walker *et al.*, 1984). Comparison of the sequences in ATP-synthase from *Escherichia coli* and photosynthetic organisms allow the prediction of arrangements of the peptidic chains for the last organisms. Sixty-six amino acid residues (Deno and Sugiura, 1984) are identical along the α and β subunits from tobacco and are positioned in sequences which participate in the binding of nucleotides in other nucleotide-binding enzymes (Kanazawa *et al.*, 1982). The percentage of homologous amino acid residues of the different subunits β is around 90% (Shinozaki *et al.*, 1983), corresponding to a conservation of their secondary structure throughout the different organisms.

Organization of the subunits has been investigated

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by chemical cross-linking (Rott and Nelson, 1984) and accessibility to the specific antibodies (Klein-Hitpass and Berzborn, 1984). Subunits α and β must be arranged alternately in an hexameric figure which is stabilized by the subunit γ forming the core of the complex. Subunits I and II are in contact with subunits δ and ε, suggesting their role in the attachment of F_1 to F_0.

Physical methods have been used for studying the general organization of the ATP-synthase. Small angle X-ray scattering has been applied in thermophilic bacterium PS_3 (Furuno *et al.*, 1983) and mitochondria (Amzel *et al.*, 1982). Neutron small angle scattering has been used with the F_1–F_0 complex from *Rhodospirillum rubrum* (Nawroth *et al.*, 1983) and with the F_1 from *Escherichia coli* (Satre and Zaccai, 1979). Circular dichroism gives some information on the conformation changes of spinach CF_1 by fixation of the substrates (Younis *et al.*, 1983). Fluorescent probes have been widely used (Pick and Finel, 1983) and allow determinations of the distances between the different sites on CF_1 (Cerione *et al.*, 1983, Snyder and Hammes, 1984).

Properties of the different subunits

In the F_1 part, subunits α and β bind the nucleotides (Deno and Sugiura, 1984; Kanazawa *et al.*, 1982). Subunit β is thought to bear the catalytic sites. Some specific sites of this subunit have been mapped. DCCD binding-sites in thermophilic bacterium PS_3 and in *Escherichia coli* or mitochondria correspond respectively to the glutamic acid 204 and 215 in the subunit β from the spinach chloroplast ATP-synthase (Yoshida and Allison, 1983). The photo-affinity probe, 8-azido ATP, binds on 3 amino acid residues of the subunit β in mitochondria (Hollemans *et al.*, 1983) corresponding to the lysine 317, isoleucine 321 and tyrosine 328 of the homologous subunit in spinach, in a part of the peptidic chain called Rossmann fold, which is essential for the catalytic activity (Kanazawa *et al.*, 1983-a). In photosynthetic organisms the photo-affinity analogues of the nucleotides label subunits α and β or β only following the favored conformation "*syn*" or "*anti*" of the probe (Czarnecki *et al.*, 1982, Czarnecki *et al.*, 1983, Abbott *et al.*, 1984). The conformation "*anti*" is required for nucleotides to be a good substrate (Czarnecki, 1984).

Subunit γ is necessary for the reconstituted α–β complex to elicit an ATP-ase activity in *Escherichia coli* (Kanazawa *et al.*, 1983b) and *Thermophilic bacterium PS*$_3$ (Kagawa *et al.*, 1984). In photosynthetic organisms such a reconstitution from isolated subunits is not achieved. In chromatophores of *Rhodospirillum rubrum* the subunit γ is necessary for photophosphorylation (Khananshvili and Gromet-Elhanan, 1982). In CF_1 from spinach subunit γ bears a disulfide bond S_1–S_2 and two sulfhydryl residues S_3, S_4 (Nalin and McCarty, 1984, Moroney *et al.*, 1984, Ketcham *et al.*, 1984). S_3 and S_4, oxidized to a disulfide bond or cross-linked, induce a proton leak through the chloroplast membrane indicating the subuit γ behaves like a proton gate. Sulfhydryl group S_3 is essential for ATP-synthase activity and is the only SH group found in all the subunits γ from the different organisms, especially from *Rhodopseudomonas blastica* (Tybulewicz *et al.*, 1984) and corresponds to the cystein 88 in *Escherichia coli* γ subunits. Reduction of the disulfide bond S_1–S_2 induces ATP-ase activity and is not found in the subunit γ from mitochondria or *Escherichia coli*, where there is no regulation of the ATPase activity. Subunit γ forms the core of the α β complex and regulates its activity by induced-changes of its conformation.

Subunit δ has been proposed for a long time as the anchorage point between F_1 and F_0. This proposal has been challenged by Berzborn *et al.* (1984) and Patrie and McCarty (1984). Subunit δ seems to be mostly necessary to prevent non-productive leak of protons through CF_0 and not to be required for photophosphorylation itself.

Subunit ε is an ATPase inhibitor but for the isolated CF_1 only (Finel *et al.*, 1984; Richter *et al.*, 1984). This CF_1 without subunit ε is a permanently Ca^{2+} ATPase, inhibited by subunit ε addition, which does not recouple CF_1 depleted thylakoids unless subunit ε is added (Richter *et al.*, 1984). Subunit ε is not required for binding CF_1 to the membrane, but its presence prevents, as subunit δ does, ineffective leak of protons through CF_0.

Coupling-factor activation

In soluble CF_1, subunit ε is the internal inhibitor of the ATPase activity. The physical treatments which induce the ATPase activity (Anthon and Jagendorf, 1983, 1984) release the subunit ε from the core of the enzyme. Heat treatment displaces subunit ε without changing the state of the disulfide bridge (Patrie and McCarty, 1984), as does ethanol treatment (Richter *et al.*, 1984) and anion-exchange HPLC (Finel *et al.*, 1984). The chemical treatments which induce ATPase activity do not release subunit ε. Thiol activation correlates with the reduction of the disulfide bridge, S_1–S_2, in subunit γ and protease activation with the cleavage of subunit α (Nalin and McCarty, 1984; Ketcham *et al.*, 1984).

With CF_1–CF_0 in the membrane we observe a dual action: the build-up of a $\Delta\tilde{\mu}H$ across the membrane induces a conformational change in the CF_1 part of the ATPase demasking the thiol residue S_3 and making the disulfide bridge S_1–S_2 accessible to the second action, reductive breakage of this S_1–S_2 bond. Conversely these thiol groups S_1, S_2 are protected against oxidative reagents in the presence of the $\Delta\tilde{\mu}H$ and become exposed when $\Delta\tilde{\mu}H$ is cancelled (Shahak, 1985). These two processes induce the ATPase activation. This is reminiscent of the dual action of the heat and disulfide reducing agents on the activation of the ATPase activity in isolated CF_1

(Nalin and McCarty, 1984). In chloroplasts the disulfide reducing agent is a thioredoxin (Mills and Mitchell, 1984). Gräber *et al.* (1984), Rumberg and Becher (1984) and Mills and Mitchell (1984) propose that the activation of the ATPase is caused by the protonation and deprotonation of the parts of the ATPase respectively, turned to the outside and the inside of the thylakoids. With CF_1–CF_0 reconstituted in liposomes, the ATPase as well as the ATP-synthase activities depend on the nature of the phospholipids used (Pick *et al.*, 1984). In photosynthetic bacteria, activation of the ATPase seems to present a different mechanism (Casadio and Melandri, 1984). The common point is that activation of the enzyme is valuable as well for dark ATP hydrolysis as for light-dependent ATP synthesis (Vallejos *et al.*, 1983).

ATP synthesis and proton flux

It is clear that ATP synthesis is coupled to a proton flux from inside to outside of the thylakoids and depends on the value of the $\Delta\tilde{\mu}H$ across the membrane.

The ratio H^+ to ATP has been reported equal to 3, during ATP synthesis in chloroplasts (Davenport and McCarty, 1984; Gräber *et al.*, 1984; Rumberg and Becher, 1984) and with CF_1–CF_0 reconstituted in liposomes (Dewey and Hammes, 1981), but ratios between 2 and 8, following the nature of the lipids used, have been found in liposomes where bacteriorhodopsin and yeast mitochondria ATP-synthase have been reconstituted (Van der Bend *et al.*, 1984). Moreover, during flash-induced ATP synthesis in pea chloroplasts, we found a ratio H^+/ATP of between 2 and 4, following the state of the chloroplasts (Lemaire *et al.*, 1984).

Several arguments have been given in favor of a localized $\Delta\tilde{\mu}H$ (Haraux and de Kouchkovsky, 1983). Double inhibitor titrations experiments (Hitchens and Kell, 1983) argue for a localized $\Delta\tilde{\mu}H$ and based on these experiments Westerhoff *et al.* (1984) propose a "mosaic coupling" in chromatophores. Yet we have to remark that not only some results must be artefactual (Cotton and Jackson, 1983) but also the principle of the double inhibitor titration is criticizable (O'Shea and Thelen, 1984). All the protons moving in thylakoids do not have the same efficiency for ATP synthesis (Hangarter and Good, 1984; Flores and Ort, 1984), but Davenport and McCarty (1984) do not find any differences for protons generated by the two photosystems excitation. Nevertheless many experimental results are interpreted by the existence of certain domains in the thylakoids, where the protons are out of equilibrium with the bulk phase (Dilley and Schreiber, 1984; Laszlo *et al.*, 1984; Hong and Junge, 1984; Junge *et al.*, 1984; Schreiber, 1984a,b).

The relation between ATP synthesis and proton flux is an open question, which would be settled only by experiments on a molecular basis. In this line Kagawa (1984) presents a model for the proton motive ATP synthesis based on an acid–base cluster hypothesis.

Nucleotide binding-sites/enzymatic mechanism and ATP synthesis

The CF_1 contains two categories of nucleotide binding-sites: "tight sites" with a K_D of 10^{-7} M or less and "loose sites" with a higher K_D (Strotmann, 1985). With the membrane bound CF_1, affinities are modified by interaction of the enzyme with the membrane of the thylakoids and by energization by light and the resulting proton motive force. It results that a so called "tight site" on isolated CF_1 can become a "loose site" on membrane bound CF_1 and activation of the ATP-synthase in the thylakoids is well reported by its ability to exchange its bound nucleotides with the nucleotides free in the medium.

Three nucleotide binding-sites are most often found with isolated CF_1 (Strotmann, 1985). Six binding-sites for ATP, two per purified β subunit, (Gromet-Elhanan and Khananshvili, 1984) or ADP (Khananshvili and Gromet-Elhanan, 1984c) are characterized on the β subunits removed from the *Rhodospirillum rubrum* membrane bound ATP-synthase. The intrinsic content of purified CF_1, even after treatment to remove residual ADP, is still 0.5 to 1.0 ADP per CF_1 (Girault *et al.*, 1982). CF_1–CF_0 contains at least 1 ADP and 1 ATP per mol (Cerione and Hammes, 1981). Illuminated thylakoids, in the presence of [^{14}C]ADP bind 0.85 (Schumann, 1984) to 1.3 mol [^{14}C]ADP (Bickel-Sandkötter, 1983) per mol of CF_1 under steady-state conditions. Beside those radioactive nucleotides, some non-exchangeable and non-radioactive nucleotides should be present.

Part of the bound nucleotides in the chloroplast-ATPase can be exchanged while the other part is not. At high medium ADP concentration, in the light, about 35% of the total enzyme molecules contain a loosely bound ADP and 65% a tightly bound ADP, while almost none of the enzyme molecules are free from ADP (Schumann, 1984). On the isolated CF_1, one ADP is difficult to exchange and two ADP or ATP are more easily exchangeable (Girault *et al.*, 1982). The magnesium is necessary for binding of one of the two ATP (Bruist and Hammes, 1981) or for the two (Girault *et al.*, 1982).

Localization of the nucleotides binding sites. After tight binding of 2-azido[^{32}P]ADP, 1.4 nmol/mg of chlorophyll, (Czarnecki *et al.*, 1983) or 2-azido [^{32}P]ATP, resulting from phosphorylation of 2-azido ADP, the radioactivity covalently bound was recovered on identical portions of the β subunit polypeptide (Abbot *et al.*, 1984).

With [^{14}C]8-azido ATP (8-N_3-ATP) the α subunits of thermophilic bacterium PS_3 were preferentially labelled (0.8–0.9 mol 8-N_3-ATP/CF_1, α/β = 4/1, Schäfer *et al.*, 1984).

Two binding-sites were characterized on isolated β

subunit of *Rhodospirillumm rubrum* F_1. One nucleotide binding-site is a Mg^{2+} independent "high affinity site" (K_D = 4.4 μM for ATP and 6.7 μM for ADP). The second is a Mg^{2+} dependent "low affinity site" (K_D = 200 μM) which seems to contain also the binding-site for P_i (Gromet-Elhanan and Khananshvili, 1984; Khananshvili and Gromet-Elhanan, 1985).

3′-0(4-Benzoyl)benzoyl ADP (Bz-ADP) interacts with ATP sites of heat-activated CF_1. Complete inactivation was shown to occur upon binding of 2.45 mol Bz-ADP/mol CF_1. Bz-ADP was bound only on subunits β of CF_1, with the isolated enzyme, (Bar-Zvi and Shavit, 1984) or α and β subunits with the membrane bound enzyme (Bar-Zvi *et al.*, 1983).

Enzymatic mechanism and role of nucleotide binding-sites. A recent review of Strotmann (1985) is available on this subject. We want to underline some outstanding aspects of this open question.

It is accepted that ATP is formed from ADP and is not the result of the direct photophosphorylation of AMP by CF_1 (Horner *et al.*, 1983). Yet the elucidation of partial reactions of photophosphorylation may be complicated by side enzyme activities (Feldman and Sigman, 1984).

The three alternating sites model, developed by Boyer (Gresser *et al.*, 1982) explains many experiments, especially those concerning the increase of water oxygen incorporation in ATP released at low ADP concentration (Kohlbrenner and Boyer, 1983; Stroop and Boyer, 1985) and increase of the off constant of ADP and P_i on one site by ATP binding on another site (O'Neal and Boyer, 1984). The non-energy requirement of the reaction ADP + $P_i \rightarrow$ ATP, which has been proposed, seems to be confirmed by the synthesis of ATP from the bound ADP and medium P_i on isolated CF_1 (Feldman and Sigman, 1982) or on a completely uncoupled ATP-synthetase (Feldman and Sigman, 1983). Contrarily, P_i:H_2O oxygen exchange could indicate that there is an energy requirement for the reversible cleavage of the enzyme bound ATP (Shermann and Wimmer, 1983).

The use of adenosine 5′(0-3-thiotriphosphate) (ATP γS) with thermophilic bacterium TF_1 has revealed that hydrolysis of ATP γS occurs with inline nucleophilic displacement (Kagawa *et al.*, 1984), which excludes possible pathways of ATP synthesis *via* a phosphorylated intermediate.

Several studies have also underlined the importance of the stereochemistry to elucidate the mechanism of ATP synthesis or hydrolysis, as is the case of ATP metal complexes. Experiments of Kagawa *et al.* (1984) show that the true substrate for ATP hydrolysis by TF_1 is the Δβγ bidentate ATP–Mg complex while Frash and Selman (1982) propose the substrate of photophosphorylation catalyzed by CF_1 be the Λ epimer of the bidentate metal–ADP complex.

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