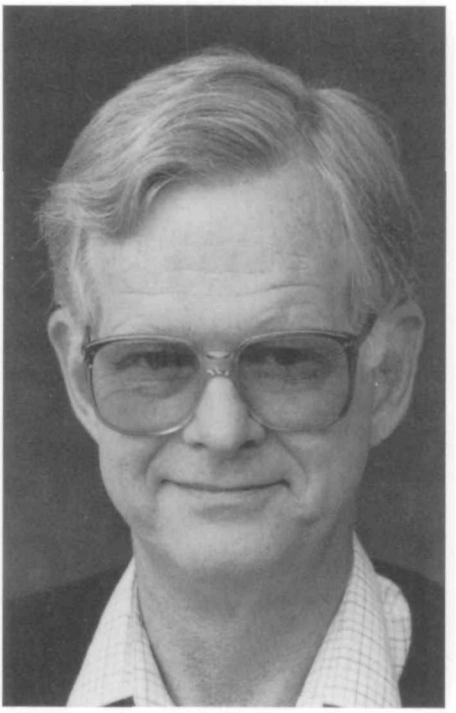

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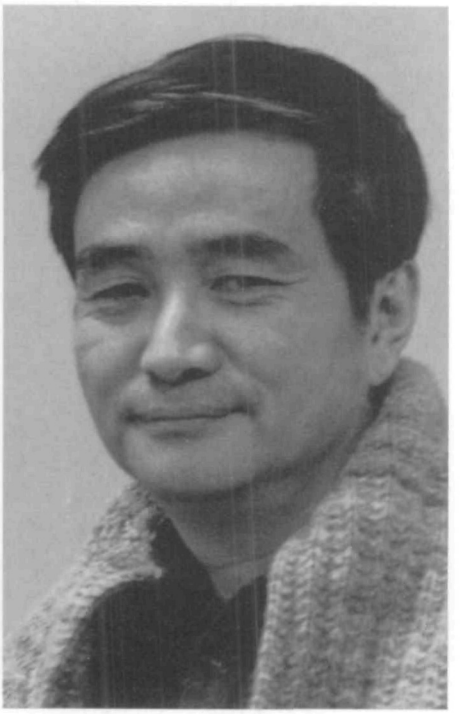

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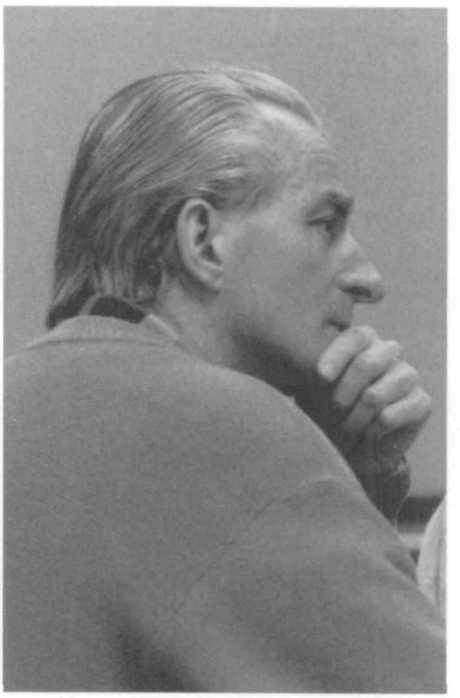

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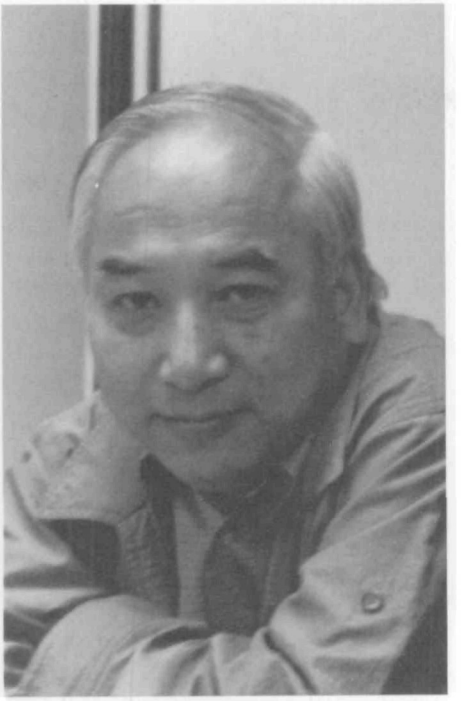

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A PLANT BIOCHEMIST'S VIEW OF H^+-ATPases AND ATP SYNTHASES

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Introduction

My twenty-five year fascination with membrane ATPases grew out of my experiences in the laboratories of André Jagendorf and Efraim Racker. André introduced me to photosynthetic phosphorylation and Ef, to whose memory this article is dedicated, convinced me that ATPases had much to do with ATP synthesis.

Astounding progress has been made in the H^+-ATPase field in just two decades. By the early 1970s, it was generally recognized that oxidative and photosynthetic ATP synthesis were catalyzed by membrane enzymes that could act as H^+-ATPases and that the common intermediate between electron transport and phosphorylation is the electrochemical proton gradient. At that time, it had been shown that a cation-stimulated ATPase activity was associated with plasma membrane preparations from plant roots. The endomembrane or vacuolar ATPases were unknown.

The application of improved biochemical methods for membrane isolation and purification, as well as membrane protein reconstitutions, led rapidly to the conclusion that there are three major classes of membrane H^+-ATPases, P, V and F. P-ATPases, which will not be considered further in this article, are phosphorylated during their catalytic cycle and have a much simpler polypeptide composition than V- or F-ATPases. The plasma membrane H^+-ATPase of plant, yeasts and fungal cells is one example of this class of enzymes (see Pedersen and Carafoli, 1987, for a comparison of plasma membrane ATPases).

Biochemical and gene sequencing analysis have revealed that V- and F-ATPases resemble each other structurally, but are distinct in function and origin. The 'V' stands for vacuolar and the 'F' for F_1F_o. F_1 was the first factor isolated from bovine heart mitochondria shown to be required for oxidative phosphorylation. F_o was so named because it is a factor that conferred oligomycin sensitivity to soluble F_1. Other F-ATPases are often named to indicate their sources. For example, chloroplast F_1 is denoted CF_1 (see Racker, 1965, for early work on F_1). Recent successes in reconstitution of vacuolar ATPase have led to a V_1V_o nomenclature for this enzyme as well.

The term 'ATP synthase' is now in general use to describe F-ATPases. This term emphasizes the facts that although F-ATPases function to synthesize ATP, they do not catalyze, normally, ATP hydrolysis linked to proton flux. In contrast, V-ATPases are very unlikely to operate as ATP synthases. Thus, F-ATPases are proton gradient consumers, whereas V-ATPases generate proton gradients at the expense of hydrolysis.

Key words: ATP synthesis, ATP hydrolysis, F_1F_o, ATP synthase.

In this brief review, I will compare the structures of F- and V-ATPases. Also, I give some insight into the mechanisms that help prevent wasteful ATP hydrolysis by the chloroplast ATP synthase (CF_1F_o).

Structure of F- and V-ATPases

Both F- and V-ATPases are structurally complex. CF_1F_o, for example, contains nine different proteins and a total of about twenty polypeptide chains (Jagendorf *et al.* 1991). The M_r of V- and F-ATPases is approximately 550 000–650 000. The complexity of these enzymes is puzzling. This is especially true in view of the fact that P-ATPases can be as simple as a single polypeptide chain of about 100 000 M_r. Genetic studies with *Escherichia coli* have established that each of the eight *E. coli* F_1F_o polypeptides is required for function (Futai *et al.* 1989).

F-ATPases may be readily separated into two parts – F_1, the catalytic sector and F_o, the proton translocating part. F_1 can be removed from many coupling membranes either by physical means or simply by diluting membranes into media of low ionic strength that contain EDTA. F_1 is, thus, an extrinsic membrane protein. Once removed from the membrane, F_1 is soluble to at least 100 mg ml^{-1}. From 2 kg of spinach leaves, 400 mg of CF_1, greater than 95 % pure, may be prepared in a day and a half. This ease of preparation makes one less reluctant to engage in calorimetric or equilibrium nucleotide binding studies that can require as much as 100 mg of CF_1 for each experiment. Depending on the source, the M_r of F_1 ranges from 365 000 to 400 000.

From all sources, mitochondrial, chloroplast and eubacterial plasma membranes, the subunit composition of F_1 is similar. There are five different proteins, labelled α–ϵ in order of decreasing M_r. There is a consensus that these polypeptides are present in a 3:3:1:1:1 stoichiometry (Table 1). The primary sequence of a large number of F_1 polypeptides has either been deduced from gene sequences or determined chemically. From sequence comparisons, three striking facts were revealed. First, the sequence of the β subunit has been remarkably conserved, even across Kingdom lines (Hudson and Mason, 1988; Walker and Cozens, 1986). For example, the β subunit of CF_1 and *E. coli* F_1 have 76 % sequence identity. Second, the α and β subunits are related and it seems quite possible that α arose by gene duplication. Third, the smaller subunits, γ, δ and ϵ, are not well conserved. In fact, ϵ of mitochondrial F_1 does not resemble CF_1 ϵ or *E. coli* F_1 ϵ

Table 1. *Subunit composition of some ATP synthases*

	Extrinsic polypeptides F_1					Intrinsic polypeptides F_o			
Source	α	β	γ	δ	ϵ	*a*	*b*	*c*	
E. coli	55	50	31	19	15	30	17	8	
						IV	I	II	III
Chloroplasts	55	54	36	21	15	27	17	14	8
Mitochondria	55	52	30	20	15	30	20	8+at least five polypeptides	

Numbers shown are M_r values$\times 10^{-3}$. (Adapted from Nelson, 1989.)

at all and mitochondrial F_1 δ is also unique (Walker and Cozens, 1986). The bacterial and chloroplast δ subunits resemble oligomycin sensitivity conferral protein (OSCP) which is considered to be an F_0 subunit. Thus, generalizations about subunit function based simply on M_r can be problematic.

From image reconstruction electron microscopy (Boekema *et al.* 1988), distance mapping by fluorescence energy transfer (McCarty and Hammes, 1987) and X-ray diffraction (Bianchet *et al.* 1991), the structure of F_1 is emerging (Fig. 1). The structure is dominated by the α/β hexamer. The α subunits are in contact with the β subunits, but the α subunits are closer to the membrane surface. The position of the γ subunit is not exactly known, but it is likely that it is centrally located in an asymmetrical manner. The ϵ subunit interacts strongly with γ, and δ is placed close to the membrane surface because of its role in F_1 binding to F_0.

The inherent structural asymmetry of F_1 is intriguing. The α and β subunits cannot be in equivalent environments. That is, the interactions of a given α or β subunit in the complex with the smaller subunits must differ from those of another α and β subunit. There is evidence to suggest that CF_1 lacking the δ and ϵ subunits retains its structural asymmetry (Shapiro *et al.* 1991). Thus, interactions of the γ subunit with α and β may be the major asymmetry inducers.

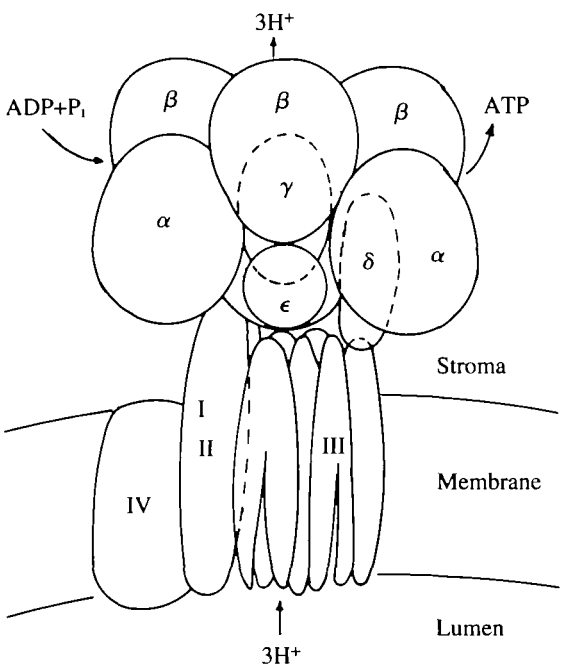


Fig. 1. A depiction of the chloroplast ATP synthase (CF_1F_0). The model of CF_1 is supported by electron microscopy and image reconstruction, fluorescence resonance energy transfer distance mapping of specific residues on various CF_1 subunits and the arrangement of the α and β subunits is in accord with the X-ray diffraction results for mitochondrial F_1. The arrangement of CF_0 subunits is entirely speculative. Courtesy of Dr Carolyn Wetzel.

The structural asymmetry of F_1 probably explains the nucleotide binding site asymmetry as well as the unusual reactivity of Lys-378 of the α subunit of CF_1. Only one of the three α subunits of CF_1 reacts at this position rapidly with Lucifer Yellow vinyl sulfone. The six nucleotide binding sites of F_1 show quite different properties. The most plausible model for the mechanism of F_1 is one in which two or three catalytic sites change their properties during the catalytic cycle (Boyer, 1989). In this alternating site or binding change mechanism, catalytic sites alternate between very high affinity and much lower affinity. The binding of substrate to one catalytic site promotes catalysis and product release from another. If this mechanism holds, and there is much evidence in its favor, F_1 cannot be permanently frozen in one asymmetrical state. The incubation of CF_1 with Mg^{2+}-ATP was found to cause two nucleotide binding sites on CF_1 to switch their properties (Shapiro and McCarty, 1990). This observation is consistent with the binding change mechanism. How substrate binding affects changes in the enzyme is unknown.

Although all five *E. coli* F_1 or CF_1 subunits are required for ATP synthesis, $\alpha_3\beta_3\gamma$ complexes exhibit the highest ATPase activity. An intact γ subunit, however, is not required for ATPase activity and some ATPase activity is seen in α/β complexes (see, for example, Kagawa *et al.* 1989). Although it probably bears the catalytic sites, isolated β is a very poor ATPase. In CF_1, γ definitely plays a regulatory role and has been proposed to be involved in proton translocation. The δ subunit is required for the binding of *E. coli* F_1 to F_0 and is necessary for the functional binding of CF_1 to CF_0. In *E. coli* and chloroplast F_1, ϵ is an inhibitory subunit, probably involved in regulation of the enzymes. These studies are summarized by Futai *et al.* (1989) and Jagendorf *et al.* (1991).

Different coupling membranes seem to have tailored the F_0 portion of the ATP synthase to suit their needs. By definition, F_0 is that part of the ATP synthase that remains after removal of F_1. Unlike F_1, F_0 is hydrophobic and can only be isolated from membranes by detergent extraction. *E. coli* F_0 (Deckers-Hebestreit and Altendorf, 1992) and chloroplast F_0 have been purified in active forms. F_0 functions in at least two ways: it binds F_1 and translocates protons across the membrane. The rates of proton conductance through CF_0 are high, suggesting that the F_0 proton translocation mechanism is a proton-selective channel (Lill and Junge, 1989; Junge *et al.* 1992).

The number of different polypeptides in F_0 varies with the source (Table 1). *E. coli* F_0 is the simplest, with just three polypeptides, labeled *a*, *b* and *c*. Spinach CF_0 has four polypeptides, denoted in Roman numerals, I–IV. Mitochondrial F_0 can have as many as eight polypeptides. All F_0 subunits contain a small (8000 M_r) hydrophobic polypeptide, referred to as the proteolipid or DCCD-binding protein. There is no lipid covalently attached to the DCCD-binding protein. This polypeptide is present in 6–12 copies per ATP synthase. Remarkably, the reaction of just one copy of the protein with DCCD (*N,N'*-dicyclohexylcarbodiimide) at a specific glutamyl or aspartyl residue is sufficient to inhibit ATP synthesis totally. ATP synthesis is inhibited by DCCD because proton transport through F_0 is blocked. All three *E. coli* F_0 subunits are required for proton translocation and at least subunits III and IV of chloroplast F_0 are necessary for proton transport.

Cross-linking studies indicate that several CF_0 subunits interact with CF_1. Some CF_0 subunits have hydrophilic domains that are predicted to extend out from the stromal side

of the membrane; that is, towards CF_1. Subunit III of CF_0 appears to bind to CF_1 with sufficient strength to allow the purification of a CF_1–subunit III complex by a chromatographic procedure (C. Wetzel and R. McCarty, unpublished observations). This complex retains some of the properties characteristic of the CF_1F_0 from which it was prepared. Probably, all CF_0 subunits are in contact with one or more CF_1 polypeptides. The dominant interactions have yet to be determined.

Like F-ATPases, V-ATPases consist of an extrinsic component, called V_1, and a membrane-associated part, now denoted V_0. The treatment of yeast, *Neurospora crassa*, or oat root vacuolar membranes and clathrin-coated vesicles with KI or KNO_3 in the presence of Mg^{2+}-ATP rapidly inactivates ATPase activity and associated proton pumping. Depending on the source, about five polypeptides are dissociated from the membrane by this treatment (see, for example, Ward *et al.* 1992). The dissociated polypeptides were devoid of ATPase activity by themselves, but, in the case of the clathrin-coated vesicles (Puopolo *et al.* 1992) and oat root systems (Ward *et al.* 1992), could be added back to the depleted membranes to give both ATPase activity and ATP-dependent proton pumping. It is of interest to note that membranes from which the V_1 components had been removed were not leaky to protons. Thus, either V_0 required a V_1 polypeptide(s) for proton transport or the attachment of V_1 helps to open a proton gating mechanism.

To an F_1 person, the subunit composition of V-ATPases is somewhat confusing. Then again, to a V_1 person, I would venture to guess that the composition of F-ATPases would also be confusing. As is the case for F-ATPases, V-ATPases seem to retain a similar subunit structure for V_1, but tailor the membrane-associated components to fit the needs of the membrane (Nelson, 1989). The situation is made even more complicated by the possibility that a given tissue within an organism could possess a V-ATPase that differs from that in another tissue.

Regardless of the apparent heterogeneity of the subunit composition of V-ATPases, there are, as is the case for F-ATPases, recurrent themes. V-ATPases are oligomeric and contain both a peripheral, catalytic component V_1 and an integral membrane part, called V_0 by analogy to F_0 (Table 2). All V-ATPases so far examined from animal, plant, fungal and yeast sources contain two easily discernable polypeptides of about 70 000 (the *A* subunit) and 60 000 M_r (the *B* subunit). Based on covalent binding of –SH alkylating reagents and photoaffinity nucleotide binding, it is very likely that the *A* subunit is catalytic. The function of the *B* subunit is unknown. The *A* subunits of various V-ATPases share remarkable amino acid sequence similarity. Remarkably, the catalytic subunits of ATP synthases of archaebacterial ATPases are more closely related to V-ATPases than to F-ATPases. Nonetheless, the *A* subunits of both archaebacterial and V-ATPases share significant sequence homology with the β subunits of F_1 (22–26 %) (Ihara *et al.* 1992). Moreover, the *B* subunits of V-ATPases are homologous with the α subunits of F_1 (20–27 %). See Nelson (1992) for a more complete description of the sequence homologies.

In addition to subunits *A* and *B*, V-ATPases from a number of membranes contain polypeptides ranging in relative molecular mass from 100 000–115 000 to 12 000–13 000. Most V-ATPases contain polypeptides (four or five) in the 30 000–45 000 M_r range. All

Table 2. *Subunit composition of some V-ATPases*

Source	Extrinsic polypeptides* V_1							Intrinsic polypeptides† V_o		
	A	*B*	*C*	*D*	*E*					
Chromatin granules	73	58	40	34	33			100	19	17
Yeast	69	60	42	36	32			100	27	17
Oat root	70	60	44	41	36	29		16	13	12
Red beet	67	55	52	44	42	32	29		16	
Archaebacterial	64	54	28						9	

*Defined by their ease of removal from vacuolar membranes by chaotropic anion treatment in the presence of Mg^{2+}-ATP. The numbers are M_r values ($\times 10^{-3}$) of the peptides.

†Subunits left behind after chaotroph/ATP treatment. The numbers are M_r values ($\times 10^{-3}$) of the polypeptides.

so far contain a 16 000 M_r protein that has the solubility properties of a 'proteolipid' and binds DCCD. As is the case for F-ATPases, DCCD inhibits V-ATPase activity and associated proton pumping. It is quite possible that the V-ATPase DCCD-binding protein arose by gene duplication. Some V-ATPases appear to contain a high M_r (100 000–115 000) polypeptide and others a low M_r (12 000–13 000 M_r) polypeptide.

V-ATPases are sensitive to inhibition by chaotrophic anions (I^-, NO_3^- or SCN^-) in a manner that is fascinatingly dependent on the presence of Mg^{2+}-ATP) (Moriyama and Nelson, 1989). A number of polypeptides dissociate from the membrane as a result of this treatment; others are left behind. Concomitant with loss of ATPase activity and associated protein pumping, more or less spherical particles of 10–12 nm are removed from vacuolar membranes (Bowman *et al.* 1992).

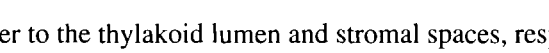
The peripheral portion (V_1) is defined as that part removed by chaotroph treatment in the presence of Mg^{2+}-ATP. The *A* and *B* subunits, probably present in a 3:3 stoichiometry with respect to the smaller polypeptides, are clearly in this class. In addition, the polypeptides in the 30 000–45 000 M_r range are removed by this treatment. Recently yeast subunit *C* (M_r 42 000) was cloned and sequenced (Beltran *et al.* 1992). Subunit *C* was shown to be required for assembly and exhibited no homology to the γ subunit of F_1. What is left after chaotroph treatment is defined as V_o. There is a consensus that the 16 000 M_r DCCD-binding protein, which is probably present in multiple copies, is part of V_o. The approximately 100 000 M_r polypeptide of some V-ATPases as well as smaller proteins (12 000–20 000 M_r) may also be part of V_o. Further analysis is required and will undoubtedly be forthcoming.

There are several observations that point to major differences between V- and F-ATPases, even though superficially they seem similar. Removal of F_1 greatly increases the proton permeability of the membrane in which it resides. This effect is not observed for V-ATPases. Perhaps some V_1 component remains associated with V_o after chaotroph treatment and blocks a proton conductance mechanism. Alternatively, a V_1 component is required for proton transport through V_o. So far V_1 has not been released from the

membrane in a form that is active in ATP hydrolysis. This result is in distinct contrast to F_1F_o. Finally, Mg^{2+}-ATP *destabilizes* V_1V_o, whereas it *stabilizes* F_1 against cold-inactivation in the presence of chaotrophic anions.

Regulation of CF_1F_o

The reaction catalyzed by the chloroplast ATP synthase is:



$$3H^+_{in}+ADP+P_i \rightleftharpoons 3H^+_{out}+ATP+H_2O,$$

where 'in' and 'out' refer to the thylakoid lumen and stromal spaces, respectively. Logic dictates that CF_1F_o should be able to catalyze the reverse reaction of ATP synthesis, ATP hydrolysis. Within chloroplasts, however, it is very unlikely that CF_1F_o catalyzes ATP hydrolysis at significant rates, even though it carries out ATP synthesis at very rapid rates.

Thylakoid membranes, as usually isolated, hydrolyze Mg^{2+}-ATP in the dark, even at 37 °C, at rates close to 0.05 μmol min^{-1} mg^{-1} protein. Much of this activity may be attributed to phosphohydrolases that contaminate the preparations. In contrast, the same membranes, when illuminated, can catalyze ATP synthesis at rates in excess of 5 μmol min^{-1} mg^{-1} protein. It is clear, then, that light dramatically activates the chloroplast ATP synthase. The ATP synthases have evolved to be specialists in ATP synthesis and, through a number of different mechanisms, potentially wasteful ATP hydrolysis by CF_1F_o is prevented.

It must be emphasized that the effect of light is indirect. Transthylakoid electrochemical proton gradients drive ATP synthesis in total darkness and, thus, are also involved in the conversion of the ATP synthase to an active form. Light-driven electron flow generates proton gradients that serve two functions in photophosphorylation. First, the proton gradient is the driving force for ATP synthesis and, second, it is required for activation.

How this unusual 'one way' regulation occurs at the molecular level is beginning to be revealed. A coherent picture, summarized in Fig. 2, of what may happen in a dark-to-light transition can now be drawn. The ϵ subunit is an inhibitor of the ATPase activity of CF_1.

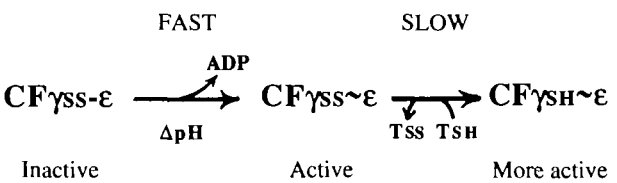


Fig. 2. A scheme for the activation of the chloroplast ATP synthase. CF stands for the CF_1 part of the ATP synthase. γSS and γSH are oxidized (disulfide) and reduced (dithiol) forms of the γ subunit, respectively. TSS and TSH are oxidized and reduced thioredoxin, respectively. In the absence of ΔpH, CF_1 contains tightly bound ADP, and ϵ and γ interact strongly, as indicated by the bold arrows. Rapid changes in the enzyme induced by ΔpH formation by electron transport in the light cause ADP release and weaken γ–ϵ interactions (as shown by ~). Although the oxidized enzyme in this state is active, further activation occurs upon reduction of the γ disulfide by reduced thioredoxin.

By necessity, it must also inhibit ATP synthesis. The ϵ and γ subunits interact and it is probable that the inhibitory effects of ϵ are mediated at least in part through the γ subunit. In the dark, ϵ interacts very strongly with γ and the ATPase activity is inhibited. Generation of the electrochemical proton gradient causes changes in CF_1 that involve both the γ and ϵ subunits (at least). A movement of ϵ relative to γ is indicated. In their new conformations, ϵ inhibition could be nullified.

Redox regulation is also involved in the physiologically significant light activation of CF_1 in chloroplasts. The γ subunit of CF_1 from photosynthetic eukaryotes contains an insert of about twenty amino acids that is absent in *E. coli* or mitochondrial F_1 subunits (Jagendorf *et al.* 1991). This insert contains a redox-active pair of Cys residues (Cys-199 and Cys-205 for spinach CF_1 γ subunit). In the dark, Cys-199 and Cys-205 form a disulfide bond, whereas in the light this disulfide (the only disulfide in CF_1) is reduced. Thioredoxin, reduced by electrons from photosystem I, is probably the physiological reductant.

Although oxidized CF_1 is active in ATP synthesis, reduced CF_1 is even more active. At physiological values of the electrochemical proton gradient, phosphorylation could be enhanced as much as tenfold (Quick and Mills, 1986). Essentially, it appears that the energy cost for activation is significantly decreased by reduction of the γ disulfide bond.

The activated state of oxidized CF_1 in thylakoids decays quite quickly in the dark. Thus, after a period of illumination, little ATPase activity is observed in the dark. After reduction of the γ disulfide bond, however, significant ATPase activity persists in the dark after a period of illumination (Bakker-Grunwald, 1977). This ATPase activity is coupled to inward proton fluxes and can generate ΔpH values of sufficient magnitude to permit ATP synthesis at a relatively low rate. Proton ionophores at concentrations that collapse the proton gradient completely abolish ATPase activity in the dark. The ΔpH generated by ATP hydrolysis in the dark by CF_1 in reduced thylakoids is, thus, sufficient to keep the enzyme, at least in part, in an active form. During illumination, ATP hydrolysis is prevented by the high value of ΔpH which drives the reaction in the direction of synthesis (Davenport and McCarty, 1986).

In intact chloroplasts, the ability of reduced CF_1 to hydrolyze ATP in the dark after a period of illumination is lost over a period of several minutes in a biphasic manner. The more rapid phase is probably a consequence of the binding of ADP to a nucleotide binding site on CF_1. ADP at micromolar concentrations rapidly inactivates the ATPase of reduced CF_1 in the dark, without causing oxidation of the γ subunit, dithiol. To reconvert the CF_1 to an active form after ADP has bound, ΔpH is required and ADP is released from the enzyme. The second, slower phase of the inactivation of CF_1F_o in intact chloroplasts in the dark is the oxidation of the dithiol by an unknown mechanism (Biaudet *et al.* 1988).

There is good evidence that the ϵ and γ polypeptides of CF_1 interact. They are physically close together (McCarty and Hammes, 1987) and alterations of the γ subunit modify ϵ–CF_1 interactions (Soteropoulos *et al.* 1992). Reduction of the γ disulfide decreases the affinity of ϵ binding to CF_1 about 20-fold and tryptic cleavage of a portion of the C terminus of γ abolishes high-affinity ϵ binding. Removal of the ϵ subunit markedly enhances the rate of reduction of γ disulfide by dithiothreitol or thioredoxin (Dann and McCarty, 1992). The γ subunit in CF_1 depleted in ϵ is easily attacked by

proteases, with the initial major cut occurring close to the γ disulfide to produce a γ fragment of about 27 000 M_r. If the γ disulfide is reduced, a second cut occurs that releases a peptide of M_r 1300. This peptide bears Cys-205.

The γ subunit of CF_1 in thylakoids in darkness is resistant to proteolysis. With the increase of ΔpH in the light, however, changes in the enzyme occur that render γ very susceptible to partial proteolysis. The γ subunit is cleaved to a fragment of 27 000 M_r and, if the disulfide is reduced, the same second cut that occurs in CF_1 deficient in ϵ is observed. Thus, with respect to the susceptibility of the γ subunit to protease attack, CF_1 in illuminated thylakoids resembles CF_1 deficient in ϵ.

Mitochondrial F_1F_o is regulated by a quite different mechanism from that of CF_1F_o. The ϵ subunit of mitochondrial F_1 has no sequence similarity to that of CF_1 or *E. coli* F_1. Mitochondrial ϵ does not appear to be an inhibitor of the ATPase activity of mitochondrial F_1. Instead, mitochondrial F_1 activity is regulated by an inhibitor protein (or proteins) that, unlike ϵ, is not considered to be part of F_1 (reviewed by Cross, 1981). The binding of the inhibitor protein is favored by ATP. In contrast, the inhibitor protein is released from the complex when the magnitude of the electrochemical proton gradient and the ADP/ATP ratio are high. Thus, ATP synthesis would be favored.

There is no evidence for an inhibitor protein other than ϵ in either *E. coli* or chloroplasts. Moreover, it is very clear that ϵ dissociation from CF_1 cannot be a part of the activation process. CF_1 deficient in ϵ binds to CF_o equally as well as CF_1, but ϵ-depleted CF_1 cannot restore ATP synthesis (Richter *et al.* 1984). When CF_1 is removed from thylakoid membranes the membranes become highly proton permeable because of proton leakage through CF_o. The permeability is so high that even extremely rapid proton translocation by light-dependent electron transport does not generate a significant ΔpH. CF_1 deficient in ϵ fails to block the CF_o proton channel and, thus, cannot restore ATP synthesis to CF_1-depleted thylakoids. If ϵ were to dissociate from CF_1 during activation, therefore, ATP synthesis would be inhibited.

The ϵ subunit is also an inhibitor of the ATPase activity of *E. coli* F_1 and has been shown to interact strongly with γ (Dunn, 1982). The *E. coli* ATP synthase can operate as an H^+-ATPase *in vivo*. For example, when respiratory chain activity is low, ATP hydrolysis by ATP synthase powers proton efflux to generate an electrochemical proton gradient. It is likely that the activity of the enzyme is regulated since the ATPase activity of the *E. coli* ATP synthase in its natural environment is much lower than the V_{max}. An energy-dependent activation mechanism which involves the overcoming of ϵ inhibition may occur in *E. coli* F_1F_o as well as in other ATP synthases.

The dual activities of the ϵ subunit of CF_1 (regulation and proton channel blocking) suggest an intriguing connection between activation and opening of a 'proton gate'. The proton conductivity through CF_1F_o is normally low when the enzyme is inactive, but is high (greater than 1 ms^{-1}) during ATP synthesis. Could it be that activation entails – at least in part – an opening of the proton gate? In which part of the ATP synthase protons induce the conformational changes involved in activation and proton gate opening is unknown. Protonation of a CF_o subunit(s) in contact with CF_1 could induce the changes. Alternatively, CF_o could act passively to deliver protons to a site on one or more of the CF_1 subunits.

To date, little is known about regulation of V-ATPases. By analogy to the F_1F_0-ATPases, I predict that one or more of the smaller polypeptides of V_1 will be a regulatory subunit. As is the case for mitochondrial F_1, a dissociable inhibitory protein could be involved.

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