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Regulation of Proton Flow and ATP Synthesis in Chloroplasts

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The chloroplast ATP synthase is strictly regulated so that it is very active in the light (rates of ATP synthesis can be higher than 5 μmol/min/mg protein), but virtually inactive in the dark. The subunits of the catalytic portion of the ATP synthase involved in activation, as well as the effects of nucleotides are discussed. The relation of activation to proton flux through the ATP synthase and to changes in the structure of enzyme induced by the proton electrochemical gradient are also presented. It is concluded that the γ and ε subunits of CF_1 play key roles in both regulation of activity and proton translocation.

KEY WORDS: CF_1; ATP synthase; proton flow; electron transport; slip.

INTRODUCTION

The ATP synthase from chloroplasts belongs to a large family of F_oF_1 type ATPases and shares a common structure with the ATP synthases from mitochondria and bacteria. The general structure of this enzyme family is a hydrophobic moiety (F_o) that translocates protons across the membrane and a hydrophilic moiety (F_1) that contains the catalytic subunits with the nucleotide binding sites. The coupling between ion movement and ATP synthesis was described by Mitchell (1961) in his chemiosmotic hypothesis. Electron transfer is coupled to vectorial proton translocation across a membrane with poor proton permeability, thus creating a proton electrochemical gradient. Coupling proton movement down the electrochemical gradient to a ATP synthesis completes the cycle. Although almost 40 years have passed since Mitchell postulated his theory, the details of the coupling between proton movement and ATP synthesis at the molecular level are not clear. Major strides toward understanding coupling were made when the structure and stoichiometry of the subunits involved were, and still are, being deciphered, and the kinetics and nucleotide binding and release are better understood.

Chloroplast $F_1(CF_1)$ is composed of five subunit types with the stoichiometry of $\alpha_3\beta_3\gamma\delta\varepsilon$. It was shown that F_1 has a hexameric structure when observed from the top (Boekema *et al.*, 1988), where alternating α and β subunits form a hexamer and the γ subunit is located at least in part within the center of the heterohexamer (Abrahams *et al.*, 1994; Snyder and Hammes, 1984). The membrane-embedded CF_o contains four different polypeptides in a stoichiometry of I, II, III_{9-12} IV (Fromme *et al.*, 1987a, b).

In this article, we discuss regulation of ATP synthesis and proton flux through the ATP synthase with particular emphasis on the effects of nucleotides and the roles of the ε and γ subunits of CF_1.

NUCLEOTIDE EFFECTS ON PROTON AND ELECTRON FLOW

Nucleotides in the medium enhance the extent of proton uptake in illuminated chloroplast thylakoids (McCarty *et al.*, 1971) and inhibit electron transport under nonphosphorylating conditions (Avron *et al.*, 1958; Higashida and Mukohata, 1976). At the steady state of illumination, the rate of proton flow into the thylakoid lumen is equal to the flux from the lumen. Since electron transport is coupled to proton translocation, the rate of proton influx is given by the rate of electron transport (*Re*) times the proton to electron stoichiometry (H^+/e). Proton efflux is first order with respect to internal proton concentration ($[H^+]_{in}$), so that the rate of efflux is given by $k[H^+]_{in}$, where k is an apparent first-order rate constant related to the permeability of thylakoid membrane to protons. Thus, at

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the steady state, $(H^+/e)Re = k[H^+]_{in}$ (Portis and McCarty, 1976).

Specific inhibitors such as N,N'-dicyclohexylcarbdiimide (DCCD) or venturicidin specifically block proton transport through CF_o and allow the evaluation of the proton leak through the membrane and the elucidation of the specific proton leak through the ATP synthase (Davenport and McCarty, 1984; Evron and Avron, 1990).

The linear relationship between the rate of electron transport (*Re*) and $[H^+]_{in}$ breaks down at high light intensity and alkaline external pH, especially in the absence of nucleotides (Portis *et al.*, 1975). In the presence of DCCD, $[H^+]_{in}$ is a linear function of *Re* at all light intensities (Davenport and McCarty, 1984). When nucleotides are absent in the medium, CF_1 undergoes some kind of energy-dependent conformational change that renders it leaky to protons. In the presence of Mg-ATP at micrometer levels, this change is largely prevented. Thus, *Re* is inhibited and $[H^+]_{in}$, ΔpH, and the extent of proton uptake are increased by ATP. Mg-ADP has similar effects, but P_i is also required (Evron and Avron, 1990; Groth and Junge, 1993).

CF_1 contains tightly bound nucleotides that function to stabilize the enzyme (Wang *et al.*, 1993) and in regulation of activity (Bar-Zvi and Shavit, 1980; Bickelsandkoetter and Strotmann, 1981). Mg-ADP is an inhibitor of the ATPase activity of CF_1 (Bar-Zvi and Shavit, 1982). In the absence of an electrochemical proton gradient, Mg-ADP binds very tightly to a site that may be both a catalytic and a regulatory site. CF_1 in darkened thylakoids is nearly totally inactive. Within a few milliseconds after illumination has begun, ATP synthesis reaches its maximum rate. Release of ADP has been correlated to the switching on of the activity of the ATP synthase in the light and binding of ADP to its switching off.

The changes in CF_1 induced by high values of ΔpH that result in increased flow of protons through the ATP synthase may also be responsible for the decrease in affinity for ADP binding to a regulatory site. Activation could involve opening of a proton gate that is regulated by nucleotides. The presence of ADP tightly bound to the CF_1 shuts the gate, whereas when the site is vacant, as in the case of thylakoids illuminated in the absence of nucleotides, the gate is open and protons flow through the ATP synthase in a nonproductive manner. The loose binding of nucleotide to this site prevents the nonproductive leak, but still permits proton efflux coupled to ATP synthesis. In view of current models for the mechanism of ATP synthases reviewed in (Richter *et al.*, 2000), nucleotide binding may restrict the movement of the γ subunit within the α/β hexamer thereby inhibiting proton flow through CF_o. The relationship between changes in the γ subunit and nucleotide binding will be discussed below. It is also interesting to note that the inhibition of proton flow through the ATP synthase by nucleotides is only partial, as is evident from the further decrease in electron transport and increase in proton uptake (Evron and Avron, 1990) and increase in ΔpH (Evron and Pick, 1997) by DCCD or venturicidin. This may indicate that a proton leak or slip occurs at high light intensities under nonphosphorylating conditions.

CHLOROPLAST ATP SYNTHASE REGULATION

The tight regulation of the activity of the chloroplast ATP synthase is a result of several mechanisms acting together to prevent ATP hydrolysis in the dark and to allow ATP synthesis at physiological ΔpH values. In addition to the release of inhibitory, tightly bound ADP, other changes within CF_1 that may be related to the regulation of activity of the ATP synthase are induced by formation of the electrochemical proton gradient (predominantly ΔpH in thylakoids) and proton flux through the synthase. Among these energy-dependent changes are greatly enhanced reactivity of Cys89 of the γ subunit with maleimides (McCarty and Fagan, 1973; Miki *et al.*, 1988; Moroney *et al.*, 1984), increased exposure of the disulfide bond in the γ subunit to reduction (Ketcham *et al.*, 1984) and the exposure of several Lys residues of the γ subunit to modification by pyridoxal phosphate (Komatsu-Takaki, 1996). The energy-dependent exposure of the disulfide bridge in the γ subunit between Cys199 and Cys205 to reduction by dithiothreitol or reduced thioredoxin is of physiological significance. The reduction of the disulfide bridge in the γ subunit, a feature unique to the chloroplast ATP synthase, allows the enzyme to synthesize ATP at high rates at values of the electrochemical gradient that are approximately 0.3 pH unit lower than those in oxidized thylakoids (Junesch and Gräber, 1987; Ketcham *et al.*, 1984). At physiological values of the proton electrochemical gradient, phosphorylation may be enhanced by as much as tenfold by reduction of the γ disulfide. Effectively, reduction lowers the energetic threshold for ATP synthesis. The mechanism of lowering the energy threshold is unclear, but could be related to proton gating. Recently, it was shown that the energetic threshold for inducing the proton leak through the ATP synthase was decreased by reduction of the disulfide bond in the γ subunit (Evron and McCarty, 2000). The presence of ATP in the medium blocked the proton leak. Opening of the proton gate appears to occur at a lower ΔpH values in reduced thylakoids, a fact that may explain the shift of ATP synthesis to lower ΔpH values after reduction.

MODIFICATIONS OF THE γ SUBUNIT AND THEIR EFFECT ON PROTON FLUX

The γ subunit is specifically labeled by *N*-ethylmaleimide (NEM) upon illumination of thylakoids (McCarty and Fagan, 1973). NEM was later shown to be on the conserved Cys residue, Cys89 (Moroney *et al.*, 1984). This modification is strictly dependent on ΔpH (Portis *et al.*, 1975) and on proton flow through the ATP synthase. ATP and ADP at low concentrations in the medium strongly inhibit the modification of γCys89 by maleimides (Magnusson and McCarty, 1975). The similarities in the properties of the effects of nucleotides on modification of γCys89 by maleimides and those on proton flux suggest that the γ subunit is part of the proton-gating mechanism. Uncouplers prevented modification of Cys89 by maleimides. Also, when proton flux through CF_o was blocked by either DCCD or venturicidin, γCys89 did not react with maleimides, even at very high ΔpH values (Evron and Pick, 1997). Thus, proton flux through the ATP synthase is a requirement for the conformational changes within the γ subunit that expose γCys89 to modification. Energy-dependent changes in the γ subunit were also detected by the increased reactivity of various Lys residues in the γ subunit to pyridoxal phosphate (Komatsu-Takaki, 1996). The nature of these conformational changes is not clear, but under conditions of proton flux, it is clear that the γ subunit is not a rigid protein. Movements of the γ subunit, both internal and with respect to other CF_1 subunits, may be important in regulation of activity as well as regulation of proton flux. Movements of the γ subunit are also likely to be involved in catalysis (Cross and Duncan, 1996; Hisabori *et al.*, 1997; Richter *et al.*, 2000; Schulenberg *et al.*, 1997).

The modification of Cys89 with NEM increases the proton leak through the ATP synthase at alkaline external pH (Evron and Pick, 1997). The leak is partially inhibited by Mg-ATP and is inhibited completely at neutral external pH, even in the absence of nucleotides. The increased leak caused by NEM modification demonstrates the importance of this region in the γ subunit for proton translocation. Alkylation of Cys89 also inhibits ATP synthesis and hydrolysis and greatly accelerates the exchange of ADP tightly bound to CF_1 with nucleotide in the medium (Soteropoulos *et al.*, 1994). Based on the crystal structures of mitochondrial F_1 and on FRET data with CF_1, γCys89 is more than 6 nm from the nucleotide-binding sites.

Another type of proton leak is induced by a cross–link within the γ subunit by bifunctional maleimides. This cross–link, probably between Cys322 and Cys89, occurs when thylakoids carrying the cross–linker, such as *o*-phenylenebismaleimide (OPBM) on Cys322, are illuminated. This intrasubunit cross–link renders the thylakoid membrane irreversibly leaky to protons, causing ΔpH to drop dramatically. Since the reaction of Cys89 is critically dependent on ΔpH, cross–linking ceases when more than 10% of the ATP synthase was cross–linked (Weiss and McCarty, 1977). Although the formation of the cross link during illumination is prevented by ATP or ADP, the leak induced by cross–linking is not blocked by nucleotides (Fig. 1). Figure 1 also shows that the leak induced by OPBM cross–linking causes both an increase in electron transport and a decrease in membrane energization, as revealed by the loss of the ΔpH-dependent quenching of the fluorescence of 9-aminoacridine (9-AA). Since venturicidin restores 9-aminoacridine fluorescence quenching, the leak induced by cross–linking is through the ATP synthase. The leak is also rapid at neutral pH in the medium. The thylakoid membranes containing cross-linked CF_1 behaves as if the ATP synthase were not attached. This leak demonstrates the importance of the γ subunit in proton gating and in coupling proton translocation to ATP synthesis.

THE INHIBITORY ε SUBUNIT AND ITS INTERACTIONS WITH THE γ SUBUNIT

The ε subunit is an inhibitor of the ATPase activity of CF_1. Removal of the ε subunit stimulates the ATPase activity tenfold or more and either native or recombinant ε subunit fully inhibits this activity (Richter *et al.*, 1984). The ε subunit is also vital to the coupling of proton flux to the synthesis of ATP. The most straightforward demonstration of this function involves the reconstitution of thylakoid membranes stripped of CF_1 with either CF_1 or with depletion of its ε subunit [$CF_1(-\varepsilon)$]. Thylakoids stripped of CF_1 are so leaky to protons that virtually no ΔpH is formed during illumination. DCCD restores the generation of high ΔpH values. Both CF_1 and $CF_1(-\varepsilon)$ bind to thylakoid membranes. Although the proton leak through CF_o is blocked by CF_1, membranes reconstituted with $CF_1(-\varepsilon)$ remain as permeable to protons as they were prior to reconstitution and are thus unable to synthesize ATP. Addition of purified ε subunit to the thylakoids reconstituted with $CF_1(-\varepsilon)$ restores low proton permeability and ATP synthesis (Richter *et al.*, 1984).

The ε subunit has been localized in the proximity of the γ subunit by fluorescence resonance energy transfer (FRET) measurements (McCarty, 1997), cross–linking (Schulenberg *et al.*, 1997), and γ-subunit labeling (Komatsu-Takaki, 1996). The strength of the interaction between the γ and ε-subunits is dependent upon the redox state of the γ disulfide (Cys199–Cys205). Reduction

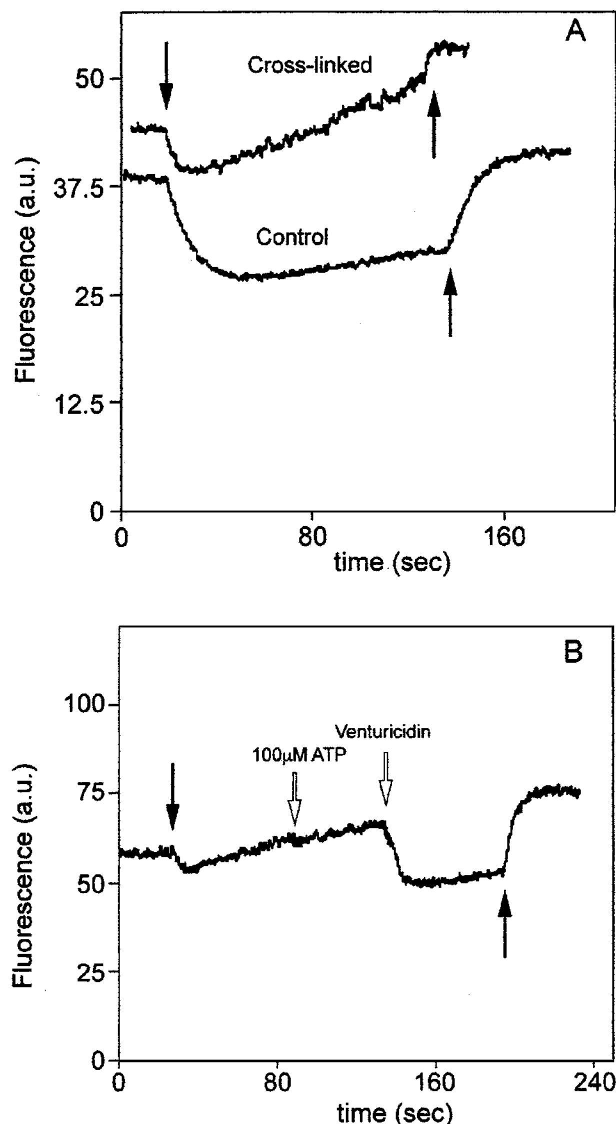

Fig. 1. Electron transport and thylakoid membrane energization in cross-linked and control thylakoids. Thylakoids were treated with *N,N′*-orthophenylene bismaleimide (OPBM) in the dark. Half the quantiy was then treated with DTT to neutralize the OPBM and half was illuminated to form a cross link (Weiss and McCarty, 1977). Electron transport and membrane energization (quenching of 9-AA fluorescence by formation of ΔpH) were measured simultaneously (Evron and McCarty, 2000) by fluorescence of 9-AA in the presence of ferricyanide (FeCy). (A) Thylakoids incubated with OPBM in the dark are compared to those incubated first in the dark and then in the light for 90 s. Rates of electron transport in units of μeq·mg Chl^{-1}·h^{-1} were 149 for dark and 450 for light. Downward arrows indicate onset of illumination; upward arrows indicate end of illumination. (B) Electron transfer and membrane energization in thylakoids cross linked with OPBM in the light. Where indicated, ATP, to a final concentration of 100 μM, and venturicidin, to a final concentration of 0.5 μM, were added. Electron transport was about 400 μeq mg $Chl^{-1}h^{-1}$ before venturicidin and 80 μeq·mg Chl^{-1}·h^{-1} after venturicidin addition.

of the γ disulfide decreases the binding affinity of ε for CF_1 by tenfold or more (Soteropoulos *et al.*, 1992). In CF_1 in thylakoids, the γ disulfide is reduced by thioredoxin, which itself is reduced by photoelectron transport. The association of CF_1 with thioredoxin was studied by FRET. Removal of the ε subunit did not affect the apparent K_d for thioredoxin binding, but did increase the extent of energy transfer from fluorescein maleimide on γCys322 to eosin attached to thioredoxin (Dann and McCarty, 1992). This result indicates that thioredoxin can approach the γ subunit more closely when the ε subunit is absent. Illumination greatly enhances the rate of reduction of the γ disulfide by either dithiothreitol or thioredoxin. The rate of reduction of the γ disulfide in CF_1 $(-\varepsilon)$ is much faster than that in CF_1. Thus, one of the conformational changes in CF_1 induced by proton flux and ΔpH probably involves a movement of the ε subunit relative to the γ subunit, such that the region bearing the disulfide bond is more exposed to the solvent. The ε subunit is an ATPase inhibitor and must also be an ATP synthesis inhibitor. ΔpH-induced alteration in the interactions between the γ and ε subunits may be part of the mechanism that overcomes inhibition by the ε subunit during active ATP synthesis. This means that the γ–ε interface undergoes an alteration during activation of ATP synthesis that is dependent upon electron transport and, hence, illumination.

Illumination of thylakoids also exposes specific portions of the γ subunit to trypsin cleavage, which are protected from trypsin cleavage in the dark (Moroney and McCarty, 1982). Limited trypsinization of the γ subunit in the light yields two large fragments that remain associated with the enzyme (Hightower and McCarty, 1996; Moroney and McCarty, 1982). Membranes that contain trypsinized CF_1 are very leaky to protons. The ATPase activity of the trypsinized CF_1 is insensitive to inhibition by the ε subunit and trypsinized CF_oCF_1 is depleted of the ε subunit. Exposure upon illumination of the regions of the γ subunit to cleavage by trypsin is independent of the reduction state of the dithiol of γ, suggesting that this conformational change between the γ and ε subunits is separate from the change seen upon reduction of the γ disulfide.

There is also evidence for change in conformation of the ε subunit upon illumination. Polyclonal antibodies raised against the ε subunit are unreactive with CF_1CF_o on thylakoid membranes until the membranes are illuminated. Upon illumination, the antibodies show strong reactivity with the ε subunit and can partially strip the ε subunit, thereby uncoupling ATP synthesis (Richter and McCarty, 1987). This result suggests that the affinity of the ε subunit is decreased by ΔpH. Clearly, however, the ε subunit cannot dissociate from the ATP synthase during

activation since dissociation would cause uncoupling. The reactivity of Lys residue at position 109 in the ε subunit of CF_1 in thylakoids becomes approximately fourfold more reactive to pyridoxal phosphate upon illumination, demonstrating that the C-terminal region of the ε subunit becomes more solvent accessible during illumination (Komatsu-Takaki, 1989).

Coupling of proton flux to ATP synthesis is dependent on maintaining the proton impermeability of CF_1CF_o under nonilluminated conditions and allowing coupled proton permeability under illuminated conditions. Alterations in either γ or ε subunits can affect this proton permeability. As discussed earlier, illumination of thylakoids in the presence of a bifunctional maleimide causes the complete loss of proton impermeability, presumably through cross–linking of γCys322 to γCys89 (Weiss and McCarty, 1977). Deletion of 10 amino acids from the C-terminus of the ε subunit (Cruz *et al.*, 1995) and truncation of as few as five amino acids from the N-terminal end (Cruz *et al.*, 1997) destroyed the ability of ε to restore proton impermeability to thylakoid membranes deficient of ε subunit. Since the N-terminally truncated ε inhibited the ATPase activity of $CF_1(-\varepsilon)$ bound to CF_o in thylakoids was inhibited by more than 50%, restoration of proton impermeability by the ε subunit may be separate from its role as an inhibitor.

These data also suggest that the γ and ε subunits act in concert to control the flux of protons through the CF_1CF_o

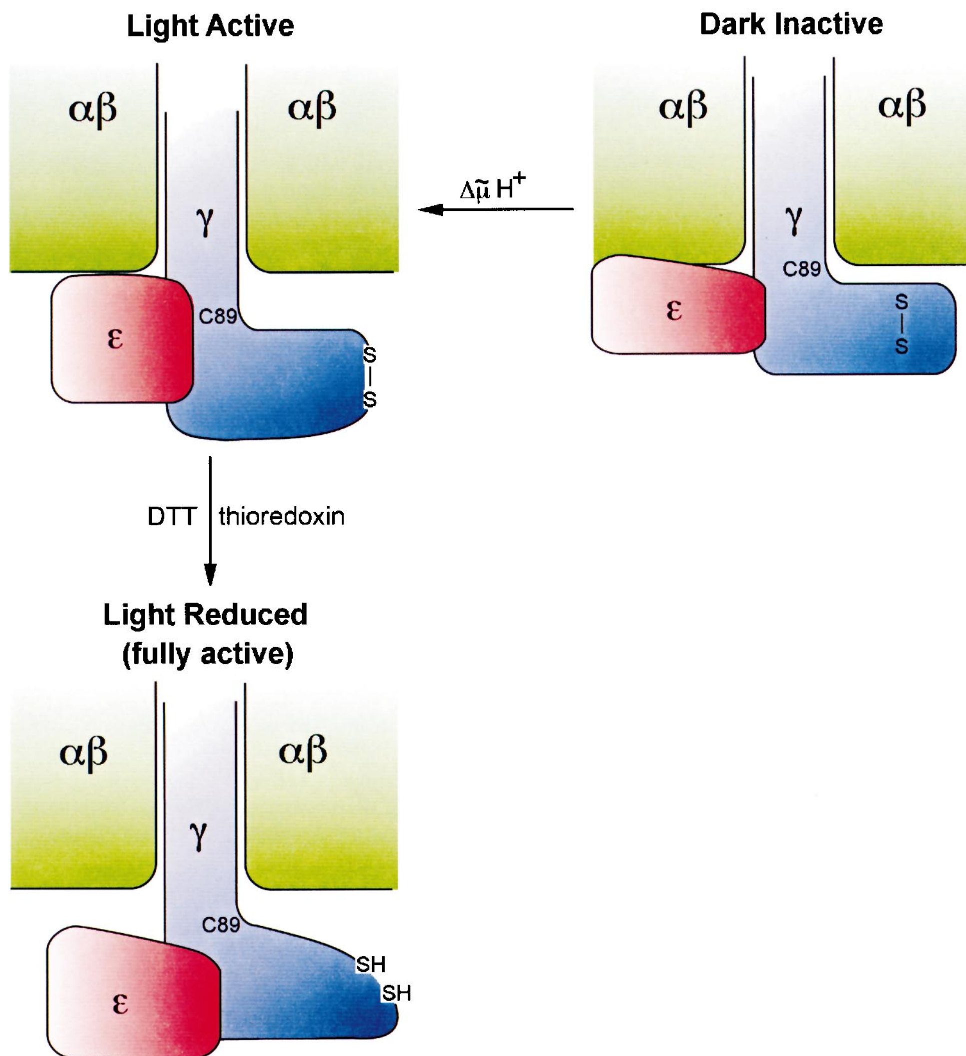

Fig. 2. Proposed model for the various activation states of the chloroplast ATP synthase. Emphasis is given to the γ and ε subunits, whose involvement in proton translocation is described above. Details are given in the text.

complex. This control is achieved by conformational shifts of these proteins relative to each other in response to the presence or absence of a transmembrane proton gradient.

In Fig. 2 the physiological effects of illumination and reduction are illustrated schematically: γ and ε subunits are hindered from movement in the dark by the $\alpha\beta$ hexamer. This inhibition is partly overcome upon illumination, when a conformational change moves γ and ε from the $\alpha\beta$ hexamer, thus exposing the disulfide to reduction and γCys89 to modification. Reduction of the disulfide bond in the γ subunit causes an additional conformational change, removing the inibition of ε movement completely and loosening the interaction between ε and γ. If we assume that proton efflux is coupled to γ and ε movement, reducing the inhibitory interaction between ε and the $\alpha\beta$ hexamer can result in larger proton flux at lower pH gradients, leading, under phosphorylating conditions, to ATP synthesis at physiological ΔpH values.

This concerted effort together with effects of bound nucleotides allow the ATP synthase to be active only on energized thylakoid membranes. Only conditions in which the inhibitory effects of the ε subunit and bound ADP are overcome can the ATP synthase be active. In addition to overcoming these inhibitions, conformational changes in the γ and ε subunits of CF_1 may also be involved in the opening of a proton gate in CF_1.

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