Woodbury, N. W., Becker, M., Middendorf, D., & Parson, W. W. (1985) *Biochemistry 24*, 7516–7521.
Zinth, W., Nuss, M. C., Franz, M. A., Kaiser, W., & Michel, H. (1985a) in *Antennas and Reaction Centers of Photosynthetic Bacteria* (Michel-Beyerle, M. E., Ed.) pp 286–291, Springer-Verlag, Berlin.
Zinth, W., Knapp, E. W., Fischer, S. F., Kaiser, W., Deisenhofer, J., & Michel, H. (1985b) *Chem. Phys. Lett. 119*, 1–4.
Zinth, W., Dobler, J., & Kaiser, W. (1986) in *Ultrafast Phenomena V* (Fleming, G. R., & Siegman, A. E., Eds.) pp 379–383, Springer-Verlag, Berlin.

Deactivation of CF_0–CF_1 ATP Synthase by Uncouplers†

Uri Pick
Department of Biochemistry, The Weizmann Institute of Science, Rehovot 76100, Israel
Received April 1, 1988; Revised Manuscript Received June 16, 1988

ABSTRACT: Energization of thylakoid membranes by illumination in the presence of dithiols induces a MgATPase activity which persists in the dark (Bakker-Grunwald & van-Dam, 1973). The relationship between the activated state of the ATP synthase (CF_0–CF_1) and $\Delta\tilde{\mu}_{H^+}$ has been investigated from effects of uncouplers on ATPase activity in reconstituted CF_0–CF_1 proteoliposomes. The results are inconsistent with the idea that persistence of the activated state requires a threshold $\Delta\tilde{\mu}_{H^+}$ for the following reasons: (1) Different uncouplers have different effects on steady-state ATPase activity: SF-6847 stimulates ATP hydrolysis at low concentration, in parallel with $\Delta\tilde{\mu}_{H^+}$ dissipation, but inhibits it at higher concentrations. Gramicidin inhibits ATP hydrolysis in parallel with $\Delta\tilde{\mu}_{H^+}$ dissipation. Nigericin just stimulates ATP hydrolysis. (2) Energization of proteoliposomes by an artificially induced $\Delta\tilde{\mu}_{H^+}$ activates ATP hydrolysis. Addition of SF-6847, gramicidin, or nigericin after energization further stimulates ATP hydrolysis. In contrast, the presence of these uncouplers during energization differentially inhibits ATP hydrolysis, and the inhibitions are not correlated with the effectiveness of the uncouplers in dissipating $\Delta\tilde{\mu}_{H^+}$. The results suggest that the inhibition results from deactivation, which is not purely energetic. (3) Deactivation of ATP hydrolysis by SF-6847 depends on the lipid composition. No deactivation is obtained without reconstitution with lipids, while glycolipids enhance the deactivation. Cholesterol inhibits H^+ uptake, but not the deactivation. (4) Low concentrations of ADP sensitize while phosphate stabilizes CF_0–CF_1 to deactivation by SF-6847, both with or without $\Delta\tilde{\mu}_{H^+}$ preactivation. The effect of ADP is not due to a direct inactivation, since by itself it does not inhibit but rather stimulates uncoupled ATP hydrolysis. It is suggested that deactivation of energized CF_0–CF_1 by certain uncouplers results from a specific interaction between the enzyme and the protonophores. This interaction is facilitated by ADP and by glycolipids and is inhibited by phosphate and during ATP hydrolysis. The results are consistent with the idea that activation involves occlusion of protons from the inner thylakoid space in hydrophobic sites of CF_0.

In energy-transducing membranes of mitochondria, chloroplasts, and bacteria, the transmembrane difference of electrochemical potential of protons ($\Delta\tilde{\mu}_{H^+}$)[1] provides the major driving force for ATP synthesis (Mitchell, 1977). The reaction is reversibly coupled to proton transport via a membrane-bound ATP synthase (F_0–F_1). In chloroplast ATP synthase (CF_0–CF_1), $\Delta\tilde{\mu}_{H^+}$ also activates the enzyme in addition to its role as a driving force for ATP synthesis (Carmeli & Liphshitz, 1972; Bakker-Grunwald & van Dam, 1974). This activation is manifested not only by the capacity of the enzyme to synthesize ATP in the light but also by its capacity to hydrolyze ATP in the subsequent dark period. The latter depends on the presence of either dithiol or trypsin during illumination (Lynn & Straub, 1969; McCarty & Racker, 1968; Mills & Mitchell, 1984; Bakker-Grunwald & van-Dam, 1973, 1974). It has been demonstrated that dithiols reduce two SH groups in the γ subunit of CF_1 which become exposed upon energization (Nalin & McCarty, 1984; Pick, 1983), while trypsin causes cleavage of the ϵ subunit of CF_1 which suppresses the activity and seems to be dislocated upon energization (Nelson et al., 1972; Richter et al., 1984; Finel et al., 1984). Marked changes in the binding and release of adenine nucleotides, particularly ADP, have suggested that high-affinity ADP binding sites in CF_1 may be involved in regulation of the activation of CF_0–CF_1 (Shulmann & Strotmann, 1981). It has also been demonstrated that the capacity to hydrolyze ATP induced upon energization persists long after complete decay of $\Delta\tilde{\mu}_{H^+}$, suggesting that maintenance of this capacity does not depend on persistence of $\Delta\tilde{\mu}_{H^+}$ (Bakker-Grunwald & van-Dam, 1974). The mechanism by which energization induces activation is not clear, but it has been suggested that it involves protonation of sites in the enzyme facing the inner thylakoid space (Schlodder et al., 1982; Mills & Mitchell, 1984). Support for this hypothesis comes from earlier studies by Jagendorff and collaborators (Ryrie & Jagendorff, 1971) that energization involves an irreversible exchange of hydrogens from water into CF_1 which persists even after isolation of the

† Supported by Binational US–Israel Grant 84-232/2.

[1] Abbreviations: CF_0–CF_1, chloroplast ATP synthase; $\Delta\tilde{\mu}_{H^+}$, transmembrane electrochemical proton gradient; SF-6847, (3,4-di-*tert*-butyl-4-hydroxybenzylidene)malononitrile; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone.

0006-2960/88/0427-8284$01.50/0

enzyme. Recently, Gräber and colleagues have demonstrated that activation by $\Delta\tilde{\mu}_{H^+}$ may be induced also in the purified enzyme reconstituted in proteoliposomes (Schmidt & Gräber, 1987a,b). In the present work, we attempted to investigate the mechanism of deactivation of the enzyme in proteoliposomes by analyzing the effects of uncouplers on ATPase activity. Our results suggest that the effects of uncouplers on the activity are inconsistent with pure energetic considerations and suggest that certain uncouplers deactivate the enzyme by direct interaction with hydrophobic protonated sites in C-F_0–CF_1.

Materials and Methods

Preparation. CF_0–CF_1 was isolated from lettuce chloroplasts as previously described (Pick, 1986).

Reconstitution. Two procedures were employed for reconstitution. Steady-state ATPase, ATP–P_i exchange, and oxonol VI absorption change measurements were carried out following reconstitution by cholate dilution (Racker et al., 1975) as follows: Soybean phospholipids (40 mg/mL) were presonicated in 30 mM Na-Tricine, pH 8, in the presence of 1.3% sodium cholate, mixed with CF_0–CF_1 (2–3 mg) and $MgCl_2$ (3 mM), and incubated for 30 min on ice. Twenty microliters of this mixture was diluted into 1 mL of reaction mixture.

Proteoliposomes used in activation measurements were reconstituted as follows: Soybean phospholipids (40 mg/mL) were sonicated in 20 mM Na-Tricine, pH 8, dialyzed 4 h at 25 °C and 12 h at 4 °C against the same buffer, resonicated with 1.3% sodium cholate, mixed with CF_0–CF_1 (0.8 mg/mL) and $MgCl_2$ (3 mM), and incubated for 30 min on ice. Sodium cholate was removed by separating 1-mL samples on Sephadex G-50 columns (15 × 1 cm), and the turbid proteoliposomes were incubated for 1–2 h with 50 mM dithiothreitol on ice before assay of activity.

Assays. ATP–P_i exchange and MgATPase activities (steady-state measurements) were carried out at 37 °C as previously described (Pick & Racker, 1979) in identical media containing 40 mM Na-Tricine, 5 mM DTT, 3 mM $MgCl_2$, 3 mM ATP, 6 mM P_i, and either [γ-^{32}P]ATP or ^{32}P; uncouplers were preincubated with the proteoliposomes (10–20 min on ice) before addition of ATP.

Activation by $\Delta\tilde{\mu}_{H^+}$ was performed as follows: 300 μL of reconstituted proteoliposomes (100 μg of protein) was preincubated for 30 s at 22 °C in acid medium by addition of 300 μL containing 60 mM sodium succinate, 4 mM $MgCl_2$, 4 mM P_i, and 2 μM valinomycin (final pH 5.2). Base medium (400 μL) containing 60 mM K-Tricine + 120 mM KCl (final pH 8.1) was added, and after another 30 s, [γ-^{32}P]ATP (1 mM final) was added, and the incubation was continued for 5 min and stopped with 5% TCA, and the released [^{32}P]P_i was determined (Avron, 1961). Defatted bovine serum albumin (5 mg/mL) was either added to the preincubation or added 15 s after the base.

Oxonol absorption changes were followed in an Aminco DW-2A dual-wavelength spectrophotometer (603–590 nm) as previously described (Admon et al., 1982). Nine aminoacridine fluorescence changes were measured in a Perkin-Elmer spectrofluorometer (MPF-44A), at 400-nm excitation and 465-nm emission (Pick et al., 1984).

Results

The effect of the protonophore SF-6847 on the time course of ATP hydrolysis catalyzed by reconstituted CF_0–CF_1 proteoliposomes is demonstrated in Figure 1. Low concentrations of SF-6847 stimulate ATPase activity. This stimulation is

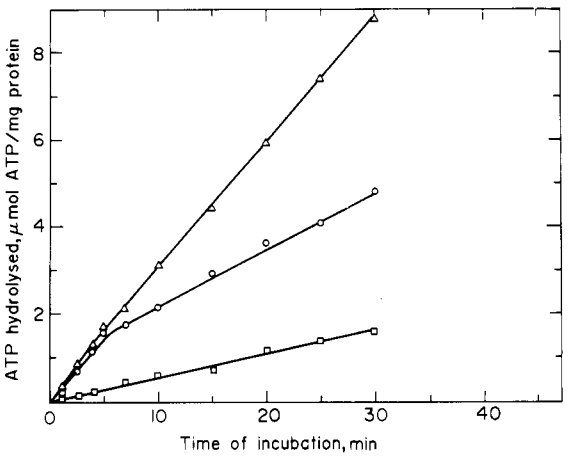

FIGURE 1: Effect of two concentrations of SF-6847 on the rate of ATP hydrolysis. SF-6847 was added to reconstituted proteoliposomes before ATP addition, and the activity was followed as described under Materials and Methods. (○) Control; (△) 0.2 μM SF-6847; (□) 10 μM SF-6847.

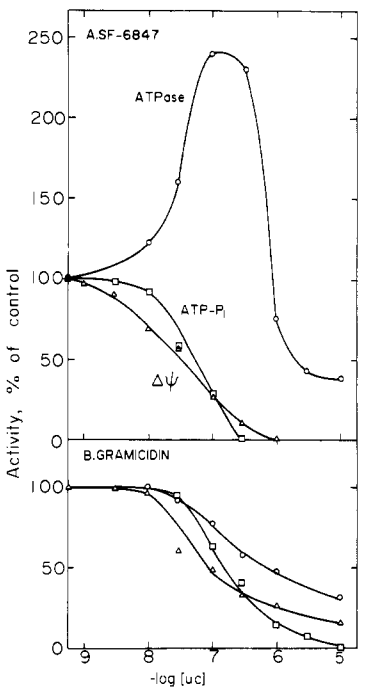

FIGURE 2: Effect of SF-6847 and gramicidin D concentration on steady-state rates of ATP hydrolysis, ATP–P_i exchange, $\Delta\psi$ formation. ATP hydrolysis and ATP–P_i exchange were measured at 37 °C under identical conditions, and steady-state $\Delta\psi$ values, estabilished after 5–10 min at 37 °C, were estimated from oxonol VI absorption changes and expressed as a percent of the control changes in the absence of uncouplers. Control activities are 110–130 and 80–90 nmol (mg of protein)$^{-1}$ min^{-1} for ATP hydrolysis and ATP–P_i exchange, respectively.

accompanied by inhibition of ATP–P_i exchange activity (which is dependent of the buildup of $\Delta\tilde{\mu}_{H^+}$) and of ATP-induced $\Delta\psi$ formation, measured by oxonol VI absorption changes (Figure 2A) as may be expected from classical uncoupling.

However, higher concentrations of the uncoupler lead to a pronounced inhibition of steady-state ATPase activity (Figures 1 and 2A). Similar biphasic effects of uncouplers on the ATPase activity of energized chloroplast thylakoids, following preillumination in the presence of dithiols, were reported earlier (Rienits, 1967; Carmeli, 1969; Bakker-Grunwald, 1974) and interpreted by a combination of uncoupling (stimulation) and a requirement for a threshold Δ pH in order to keep CF_0–CF_1 in an energized conformation, which is dissipated by high uncoupler concentrations (inhibition).

Table I: Kinetic Parameters for Inhibition or Stimulation by Different Uncouplers on Steady-State ATP Hydrolysis and ATP–P_i Exchange[a]

	ATP–P_i exchange	MgATPase		CaATPase	
uncoupler	K_i^{50} (μM)	K_s^{50} (μM)	K_i^{50} (μM)	K_i^{50} (μM)	I_{max} (%)
SF-6847	0.06	0.05	0.2	0.2	34
FCCP	0.8	0.5	6	4	32
nigericin	1.5	2	>50		
K^+-valinomycin	0.02	0.02	0.5		
gramicidin	0.2		0.15	0.5	38
atebrin	30		80	20	49

[a] ATP hydrolysis and ATP–P_i exchange were measured as in Figures 2 and 3. K_i^{50}, concentration for 50% inhibition; K_s^{50}, concentration for 50% stimulation; I_{max}, maximal extent of inhibition expressed as percent of control.

This interpretation seems inconsistent with the kinetics of ATP hydrolysis in reconstituted CF_0–CF_1: First, because in this system there is no preenergization, and if the $\Delta\tilde{\mu}_{H^+}$ created by ATP hydrolysis is required to sustain the energized state, then one would expect to obtain an increase in ATP hydrolysis with time (autocatalytic activation) rather than the observed decrease (Figure 1, control), which seems to reflect the time required for the buildup of $\Delta\tilde{\mu}_{H^+}$.

Furthermore, if the reason for the inhibition of ATPase activity by SF-6847 is purely energetic, then it would be expected that every protonophore (uncoupler) should have a similar biphasic effect on the activity. However, as is demonstrated in Figure 2B and summarized in Table I, this does not seem to be the case. Gramicidin D, for example, does not stimulate ATPase activity at all but rather inhibits it in parallel with inhibition of ATP–P_i exchange and $\Delta\psi$ formation. Atebrin has a similar effect, while nigericin stimulates ATPase activity but does not inhibit it at high concentrations.

Energized chloroplasts catalyze in the light a CaATPase activity (Avron, 1962). We have recently demonstrated that this activity has an obligatory dependence on energization ($\Delta\tilde{\mu}_{H^+}$) but is not coupled to proton translocation (Pick & Weiss, 1988). We have observed that SF-6847 does not stimulate CaATPase activity in reconstituted CF_0–CF_1 but partially inhibits the activity at high concentrations (Table I; Pick & Racker, 1979). This inhibition is not unique to SF-6847 (Table I); also, other uncouplers (FCCP, gramicidin, atebrin) inhibit CaATPase activity at relatively high concentrations roughly in parallel with the inhibition of MgATPase activity. These results suggest that the CaATPase activity resembles the MgATPase activity in its susceptibility to inhibition by high uncoupler concentration but differs from it in the lack of stimulation by lower uncoupler concentration, as may be expected if CaATPase is not coupled to H^+ translocation.

From the above consideration, it appears that the uncoupler-dependent ATPase inhibition does not result from deenergization but may reflect a more specific interaction of certain uncouplers with CF_0–CF_1. In a search for a better experimental system to resolve between effects of uncouplers on the activation state, and on steady-state kinetics of ATP hydrolysis by CF_0–CF_1, we have adapted an artificially induced $\Delta\tilde{\mu}_{H^+}$ activation system of the reconstituted enzyme, developed by Gräber (Schmidt & Gräber, 1987a,b). This two-stage system enables one to check separately the effect of uncouplers on the activation and on the steady-state kinetics. By comparing different reconstitution methods, we found that the most efficient activation of ATP hydrolysis is obtained by reconstitution in the presence of cholate followed by gel filtration on Sephadex G-50 columns (see Materials and Methods). Under these conditions, the ATPase activity of the enzyme is stimulated 80–150% by artificially induced $\Delta\tilde{\mu}_{H^+}$ (in the absence of uncouplers), and the induced activation persists for at least 10 min. SF-6847 in this system may either inhibit or stimulate

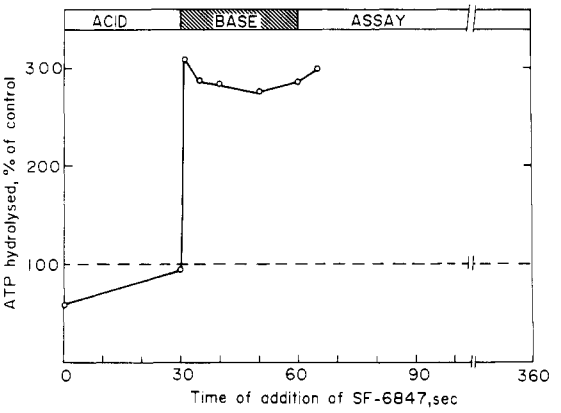

FIGURE 3: Dependence of the ATPase activity of activated CF_0–CF_1 on the stage of addition of SF-6847. Activation of CF_0–CF_1 proteoliposomes was performed as described under Materials and Methods. SF-6847 (3 μM) was added at different stages as indicated. Control activity, 0.32 μmol of ATP (mg of protein)$^{-1}$ min^{-1}.

Table II: Effects of SF-6847 on the Activation and on Steady-State ATP Hydrolysis When Added before or after the Acid–Base Activation Stage[a]

		ATPase act. [μmol (mg of protein)$^{-1}$ min^{-1}] for additions to assay of		
A/B activation	SF-6847 stage of addition	none	albumin	phospholipids
+	no addition	0.31	0.27	0.29
+	activation	0.25	0.17	0.24
+	assay	1.03	0.32	0.36
–	no addition	0.16	0.14	0.16

[a] SF-6847 (3 μM) was added either immediately before the acid (activation) or 1 s after ATP (assay). Where indicated, defatted bovine serum albumin (5.2 mg) or sonicated soybean phospholipids (15 mg) were added 15 s before the addition of ATP in order to neutralize the uncoupler during the assay. Other experimental details are described under Materials and Methods. A/B, acid–base activation treatment.

ATP hydrolysis, depending on the stage of its addition; if added during the activation stage, it leads to inhibition, while addition of the same uncoupler concentration immediately after the activation (to the assay only) leads to a pronounced stimulation (Figure 3, Table II). In order to test the effect of SF-6847 on the activation only, we attempted to neutralize it following activation by binding to defatted serum albumin or excess sonicated phospholipids. As is demonstrated in Table II, both albumin and sonicated lipids efficiently neutralize the uncoupler as indicated by the prevention of the SF-6847-induced stimulation of ATP hydrolysis when added to the assay only. When added during the activation and neutralized by albumin before addition of ATP, SF-6847 induced a pronounced inhibition of the activity. It seems, therefore, that the smaller inhibition observed when the uncoupler is present both during activation and during assay results from a combination of inhibition during activation and partial stimulation in the assay

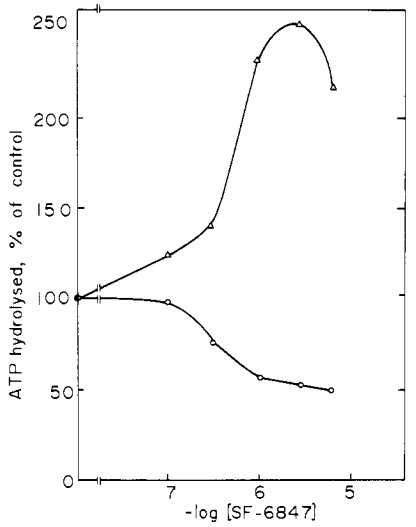

FIGURE 4: SF-6847 concentration dependence for stimulation and deactivation. SF-6847 was either added to the acid preincubation medium, and neturalized by addition of albumin 15 s before ATP (O), or added immediately subsequent to ATP addition (Δ). Control activity, 0.34 μequiv (mg of protein)$^{-1}$ min^{-1}.

Table III: Deactivation and Stimulation of ATPase Activity by Different Uncouplers[a]

uncoupler	concn	ATPase act. (% of control), stage of addition of uncoupler: activation	assay
SF-6847	3 μM	54	280
gramicidin	10 μM	67	217
nigericin	10 μM	95	269
NH_4^+	25 mM	72	225

[a] Uncouplers were added either before the acid stage and neutralized by albumin 15 s before ATP (activation) or 1 s after ATP (assay). Control activity, 0.33 μmol of ATP hydrolyzed (mg of protein)$^{-1}$ min^{-1}.

phase. This system seems, therefore, appropriate to distinguish between deactivation and stimulation of ATPase activity of CF_0–CF_1. It may be noted that also without $\Delta\tilde{\mu}_{H^+}$ activation, SF-6847 induces a slight inhibition when preincubated with the enzyme before ATP addition, (10–30%) and a significant but smaller stimulation when added after ATP (40–80%, Table IV).

The time of addition of SF-6847 is critical for its final effect on ATP hydrolysis as is demonstrated in Figure 3. Addition at the onset of $\Delta\tilde{\mu}_{H^+}$ induction produces inhibition while addition 1 s after the onset of $\Delta\tilde{\mu}_{H^+}$ produces maximal stimulation.

The concentration dependence for SF-6847 deactivation (when present during activation) and stimulation (when present in assay) is similar as is demonstrated in Figure 4. It may be noted that the optimal concentration for both effects depends almost proportionally on the lipid concentration. This seems to be the reason for the difference in the concentration dependence for stimulation of ATP hydrolysis in Figure 4 and in Figure 2A in which the lipid concentration was 7 times smaller.

A comparison between the effects of different uncouplers on the deactivation and stimulation of ATP hydrolysis in this system is summarized in Table III. Gramicidin D, in contrast to the steady-state system (Figure 2B), stimulates when added after ATP, and inhibits when present during activation, similar to SF-6847. In contrast, nigericin, which also stimulates ATP hydrolysis when added during the assay, does not inhibit when present in the activation phase, consistent with the lack of

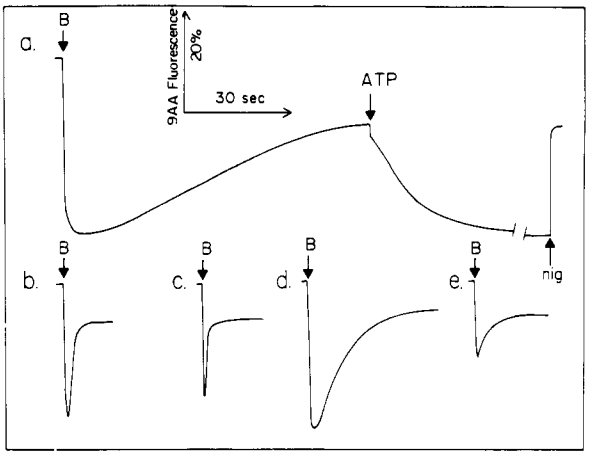

FIGURE 5: Comparison between different uncouplers in the acceleration of proton efflux from acidified proteoliposome at low temperature. Conditions except for the addition of 5 μM 9-aminoacridine (9AA), ATP (0.5 mM), and nigericin (5 μM, nig) were added where indicated. 1.5 μM SF-6847 (b), 5 μM nigericin (c), 5 μM gramicidin D (d), or 15 mM NH_4Cl (e) was added to the acid incubation medium.

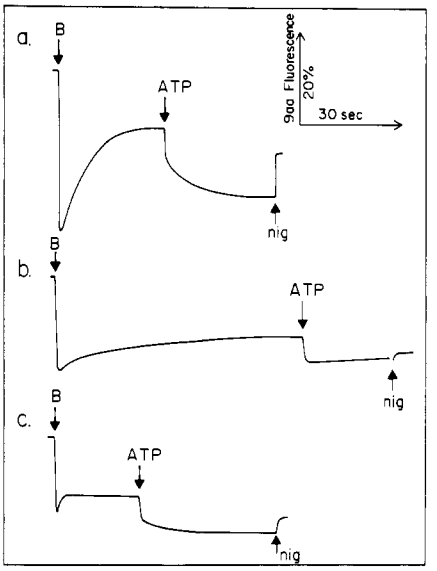

FIGURE 6: Effect of lipid composition on artificial and ATP-induced ΔpH in CF_0–CF_1 proteoliposomes. CF_0–CF_1 proteoliposomes composed of soybean phospholipids (a), a 3:1 mixture of soybean phospholipids with cholesterol (b), or thylakoid glycolipids (c) were prepared as in Table IV. Measurements and other details are as in Figure 5, except for the temperature which was 21 °C.

inhibition under steady-state conditions by this ionophore (Table I). If deactivation by uncouplers were due to prevention of the $\Delta\tilde{\mu}_{H^+}$ activation, than it should be expected to find a correlation between the efficiency of different uncouplers in dissipating the induced $\Delta\tilde{\mu}_{H^+}$ and their effectiveness as deactivators. We have, therefore, tried to measure the induced ΔpH by following 9-aminoacridine (9AA) fluorescence changes. As demonstrated in Figures 5 and 6, this method enables one to follow the dissipation of both the artificially induced and the ATP-induced ΔpH. In the absence of acid–base activation, ATP induces a much lower rate of 9-aminoacridine fluorescence quenching (about 10 times slower), indicating slower H^+ uptake (not shown).

The effect of uncouplers on the dissipation of the induced ΔpH, at concentrations which produce maximal effects on ATPase activity, is too fast to be measured under these conditions due to the limited time resolution and the probe. However, a relative comparison can be made at low temperature (5 °C), and at somewhat lower uncoupler concentrations

Table IV: Effect of Lipid Composition on CF_0–CF_1 Deactivation and Stimulation by SF-6847[a]

A/B treatment	SF-6847 stage of addition	ATPase act. [μmol (mg of protein)$^{-1}$ min^{-1} (%)] for lipid composition: none	phospholipids	phospholipids/cholesterol (3:1)	glycolipids
+	none	0.290 (100)	0.304 (100)	0.160 (100)	0.488 (100)
+	activation + assay	0.285 (98)	0.288 (95)	0.087 (54)	0.078 (16)
+	activation	0.295 (99)	0.187 (61)	0.085 (53)	0.248 (51)
+	assay	0.278 (98)	0.851 (280)	0.166 (104)	0.165 (34)
–	none	0.310 (100)	0.178 (100)	0.083 (100)	0.310 (100)
–	preincubation	0.320 (103)	0.155 (87)	0.074 (89)	0.218 (70)
–	assay	0.318 (103)	0.259 (145)	0.076 (92)	0.086 (28)

[a] Proteoliposomes were prepared from soybean phospholipids, phospholipids + cholesterol, or lettuce thylakoid glycolipids as described under Materials and Methods. Soluble CF_0–CF_1 (not reconstituted) was assayed in the presence of 5 mM octyl glucoside. SF-6847 (3 μM) was added either before A/B activation (activation + assay), immediately after ATP (assay), or before A/B activation followed by albumin quencher before the assay (activation) as in Table II. In control (unactivated) proteoliposomes, SF-6847 was either preincubation with the vesicles for 30 s before the assay and neutralized with albumin or added after ATP as above. Numbers in parentheses represent percent of control activities.

which produce similar stimulations of ATPase activity. Such a comparison (Figure 5) suggests that relative to SF-6847, nigericin, which does not cause deactivation, dissipates ΔpH faster while gramicidin, which produces a similar deactivation, is far less effective in dissipating the induced ΔpH. We have also measured the ΔpH decay rate in a rapid mixing system connected to a spectrofluorometer, using trapped pyranine as an intravesicular pH indicator (Pick et al., 1984). The first-order rate constants for the intravesicular pH change at 20.5 °C, under identical conditions used for ATPase activation, were 0.1, 0.55, 0.17 and 11 s^{-1} in the absence of uncouplers, with 3 μM SF-6847, with 10 μM gramicidin D, and with 10 μM nigericin, respectively. These results show that there is no correlation between the effectiveness of these uncouplers in dissipating $\Delta\tilde{\mu}_{H^+}$ and ATPase deactivation and suggest that the deactivation does not result from prevention of energization.

Reconstitution of CF_0–CF_1 with lipids appears to be essential for the effects of SF-6847 on its catalytic activity since in the absence of lipids the ATPase activity of solubilized CF_0–CF_1 as well as of water-soluble CF_1 is neither stimulated nor inhibited by SF-6847 (10^{-8}–10^{-4} M; not shown). Therefore, and also since the activity of CF_0–CF_1 is influenced by the type of lipid with which it is reconstituted (Pick et al., 1986), it seemed of interest to check whether lipid composition also affects the deactivation of CF_0–CF_1 by SF-6847. Table IV summarizes a comparison between CF_0–CF_1 reconstituted with three lipid mixtures—phospholipids, phospholipids with cholesterol (3:1 ratio), and chloroplast thylakoid glycolipids—and nonreconstituted CF_0–CF_1 with respect to the sensitivity to SF-6847 in deactivation and stimulation of ATP hydrolysis. Cholesterol significantly inhibits ATPase activity of the enzyme but does not prevent the stimulatory effect by induced $\Delta\tilde{\mu}_{H^+}$, indicating that the enzyme is well incorporated into the liposomes since it is responsive to the transmembrane $\Delta\tilde{\mu}_{H^+}$. The deactivating effect of SF-6847 during activation is even more pronounced in cholesterol-containing vesicles than in pure phospholipids; however, there seems to be no stimulation of ATP hydrolysis by SF-6847 when added after ATP in contrast to the situation with pure phospholipids. Figure 6b demonstrates that the decay of Δ pH in cholesterol-containing vesicles is slower than in phospholipid vesicles, indicating that the absence of stimulation by the uncoupler is not a result of a high permeability to protons. It is also evident that addition of ATP hardly induces any H^+ uptake. We have also observed that cholesterol-containing proteoliposomes have a very low ATP–P_i exchange activity (not shown). These results seem to suggest that CF_0–CF_1 in cholesterol-containing vesicles is intrinsically uncoupled; namely, ATP hydrolysis is not coupled to H^+ uptake and therefore is also not stimulated by uncouplers.

Table V: Effect of ADP and Inorganic Phosphate on SF-6847 Inactivation and Stimulation of ATPase Activity[a]

A/B activation	SF-6847 stage of addition	ATPase act. [μmol (mg of protein)$^{-1}$ min^{-1} (%)] for addition to activation of: P_i	P_i + ADP	none
+	none	0.30 (100)	0.24 (100)	0.16 (100)
+	activation	0.17 (58)	0.07 (29)	0.08 (50)
+	assay	0.92 (307)	1.03 (429)	0.53 (330)
–	none	0.16 (100)	0.14 (100)	0.09 (100)
–	preincubation	0.14 (91)	0.08 (52)	0.05 (55)
–	assay	0.33 (210)	0.53 (385)	0.15 (162)

[a] ADP (15 μM) or phosphate (1 mM) was added to the preincubation or activation medium. Other details are as in Table III.

In proteoliposomes made of chloroplast thylakoid glycolipids, the deactivating effect of SF-6847 is particularly dominant, both at the activation and at the assay phases, and when present in both, an additive inhibition is obtained. SF-6847 also similarly inhibits the activity in the absence of $\Delta\tilde{\mu}_{H^+}$ preactivation. It appears that the absence of stimulation of ATP hydrolysis in glycolipid proteoliposomes results from their high passive permeability to protons, as is demonstrated in Figure 6c [see also Pick et al. (1984, 1987)]. This makes the deactivating effect of SF-6847 more prominent also under steady-state hydrolysis (assay) conditions. It may be noted that at a limiting lipid concentration glycolipids are far more efficient in reconstitution than phospholipids and increase the V_{max} and the affinity for ATP in ATP hydrolysis (Pick et al., 1984). On the whole, these results suggest that the lipid composition affects not only the activity of CF_0–CF_1 but also its sensitivity to deactivation by SF-6847.

From previous studies in chloroplasts, it is known that phosphate stabilizes while ADP labilizes the activated state of CF_0–CF_1, as manifested by its capacity to hydrolyze ATP following preillumination in the presence of dithiols. We have, therefore, tested the effects of these ligands on the activation of reconstituted CF_0–CF_1 by the acid–base treatment, and on its susceptibility to SF-6847 (Table V).

ADP by itself partially inhibits the activation and sensitizes CF_0–CF_1 to deactivation by SF-6847. Moreover, also without $\Delta\tilde{\mu}_{H^+}$ activation, ADP substantially facilitates deactivation by SF-6847. Conversely, the stimulation of ATP hydrolysis by SF-6847, when added after ATP, is in fact increased by ADP, particularly in the absence of activation. The omission of phosphate during activation also produces by itself a significant inhibition, and it also sensitizes the enzyme to SF-6847 deactivation, particularly in the absence of activation. However, in contrast to ADP, the absence of phosphate does not enhance but rather inhibits the potential stimulation by SF-6847 when added after ATP. Thus, while both ADP and the absence of phosphate similarly sensitize deactivation by

the uncoupler, their effects on the catalytic potential of the enzyme are different.

Discussion

It has been previously demonstrated that protonophores have a dual effect on light-triggered MgATPase in chloroplasts, with respect to both time and uncoupler concentration: initial stimulation and subsequent inhibition, which is particularly pronounced at high uncoupler concentrations (Carmeli, 1969; Bakker-Grunwald & van-Dam, 1973, 1974; Bakker-Grunwald, 1974). The stimulation was interpreted by uncoupling (energetic) and the inhibition by a drop of $\Delta\tilde{\mu}_{H^+}$ below the threshold level which is required in order to keep the enzyme at the active conformation (deactivation). It has also been demonstrated that uncouplers accelerate the decay of the activated state in the dark, and it was mentioned that the acceleration is observed only when the uncoupler is added before ATP (Bakker-Grunwald & van-Dam, 1974). It has been suggested that the uncouplers decrease the ΔpH created during ATP hydrolysis below a threshold level, which is required to sustain the enzyme in the active conformation.

The present work carried out with purified CF_0–CF_1 proteoliposomes confirms the basic observations made on chloroplasts but is inconsistent with the interpretation of the mechanism of deactivation in the following issues. (a) Uncoupler specificity: From pure energetic consideration, it is expected that every uncoupler should have the same effect on the ATPase activity of the reconstituted enzyme. Yet, different uncouplers added to the enzyme prior to ATP have completely different effects on the steady-state ATP hydrolysis rates (Figure 2, Tables I and III). (b) If deactivation by uncouplers were a consequence of deenergization, a correlation should be observed between the efficiency of dissipation of $\Delta\tilde{\mu}_{H^+}$ and of deactivation. This is not the case (Table III, Figure 5). (c) Preincubation with SF-6847 without $\Delta\tilde{\mu}_{H^+}$ activation, followed by its neutralization before ATP addition, should not inhibit ATP hydrolysis, according to the above interpretation. Yet, SF-6847, as well as other protonophores, does inhibit activity under these conditions, and the inhibition is particularly pronounced in the presence of ADP, in the absence of P_i, and with glycolipids. Moreover, one cannot interpret from energetic considerations the difference between addition of SF-6847 before or 1 s after ATP (in the absence of $\Delta\tilde{\mu}_{H^+}$ preactivation) as prevention of autocatalytic activation, since there is no appreciable ΔpH formation under these conditions (data not shown). It should be noted that the experiments were conducted in the presence of valinomycin, to avoid formation of significant $\Delta\psi$. A possible interpretation for the sharp dependence on the time of addition of uncoupler may be that under turnover conditions or in the presence of ATP the enzyme is less accessible to deactivation by the uncoupler. (d) Uncouplers which deactivate the enzyme also partially inhibit CaATPase activity which is not coupled to H^+ translocation (Pick & Weiss, 1988), and therefore cannot be related to $\Delta\tilde{\mu}_{H^+}$ energization.

An alternative interpretation of our results is that uncouplers may have two different effects on the enzyme: a pure energetic uncoupling, which stimulates ATP hydrolysis, and a deactivation, namely, a specific interaction of certain uncouplers with the enzyme, causing relaxation of the enzyme molecules to inactive or partially active conformation. Deactivating uncouplers include SF-6847, FCCP, gramicidin D, and atebrin, but not nigericin. The final effect of the uncoupler on the activity will be determined by its relative efficiency as a deactivator or as a protonophore. For gramicidin and atebrin, the deactivation seems dominant throughout the concentration range when added prior to ATP. Nevertheless, gramicidin stimulates ATP hydrolysis when added after ATP (Table III). This may indicate that the deactivating interaction of gramicidin with the enzyme is slow or that it is inhibited under turnover conditions or by ATP. SF-6847, FCCP, and valinomycin have a biphasic effect, probably resulting from dominant uncoupling at low concentrations and dominant deactivation at high concentrations. With nigericin, deactivation seems to be absent altogether.

The deactivation of CF_0–CF_1 by SF-6847 and other uncouplers is facilitated by preincubation with the enzyme (see the time dependence in Figure 3), is partly protected when the enzyme hydrolyzes ATP, depends on the presence of lipids, and is influenced by the lipid composition. The latter observation suggests that the site of interaction with the uncoupler is located in a hydrophobic domain in CF_0.

An interesting side issue is the effect of cholesterol on the activity of CF_0–CF_1. The observation that ATP hydrolysis in cholesterol proteoliposomes is not stimulated by SF-6847 and does not generate ΔpH indicates that the enzyme is functionally uncoupled. Yet, it is activated by imposed $\Delta\tilde{\mu}_{H^+}$ and sensitive to deactivation by SF-6847, suggesting that the enzyme is properly incorporated into the vasicles. Since cholesterol rigidifies phospholipid bilayers, and makes them relatively impermeable to protons, it seems that the proton pumping activity of CF_0–CF_1 is either perturbed by the membrane rigidification or specifically inhibited by cholesterol.

The effects of ADP and inorganic phosphate on the deactivation are particularly interesting. It has been known for a long time (Carmeli & Lifshitz, 1972) that ADP at low concentrations accelerates the decay of the activated state in thylakoids following light triggering. It has been suggested, based on changes in ADP binding properties following energization, that ADP plays a critical role in activation/deactivation (Schulmann & Strotmann, 1981). However, the role of ADP is still controversial, and other workers support the idea that the tight sites, which bind ADP, are the catalytic, not the regulatory, sites (Feldman & Boyer, 1985). The present results indicate that ADP increases the susceptibility of CF_0–CF_1 to deactivation by SF-6847. On the other hand, ADP does not perturb the catalytic potential of the enzyme (uncoupled ATP hydrolysis when SF-6847 is added after ATP) but, on the contrary, improves it. These results are consistent with the idea that ADP has a major role in regulating the activation/deactivation of CF_0–CF_1. Phosphate, which was reported previously to stabilize CF_0–CF_1 in the activated state, also seems to stabilize the enzyme against deactivation by SF-6847. In contrast, Mg, which was previously claimed to destabilize light-triggered ATPase in thylakoids (Bakker-Grunwald & van Dam, 1974), has no effect on the deactivation with or without SF-6847 in the purified enzyme (data not shown).

What is the exact change induced in CF_0–CF_1 upon activation is still an open question. It has been suggested that $\Delta\tilde{\mu}_{H^+}$ activation involves protonation of sites facing the inner space of thylakoids (Schlodder et al., 1982; Mills & Mitchell, 1984). Since the activated state of the enzyme remains long after the dissipation of $\Delta\tilde{\mu}_{H^+}$, this implies that such putative sites are not in equilibrium with the intrathylakoid pH, namely, are occluded in CF_0–CF_1. The observation that protonophores accelerate deactivation is consistent with this hypothesis and suggests that deactivation results from release of protons from cryptic sites in hydrophobic domains of the enzyme.

Acknowledgments

I thank Prof. Mordhay Avron for helpful comments and criticism and S. Bassilian for technical assistance.

REFERENCES

Admon, A., Shahak, Y., & Avron, M. (1982) *Biochim. Biophys. Acta 681*, 405–411.
Avron, M. (1961) *Anal. Biochem. 2*, 535–543.
Avron, M. (1962) *J. Biol. Chem. 237*, 2011–2017.
Bakker-Grunwald, T., van-Dam, K. (1973) *Biochim. Biophys. Acta 292*, 808–814.
Bakker-Grunwald, T., & van-Dam, K. (1974) *Biochim. Biophys. Acta 347*, 290–298.
Carmeli, C. (1969) *Biochim. Biophys. Acta 189*, 256–266.
Carmeli, C., & Liphshits, Y. (1972) *Biochim. Biophys. Acta 267*, 86–95.
Feldman, R. I., & Boyer, P. (1985) *J. Biol. Chem. 260*, 13088–13094.
Finel, M., Rubinstein, M., & Pick, U. (1984) *FEBS Lett. 166*, 85–89.
Lynn, W. S., & Straub, K. D. (1969) *Proc. Natl. Acad. Sci. U.S.A. 63*, 540–547.
McCarty, R. E., & Racker, E. (1968) *J. Biol. Chem. 243*, 129–137.
Mills, J. D., & Mitchell, P. (1984) *Biochim. Biophys. Acta 764*, 93–104.
Mitchell, P. (1979) *Science (Washington, D.C.) 206*, 1148–1159.
Nalin, C. M., & McCarty, R. E. (1984) *J. Biol. Chem. 259*, 7275–7279.
Nelson, N., Nelson, H., & Racker, E. (1972) *J. Biol. Chem. 247*, 7657–7662.
Pick, U. (1986) *Methods Enzymol. 126*, 512–520.
Pick, U., & Racker, E. (1979) *J. Biol. Chem. 254*, 2793–2799.
Pick, U., & Bassilian, S. (1982) *Biochemistry 21*, 6144–6152.
Pick, U., & Weiss, M. (1988) *Eur. J. Biochem. 173*, 623–628.
Pick, U. Gounaris, K., Admon, A., & Barber, J. (1984) *Biochim. Biophys. Acta 765*, 12–20.
Pick, U., Weiss, M., Gounaris, K., & Barber, J. (1987) *Biochim. Biophys. Acta 891*, 28–39.
Racker, E. Chien, T. E., & Kandrach, A. (1975) *FEBS Lett. 57*, 14–18.
Richter, M. L., Patrie, W. J., & McCarty, R. E. (1984) *J. Biol. Chem. 259*, 7371–7373.
Rienits, K. G. (1967) *Biochim. Biophys. Acta 143*, 595–605.
Ryrie, I., & Jagendorf, A. T. (1971) *J. Biol. Chem. 246*, 582–588.
Schlodder, E., Gräber, P., & Witt, H. T. (1982) in *Topics in Photosynthesis* (Barber, J., Ed.) pp 105–175, Elsevier, Amsterdam.
Schmidt, G., & Gräber, P. (1987a) *Biochim. Biophys. Acta 890*, 393–394.
Schmidt, G., & Gräber, P. (1987b) *Z. Naturforsch., C: Biosci. 42C*, 231–236.
Schulmann, J., & Strotmann, H. (1981) in *Proceedings of the 5th International Congress of Photosynthesis* (Akoyonoglu, G., Ed.) pp 881–892, Balaban Ins. Science, Philadelphia.

Empirical Estimation of Interaction Energies for Ligands Binding in the Isolated β-Subunit of F_0F_1 ATP Synthase from *Rhodospirilum rubrum*†

Daniel Khananshvili

Department of Biochemistry, Weizmann Institute of Science, P.O. Box 26, Rehovot 76100, Israel

Received December 21, 1987; Revised Manuscript Received May 18, 1988

ABSTRACT: Under standard experimental conditions one site of the isolated β-subunit of F_0F_1 from *Rhodospirilum rubrum* binds ATP or ADP with $\Delta G°_{ATP}(\beta_1) = -7.6$ kcal/mol and $\Delta G°_{ADP}(\beta_1) = -7.4$ kcal/mol, while the second site binds ATP, ADP, or P_i with $\Delta G°_{ATP}(\beta_2) = -5.1$ kcal/mol, $\Delta G°_{ADP}(\beta_2) = -5.6$ kcal/mol, and $\Delta G°_{P}(\beta_2) = -4.9$ kcal/mol. The synthesis–hydrolysis of ATP on the second site of β can be described by

$$ADP + P_i + E \overset{\Delta G°_{DP}}{\rightleftharpoons} E{\cdot}ADP{\cdot}P_i \overset{\Delta G°_{B}}{\rightleftharpoons} E{\cdot}ATP \overset{\Delta G°_{ATP}}{\rightleftharpoons} E + ATP \overset{\Delta G°_{N}}{\rightleftharpoons} E + ADP + P_i \quad (1)$$

in which $\Delta G°_{DP} = -7.1$ kcal/mol, $\Delta G°_{B} = +9.6$ kcal/mol, $\Delta G°_{ATP} = +5.1$ kcal/mol, and $\Delta G°_{N} = -7.6$ kcal/mol. This suggests that the binding energy of both ATP ($\Delta G°_{ATP}$) and ADP + P_i ($\Delta G°_{DP}$) is weak, causing a very unfavorable enzyme-bound ATP synthesis, $\Delta G°_{B} = +9.6$ kcal/mol. This value of $\Delta G°_{B}$ is very different from the value of $\Delta G°_{B} = -0.4$ kcal/mol observed in the single catalytic site of F_1. This large difference in $\Delta G°_{B}$ values ($\Delta\Delta G°_{B} = +10$ kcal/cal) is caused by the difference in ATP binding ($\Delta\Delta G°_{ATP} = +11.2$ kcal/mol). The overall binding energy of ADP + P_i is not so different in two experimental systems, $\Delta\Delta G°_{DP} = +1.2$ kcal/mol, and it cannot account for such a large difference in $\Delta G°_{B}$ values. It is postulated that the strong binding site for ATP in F_1 is formed by two weak "half-sites", located on the different and neighboring copies of β-subunit. The ATP binding to two half-sites with $\Delta G°_{ATP}(\beta_1) = -7.6$ kcal/mol and $\Delta G°_{ATP}(\beta_2) = -5.1$ kcal/mol could give a strong binding to F_1 with $\Delta G°_{ATP}(F_1) = -16.3$ kcal/mol if we assume that the increase in binding energy caused by a two-step binding to two half-sites is $\Delta G^{S}(\beta_{12}) = (-7.6) + (-5.1) - (-16.3) = +3.6$ kcal/mol. This estimated value of $\Delta G^{S}(\beta_{12})$ is reasonably close to the observed value of $\Delta G^{S}(F_1) = +2.9$ kcal/mol in F_1, supporting the model in which a catalytic unit in F_1 is formed between two β-subunits.

The membrane-bound H^+-ATPase (F_0F_1 complex) of energy-transducing membranes (bacteria, chloroplasts, and mitochondria) is able to use an electrochemical gradient of protons ($\Delta\mu H^+$) for steady-state ATP synthesis (Mitchell, 1966; Racker, 1977; Kagawa et al., 1978; Kagawa, 1984). The H^+-ATPase has two main portions, a catalytic component F_1 composed of five subunits (α, β, γ, δ, ϵ) with a stoichiometry

† This work was supported by a grant from Minerva Foundation to Prof. Z. Gromet-Elhanan.

0006-2960/88/0427-8290$01.50/0
