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Anomalous Uncoupling of Photophosphorylation by Palmitic Acid and by Gramicidin D†

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ABSTRACT: Palmitic acid and gramicidin D at low concentrations uncouple photophosphorylation in a mechanism that is inconsistent with classical uncoupling in the following properties: (1) ΔpH, H^+ uptake, or the transmembrane electric potential is not inhibited. (2) O_2 evolution is stimulated under nonphosphorylating conditions but slightly inhibited in the presence of adenosine 5′-diphosphate + inorganic phosphate (P_i). (3) Light-triggered adenosine 5′-triphosphate (ATP)–P_i exchange is hardly affected, and ATPase activity is only slightly stimulated. (4) ATP-induced ΔpH formation is selectively inhibited. This characteristic uncoupling is observed only when the native coupling sites of the electron transport system are used for energization such as for methylviologen-coupled phosphorylation. With pyocyanine, which creates an artificial coupling site, 1000-fold higher gramicidin D and higher palmitic acid concentrations are required for inhibition, and the inhibition is accompanied by a decrease in ΔpH. Moreover, comparison between photosystem 1 and photosystem 2 electron transport and the effects of membrane unstacking suggest that low gramicidin D preferentially inhibits photosystem 2, while palmitic acid inhibits more effectively photosystem 1 coupling sites. The inhibitory capacity of fatty acids significantly drops when the chain length is reduced below 16 hydrocarbons or upon introduction of a single double bond in the hydrocarbon chain. It is suggested that palmitic acid and gramicidin D interfere with a direct H^+ transfer between specific electron transport and the ATP synthase complexes, which provides an alternative coupling mechanism in parallel with bulk to bulk $\Delta\tilde{\mu}_{H^+}$. The sites of inhibition seem to be located in chloroplast ATP synthase, photosystem 2, and the cytochrome b_6f complexes.

Several mechanisms have been proposed to explain energy transduction leading to adenosine 5′-triphosphate (ATP) formation in photosynthetic and respiratory systems. According to the chemiosmotic hypothesis (Mitchell, 1966, 1979), which has been widely accepted, ATP formation is mediated by a transmembrane proton electrochemical gradient ($\Delta\tilde{\mu}_{H^+}$) that is generated by electron (e^-) transport reactions. An alternative hypothesis, proposed by Williams (Williams 1959, 1961, 1976), suggested that protons are released within the

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membrane from e^- transport to the ATPase complexes.

Although the basic concept of the chemiosmotic hypothesis, namely, coupling by bulk to bulk $\Delta\tilde{\mu}_{H^+}$, has been unambiguously verified in many systems, there are several studies on the kinetics and energetics of oxidative phosphorylation and photophosphorylation that are inconsistent with the idea that $\Delta\tilde{\mu}_{H^+}$ is the only driving force for ATP formation [reviewed by Ferguson and Sorgato (1982), Westerhoff et al. (1984), Ferguson (1985), and Rottenberg (1985)].

The groups of Dilley (Prochaska & Dilley, 1978a,b; Laszlo et al., 1984; Dilley & Schreiber, 1984), Homann (Theg & Homann, 1982) and Junge (Theg & Junge, 1983) have demonstrated the existence, in chloroplasts, of an intramembranal proton pool, originating in PS-II,[1] which exists also in the dark after complete collapse of ΔpH and can be inhibited by acetic anhydride derivation of membrane proteins, by release of Cl^- from PS-II, by removal of CF_1, by osmotic changes, or by uncouplers. It has also been demonstrated that this pool may serve as an energy source for ATP formation (Dilley & Schreiber, 1984). Light-dependent labeling of the 9-kDa polypeptide of CF_0 pointed to interactions between PS-II and the ATP synthase (Prochaska & Dilley, 1978a,b). These results suggest the existence of an intramembranal proton pool in thylakoids, made of protein primary amino groups, that may compose a functional linkage in a direct transfer of protons from PS-II to the ATP synthase.

Another example of a clear deviation from predictions of the chemiosmotic hypothesis is the effects of certain unusual uncouplers of ATP formation. According to the chemiosmotic hypothesis uncouplers inhibit phosphorylation and stimulate coupled e^- transport by dissipation of $\Delta\tilde{\mu}_{H^+}$. Yet, in mitochondria a group of chemicals, including halothane and chloroform (Rottenberg, 1983), fatty acids (Rottenberg & Hashimoto, 1986; Rottenberg & Steiner-Mordoch, 1986), and detergents (Hashimoto and Rottenberg, unpublished results) inhibit ATP formation and stimulate e^- transport without affecting $\Delta\tilde{\mu}_{H^+}$. These and previous results suggest an alternative coupling mechanism by a direct H^+ transfer between e^- transport and the ATP synthase complexes (Rottenberg, 1985). Similarly, in chloroplasts, gramicidin D at very low concentrations inhibits selectively the intramembrane deposition of protons from PS-II (Baker et al., 1981; Theg & Junge, 1983) and ATP formation coupled to e^- transport via PS-II + PS-I, but not to cyclic e^- transport with PS-I alone (Opanasenko et al., 1985). These results suggest a selective effect of gramicidin D on the coupling between PS-II and ATP formation.

In the present work we analyzed the effects of several inhibitors of photophosphorylation, in particular, palmitic acid (PA) and gramicidin D, on several energy-transducing reactions in lettuce thylakoids, namely, ATP formation, e^- transport, H^+ uptake, ΔpH and $\Delta\psi$ formation, light + dithiothreitol (DTT) triggered ATPase and ATP–P_i exchange reactions, and on the dependence of inhibition on the source of e^- transport. The results clearly suggest that PA and gramicidin D inhibit ATP formation by a mechanism distinctly different from uncoupling or energy-transfer inhibition, depending on specific interactions between CF_0–CF_1 and protein complexes of the e^- transport system. These results support the notion of two alternative coupling pathways between e^- transport and phosphorylation: one via a bulk to bulk $\Delta\tilde{\mu}_{H^+}$ and another by direct intramembrane proton transfer from e^- transport to the ATP synthase complexes.

[1] Abbreviations: CF_0–CF_1, chloroplast ATP synthase; PS-I, photosystem 1; PS-II, photosystem 2; PA, palmitic acid; NADP, nicotinamide adenine dinucleotide phosphate; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; SF 6847, (3,5-di-*tert*-butyl-4-hydroxybenzylidene)malonitrile; Chl, chlorophyll; ΔpH, transthylakoid pH gradient; $\Delta\psi$, transthylakoid electrical potential gradient.

Materials and Methods

Chloroplast thylakoids were prepared from lettuce leaves as previously described (Avron, 1960). ATP formation and ATP–P_i exchange were measured by incorporation of ^{32}P into ATP, and ATP hydrolysis was followed by the release of ^{32}P from [γ-^{32}P]ATP. [^{32}P]P_i and [γ-^{32}P]ATP were separated by extraction of phosphomolybdate complexes with 2-methyl-1-propanol–xylene as described earlier (Avron, 1961; Shahak, 1982). Activation and assay of light-triggered ATPase and ATP–P_i exchange were performed essentially according to the method of Shahak (1982). Methylviologen reduction was measured with an oxygen electrode (Karlish & Avron, 1968). Measurements of 9-aminoacridine fluorescence changes for calculation of ΔpH (Schuldiner et al., 1972) were performed in a Perkin-Elmer MPF-44A spectrofluorometer, with excitation and emission wavelength set on 400 and 458 nm and slits set at 1 and 20 nm, respectively. Illumination was provided by a 24-V halogen lamp projector filtered through a Schott-RG-645 filter. Measurements of proton uptake with and without imidazole and calculation of ΔpH from the imidazole-stimulated proton uptake were performed as previously described (Pick & Avron, 1976). Oxanol VI absorption changes (603–590 nm) were measured in an Aminco DW-2 dual-wavelength spectrophotometer (Admon et al., 1982) for evaluation of the transmembrane electrical potential ($\Delta\psi$). The basic reaction mixtures used for most measurements contained 30 mM KCl, 30 mM Na–tricine, pH 8, 2 mM $MgCl_2$, 2 mM P_i, 0.5 mM adenosine 5′-diphosphate (ADP), 100 μM methylviologen, and 20 μg of Chl/mL. Illumination was provided for 2 min (at saturating light intensity) at 21 °C. Oxygen evolution measurements were performed in the presence of 5 mM sodium azide, 9-aminoacridine fluorescence changes in the presence of 2 μM 9-aminoacridine, and oxanol VI absorption changes with 2 μM oxanol VI. Basal e^- transport rate (O_2 evolution with methylviologen) was measured in the absence of ADP with 100 μM ATP. Inhibitors were always added after the chloroplast membranes and preincubated for 10–30 min on ice before the assay. The length of the incubation (from 5 to 60 min) did not affect the extents of inhibition. Fatty acids were prepared as dilute ethanol solutions (0.5–5 mM) under N_2 and injected into a stirred membrane solution to avoid the formation of lipid micelles.

Chemicals were obtained from Sigma Chemical, and ^{32}P was obtained from the Negev Radioactive Centre in Israel. [γ-^{32}P]ATP was prepared by photophosphorylation of ADP by ^{32}P and separation on poly(ethylene imine)–cellulose columns (Magnusson et al., 1976).

Results

(*A*) *Comparison of the Effects of Different Photophosphorylation Uncouplers and Inhibitors on ATP Formation, Electron Transport, and ΔpH.* The effect of six uncouplers and inhibitors of photophosphorylation on ATP formation, on methylviologen-mediated electron transport, and on ΔpH formation (measured by 9-aminoacridine fluorescence quenching) is demonstrated in Figure 1. Electron transport was measured under both phosphorylating and nonphosphorylating ("basal") conditions. In the latter reaction a low concentration of ATP (100 μM) was added to block H^+ leakage through the ATP synthase (McCarty et al., 1971).

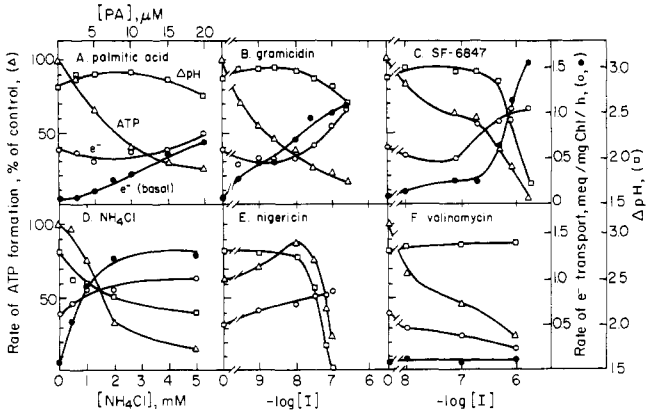

FIGURE 1: Comparison of the effects of different inhibitors of photophosphorylation on e^- transport and ATP and ΔpH formation. Assays of ATP formation (Δ), coupled e^- transport (O), and ΔpH (□) were performed in media containing 30 mM Na–tricine (pH 8), 30 mM KCl, 2 mM $MgCl_2$, 0.5 mM ADP, 2 mM phosphate, 100 μM methylviologen, and 40 μg of Chl in 2 mL. Illumination was provided for 2 min. ^{32}P, 5 mM NaN_3, or 2 μM 9-aminoacridine were added to phosphorylation, e^- transport, or ΔpH assay media, respectively. Basal e^- transport (●) was measured in the presence of 100 μM ATP instead of ADP. Other experimental details are described under Materials and Methods. Control phosphorylation rates are 190, 190, 202, 190, 168, and 195 μmol of ATP (mg of Chl)$^{-1}$ h^{-1} for A–F, respectively.

In contrast to typical uncouplers like NH_4Cl, which inhibit photophosphorylation in parallel with a decrease of ΔpH and a stimulation of e^- transport in both the presence or absence of ADP (Figure 1D), it appears that palmitic acid (PA) or low concentrations of gramicidin ($<10^{-8}$ M) inhibit ATP formation without decreasing ΔpH or stimulating e^- transport under phosphorylating conditions (Figure 1A,B). However, basal e^- transport is significantly enhanced to about the rate under phosphorylating conditions. A similar behavior is also obtained with low concentrations of SF 6847 ($<10^{-7}$ M) (Figure 1C). In fact, all three uncouplers at low concentrations induce a slight increase in ΔpH and a slight inhibition of electron transport under phosphorylating conditions, somewhat resembling the action of an energy-transfer inhibitor of photophosphorylation, like valinomycin (Figure 1F; Karlish & Avron, 1971). The ionophore nigericin stimulates ATP formation at low concentrations in parallel with a stimulation of e^- transport (Figure 1E). The latter phenomenon has been described before (Giersch & Meyer, 1984) and results from an indirect effect on the e^- transport system as will be described in another publication (Pick and Weiss, unpublished results). Inhibition of ATP formation by higher nigericin concentrations is associated with a pronounced decrease in ΔpH.

The correlation between the reduction of the ATP/2 e^- ratio and ΔpH for the different uncouplers and inhibitors is better demonstrated in Figure 2: The drop in coupling ratio from 0.65 to 0.25 observed with palmitic acid or gramicidin is not associated with a drop in ΔpH, unlike the situation with nigericin, NH_4Cl, or SF 6847. Triton X-100 at low concentrations (10–30 μM) also inhibits ATP synthesis without stimulating e^- transport (phosphorylating conditions) or inhibiting ΔpH formation or proton uptake (see also Figure 5). Therefore, the mechanism of Triton X-100 inhibition seems to be similar to that of PA. The milder slope obtained for nigericin may be due to a partial substitution of ΔpH by a transmembrane electrical potential (Δψ) as a driving force for ATP formation due to an electroneuntral H^+/K^+ exchange. These results suggest that the uncoupling of photophosphorylation by PA or by gramicidin D ($<10^{-8}$ M) is not due to the collapse of ΔpH.

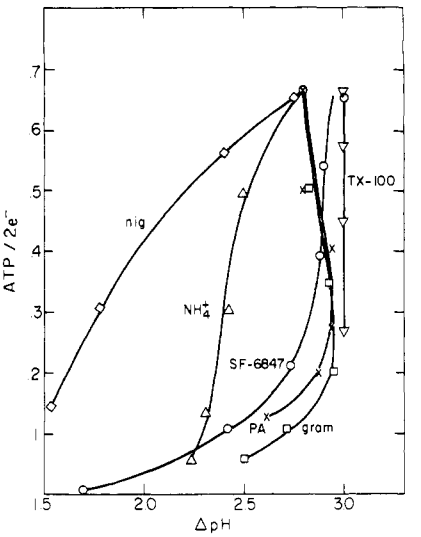

FIGURE 2: Comparison of ATP/2 e^- ratios for different inhibitors of photophosphorylation. ΔpH and ATP/2 e^- ratios were calculated from the data in Figure 1, and similar determinations were performed and analyzed for Triton X-100 (10–30 μM). nig, gram, and TX-100 stand for nigericin, gramicidin D, and Triton X-100, respectively.

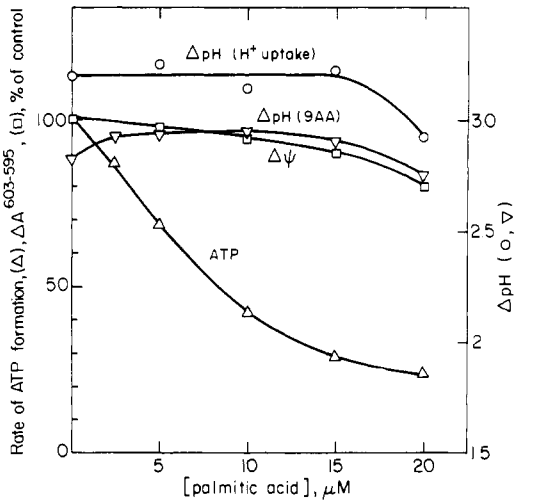

FIGURE 3: Effect of PA on ATP, ΔpH, and Δψ formation. ATP and ΔpH formation (from 9-aminoacridine fluorescence quenching) were measured as in Figure 2. ΔpH measurements, calculated from the imidazole-stimulated H^+ uptake, were carried out in a medium containing 60 mM KCl, 0.5 mM Na–tricine (pH 7.7), 3 mM $MgCl_2$, 30 μM methylviologen, and 40 μg of Chl/2 mL with or without 100 μM imidazole. Oxanol VI absorption changes (603–595 nm), taken as a measure for Δψ, were measured in media containing 10 mM K_2SO_4, 5 mM $MgSO_4$, 10 mM Na–tricine (pH 8), 5 μM phenazine methosulfate, 2 μM oxanol VI, and 40 μg of Chl/2 mL. The results are expressed in percent from steady-state absorption changes in the light relative to the control. 9AA stands for 9-aminoacridine. Other experimental details are given under Materials and Methods.

(B) Effect of Palmitic Acid on ΔpH, Δψ, and H^+ Uptake in Lettuce Thylakoids. In order to ascertain if the insensitivity of 9-aminoacridine fluorescence quenching to PA indeed reflects lack of changes in the proton conductance of thylakoid membranes by the uncouplers, we have used three alternative methods to estimate the effect of PA on the permeability of thylakoid membranes to protons: (1) direct measurements of ligh-induced proton uptake and dark decay kinetics; (2) calculation of ΔpH from imidazole-stimulated H^+ uptake (Pick & Avron, 1976); and (3) Δψ measurements by oxanol VI absorption changes. As is demonstrated in Figure 3, PA up to 15 μM, which inhibited ATP formation by 75%, hardly affected $\Delta\tilde{\mu}_{H^+}$ as measured by any of the three methods under similar conditions.

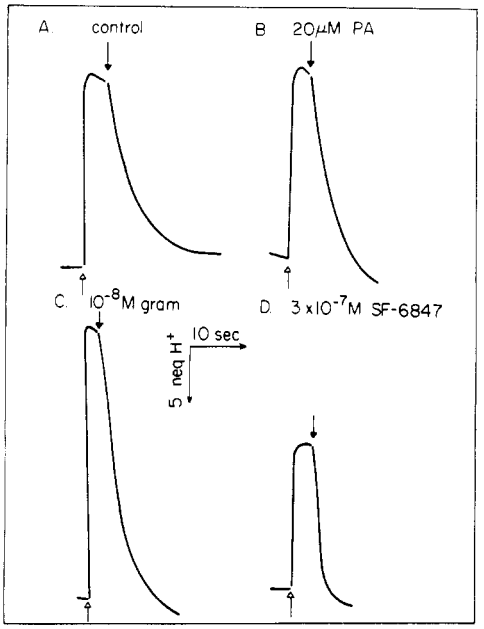

FIGURE 4: Effect of PA, gramicidin, D, and SF 6847 on H^+ uptake. H^+ uptake was measured as described in Figure 3. Arrows indicate onset (open) and termination (solid) of illumination. gram stands for gramicidin D.

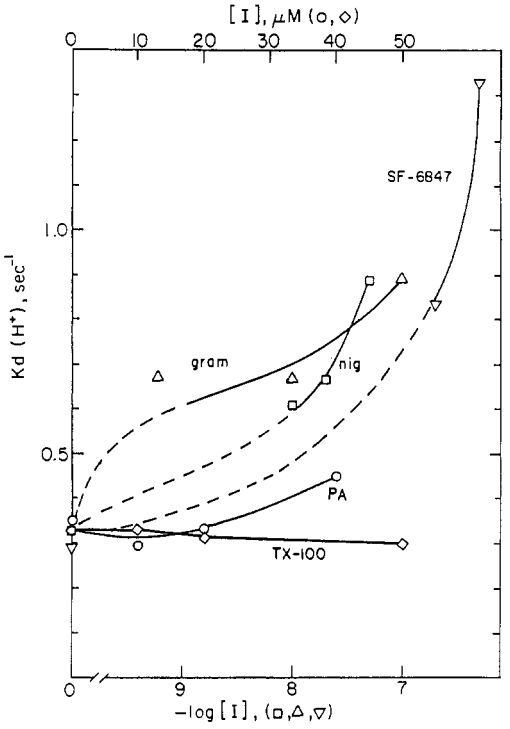

FIGURE 5: Effect of photophosphorylation inhibitors on the rate of H^+ efflux. Experiments were performed as in Figure 4. Kd, reciprocals of the half-time for H^+ efflux in the dark; [I], inhibitor concentration; gram, gramicidin D; nig, nigericin, TX-100, Triton X-100.

Similarly, 20 μM PA has no effect on the extent of H^+ uptake (Figure 4) and only slightly stimulated the rate of proton efflux (Figure 5). Also Triton X-100 (up to 50 μM), which strongly inhibits photophosphorylation, does not affect H^+ uptake, suggesting a similar mechanism of inhibition. In contrast, 10^{-8} M gramicidin stimulated H^+ uptake while accelerating the dark decay rate. For comparison, SF 6874, at concentrations that induce a similar inhibition of ATP formation, significantly inhibits H^+ uptake and greatly stimulates the dark decay rate. The fact that these agents affect neither proton uptake nor efflux indicates that uncoupling is not due

Table I: Comparison of the Effect of Inhibitors on Light-Activated ATPase and ATP–P_i Exchange

addition	ATPase μmol (mg of Chl)$^{-1}$ h^{-1}	%	ATP–P_i exchange μmol (mg of Chl)$^{-1}$ h^{-1}	%
none	101	(100)	41.0	(100)
20 μM PA	123	123	36.6	89.2
10^{-8} M gramicidin D	136	135	28.7	70.2
10^{-7} M nigericin	152	151	11.8	28.9
3 mM NH_4Cl	268	265	3.3	8.1

[a] Assay conditions are as described in Figure 6A.

to an increase in the permeability of thylakoid membranes to protons and a consequent dissipation of transmembrane pH or electrical gradients.

(C) Effects of Palmitic Acid on Light-Activated ATPase and on ATP-Induced ΔpH Formation. In order to further characterize the mechanism of PA inhibition, we have studied the effect of PA on dithiothreitol plus light-activated ATP hydrolysis, ATP–P_i exchange, and ATP-dependent ΔpH formation in spinach thylakoids.

PA induces only a slight inhibition of ATP–P_i exchange and a mild stimulation of ATPase activity (Figure 6A). In contrast with nigericin (10^{-7} M) or NH_4Cl (3 mM), which strongly inhibit ATP–P_i exchange and stimulate ATPase activity, PA (20 μM) and gramicidin (10^{-8} M) induced comparatively mild inhibitions (ATP–P_i exchange) or stimulations (ATPase) of dithiothreitol + light-activated reactions (Table I).

Another difference between classical uncouplers and nonclassical uncouplers (decouplers) is the selective inhibition of ATP-induced ΔpH formation (measured by 9-aminoacridine fluorescence quenching following dithiothreitol + light energization) by PA and by low concentrations of gramicidin (Figure 6B). Whereas NH_4Cl and nigericin inhibit both light-induced and ATP-induced ΔpH formation, PA and gramicidin ($<10^{-8}$ M) selectively inhibit ATP-induced but not light-induced ΔpH formation (Figure 6C). These results suggest a specific effect of both uncouplers on the ATP synthase complex.

(D) Specificity of PA and Gramicidin Inhibition of ATP Formation for the Electron Transport System. Since the results described above indicate that PA and gramicidin affect the coupling between e^- transport and ATP formation in an unusual way, it seemed of interest to compare the effects of these inhibitors on photophosphorylation in the presence of different e^- transport carriers that separately activate the PS-II coupling site ($H_2O \rightarrow$ diaminodiurene $\rightarrow$ ferricyanide) or the PS-I coupling site (DCMU + ferredoxin + NADPH). For comparison, the PS-II plus PS-I coupling sites ($H_2O \rightarrow$ methylviologen) as well as an artificial coupling site created by pyocyanine (cyclic e^- transport in PS-I) were also tested.

As is demonstrated in Figures 7 and 8 the sensitivity of ATP formation to PA and gramicidin is dependent on the e^- transport segment utilized. Uncoupling of photophosphorylation with pyocyanine requires about 1000-fold higher gramicidin D concentrations than for uncoupling with methylviologen, diaminodiurene + ferricyanide, or ferredoxin + NADPH (Figure 7B), and it is correlated with a drop in ΔpH (Figure 8, solid triangles). Similarly, the sensitivity to PA is lowest with pyocyanine, higher for diaminodiurene + ferricyanide, and highest with ferredoxin + NADPH (Figure 7A). It is also worthwhile to note (Figure 8) that the control phosphorylation rate with methylviologen is far greater than that obtained from the same ΔpH with pyocyanine, and the shapes of the curves are strikingly different. These results

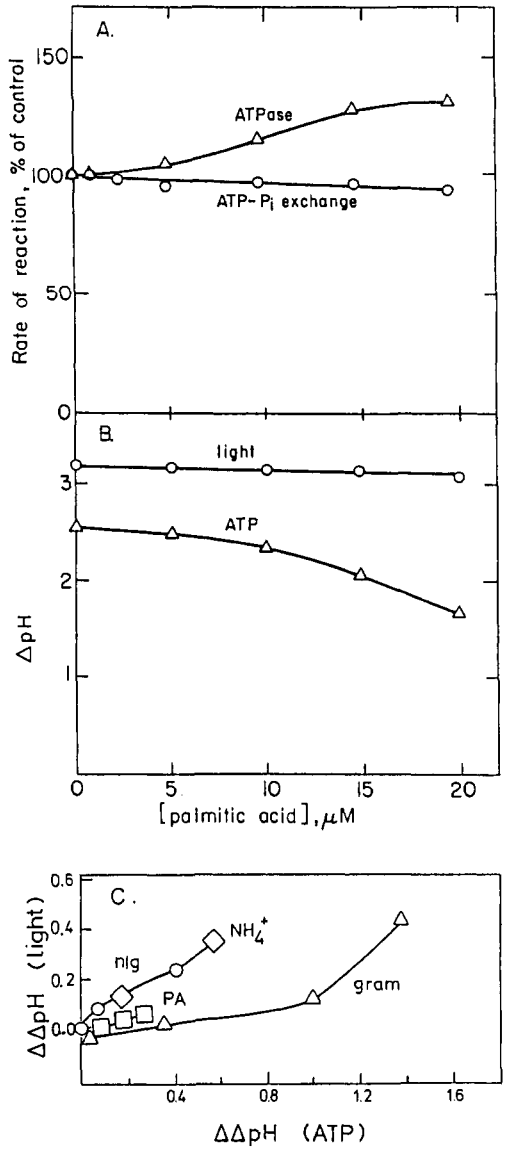

FIGURE 6: Effects of PA on light + dithiothreitol triggered CF_0–CF_1 activities. (A) ATPase and ATP–P_i exchange. Chloroplast thylakoids (40 μg of Chl) were preilluminated for 4 min at 22 °C in 0.1 mL containing 40 mM Na–tricine (pH 8), 40 mM sucrose, 10 mM dithiotheritol, 10 mM $MgCl_2$, 1 mM sodium pyrophosphate, and 20 μM phenazine methosulfate. After 15 s in the dark, reaction mixtures (0.9 mL) were added containing 30 mM Na–tricine (pH 8), 5 mM dithiothreitol, 5 mM $MgCl_2$, 3 mM ATP, 2 mM phosphate, and either [γ-^{32}P]ATP (5×10^5 cpm/mL, ATPase reaction) or [^{32}P]P_i (2×10^6 cpm/mL, ATP–P_i reaction). Palmitic acid was added immediately after addition of reaction mixtures, the reaction was allowed to proceed for 10 min in the dark and then stopped with 5% trichloracetic acid, and ^{32}P was extracted as described under Materials and Methods. Control activities were 49 and 72 μmol (mg of Chl)$^{-1}$ h^{-1} of P_i exchanged or ATP hydrolyzed, respectively. (B) Comparison between effects of PA on ATP and on light-induced ΔpH formation. Chloroplast thylakoids (40 μg of Chl/2 mL) were preilluminated in the presence or absence of inhibitors for 2 min in a medium containing 30 mM Na–tricine (pH 8), 30 mM KCl, 2 mM $MgCl_2$, 2 mM P_i, 5 mM dithiothreitol, 100 μM methylviologen, and 2 μM 9-aminoacridine. Twenty seconds after turning off the light, ATP (0.15 mM) was added, and after another 2 min, the reaction was stopped with nigericin (5×10^{-7} M). ΔpH values were calculated from the light- and ATP-induced fluorescence quenching of 9-aminoacridine fluorescence as described before (Schuldiner et al., 1972). ΔΔpH (C) represents the inhibitor-induced decrease in calculated values of ΔpH measurements in the light or in response to ATP. nig, nigericin; gram, gramicidin D.

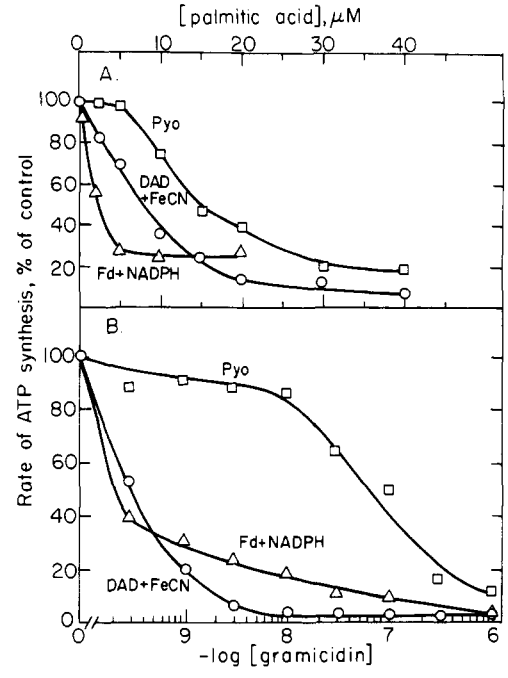

FIGURE 7: Comparison of inhibitions by PA and gramicidin D between different electron transport mediators. ATP formation was measured in the presence of 30 μM pyocyanine (pyo, □), 200 μM diaminodiurene and 1 mM ferricyanide (DAD + FeCN, ○), or 20 μM ferredoxin (Fd), 0.5 mM NADPH, 3 mM glucose 6-phosphate, 2 units/mL glucose-6-phosphate dehydrogenase, and 5 μM DCMU (△). Control rates of ATP formation were 880, 525, and 72 μmol of ATP (mg of Chl)$^{-1}$ h^{-1} with pyocyanine, diaminodiurene + ferricyanide, or ferredoxin + NADPH, respectively.

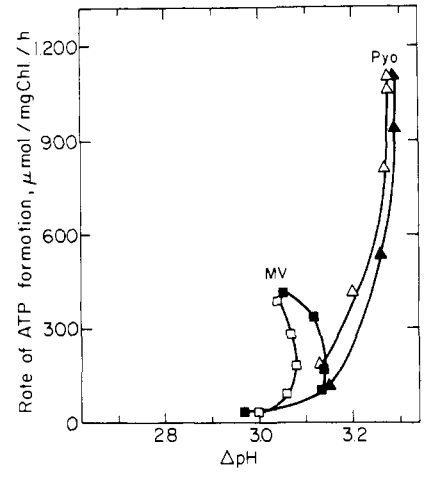

FIGURE 8: Comparison of effects of PA and gramicidin D on ATP and ΔpH formation with methylviologen and pyocyanine. ATP and ΔpH formation were measured in the presence of methylviologen (MV, 100 μM) or pyocyanine (pyo, 30 μM). The concentrations of PA (□, △) were 5–40 μM and of gramicidin D (■, ▲) 10^{-8}–10^{-6} and 3×10^{-10}–10^{-7} μM for pyocyanine and methylviologen, respectively.

suggest that in the presence of pyocyanine, gramicidin D and PA inhibit ATP formation only at high concentrations, acting as classical uncouplers, while with the native coupling sites of both PS-I and PS-II the uncouplers interfere in ATP formation by a different mechanism and at lower concentrations.

In order to verify that the differential sensitivities to PA between different e^- transport mediators do not result from different levels of energization or of phosphorylation rates, which are highest with pyocyanine, we measured the dependence of PA inhibition on pyocyanine concentration (Figure 9B). The extent of PA inhibition is essentially constant down to 20% of the phosphorylation rate at low pyocanine concentrations. Similarly, decreasing the light intensity with methylviologen down to 10% of the phosphorylation rate hardly affects the sensitivity to PA (Figure 9A). It may be noted also

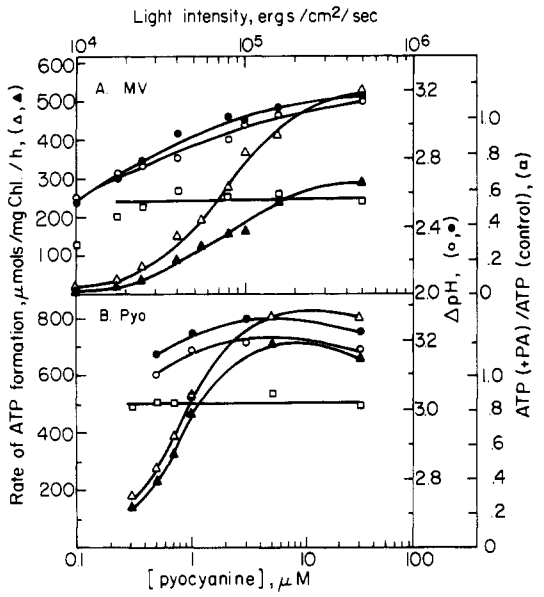

FIGURE 9: Effects of light intensity and e^- mediator concentration on the sensitivity to PA. ATP (△, ▲) and ΔpH (○, ●) formation were measured in the absence (○, △) or presence (●, ▲) of 10 μM PA. In (A) 100 μM methylviologen (MV) was the e^- mediator, and the light intensity was varied. In (B) pyocyanine (pyo) concentration was varied at saturating light intensity. The extent of inhibition of ATP formation by PA is also indicated (□).

that in both cases ΔpH is not decreased but rather slightly increased by PA also at reduced e^- transport rates.

The results presented in Figure 7 suggest that the inhibition of ATP formation by PA and gramicidin ($<10^{-8}$ M) may result from interference in direct energetic coupling between CF_0–CF_1 and specific elements of the e^- transport system in both PS-I and PS-II. Moreover, there seem to exist differences in the relative effectiveness of inhibition of PS-I and PS-II coupled phosphorylations between PA and gramicidin D, suggesting different specificities for the two coupling sites. If indeed specific interactions between CF_0–CF_1 and e^- transport complexes take place in thylakoid membranes, then it could be expected that perturbation of the organization of the system should also affect these interactions. Disorganization of the thylakoid e^- transport system can be obtained by decreasing the Mg and salt concentration. Under these conditions the membranes unstack and the PS-II and PS-I + CF_0–CF_1 that are normally segregated into appressed and exposed membrane domains, respectively, intermix (Barber, 1980). As is demonstrated in Table II, unstacking of thylakoids (performed in the presence of sucrose to maintain the osmotic balance) considerably decreases the sensitivity of photophosphorylation to PA but increases the sensitivity to 10^{-8} M gramicidin. The sensitivity to gramicidin is particularly pronounced with pure PS-II e^- transport (diaminodiurene + ferricyanide). These observations are consistent with a preferential effect of gramicidin D on PS-II/CF_0–CF_1 coupling and of PA on PS-I/CF_0–CF_1 coupling.

(*E*) *Fatty Acid Specificity for Uncoupling of Photophosphorylation.* Analysis of the uncoupling efficiency of a series of fatty acids reveals that both the chain length and the saturation level of the hydrocarbon chains affect the inhibition of photophosphorylation: Fatty acids shorter than 16 carbons show a pronounced decrease in the inhibitory effectiveness (Figure 10A). Similarly, a single double bond either in 18-carbon (oleic acid) or in 16-carbon fatty acids (palmitoleic acid) significantly decreases the inhibitory efficiency (Figure 10B). These results suggest that fatty acid inhibition of photophosphorylation requires a minimal chain length of 16 carbons and complete saturation of the hydrocarbon chain.

Table II: Effect of Thylakoid Unstacking on the Sensitivity to PA and to 10^{-8} M Gramicidin[a]

		rate of ATP formation			
		5 mM Mg^{2+}		0.25 mM Mg^{2+}	
e^- carrier	inhibitor	μmol (mg of Chl)$^{-1}$ h^{-1}	%	μmol (mg of Chl)$^{-1}$ h^{-1}	%
methylviologen		399	(100)	101	(100)
	PA	203	51	85	84
	gramicidin	133	33	15	15
pyocyanine		568	(100)	395	(100)
	PA	506	89	286	72
	gramicidin	430	75		
diaminodiurene + ferricyanide		323	(100)	235	(100)
	PA	151	47	165	70
	gramicidin	20	6	6	3

[a] Chloroplast thylakoids (40 μg of Chl/2 mL) were preincubated for 15 min on ice and for 5 min at 22 °C in media containing 20 mM Na–tricine (pH 8), 2 mM phosphate and $[^{32}P]P_i$, 0.5 mM ADP, 100 μM methylviologen, and either 0.25 mM $MgCl_2$, 10 mM KCl, and 0.2 M sucrose or 5 mM $MgCl_2$ and 30 mM KCl. Methylviologen (100 μM), pyocyanine (30 μM), or diaminodiurene (200 μM) + ferricyanide (1 mM) as well as PA (10 μM) or gramicidin D (10^{-8} M) were also present during the preincubation. Other experimental details are described under Materials and Methods.

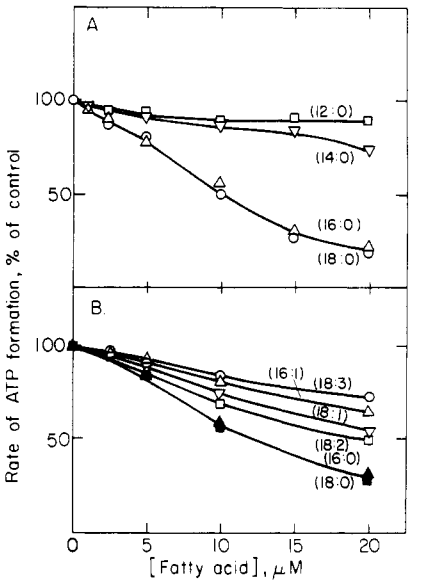

FIGURE 10: Fatty acids specificity for inhibition of photophosphorylation. Photophosphorylation in the presence of methylviologen was measured in the presence of (A) lauric (□), myristic (▽), pamitic (△), or stearic (○) acids or (B) oleic (▽), linoleic (□), linolenic (○), palmitoleic (△), palmitic (▲), or stearic (●) acids.

Discussion

The mechanism of inhibition of ATP formation by PA and by gramicidin ($<10^{-8}$ M) is inconsistent with classical (chemiosmotic) uncoupling because (1) there is no dissipation of $\Delta\tilde{\mu}_{H^+}$, (2) there is no stimulation of e^- transport under phosphorylating conditions, and (3) there is hardly any inhibition of ATP–P_i exchange. Nevertheless, the effects of PA and gramicidin show the basic indications of uncoupling (i.e., stimulation of basal e^- transport, inhibition of ATP synthesis, reducton of P/2 e^- ratios, and stimulation of ATPase activity). Other effects resemble some effects of energy-transfer inhibitors: a slight increase in ΔpH and selective inhibition of

ATP-induced ΔpH formation. The latter results seem to suggest a direct interaction with the ATP synthase. On the other hand, the specificity for the e^- transport source suggests specific interactions with e^- transport components.

Although these results are inconsistent with chemiosmotic coupling mechanisms, they can be interpreted by assuming direct coupling either by conformational interaction or by an intramembrane proton transfer between specific e^- transport complexes and the ATPase complex that are selectively blocked by PA and low gramicidin D concentrations.

Similar inhibitions of ATP formation without dissipation of $\Delta\tilde{\mu}_{H^+}$ or stimulation of coupled e^- transport in mitochondria were reported recently for general anaesthetics (Rottenberg, 1983), free fatty acid, and gramicidin D, and this mechanism of inhibition was termed decoupling (Rottenberg & Hashimoto, 1986). Also Beard and Dilley reported recently that the onset of flash-induced ATP formation in chloroplasts in the absence of KCl is unaffected by permeable buffers, consistent with direct coupling between e^- transport and CF_0–CF_1 complexes (Beard & Dilley, 1986).

There are several reports indicating the existence of intramembrane proton buffering domains in thylakoid membranes that are connected to PS-II and are not in equilibrium with the intrathylakoid bulk phase (Prochaska & Dilley, 1978a,b; Theg & Homann, 1982; Theg & Junge, 1983; Lazlo et al., 1984). Indications that these proton pools may be utilized for ATP formation has also been presented (Dilley & Schreiber, 1984). Gramicidin D, at extremely low concentrations, was demonstrated to specifically inhibit the PS-II-linked intramembranal deposition of protons (Theg & Junge, 1983) and more recently was shown to preferentially inhibit ATP formation coupled to methylviologen as compared to phenazine methosulfate catalyzed e^- transport (Opanasenko et al., 1985). The results presented in Figure 7 and in Table II are consistent with these reports and suggest that gramicidin D ($<10^{-8}$ M) may preferentially interfere in direct proton transfer between PS-II and CF_0–CF_1, while PA may decouple better PS-I to CF_0–CF_1 proton transfer. The intramembrane proton pools in thylakoid membrane may be created by aggregations of CF_0–CF_1 with specific e^- transport complexes (cytochrome b_6f, PS-II?) in the membrane. PA may have higher affinity for CF_0–CF_1, while gramicidin may have higher affinity for the CF_0–CF_1:PS-II aggregate.

At present there is no direct evidence for intramembrane proton transfer between electron transport and ATP synthase complexes in thylakoid or mitochondrial membranes, and the anomalous uncoupling by PA and gramicidin may be explained also by alternative mechanisms such as direct conformational coupling (Boyer, 1984). Nevertheless, the existence of intramembrane proton buffering groups in chloroplasts that are involved in ATP formation and in particular the extreme sensitivity of these proton pools to gramicidin suggest direct coupling via intramembrane H^+ transfer.

The possibility of direct energetic coupling between e^- transport components and CF_0–CF_1 in chloroplasts raises several questions concerning its mechanism, the organization of enzyme complexes in thylakoid membranes, and the concept of a H^+ channel.

(*1*) *Interrelations between Chemiosmotic and Direct Coupling.* If indeed two alternative and distinct coupling routes between e^- transport and ATP synthesis coexist, namely, bulk to bulk transmembrane and direct intramembrnae proton transfers, then it could be expected that selective inhibition of the intramembrane pathway should only partially inhibit ATP formation. The results in Figures 1 and 2 (incomplete or biphasic inhibitions) seem to indicate that this may be the case. Similarly, ATP synthesis driven by artificially generated $\Delta\tilde{\mu}_{H^+}$ should not be sensitive to these agents. This is demonstrated here by the insensitivity of pyocyanine-driven ATP formation to PA and low gramicidin concentrations. Similarly, in the mitochondria PA does not inhibit ATP synthesis driven by artificially generated $\Delta\tilde{\mu}_{H^+}$ (Rottenberg & Steiner-Mordoch, 1986).

(*2*) *Organization of the Electron Transport System in Thylakoid Membranes.* The results presented in Figure 7 and in Table II suggest that direct energetic coupling takes place between CF_0–CF_1 and both PS-I and PS-II e^- transport complexes. The segregation of protein complexes in stacked thylakoid membranes (in the presence of Mg^{2+} and slat) into appressed domains, containing mostly PS-II, and exposed domains, containing mostly PS-I and CF_0–CF_1 (Barber, 1980), seems inconsistent with direct interactions between PS-II and CF_0–CF_1 complexes. However, this organization does not exclude the possibility of interaction of CF_0–CF_1 with PS-II units at the contact area between appressed and exposed membrane domains and also with the PS-II that is present in the exposed domains (Andersson et al., 1982). The increased effectiveness of gramicidin inhibition upon unstacking, which supposedly increases the availability for contacts between PS-II units and CF_0–CF_1, also supports the idea of direct energetic coupling between CF_0–CF_1 and PS-II.

(*3*) *Existence of a Proton Channel.* Another important implication of these results concerns the concept of a proton channel in the ATP synthase. Although it has been convincingly demonstrated that the ATP synthase itself (McCarty, 1984; Schönfeld & Neumann, 1977) as well as the purified dicyclohexylcarbodiimide binding proteolipid subunit (Nelson et al., 1977) enhances proton conductance through membranes, there is no evidence that protons are transported through a hydrophilic channel-like structure inside CF_0, as is generally assumed. The possibility of direct proton transfer between e^- transport and the ATP synthase complex is clearly inconsistent with this view and suggests that the transfer of protons takes place along the outer surface of the protein complexes, facing the lipid. Hypothetical models for such a mechanism have been suggested before [for example, Boyer (1984)].

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Registry No. PA, 57-10-3; ATP, 56-65-5; ATPase, 9000-83-3; H^+, 12408-02-5; O_2, 7782-44-7; gramicidin D, 1393-88-0; lauric acid, 143-07-7; myristic acid, 544-63-8; stearic acid, 57-11-4; oleic acid, 112-80-1; linolenic acid, 463-40-1; linoleic acid, 60-33-3; palmitoleic acid, 373-49-9.

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Characterization of Long-Range Electron Transfer in Mixed-Metal [Zinc,Iron] Hybrid Hemoglobins†

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ABSTRACT: Measurements characterizing electron transfer from a photoexcited zinc protoporphyrin triplet (^{3}ZnP) to a ferriheme electron acceptor within the $[\alpha_1,\beta_2]$ electron-transfer complex of [Fe^{III},Zn] hybrid hemoglobins are reported. Analytical results demonstrate that the hybrids studied are pure, homogeneous proteins with 1:1 ZnP:FeP content. Within the T quaternary structure adopted by these hybrids, the optical spectrum of a Fe^{III}P is perturbed by the protein environment. Room temperature kinetic studies of the rate of ^{3}ZnP decay as a function of the heme oxidation and ligation state demonstrate that quenching of ^{3}ZnP by $Fe^{III}(H_2O)$P occurs by long-range intramolecular electron transfer with rate constant k_t = 100 (±10) s^{-1} and is not complicated by spin-quenching or energy-transfer processes; results are the same for α(Zn) and β(Zn) hybrids. Replacement of H_2O as a ligand to the ferriheme changes the ^{3}ZnP → Fe^{III}P electron-transfer rate constant, k_t, which demonstrates that electron transfer, not conformational conversion, is rate limiting. However, the trend is not readily explained by simple considerations of spin-state and bonding geometry: k_t decreases in the order imidazole > H_2O > F^- ~ CN^- ~ N_3^-. The reverse electron-transfer process $Fe^{II}P \rightarrow ZnP^+$ has not been observed directly but has been shown to be much more rapid, with rate constant $k_b > 10^3$ s^{-1}, consistent with the possible importance of "hole" superexchange in electron tunneling within protein complexes.

Long-range electron transfer can be studied without the complication of second-order processes by use of modified proteins that hold an electron donor/acceptor redox pair held at fixed distance (Marcus & Sutin, 1985; Mayo et al., 1986; Tollin et al., 1986). For example, Gray and co-workers (Winkler et al., 1982; Scott et al., 1985; Crutchley et al., 1985) and Isied and co-workers (Isied et al., 1982, 1984) have developed a technique for studying electron transfer *within* a protein. They show that $[(L)_5Ru]^{2+}$, bound to a histidine residue on the outside of a redox protein, such as ferricytochrome *c*, can exchange an electron with a redox center

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