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The mechanism of stimulation of photophosphorylation by amines and by nigericin

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The anomalous stimulation of photophosphorylation by low concentrations of nigericin and hydrophylic amines has been reinvestigated. It is demonstrated that this stimulation is: (1) Closely linked to an inhibitory effect of sugars on electron transport which is reversed by nigericin and amines. Both sugar inhibition and nigericin (or amine) stimulation involve minor changes in ATP/2e$^-$ ratios. (2) Inhibited by high salt. (3) Dependent on the presence of chloride. (4) Associated with a slight decrease in ΔpH, a slight increase in Δψ and an overall minor decrease in $\Delta\tilde{\mu}_{H^+}$. There is no correlation between the stimulation of photophosphorylation and Δψ, suggesting that the two effects are unrelated. (5) Blocked by low concentrations of palmitic acid and gramicidin D which inhibit ATP formation without affecting $\Delta\tilde{\mu}_{H^+}$ (decoupling). (6) Maximal effects of sugars and nigericin or amines are obtained when ATP formation is driven by electron transport through both photosystems, partial effects are obtained when only Photosystem I is operative, while hardly any effects are obtained when only Photosystem II is operative. These results suggest that the stimulation of photophosphorylation by nigericin or amines results from salt accumulation which affects electron transport and the coupling between ATP formation and electron transport. Conditions of external sugar and internal high salt may induce in thylakoid membranes a shift from bulk-bulk $\Delta\tilde{\mu}_{H^+}$ to localized H^+ coupling, probably reflecting intramembranal proton transfers between electron transport and ATP synthase complexes.

Introduction

It is generally accepted that the major mechanism leading to ATP formation in energy transducing membranes such as mitochondrial inner membranes, chloroplast thylakoids and bacterial membranes involves a transmembrane proton electrochemical gradient [1,2]. However, several striking deviations from the predictions of the chemiosmotic hypothesis have been reported recently [3–6], and gave rise to alternative hypotheses which basically suggest a more direct route of coupling between electron transport and ATP synthesis [7].

One example is the stimulation of ATP formation in chloroplasts by low concentrations of certain uncouplers, which decrease the ΔpH. This phenomenon, first observed with amines [8] and certain ionophores [9], was extensively studied by Giersch and collaborators [10–13]. The conclusion from these studies is that low concentrations of

Abbreviations: ΔpH, transthylakoid pH gradient; Δψ, transthylakoid electrical potential gradient; $\Delta\tilde{\mu}_{H^+}$, transthylakoid electrochemical potential of protons; Chl, chlorophyll; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; MV, methyl viologen; PS I, Photosystem I; PS II, Photosystem II; DAD, diaminodiurene; FeCn, ferricyanide; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; Fd, ferredoxin; NADP, nicotinamideadeninedinucleotide phosphate; PA, palmitic acid; P_i, inorganic phosphate; SF-6847, 3,5-di(*tert*-buthyl)-4-hydroxybenzylidenemalonitrile.

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amines and carboxylic ionophores like nigericin, enhance ATP formation, while slightly decreasing Δ pH without significantly affecting the ATP/$2e^-$ ratio.

Another example comes from studies by the groups of Prochaska and Dilley [14–16], Theg and Homann [17] and Theg and Junge [18] who demonstrated the existence in chloroplasts of intramembrane proton pools which are charged by electron transport, and can be utilized for ATP formation [16]. Recently, Beard and Dilley [19] have demonstrated that exposure of chloroplast thylakoid to sucrose medium is essential to obtain these phenomena, which are abolished by KCl.

We have recently demonstrated that palmitic acid and gramicidin D at low concentrations uncoupled photophosphorylation without decreasing $\Delta\tilde{\mu}_{H^+}$, that this unusual uncoupling is specific to the two native coupling sites of the electron-transport system. We suggested that these uncouplers interfere with a transfer of protons between intramembranal protein complexes of electron transport and the ATP synthase [20].

In this work we reinvestigate the anomalous stimulation of photophosphorylation by amines and by nigericin. We demonstrate that this stimulation is closely linked to an inhibitory effect of sugars on electron transport, which is reversed by salt accumulation inside thylakoids. From the fact that the stimulation of ATP formation is accompanied by a drop in $\Delta\tilde{\mu}_{H^+}$, is blocked by palmitic acid and gramicidin D, and shows specificity for electron transport through Photosystem I, we propose that this phenomenon may reflect a partial shift from a delocalized to a more localized H^+ coupling mechanism between electron transport and ATP formation.

Materials and Methods

Chloroplast thylakoids were prepared from lettuce leaves as previously discribed [21]. ATP formation was measured by the incorporation of ^{32}P into ATP, followed by separation of [γ-^{32}P]ATP by extraction of the phosphomolybdate complex with isobutanol-xylene as described by Avron [22]. Oxygen uptake and oxygen evolution measurements were performed with an oxygen electrode as described by Karlish and Avron [23]. Measurements of 9-aminoacridine fluorescence changes for calculation of ΔpH [24] were performed in a Perkin-Elmer MPF-44A spectrofluorimeter, with excitation and emission wavelengths set on 400 and 458 nm and slits set at 1 nm and 20 nm, respectively. A calculation of ΔpH from fluorescence quenching measurements was made according to the equation:

$$\Delta\text{pH} = -\log\frac{Q}{1-Q}\cdot 125\ C$$

Q being the fractional fluorescence quenching and C the chlorophyll concentration in μg Chl/ml (see Discussion for explanation). Oxonol VI absorption changes (603–590 nm) and calibration of the absorbance change for estimation of the steady-state transmembrane electrical potential ($\Delta\psi$) performed by pH shifts, were measured in an Aminco-Chance DW-2 spectrophotometer according to Admon et al. [25]. Illumination was provided by a 24 V halogen lamp projector filtered through a Schott RG-645 filter. The basic reaction mixture contained: 20 mM sodium Tricine (pH 7.8), 10 mM KCl with or without 200 mM sucrose, 2 mM $MgCl_2$, 0.5 mM ADP, 2 mM phosphate, 100 μM methyl viologen and chloroplast thylakoids containing 20 μg Chl/ml.

Results

The effect of sucrose, KCl and Mg^{2+} on the stimulation of photophosphorylation by NH_4^+ and by nigericin

It has been reported previously that NH_4^+ as well as nigericin, at relatively low concentrations, stimulate ATP formation in chloroplasts with methyl viologen as electron acceptor [8–13]. This stimulation is not unique to NH_4^+ and is obtained also by other hydrophylic amines such as methylamine and imidazole (Fig. 1). Amines which are more effective uncouplers, and inhibit ATP formation at lower concentrations, stimulate photophosphorylation to a smaller extent (benzylamine) or not at all (9-aminoacridine). Extensive elimination of Cl^- from the medium, by washing thylakoids in Cl^--free solutions, and substitution with impermeable anions, prevented the NH_4^+-dependent stimulation of photophosphorylation

(Table I). Since accumulation of amines by illuminated thylakoids depends on the availability of permeable anions [26] these results seem to suggest that the stimulation of ATP formation is dependent on the massive uptake of ammonium chloride.

According to previous reports, the maximal stimulation of ATP formation by amines or nigericin is approx. 30%. However, as is demonstrated in Fig. 2, ATP formation may be stimulated over 100% by NH_4^+ depending on the presence or absence of sucrose, the concentrations of KCl, Mg and NH_4^+ (optimal at 0.25–0.5 mM). The presence of 200 mM sucrose increases the relative NH_4^+-dependent stimulation of ATP formation under all conditions (Fig. 2A–C). Conversely, high KCl concentration decreases the stimulation both at low Mg^{2+} (0.25 mM, 2B) and at high Mg^{2+} (5 mM, see Fig. 3). Consequently, the largest stimulation of ATP formation is obtained in the presence of sucrose and low salt (10 mM KCl). In the presence of low Mg^{2+} and low KCl (Fig. 2A) the rate of ATP formation is very low mostly due to unstacking and not to Mg^{2+} limitation, since addition of salt alone, which prevents unstacking, greatly increases the rate of ATP formation (Fig. 2B).

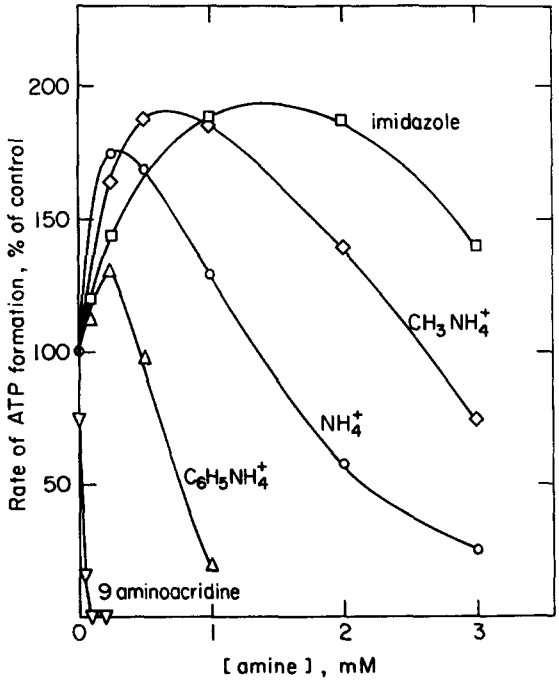

Fig. 1. Effect of different amines on ATP formation. ATP formation was measured in media containing 20 mM sucrose, 20 mM KCl, 100 μM methyl viologen, 5 mM $MgCl_2$, 10 mM sodium Tricine (pH 7.8), 2 mM phosphate+$^{32}P_i$, 0.5 mM ADP, thylakoids (20 μg Chl/ml) and the chloride salt of the indicated amines. Illumination was provided for 2 min. Control rates of ATP formation were 220–245 μeq. ATP per mg Chl per h. Other details are described in Materials and Methods.

TABLE I

Cl^- DEPENDENCE FOR NH_4^+-INDUCED STIMULATION OF ATP FORMATION

Lettuce thylakoids were washed once and resuspended in a chloride-free, sucrose medium. The phosphorylation medium contained: 200 mM sucrose; 5 mM sodium-Hepes (pH 7.8); 0.5 mM ADP, 2 mM sodium phosphate+^{32}P; 1 mM $MgSO_4$, 100 μM methyl viologen, thylakoids (20 μg Chl/ml) and 10 mM of the indicated salts. Ammonium sulfate concentration was 0.5 mM.

Salt (10 mM)	Rate of ATP formation (μmol per mg Chl per h)		Stimulation (% of control)
	control	$+(NH_4)_2SO_4$	
NaCl	200	265	33
Sodium-Hepes	206	208	1
Sodium glycin	207	207	0

The effect of KCl concentration on the NH_4^+ and nigericin-induced stimulation of ATP formation is better demonstrated in Fig. 3. Although KCl concentration (from 3 to 300 mM) has very little effect on the unstimulated rate of ATP formation the higher concentrations clearly inhibit the NH_4^+ and nigericin-induced stimulation both in the presence or absence of sucrose. The minor enhancement of nigericin-stimulated ATP formation by low KCl concentrations (3–30 mM) probably reflects K^+ limitation for K^+/H^+ exchange. The apparent stimulation of NH_4^+-induced phosphorylation in the absence of sucrose by KCl probably reflects an osmotic effect on chloroplast swelling which results from massive NH_4Cl uptake in the absence of sufficient external osmoticum.

Since the pronounced effect of sucrose on the relative stimulation of ATP formation by amines and nigericin seems to be partly due to a net inhibitory effect of sucrose itself on ATP formation, we have further investigated the effect of

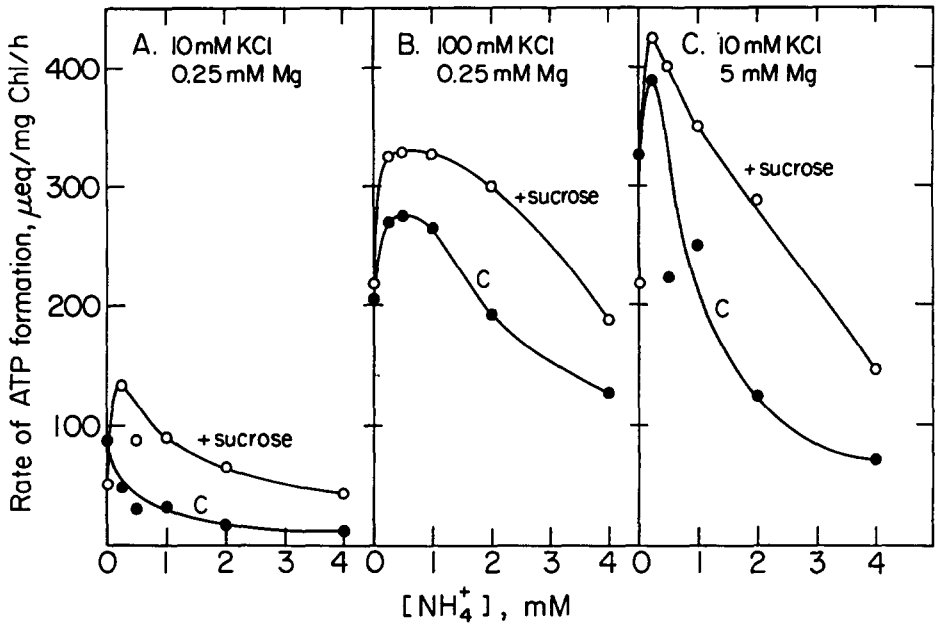

Fig. 2. Effect of sucrose, KCl and Mg on the stimulation of photophosphorylation by NH_4Cl. ATP formation was measured in media containing 10 mM KCl and 0.25 mM $MgCl_2$ (A), 100 mM KCl and 0.25 mM $MgCl_2$ (B) or 10 mM KCl and 5 mM $MgCl_2$ (C) with or without 200 mM sucrose and the indicated ammonium chloride concentration. C, control without sucrose.

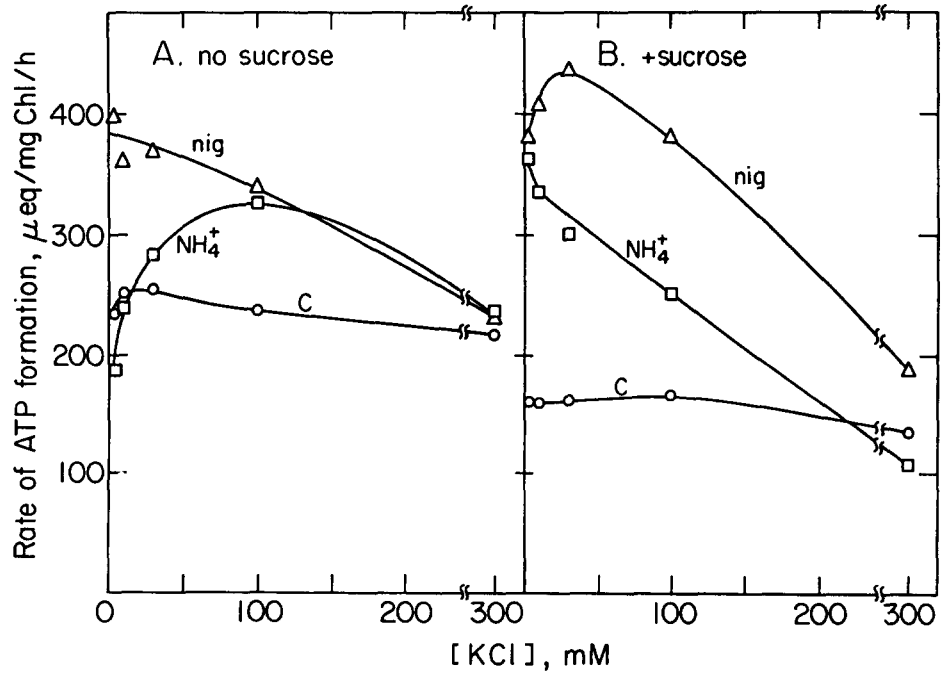

Fig. 3. The dependence of nigericin and NH_4^+ stimulation of photophosphorylation on KCl concentration. ATP formation was measured in the absence (A) or presence (B) of 200 mM sucrose, 2 mM $MgCl_2$, the indicated KCl concentrations with or without 1 mM NH_4Cl or 10^{-8} M nigericin. C, control.

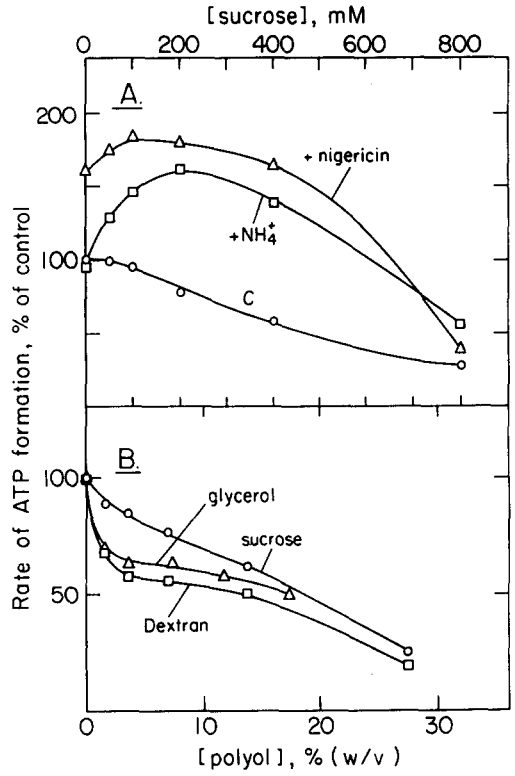

Fig. 4. The effects of sucrose and other polyols on photophosphorylation. ATP formation was measured in the presence of the indicated sucrose concentration with or without 1 mM NH_4Cl or 10^{-8} M nigericin (A). In B glycerol, sucrose or Dextran-10000 were added to give the indicated weight/volume concentrations.

sucrose on ATP formation. Fig. 4A demonstrates that the stimulation by both nigericin and NH_4^+ is optimal at 200–400 mM sucrose, while higher sucrose concentrations inhibit not only basal ATP formation but also the stimulated photophosphorylation. Similar inhibitions are obtained also by the polysaccharide Dextran 10000 and by glycerol (Fig. 4B). Dextran and glycerol also enhance the nigericin and NH_4^+-induced stimulation of ATP formation similar to sucrose (not shown). These results suggest that the effects are not osmotic, but more general polyol effects.

The effect of NH_4^+ and of nigericin on electron transport

A possible reason for the stimulation of ATP formation by amines and nigericin is a specific enhancement of coupled electron transport. Table II demonstrates that nigericin and NH_4^+ indeed stimulate electron transport, but that the relative stimulation, particularly in sucrose medium, is similar to the relative stimulation of ATP formation, resulting in only a slight drop in ATP/2e$^-$ ratio, in agreement with previous results of Giersch and Meyer [13]. The analysis also shows that sucrose inhibits while KCl slightly stimulates e$^-$ transport, similar to their effects on ATP formation. In order to check whether the stimulation of electron transport and of ATP formation under these conditions is not a general result of uncouplers we compared the effect of NH_4^+ and nigericin on electron transport with the effect of a pure protonophore (SF-6847) which would be expected to stimulate electron transport solely by uncoupling. As is demonstrated in Fig. 5, for the same drop in ATP/2e$^-$, NH_4^+ and nigericin enhance electron transport much more than SF-6847. These results seem to suggest that the sucrose-induced inhibition, as well as the NH_4^+ and nigericin-induced stimulation of ATP formation are primarily effects on the electron-transport system.

TABLE II

EFFECT OF NH_4^+ AND NIGERICIN ON ATP FORMATION, ELECTRON TRANSPORT AND ATP/2e$^-$ IN DIFFERENT IONIC MEDIA

Phosphorylation and O_2 uptake were measured in media containing either 10 mM KCl (low salt), 10 mM KCl+200 mM sucrose (sucrose) or 100 mM KCl (KCl). NH_4Cl (0.5 mM) or nigericin (10^{-8} M) were added where indicated.

Medium Addition	ATP formation (μeq per mg Chl per h)	O_2 uptake	ATP/2e$^-$
Low KCl			
–	335	490	0.72
NH_4^+	410	737	0.56
Nig	470	758	0.62
Low KCl + sucrose			
–	240	315	0.76
NH_4^+	420	637	0.66
Nig	540	761	0.71
High KCl			
–	350	560	0.63
NH_4^+	390	775	0.50
Nig	420	859	0.49

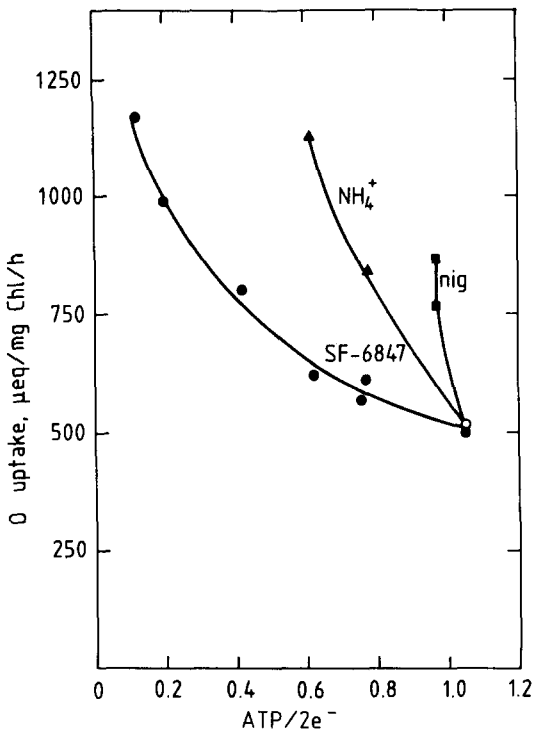

Fig. 5. Correlations between uncoupling and stimulation of electron transport for different uncouplers. ATP formation and oxygen evolution were measured in parallel under identical conditions in media containin 200 mM sucrose, 10 mM KCl, 2 mM $MgCl_2$ and either SF-6847 (10^{-8}–$5 \cdot 10^{-7}$ M) or nigericin ($5 \cdot 10^{-9}$–$2 \cdot 10^{-8}$ M) or NH_4Cl (0.25–1 mM). The untreated control is indicated by the open circle.

The involvement of a transmembrane electrical potential and of direct coupling in the stimulation of photophosphorylation by amines and nigericin

Since both nigericin and amines slightly decrease the steady-state ΔpH across thylakoid membranes in the light (see Table III), it is evident that the driving force for the stimulation of ATP formation cannot be ΔpH. There are at least two alternative mechanisms which may cause the stimulated ATP formation: generation of a significant transmembrane electrical potential ($\Delta\psi$), or a more direct energetic coupling between electron transport and ATP synthesis, which probably takes place in photosynthesis, as is implied by recent results [19,20,27]. The possibility that NH_4^+ and nigericin enhance $\Delta\psi$ was checked both directly, by following Oxonol VI absorption changes (Table III). NH_4^+ and nigericin slightly increase the steady-state $\Delta\psi$ values in illuminated thylakoids but the contribution of $\Delta\psi$ to the overall $\Delta\tilde{\mu}_{H^+}$ is insignificant under all conditions, and the calculated $\Delta\tilde{\mu}_{H^+}$ is always somewhat decreased. Higher concentrations of nigericin and NH_4^+ induce a more pronounced increase in steady-state $\Delta\psi$ (20–30 mV) but a further decrease of ΔpH and the overall $\Delta\tilde{\mu}_{H^+}$, Table III also demonstrates that the increase in $\Delta\psi$ in a sucrose medium by NH_4^+ and nigericin is smaller than in the low salt medium or in a glycine medium, demonstrating that there is no correlation between the enhancement of photosphosphorylation and of $\Delta\psi$.

These results are consistent with previous reports of Giersh [11,12] who found little or no effects of nigericin and amines on the steady-state carotenoid absorption change, and therefore suggested that the transmembrane electrical potential is not involved in the stimulation of ATP formation by amines and nigericin.

Since $\Delta\tilde{\mu}_{H^+}$ is hardly affected by low concentrations of nigericin and NH_4^+, it is evident that the enhanced rate of ATP formation should reflect a different coupling mechanism. The large stimulation of electron transport under these con-

TABLE III

ATP formation, ΔpH (measured with 9-aminoacridine, results of 2–4 measurements) and $\Delta\psi$ (estimated from Oxonol VI absorption changes, average of two measurements) were measured in media as in Table II or in the presence of 100 mM sodium glycine (pH 7.8).

Medium Additions	ATP formation (μmol per mg Chl per h)	ΔpH	$\Delta\psi$ (mV)	$\Delta\mu_{H^+}$ (pH units)
Low KCl	296	3.00 ± 0.05	3	3.05
0.25 mM NH_4^+	373	2.90 ± 0.05	7	3.02
1.00 mM NH_4^+	145	2.64 ± 0.06	9	2.79
10^{-8} M nig	450	2.90 ± 0.08	5	2.98
Low KCl + sucrose	222	3.10 ± 0.05	3	3.15
0.25 mM NH_4^+	344	2.94 ± 0.02	6	3.03
1.00 mM NH_4^+	284	2.75 ± 0.07	7	2.86
10^{-8} M nig	480	2.99 ± 0.03	6	3.09
High KCl	312	3.05 ± 0.05	1	3.07
0.25 mM NH_4^+	337	2.95 ± 0.04	4	3.02
1.00 mM NH_4^+	315	2.80 ± 0.06	6	2.91
10^{-8} M nig	347	2.82 ± 0.05	4	2.88
Glycine	387	3.05 ± 0.06	2	3.10
1 mM NH_4^+	400	2.80 ± 0.08	9	2.95

TABLE IV

EFFECTS OF PALMITIC ACID AND GRAMICIDIN D ON NIGERICIN AND AMINE STIMULATED ATP FORMATION

Phosphorylation was measured in the presence of 200 mM sucrose, 10 mM KCl, 3 mM $MgCl_2$ and the indicated additions.

Addition	ATP formation (μeq per mg Chl per h)	%
No additions	280	100
10 nM gramicidin	140	50
20 μM palmitic acid	129	46
10 nM nigericin	400	142
10 nM nigericin + 10 nM gramicidin	120	43
10 nM nig + palmitic acid	117	42
2 mM methylamine	361	129
2 mM methylamine + 10 nM gramicidin	134	48
2 mM methylamine + 20 μM palmitic acid	126	45

ditions suggests that the stimulation of ATP formation results from a direct energy coupling between electron transport and ATP formation. We have recently demonstrated [20] that palmitic acid and gramicidin D at low concentrations inhibit ATP formation and decrease $P/2e^-$ without decreasing $\Delta\tilde{\mu}_{H^+}$, and interpreted these results as reflecting a direct coupling between electron transport and ATP synthesis which is specifically blocked by these inhibitors. As is demonstrated in Table IV both palmitic acid and 10^{-8} M gramicidin D completely block the stimulation of ATP formation which is induced by nigericin or by methylamine. These results are consistent with the suggestion that the stimulation of ATP formation may reflect stimulation of a direct coupling between ATP synthesis and electron transport.

TABLE V

THE EFFECTS OF SUCROSE AND KCl ON ELECTRON TRANSPORT IN PS I AND IN PS II

Oxygen uptake (1, 3) or evolution (2) was measured in the presence of 100 μM methyl viologen (1), 100 μM diaminodiurene and 1 mM potassium ferricyanide (DAD + FeCN), (2), or with 30 μM dichlorophenolindophenol, 100 μM methyl viologen. 1 mM sodium ascorbate and 10 μM DCMU (DCPIP + MV + DCMU), (3). Low salt, sucrose and KCl media are as in Table II.

e^- mediators	Rate of oxygen uptake or evolution (μeq per mg Chl per h)		
	low salt	KCl	sucrose
(1) MV	520	567	281
(2) DAD + FeCN	790	632	814
(3) DCPIP + MV DCMU	585	667	325

TABLE VI

THE EFFECTS OF NIGERICIN AND NH_4^+ ON ATP FORMATION IN THE PRESENCE OF DIFFERENT ELECTRON-TRANSPORT MEDIATORS

ATP formation was measured in the presence of 100 μM methyl viologen (1) or 20 μM lettuce ferredoxin + 200 μM NADP (2) or 200 μM diaminodiurene + 1 mM ferrycyanide (3) or 20 μM ferredoxin + 200 μM NADPH + 3 mM glucose-6-phosphate + 5 units/ml glucose 6 phosphate dehydrogenase + 10 μM DCMU (4) or 30 μM pyocyanine (5). Sucrose (200 mM), nigericin (10^{-8} M) or NH_4Cl (0.5 mM) were added where indicated.

e^- mediator	Rate of ATP formation (μmol per mg Chl per h)	(% of control)		
		+ sucrose	sucrose + nigericin	sucrose + NH_4^+
(1) MV	238	71	132	133
(2) Fd + NADP	221	59	129	155
(3) DAD + FeCN	280	86	83	65
(4) Fd + NADPH + DCMU	19	88	100	103
(5) Pyocyanine	634	85	102	102

Attempts to localize the section of the electron-transport chain which is affected by sucrose, NH_4^+ and nigericin

Since the stimulation of photophosphorylation by NH_4^+ and nigericin as well as the inhibition by sucrose seem to be primarily effects on electron transport, it seemed of interest to identify which section of electron-transport system is being affected. Analysis of the effect of sucrose on electron transport through Photosystem II ($H_2O \rightarrow$ diaminodiurene $\rightarrow$ ferricyanide), Photosystem I (ascorbate $\rightarrow$ dichlorphenolindophenol $\rightarrow$ methyl viologen) and through both photosystems ($H_2O \rightarrow$ methyl viologen) shows that sucrose inhibits electron transport through Photosystem I to the same extent observed for the overall e^- transport chain, whereas e^- transport through Photosystem II is not inhibited (Table V).

Analysis of the effect of sucrose and of NH_4^+ and nigericin on ATP formation driven by e^- transport through Photosystem I, Photosystem II or both is summarized in Table VI. ATP formation driven by Photosystem II is slightly inhibited by sucrose and further inhibited by nigericin. Conversely, NH_4^+ or nigericin seem to reverse the sucrose inhibition of ATP formation in the presence of cyclic electron transport in Photosystem I. However, the full extent of sucrose-inhibition or NH_4^+ (or nigericin) -induced stimulation is obtained only when ATP formation is driven by linear electron transport through both Photosystem II and Photosystem I. It seems, therefore, that the effects of sucrose, NH_4^+ and nigericin on ATP formation are partly due to a specific effect on Photosystem I and partly to integration of the activity of both photosystems.

Discussion

The anomalous stimulation of ATP formation by the uncouplers nigericin and amines, which is accompanied by a slight decrease in $\Delta\tilde{\mu}_{H^+}$, was previously suggested to result from $\Delta\psi$-induced activation of the ATP synthase [11], a switch for kinetic to energetic control of ATP formation [10,13] or an inhomogenous proton electrochemical potential distribution in the thylakoid lumen [11]. Our results are consistent only with the latter mechanism, and suggest that the stimulation of ATP formation may be a consequence of an enhanced direct transfer of energy, possibly in the form of localized protons, in intrathylakoid membrane domains between electron transport and the ATP synthase complexes. Such a mechanism is supported by the following observations, (a) the phenomenon is particularly evident in the presence of sucrose and inhibited by high salt concentrations. These observations are consistent with recent results of Dilley and coworkers that localized proton coupling phenomena in thylakoids are observed only in chloroplasts which have been exposed to sucrose and not to KCl media [19]. (b) The stimulated ATP formation is blocked by palmitic acid and by gramicidin D, which uncouple photophosphorylation without affecting $\Delta\tilde{\mu}_{H^+}$, presumably by blocking direct proton transfer between electron transport and the ATP synthase complexes. (c) There seems to be a specificity for the source of protons to obtain these phenomena, namely, PS I + PS II > PS I > PS II. These results point to the coupling site in PS I as the major source of energy responsible for the stimulation of ATP formation.

A kinetic mechanism, namely specific activation of CF_0-CF_1, which has been suggested before to explain this stimulation [13] seems inconsistent with the stimulation of electron transport and the slight decrease in ATP/$2e^-$. Also, kinetic activation of CF_0-CF_1 via either one of the two mechanisms which have been described seems highly unlikely – thiol modulation via the thioredoxin system would be completely inhibited by methyl viologen [28] while $\Delta\tilde{\mu}_{H^+}$ activation cannot contribute, since it is decreased, if anything, under these conditions.

A note should be made concerning the calculation of ΔpH from 9-aminoacridine fluorescence changes. In the original formulation [24] it has been assumed that 9-aminoacridine accumulates in the internal osmotic space of illuminated thylakoids similar to other amines in response to internal acidification and that its fluorescence is completely quenched inside thylakoids. ΔpH has been calculated according to the equation

$$\Delta pH = -\log \frac{Q}{1-Q} \frac{V}{V_i}$$

(where Q is the fractional fluorescence quenching and V/V_i the volume ratio outside and inside the thylakoids [24]. This conclusion has been criticized by several groups who found for measurements with different acridines deviations from theoretical expectations such as overestimated ΔpH values in comparison to other techniques [29–31], little dependence on the internal osmotic volume [32], dependence of fluorescence quenching on surface charge [31–33] and partial fluorescence quenching with impermeable acridine derivatives [34].

A modified, more realistic mechanism which we favour to interpret the the acridine fluorescence quenching in illuminated thylakoids, assumes that the majority (approx. 90%) of the internalized acridine molecules form dimers or higher aggregated forms inside thylakoids that this process is facilitated by interaction with fixed negative changes in the inner thylakoid space and is in equilibrium with the internal pH. The internal capacity factor in the equation, according to such a model, will not be determined solely by the internal volume, but mainly by internal binding sites for acridine. Such a model is consistent with experimental results of others [32] and is supported also by our observations of cross inhibitions in fluorescence quenching between different probes indicative of competition for internal binding sites in illuminated thylakoids (Pick U., unpublished observations). We have therefore modified the equation for calculation of ΔpH from acridine fluorescence quenching by replacing the volume ratio factor by an empirical factor, derived from comparisons with ΔpH measurements obtained from two other methods [35,36]. This factor which is proportional to chlorophyll concentration ($F = 125C$, C in μg Chl/ml) was found to yield ΔpH values which are consistent with other techniques under a wide range of ΔpH, osmolarity and ionic strength conditions.

A question that arises is: what is common in the uncoupling mechanisms of nigericin and amines which brings about this phenomenon? Although the uncoupling mechanisms of nigericin and of NH_4^+ seem a priori very different, the end result of the action of these uncouplers is quite similar. Nigericin enhances in illuminated thylakoids an electroneutral H^+/K^+ exchange which initially results in transformation of ΔpH into Δψ and a net accumulation of $KCl \cdot NH_4Cl$, which passive diffuses into thylakoids as NH_3, acts as an internal pH buffer, decreasing ΔpH while increasing the overall uptake of H^+ and of positive charges and, therefore, also initially transforms ΔpH into Δψ and results in net accumulation of NH_4Cl. In both cases, therefore, the effect of suboptimal uncoupler concentrations is expected to result in a decreased ΔpH, increased Δψ and in net salt uptake. It is not surprising, therefore, that the effects of nigericin and ammonium on ATP formation, e^- transport, ΔpH and Δψ are almost identical.

The observation that nigericin and NH_4^+ directly stimulate coupled e^- transport, has not been observed before probably due to the large stimulation of e^- transport due to uncoupling (energetic effect) which masks the direct effect on coupled e^- transport. Although the mechanism of stimulation of e^- transport is not clear it seems to be due mainly to the accumulation of salt and the resulting increase in the intrathylakoid ionic strength. This is supported by the Cl^- requirement for these phenomenon (Table I) and by the absence of stimulation in a glycine medium (Table II). The increase in intrathylakoid osmolarity, and consequent swelling, seems to be of minor importance, since the effects of different osmotic elements (sugars, monovalent or zwitterionic salts) are consistent with an osmotic mechanism.

The results which are presented here suggest that there is a close linkage between the inhibitory effect of sugars and the stimulation induced by nigericin and amines due to salt accumulation. Both effects are associated with minor changes in $ATP/2e^-$ ratio and show similar specificity for the electron-transport partial reactions. Another interesting consequence of our results is the sidedness of the thylakoid membrane with respect to the salt and polyol effects. The observation that maximal stimulation of ATP formation and e^- transport is obtained by nigericin and NH_4^+ in the presence of low salt suggests that the optimal condition for e^- transport are high internal and low external salt. Conversely, the effect of sugar seems to be primarily on the outer side of the membranes, since glycerol, which permeates the chloroplast membrane (Pick, U., unpublished re-

sults), has the same inhibitory effect as sucrose and dextran. Therefore it seems that salt and polyols differentially affect the inner and outer faces of the thylakoid membrane.

According to this view the stimulation of ATP formation by nigericin and amines results from: (1) enhancement of electron transport by the increased salt accumulation in thylakoids, which is induced by nigericin and amines; (2) a shift from more delocalized to more localized H^+ coupling in the presence of sugar – low salt outside, and high salt inside the thylakoids.

The mechanism by which sugars and salt accumulation modify the electron transport and coupling properties in thylakoid membranes are still obscure. However, it is conceivable that differential changes in the ionic strength between the inside and outside of thylakoid membranes, and the presence of sugar could modify specific interactions between protein complexes in the thylakoid membrane by strengthening of loosening ionic and hydrogen bonds. As a consequence, electron transport might be affected by modified interactions of extrinsic components of the system (plastocyanin, ferredoxin) with protein complexes or due to changes in the stacking or in the spill-over characteristics. Similarly, direct interactions between the ATP synthase and components of the electron-transport complexes, which could affect the efficiency of localized proton coupling, may be grossly influenced.

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