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Nigericin and hexylamine effects on localized proton gradients in thylakoids

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Low concentrations of nigericin and amines such as hexylamine have been suggested to convert chloroplast thylakoid membrane localized proton gradients to a delocalized mode by transferring H^+ ions into the lumen (De Kouchkovsky, Y. et al. (1986) VII Int. Congr. Photosynth. III, 169). Experiments to test that hypothesis have been done using two assay systems that provide robust indicators of a shift from localized to delocalized energy coupling. Thylakoids stored in low salt medium normally show localized coupling (lumen pH remains considerably more alkaline than the pK_a of pyridine (5.44)) in both assays and storage in high salt medium yields a delocalized coupling pattern (lumen pH drops to values close to the pK_a of pyridine). Therefore, applying those assays to low-salt-stored thylakoids, with and without nigericin or hexylamine, provided the test for whether those compounds caused delocalization of the H^+ gradient. The assays were: (A) the effect of pyridine (a membrane-permeable buffer) on the ATP formation onset lag in flashing light; (B) permeable buffer (pyridine or hydroxyethylmorpholine) effects on H^+ uptake into the lumen in steady illumination under coupled compared to basal conditions. By both criteria, it was found that 1–3 nM nigericin caused much more acidification of the lumen compared to the control (absence of nigericin) in low-salt-stored thylakoids. The apparent delocalization of domain H^+ gradients into the lumen occurred at nigericin concentrations noted by earlier work to cause stimulation of both electron transport and ATP formation (Giersch, G. (1983) Biochim. Biophys. Acta 725, 309–319). In contrast, hexylamine at 50–100 μM did not cause acidification of the lumen, although it clearly had perturbing effects on the membrane domain metastable H^+ pool. At hexylamine concentrations less than 100 μM there may be transfer of protonated hexylamine into the lumen, although we could not detect it. The high pK_a (10.2) of hexylamine would keep the amine in the protonated form and not contribute to lumen acidification.

Introduction

Uncouplers have been important tools for studying membrane bioenergetic mechanisms, and their action is generally compatible with the chemiosmotic view of energy coupling [1], although notable exceptions have been reported [2–5]. One example of 'anomalous' uncoupler behavior is the effect of low concentrations of nigericin and some amines which cause a stimulation of electron transport and ATP formation [4,6]. Pick and Weiss [4] suggested that this anomalous uncoupling is brought about through effects on salt concentrations within the thylakoid membrane which result in enhanced proton flow to the CF_0-CF_1 complex via a localized energy coupling mechanism. It seems clear from other recent work that changes in the ionic environment of thylakoids can reversibly shift the energy coupling proton gradient from localized to delocalized coupling gradients [7–11], thus any effects of uncouplers on ion concentrations (other than H^+) in membranes could have effects on the expression of localized or delocalized energy coupling.

It must be stressed that, while the concept of membrane-localized proton gradient energy coupling is use-

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenyl hydrazone; Epps, *N*-(2-hydroxyethyl)piperazine-*N'*-3-propanesulfonic acid; HA, hexylamine; HEM, hydroxyethylmorpholine; Hepes, *N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid; HS, thylakoids washed then stored under high KCl conditions; LS, thylakoids washed and stored under low KCl conditions; Mes, 2-*N*-morpholinoethanesulfonic acid.

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ful (and we believe necessary) for interpreting certain results [12,13], virtually nothing is known about the mechanisms for formation and utilization of localized proton gradients. Some workers suggest that protons are in some way constrained at the membrane/bulk phase interface in a localized space defined by kinetic constraints [14–17]. Another view is that thylakoids have sequestered, localized domains in which protons can diffuse (in a space physically separated from the lumen by a barrier, cf. Refs. 12 and 18 for speculations on this point) from the redox sources to the CF_0-CF_1 sinks and/or exchange between mobile complexes via localized interactions [19]. Obviously, much remains to be done before we understand the membrane–proton interactions that are part of the energy coupling process. In this context, uncoupling agents are one of the experimental tools around which to design tests of various hypotheses.

We report here the testing of a prediction suggested by de Kouchkovsky et al. [15,20] that low concentrations of either amines such as hexylamine or the ion exchange uncoupler nigericin can delocalize protons from membrane-associated domains into the lumen. This is an important issue for two reasons: (A) such an action of amines or nigericin would reveal interesting properties of the thylakoid energy coupling and membrane structure/function relationships and (B) because hexylamine (at low levels, near 25 μM) distribution has been used for ΔpH determinations [21]. If such a delocalizing effect occurred at the low hexylamine concentrations used for ΔpH determinations, it would obscure the possibility of detecting anything other than delocalized proton gradients. Using permeable buffers in two types of assay for measuring localized or delocalized energy coupling effects, we have a straightforward way to determine whether hexylamine or nigericin delocalizes membrane phase proton gradients into the lumen. One assay involves the effect of a non-uncoupling permeable buffer (pyridine, for example) on the length of the ATP formation onset lag [22]. When that parameter is unaffected by permeable buffers it is taken as an indication of localized domain energy coupling [8,9,22] and when clear effects of a buffer are recorded as increasing the length of the ATP onset lag, a delocalized energy coupling mode is indicated [8,9,13]. The second criterion is the magnitude of the H^+ uptake and the effect of a permeable buffer on the extent of the H^+ uptake [23]. Protons accumulating in the lumen to an extent needed to energize ATP formation (pH 8 external) protonate a lumenally-located buffer having an appropriate pK_a and this gives rise to additional H^+ uptake. Previous work, including the accompanying paper [24] and [25], has correlated localized or delocalized energy coupling modes with the small or large effects, respectively, of permeable buffers on the extent of H^+ uptake.

Materials and Methods

Thylakoid preparation

Thylakoids were prepared from growth chamber spinach and chlorophyll content determined as described in Ref. 8. Thylakoids stored in 200 mM sorbitol, 3 mM $MgCl_2$, and 5 mM Hepes-KOH (pH 7.5), referred to as low-salt-stored thylakoids, expressed localized energy coupling characteristics by the criteria of permeable buffer (pyridine) or hydroxyethylmorpholine effects on the energization onset lag of ATP formation and on postillumination phosphorylation [8,9,24]. Thylakoids stored with 100 mM KCl replacing the sorbitol, referred to as high-salt-stored thylakoids, expressed delocalized energy coupling in a subsequent ATP formation assay.

ATP formation

Flash-induced. Photophosphorylation energized by a train of single-turn over flashes (5 Hz) was measured by the luciferin/luciferase luminescence assay as previously described [8,9]. Unless otherwise noted, the reaction medium (at 10°C) was 10 mM sorbitol, 50 mM Tricine-KOH (pH 8.0), 3 mM $MgCl_2$, 1 mM phosphate, 5 mM dithiothreitol, 0.1 mM methylviologen, 5 μM diadenosine pentaphosphate (to inhibit adenylate kinase), 0.1 mM ADP (purified to remove contaminating ATP using Dowex AG-1, cf. Ref. 8), 400 nM valinomycin, 10 μl of the LKB luciferin-luciferase assay kit prepared as specified in Ref. 8 and $\pm$5 mM pyridine as indicated.

Continuous illumination ATP formation. Steady state phosphorylation rates were determined using the above luciferin-luciferase method as described in detail in the accompanying manuscript [24].

H^+ uptake

The H^+ electrode method was used with an Orion combination pH electrode and a Corning Model 12 pH meter. H^+ uptake under coupling conditions was measured in a medium consisting of 100 mM sorbitol, 5 mM $MgCl_2$, 5 mM K_2HPO_4, 0.1 mM ADP, 0.1 mM methylviologen, hexokinase at 15 units ml^{-1}, 10 mM glucose (see below), and thylakoids equivalent to 40 μg Chl ml^{-1}. Illumination was with saturating intensity of $CuSO_4$ filtered white light (Fig. 6) or for Fig. 3, red (Corning 2-64) light at saturation or at reduced intensity (240 mW cm^{-2}, close to that given to the thylakoids in the phosphorylation assay using luciferin-luciferase). Temperature was 10°C. Hexokinase and glucose were included as an ADP-regenerating system, permitting the recording of pH changes caused by the redox-driven H^+ accumulation without interference from pH increases that accompany net ATP formation.

H^+ efflux from membrane domains

Protons held in membrane domains can be measured by observing uncoupler-dependent H^+ efflux into an alkaline medium (pH ≈ 8.4) in the dark. Previous work [18,26,27] has shown that the protocol used allows the lumen pH to come very close to equilibration with the external phase pH in times less than 2 min and the uncoupler-dependent H^+ efflux can be taken as coming from metastable, localized, membrane domains. For measurement of dark proton efflux the thylakoids were prepared as described above except that for the storage medium Mes buffer at pH 6.5 was substituted for Hepes at pH 7.5, thus avoiding lumenal proton contribution to the observed H^+ efflux by the residual Hepes buffers [27]. Proton efflux was determined with a pH electrode as previously described [28] in a medium of 0.3 mM Epps (pH 8.4), 100 mM sorbitol, 3 mM $MgCl_2$ and 20 mM KCl.

Results

Nigericin and hexylamine effect on ATP energization lags

The hypothesis put forward by De Kouchkovsky's group [15], that nigericin and amines cause a shift from localized to bulk-phase proton gradient energy coupling, can be tested quite easily with the ATP formation onset lag assay we routinely use [8,9]. When ATP formation is initiated by single-turnover flashes, delocalized proton gradient coupling is indicated when 5 mM pyridine present in the assay causes a 10–15 flash lag increase for ATP formation energization [8,9]. Such delocalized coupling is routinely observed in thylakoids stored in 100 mM KCl-containing medium or in low-salt-stored thylakoids given membrane permeable Ca^{2+} chelators [10]. Thylakoids stored in a low-salt medium (200 mM sorbitol or sucrose in place of the KCl) show little or no effect of 5 mM pyridine on either of the phosphorylation parameters, and that has been interpreted as owing to a localized proton gradient coupling mode [8,9]. Regardless of the storage conditions, the ATP formation assay was always in a single, defined assay buffer with only a slight carry-over of the storage buffer (the dilution of the stock chlorophyll (more than 2 mg ml^{-1}) was 100-fold or more, giving K^+ carryover of 1 mM or less final concentration).

If low concentrations of hexylamine or nigericin cause the putative localized proton gradient to equilibrate rapidly with the lumen (rapidly here implies fast enough to acidify the lumen to pH 5.7 or below in the lag period before the onset of ATP formation), then a pyridine-induced increase in the onset lag flash number should occur with either uncoupler present in the ATP formation assay using low-salt-stored thylakoid samples. In this experiment, thylakoids were prepared in the usual way by suspending the stock membranes in either a low-salt or high-salt storage buffer, then after diluting into the assay buffer (see above), and testing the effect of nigericin and hexylamine on the ATP formation onset lag in the absence or presence of 5 mM pyridine.

Low concentrations of nigericin (far below the level needed for uncoupling), caused a pronounced pyridine-dependent increase in the ATP formation onset lag with the low-salt-stored thylakoids (Table I). With as little as 3.5 nM nigericin there was a significant, pyridine-dependent increase in the onset lag (≈ 10/11 flashes), and with 7.0 nM nigericin the pyridine effect increased to 18/23 flashes. High-salt-stored thylakoids in the presence of 5 mM pyridine gave the usual 13/18 flash lag increase, but 3.5 nM nigericin did not change the pyridine effect significantly (Table I). In both the low and high-salt-stored thylakoid preparations 3.5 nM nigericin caused a measurable decrease (38% and 26% inhibition, respectively) in the ATP yield per flash, but in a steady illumination assay only 15% inhibition occurred (data not shown). This is a reasonable result, inasmuch as the 200 ms dark times between the 10 μs flashes gives time for the low nigericin concentration to dissipate some of the protons produced by the flash-driven redox turnover. A significant amount of the ATP produced after each flash is expected to occur during the dark interval (200 ms in this case), as clearly shown by Horner and Moudrianakis [11].

In the absence of pyridine, nigericin addition caused a 5- to 10-flash increase in the ATP onset lag, for both low- and high-salt-stored membranes, as expected for any uncoupler which can dissipate the metastable acidic domain in the dark [26,29,30]. Nigericin by itself should dissipate the metastable H^+ pool and thus increase the onset lag for both the high-salt-stored case as well as the low-salt-stored case, because previous results showed that in the absence of the uncoupler the metastable domain H^+ pool remains acidic in the dark for both thylakoid storage treatments [28]. It is only after excitation flashes begin that, in the high-salt case, the H^+ gradient readily equilibrates with the lumen [28]. High salt-stored thylakoids with or without 3.5 nM nigericin gave the usual pyridine-induced 10–15 flash lag increase [8,9], indicating that the expected delocalized gradient coupling response was present.

However, concentrations of hexylamine up to 100 μM gave a different result with regard to the ATP onset lag number and pyridine effects. For the low-salt-stored thylakoids 5 mM pyridine resulted in virtually no increase in the flash lag number, whether or not 100 μM or less hexylamine was present (Table II). Such results are typical of low-salt-stored thylakoids [8,9], and are consistent with a localized energy coupling mode being maintained in both the control and the plus-hexylamine cases. High salt-stored membranes, on the other hand, showed the usual response

TABLE I

Effects of low concentration of nigericin on thylakoid ATP formation onset lags

See Materials and Methods for details of thylakoid storage and assay conditions for the luciferin-luciferase ATP formation assay. The ATP formation onset lag was measured as in Ref. 8 by two parameters (e.g., 32/37 on line 1, where the first detectable rise in the luminescence signal is 32 flashes, and the extrapolated steady rise in the luminescence signal back to the *X*-axis is 37 flashes). The ΔLag column represents the effect of pyridine on the ATP formation onset lag parameters. Thylakoids were incubated in the 10°C reaction medium ± nigericin and ± pyridine for 3 min prior to starting the 5 Hz flash train.

Thylakoid storage	Pyridine (mM)	Nigericin (nM)	ATP formation onset lag	ΔLag +Pyr/(−Pyr)	ATP formation yield per flash (nmol ATP(mg Chl)$^{-1}$)
Low salt	–	–	32/37 [a]	–	0.58 [b]
	5	–	34/40	2/3	0.54
	–	3.5	37/43	5/6 [c]	0.36
	5	3.5	47/54	10/11	0.32
	–	7.0	42/48	10/11 [c]	0.34
	5	7.0	60/71	18/23	0.20
High salt	–	–	40/48	–	0.46
	5	–	53/66	13/18	0.41
	–	3.5	49/55	9/7 [d]	0.34
	5	3.5	64/76	15/21	0.25

[a] Standard error of the mean for the flash lags was generally ±1 or 2 flashes, occasionally ±3 flashes.
[b] Standard error of the mean for ATP formation yield per flash was generally less the ±0.07 nmol ATP (mg Chl)$^{-1}$.
[c] Compare to the low-salt control; i.e., this is the nigericin effect.
[d] Compare to the high-salt control; i.e., the nigericin effect.

to 5 mM pyridine without or with 50 μM hexylamine; i.e., a 10–15 flash increase in the ATP formation onset lag, indicating delocalized energy coupling [8,9].

Methylamine, a commonly used ΔpH probe, was also tested for its effects on the ATP formation onset lag. Results similar to those reported here for hexylamine were obtained for methylamine at 30 and 100 μM (data not shown).

For both low- and high-salt-stored thylakoids, it must be noted that 50 μM hexylamine caused a significant increase in the ATP formation onset flash number, even though pyridine had no further effect on the onset lag in the low-salt-stored thylakoids (Table II). Simple uncoupling is not the likely explanation because up to 50 μM hexylamine did not cause a decrease in the ATP yield per flash (Table II), a parameter quite sensitive to uncoupler action in single-turnover flash-driven ATP formation (owing to the 200 ms dark time between flashes). This hexylamine effect is reminiscent of 'reversible uncoupler' treatments shown earlier to cause a 10–12 flash increase in the ATP onset lag [29,30]. This similarity was studied further and the results are reported below, after considering nigericin and hexylamine effects on H^+ uptake into the lumen

TABLE II

Comparison of hexylamine effects on ATP formation onset lag number of low- and high-salt-stored thylakoids

Conditions were as in Table I. Standard error of the means for the ATP onset lag and ATP yield per flash were similar to those given in Table I.

Thylakoid Storage	Pyridine (mM)	Hexylamine (μM)	ATP formation onset lag	ΔLag (+Pyr/(−Pyr))	ATP formation yield per flash (nmol (mg Chl)$^{-1}$)
Low Salts	–	–	28/34	–	0.43
	5	–	28/38	0/4	0.38
	–	25	30/42	–	0.38
	5	25	33/46	3/4	0.34
	–	50	39/49	–	0.41
	5	50	41/53	2/3	0.40
	–	100	48/63	–	0.35
	5	100	50/66	2/3	0.32
High Salt	–	–	45/54	–	0.30
	5	–	59/77	14/23	0.28
	–	50	73/99	–	0.24
	5	50	87/118	14/19	0.18

compared to H^+ accumulation into membrane domain buffering domains.

Effects of nigericin and hexylamine on H^+ uptake

As described in the accompanying paper [24], the effect of permeable buffers such as pyridine or hydroxyethylmorpholine on the extent of H^+ uptake is a criterion for discriminating between H^+ accumulation mainly in membrane-localized domains from H^+ uptake which equilibrates with the lumen. Low salt-stored thylakoids under coupling conditions can accumulate up to 200 nmol H^+ (mg Chl)$^{-1}$ with only a slight stimulation of H^+ uptake in the presence of hydroxyethylmorpholine (pK_a 6.2) or pyridine (pK_a 5.4) [24]. That implies that the H^+ ions in those conditions are accumulated mainly in localized domains (to at least a pH $\leq$ 5.7, sufficient to energize ATP formation [8,9]), the logic being that if the lumen had reached a pH near 6, particularly hydroxyethylmorpholine but also pyridine would significantly buffer the lumen, leading to additional H^+ uptake.

Nigericin (1 nM) added to the H^+ uptake assay medium using low-salt-stored thylakoids under coupling conditions with and without pyridine gave a large (nearly 2-fold) stimulation of H^+ uptake as shown in Fig. 1, right-hand side. In the absence of nigericin, the thylakoids did not give a pyridine-dependent increase in H^+ uptake, in agreement with the previous results with thylakoids in a localized energy coupling mode (low-salt-stored) [24]. Fig. 1 also shows, for comparison, the effect of valinomycin and nigericin separately and together. Even in the absence of valinomycin, nigericin gave a significant pyridine-dependent stimulation of H^+ uptake. This experiment supports the notion that nigericin at these very low concentrations transfers H^+ ions into the lumen where the pyridine buffer interacts with them, leading to greater H^+ uptake. Based on our previous results and the localized domain $\Delta\tilde{\mu}_{H^+}$ hypothesis [9,18,24], we suggest that the additional pyridine uptake owing to nigericin is driven by H^+ ions exchanged by the antibiotic into the lumen from localized domains. Hexylamine at up to 50 μM in the same type of H^+ uptake assay with low-salt-stored thylakoids did not induce a greater H^+ accumulation with either 5 mM pyridine or 0.5 mM hydroxyethylmorpholine (data not shown). This result argues against the hexylamine acting to transfer significant amounts of H^+ ions from the putative membrane domain H^+ gradient into the lumen.

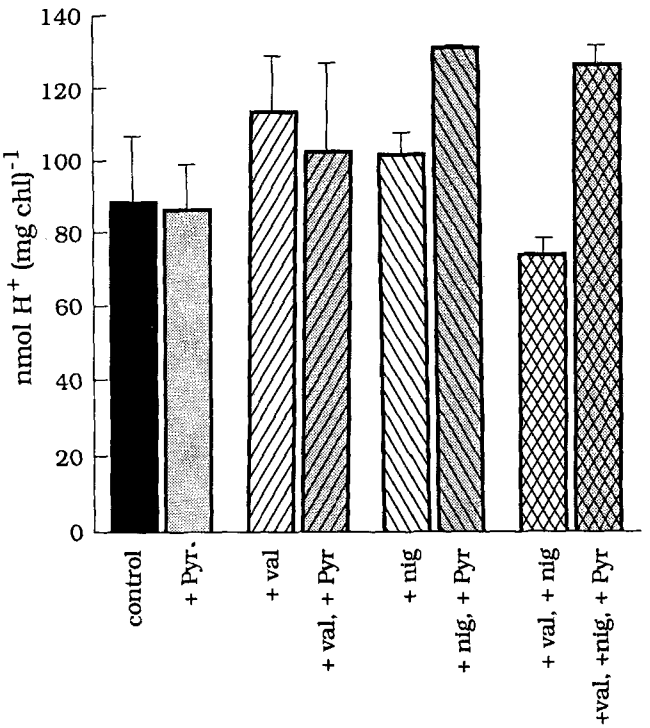

Fig. 1. Effect of nigericin ± pyridine on H^+ uptake in low-salt-stored thylakoids under coupled conditions. Conditions for measuring H^+ uptake under coupled conditions were as stated in Materials and Methods. Concentrations of additional reagents, added as indicated, were: valinomycin, 200 nM; nigericin, 1 nM; and pyridine, 5 mM.

Effects of hexylamine on the metastable H^+ buffering domains, and the 'Reversible Uncoupler' experiment

The dark, reversible uncoupler treatment referred to above (i.e., CCCP given for 30 s under high (8.5) pH conditions, followed by BSA which binds the CCCP) causes a depletion of the metastable, localized domain proton pool resulting in an increase of about 10–15 flashes in the lag before ATP formation begins [29,30]. Refilling the domain proton pools after the CCCP and BSA depletion treatment is accomplished by 12–14 flashes, without ADP and P_i present, and results in the return to the original, shorter flash requirement [29,30]. Those results have been interpreted as consistent with the notion that the domains must be filled with protons before energization can be reached [30]. That interpretation was strengthened by the finding that high-salt-stored thylakoids, which we suggest have the delocalized energy coupling mode operating, did not show any lag increase after the CCCP, BSA treatment [30]. We tested whether hexylamine depletes the membrane domain H^+ pool in two related ways: (A) by measuring (high concentration) uncoupler-induced H^+ efflux into pH 8.4 media without or with prior low hexylamine treatment; and (B) comparing hexylamine effects to the reversible CCCP uncoupling effects on the ATP formation onset lag.

In an experiment of type 'A', 5 μM nigericin added to low-salt-stored thylakoid suspensions in the dark at pH 8.4 causes the efflux of about 20–40 nmol H^+ (mg Chl)$^{-1}$ from membrane-localized buffering domains [27,28]. With the thylakoids used in this study, addition of 5 μM nigericin gave 20–25 nmol H^+ efflux (mg Chl)$^{-1}$ in both the control samples and in samples to

which 25 μM hexylamine had been added prior to the nigericin (Fig. 2). With 50–100 μM hexylamine added prior to nigericin the nigericin-induced H^+ efflux was only 10–12 nmol (mg Chl)$^{-1}$, suggesting that adding at least 50 μM hexylamine caused the partial dissipation of the domain H^+ pools before addition of the high nigericin concentration. In those experiments the controls and the hexylamine-treated samples were given a 3 min dark incubation prior to addition of 5 μM nigericin (these high levels of nigericin induce fast equilibration of H^+ between the membrane domains and the external medium).

The 'reversible uncoupler' experiment, type 'B' referred to above, reveals the effects of domain protons on the length of the ATP formation onset lag. As found earlier [29,30], our data show that depleting the domain H^+ pools with CCCP followed by BSA addition causes the ATP onset lag to increase from 21/33 to 40/48 flashes (lines 3 and 4, Table III). A second flash series given to the CCCP, BSA treatment (line 4) resulted in the ATP formation onset lag returning to 23/34, which is consistent with the domains being refilled during the first flash cycle [29,30]. Hexylamine (25 μM) addition by itself (line 5) increased the lag by 17/27 flashes (compared to the line 1 control) and hexylamine given to the BSA/CCCP treatment (line 6) caused a similar, 16/18 flash increase, but the hexylamine effect was not reversed by the second cycles. The latter point is consistent with hexylamine not being absorbed by BSA. Hexylamine has a perturbing effect on the ATP formation onset lags consistent with the Table II data, but the effect is independent of the protophore effect of CCCP. This is shown also by comparing the data of lines 6 and 7, Table III. With 25 μM hexylamine present the CCCP effect of increasing the first cycle onset lags from 37/51 to 54/66 was reversed in a second cycle to a lag of 41/48 flashes. Those results are consistent with the notion that 25 μM hexylamine does not significantly promote localized domain H^+ ions being delocalized into the lumen.

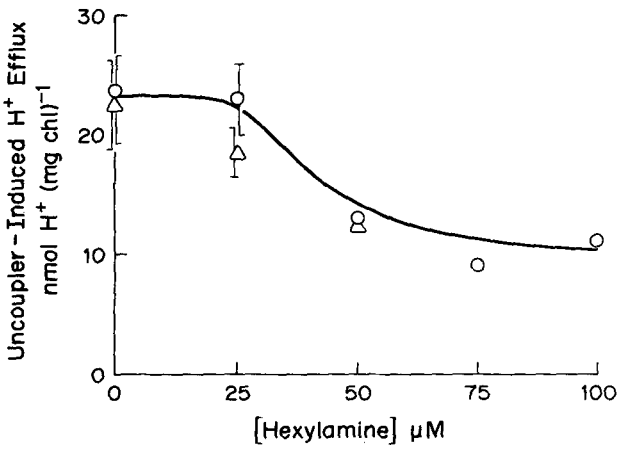

Fig. 2. Hexylamine effect on 5 μM nigericin-induced H^+ release from low-salt-stored thylakoid suspensions. Thylakoids were prepared as stated in Methods, using 5 mM Mes (pH 6.2) buffer in the storage medium in place of 5 mM Hepes. The reaction medium contained 100 mM sorbitol, 3 mM $MgCl_2$, 20 mM KCl, 20 Kg Chl ml^{-1}, 1.0 mM Tricine, 400 nM valinomycin, and variable amounts of HA in a thermostated cuvette at 10°C and carefully kept dark. After titrating the pH to 8.4 using a glass pH electrode, and after a 3 min equilibration period, aliquots of nigericin (5 μM final concentration) were added and pH changes were recorded. Open circles and triangles represent experiments done on different days. Standard HCl addition calibrated the system for calculation of the nigericin-induced H^+ efflux.

TABLE III

Comparison of hexylamine (HA) to CCCP in dissipating metastable proton pools in low salt-stored thylakoids

The 'reversible uncoupling' protocol [29,30] involved adding CCCP (i.e., to release H^+ ions from metastable localized domains) to a thylakoid suspension maintained at pH 8.4 in a phosphorylation medium as specified in the Materials and Methods. After 30 s in the dark BSA was added to 1 mg/ml to adsorb the CCCP, and 30 s later, a 5 Hz excitation flash sequence was given to energize ATP formation. BSA added before the CCCP binds the uncoupler before it reaches the thylakoids in significant levels. Standard errors of the means for ATP formation onset lag and ATP yield per flash were similar to those given in Table I.

Treatment	ATP formation onset lag		ATP yield per flash (nmol ATP (mg Chl)$^{-1}$) (1st cycle)
	1st cycle	2nd cycle	
1 Control	13/19		0.42
2 +BSA	15/20		0.39
3 +BSA, then CCCP	21/33	15/26	0.37
4 +CCCP, then BSA	40/48	23/34	0.34
5 +HA[a]	30/46	27/35	0.22
6 +BSA, then CCCP, HA	37/51	37/49	0.36
7 +CCCP, then BSA, HA	54/66	41/48	0.31

[a] HA 25 μM.

Discussion

The goal of this work was to test whether nigericin and hexylamine (at concentrations too low to result in uncoupling) cause what is believed to be (initially and in the absence of those compounds) a localized energy coupling proton gradient to equilibrate with the lumen, producing a delocalized protonmotive force. The key assumption (discussed in previous work [8,9,13]) is that low-salt-stored thylakoids maintain a localized protonmotive force during efficient energy coupling (ATP formation) conditions, whereas high-salt-stored membranes, shown to be equally efficient in ATP formation [8,9], allow equilibration of the $\Delta\tilde{\mu}_{H^+}$ with the lumen.

By two of the criteria we used to detect either localized or delocalized energy coupling, (A) perme-

able buffer (pyridine at 5 mM being a permeable but not an uncoupling amine) effects on the ATP formation onset lag driven by a 5 Hz single-turnover flash train (Table I) and (B) permeable buffer effects on continuous light-driven H^+ uptake (Fig. 1), it was clear that low levels of nigericin cause low-salt-stored thylakoids to shift from a localized to a delocalized $\Delta\tilde{\mu}_{H^+}$. It should be noted that 3.5 nM nigericin present in the flash-driven ATP formation onset experiment is having a more subtle effect than the complete $\Delta\tilde{\mu}_{H^+}$ dissipation which would occur at potent (≈ 1 μM) uncoupling concentrations. The complete inhibition of ATP formation by high nigericin concentrations occurs via complete inhibition of H^+ accumulation [31]. Some dissipation of accumulated H^+ ions to the external medium may occur at the 3 to 7 nM nigericin range used here, suggested by the decrease in the ATP yield per flash from 0.58 to near 0.36 nmol ATP per mg Chl per flash (Table I). However, the yield of 0.36 is still quite respectable, indicating a not very significant loss of $\Delta\bar{\mu}_{H^+}$. What is most significant is that the low nigericin apparently allows the H^+ ions in the putative localized domains to exchange into the lumen where the pyridine located there can interact with them. Drawing on the data and arguments of the accompanying paper [24] concerning the equivalent pyridine concentration in the lumen for either low- or high-salt-stored thylakoids, we conclude that for low-salt-stored membranes, pyridine is adequately present in the lumen under all conditions, but only when nigericin was added did the energy coupling $\Delta\bar{\mu}_{H^+}$ equilibrate with the lumen during the development of the threshold $\Delta\bar{\mu}_{H^+}$ needed to energize ATP formation. Hence, the observed pyridine-dependent extension of the ATP formation onset lag (Table I).

Consistent with that argument, nigericin also caused the total H^+ uptake extent, under coupling conditions, to be increased by pyridine in low-salt-stored thylakoids, in contrast to the lack of a pyridine effect in the control case without nigericin (Fig. 1). This seems to strongly support the hypothesis that low nigericin concentrations delocalize H^+ ions from a domain-limited $\Delta\tilde{\mu}_{H^+}$ into the lumen, thereby forming a delocalized $\Delta\bar{\mu}_{H^+}$ competent to drive ATP formation.

However, by both criteria used to assess delocalized $\Delta\tilde{\mu}_{H^+}$ hexylamine up to 100 μM *did not* delocalize the energy coupling gradient in low-salt-stored thylakoids (Table II). An argument to rationalize this lack of a delocalizing effect in contradistinction to the noticeable nigericin effects will be offered below, after some comments on the effects that hexylamine has on other parameters related to domain H^+ ions. Hexylamine has some effect on the membrane domain proton gradient because at 50 μM it dissipates the metastable acidic domain assayed by measuring the dark H^+ efflux into pH 8.4 media, induced by high nigericin concentrations (Fig. 2). It is likely that neutral hexylamine diffuses into the domains in which it would become protonated. Protonation of hexylamine in the domains could explain why fewer H^+ ions were detected on addition of nigericin to dark-equilibrated membranes (Fig. 2). Perhaps the hexylamine cations remain trapped in the domain structures, although this cannot be specified at present. Moreover, if some of the domain metastable H^+ ions were absorbed by hexylamine, that would explain the noticeable increases in the ATP onset lag caused by the amine (i.e., the charged amine would not be able to carry protons along the putative H^+ flux pathway leading to the CF_0-CF_1). More redox turnovers would then be necessary to refill the domains to replace H^+ ions trapped on the amine, therefore increasing the ATP formation onset lag [30]. Hexylamine caused a similar increase in the number of flashes required to reach the energetic threshold for ATP formation when high-salt-stored thylakoids were used (Table II), and there also the effect was independent of the presence or absence of pyridine.

The absence of a synergistic effect of hexylamine and pyridine or hydroxyethylmorpholine in the H^+ uptake assay (data not shown) and the failure of hexylamine to induce a pyridine-dependent increase in the number of flashes required to begin ATP formation (Table II) are so clearly different from the nigericin effects on those parameters that it seems justified to conclude that the hexylamine, at concentrations less than 100 μM, does not act to transfer domain protons to the lumen in sufficient quantity to significantly drive the pH into the acidic range.

The hexylamine-induced increase in the ATP formation onset lag (Table II) together with the effect of hexylamine in dissipating the metastable domain proton pool (Fig. 2) implies that the amine perturbs the domains of the low-salt-stored thylakoids, but not in a way that leads to the redox-pumped protons significantly acidifying the lumen.

The quite different effects of nigericin and hexylamine may be explained by the following model: nigericin-K^+ reaches the localized acidic domains, is converted to nigericin-H^+ by mass action and the nigericin-H^+ diffuses to the lumen to exchange the H^+ for a lumen K^+, thus effectively equilibrating the acidic, localized compartment with the lumen. The proposed shuttling of domain H^+ ions into the lumen clearly (according to the data of Fig. 1) drops the lumen pH low enough to significantly protonate pyridine (pK_a 5.44), and, following our hypothesis [9,24], sufficiently low to open the putative CF_0 gating site to allow lumen protons to flow through the CF_0-CF_1 complex and energize ATP formation. The gating site has been suggested to be a Ca^{2+} ion bound to the 8 kDa CF_0 subunit, part of the CF_0 H^+ channel [10,32]. According to this model, the pH in the lumen must reach an

acidity sufficient to protonate the (suggested) carboxyl binding sites which bind the gating Ca^{2+} ion.

Hexylamine apparently reaches the acidic domains in the neutral form, where it would be protonated, but even if it diffused into the lumen its high pK_a (≈ 10) means that at pH 8 more than 99% and at pH 6 more than 99.99% would remain protonated. For a significant acidification of the lumen to result solely from hexylamine cation diffusion into the lumen from the domains, an enormous amine uptake would be required. While hexylamine may carry H^+ ions from the domains to the lumen, acidifying the lumen to near the pK_a of hydroxyethylmorpholine ($pK_a \approx 6.2$) would not be expected if, as can be calculated, only 0.01% of the amine cation dissociates. However, most of the nigericin-H^+ entering the lumen could lose the H^+ in exchange for K^+, making a much more significant contribution to acidification compared to the acid form of hexylamine. Bizouarn et al. [36], using higher hexylamine concentrations (between 300–500 μM) have described a very interesting effect of the amine as acting to buffer otherwise rapid changes in the lumen of thylakoids upon addition of ADP to elicit ATP formation. Those effects were obtained with thylakoids stored and assayed with 50 mM KCl present, and it is expected that a delocalized $\Delta\tilde{\mu}_{H^+}$ would have been induced [8,9].

However, if some of the protonated hexylamine [37] (or other amines such as methylamine [38] used for ΔpH determinations) formed in the putative domains goes to the lumen, there could result an overestimation of the lumen acidity compared to that which would have registered in the absence of hexylamine. Thus, the lumen pH estimated with [^{14}C]methylamine for the low-salt-stored thylakoids under coupling conditions (Fig. 4 of the accompanying paper [24]) may be too acidic a value. This possibility perhaps can be resolved by employing other pH indicator probes such as the anionic carboxyfluoracein derivatives now commercially available (for their use in mitochondria see Ref. 39). This line of speculation, if correct, would imply that the suggestion of Sigalat et al. [20] concerning the possible erroneous amine probe ΔpH determination for the 'localized' case should be taken seriously. The best way to resolve this question is to use the other pH indicator probes referred to above.

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References

1 Ferguson, S. (1985) Biochim. Biophys. Acta 811, 47–95.
2 Hatefi, Y. (1975) J. Supramol. Struct. 3, 201–213.
3 Hanstein, W.G. and Hatefi, Y. (1974) J. Biol. Chem. 249, 1356–1362.
4 Pick, U. and Weiss, M. (1988) Biochim. Biophys. Acta 934, 22–31.
5 Opanasenko, V.K., Red'ko, T.P., Kuz'mina, V.P. and Yaguzhinsky, L.F. (1985) FEBS Lett. 187, 257–269.
6 Giersch, C. (1983) Biochim. Biophys. Acta 725, 309–319.
7 Sigaiat, C., Haraux, F., De Kouchkovsky, F., Hung, S.P.N. and De Kouchkovsky, Y. (1985) Biochim. Biophys. Acta 809, 403–413.
8 Beard, W.A. and Dilley, R.A. (1986) FEBS Left. 201, 57–62.
9 Beard, W.A. and Dilley, R.A. (1988) J. Bioenerg. Biomembr. 20, 129–154.
10 Chiang, G. and Dilley, R.A. (1987) Biochemistry 26, 4911–4916.
11 Horner, R. and Moudrianakis, E.N. (1983) J. Biol. Chem. 258, 11643–11647.
12 Dilley, R.A., Theg, S.M. and Beard, W.A. (1987) Annu. Rev. Plant Physiol. 38, 347–389.
13 Dilley, R.A. (1991) Curr. Topics Bioenerg., in press.
14 Haraux, F. and De Kouchkovsky, Y. (1983) Physiol. Vég. 21, 563–576.
15 De Kouchkovsky, Y., Sigalat, C. and Haraux, F. (1986) VIIth Int. Congr. Photosynthesis (Biggins, J., ed.), Vol. III, 169–172, Kluwer, Dordrecht.
16 Westerhoff, H.V., Kell, D.B., and Van Dam, K. (1988) in Microcompartmentation (Jones, D.P., ed.), CRC Press, Cleveland.
17 Kamp, F. (1990) Ph.D. thesis, University of Amsterdam.
18 Allnutt, F.C.T., Atta-Asafo-Adjei, E. and Dilley, R.A. (1989) J. Bioenerg. Biomembr. 21, 535–551.
19 Pick, U., Weiss, M. and Rottenberg, H. (1987) Biochemistry 26, 8295–8302.
20 Sigalat, C., De Kouchkovsky, Y., Haraux, F. and De Kouchkovsky, F. (1988) Biochim. Biophys. Acta 934, 375–388.
21 Portis, A.R. and McCarty, R.E. (1976) J. Biol. Chem. 251, 1610–1617.
22 Graan, T., Flores, S. and Ort, D.R. (1981) in Energy Coupling in Photosynthesis (Selman, B.R. and Selman-Reimer, S., eds.), pp. 25–34, Elsevier/North-Holland, New York.
23 Nelson, N., Nelson, H., Naim, Y. and Neumann, J. (1971) Arch. Biochem. Biophys. 145, 263–267.
24 Renganathan, M., Pan, R.S., Ewy, R., Theg, S.M., Allnutt, F.C.T. and Dilley, R.A. (1991) Biochim. Biophys. Acta 1059, 16–27.
25 Beard, W.A., Chiang, G. and Dilley, R.A. (1988) J. Bioenerg. Biomembr. 20, 107–128.
26 Baker, G.M., Bhatnagar, D. and Dilley, R.A. (1981) Biochem. 20, 2307–2315.
27 Pfister, V.R. and Homann, P.H. (1986) Arch. Biochem. Biophys. 246, 525–530.
28 Allnutt, F.C.T., Dilley, R.A. and Kelly, T. (1989) Photosyn. Res. 20, 161–172.
29 Dilley, R.A. and Schreiber, U. (1984) J. Bioenerg. Biomembr. 16, 173–193.
30 Theg, S.M., Chiang, G. and Dilley, R.A. (1988) J. Biol. Chem. 263, 673–681.
31 Shavit, N., Dilley, R.A. and San Pietro, A. (1968) Biochemistry 7, 2356–2363.
32 Dilley, R.A. and Chiang, G. (1989) Annals N.Y. Acad. Sci. 574, 246–267.
33 McCarty, R.E. and Coleman, C.H. (1970) Arch. Biochem. Biophys. 141, 198–206.
34 Davenport, J.W. and McCarty, R.E. (1986) Biochim. Biophys. Acta 851, 136–145.

35 Davenport, J.W. and McCarty, R.E. (1980) Biochim. Biophys. Acta 589, 353–357.
36 Bizouarn, T., De Kouchkovsky, Y. and Haraux, F. (1989) Biochim. Biophys. Acta 974, 104–113.
37 Gaensslen, R.E. and McCarty, R.E. (1972) Anal. Biochem. 48, 504–514.
38 Schuldiner, S., Rottenberg, H. and Avron, M. (197 Biochem. 25, 64–70.
39 Jung, D.W., Davis, M.H. and Brierley, G.P. (1989) Anal. 178, 348–354.