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NIGERICIN-INDUCED STIMULATION OF PHOTOPHOSPHORYLATION IN CHLOROPLASTS

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Amines have been shown recently to stimulate at low concentrations the steady-state rate of photophosphorylation by unbroken chloroplasts (Giersch, C. (1982) Z. Naturforsch. 37c, 242–250). In the present contribution it is demonstrated that not only amines but also the carboxylic ionophores nigericin and monensin at concentrations of 10 and 150 nM, respectively, stimulate the phosphorylation rate. The ATP/2e ratio is not decreased upon the addition of nigericin at concentrations that stimulate phosphorylation. Nigericin-induced stimulation is observed only in the presence of sufficient external potassium, indicating that the observed stimulation is unlikely to be a side-effect of the uncoupler but is related to H^+-K^+ exchange. The proton permeability of the thylakoid membrane is increased and the proton gradient decreased by amounts of nigericin that stimulate phosphorylation. The membrane potential is not affected in the steady state, indicating that the proton-motive force is slightly reduced upon addition of the ionophore. Data on the proton-motive force were related to maximum values of the phosphorylation potential, which was 45000–50000 M^{-1} in the absence and 30000–35000 M^{-1} in the presence of 10 nM nigericin. The observation that the ATP/2e ratio is not decreased in the presence of uncoupler-induced proton leakage is suggested to indicate that the thylakoid lumen does not represent a homogeneous phase of constant proton electrochemical potential. The results presented here are in agreement with the chemiosmotic concept as far as energetic aspects are concerned but seem to be at variance with the postulated free mobility of protons inside the thylakoids. A tentative model of uncoupler-induced stimulation of phosphorylation is presented.

Introduction

Illuminated chloroplasts are capable of synthesizing ATP from added ADP and P_i. The rate of photophosphorylation is generally assumed to increase with increasing energization of the thylakoid system, i.e., with increasing proton-motive force, which according to the chemiosmotic concept, is the driving force of ATP production [1]. Recently, some exceptions from this type of dependence of phosphorylation rate on the proton-motive force have been observed in this laboratory. CO_2-dependent oxygen evolution by intact chloroplasts, which is an ATP-consuming process, was stimulated upon the addition of ammonium chloride that deminished the proton-motive force [2]. Low concentrations of ammonium chloride or methylamine (less than 1 mM) lead to increased phosphorylation of exogenous ADP by intact chloroplasts, the envelope of which had been made permeable for adenylates and P_i by osmotic stress [3,4]. Again, the proton motive force was lowered upon addi-

Abbreviations: $CF_{1(0)}$, chloroplast coupling factor 1(0); Chl, chlorophyll; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; Hepes, 4-(2-Hydroxyethyl)-1-piperazineethanesulphonic acid; $\Delta G'_{ATP}$, phosphoryl group transfer potential; ΔpH, pH gradient across the thylakoid membrane; $\Delta\psi$, electrical potential difference across the thylakoid membrane.

tion of the uncoupler *. A number of related observations can be found in the literature [5–10]. In all cases phosphorylation was observed to be stimulated under conditions where the proton-motive force was expected to be decreased. However, most of these authors did not comment on this particular finding, and interpretations were offered only in two reports [6,9]. However, these interpretations either do not apply to chloroplasts [6] or are of restricted value [9] when compared to other findings [11] of these authors. Apparently, uncoupler-induced stimulation of phosphorylation, though repeatedly observed, has never been studied systematically. Understanding of this effect is not only interesting per se, but will also help to clarify molecular aspects of ATP production in chloroplasts. In this contribution it is demonstrated that in addition to amines also the carboxylic ionophores nigericin and monensin cause enhancement of photophosphorylation when used at proper concentrations.

Material and Methods

Intact chloroplasts were isolated from spinach leaves of greenhouse plants according to a modification [12] of the procedure of Jensen and Bassham [13]. Bovine serum albumin (0.3%, w/v) was added to the grinding medium instead of ascorbate. Chlorophyll was determined according to the method of Arnon [14]. If not indicated otherwise, intact chloroplasts (33 μg/ml) were suspended in an assay medium containing 0.1 M sorbitol, 5 mM $MgCl_2$, 10 mM KCl, 10 mM NaCl, 1 mM KH_2PO_4, and 10 or 40 mM Hepes, pH adjusted to 8.0 with NaOH. The low osmolarity of this assay medium causes swelling of chloroplasts, the envelope of which becomes permeable to small molecules like the adenylates or P_i by this treatment. These chloroplasts are referred to as unbroken (type 'B' in the nomenclature of Reeves and Hall [15]). The electron acceptor was 25 μM methyl viologen. Actinic light was provided by passing a beam of white light through a 2 mm RG 630 glass filter (Schott) and a heat-reflecting interference filter (Calflex C, Balzer). Oxygen evolution and 9-aminoacridine fluorescence were measured as described in Ref. 4, absorption changes of chloroplast suspensions as described in Ref. 3. Phosphorylation rates were determined by incorporation of $^{32}P_i$ into ATP [3] or by following the rate of alkalinization of a weakly buffered (10 mM Hepes) chloroplast suspension [16]. Buffering capacity of the assay medium was determined by addition of known amounts of HCl. To estimate phosphorylation potentials, limiting amounts of [^{14}C]ADP ([8-^{14}C]ADP, ammonium salt, 2.07 GBq/mmol) were added to illuminated chloroplasts. Before use, NH_4^+ present in the [^{14}C]ADP solution was exchanged for Na^+ on a small cation-exchange column (Dowex 50 WX2, 100–200 mesh). Phosphorylation was stopped by injecting aliquots of the sample into $HClO_4$, 0.8 M final concentration. The samples were immediately neutralized and analyzed by high-performance liquid chromatography. Conditions of chromatography were: 30 cm $\times$ 4.6 mm Partisil SAX 10 (strong basic anion exchanger, Whatman), elution medium of 0.7 M KH_2PO_4, pH(H_3PO_4) 3.0, flow rate 0.75 ml/min. Generally, three peaks were found in the chromatogram, corresponding to AMP, ADP and ATP, respectively. Label recovered in the peaks was quantified by means of an on-line β-monitor and a multi-channel analyzer as described in Ref. 17. The ATP/ADP ratio was calculated from the net counts of the corresponding peaks.

Results

Electron transport and photophosphorylation

The carboxylic ionophore nigericin catalyzes an electroneutral H^+-K^+ exchange [18]. In chloroplasts, this exchange abolishes the pH gradient across the thylakoid membrane which is thought to be the main driving force of photophosphorylation [1]. A decrease in the rate of ATP production and an increase in electron transport upon the addition of this uncoupler are therefore expected; this is indeed observed at higher nigericin concentrations (Fig. 1). The rate of phosphorylation is decreased to 8% and that of electron transport increased to 148% compared to the control by addition of 0.5 μM nigericin; concentrations ex-

* The term 'uncoupler' is used also under conditions where compounds like nigericin or methylamine do not uncouple ATP production from electron transport but increase the rate of phosphorylation without impairing the ATP/2e ratio.

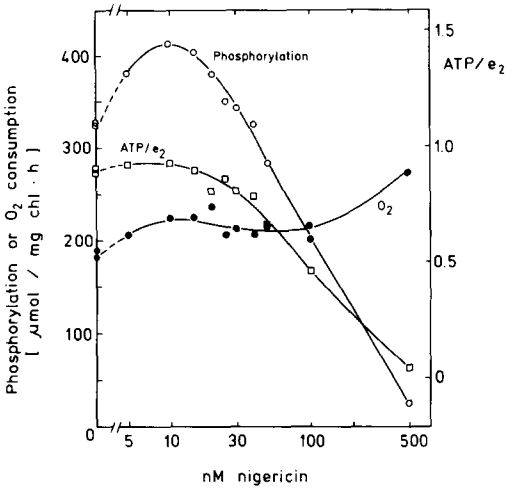

Fig. 1. Dependence of the rate of phosphorylation, electron transport and the ATP/2e ratio on the nigericin concentration. Phosphorylation was measured by the pH method [16]. Intact chloroplasts corresponding to 66 µg Chl were suspended in 2 ml assay medium containing 10 mM Hepes; 1 mM ADP, 1 mM NaCN, 25 µM methyl viologen and nigericin at the indicated concentrations were added. The pH of the assay was brought to 8.05 ± 0.02 by addition of KOH. The buffering capacity of the suspension was determined by adding 0.3 µval H^+ in the dark after illumination by 200 W/m^2 red light for 2 min. Phosphorylation rates and rates of electron transport were determined from the slopes of the respective traces 1 min after the onset of illumination. The ATP/2e ratio was calculated from these rates.

ceeding 1 µM generally abolish ATP production completely. However, phosphorylation is stimulated at uncoupler concentrations between 5 and 20 nM (Fig. 1). The nigericin concentration causing maximum stimulation varies somewhat with the preparation and is about 10 nM under saturating illumination. Even more variable is the extent of stimulation induced by addition of 10 nM nigericin: in 10 experiments, the uncoupler-induced stimulation of the phosphorylation rate varied between 19 and 60%. Mean stimulation for these 10 preparations was 32%. Control experiments where the phosphorylation rate was monitored by incorporation of $^{32}P_i$ into ATP showed the same dependence of the phosphorylation rate on the concentration of nigericin.

The rate of electron transport is slightly stimulated upon addition of the uncoupler at concentrations not exceeding about 10 nM and practically independent of the amount of added uncoupler for 10–100 nM nigericin (Fig. 1). Increase in electron transport typical for uncouplers is observed at still higher concentrations. This complex triphasic dependence of electron transport on uncoupler concentration is similar to that observed with methylamine [4]. The ATP/2e ratio is not decreased upon the addition of nigericin at concentrations that stimulate phosphorylation; rather, it is slightly increased at about 5–15 nM nigericin with most preparations. Nigericin at concentrations exceeding 15–20 nM drastically lowers the ATP/2e ratio. In the absence of the uncoupler, ATP/2e ratios were generally about 0.9–1.1; values close to 2 as reported by other groups [19,20] were not observed.

Monensin, which like nigericin belongs to the carboxylic ionophores, catalyzes H^+-Na^+ exchange [18]. Phosphorylation by chloroplasts suspended in the medium described in Fig. 1 (this medium contains K^+ and Na^+ at comparable concentrations) is stimulated by 100–200 nM monensin. Maximum stimulation occurs at about 150 nM and exceeds the control by 27% (mean of five experiments). It should be noted that monensin-induced stimulation of photophosphorylation was observed with thylakoid lamellae by Degani and Shavit [8] some years ago. These authors failed to observe nigericin-induced stimulation, probably because the nigericin concentration of 90 nM used in that study was too high.

A number of other uncouplers were checked but failed to induce enhancement of the phosphorylation rate. Among these were dinitrophenol, FCCP, gramicidin and the K^+ carrier valinomycin, which were used at minimal concentrations of 10, 20, 0.1 and 0.3 nM, respectively. Simultaneous addition of FCCP and valinomycin at proper concentrations should lead to H^+-K^+ exchange across the thylakoid membrane comparable to the effect of nigericin. However, when in the presence of low valinomycin concentrations which by itself did not affect phosphorylation (2–5 nM) the FCCP concentration was stepwise increased from non-inhibitory levels, no stimulation of phosphorylation as for nigericin (Fig. 1) was observed. This indicates that a fixed H^+-K^+ stoicheiometry is necessary for an increase of phosphorylation.

The rate of phosphorylation by unbroken chloroplasts is known to be enhanced by methylamine

TABLE I

EFFECTS OF LOW CONCENTRATIONS OF NIGERICIN AND METHYLAMINE ON THE RATE OF PHOTOPHOSPHORYLATION

Conditions as for Fig. 1, except that NaCN was replaced by KCN, and that methylamine was added at the concentrations indicated in the table.

Addition	Phosphorylation rate (μmol/mg Chl per h)	% stimulation
None	298	–
Nigericin		
5 nM	335	12.4
10 nM	366	22.8
Methylamine		
0.25 mM	324	8.7
0.5 mM	347	16.4
0.25 mM methylamine +5 nM nigericin	333	11.7

TABLE II

DEPENDENCE OF NIGERICIN-INDUCED STIMULATION OF PHOTOPHOSPHORYLATION ON THE AMOUNT OF ADDED KCl

Isolated intact chloroplasts were suspended in a medium containing 0.1 M sorbitol, 1 mM NaCN, 1 mM Na_2ADP, 5 mM $MgCl_2$, 10 mM NaCl, 1 mM NaH_2PO_4, 25 μM methyl viologen and 10 mM Hepes to give a chlorophyll concentration of 33 μg/ml. Experimental produce as for Fig. 1, except that the initial pH value was adjusted by addition of NaOH instead of KOH and that nigericin and KCl were added as indicated.

KCl added (mM)	Phosphorylation rate (μmol/mg Chl per h)		% stimulation by nigericin
	Control	+10 nM nigericin	
–	270	270	0
0.5	270	288	6.7
1	254	304	20
2	241	301	25
5	246	311	26.4
10	251	324	29
25	261	335	28.4

(Ref. 4; see also Table I). When suboptimal concentrations of nigericin were added to the assay medium in the presence of suboptimal concentrations of methylamine, the effects of these uncouplers on the phosphorylation rate failed to be additive (Table I). Stimulation of phosphorylation due to the addition of both uncouplers did not exceed that observed on separate addition of each of the compounds. This suggests that, on a molecular level, the events leading to increased phosphorylation are not identical for the two types of uncouplers.

The effect of the external KCl concentration on photophosphorylation in the absence and presence of 10 nM nigericin is shown in Table II. Intact chloroplasts as used in this study contain about 2.5 μmol K^+/mg Chl [21]; their osmotic volume is about 30 μl/mg Chl [22]. The K^+ concentration in the medium resulting from K^+ release by the osmotically stressed chloroplasts, therefore, does not exceed 80 μM; it is probably considerably lower, as part of the K^+ may be bound. Nigericin has no effect on photophosphorylation (or electron transport, not shown) in the presence of this low potassium concentration (Table II, no KCl added). The effectiveness of nigericin in enhancing the phosphorylation rate increases with the amount of added KCl and is saturated at about 5 mM. Concentrations of K^+ exceeding 25 mM gradually decrease phosphorylation rates both in the absence and presence of nigericin, but have practically no effect on the uncoupler-induced stimulation (not shown). The results shown in Table II suggest that nigericin-induced stimulation of photophosphorylation is related to the ability of the ionophore to catalyze K^+-H^+ exchange. Apparently, factors different from the external level of potassium determine the extent of stimulation at K^+ concentrations exceeding about 5 mM. Interestingly, Shavit et al. [23] observed that nigericin-catalyzed K^+ movements across thylakoid membranes were saturated at 4–5 mM KCl.

The dependence of the nigericin effect on H^+ concentrations in the medium and in the thylakoid lumen is expected to be complex as variation of the pH gradient across the thylakoid membrane will affect both H^+-K^+ exchange and the protonmotive force. In the following two experiments, the pH gradient was varied by external pH and by light intensity, respectively. The ΔpH is known to be maximal for external pH values between 8 and 9 [24]. Below pH 8, it is reduced with decreasing external pH. Correspondingly, phosphorylation rates are decreased when the pH value of the assay

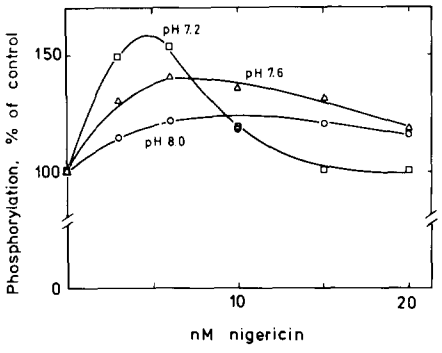

Fig. 2. Dependence of the rate of photophosphorylation on the nigericin concentration for three different pH values of the assay medium. For pH 8.0, assay conditions were as for Fig. 1. The concentration of Hepes was reduced to 5 mM for the other two assays. Phosphorylation rates of the control were 315 (pH 8.0), 220 (pH 7.6) and 132 (pH 7.2) μmol/mg Chl per h.

medium is lowered (Ref. 25; see also legend to Fig. 2). Nigericin-induced stimulation is, on a percentage basis, relatively independent of the pH value; with some preparations even an increase of relative stimulation is observed when the pH of the medium is lowered (Fig. 2). Nigericin concentrations inducing maximum stimulation generally decrease with lowering the external pH.

Fig. 3 depicts the effect of nigericin on light-driven ATP production under high and low light illumination. At low intensities which do not saturate the pH gradient [4] both the extent of

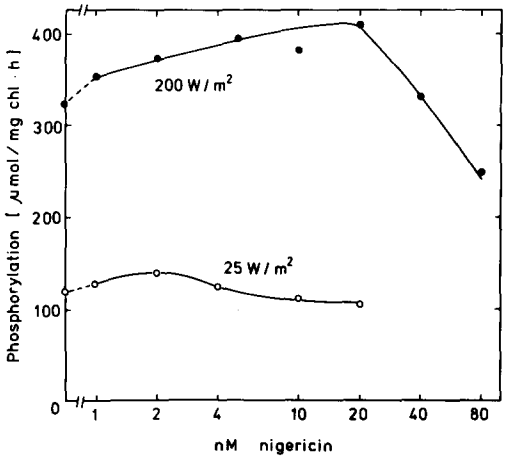

Fig. 3. Effect of nigericin on the phosphorylation rate at saturating and limiting light intensities. Conditions as for Fig. 1, except that light intensities were as indicated.

stimulation and optimal uncoupler concentration are lower than at high intensities. Under saturating illumination, the rate of phosphorylation is increased from 322 to 410 μmol/mg Chl per h upon the addition of 20 nM nigericin; under limiting light, the rate is 120 and 140 μmol/mg Chl per h in the control and in the presence of 2 nM nigericin, respectively. Whereas increase in absolute rates differs by a factor of more than 4, increase on a percentage basis shows considerably less difference, namely, 27% (200 W/m^2) compared with 17% (25 W/m^2).

Figs. 2 and 3 indicate that the extent of nigericin-induced stimulation of photophosphorylation is – within certain limits – proportional to the rate of the control in the absence of the uncoupler. This may imply that nigericin increases the capacity of a given proton-motive force to drive phosphorylation, and that this increase is proportional to the magnitude of the proton-motive force itself. Moreover, the amount of nigericin necessary to induce optimal stimulation decreases under conditions of a reduced ΔpH. This again suggests that nigericin-induced stimulation of phosphorylation is caused by a proper modification of an existing energization. Regarding the energetics of phosphorylation it is remarkable that phosphorylation is stimulated by nigericin also under limiting illumination (Fig. 3). The same phenomenon was observed before with methylamine [4]. Enhancement of phosphorylation at limiting light intensities means that the quantum efficiency of photophosphorylation is increased. This is a rather unexpected finding of view of the effects uncouplers are agreed to have on the proton-motive force.

Effects of nigericin on the proton-motive force

Electroneutral H^+-K^+ exchange catalyzed by nigericin is reported to reduce the proton-motive force by lowering the ΔpH and having no effect on the membrane potential [26]. However, those uncoupling concentrations of the ionophore are in 10–100-fold excess of the concentrations used here; data on the effect of low nigericin concentrations on the proton-motive force were apparently not published.

The pH gradient is frequently monitored by 9-aminoacridine fluorescence quenching [27]. Though people agree that quenching of 9-

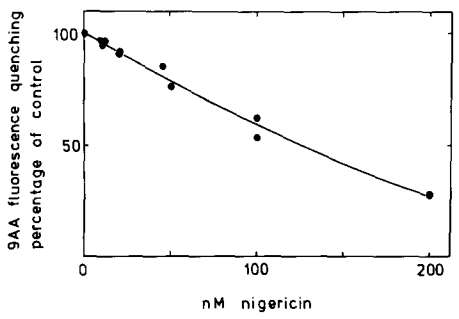

Fig. 4. Dependence of 9-aminoacridine (9AA) fluorescence quenching on the nigericin concentration. Intact chloroplasts containing 0.1 mg Chl were added to 3 ml assay medium (40 mM Hepes, pH 8.0) containing 1 mM KCN and 25 μM methyl viologen. Concentration of 9-aminoacridine was 5 μM. Data shown in the figure correspond to the recovery of 9-aminoacridine fluorescence quenching observed upon the addition of the indicated nigericin concentrations.

aminoacridine fluorescence can be due to a variety of processes [28,29], this method seems to be reliable for relative ΔpH measurements with intact [2] or broken [30] chloroplasts.

Fig. 4 shows the dependence on nigericin concentration of light-induced 9-aminoacridine fluorescence quenching of chloroplasts under non-phosphorylating conditions. Apparently, the pH gradient is reduced with increasing uncoupler concentrations, and even low nigericin concentrations cause a slight lowering: 10 and 20 nM nigericin decrease the ΔpH by 0.07 and 0.12 pH units, respectively (calculated from the data of Fig. 4). For comparison, 0.1 μM decreases the ΔpH by 0.5 pH units. This is somewhat more than reported by Hope et al. [11], who observed a decrease of the ΔpH by about 0.3 pH units upon addition of 0.1 μM nigericin.

Adenylates are known to interfere with 9-aminoacridine fluorescence emission [31], and only part of the light-induced quenching is recovered after switching off the light. Nevertheless, it was evident that nigericin did not increase but rather decrease the pH gradient under phosphorylating conditions (not shown). In conclusion, the 9-aminoacridine fluorescence data indicate that nigericin at concentrations that cause stimulation of the phosphorylation rate decreases the proton gradient by about 0.1 pH units or 6 mV. For comparison, stimulating concentrations of methylamine or NH_4Cl decrease the proton gradient by 0.2–0.3 pH units [3].

Additional information on the energy status of the chloroplasts is obtained from measurement of the H^+ permeability of the thylakoid membrane. The rate of proton extrusion following a light-dark transition is significantly increased by addition of 10 nM nigericin (Fig. 5). Concomitantly, the extent of proton uptake at the onset of illumination is reduced by 10 or 200 nM nigericin. Both the increase in the rate of proton extrusion and the decrease of the extent of proton uptake indicate that the thylakoid membrane becomes more leaky towards protons by addition of the uncoupler. As the rate of electron transport is only slightly increased by 10 nM nigericin, these data support the view that also the pH gradient is decreased by the uncoupler.

The membrane potential was estimated by means of the carotenoid shift [32]. When the light

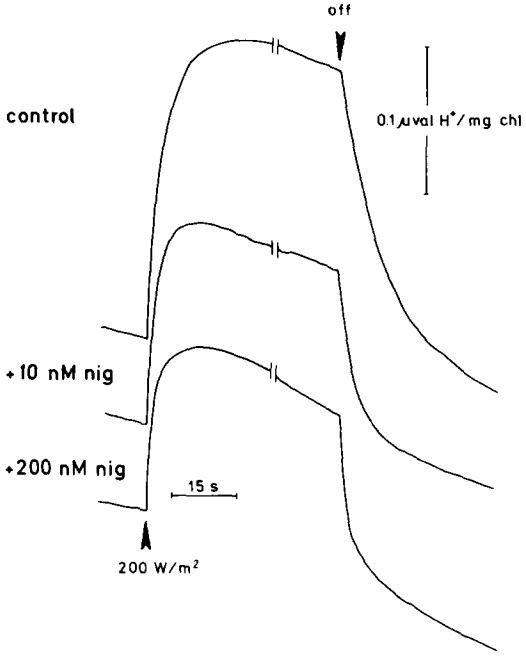

Fig. 5. Effect of nigericin (nig) on light-induced pH changes of a weakly buffered chloroplast suspension. Experimental conditions as for Fig. 1, except that Hepes and ADP were omitted from the assay medium. The buffering capacity was determined by addition of 5 μl of 1 mM HCl. Illumination time was 1 min. Phosphorylation rates of this preparation were 301 and 391 μmol/mg Chl per h in the absence and presence of 10 nM nigericin, respectively.

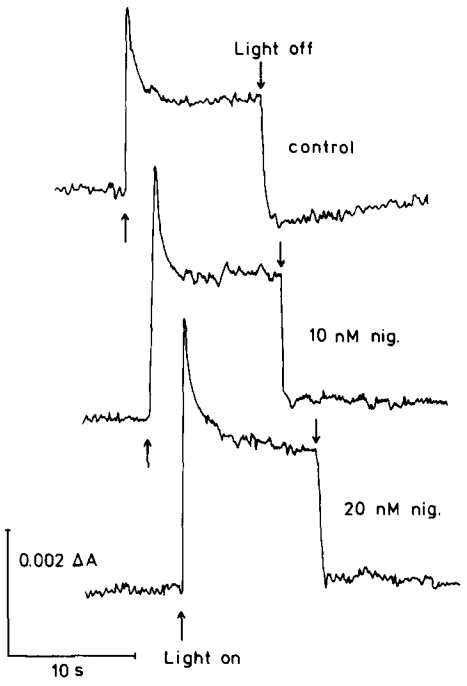

Fig. 6. Effects of low nigericin (nig) concentrations on the apparent light-induced absorption change at 518 nm of a suspension of chloroplasts. Reference wavelength was 540 nm. Assay conditions were as for Fig. 4, except that 1 mM ADP was added and 9-aminoacridine was omitted. Phosphorylation rates of this preparation were 326 and 392 μmol/mg Chl per h in the control and in the presence of 10 nM nigericin, respectively.

is switched off after several seconds of illumination, a rapid decrease in absorption is observed which is generally followed by a slow change (Fig. 6) that reflects light scattering and diffusion potentials [33,34]. Absorption changes observed upon darkening and corrected for the slow components allow estimation of the relative magnitude of the membrane potential in the steady state. Fig. 6 indicates that the steady-state membrane potential is not much affected by the addition of 10 or 20 nM nigericin. Depending on the preparation, nigericin at these low concentrations either has no effect on the membrane potential or induces a small increase as in Fig. 6. However, enhancement of the phosphorylation rate by nigericin was also observed with preparations which showed no increase of the steady-state ΔA_{518} upon addition of the ionophore. Therefore, this increase cannot be responsible for stimulation of phosphorylation. Higher concentrations (0.2 or 1 μM) reproducibly increased the steady-state $\Delta\psi$ in agreement with results obtained by Hope et al. [11] and by Kraayenhof and co-workers [35]. In contrast, the membrane potential observed during the first 1–3 s after the onset of illumination is significantly increased even by low nigericin concentrations (Fig. 6). This initial increase is caused also by higher nigericin concentrations (0.1 or 1 μM, data not shown). In conclusion, nigericin at low concentrations (5–20 nM) increases the membrane potential transiently during the first few seconds after the beginning of illumination and has no significant effect on its steady-state value.

Phosphorylation potentials

The phosphorylation rate depends both on the kinetic and energetic competence of the energy-transforming system. It is generally affected in a complex manner by a number of factors such as light intensity, ΔpH, the pH value in

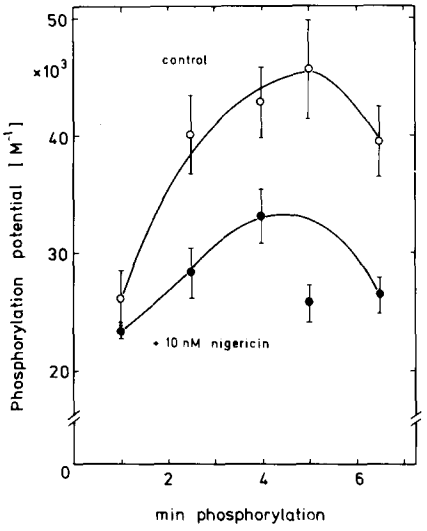

Fig. 7. Phosphorylation potential of chloroplasts in the absence and presence of 10 nM nigericin. Chloroplasts were suspended in the assay medium of Fig. 4 from which 9-aminoacridine was omitted. After illumination for 15 s at 200 W/m², 27.4 μM [^{14}C]ADP was added. Aliquots of 120 μl were withdrawn and injected into 35 μl of 3.5 M HCl at the times indicated. The neutralized samples were analyzed by high-performance liquid chromatography. Phosphorylation potentials were calculated from the measured ATP/ADP ratios and the known P_i concentrations. Error bars give the effect of the statistical error of the background on the peaks and correspond to mean values ± S.E. Phosphorylation rates were 336 μmol/mg Chl per h (control) and 378 μmol/mg Chl per h (+10 nM nigericin).

the thylakoid lumen, $\Delta\psi$, and adenylate and phosphate concentrations [36]. In contrast, the phosphorylation potential, $[ATP]/[ADP] \cdot [P_i]$, is thought to be determined only by the energetic competence of the chloroplasts [1,37]. The maximum value of the phosphorylation potential obtained under saturating illumination in the presence of limiting ADP concentrations reflects the energy provided by the proton-motive force. Fig. 7 shows the time course of the phosphorylation potential in the presence and absence of 10 nM nigericin. A rapid increase of the phosphorylation potential is observed initially; maximum values are reached after about 5 min. Maximum phosphorylation potentials in the absence of the ionophore are $45–50 \cdot 10^3\ M^{-1}$, corresponding to $\Delta G'_{ATP}$ values of about -58 kJ/mol. This figure is an agreement with values reported by Kraayenhof [38] for thylakoids and somewhat less than those observed with osmotically disrupted chloroplasts [39]. In the presence of nigericin, both the rate of increase and the final value of the phosphorylation potential are reduced. Maximum values in the presence of the ionophore are $30–35 \cdot 10^3\ M^{-1}$. Comparable results were obtained upon the addition of 0.5 mM methylamine, which reduced the phosphorylation potential from 43000 to 38000 M^{-1}. As the rate of phosphorylation is stimulated by nigericin, initial values of the phosphorylation potential should be higher in the presence of the ionophore than those in the control. This is indeed observed for times up to about 30 s from the beginning of phosphorylation. Subsequently, phosphorylation rates are reduced, most likely by the low, still decreasing ADP concentration.

Discussion

(a) Energetics

The 9-aminoacridine fluorescence data (Fig. 4), the relative rates of H^+ extrusion (Fig. 5) and the electrochromic shift (Fig. 6) indicate that the effect of nigericin at low concentrations is not principally different from that expected and observed at higher concentrations of this ionophore: the pH gradient is slightly reduced and the membrane potential only marginally affected. The membrane potential, on the other hand, is increased upon the addition of 10 or 20 nM nigericin during the first few seconds of illumination. A slight ionophore-induced increased of the potential may be present also in the steady state. The increase of the steady-state $\Delta\psi$ does not exceed about 5% of the potential in the control, which is reported to be 30–50 mV [11,40,41]. Thus, the potential in the steady state is increased by maximally about 3 mV with some preparations. However, as pointed out above, increase in the steady-state membrane potential is not related to the observed enhancement of the phosphorylation rate. Under steady-state conditions, nigericin at 10–20 nM lowers the ΔpH by maximally 0.1 pH units, and part of this decrease may be compensated by an increased $\Delta\psi$. The proton-motive force is therefore decreased maximally 2% upon the addition of 10–20 nM nigericin.

The phosphorylation potential is reduced upon the addition of the uncoupler (Fig. 7). Both equilibrium thermodynamics [36] and the non-equilibrium treatment [42] predict proportionality between the proton-motive force and $\Delta G'_{ATP}$. A reduced phosphorylation potential should therefore be indicative of a reduced proton-motive force. The extent of alteration of the phosphorylation potential (Fig. 7) may be surprising in view of the estimated small maximum decrease of the proton-motive force. However, the ratio of the phosphorylation potential P at a given proton-motive force, P_{pmf}, to that resulting from a 2% decrease of the proton-motive force, can be expressed [39] under the assumption of equilibrium as:

$$P_{pmf}/P_{pmf-2\%} = 10^{n \cdot 0.02 \cdot pmf}$$

where n is the number of protons which co-operate in the synthesis of one molecule of ATP via the CF_0/CF_1. For $n = 3$ and pmf = 3 pH units, the equilibrium phosphorylation potential is decreased by a factor of 1.51 by a 2% reduction of the proton-motive force. Thus, also the data of Fig. 7 imply that the proton-motive force is reduced upon the addition of low nigericin concentrations in agreement with the direct measurement of the proton-motive force. Simultaneous decrease of the phosphorylation potential and the proton-motive force is consistent with the role of the proton-motive force as the driving force of ATP production.

Likewise, increase of the phosphorylation rate in the presence of a diminished proton-motive force does not contradict the chemiosmotic concept, as the rate of phosphorylation has been demonstrated to be limited kinetically [3]. Low concentrations of proper uncouplers apparently remove the kinetic barrier, thereby stimulating phosphorylation. Obviously, addition of low concentrations of nigericin and methylamine affects the energetics (proton-motive force, $\Delta G'_{ATP}$) and the kinetics (phosphorylation rate) of energy conversion in opposite directions. This stresses the not always noticed [43] necessity to separate energetic and kinetic aspects in studies on energy transformation.

(b) Proton fluxes and the efficiency of photophosphorylation

Nigericin and methylamine increase the passive proton efflux across the membrane of illuminated thylakoids. This pathway is denoted by v_b in Fig. 8. That even low concentrations of methylamine increase proton leakage is documented in Ref. 4; the initial rate of proton extrusion was increased by 30% upon the addition of 0.2 mM methylamine under non-phosphorylating conditions ($-$ADP, $+P_i$). Increased proton leakage by addition of 10 nM nigericin is documented in Fig. 5. Also the decrease in ΔpH observed upon addition of this ionophore under non-phosphorylating conditions (Fig. 4) is likely to reflect an increase of v_b. Increased proton leakage should reduce the efficiency of phosphorylation, i.e., it should lower the ATP/2e ratio. However, this was not observed for nigericin (Fig. 1) or methylamine [4]. The ATP/2e ratio rather remained constant or was even increased.

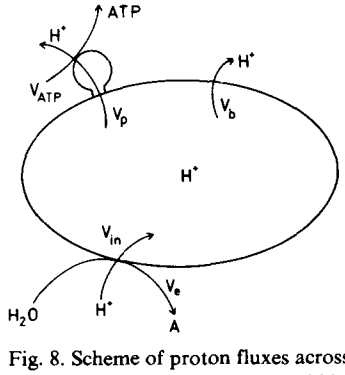

Fig. 8. Scheme of proton fluxes across the thylakoid membrane. Protons pumped into the thylakoid lumen by electron transport (pathway v_{in}) can leak through the membrane (v_b) or can give rise to ATP synthesis (v_{ATP}) by passing the coupling factor CF_0/CF_1 (v_p); v_e is the rate of light-driven electron transport from water to a proper acceptor A.

Assuming that the ratio v_{in}/v_e (Fig. 8) is not affected by these uncouplers, a constant ATP/2e ratio in the presence of increased v_b indicates an increased ratio of v_{ATP}/v_p. Increase in the latter ratio means that phosphorylation operates more efficiently, i.e., that the number of protons co-operating in the synthesis of one molecule of ATP decreases upon the addition of the uncoupler. Though this possibility cannot be excluded definitely an uncoupler-induced increase in v_{ATP}/v_p is not considered here. Under this assumption, the insensitivity of the ATP/2e ratio to increased proton leakage suggests that the coupling of v_{in} to v_p is not impaired by this leakage. This implies that protons delivered by electron transport and channelled through the coupling factor are not in rapid equilibration with those crossing the membrane by pathway v_b. This more direct type of coupling between v_{in} and v_p which does not necessarily involve the bulk thylakoid lumen is at variance with the classical chemiosmotic hypothesis [1] that postulates the existence of isopotential bulk phases and denies a possible involvement in energy transduction of localized proton flux pathways within the membrane. The latter possibility has been suggested by Williams [44] some years ago and, since that time, discussed [45], modified [37] and experimentally tested [46–49]. Apparently, the findings reported in this paragraph can be explained by assuming the existence of local proton fluxes rather than by the orthodox chemiosmotic concept.

(c) Conclusion and a hypothesis on the mechanism of uncoupler-induced stimulation

The results presented here suggest that electroneutral H^+-X^+ exchange is a necessary condition for the uncoupler-induced stimulation of phosphorylation; X^+ can be K^+ (nigericin), Na^+ (monensin) or R-NH_3^+ (amines). It is likely that other uncouplers will cause comparable effects.

Both groups of uncouplers alter the membrane potential: $\Delta\psi$ is increased upon the addition of methylamine not only in the steady state [3] but also during the initial stage of illumination (Giersch and Meyer, unpublished data), and it is increased

at least initially by nigericin (Fig. 6). If present, the increase in steady-state $\Delta\psi$ occurs at a diminished proton-motive force as outlined above. Uncoupler-induced stimulation, therefore, cannot be explained by merely correlating the increase in the rate of phosphorylation with an observed increase in $\Delta\psi$ without further assumptions on the individual contribution of ΔpH and $\Delta\psi$ to the regulation of phosphorylation. In this respect, the interpretation given by Hope et al. [9] is not satisfactory. These authors observed an 18% increase in the phosphorylation rate upon the addition of 0.4 μM nigericin; this increase was ascribed to an observed increase of $\Delta\psi$ by 8 mV though the total proton-motive force was reduced by about 35 mV (Fig. 8 in Ref. 11) upon addition of nigericin.

The membrane potential has been suggested to be involved in the activation of the chloroplast coupling factor [36,50]. Thus, the observed increase in the membrane potential may be important with respect to the regulation of phosphorylation. Uncoupler-induced increase of $\Delta\psi$ may increase the number of active CF_1 molecules, which results in an increase of the rate of phosphorylation. Enhanced phosphorylation implies that the proton flux via the coupling factor is increased (v_p, Fig. 8), and this increase is likely to decrease the ΔpH and to stimulate electron transport in addition to the uncoupler-induced increase in v_b that is demonstrated in Fig. 5. It should be pointed out that also this model has to assume the existence of localized proton pathways as outlined in section b, as activation of additional CF_1 molecules per se should increase the ATP/2e ratio only transiently, not in the steady state (which would be necessary in order to explain the constant ATP/2e ratio in the presence of increased proton leakage). It is known that activation of the CF_1 is related to the release of tightly bound nucleotides and that the light-induced activation of photophosphorylation can be followed by measuring the amount of tightly bound adenylates [51,52]. Preliminary experiments indicate that low concentrations of nigericin affect the kinetics and extent of nucleotide binding to the CF_1 (Schumann and Giersch, unpublished data). Further experiments are required to decide whether this effect of uncouplers on nucleotide binding can account for the observed stimulation of phosphorylation.

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References

1 Mitchell, P. (1976) Biochem. Soc. Trans. 4, 399–430
2 Tillberg, J.-E., Giersch, C. and Heber, U. (1977) Biochim. Biophys. Acta 462, 31–47
3 Giersch, C. (1981) Biochem. Biophys. Res. Commun. 100, 666–674
4 Giersch, C. (1982) Z. Naturforsch. 37c, 242–250
5 McCarty, R.E. (1968) Biochem. Biophys. Res. Commun. 32, 37–43
6 McCarty, R.E. (1969) J. Biol. Chem. 244, 4292–4298
7 Gromet-Elhanan, Z. and Avron, M. (1965) Plant Physiol. 40, 1053–1059
8 Degani, H. and Shavit, N. (1972) Arch. Biochem. Biophys. 152, 339–346
9 Hope, A.B., Ranson, D. and Dixon, P.G. (1982) Aust. J. Plant Physiol. 9, 399–407
10 Wei, J.M., Shen, Y.G., Li, D.Y. and Zhang, X.X. (1980) Acta Phytophysiol. Sin. 6, 393–398
11 Hope, A.B., Ranson, D. and Dixon, P.G. (1982) Aust. J. Plant Physiol. 9, 385–397
12 Heber, U. (1973) Biochim. Biophys. Acta 305, 140–152
13 Jensen, R.G. and Bassham, J.A. (1966) Proc. Natl. Acad. Sci. U.S.A. 56, 1095–1101
14 Arnon, D.I. (1949) Plant Physiol. 24, 1–15
15 Reeves, S.G. and Hall, D.O. (1980) Methods Enzymol. 69, 85–94
16 Nishimura, M., Ito, T. and Chance, B. (1962) Biochim. Biophys. Acta 59, 177–182
17 Giersch, C. (1979) J. Chromatogr. 172, 153–161
18 Pressman, B.C. (1976) Annu. Rev. Biochem. 45, 501–530
19 Robinson, S.P. and Wiskich, J.T. (1976) Biochim. Biophys. Acta 440, 131–146
20 Reeves, S.G. and Hall, D.O. (1973) Biochim. Biophys. Acta 314, 66–78
21 Barber, J. and Nakatani, H.Y. (1980) Methods Enzymol. 69, 585–604
22 Heldt, H.W. and Sauer, F. (1971) Biochim. Biophys. Acta 234, 83–91
23 Shavit, N., Degani, H. and San Pietro, A. (1970) Biochim. Biophys. Acta 216, 208–219
24 Rottenberg, H. and Grunwald, T. (1972) Eur. J. Biochem. 25, 71–74
25 Pick, U., Rottenberg, H. and Avron, M. (1974) FEBS Lett. 48, 32–36
26 Jagendorf, A.T. (1975) in Bioenergetics of Photosynthesis (Govindjee, ed.), pp. 413–492, Academic Press, New York
27 Pick, U. and McCarty, R.E. (1980) Methods Enzymol. 69, 538–546
28 Barber, J. (1982) Annu. Rev. Plant Physiol. 33, 261–295
29 Kraayenhof, R. (1980) Methods Enzymol. 69, 510–520

30 Haraux, F. and De Kouchkovsky, Y. (1980) Biochim. Biophys. Acta 592, 153–168
31 Pick, U., Rottenberg, H. and Avron, M. (1973) FEBS Lett. 32, 91–94
32 Witt, H.T. (1979) Biochim. Biophys. Acta 505, 355–427
33 Junge, W. (1977) Annu. Rev. Plant Physiol. 28, 503–536
34 Thorne, S.W., Horvath, G., Kahn, A. and Boardman, N.K. (1975) Proc. Natl. Acad. Sci. U.S.A. 72, 3858–3862
35 Schuurmans, J.J., Peters, A.L.J., Leeuwerik, F.J. and Kraayenhof, R. (1981) in Vectorial Reactions in Electron and Ion Transport in Mitochondria and Bacteria (Palmieri, F., Quagliariello, E., Siliprandi, N., and Slater, E.C., eds.), pp. 359–369, Elsevier, Amsterdam
36 Schlodder, E., Gräber, P. and Witt, H.T. (1982) in Electron Transport and Photophosphorylation (Barber, J., ed.), pp. 105–175, Elsevier, Amsterdam
37 Kell, D.B. (1979) Biochim. Biophys. Acta 549, 55–99
38 Kraayenhof, R. (1969) Biochim. Biophys. Acta 180, 213–215
39 Giersch, C., Heber, U., Kobayashi, Y., Inoue, Y., Shibata, K. and Heldt, H.W. (1980) Biochim. Biophys. Acta 590, 59–73
40 Vredenberg, W.J. and Tonk, W.J.M. (1975) Biochim. Biophys. Acta 387, 580–587
41 Huber, H.-L., Rumberg, B. and Siggel, U. (1980) Ber. Bunsenges. Phys. Chem. 84, 1050–1055
42 Stucki, J.W. (1980) Eur. J. Biochem. 109, 269–283
43 Sorgato, M.C., Branca, D. and Ferguson, S.J. (1980) Biochem. J. 188, 945–948
44 Williams, R.J.P. (1961) J. Theor. Biol. 1, 1–17
45 Williams, R.J.P. (1978) Biochim. Biophys. Acta 505, 1–44
46 Ort, D.R. and Dilley, R.A. (1976) Biochim. Biophys. Acta 443, 95–107
47 Tandy, N.E., Dilley, R.A., Hermodson, M.A. and Bhatnagar, D. (1982) J. Biol. Chem. 257, 4301–4307
48 Hing, Y.Q. and Junge, W. (1983) Biochim. Biophys. Acta 722, 197–208
49 Hitchens, G.D. and Kell, D.B. (1983) Biochim. Biophys. Acta 723, 308–316
50 Harris, D.A. and Crofts, A.R. (1978) Biochim. Biophys. Acta 502, 87–102
51 Gräber, P., Schlodder, E. and Witt, H.T. (1977) Biochim. Biophys. Acta 426–440
52 Strotmann, H., Bickel-Sandkötter, S., Franek, U. and Gerke, V. (1981) in Energy Coupling in Photosynthesis (Selman, B.R. and Selman-Reimer, S., eds.), pp. 187–196, Elsevier, Amsterdam