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STIMULATION OF PHOTOPHOSPHORYLATION BY LOW CONCENTRATIONS OF UNCOUPLING AMINES

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SUMMARY: Steady-state photophosphorylation by broken chloroplasts is stimulated by 10-50% on addition of low (< 1 mM) concentrations of NH_4Cl or methylamine. Stimulation of photophosphorylation by these compounds is accompanied by a decrease of the transmembrane proton gradient and an increase in the membrane potential. However, the increase in the latter is not equivalent to the decrease in the proton gradient. Thus, in the presence of uncoupling amines, the proton motive force is reduced. The results suggest that uncoupling amines remove a kinetic limitation of the energy-converting process, thereby stimulating the rate of phosphorylation.

The chemiosmotic hypothesis (1) suggests that photophosphorylation is driven by a light-induced proton gradient, ΔpH, and a transmembrane potential difference, $\Delta\psi$, which together form the proton motive force (pmf). A large volume of evidence in favour of the concept of chemiosmosis has accumulated. However, recent work dealing with intact mitochondria (2) and chloroplasts (3) yielded results which cannot be accomodated easily within the framework of the chemiosmotic hypothesis. The work with intact chloroplasts (3) has been extended using broken chloroplasts because the latter are much less complex systems. These osmotically ruptured ("Type B", ref.4) chloroplasts are, in contrast to intact chloroplasts, capable of photophosphorylating exogenous ADP at high rates. It is well documented that ammonium chloride at concentrations of 10 mM causes uncoupling, i.e. abolishes phosphorylation and drastically stimulates the rate of electron transport, most likely by increasing the permeability of the thylakoid membrane for protons (5). However, as will be shown here, NH_4Cl or methylamine in low concentrations do not de-

ABBREVIATIONS: Ap_5A, P^1,P^5-di(adenosine-5'-)pentaphosphate; 9-AA, 9-aminoacridine; FCCP, carbonyl cyanide p-trifluoromethoxyphenylhydrazone; ΔpH, transmembrane proton gradient; $\Delta\psi$, transmembrane potential difference; pmf, proton motive force.

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crease, but rather increase the rate of phosphorylation. The present contribution describes some aspects of this puzzling observation and attempts to correlate the uncoupler-induced stimulation of phosphorylation with changes in the proton motive force.

MATERIAL AND METHODS: Intact chloroplasts were isolated from greenhouse or field-grown spinach leaves by a modification (6) of Jensen and Bassham's procedure (7). Chlorophyll was determined according to Arnon (8). Oxygen evolution was measured in a Clark-type electrode. Intact chloroplasts were suspended in an assay medium containing 0.1 M sorbitol, 5 mM $MgCl_2$, 10 mM NaCl and 50 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES), adjusted to pH 7.5 with NaOH. The electron acceptor was 1 mM ferricyanide. Quenching of 9-AA fluorescence was measured with a photomultiplier as described previously (3). Absorption changes of chloroplast suspensions were recorded with a sensitive dual wavelength spectrophotometer (Aminco, DW 2) equipped with cross-beam illumination. The photomultiplier was protected by a Corning 4-96 filter. Actinic light intensities were determined by a radiometer (Yellow Springs Instruments Co., model 65A). The actinic light ($200W/m^2$) was provided by passing a beam of white light through 1 mm Calflex C (Balzer) and 2 mm RG 630 (Schott) glass filters. Photophosphorylation was measured by enzymic determination of ATP or by incorporation of $^{32}P_i$. For the enzymic ATP-test, activity of adenylate kinase (EC 2.7.4.3) in the assay was inhibited by addition of 0.15 mM Ap_5A (9) which does not affect the rate of phosphorylation as measured by incorporation of $^{32}P_i$. For measurement of incorporation of $^{32}P_i$, 0.5 mM $KH_2{}^{32}PO_4$ (5 mCi/mmol) was added. The label recovered in phosphate and in ATP was determined by high performance liquid chromatography. Conditions of chromatography were: 30 cm x 4.6 mm Partisil SAX 10 (Whatman), elution medium of 0.7 M KH_2PO_4, pH 3.0 (H_3PO_4), flow rate of 1 ml/min. Label was quantified by means of an on-line ß-monitor and a multi channel analyzer as described in ref. 10. The rate of phosphorylation was calculated from the percentage of total label recovered in the ATP peak, the amount of added P_i, and the chlorophyll concentration.

RESULTS AND DISCUSSION: When intact chloroplasts are suspended in a hypotonic assay medium, the chloroplast envelope is osmotically disrupted. Fig. 1 shows the dependence of the rate of phosphorylation by these chloroplasts on the concentration of the uncoupling agent NH_4Cl. Low increasing concentrations of ammonium chloride gradually stimulate phosphorylation. At higher concentrations of NH_4Cl, the stimulation is decreased. In the presence of about 1 mM NH_4Cl, the rate of phosphorylation is the same as in the absence of the uncoupler, and higher concentrations of NH_4Cl drastically decrease phosphorylation. In the experiment of Fig. 1, 0.4 mM NH_4Cl caused maximum stimulation corresponding to an increase of phosphorylation by about 30% as compared to the control rate in the absence of NH_4Cl. Both the extent of stimulation and the concentration of NH_4Cl causing maximum stimulation varied with chloroplast

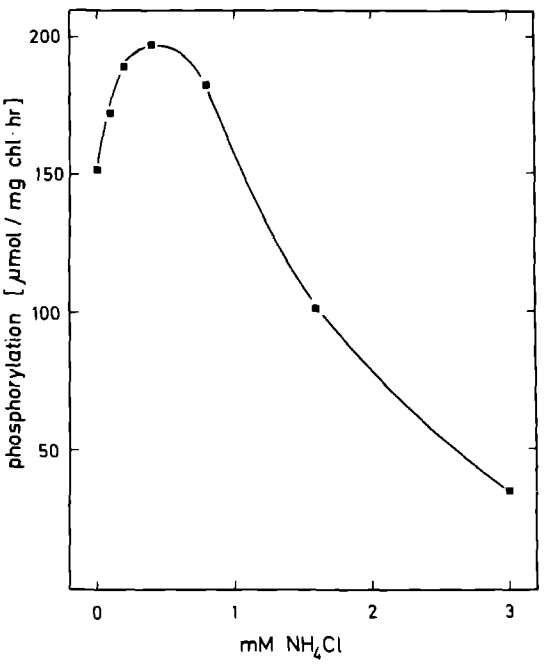

Fig. 1 Dependence of the rate of photophosphorylation on the concentration of NH_4Cl. Chloroplasts (33 µg chlorophyll/ml), 1 mM ferricyanide, and NH_4Cl as indicated were added to 1 ml of assay medium. Phosphorylation was started by simultaneous addition of 0.2 mM ADP and 0.5 mM $KH_2{}^{32}PO_4$ in the light and stopped after 30 s by addition of $HClO_4$ to a final concentration of 0.3 M. The acidified samples were immediately analyzed by high performance liquid chromatography.

preparations and were usually 10-30% and 0.2-0.5 mM, respectively. The mean stimulation (7 experiments) was 18%. With some preparations, no stimulation could be detected on addition of NH_4Cl. Concentrations in excess of 1 mM generally decreased the rate of photophosphorylation. The decrease of phosphorylation at higher concentrations of NH_4Cl is a well-documented phenomenon and is thought to be due to a decrease of the light-generated Δ pH across the thylakoid membrane (5). The increase of phosphorylation, as observed after addition of low concentrations of NH_4^+, can obviously not be explained by this mechanism.

Stimulation of the rate of phosphorylation is not only found with ammonium chloride. Methylamine caused an even more pronounced stimulation (Fig. 2). The extent of stimulation was 39% (mean of 14 experiments), and

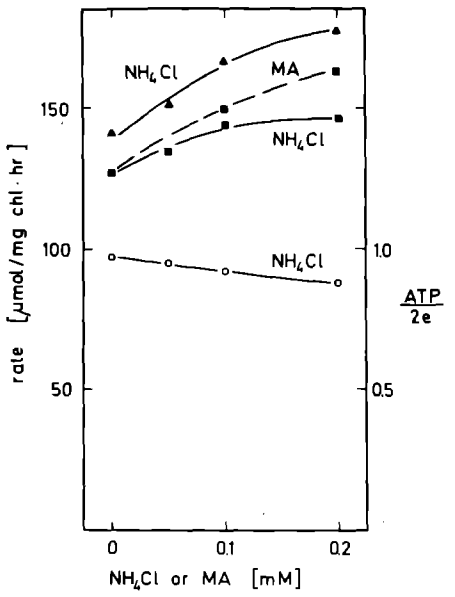

Fig. 2 Effect of NH_4Cl and methylamine (MA) on the rate of phosphorylation (■), on electron transport ($2e^-$, ▲) and on the ATP/2e ratio (o). Experimental conditions were the same as in Fig. 1, except that chloroplasts corresponding to 66 µg chlorophyll were suspended in 2 ml of the assay medium, Ap_5A (0.15 mM) and 1 mM KH_2PO_4 were added before the onset of illumination; phosphorylation was started by addition of 0.45 mM ADP in the light, and stopped after 90 s. The ATP content of the neutralized samples was determined enzymically. ATP/2e ratios were calculated from the simultaneous recording of oxygen evolution.

the concentration of methylamine causing maximum stimulation was higher than that of NH_4Cl; with some preparations concentrations as high as 1 mM methylamine led to maximum stimulation. Addition of methylamine stimulated phosphorylation in all preparations tested. In contrast, addition of 0.1-5 µM FCCP, which is known to be a potent uncoupler of oxidative and light-driven phosphorylation (5), decreased the rate of photophosphorylation (data not shown).

The rate of electron transport was increased by concentrations of NH_4Cl that stimulated phosphorylation. The extent of stimulation of electron transport exceeded that of phosphorylation. Therefore, the ATP/2e ratio decreased with increasing concentrations of NH_4Cl (Fig. 2). ATP/2e ratios in the absence of uncouplers were usually 1.2-0.8. Values close to 2 as

reported by Reeves and Hall (11) and Robinson and Wiskich (12) were not observed.

From linear non-equilibrium thermodynamics (13) and experimental observations (14) one should expect that an increase in the rate of phosphorylation is due to an increase in the proton motive force, which is believed to be the driving force of phosphorylation (1). Stimulation by low concentrations of methylamine or NH_4^+ therefore suggests an increase of the proton motive force by a reputed uncoupler. The two components of the pmf were estimated by quenching of 9-AA fluorescence which monitors the transmembrane ΔpH (15), and by fast apparent absorption changes at 518 nm, that are related to the transmembrane potential difference $\Delta\psi$ (16). Table 1 shows the dependence of light-induced quenching of 9-AA fluorescence on the concentrations of methylamine and NH_4Cl. Quenching of 9-AA fluorescence is decreased also at those concentrations of uncouplers that stimulate phosphorylation. This decrease is not drastic in energetic terms: addition of 0.8 mM methylamine (1.5 mM NH_4Cl) decreases the ΔpH by about 0.3 (0.6) pH units (Table 1), as can be calculated from the formula given by Schuldiner et al. (15).

The reliability of the fluorescence of 9-AA for monitoring the transmembrane ΔpH has been repeatedly questioned (17). With respect to the data shown in Table 1, however, it should be pointed out that at least two main arguments raised against the use of 9-AA as a probe for ΔpH do not apply: first, only a small percentage of 9-AA seems to be bound to the membrane (3), and an increase in the fluorescence due to the removal of 9-AA from the binding sites by addition of ammonium chloride or methylamine is small and can be neglected. Second, uncoupling amines cause thylakoid swelling (18). This leads to an underestimation of the decrease in ΔpH calculated from the 9-AA signal according to Schuldiner and coworkers (15); thus, an increase of 9-AA fluorescence upon the addition of NH_4^+ or methylamine should indeed indicate a decrease in ΔpH.

Table 1. Effect of NH_4Cl and methylamine on the transmembrane proton gradient

Experimental conditions were identical to those for Fig. 2, except that 5 μM 9-AA was included. Q is the percentage of 9-AA fluorescence that is quenched by illuminating the sample with 200 W/m^2. The decrease in ΔpH is calculated according to (15) from: $\Delta(\Delta pH) = \log \frac{Q^o(1-Q)}{Q(1-Q^o)}$, where Q^o is the quenching in the absence of uncoupler.

methylamine (mM)	0	0.2	0.4	0.8	1.5	3.0
Q, % of control	100	85.7	74.9	64.4	55.3	39.2
decrease in ΔpH	0	0.11	0.20	0.29	0.38	0.56
NH_4Cl (mM)	0	0.2	0.4	0.8	1.5	3.0
Q, % of control	100	83.9	74.0	56.0	36	16
decrease in ΔpH	0	0.12	0.19	0.35	0.58	0.97

The transmembrane potential difference, on the other hand, is increased by the addition of methylamine (Fig. 3). Data shown in the figure correspond to the fast absorbance change at 518 nm observed when the light is turned off. This fast signal is gradually decreased by adding increasing concentrations of valinomycin in the presence of 10 mM KCl (data not shown). The observed increase of the membrane potential by methylamine (Fig. 3) is at variance with current concepts of uncoupling by amines (5, 19), according to which $\Delta\psi$ should be unaffected or decreased in the presence of uncoupler.

The results given above would have a straightforward explanation if the increase in the membrane potential on addition of the uncoupler is higher, in energetic terms, than the decrease in ΔpH. The latter is decreased on addition of 0.4 mM methylamine by 0.2 pH units (Table 1), which is equivalent to 12 mV. As the $\Delta\psi$ is proportional to the 518 nm signal (16), the membrane potential is increased by about 14% upon addition of 0.4 mM methylamine (Fig. 3). According to observations made by several investigators (20-22), the $\Delta\psi$ in the steady-state (and in the absence of uncouplers) is 10-30 mV. The increase in membrane potential shown in Fig. 3 would then be at most about 4 mV. Hence, it is unlikely that the increase in the rate of

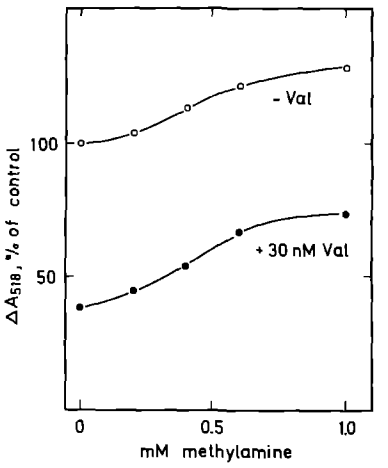

Fig. 3 Dependence of the light-induced absorption change at 518 nm on the concentration of methylamine in the absence and presence of 30 nM valinomycin (Val). Reference wavelength was 540 nm. NaCl in the assay medium was replaced by 10 mM KCl. Experimental conditions were the same as described for Fig. 2 with the exception that Ap_5A was omitted and that ADP, methylamine and valinomycin were added before the onset of illumination. Data shown in the figure correspond to the fast absorption change seen when the light was turned off after illumination for 15 s with $200 W/m^2$ red light.

phosphorylation observed on addition of the uncoupler can be ascribed to an increased pmf.

Moreover, the membrane potential can be decreased by addition of 30 nM valinomycin (Fig. 3). The proton gradient is not affected (not shown). Nevertheless, the rate of phosphorylation is only marginally decreased or not affected at all (Fig. 4). If, in the absence of valinomycin, the increase in the membrane potential caused by methylamine (Fig. 3) is responsible for the increase in phosphorylation (Fig. 4), it is difficult to understand why the rate of phosphorylation is practically not affected when the membrane potential is drastically reduced by addition of 30 nM valinomycin (Fig. 3). Thus, although addition of amines increases the membrane potential, it is insufficient to account for the stimulation of photophosphorylation.

CONCLUSION: The steady state rate of photophosphorylation depends both on the energetic and kinetic competence of the chloroplasts. The decrease of

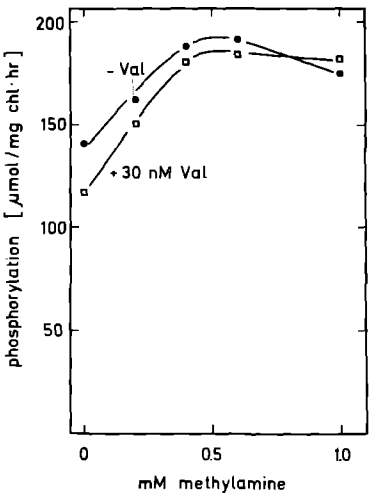

Fig. 4 Stimulation of photophosphorylation by methylamine in the absence and presence of 30 nM valinomycin (Val). The assay medium contained 10 mM KCl instead of NaCl. Otherwise, experimental conditions were as described for Fig. 1, except that the concentration of ADP was 0.45 mM, that methylamine was added instead of NH_4Cl and that phosphorylation was stopped after 1 min.

the pmf under conditions where phosphorylation is stimulated suggests that uncoupling amines like NH_4Cl or methylamine remove a kinetic limitation of phosphorylation. Removal of this unknown kinetic restriction by low concentrations of amines, when the pmf is not drastically decreased, causes an increase in the rate of phosphorylation. However, stimulation is only possible when chloroplasts are overenergized, i.e., when even a decreased pmf provides sufficient energy to allow phosphorylation at an unaffected or even increased rate. Supporting evidence for this conclusion comes from studies on the phosphorylation potential in intact and broken chloroplasts (23). Overenergization of chloroplasts was also discussed by Slovacek and Hind (24), who demonstrated that under a variety of assay conditions the rate of CO_2-dependent oxygen evolution in intact chloroplasts can be stimulated by 0.33 mM NH_4Cl. The authors ascribe the stimulatory effect of ammonium chloride to imbalances between the rates of ATP production and utilization. In their opinion, the partial decrease in ΔpH caused by addition of the un-

coupler results in a decrease of phosphorylation that would overcome such imbalances. However, this conclusion seems questionable in view of the observation that low concentrations of NH_4Cl may stimulate rather than decrease photophosphorylation in spite of a lowered ΔpH.

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