# The effect of gramicidin on ATP synthesis in pea chloroplasts: two modes of phosphorylation

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The effect of gramicidin on the phosphorylation rate, electron flow and light-induced $H^+$ uptake ($\Delta H^+$) in chloroplasts with methyl viologen (MV) or phenazine methosulfate (PMS) added has been studied. In the presence of MV low concentrations of gramicidin ($\sim 10^{-9}$ M) were shown to inhibit the phosphorylation rate up to 80–90% of the initial value, without changing either the electron transport rate or $\Delta H^+$ value. Two components of phosphorylation have been identified in the presence of PMS – the first is DCMU-sensitive, which was suppressed by low gramicidin concentrations ($< 10^{-9}$ M) and the second – DCMU-insensitive component, which was suppressed by high gramicidin concentrations ($\sim 10^{-7}$ M) exclusively, the high gramicidin concentration inducing the $\Delta H^+$ value fo fall.

*Thylakoid membrane* *Photophosphorylation* *Protein uptake* *Proton-binding domain* *Photosystem II* *Electron flow* *(Pea chloroplast)*

## 1. INTRODUCTION

The possibility of proton transfer from the redox chain to the ATP-synthetase complex without equilibration with the aqueous phase of the thylakoid interior is being widely discussed. Some works [1–3] point to the existence of an $H^+$-binding domain whose protons are not in equilibrium with those in the bulk phase. The domain-bulk phase system comes to equilibrium in the presence of low gramicidin concentrations ($\sim 10^{-9}$ M). These gramicidin concentrations had no effect on the transmembrane pH value at high frequency light flashes [4]. The domain buffer system has been shown by kinetic studies to be functionally dependent on PS II [4,5].

Here, conditions were chosen in which in pea chloroplasts two modes (two components) of steady-state phosphorylation were observed; these modes differed strongly in sensitivity to inhibitors – gramicidin D being an uncoupler, and DCMU being an electron-transfer inhibitor. It was shown that low gramicidin concentrations ($\sim 10^{-9}$ M) exclusively suppress the DCMU-sensitive component of the phosphorylation process. Both these data and those from [4] support the suggestion that the phosphorylation component sensitive to DCMU and low concentrations of gramicidin involves the $H^+$-binding domain and PS II.

*Abbreviations:* PS II, photosystem II; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; MV, methyl viologen; PMS, phenazine methosulfate

## 2. MATERIALS AND METHODS

This study was carried out with isolated pea chloroplasts. The isolation medium contained 400 mM sucrose, 10 mM NaCl, 5 mM $MgCl_2$ and 20 mM Tris-HCl buffer, pH 7.5. The homogenate was filtered and centrifuged for 10 min at 1300 × *g*. The chloroplasts were washed with a medium containing 100 mM sucrose, 10 mM NaCl, 20 mM Tris-HCl buffer, pH 7.5, and re-centrifuged in the same manner. The chloroplasts thus obtained were

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resuspended in a medium containing 100 mM sucrose, 50 mM NaCl, 2 mM $MgCl_2$ and 1 mM Hepes, pH 7.5, and stored at 0°C. The chlorophyll concentration was assayed according to Arnon [6]. The phosphorylation rate was determined by the luciferin-luciferase luminescence method [7]. The oxygen consumption in the presence of MV was monitored by a platinum Clark-type electrode. These reactions were performed in medium I, containing 100 mM sucrose, 50 mM NaCl, 2 mM $MgCl_2$, 5 mM $Na_2HPO_4$, 1 mM ADP and 0.05 mM MV or PMS, 5 mM Tris-HCl buffer, pH 7.5, and chloroplast – 15 $\mu$g chlorophyll/ml medium. pH changes of the medium in the course of the light-dependent uptake of protons by thylakoids ($\Delta H^+$) were registered by an OP-208 pH-meter (Hungary) and computerized by Electronika D3-28 (USSR) media II and III for pH-metric measurements were used, which contained the same concentrations of sucrose and chlorides as medium I and 0.2 mM ADP, 0.5 mM Tris-HCl buffer. Medium II also contained 0.5 mM $NaH_2AsO_4$; medium III – 0.5 mM $Na_2HPO_4$, hexokinase and 20 mM glucose. The pH of medium III was maintained at a steady level during ATP synthesis. The chlorophyll content was 30 $\mu$g/ml medium. All reactions were performed in thermostatted cells at 20°C and under white light illumination. The pH value (7.5) was selected in such a manner as to reduce both the inactivation of the samples during storage and the effect of thylakoid protonation in darkness on the measured $\Delta H^+$. Hepes, DCMU and gramicidin D were from Serva (FRG); ADP, ATP and hexokinase were from Reanal (Hungary).

## 3. RESULTS

Curve I in fig.1 clearly shows (in the presence of PMS) the existence of two components of the phosphorylation process which differ greatly in gramicidin sensitivity. One is sensitive to low gramicidin concentrations (~$10^{-9}$ M) and is specifically inhibited by DCMU (fig.1, curve 2). The second is sensitive to higher concentrations ($10^{-8}$–$10^{-7}$ M), and is not inhibited by DCMU (fig.1, curves 1 and 2). Gramicidin at low concentrations does not decrease the level of light-dependent proton uptake ($\Delta H^+$) by thylakoids during phosphorylation (fig.1, curve 3).

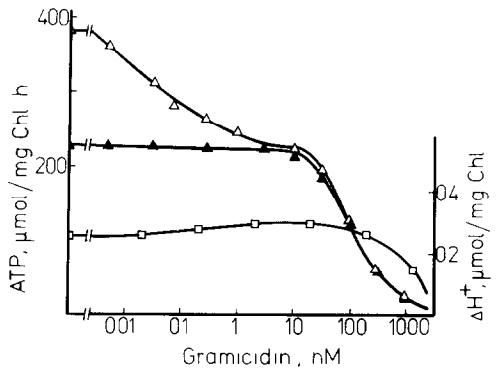


Fig.1. The effect of gramicidin D on PMS-catalyzed processes. Phosphorylation in medium I: 1, without DCMU (△); 2, in the presence of 2 × $10^{-6}$ M DCMU (▲); 3, light-dependent proton uptake in medium II (□).

Measurements of ATP synthesis rate in the presence of MV show that under these conditions the phosphorylation component which is mostly realized is that which is sensitive to low gramicidin concentrations (fig.2, curve 1). The low concentrations of gramicidin, while inhibiting ATP synthesis in the presence of MV by 70–90%, had no effect on the electron-transfer rate and on the $\Delta H^+$ value under phosphorylation conditions (fig.2, curves 2 and 4). The low concentrations of gramicidin do not alter the rate of basal electron transfer without

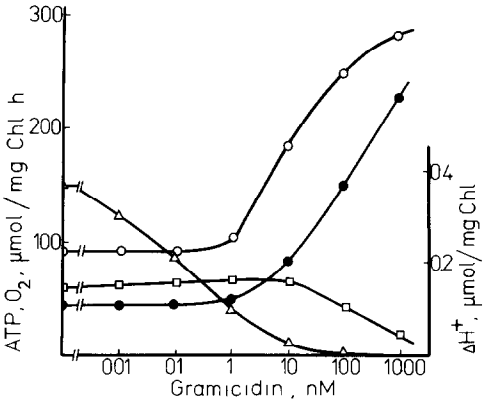


Fig.2. The effect of gramicidin D on MV-catalyzed processes. 1, phosphorylation (△); 2, oxygen consumption during phosphorylation (○); 3, oxygen consumption in the absence of ADP (●) in medium I; 4, light-dependent proton uptake in medium II (□) ($\Delta H^+$ values in media II and III were equal at all gramicidin concentrations).

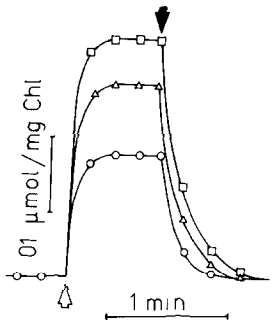


Fig.3. Light-dependent proton uptake in the presence of 1, MV (○); 2, PMS (□); 3, PMS + DCMU ($2 \times 10^{-6}$ M) (△); medium II.

ADP added (fig.2, curve 3). In the presence of MV, diurone ($2 \times 10^{-6}$ M) completely inhibits the phosphorylation process and the light-dependent proton uptake ($\Delta H^+$). A comparison of the $\Delta H^+$ values shows that under these conditions $\Delta H^+$ is approximately twice as high in the presence of PMS as in the presence of MV (figs 1–3). The addition of $2 \times 10^{-6}$ M DCMU to the reaction medium completely inhibits the proton uptake if MV is present. In experiments involving PMS, DCMU ($2 \times 10^{-6}$ M) decreases $\Delta H^+$ up to 15–20% (fig.3) of the initial value.

## 4. DISCUSSION

In the experiments with PMS we observed two components of the phosphorylation process which are clearly distinct in their sensitivity to gramicidin D, a phosphorylation uncoupler. Diurone induced inhibition of the first component indicates its relation to PS II functioning. The analysis of the curves in figs 1 and 2 allows us to conclude that the second component makes a significant contribution to ATP synthesis only in the case of intensive operation of PS I (in the presence of PMS). When the operation of PS I is limited by electron transfer between the photosystems (in the presence of MV) the only active process is that which is sensitive to low gramicidin concentrations (fig.2).

One can see that DCMU-sensitive phosphorylation is inhibited by the same concentrations of gramicidin which, according to [4], specifically shunt the PS II related $H^+$-binding domain. This allows one to suggest that the $H^+$-binding domain is included in the PS II-related phosphorylation component.

The following detailed study enables some of the features of the DCMU-sensitive component of the phosphorylation process to be described.

(i) Inhibition of ATP synthesis by low concentrations of gramicidin (in the presence of MV) is not accompanied by a change in $\Delta H^+$ value and the coupled electron-transfer rate (fig.2); therefore the proton flow coupled to electron transfer should also exhibit no changes. Consequently, a decrease of the phosphorylation $H^+$ flow via ATP synthetase under the experimental conditions is rigorously compensated by additional proton flow from the thylakoid via gramicidin channels.

(ii) In our experiments under stationary illumination and high ionic strength of media, the transmembrane potential $\Delta\psi$ was found to be low (4–6 mV) [9,10]. The inhibition of the second component of ATP synthesis which was observed at high gramicidin concentrations caused an increase in the rate of electron transfer from water to MV and decrease of the $\Delta H^+$ value (fig.1). In this case the gramicidin effects are easily explained in terms of the classical chemiosmotic model [11].

(iii) The properties of the PS II-related phosphorylation component depend on the ionic strength of the medium. Karlish and Avron [8], using low ionic strength, obtained an alternative picture of the effect of low gramicidin concentrations on the electron-transfer rate in pea chloroplasts. We succeeded in reproducing the results of this study using low ionic strength in chloroplast samples with low phosphorylating activity.

(iv) As to the quantitative differences between the two phosphorylation components in chloroplasts, it should be noted that DCMU-sensitive phosphorylation (in the presence of PMS) is realized at higher $\Delta H^+$ values than ATP synthesis in the presence of MV. These results are consistent with the data of Junge [12]. It could be seen that the transmembrane phosphorylation functions at higher $\Delta H^+$ values than phosphorylation sensitive to low gramicidin concentrations (figs 1–3).

Thus, the available data show that under steady-state conditions ATP-synthetase complexes in chloroplasts exhibit two qualitatively different modes of operation. The first is connected with PS II and possibly with the $H^+$-binding domain; it can operate at relatively low $\Delta H^+$ values. The other is realized at high $\Delta H^+$ values, and in this

case ATP synthesis proceeds at the expense of $\Delta\bar{\mu}H^+$ energy. The low gramicidin concentration selectively shunts the PS II-related component of phosphorylation.

It was essential that our experiments were performed under conditions such that a considerable part of the ATP-synthetase complexes and PS II proteins were spatially separated within the thylakoid membrane [13]. The model of the direct PS II–domain–ATP-synthetase interaction considered here implies that under our experimental conditions a definite part of the ATP-synthetase complexes remains in proximity to PS II.

On the other hand, the observed spatial separation of PS II and ATP-synthetase [13] leaves possibilities for alternative explanations. In discussion of the present results with Professor V.P. Skulachev he suggested that in all cases in chloroplasts, phosphorylation proceeds in accordance with the chemiosmotic scheme [11], but gramicidin acts as a stronger uncoupler in the case of the PS II–ATP-synthetase system than in the case of the PS I–ATP-synthetase system due to significant spatial separation of the pools of PS II and ATP-synthetase. This supposition needs an additional explanation of the fact that under conditions of PS II operation, low gramicidin concentrations change neither $\Delta H^+$ nor the electron-transfer rate.

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