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Effects of monofunctional sulfhydryl reagents on the proton permeability of pea thylakoid membranes

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Abstract

The effects of dark and light labeling of the sulfhydryl groups within the CF_i complex with monofunctional maleimides on the dark proton efflux from pea chloroplasts have been studied by measuring the kinetics decay of the proton gradient in the dark. It was found that the steady-state value of the light-induced proton gradient (ΔpH) under nonphosphorylating conditions markedly decreases when both NEM and SLM bind light accessible thiol groups, even at very low (10 μM) maleimide concentrations. Concomitant with this was a considerable increase of the decay rate constant k_d of the dark proton leakage. Resolving non-coupled proton efflux mediated by CF_1 complex from the passive proton diffusion on a kinetic basis, it is suggested that the increased dark proton efflux could be attributed to the stimulation of proton permeability through the CF_1 complex.

ABBREVIATIONS

9Aa	9-aminoacridine
CF_1	chloroplast coupling factor 1
DTT	dithiothreitol
NEM	*N*-ethylmaleimide
SLM	*N*-(2,2,6,6-tetramethylpiperidine-1-oxyl-4-yl)maleimide
$[H^+]_i$,$[H^+]_0$	internal and external proton concentrations
H_x	number of protons accumulated by thylakoids
C_H	proton binding capacity of thylakoids
J_H	rate of proton efflux
k_d	rate constant for dark proton efflux
P_H	proton permeability of the thylakoid membranes

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INTRODUCTION

Two general types of sulfhydryl groups were recognized on the γ subunit of CF_1, i.e., one is freely accessible for maleimides in the dark and the second is exposed during light-induced conformational transitions in chloroplast CF_1 [1–3]. The light accessible thiol sites in the γ subunit have been generally considered to play an essential role for the synthesis and hydrolysis of ATP [2,3] and in the proton gating mechanism through CF_1 [4].

Although the modification of the light accessible thiol group in the γ subunit of CF_1 complex with mono- and bifunctional alkylating reagents resulted in an effective inhibition of photophosphorylation [1–7], Moroney et al. [6] have proposed that NEM is largely energy transfer inhibitor as distinct from the bifunctional maleimides which exhibit predominantly uncoupling activity [4–7]. However, in an earlier study Portis et al. [8] suggested that the energy-dependent conformational changes within the CF_1 complex may result in an increased H^+ efflux from the chloroplasts possibly through CF_1 complex. Underwood and Gould [9] also showed in electron transport measurements under non-phosphorylating conditions, that pretreatment of chloroplasts in the light with NEM caused H^+ efflux through the CF_1 complex, thus implying an intra-enzyme uncoupling effect of NEM. More recently it has been reported that labeling of light accessible sulfhydryl group in the γ subunit of CF_1 complex might be responsible for the ΔpH shift towards lower values when the chloroplasts were pretreated with monofunctional maleimides in the light [10]. Despite these data the specific role of sulfhydryl residues within CF_1 complex, and particularly in its γ subunit in H^+ transfer remains not fully elucidated. In the present study the effects of monofunctional sulfhydryl reagents (NEM and SLM) on the proton permeability were examined by direct measurement of the kinetics decay of the proton gradient in the dark, by the procedure essentially as described in ref. 11.

EXPERIMENTAL

Pea chloroplasts were prepared as described previously (12). Dark and light modifications of thylakoid membranes with various concentrations of NEM and SLM were performed essentially as described in ref. 10. A two-fold excess of DTT was added after the incubation to bind the unchanged maleimides. Finally the labeled chloroplasts were washed twice in the incubation buffer. Light-induced 9-aminoacridine (9Aa) fluorescence quenching and estimation of ΔpH and $[H^+]_i$ were carried out as described by to Schuldiner et al. [13] using a Jobin Yvon JY3 spectrofluorimeter. The red actinic illumination (180 $\mu E\ m^{-2}\ s^{-1}$, > 600 nm, Schott RG 11 cut-off filter) was provided on the top of cuvette by a 1000 W halogen lamp (NARVA) linked with the fluorimeter by a fiber optic light source. The 9Aa fluorescence was excited at 390 nm and measured at 450 nm (slits = 4 nm). The reaction medium contained 50 mM Tricine (pH 7.8), 0.33 M sucrose, 50 mM NaCl, 5 mM $MgCl_2$, 50 μM PMS, 3.5 μM 9Aa and thylakoid concentration corresponded

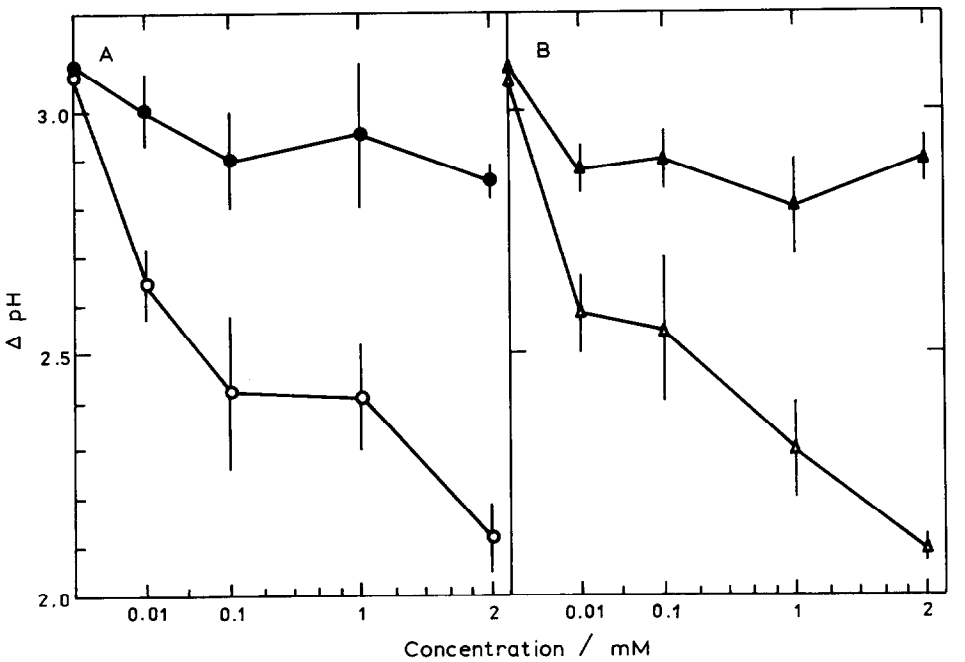

Fig. 1. Effects of dark (●, ▲) and light (○, △) labeling of pea thylakoid membranes with sulfhydryl modifying reagents NEM (A) and SLM (B) on the light-induced proton gradient (ΔpH) in photosystem 1 (+PMS) electron flow system. The time of incubation with either NEM or SLM was 2 min. Other experimental conditions are given in the Experimental.

to 25 μg Chl/ml. A thylakoid osmotic volume of 0.0067 m^3 (mol Chl)$^{-1}$ [14] was used to calculate the ΔpH. Estimated ΔpH values were corrected for binding of 9Aa as described by Slovaček and Hind [15]. Proton uptake was measured simultaneously with a glass combination electrode in a magnetically stirred cuvette using a Radelkis pH meter (Type OP-207). The amount of H^+ taken up (in nmol/mg Chl) were estimated by titration with 5 mM HCl as in described in ref. 16.

RESULTS AND DISCUSSION

The effects of dark and light labeling of the sulfhydryl groups within CF_1 complex on the light-induced difference between the external and internal proton concentrations (ΔpH) of pea thylakoids were examined under non-phosphorylating conditions using the 9Aa fluorescence quenching technique as proposed previously [13].

Fig. 1A, B indicates that in PMS-mediated (Photosystem 1) electron flow the dark labeling with monofunctional alkylating reagents (NEM and SLM) does not affect markedly the ΔpH values at any of the maleimide concentration tested. It is also seen that the modification of the light accessible thiol group(s) with both NEM

and SLM causes a sharp decrease (about 0.5 pH unit) of the ΔpH values even at very low maleimide concentrations (10 μM), and drop to 2.0 at concentrations of 2 mM, the differences between dark and light labeled thylakoids being statistically significant ($p < 0.01$, $n = 5$). The extent of the ΔpH decrease is almost equal in the NEM and the SLM labeled thylakoids, implying that this effect does not depend on the chemical nature of *N*-substituents of the maleimides used.

These results are similar to the effect of bifunctional maleimides on the ΔpH [4–7], but contradict the earlier data, indicating that NEM has no effect on the light-induced proton uptake [1] or on the magnitude of the ΔpH [8]. We are unable to explain this discrepancy at present. However, it should be mentioned that Underwood and Gould [9] have reported that NEM stimulates the rate of the non-phosphorylating electron flow and this effect has been attributed to the decrease in the level of thylakoid energization. Although the assumption of Moroney et al. [6] that monofunctional maleimides are energy transfer inhibitors, their exact mode of action on the inhibition of photophosphorylation is not fully understood. Since the ATP synthesis is coupled to the proton efflux driven by the pH difference accross the thylakoid membranes [17], the direct measurement of the proton efflux presents the possibility of clarifying the effect of modification of the light accessible SH group in the γ subunit on the proton translocation.

It is well established that the proton efflux occurs by three separate leakage pathways: (1) passive diffusion through the lipid bilayer of the thylakoid membranes; (2) uncoupled proton leakage through the CF_1 complex and (3) facilitated transfer by the CF_1 complex in a reaction coupled to the formation of ATP [18–20]. Since under non-phosphorylating conditions only the two first pathways operate [11,19], the dark proton efflux was given by [20]:

$$J_H = P_H([H^+]_i - [H^+]_0) + k[H^+]_i/[H^+]_0 \quad (1)$$

where the first term denotes the passive (diffusion) mediated proton efflux and the second the uncoupled proton leakage through CF_1 complex. The dark proton efflux on the other hand decays with apparent first-order rate constant [21] and is found to be proportional to the total number of protons accumulated (H_x) by the thylakoids [11]:

$$J_H = k_d H_x \quad (2)$$

where k_d is the first-order rate constant of the dark proton efflux. Proton influx (determined by the rate of the electron transport multiplied by H^+/e) and efflux under non-phosphorylating conditions are equivalent at steady state illumination [8,22]:

$$(H^+/e)R_e = k_d H_x \quad (3)$$

where R_e is the rate of electron flow. In view of this the dark decay of the proton gradient was examined as a measure of the proton fluxes in maleimide modified pea thylakoids.

The data presented in Fig. 2A, B indicated that light labeling with both NEM and SLM resulted in a considerable increase of the first-order rate constant of the

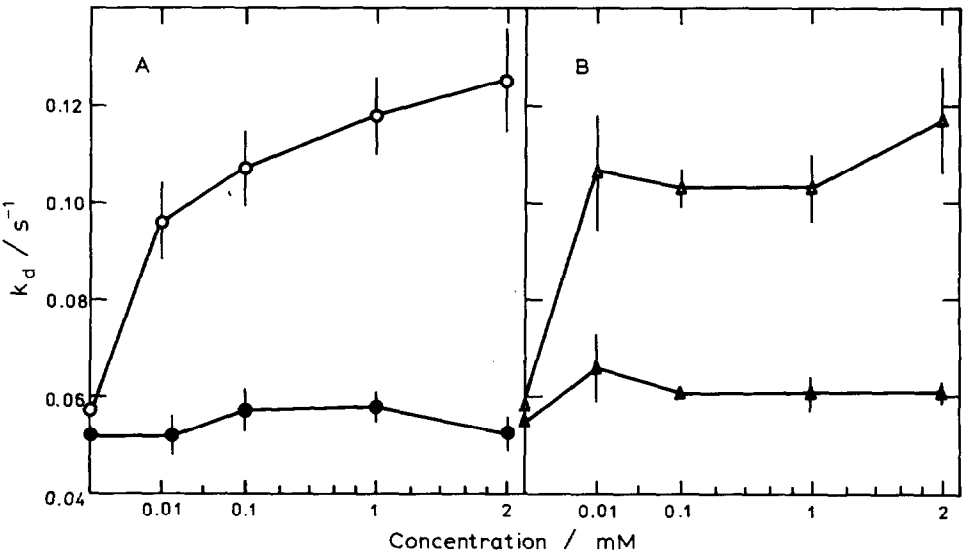

Fig. 2. First order rate constant (k_d) of dark proton efflux in pea thylakoids labeled with NEM (A) and SLM (B). (●, ▲) dark labeled; (○, △)light labeled. k_d was estimated from the kinetic analysis point by point of ΔpH decay time curves after turning off the actinic light as described previously [23].

dark proton efflux. Schönfeld and Kopeliovitch [11] have previously shown that k_d could be used as a measure of proton permeability using a simple relation of proportionality:

$$k_d = P_H/C_H \tag{4}$$

where C_H denotes the proton binding capacity. According to this, it appears very likely that maleimide modification of the light accessible thiol group within the γ subunit resulted in an increase of the proton permeability. However, as mentioned in ref. 11, k_d could be directly related to the P_H only if the C_H was not affected. In our experimental conditions the C_H values of 6.22×10^{-4} cm calculated from the slope of the line in Fig. 3 B (inset) remains constant and is very close to that in ref. 11. This is also consistent with the constant ratio between H_x and $[H^+]_i$ reported previously [23]. Hence, it is evident from Fig. 3A that the increased values of P_H reflect an increased proton leakage from the thylakoid membranes as a result of light labeling with NEM or SLM. Bearing in mind that under non-phosphorylating conditions and $pH_0 > 7.0$ (as in our experiments) the non-specific diffusion does not contribute practically to the proton efflux [24], it seems reasonable to assume that the increased P_H is due to the enhanced uncoupled proton leakage through the CF_1 complex. The fact that this leakage depends linearly on $[H^+]_i/[H^+]_0$ supports our assumption.

The results presented here are in full agreement with the published data [9] indicating that illumination of thylakoids in the presence of NEM enhanced the

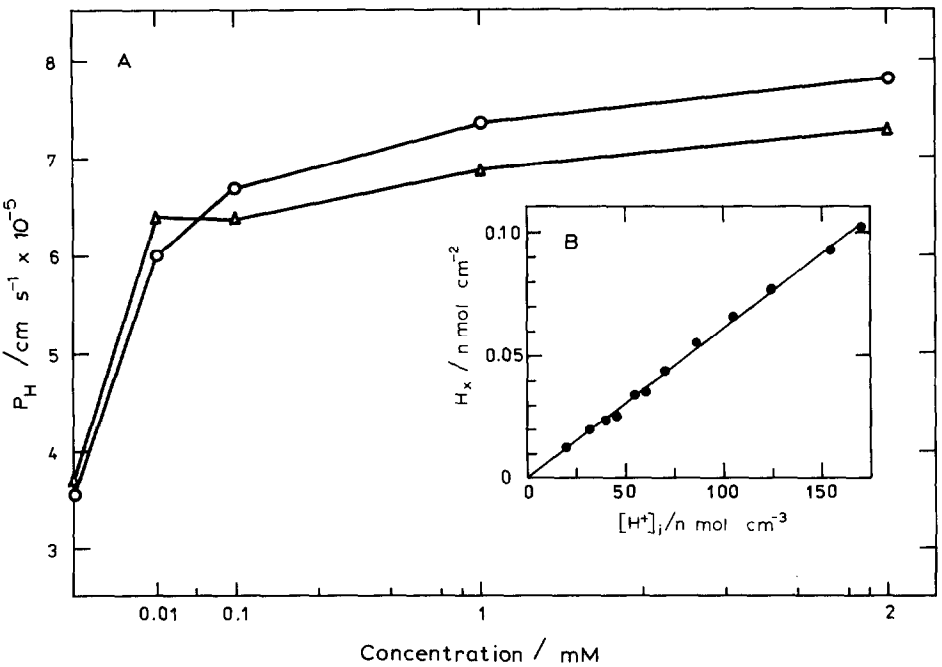

Fig. 3. (A) Effect of light labeling with NEM (○) and SLM (△) on the proton permeability (P_H) of pea thylakoid membranes. (B) Dependence of the extent of proton uptake H_x on $[H^+]_i$. H_x and $[H^+]_i$ were measured at different maleimide (NEM and SLM) concentrations. C_H was calculated essentially as described previously [11] using the eqn. $C_H = H_x/[H^+]_i$.

proton efflux through the CF_1 complex, due to the intraenzyme uncoupling, although this suggestion is based on the indirect measurements of the proton fluxes. Furthermore, in view of the data discussed above it appears that the monofunctional alkylating reagents cannot be regarded as pure energy transfer inhibitors as proposed previously [6].

Although the extent to which uncoupled proton efflux could operate simultaneously with the coupled efflux is questionable [19], it is quite possible to assume that intraenzyme uncoupling occurring by monofunctional maleimide modification of the light accessible thiol site in the γ subunit play an essential role, and might be involved (at least partially) in the proton translocation through the CF_1 complex and inhibition of photophosphorylation. The exact mechanisms however remain to be clarified. This requires direct kinetic analysis of the proton fluxes under phosphorylating conditions.

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