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Chloride-driven 3,3′-dipropylthiodicarbocyanine ($DiSC_3$-(5)) and tetraphenylphosphonium cation (TPP^+) uptake by thylakoids: inhibition of uptake by antibodies raised to the major polypeptides of the chloride efflux active particle(s)

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The membrane potential probes, 3,3′-dipropylthiodicarbocyanine ($DiSC_3$-(5)) and tetraphenylphosphonium cation (TPP^+), were employed as qualitative sensors of Cl^- translocation through the electrogenic Cl^- channel in thylakoid membranes. $DiSC_3$-(5) fluorescence was quenched in media containing Cl^- (outside > inside), suggesting that an inward-directed Cl^- diffusion potential resulted in additional uptake of $DiSC_3$-(5). Tri-*n*-butyltin chloride, which abolishes Cl^- gradients, largely abolished the Cl^--induced $DiSC_3$-(5) fluorescence quenching. Imposing a Cl^- gradient across the thylakoid membrane resulted in uptake of the radiolabeled membrane potential probe, TPP^+. The Cl^--induced [^{3}H]TPP^+ uptake was inhibited by polyclonal antibodies raised to the 62-kDa polypeptide of the Cl^--efflux active particle(s). These results suggest that the 62-kDa polypeptide may constitute an integral part of the Cl^- channel in thylakoid membranes.

Introduction

Our earlier studies on Cl^- transport involved isolation from detergent-treated thylakoid membranes of a particulate fraction which, upon reconstitution into artificial phospholipid vesicles, enhanced Cl^- efflux from vesicles [1]. Antibodies raised to these Cl^- efflux-active particle(s) which consist of four major polypeptides inhibited the K^+-driven Cl^- uptake by thylakoids. Only the antisera raised against the 62- and the 57-kDa polypeptides of the particle(s) inhibited Cl^- uptake [2]. These results suggest that a Cl^- channel may be present in thylakoid membranes and, furthermore, that the unidirectional influx of Cl^- via this channel might generate a negative transmembrane potential. In other plant systems for example, salt secretion from the *Limonium* salt gland leads to a negative secretory potential [3] while, in the excitable algae, the outward current is carried by Cl^- [3].

In the current study, electrogenic fluxes of Cl^- were established by adding the impermeant/permeant ion couple, choline chloride, to the reaction mixtures. The impermeant/impermeant couple, choline-Hepes, served as a control. The flu-

Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; $DiSC_3$-(5), 3,3′-dipropylthiodicarbocyanine, TPP·HBr, tetraphenylphosphonium bromide; SDS, sodium dodecyl sulfate.

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orescence quenching of $DiSC_3$-(5) [4] and the uptake of radiolabeled tetraphenylphosphonium cation (TPP^+) [5] induced by choline chloride gradients are interpreted as indicating that Cl^- diffuses across the membrane. Furthermore, the Cl^--induced TPP^+ uptake was inhibited by polyclonal antibodies raised to a 62-kDa polypeptide of the Cl^- efflux-active particle(s). These studies provide a new assay for measuring Cl^- movements across cell membranes independent of functioning cation channels.

Materials and Methods

Isolation of chloroplasts. Chloroplasts, after isolation [6], were swollen in a large volume of 5–10 mM choline-Hepes buffer (pH 7.7) and stored in a buffer containing 5 mM choline-Hepes (pH 7.7); 0.2 M sucrose and 5 mM KCl at a chlorophyll concentration of about 1 mg/ml. The transmembrane potential resulting from efflux or influx of Cl^- was monitored by the fluorescence quenching of $DiSC_3$-(5). For Cl^--driven [^{3}H]TPP^+ uptake measurements, chloroplasts, after a swelling step, were suspended in: 0.2 M mannitol, 10 mM choline-Hepes (pH 7.7) and 0.1 mM $MgCl_2$.

Buffers. A stock buffer containing 5 mM choline-Hepes (pH 7.7) and 0.2 M sucrose was supplemented either with concentrated choline chloride or choline-Hepes (pH 7.7) to obtain a series of buffers in which the choline chloride and choline-Hepes concentration each ranged from 1.0 to 25 mM.

Fluorescence measurements. For $DiSC_3$-(5) fluorescence measurements, excitation was set at 622 nm and emission was measured at 665 nm [4]. In order to retain constant initial fluorescence, stock $DiSC_3$-(5) was protected from light and a fresh pipette tip was used for each dye addition. After addition of $DiSC_3$-(5), the fluorimeter was set to 90% fluorescence. Thereafter, the instrument settings were not changed for the duration of the experiment. Fluorescence changes upon addition of KCl-equilibrated chloroplasts were expressed as percent fluorescence quenching calculated using the following relationship:

$$\frac{\text{initial fluorescence} - \text{final fluorescence}}{\text{initial fluorescence}} \times 100$$

= percent fluorescence quenching

Chloroplasts were mixed with the dye by one inversion of the cuvette and the extent of fluorescence quenching was measured. Each point plotted in Fig. 1b and c represents an average of triplicate determinations.

Immunoblotting. Analytical SDS-gel electrophoresis was performed on slab gel, 12 × 17.5 cm, 1.2-mm thick, as described previously [2] except that the running gel was 10% acrylamide and electrophoresis was carried out overnight at constant current of 12 mA.

After separation of polypeptides, the gel was cut into strips and the denatured polypeptide bands were electroblotted in a Bio-Rad apparatus on nitrocellulose strips. Transfer time was 3 h at 200 mA (50 V). The transfer buffer used contained per/l: 3 g Tris base (Bio-Rad), 9 g glycine (Bio-Rad), 20% methanol and 0.02% SDS (Bio-Rad). One nitrocellulose strip was stained with Amido black [7] to visualize the transferred proteins, the other strips were reserved for reactions with specific antibodies. Antigen-antibody interactions were carried out as directed in the Bio-Rad Immunoblot assay kit and the interactions were visualized with goat-anti-rabbit IgG horse radish peroxidase conjugate (Bio-Rad). Bovine serum albumin was used as a blocking agent and the first antibody incubation with appropriate nitrocellulose strips was carried out overnight in the presence of 0.1 mM NaN_3 and 0.1% Triton X-100.

Analytical methods. Chlorophyll was determined as described by Arnon [8]. Protein concentration was determined by the method of Lowry et al. [9] using bovine serum albumin as a standard.

Materials. $DiSC_3$-(5) was purchased from Molecular Probes, Inc. Hepes, choline base and methyl viologen were from Sigma; [^{3}H]TPP^+, 35.5 Ci/mmol, was purchased from New England Nuclear; disposable acryl cuvettes for fluorescence measurements were from Sarstedt.

Results

The presence of an electrogenic Cl^- channel in thylakoid membranes should permit the measurement of Cl^- translocation across the membrane in the presence of concomitant cation channel function. In principle, Cl^- diffusion through the elec-

trogenic Cl^- channel should lead to an imbalance of charges and, consequently, a development of a transmembrane potential, detectable with probes of membrane potential. The probes chosen were a positively charged fluorescent molecule, $DiSC_3$-(5) and radiolabeled TPP^+.

For each experiment, chloroplasts were washed with 5 mM choline-Hepes buffer (pH 7.7) and resuspended in 5 mM choline-Hepes buffer (pH 7.7) containing 0.2 M sucrose and 5 mM KCl. After resuspension, chloroplasts were incubated for 30 min to equilibrate the KCl concentration in the interior of the thylakoid with that of the medium. The KCl-equilibrated thylakoids were then added to media containing an equivalent amount of sucrose and buffer, but concentrations of Cl^- varying from 1–25 mM. In this manner, positive or negative ion diffusion potentials were imposed across the thylakoid membranes.

An increased quenching of $DiSC_3$-(5) fluorescence was observed at a 25-mM concentration of choline chloride when compared to the quenching observed in the same concentration of choline-Hepes medium (Fig. 1a). A summary of these experiments at various concentrations of ions are presented in Fig. 1, b and c. We interpret these results as indicating that the increased $DiSC_3$-(5) uptake results from the diffusion of Cl^- through the Cl^- channel. Fluorescence quenching of $DiSC_3$-(5) in the presence of the impermeant ion couple, choline-Hepes, served as the control for these experiments. The observed fluorescence quenching in the presence of choline-Hepes was probably due to the binding of the positively charged probe to the negatively charged thylakoids.

If the $DiSC_3$-(5) fluorescence quenching observed in the presence of choline chloride is indeed due to Cl^- diffusion potential, then dissipation of the Cl^- gradient should eliminate this diffusion potential and reduce the $DiSC_3$-(5) fluorescence quenching. Hence, the effects of tri-*n*-butyltin chloride, shown previously to equilibrate Cl^- across the membranes of *Halobacterium halobium* and thus dissipate the Cl^- gradient [10], were tested in thylakoids. Addition of tri-*n*-butyltin chloride to reaction mixtures containing 'swollen', KCl-equilibrated thylakoids reduced the fluorescence quenching in the presence of 25 mM choline chloride to the levels observed in the presence of 25 mM choline-Hepes (Fig. 1a, b and c).

TPP^+, the positively charged probe of trans-

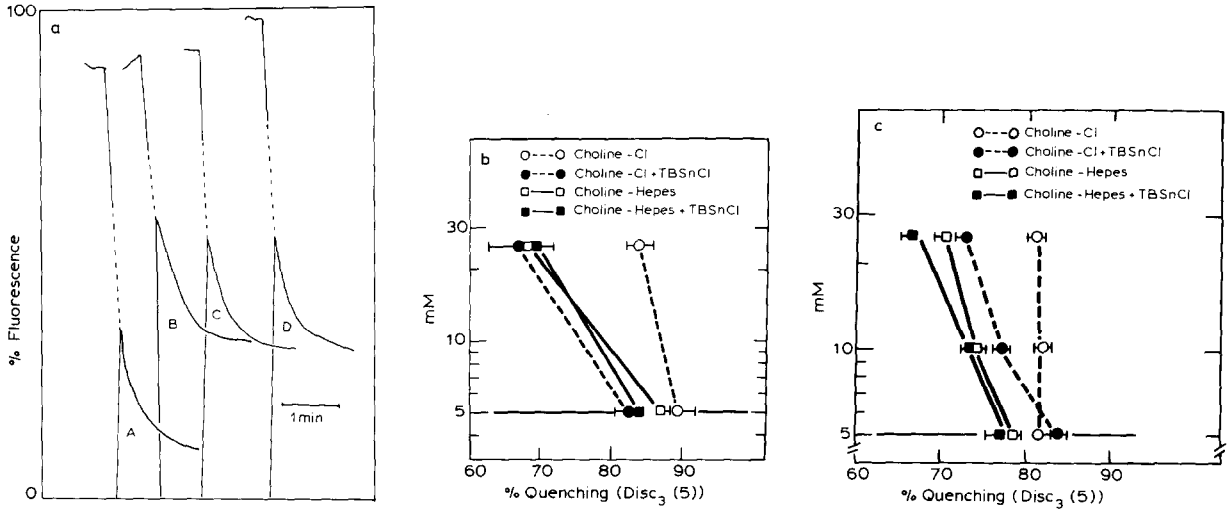

Fig. 1. Tri-*n*-butyltin chloride effect on $DiSC_3$-(5) fluorescence changes induced by addition of KCl-equilibrated chloroplast into media containing choline chloride. (1, b), $DiSC_3$-(5) concentration was 2.16 μM, the chlorophyll concentration was 1.70 μg/ml. In a: A, 25 mM choline chloride; B, 25 mM choline chloride + 9 μM tri-*n*-butyltin chloride; C, 25 mM choline-Hepes (pH 7.7); D, 25 mM choline-Hepes (pH 7.7) + 9 μM tri-*n*-butyltin chloride. (c) The $DiSC_3$-(5) concentration was 2.0 μM, chlorophyll concentration was 2.0 μg/ml and tri-*n*-butyltin chloride was 7.4 μM.

membrane potential was also used to detect the presence of a Cl^- diffusion potential. The results summarized in Fig. 2a show that the addition of choline chloride to thylakoids preequilibrated with [^{3}H]TPP^+ induced a rapid uptake of the labeled probe. Assuming 20 μl/mg chlorophyll to be the intrathylakoid volume, the [^{3}H]TPP^+ concentration within the thylakoid (Fig. 2a) would exceed the concentration of the medium by 160-fold. This [^{3}H]TPP^+ gradient remained stable for about a minute, after which time it decreased. The decline is probably due to the decay of the transmembrane potential which, in turn, results in a TPP^+ efflux. The [^{3}H]TPP^+ efflux is probably compensated by proton influx. A recent report indicates that there is a pathway which keeps the protons in the lumen of the thylakoid at equilibrium with the protons in the outside medium when the medium pH is above 7.5 [11].

The effects of the antibodies raised against the 62- and 57-kDa proteins on TPP^+ uptake induced by a Cl^- gradient were also tested. The antisera against the 62-kDa polypeptide completely inhibited Cl^- uptake, while the preimmune serum had no effect (Fig. 2b). Antibodies raised to soluble CF_1 had no effect on TPP^+ uptake.

Antibody effects on Cl^--driven TPP^+ uptake were examined also by flow dialysis technique [1]. Antiserum against the 62-kDa polypeptide inhibited Cl^--driven TPP^+ uptake. Maximal inhibition was achieved by 0.27 μl of serum/μg chlorophyll. Immune serum raised against the 57-kDa polypeptide was only slightly inhibitory at 0.62 μl of serum/μg chlorophyll. No inhibition was detected by antibodies raised against the isolated CF_1 at 0.77 μl of serum/μg chlorophyll. The size of choline chloride gradients in these experiments was 0.5–20 mM. Accurate calculations of the extent of inhibition by antibodies were not possible, since TPP^+ efflux rapidly followed TPP^+ uptake in runs with control sera.

Immunoblots were performed to confirm the presence of specific antibodies in the sera of rabbits immunized with the 62- and the 57-kDa polypeptides (Fig. 3). The Cl^- efflux-active particle(s) in one lane and proteins which were ammonium sulfate precipitated from crude membrane extracts in the other lane were subjected to electrophoresis

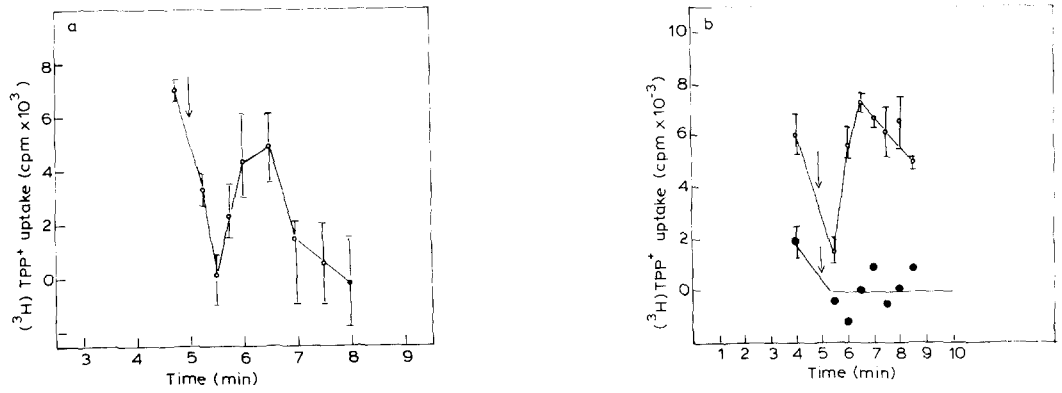

Fig. 2. [^{3}H]TPP^+ uptake and its inhibition by antibodies raised against the 62-kDa polypeptide. (a) Chloroplasts, after a swelling step, were stored in the reaction mixture from which TPP^+ was omitted. 2 ml of reaction mixture, containing 0.2 M mannitol, 10 mM choline-Hepes (pH 7.7), 0.1 mM $MgCl_2$ and 10 μM [^{3}H]TPP^+ ($4 \cdot 10^4$ cpm/nmol) were stirred slowly at 22° C for 4 min with thylakoids, 152 μg of chlorophyll. Two 200-μl aliquots were withdrawn and added to BF/C filters under vacuum and pre-wetted with unlabeled reaction mixture. The gradient was imposed (at the arrow) by addition of 400 μl of 0.25 M choline chloride. An aliquot was withdrawn immediately and filtered. Thereafter, aliquots were withdrawn at 15- or 30-s intervals. Unwashed filters were transferred to counting vials. Thylakoids on filters were solubilized with 10% SDS (200 μl). Chlorophyll was bleached by addition of 100 μl of 20% trichloroacetic acid. Vials were counted 8–16 h after addition of 10 ml of liquiscint, Counts trapped by filters (reactions without chloroplasts) were subtracted from reactions containing chloroplasts. (a) An average from triplicate determinations is plotted. (b) Immune or control sera, 125 μl, dialyzed against 10 mM choline-Hepes buffer (pH 7.7) which contained 1 mM NaN_3 were slowly stirred for 3 min at 22° C with 202 μl of chlorophyll prior to addition of reaction mixture. Thereafter, the experiments were carried out as in (a). Control serum, ○; serum raised against the 62-kDa polypeptide, ●.

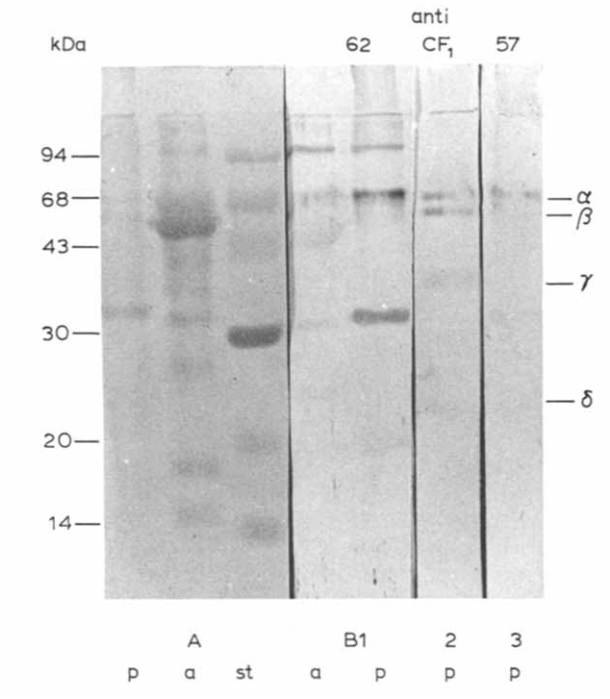

Fig. 3. Immuno reactions of antibodies raised against the 62-kDa, the 57-kDa and the CF_1 polypeptides with proteins of thylakoid membranes. A. Protein blotted on nitrocellulose stained with amido black; p, 76 μg Cl^--efflux active particle(s); a, 69-μg membrane proteins precipitating at 24–50% saturations of ammonium sulfate; st, protein standard, 94, 68, 43, 30, 20 and 14 kDa. B. Proteins blotted on nitrocellulose were reacted with antiserum against (1) 62-kDa polypeptide (1 : 25 dilution) as described in Materials and Methods; a, 104-μg membrane proteins precipitating at 24–50% saturations of ammonium sulfate; p, 114-μg Cl^--efflux active particle(s); (2) CF_1 (1 : 50 dilution); (3) 57-kDa polypeptide (1 : 25 dilution).

and immunoblotting using the antisera against the 62- and 57-kDa proteins as well as antisera against CF_1 as described under Materials and Methods. Antibodies raised against the 62-kDa polypeptide, in addition to reacting with the polypeptide of its own mass, reacted also with one large polypeptide of approx. 95 kDa and with one smaller polypeptide of about 32–35 kDa. These two additional polypeptides were present both in the crude extract and in the partially purified particle(s) (Fig. 3, lanes a and p under B1).

The antiserum against the 57-kDa polypeptide did not react with a protein of this molecular weight but did react weakly with the 62-kDa polypeptide (Fig. 3, lane B3). The presence of low levels of antibodies against the 62-kDa polypeptide in the antiserum raised against the eluted protein with a molecular weight of 57 kDa is consistent with partial inhibition of TPP^+ uptake by the antiserum against 57 kDa.

Cl^- efflux-active particle(s) which precipitated between 24–37% saturations of ammonium sulfate were contaminated by CF_1-CF_0 [12] which precipitates between 37–45% ammonium sulfate saturations. The immunoblots obtained with CF_1 antiserum clearly show that the subunits of CF_1 are present in the particle preparation (Fig. 3, lane B2). However, the antibodies raised against the 62-kDa polypeptide, isolated from the Cl^- efflux-active particle(s) [1], clearly react with polypeptides which are not recognized by antibodies against CF_1. The gel indicates that the 62-kDa protein has a slightly slower migration rate than the α subunit of CF_1.

Discussion

The data obtained using the positively charged membrane potential probe $DiSC_3$-(5) indicate that Cl^- can cross the membrane independently of cation movement. $DiSC_3$-(5) fluorescence quenching increased in choline chloride media (outside > inside) suggesting an inward directed Cl^- diffusion potential and an additional uptake of $DiSC_3$-(5). Tri-*n*-butyltin chloride, which causes Cl^-/OH^- exchange [10], decreased $DiSC_3$-(5) fluorescence quenching, suggesting destruction of the Cl^- diffusion potential.

The potassium gradient-driven chloride transport was inhibited to about the same extent by the antibodies raised against the 62-kDa and the 57-kDa polypeptides, each at a concentration of 0.45 μl of serum/μg of chlorophyll [2]. The chloride-driven TPP^+ uptake was strongly inhibited only by the antiserum against the 62-kDa polypeptide. Maximum inhibition was achieved by 0.27 μl serum/μg chlorophyll. The antiserum raised against the 57-kDa polypeptide was only slightly inhibitory. Contamination of the 57-kDa antiserum with the 62-kDa antiserum would account for the observed slight inhibition of the Cl^--driven TPP^+ uptake (Fig. 3). Since the same batches of sera were employed for the assays of both reac-

tions, the results may be interpreted to indicate that the 62-kDa polypeptide was involved in the catalysis of both the Cl^--driven TPP^+ and the K^+-driven Cl^- uptake. How could such a result be accounted for mechanistically: In the Cl^--driven TPP^+ uptake, which is insensitive to loop diuretics (unpublished observations), TPP^+ should diffuse through lipids and Cl^- through a proteinaceous Cl^- channel. The potassium-driven Cl^- transport, on the other hand, is inhibited by the loop diuretics [1], which inhibit KCl cotransport systems, and by the antibody raised against the 62-kDa polypeptide. Our diverse observations may become understandable if the KCl cotransport system were to involve parallel (coupled) operation of a cation and an anion channel. On the other hand, an asymmetric, electrically coupled $K^+/2Cl^-$ carrier [15], susceptible to loop diuretics, could also account for the inhibition of both reactions by the antibody against the 62-kDa polypeptide.

Furthermore, while the Cl^- efflux-active particle(s) are contaminated by proteins of CF_1, the antiserum against the 62-kDa protein which inhibits Cl^- movements recognized polypeptides not recognized by antibodies to CF_1. The antiserum against CF_1 did not inhibit Cl^- movement [2].

Chloride movements are widespread in nature. Band 3 (molecular weight 95 000) in red blood cell membranes catalyzes a neutral anion exchange [13]. Halorhodopsin is a light-dependent Cl^- pump [10]. Movement in *Mimosa pulvini* is associated with Cl^- efflux from motor cells [3]. Salt secretory activity of *Limonium* salt gland develops on exposure of leaves to the salt. Inhibitors of RNA and protein synthesis prevent the appearance of secretory activity, suggesting that the secretory apparatus is inducible [3]. Proton pumping by the purified uncoupler protein (molecular weight 30 000) isolated from the mitochondria of adipose tissue, requires added Cl^- [14]. Further studies are needed in order to assess possible similarities between the anion channels in thylakoid membranes and these others systems.

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