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Spermine is a potent modulator of proton transport through LHCII

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ABSTRACT

The effect of spermine on proton transport across large unilamellar liposomes containing incorporated complexes of the PSII antenna has been studied with the application of a pH-sensitive dye entrapped inside the vesicles. Both monomeric LHCbs and trimeric LHCII increased the permeability of proteoliposomes to protons when in a partly aggregated state within the lipid membrane. We have previously shown that a spermine-induced conformational change in LHCII results in its aggregation and ultimately in the enhancement of excitation energy as heat (*qE*). In this paper, spermine-induced aggregation of LHCII was found to facilitate proton transport across the proteoliposomes, indicating that a second protective mechanism (other than *qE*) might exist and might be regulated *in vivo* by polyamines when photosynthesis is saturated in excess light.

Introduction

The chloroplast thylakoid membrane of photosynthetic organisms is the site where light energy is primarily converted. An extensive system of membrane-associated light-harvesting pigment–protein complexes (LHCs) serve as the antenna that absorbs and delivers solar energy to the reaction centres of photosystems II (PSII) and I (PSI), where primary charge separation takes place. The two photosystems energize the electron transfer chain (ETC) from water to $NADP^+$ which is coupled with a proton flow across the photosynthetic membrane. The accumulation of these protons in the inter-thylakoid membrane space (lumen) generates a transmembrane gradient of protons, called the proton motive force (*pmf*) which comprises two components, the proton concentration gradient (ΔpH) and the membrane potential ($\Delta\psi$). *Pmf* is converted into ATP through ATP synthase according to the chemiosmotic hypothesis (Mitchell, 1966; Ioannidis and Kotzabasis, 2014). In addition to its role in driving ATP synthesis, the ΔpH component of *pmf* acts as a key signal in regulating energy dependent quenching (*qE*) in the photosynthetic antenna (Wraight and Crofts, 1970; Briantais et al., 1979; Muller et al., 2001).

qE is the major component of non-photochemical chlorophyll fluorescence quenching (NPQ), a photoprotective mechanism that dissipates the excess absorbed light energy as heat within the PSII antenna (LHCII), preventing photoinhibition. Under light-saturating conditions, overaccumulation of protons in the lumen occurs and a high ΔpH is formed. Low lumen pH changes LHCII antenna conformation/organization activating the quenching pigment(s). *qE*-related spectroscopic signatures *in vivo* have been correlated with those observed when the purified LHCII adopt quenched states upon aggregation *in vitro* (Horton et al., 1991; Ruban et al., 1991, 1993a,b, 1998; Miloslavina et al., 2008; Ballottari et al., 2010). In addition to the protective energy dissipation, a physiological role of LHCII aggregation in controlling the ion fluxes across the thylakoid membrane under high-light conditions has also been suggested (Wardak et al., 2000; Iwaszko et al., 2004). It is known that proton permeability of LHCII-containing membranes is twice as high compared to control pure lipid vesicles (Iwaszko et al., 2004).

A number of studies from our group have implicated polyamines (PAs) in the stimulation of *qE* (Ioannidis and Kotzabasis, 2007; Ioannidis et al., 2009, 2011; Tsiavos et al., 2012). The main PAs [putrescine (Put), spermidine (Spd) and spermine (Spm)] are found *in vivo* in chloroplast and bound to LHCII (Navakoudis et al., 2007), while upon illumination they accumulate in the lumen (Ioannidis et al., 2012). Based in previous work with amines it is anticipated that amines could reach 90 mM in the lumen for a concentration

Abbreviations: LHCII, light-harvesting complex II; Chl, chlorophyll; PAs, polyamines; Spm, spermine; Spd, spermidine; Put, putrescine; ΔpH, thylakoid proton gradient; k_{H^+}, rate constant for proton permeability; *qE*, ΔpH-dependent quenching of chlorophyll fluorescence; β-DM, dodecyl-β-D-maltoside; PTS, 8-hydroxypyrene-1,3,6-trisulfonic acid; Val, valinomycin; H^+, protons; PC, phosphatidylcholine.

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of about 2 mM in the stroma (Gaensslen and McCarty, 1971). Upon shuttering of actinic light it is anticipated that ΔpH between stroma and lumen will be minimized and polyamines will "return" to the stroma. Recently, PAs were found to stimulate, at physiological pH, fluorescence quenching of both trimeric LHCII and monomeric LHCb complexes *in vitro*, mimicking to a great extent the action of protons (Tsiavos et al., 2012). Spm was the most potent quencher and induced aggregation of LHCII trimers, due to its highly cationic character. In the present work, we studied the effect of the aggregation state of LHCII on non-specific proton permeation across lipid membranes of liposomes. Based in the results obtained, a new protective role of Spm is discussed.

Materials and methods

Materials

Egg phosphatidylcholine (egg-PC) and cholesterol of the highest purity were purchased from Avanti (Avanti Polar Lipids Inc., Alabaster, Alabama). The non-ionic surfactant *n*-dodecyl-β-D-maltoside (β-DM), spermine, valinomycin, 8-hydroxypyrene-1,3,6-trisulfonic acid (PTS) and sephadex G-100 were obtained from Sigma (Sigma-Aldrich, St. Louis, MO). SM2 BioBeads were purchased from Bio-Rad (Bio-Rad Laboratories Inc., Hercules, CA) and polycarbonate filters from Avestin (Avestin Inc., Ottawa, Canada). All other reagents were of analytical grade.

Preparation of LHCII complexes

Thylakoids from spinach leaves were isolated according to Bassi et al. (1985). The LHCII was isolated as described in Tsiavos et al. (2012). The Chl *a*/*b* ratio of the trimeric LHCII preparation was 1.36, indicating that each monomer contained about 7 Chl *a* and 5 Chl *b* molecules (Kühlbrandt et al., 1994; Ruban et al., 1999). The Chl *a*/*b* ratio of LHCbs was 1.8.

Preparation of vesicles

The production of unilamellar liposomes and proteoliposomes with a low ionic permeability is illustrated in Fig. 1 and was carried out in four stages according to Rigaud and Lévy (2003): (1) preparation of preformed large, unilamellar liposomes, (2) solubilization of liposomes, (3) membrane protein reconstitution and (4) proteoliposome formation upon detergent removal.

Liposome preparation

At first, large homogeneous and unilamellar liposomes were prepared from egg-PC and cholesterol in a molar ratio of 9:1. Lipids were dried from chloroform in a stream of nitrogen gas to give a thin film in a glass flask. To this thin film a solution of 10 mM Tricine (pH 7.5), 50 mM KCl containing 4×10^{-4} M PTS, a fluorescent pH-sensitive probe, was added. The solution was mixed in a vortex mixer to form multilamellar vesicles. The suspension was then passed through an extruder (21 times) using a 0.2 μm pore size polycarbonate filter in a LiposoFast extrusion system to form large unilamellar vesicles of 180–200 nm diameter (Mayer et al., 1986; Reimhult et al., 2003).

Solubilization of liposomes by dodecyl maltoside

The higher β-DM concentration needed to be added to the liposome suspension in order to reach the onset of solubilization, was calculated by the equation given by Lambert et al. (1998): $D_{total} = D_{water} + R_{sat} \times [lipid]$, in which D_{total} is the concentration of the detergent to be added, [lipid] is the lipid concentration, D_{water} is the aqueous monomeric detergent concentration and R_{sat} is the detergent-to-lipid ratio in detergent-saturated liposomes. Given that D_{water} of β-DM in the presence of lipids is 0.3 mM (or 0.15 mg/ml), R_{sat} is 1 mol β-DM/mol lipid (or 0.625% w/w) and the lipid concentration 1.25 mg/ml, the final concentration of β-DM added was 0.65 mM; this is far below the critical concentration for lipid–detergent micelle formation. The liposomal suspension was stirred for 1 h at room temperature before protein addition.

Incorporation of LHCII proteins into liposomes

To test the effect of LHCII aggregation on proton permeability, we prepared proteoliposomes containing preincubated and non preincubated LHCII samples with SM2 BioBeads or Spm (Fig. 1). More particularly, in order to obtain proteoliposomes with integrated quenched LHCII, we preincubated LHCII samples for 1 h with SM2 BioBeads or for 15 min with 100 μM Spm. Unquenched or quenched LHCII (after the removal of the polystyrene beads) was added to the lipid–detergent suspension at a lipid-to-protein ratio of 80 (w/w) and was incubated for 1 h at room temperature before detergent removal and proteoliposome reconstitution. Note that an equal volume of buffer containing 14 mM Hepes (pH 7.5) without LHCII, was also added to the liposomal suspension before liposome reconstitution for control measurements.

Liposome and proteoliposome reconstitution

Liposome and proteoliposome reconstitution was performed by detergent removal using adsorption onto polystyrene BioBeads SM2. 0.05 g SM2 BioBeads were added per 0.5 ml of suspension and stirred for 1 h at room temperature.

Removal of external probe

In the end, gel chromatography was applied to separate the PTS that had not been incorporated into liposomes and proteoliposomes. The used Sephadex G-100 packed column, had an internal diameter of 0.8 cm and 10.5 cm length. A solution of 10 mM Tricine (pH 7.5), 50 mM KCl was used as a mobile phase while the flow rate was adjusted to 0.27 ml/min. Combined detection of UV–vis absorption spectra was applied to identify the fractions of liposomal and proteoliposomal suspensions.

Kinetic measurements

Proton permeability across lipid membranes was monitored fluorometrically using a LS-50B spectrophotometer luminometer of Perkin Elmer (Perkin Elmer, Waltham Massachusetts). The PTS-containing liposomes and proteoliposomes were subjected to an external acidic pulse and changes in internal fluorescence were monitored as a function of time. The emission was detected at 513 nm and the excitation was set at 402 and 452 nm (at the main maxima of the absorption bands of protonated and non-protonated forms of PTS, respectively). The fluorescence intensity of PTS at 402 nm (F_{402}) and 452 nm (F_{452}) was used to monitor the acidification kinetics of the liposome and proteoliposome interior. The build-up of a potential counteracting H^+ release in the liposome interior was prevented by addition of valinomycin (a K^+ ion carrier, in an ethanolic solution) 40 s after the beginning of the recording at a final concentration of 3 μM. The proton gradient across the membranes was generated 2 min after the valinomycin addition by injection of a small volume of 0.67 N HCl into the cuvette. The suspension was acidified to a pH level of 5.7 (*versus* pH 7.3 inside the vesicles). The pH changes inside liposomes and proteoliposomes were calculated on the basis of a calibration curve (Fig. 2). The fluorescence intensity of the main band of PTS at 402 nm was found to reach its maximum at pH 6.45 and therefore could not be used for the estimation of the exact pH values inside vesicles (Fig. 2). On the contrary, the fluorescence intensity of the non-protonated form of PTS at 452 nm showed a higher sensitivity to acidic pH