Small heat shock protein-membrane interaction

Summary of the Ph.D. Thesis

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INTRODUCTION

All organisms are known to interact with their environment. This may involve a considerable stress to the organism that has to respond to this challenge by means of some specific cellular processes. A number of cellular constituents can be damaged, e.g. DNA, proteins, membrane-forming lipids. **Membranes** are engaged in the preservation of cell integrity, in the structural and functional organization of cell components as well as in signalling processes. On the other hand, as judged from their intracellular location, structure and chemical composition, they are potential targets of various kinds of stress. They **are**, however, **not just targets of stress, but also function as active sensors of changes in the physiological condition of the cell.** As such they initiate stress responses such as expression of heat shock proteins and changes in the composition and dynamics of membrane lipids, ensuring thereby cell acclimatization. **Heat shock proteins (Hsps) have been proved to bind to and stabilize membranes. Among Hsps small Hsps (sHsps) are particularly efficient in membrane protection.**

The aim of this study was to investigate the mechanism and the functional importance of interaction between sHsps and membranes (focused on **mainly membrane lipids).** For this purpose we used two prokaryotes as experimental objects:

a/ a photosynthetic cyanobacterium, ***Synechocystis* PCC 6803**, because (i) it has but one sHsp, (ii) the beneficial effect of which is easily detectable by measuring photosynthetic activity and (iii) it is an excellent model of higher plant organisms;

b/ ***Escherichia coli*** (i) to explore the general nature of sHsp function on cellular membranes and (ii) to reveal the possible importance of having two existing sHsps instead of one as in *Synechocystis* PCC 6803.

LITERATURE DATA AND AIMS

I. The only sHsp of ***Synechocystis* PCC 6803**, **Hsp17**, is known to interact with several proteins during heat stress, keeping them in a refolding competent state for subsequent recovery (Giese and Vierling, 2002 and 2004). This sHsp also **binds to and stabilizes thylakoid membranes protecting them against heat and photoinhibitory effects of light exposure**, by which it confers higher stability on photosynthetic function (Török et al., 2001; Asadulghani et al., 2003; Nitta et al., 2005). By means of the selective modulation of the membrane lipid phase it was concluded that **the expression and membrane association of Hsp17 are correlated with the physical state (fluidity) of thylakoid membranes** (Horváth et al., 1998).

1/a In order to get further knowledge about the Hsp17-membrane interaction as a mean of heat acclimation we aimed at **revealing the factors that are involved in the acquisition of higher thermal stability of thylakoid membranes** paralleled with **the development of acquired thermotolerance of the photosynthetic apparatus**.

We aimed:

− to investigate the role of heat/light preconditioning in the acquisition of thermotolerance (by measuring the photosynthetic activity of heat/light or dark-acclimated cells),

− to test the changes in composition, physical properties, structure and organization of thylakoid membranes during heat acclimation,

− to characterize the changes in membrane lipid phase, and

− to analyze the mechanism of action of potential stabilizing factors and probable specific/ selective sHsp-lipid interactions.

1/b To get an insight into **the molecular mechanism of Hsp17-lipid interaction** we **modified** the head group and/or fatty acid region of the **lipid component** used in the ***in vitro*** experiments.

2. By modifying the oligomeric state and/ or potential lipid interactive motifs of Hsp17 we intended to get detailed knowledge about the mechanism of interaction between the sHsp and membrane (lipids) both *in vivo* and *in vitro*. Meanwhile we

addressed the question **whether the sHsp-lipid interaction could be engineered**. In collaboration with the laboratory of E. Vierling (Tucson, AZ, USA) we tested cells expressing point mutants of Hsp17 and the purified recombinant proteins themselves. These cells and proteins differed from their wild type counterparts in terms of their thermotolerance and their oligomeric state as well as *in vitro* chaperone activity, respectively (Giese and Vierling, 2002 and 2004; Giese et al., 2005). In addition to the WT controls we investigated a series of point mutant cells and the purified proteins themselves (S2Y, L9P, Q16R, L66A, F102S, V143A), which gave us an excellent oppurtunity to study both the direct and indirect effects of protein modification on its physical properties and its lipid interaction.

II. The ***Escherichia coli*** sHsps **IbpA and IbpB** are known to protect soluble proteins from aggregation caused by extreme, long term heat effects *in vivo* (Kuczynska-Wisnik et al., 2002). Overexpression of these sHsps, however, was shown to confer thermotolerance on cells even upon short term heat exposure (Kitagawa et al., 2000). This implies that **IbpA/B may protect essential cellular components or compartments other than soluble proteins**. ***E. coli*** **sHsps are** believed to be **localized to membranes** (Miyake et al., 1993; Laskowska et al., 1996; Kuczynska-Wisnik et al., 2002). This is supported by the finding that **selective modulation of the membrane lipid phase triggers heat shock response and leads to the acquisition of thermotolerance** (Shigapova et al., 2005). Based on the above data **we decided to check the potential role of IbpA and IbpB in the heat acclimation of *E. coli* cell membranes**. To this end **we compared the membrane phenotype** (permeability, fluidity and fatty acid composition) **of wild type cells with that of ΔIbpAB cells *in vivo* and tested the interaction of lipids and purified recombinant proteins** (IbpA and IbpB) ***in vitro*.** Moreover, **to define the direct effect of sHsps on heat acclimation of cellular membranes, to differentiate between their function and to see whether or not they cooperate in membrane protection, we investigated the membrane phenotype of ΔIbpAB cells expressing exogenous IbpA, IbpB or IbpAB.**

METHODS

1. *Synechocystis* PCC 6803

Synechocystis cells were grown photoautotrophically in BG-11 media at 30 °C, and cultures in exponential growth phase were used in the experiments. Cells were kept at 30 °C as controls, and exposed to 42 °C for 3 h in the light or in the dark to explore light and heat acclimatization. Cells expressing point mutant Hsp17s were created by introducing integratively pNaive plasmids bearing the actual hsp17 into the double mutant cells (Δ*hsp17*, Δ*clpB*).

Heat inactivation of photosynthesis was followed by measuring the oxygen evolution of cells. Heat stability of protein complexes of PSII was tested by visualizing the heat-induced profile of steady-state chloropyll *a* fluorescence in intact cells. UV-B tolerance of PSII complexes in heat-hardened cells expressing point mutant Hsp17s was tested by measuring photosynthetic oxygen evolution.

Fluidity vs. temperature profile of isolated thylakoid membranes was determined by measuring steady-state anisotropies. Microheterogenous organization of these samples was characterized by lifetime analysis of DPH probes embedded in membranes.

Recombinant proteins of wild type and point mutant Hsp17s were expressed in *E. coli*. Cells were disrupted by sonication, then sHsps were purified by means of ammonium-sulfate precipitation, sucrose gradient cenrifugation and chromatography (DEAE ion exchange and „HiTrap Butyl" hydrophobic columns). Protein concentration was determined by the microBCA method.

In sHsp-lipid binding experiments liposomes (LUVs) and purified proteins were incubated together. The liposomes were pelleted and the protein content of lipid bound and not bound fractions was analysed by SDS-PAGE. In experiments carried out by using ^{14}C-labelled LUVs, the lipid content of the lipid bound proteins analysed by SDS-PAGE was revealed by autoradiography.

2. *Escherichia coli*

E. coli wild type (MC4100), ΔIbpAB or ΔIbpAB cells carrying plasmids expressing IbpA, IbpB, IbpAB were grown at 30 °C in LB media. Cell cultures in exponential growth phase were used in the experiments. Heat shocked cells were exposed to 50 °C for 15 min, unless membrane permeability was measured. In this case cells were incubated at a temperature range of 30-70 °C for 15 min.

After cell disruption with „French press", outer and inner membranes were isolated by a variety of biochemical separation methods (e.g. 3-step sucrose gradient centrifugation).

Heat-induced damage to the membrane permeability barrier was followed by NPN fluorescence measurements. Each of the different cell lines was compared to the ΔIbpAB cells.

Membrane (peripherial and hydrophobic) fluidities of wild type and ΔIbpAB cells kept at 30 °C or heat shocked were determined by measuring steady-state TMA-DPH and DPH anisotropies.

General methods

Protein composition of different samples was analyzed by SDS-polyacrylamide gelelectrophoresis (SDS-PAGE) by using Coomassie staining.

Total cell lysates and membraneous or cytosolic fractions were probed by anti-Hsp17, anti-GroEL/Cpn60, anti-DnaK and anti-IbpAB antibodies.

Lipids were extracted from cells, isolated membranes and liposomes by using a mixture of chloroform: methanol (1:2) as an organic solvent. Total neutral lipids (TNL), total polar lipids (TPL) or individual lipid classes were separated by thin layer chromatography (TLC). Fatty acid composition of lipid samples was determined by GC-MS analysis. Analysis of the molecular species of lipids in the samples was carried out by LC-MS technique.

^{14}C-labelled total polar lipids were prepared from cells grown in BG-11 media containing ^{14}C-$NaHCO_3$. 1-oleyl-2-palmitoyl-phosphatidyl-glycerol (OPPG) was

synthetized from 1-oleyl-2-palmitoyl-phosphatidyl-choline (OPPC) by phospholipase D-catalyzed heterogenous reaction. Isolated lipids were fully hydrogenated by PdRh-PVPP catalyst-assisted heterogenous reactions.

As model membranes multilamellar vesicles (MLV), small unilamellar vesicles made of MLVs by sonication or large unilamellar vesicles (LUVs) made of MLVs by extrusion method were used.

I*n vitro* sHsp-lipid interaction and the stability of pure lipids were characterized (i) by testing monomolecular lipid layers or (ii) by measuring the fluidity of liposomes at their lipid head group (TMA-DPH anisotropy) or hydrophobic region (DPH anisotropy). (i) To examine the physical properties of pure lipids, the area vs. temperature profile of monomolecular lipid films spread on the buffer-air interphase was followed at constant lipid pressure. To explore protein-lipid interaction the initial surface pressure of lipid layers was adjusted to desired values. Proteins were injected underneath the lipid films, and subsequent surface pressure change proportional to the extent and typical of the kinetics of interaction was detected. (ii) Membrane fluidity of hydrophobic interior of lipid membranes (SUVs) made of lipids isolated from *Synechocystis* and liposomes (LUVs) incubated with Hsp17s was followed by DPH anisotropy measurements. Fluidity of LUVs incubated with purified recombinant proteins (IbpA, IbpB and IbpAB) was investigated by applying both TMA-DPH and DPH fluorescent dyes.

RESULTS AND CONCLUSIONS

I. *Synechocystis* PCC 6803

1/a We have studied the heat/light acclimation of ***Synechocystis*** cells, thylakoid membranes and photosynthetic function.

(**i**) We concluded that **heat-primed acclimation** (3 h, 42 °C) of *Synechocystis* cells **resulted in the thermal stabilization of photosynthesis** (PSII activity) **exclusively in the light**. In parallel, a great **increase in thermal stability and microdomain reorganization of thylakoid membranes** was observed only in cells challenged by heat stress in the light.

(**ii**) Analysis of membranes isolated from cells exposed to a sublethal heat stress in the light revealed that changes both in protein and lipid composition are likely to be involved in a short term acclimation process. In addition to a general lowering of the double bond content of lipids, **a highly saturated MGlcDG, "heat shock lipid" appeared in the heat/light-acclimated membranes**. This lipid is able to stabilize membrane (lipid phase) by its special physical properties (its head group and extremely saturated fatty acid regions). Thylakoid membranes could also be stabilized by **Hsp17** that **preferentially interacts with non-bilayer phase forming lipids (MGlcDG, MGDG)**. All in all, the "heat shock lipid" and Hsp17 are effective members of the stress defensive machinery of cyanobacteria. The highly saturated **MGlcDG and Hsp17** are probably involved in the retailoring of membrane ultrastructure in heat/light-hardened cells **confer**ring **higher thermal stability on thylakoid membranes and related photosynthetic function**.

(**iii**) By analyzing the mechanism of action of the potential stabilizing factors we concluded that both **Hsp17 and the "heat shock lipid"** modify the fluidity and the transition temperatures of (lipid) membranes in a way to ensure extension of membrane physiological state. To this end both **are able to hinder membrane hyperfluidization and the formation of non-bilayer strutures**.

1/b Analysis of the mechanism of Hsp17-lipid interaction by the means of modifying the interactive lipid component revealed that **Hsp17** differentiates between lipid molecular

species. This **"species recognition" depends mainly on the nature of the lipid head groups, but is also influenced by the fatty acid length, position and unsaturation level**.

2. To get a deeper insight into the mechanism of interaction between *Synechocystis* Hsp17 and membrane (lipids), we tested cells expressing point mutants of sHsp and the purified recombinant proteins themselves. These cells and proteins differed from their wild type counterparts in terms of their thermotolerance and their oligomeric state as well as *in vitro* chaperone activity, respectively.

(**i**) When mutations resulting either in a stable dissociation of sHsp oligomers to dimers (L66A, F102S, V143A) or in a sHsp oligomer with reduced abilility to dissociate (S2Y) were tested, it turned out that **dissociation and reassociation of the Hsp17 oligomer is necessary to enable sHsp to stably interact with membrane (lipid)s**.

(**ii**) **Dimers (possibly monomers) appear to associate with lipid membranes.**

(**iii**) N-terminal point mutant proteins (L9P and Q16R), which did not differ from the WT counterpart in terms of their oligomeric state, proved to behave differently with respect to their ability to associate with lipids and to protect thylakoid function. L9P had a lesser, Q16R a higher membrane activity than the WT protein. These results led us to conclude that **the N-terminal part of the protein is responsible for membrane (lipid) interaction**.

(**iv**) **In heat/light-acclimated cells the Q16R protein was localized exclusively to thylakoid membranes. By its superior membrane activity** this mutant **protects thylakoid (PSII) function against damages caused by photoinhibitory UV-B stress.**

(**v**) Results obtained from *in vitro* protein-lipid interaction studies are correlated with those describing the *in vivo* behaviour (membrane localization and protection) of Hsp17s. All things considered, **the *in vivo* membrane activity of Hsp17 is likely governed (at least partly) by sHsp-lipid interactions**.

II. *Escherichia coli*

To explore the general nature of sHsp function on cellular membranes and to reveal the possible importance of having two existing sHsps we have studied the potential role of *Escherichia coli* sHsps, IbpA and IbpB in the heat acclimation of bacterial membranes.

(**i**) We found that **IbpA/B gets localized to cell membranes** (especially to the outer membrane) **during heat stress**.

(**ii**) By comparing the membrane phenotype (permeability and fluidity) of wild type cells with that of ΔIbpAB cells we concluded that, in parallel with the membrane localization of IbpA/B, **ΔIbpAB cell membranes are more permeable and more fluid at the membrane periphery as compared to the WT during extreme heat stress**.

(**iii**) Since **IbpA, IbpB and IbpAB markedly decreased the fluidity of lipid membranes at the head group region *in vitro***, deletion of these sHsps could indeed result in an altered membrane phenotype observed *in vivo*. **Exogenous expression of IbpA, IbpB or IbpAB in ΔIbpAB cells partly restored the WT membrane phenotype.** This finding **also suggests the direct role of sHsps in membrane protection**.

(**iv**) Unlike their rather slow protein protection IbpA/B proved to preserve membrane integrity during even short term, exterme heat stress.

(**v**) Our ***in vivo* and *in vitro* results show that IbpA must play more decisive role than IbpB in membrane protection**. IbpB is, however, more effective in protein protection, which implies a highly organized IbpA-IbpB cooperation in different cellular compartments.

(**vi**) **Deletion of ibpAB resulted in the fluidization of membrane hydrophobic interior.** Although sHsps are slightly able to reduce the fludity of lipid membranes at the hydrophobic core, control membranes of ΔIbpAB cells were still more fluid than those of WT cells. This suggests that the presence of sHsps in the membranes and the regulation of the fludity of membrane interior are not directly correlated. We speculate that **this is rather regulated by changes in the fatty acid composition. Deletion of ibpAB resulted in a unique change in the fatty acid composition of cell membranes (an increase in the amount of 18:1v)** irrespective of heat conditions. Fatty acid composition of cell membranes was influenced by all the expression of IbpA, IbpB or IbpAB. This implies that **the regulation of fatty acid composition and fluidity of hydrophobic region of cell membranes is affected by sHsps** at least in an indirect manner.

Possible practical importance of our results

The present study showed that by revealing the function and the mechanism of sHsp-membrane (lipid) interaction, **modifications either in protein structure** (see point mutations) **or lipid composition** (see e.g. MGlcDG) **could be engineered**. **The effect of these modifications could be predicted on thylakoid membrane function or**, in general, **cell acclimatization**. Such basic information (e.g. Q16R mutant) may help in the development of **transgenic plants** characterized with e.g. **higher drought resistance**.

Since sHsps and their association with membranes are apparently involved in a number of phenomena of practical importance (drought resistance of plants, infections, autoimmune, degenerative, cardiovascular diseases, cancer, signalling processes, etc.), controlled modification of sHsp-membrane interaction may lead to a breakthrough in the solution of at least some of the above problems.

LIST OF PUBLICATIONS

(*Articles and manuscripts the Ph.D. thesis is based on)

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