Ph.D. Thesis

Alterations of membrane physical state regulate the *E.coli* heat shock response

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INTRODUCTION AND AIMS OF THE STUDY

Membranes represent one of the most thermally sensitive macromolecular structures, however, they are not simply the targets of heat-induced damage. Recent data support the notion that changes in the physical state of biological membranes influence the expression of heat shock and others genes (Vigh et al, 1998). It is also now evident that the specific composition of membrane lipids is affected during thermal acclimation to compensate for the fluidizing/rigidifying effects of temperature. Such data strongly suggest that the lipid matrix of membranes, acting as “cellular thermometers”, play an essential role in stress signalling and adaptation.

In order to test the validity of the “membrane sensor” hypothesis, Escherichia coli, a gram-negative bacterium, was used as a cellular model to re-examine critically the evidence for and against the involvement of membranes (both outer and inner) in determining the response of cells to heat stress and stress caused by the membrane fluidizing agent benzyl alcohol (BA).

The major goal of this work was to correlate the physio-chemical properties of cell membranes to the adaptation and resistance of E. coli, to both physical (heat) and chemical (BA) stress. The study aimed to elucidate the common and differing elements of the stress response occurring in cellular membranes caused by external stress signals of a different nature (high temperature and membrane fluidizing agent), by observing changes at the membrane level. It was expected that signals generated within the membranes might trigger the heat shock response (HSR) and acquisition of cellular thermotolerance in a similar manner. Our studies, therefore, addressed the validity of the “membrane sensor” hypothesis in E.coli, which was chosen as our model organism due to its simplicity and because it is biochemically and genetically well characterized. The fact that E. coli possesses two membranes also allowed us to investigate its different cellular compartments. A further aim of this study was to promote methodology available to test such stress sensing systems. Consequently, a reporter system was developed to study the transcription of heat shock genes, including heterologous promoter sequences of cyanobacterial heat shock genes, in an E. coli host. Moreover, we examined whether “foreign” genes were still under membrane fluidity control of the host.

Ultimately, we aimed to re-examine the evidence for and against the involvement of membranes in determining the response of cells to stress and cellular acquisition of
thermotolerance. We tried to apply several biophysical techniques to carry out a comparative study of the effect of both stressors on the membrane, namely:

- membrane fluidity was measured using the hydrophobic fluorescent probe 1, 6-dyphenyl-1, 3, 5-hexatriene (DPH).

- membrane permeability was investigated using the lipophilic fluorescent probe 1-N-phenyl-naphthylamine (NPN)

- atomic absorption was used to estimate potassium ion efflux

- FACS (Flow cytometry) examined the overall inner membrane integrity

- both outer and inner membranes separated by ultracentrifugation in a sucrose density gradient were used in differential scanning calorimetry (DSC) measurements to analyze the thermotropic phase behaviour of the membrane lipids

- membrane lipids were separated on thin layer chromatography and fatty acid composition was analysed by gas chromatography

- reporter systems were developed to investigate the expression of cyanobacterial heat shock promoters in an *E. coli* host.

**RESULTS**

**A. Membrane involvement in stress adaptation management: physico-chemical characteristics of membrane properties upon heat and BA treatment**

Gram-negative bacteria like *E. coli* possess two membranes, the inner or cytoplasmic membrane and an outer membrane with differing lipid and protein composition. Cytoplasmic and outer membranes were isolated and the composition of their proteins, lipids and fatty acids as well as their lipid phase state was determined.
A consistent relationship could be established between specific fatty acid changes and the capacity of cells to acquire cellular thermotolerance. Augmentation of saturated fatty acids both in wild type *E.coli* and its σ\^{32} mutant, grown either at high temperature (43°C) or in the presence of BA, supported the view that such alterations in membrane lipids account at least partially for heat adaptation.

Notably, a very pronounced basal level difference was also detected in the fatty acid composition of the σ\^{32} mutant compared to its isothermally grown wild type counterpart. Altered membrane lipid phase states, determined *in vitro* by measuring thermal transition of lipids from gel to fluid phase using DSC, could be seen both upon the exposure of isolated membranes to BA or in membranes isolated from cells grown at different temperatures or pre-adapted to BA. Changes observed in the temperature of gel-to-fluid lipid phase transitions could be interpreted as thermoadaptive responses by highlighting typically different tendencies in the outer and inner membranes. The effects of BA were more pronounced in the outer membrane, either with preadapted BA grown cells or with cells instantaneously treated with BA. Notably, the kinetics of BA-induced “hyperfluidization,” which was followed by a slow recovery period, and was measured on whole cells by DPH anisotropy assay was in good accordance with DSC data.

By using two complement *in vivo* assays (FACS and K\(^{+}\) efflux determination), the permeability of cell membranes was investigated. We showed that a broad temperature range or prompt administration of BA challenges the passive permeability of both the outer and inner membranes in a similar way to heat stress. However, evidence of adaptive membrane rearrangements was seen mostly in the outer membranes. Both heat and BA treatments resulted in “hyperfluidization,” a partial disruption of the permeability barrier and induced the transcriptional activation of heat shock genes. It is noted, that the maximal activation of specific heat shock genes (*dnaK, groESL, ibpA and ibpB*) was obtained at different levels of the disorganizations of membranes. However, it was also noted that although BA treatment caused transcriptional induction of heat shock genes, unlike heat, it failed to induce heat shock protein (Hsp) synthesis. This might be explained by the fact that BA did not produce significant effects on protein stability and denaturisation as observed with heat treatment. These findings not only support the “membrane sensor” model, but might also imply that differently localized “sensory membrane domains” are engaged in the activation of different Heat shock genes.
B. Heat shock response and acquired thermotolerance

To understand the relationship between the HSR, synthesis of Hsps and the acquisition of thermotolerance we investigated the survival of cells preadapted to a specific growth temperature and then briefly exposed to elevated temperature. As with Synechocystis PCC 6803 (Synochosystis from here on), E. coli cells grown at elevated temperature showed increased resistance to thermal stress. Moreover, pre-treatment with BA also induced acquired thermotolerance mimicking the effect of sub-lethal heat treatment. Sublethal heat treatment of E. coli induced Synthesis of Hsps (GroEL and DnaK), while BA induced only transcription from such genes and accumulation of Hsp protein was not evident. Since the amount of the two most abundant heat shock proteins, GroEL and DnaK, remained basically unchanged during BA treatment, one might speculate that the perturbant acted at the level of membrane fluidity, rather than protein denaturation. Indeed, the DPH-anisotropy-time profile on whole cells showed that development and subsequent decay of BA-induced membrane hyperfluidization was consistent with the operation of fluidity compensatory membrane rearrangements that are analogous to thermal shift induced homeostatic optimisation of membrane fluidity. Our data support the idea that thermotolerance can be acquired in the absence of Hsp synthesis.

C. Development of the novel reporter system to study heterologous regulation of the cyanobacterial heat shock gene promoters in E.coli host

Using an extreme thermostable enzyme, 1,3-1,4 beta glucanase or “lichenase”, as a novel reporter system we showed, that the promoter regions of both Synechocystis chaperonins, groESL and cpn60, containing no obvious $\sigma^{32}$ recognition sequences, are able to direct the expression of lichenase in E.coli. Moreover, heat stress and the membrane fluidizer BA are equally able stimulate the operation of these cyanobacterial heat shock gene promoters in E.coli, especially that of groESL. The regulation of such $\sigma^{32}$ independent promoters supports the idea that fluidizing affects at the membrane trigger another mechanism(s) that can influence the activity of heat shock promoters.
SUMMARY AND CONCLUSIONS

A comparative study of HSR upon high temperature growth and BA treatment, on membrane fluidization was carried out in the gram-negative bacterium *E. coli*. The evidence for and against membrane involvement in HSR and acquired thermotolerance caused by both physical (heat) and chemical (BA) stress has been re-examined.

1. Both sublethal heat exposure and BA treatment led to the acquisition of thermotolerance but seemingly the mechanisms involved appear to be different. Notably BA did not cause protein denaturation and there was no induction of major Hsps as observed with heat treatment.

2. However, both stressors had a strong fluidizing effect, which triggered a stress adaptive response that lead to a recovery of the membrane fluidity back to its basal level.

3. Heat and BA caused analogous changes in fatty acid composition and in the ratio of the lipid classes, as detected by gas chromatography. The amount of saturated fatty acids among all lipid classes increased significantly at the expense of their unsaturated counterpart. There was a significant decrease in the ratio of the non-bilayer forming lipid, phosphoethanolamine. Notably, analogous changes in lipid/fatty acid composition occurred in the $\sigma^{32}$ mutant indicating that membrane adaptive changes occur independently of Hsps in the cell.

4. Examination of isolated outer and inner membranes revealed that the functionality of both membranes was affected upon heat and BA treatment. As with heat, BA had a direct effect in both types of cells (WT and its $\sigma^{32}$ mutant). It was noted that the outer membrane permeability barrier was disturbed and the inner membrane became leaky for potassium ions, however, these changes were not reflected in the survival of cells and the overall inner membrane integrity.

5. DSC measurements with heat and BA treated cells showed that similar changes in the thermotropic phase behaviour of membrane lipids in both outer and inner membranes. Membrane lipids of *E.coli* adapted to heat or BA showed a broad
temperature phase transition reflecting cell membrane adaptation, whereas BA non-adapted cells did not reveal such an adaptive response when exposed to BA.

6. Analysis of cellular compartments (cytosol, periplasm and membranes) revealed that a common set of proteins was induced in response to heat and BA treatment. However, proteins specifically induced by each stressor were also seen. The proteins identified had very diverse cellular functions and included Hsps, transcription regulating factors and metabolic enzymes.

7. Despite the fact that the HSR was not equally induced by heat and BA at the translational level, a Northern blot analysis showed that heat shock genes were still activated even in the σ^{32} mutant, although the precise spectrum of genes activated did vary.

8. Methodology to study heat shock gene regulatory elements upon various stress inducers was developed. A novel reporter system was constructed using an extremely thermostable enzyme 1,3-1,4 glucanase (lichenase) from the thermophilic bacterium Clostridium thermocellum. It was demonstrated that Synechocystis heat shock promoter elements were recognized in E. coli and, moreover, they were under membrane fluidity control in this host.

Our results demonstrate that selective perturbation of the membrane’s physical state (fluidity, permeability, lipid phase transitions) appears to be sufficient criterion for triggering acquired thermotolerance and transcriptional activation of heat shock genes in E. coli. It was also shown that E. coli is an appropriate host to study the membrane physical state dependent expression of “foreign”, cyanobacterial chaperonin genes. Our data is additional evidence to the operation of a more complex, multicomponent sensory system in which membranes represent one of the cellular “thermosensors”.

We assume, that documented association of certain Hsps with membranes by causing membrane rigidification (Török et al, 1997 and 2001; Tsvetkova et al, 2002) may also result in an inactivation of the membrane-perturbation signal, thereby turning off heat shock genes in a negative feedback loop. On the basis of the model proposed above, the modulation of membrane physical order may repress
transcription of heat shock genes in the heat-modified state, explaining the known temporary nature of induction of the stress response. Therefore, such proposed “cross-talk” between the membrane-located sensor(s) and the HSR suggests the existence of an as yet unknown feedback mechanism of heat shock gene regulation. We believe that this proposed mechanism of transcriptional heat shock gene activation and attenuation mediated by membrane physical state may also be operative for other genes.

LIST OF REFERENCES, PUBLICATIONES, POSTERS AND ORAL PRESENTATIONS

References:


**List of publications:**


Posters:


Oral presentation: