Chapter III

Studies on the role of the membrane perturbants CCCP and ethanol on the translocation of the periplasmic protein alkaline phosphatase in Escherichia coli
Chapter III

Introduction

3.1 Study on the induction of AP in *E.coli*, grown in the presence of CCCP or ethanol

3.2 Study on the location of AP (if induced) in *E.coli* grown in the presence of CCCP or ethanol

3.3 Study on the role of CCCP and ethanol on the folding of cytosolic AP precursor

3.4 Conclusion

PAGE

58

58

61

63

65
Introduction

This chapter of the thesis deals with our observations on the role of membrane perturbants CCCP and ethanol on Escherichia coli at the level of translocation of membrane proteins from the cytoplasm (the region of their synthesis) to the different specific membrane locations (the regions of their functional activities). In E. coli, the source of energy for translocation is both the ATP and the PMF across the cytoplasmic membrane [Eichler & Wickner, 1997; Manting & Driessen, 2000; Mori & Ito, 2001]. The PMF is resulted from a combination of the membrane potential [$\Delta \psi$] and the pH gradient [$\Delta p$] [Mitchell & Moyle, 1969]. CCCP is a well-known protonophore i.e. it has proton-uncoupling activity, in consequence of which it dissipates the PMF across the energized cell membrane [Zoratti & Szabo, 1995; Ichas & Mazat, 1998; Akiyama, 2002]. The well-known membrane perturbant ethanol, besides having multiple actions on cell membrane altering the membrane structure and function [Ingram & Butke, 1984; Ingram, 1986], is also believed to be a PMF-dissipator [Enekist et al., 1981]. Therefore, both CCCP and ethanol, by their PMF-dissipating function, are supposed to affect the transport of the membrane proteins across the E. coli plasma membrane. In order to study, in depth, the role of CCCP and ethanol on the translocation or export of the membrane proteins in vivo, the inducible periplasmic protein alkaline phosphatase (AP) of E. coli was chosen. The reasons behind the selection of AP were that due to the inducible nature of the AP gene, (i) its synthesis could be regulated as-and-when basis and (ii) the action of CCCP or ethanol could be studied even at the level of onset of expression of a gene.

3.1 Study on the induction of AP in E. coli, grown in the presence of CCCP or ethanol:

AP, a nonspecific phosphomonoesterase, can generate inorganic phosphate from a variety of phosphorylated derivatives. Its synthesis is regulated by the end product repression i.e. the addition of phosphate to the media inhibits the expression of AP gene;
in other words, phosphate starvation induces AP synthesis [Brickman & Beckwith, 1975]. Therefore, growth of E.coli cells in the phosphate-free medium induces the expression of AP gene.

For the induction of AP, cells of E.coli MPh42 strain were initially grown to the log phase (up to \( [\text{O.D}]_{600\text{nm}} = 0.3 \) i.e. \( 1.5 \times 10^8 \text{ cells/ml} \)) at 30°C in MOPS medium, washed two times with and finally suspended in the same volume of phosphate-less MOPS medium. The cells were then distributed in different parts to treat with the various concentrations (0, 10, 30, and 50 \( \mu \text{M} \)) of CCCP or (0, 2.5, 5.0, 7.5 and 10.0 \% v/v) of ethanol and were further allowed to grow at 30°C for the induction of AP. At different times of growth, a 2.0 ml aliquot was withdrawn from each of the samples; 1.0 ml was collected over 0.2 ml toluene to assay the AP activity, as described in section 2.4, and the remaining 1.0 ml was used to measure the bacterial O.D. at 600 nm. The figures 3.1A-D are obtained by plotting the experimental data.

![Fig. 3.1A: Bacterial growth curve in presence of different concentrations of CCCP](image)

![Fig. 3.1B: Effect of CCCP on the induction of active AP](image)

Fig.3.1A shows the growth inhibition pattern of E.coli MPh42 grown in phosphate-free medium in the presence of different concentrations of CCCP, the growth practically stopped in the presence of 50 \( \mu \text{M} \) CCCP. Fig.3.1B shows that at any instant of growth, the amount of induced active AP appeared to fall gradually by the presence of increasing concentrations (0 – 50\( \mu \text{M} \)) of CCCP. No active AP was found in cells grown in the presence of 50\( \mu \text{M} \) CCCP, the bacteriostatic concentration (as observed from fig. 3.1A). The figures 3.1A and 3.1B also indicate that both the growth rate as well as the
amount of active AP decreased considerably in the 30μM CCCP-treated cells compared to the untreated control cells; the effects were not so severe in cells grown in the presence of 10μM CCCP.

Similar results, as shown in figures 3.1C and 3.1D, were obtained by the growth of cells in the presence of different concentrations (0, 2.5, 5.0, 7.5 and 10.0% v/v) of ethanol. With the increase of ethanol concentration in the phosphate-less growth medium, bacterial growth was gradually inhibited, causing bacteriostatic condition at 10% v/v ethanol (fig.3.1C). At the same time when the AP was assayed, the amount of induced active AP was found to be reduced gradually with the increase of the concentration (0 – 10% v/v) of ethanol; at 10% v/v ethanol, no active AP was observed (fig. 3.1D).

It is known that AP is synthesized in the cytoplasm as the precursor molecule having an N-terminal signal sequence of 21 amino acids, which guides the nascent polypeptide to the cytoplasmic membrane; the signal sequence is ultimately removed proteolytically by signal peptidase after the protein is translocated out through the inner membrane [Kumamota et al., 1984]. After cleavage of the signal sequence, the enzymatically active AP is localised in the periplasm. Therefore, the CCCP- or ethanol-mediated fall of cellular AP unit might arise due to either of the following two reasons: 1) CCCP or ethanol acted at the level of AP induction i.e. the increase of the concentration of CCCP/ethanol in the growth medium gradually reduced the induction
of AP, ultimately causing no induction at a concentration of 50 \( \mu \text{M} \) CCCP or 10% \( v/v \) ethanol; 2) CCCP/ethanol not at all hindered the induction, but reduced the translocation of the induced AP precursors across the cytoplasmic membrane, ultimately causing total block of translocation at a concentration of 50 \( \mu \text{M} \) CCCP or 10% \( v/v \) ethanol. To find out the exact reason, the following western blot study of the different cell fractions (periplasmic, cytoplasmic and membrane) was performed using AP antibody. In all the subsequent experiments, only 50\( \mu \text{M} \) CCCP or 10% ethanol were used.

3.2 Study on the location of AP (if induced) in \textit{E.coli} grown in the presence of CCCP or ethanol:

Log phase grown cells of \textit{E.coli} MPh42 ([O.D]_{600nm} = 0.3 i.e. 1.5 \( \times \) \( 10^8 \) cells/ml) in MOPS medium were transferred to phosphate-free MOPS medium. The cell suspension in the phosphate-less medium was then divided in three parts – in one part 50 \( \mu \text{M} \) CCCP was added, in another part 10% ethanol was added, while the third part was treated as control. All the three parts were allowed to shake at 30\(^\circ\)C for induction of AP. After allowing induction for 20 minutes, different cell fractions (cytoplasmic, periplasmic and membrane) were isolated from 1.0 ml cell culture according to the method described in section 2.9. The fractions were then electrophoresed in 12% SDS-polyacrylamide gel, as described in section 2.6 and the 'western blot' study using rabbit antibody to AP.

![Fig 3.2: Western blot of the different fractions of E.coli MPh42 cells with antibody to AP.](image)

- lane a: pure AP; lanes b,e,h: periplasmic fraction, lanes c,f,i: cytoplasmic fraction and lanes d,g,j: membrane fraction of the untreated, CCCP treated and ethanol treated cells respectively.
anti-AP antibody (raised in our laboratory against E. coli AP, which was purchased from Sigma) was performed according to the method described elaborately in section 2.11. Protein bands in the gel were blotted onto nitrocellulose membrane, which was subsequently tested for the presence of AP using antiserum to AP as the primary antibody, HRP-conjugated goat anti-rabbit IgG as the secondary antibody and 4-chloro, 1-napthol as the HRP substrate. A part of each fraction was also assayed for AP activity, as mentioned in section 2.4.

The 'western-blot' result, shown in fig.3.2, clearly implied that the induction of AP was not at all impaired by the action of CCCP or ethanol. Fig.3.2 indicate that the induced AP, isolated from the 50μM CCCP-treated cells as well as from the 10% ethanol-treated cells respectively, was found in the cytoplasmic fraction (lane f & i respectively). No AP band was observed in the lanes c & g, where the periplasmic and the membrane fractions of CCCP treated cells and in the lanes h & j, where the periplasmic and the membrane fractions of ethanol treated cells were respectively loaded. On the other hand, in CCCP/ethanol-untreated control cells, majority of the induced AP was found in the periplasmic fraction (lane b, fig.3.2), a minor pool in the cytoplasmic fraction (lane c) and no trace of AP in the membrane fraction (lane d). The trace of little AP in the cytoplasm of the control cells (lane c, fig.3.2), perhaps, represented the amount of AP that was yet to be translocated to the periplasm. However, this result implied that CCCP or ethanol hindered the translocation of AP from the cytoplasm (the region of its synthesis) to the periplasm. The CCCP/ethanol-mediated inhibition of translocation was justifiable from the fact that both the CCCP and the ethanol, by their common proton uncoupling function, dissipated the pmf across the E. coli plasma membrane, in consequence of which the energetics for translocation was affected.

Table 3.1 : Units of AP present in the different fractions of the cells of E. coli MPh42 grown in phosphate-less medium for 30min for induction of alkaline phosphatase.

<table>
<thead>
<tr>
<th>Control (cells grown in absence of CCCP or ethanol)</th>
<th>Cells grown in the presence of 50μM CCCP</th>
<th>Cells grown in the presence of 10% Ethanol</th>
</tr>
</thead>
<tbody>
<tr>
<td>P</td>
<td>M</td>
<td>C</td>
</tr>
<tr>
<td>3.4432</td>
<td>0.0021</td>
<td>0.0546</td>
</tr>
</tbody>
</table>

* P, M and C stand for periplasmic, membrane and cytoplasmic fraction respectively.
Chapter III

The result of the AP assay of the fractions, shown in table 3.1, was in conformity with the result of the above immuno-blot study. Table 3.1 shows that, excepting the periplasmic fraction of the untreated control cells, no other fractions of the control cells and of the CCCP/ethanol-treated cells showed any considerable AP activity. The enzymatic active form of the signal-sequence-cleaved AP, localised in the periplasm, is a dimer; dimerization occurs through the formation of intra-chain disulphide bonds [Kim & Wyckoff, 1991]. AP cannot be enzymatically active when it retains in the cell cytoplasm specifically because its disulphide bond can not ordinarily be formed in the reducing environment of the cell cytosol [Derman & Beckwith, 1991; Derman et al., 1993]. Since AP can not take its active conformation in the reducing atmosphere of the cytoplasm, therefore, even after the expression of AP in the CCCP or ethanol-treated cells, the induced, non-exported AP showed no enzymatic activity (fig.3.1B & 3.1D and table 3.1).

3.3. **Study on the role of CCCP and ethanol on the folding of cytosolic AP precursor**:

In the previous study it was observed that by the growth of the E.coli cells in the presence of CCCP or ethanol, the induced AP had been accumulated in the cytoplasm. This internalized cytosolic AP had no enzymatic activity. The absence of AP activity was supposed to be due to non-attainment of the active conformation of AP in the reducing environment of the cell cytosol. To check whether due to the action of CCCP or ethanol, the AP was accumulated in the cell cytoplasm as the unfolded precursor form, the pulse-chase experiment with 35S-methionine and the subsequent immunoprecipitation study with AP antibody, were performed.

Cells of E.coli MPh42 were initially grown to log phase [up to (O.D)\textsubscript{600nm} \approx 0.3 i.e. \( \sim 1.5 \times 10^8 \) cells/ml] at 30°C in MOPS medium, where the methionine concentration was 1/10\textsuperscript{th} of that in normal MOPS medium. The grown cells were centrifuged, washed with, and finally suspended in phosphate-free and methionine-free MOPS medium. This cell suspension was divided in three parts – in one part 50 \( \mu \)M CCCP was added, in another part 10% ethanol was added, while the third part was treated as control. All the three
parts were allowed to shake at 30°C for induction of AP. After allowing induction for 30 minutes, 35S-methionine was added to the cells of each part at a concentration of 30μCi/ml. The cells were then allowed to grow and label at 30°C for a pulse of 90 seconds and the labeling was subsequently chased by 0.2M cold methionine. At 10 minutes of chasing, cell aliquots of 0.5ml were withdrawn to extract proteins for immunoprecipitation, according to the method of Oliver and Beckwith [Oliver and Beckwith, 1982, described in section 2.10]. To each extract, 3.0μl of antiserum to AP was added and the mixture was kept on ice to incubate overnight at 0°C. To this incubated sample, 60μl of protein A-CL agarose was added and further kept on ice for 20 minutes. The immunocomplex was washed and finally suspended in 50μl DTT-free gel loading buffer to keep and run the protein in 10% SDS-polyacrylamide gel under non-reducing condition. For autoradiography (as described in section 2.8) the gel after electrophoresis was fixed in 12% glacial acetic acid and 30% methanol solution for 30 minutes, rinsed in distilled water and soaked in 1M sodium salicylate (E.Merck) for 30 minutes. Gels were then dried and allowed to expose X-ray film (Kodak X-Omat) at -70°C for 48-72 hours.

The maturation of AP i.e. the cleavage of signal peptides well as the intra-chain disulphide bond formation was reported to occur only after a protein precursor had translocated through the plasma membrane. Therefore, by the run of the immunoprecipitates in the SDS-Polyacrylamide gel under non-reducing condition, shown in fig.3.3, the mature form (signal sequence-less and folded by intra-chain disulfide bond) moved faster than the precursor form (signal sequence-containing and having no intra-chain disulfide bond). Fig.3.3 signifies that when E.coli MPh42 cells, grown in phosphate-less medium in the presence of 50μM CCCP or 10% v/v ethanol, were allowed to label with 35S-methionine for a pulse of 90 s and then the labeling was subsequently chased with cold methionine for 10 min, the nascent AP precursor of the CCCP- or ethanol-treated cells was not matured during the chase period, while the

![Fig. 3.3. Autoradiograph of pulse chase and immunoprecipitation study. lane a: CCCP & ethanol untreated control cell; lane b: CCCP treated cell; lane c: ethanol treated cell, after chasing for 10 minutes.](image-url)
maturation occurred in the untreated control cells. Thus the above result implied that by the presence of 50μM CCCP or 10% v/v ethanol in the growth medium, AP was induced in E.coli, but the induced unfolded AP precursor could not be exported out from the cell cytosol. This result not only supplemented the result obtained from the 'western blot' study, but also corroborated the result of AP activity (fig.3.1) i.e. the zero activity of induced AP in 50μM CCCP / 10% ethanol-treated cells was due to the lack of maturation of AP.

3.4 Conclusion:

The above experimental results indicate that –

i) When the periplasmic protein AP was allowed to induce in E.coli by growing in phosphate-less medium, the presence of the increasing concentrations of CCCP (0 – 50 μM) or ethanol (0 – 10% v/v) in the growth medium gradually reduced both the cell growth rate and the activity of induced AP; in the presence of 50μM CCCP / 10% v/v ethanol, the growth rate as well as the AP activity were found to be nil.

ii) The zero activity of AP, induced in the presence of 50 μM CCCP or 10% v/v ethanol, was due to the storage of partially folded inactive AP precursor in the cell cytoplasm in stead of being transported out to the periplasmic space to attain the active dimeric conformation.

iii) The cytosolic storage of AP was caused by the CCCP- or ethanol-mediated dissipation of the pmf across the E.coli plasma membrane for which the translocation process was de-energized and the export of AP precursor from cytoplasm to periplasm was inhibited.

iv) Moreover, not only of inducible AP, the translocation of many other membrane proteins – inducible or constitutive - should be inhibited by the CCCP/ethanol-mediated dissipation of pmf. Studies, from other laboratories, in intact bacterial cells provided evidence that the dissipation of pmf blocked the translocation of
several outer membrane and periplasmic proteins [Baker & Randall, 1984; Zimmermann & Wickner, 1983; Daniel et al., 1981; Enequist et al., 1981].