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**Role of the HSP70 Homologue from Chloroplasts in
the Assembly of the Photosynthetic Apparatus**

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Title

Role of hsp70 homologue from chloroplasts in the assembly of
the photosynthetic apparatus

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C. ABSTRACT

The major goal of the proposed research was to study the role of a 70-kDa heat shock cognate protein from chloroplasts (ct-HSP70) in the assembly of chlorophyll-protein complexes. The latters are mostly important in allowing photosynthesis to occur. Photosynthesis is at the heart of crop productivity and the knowledge of the biogenesis of the photosynthetic apparatus is essential to manipulate the efficiency of photosynthesis.

The characterization of the function of the ct-HSP70 was planed to be studied in vitro by assaying its capability to physically interact with the thylakoid proteins and to assist their assembly into thylakoid membranes. We planed to identify regions in the light-harvesting complex protein (LHCP) that interact with the ct-HSP70 and characterize the interaction between them. We also intended to isolate cDNA clones encoding ct-HSP70s, sequence them, express one of them in *E. coli* and use the purified protein for functional assays.

The research in this BARD proposal aimed at providing insights and aid in understanding the mechanism by which plants may respond to the heat stress. Since plants often experience increased temperatures.

D. OBJECTIVES OF THE ORIGINAL RESEARCH PROPOSAL

The original proposal aimed to study the function of ct-HSP70 in the assembly of the photosynthetic apparatus in the thylakoid membranes. The specific objectives of the proposed research were:

1. Studying the role of ct-HSP70 in the assembly of pigment-protein complexes. Previous work in our laboratories has demonstrated the requirement of ct-HSP70 for insertion of the precursor of the major light-harvesting chlorophyll a/b-binding protein into thylakoid membranes. In the framework of this BARD proposal, we planned to further investigate the role of ct-HSP70 in the assembly of other thylakoid proteins and multiprotein complexes.
2. Mutational dissection of the interaction between ct-HSP70 and LHCP. We have developed a number of in vitro assays to characterize effects of ct-HSP70 on the refolding of denatured LHCP. We planned to take advantage of these systems and to identify regions of LHCP that are required for these interactions by using deletion mutants of LHCP. Site-directed mutations in the domains interacting with LHCP will be used to infer the nature of these interactions in more detail.
3. Molecular cloning, expression and mutagenesis of ct-HSP70. We planned to isolate the cDNAs for ct-HSP70s from cDNA libraries using oligonucleotide probes. We hoped that one of the isolated cDNA clones will be used for expression of HSP70 in *E. coli*, the protein will be purified and used for functional assays. We also hoped that the cDNA will be mutagenized to produce ct-HSP70 in which various regions of the protein are altered. Effect of these changes on the ability of ct-HSP70 to assist insertion of pLHCP and other proteins into the thylakoid membranes will be studied in the in vitro assay developed in our labs.

E. RELEVANT DATA

E1. Introduction:

Heat stress is one of the major affects that cause the decrease in photosynthetic activity. It was shown that the later is caused by a damage to the photosynthetic apparatus. The research proposed in this BARD grant aimed at the examination of the effects of temperature on the biogenesis and organization of the photosynthetic apparatus on the molecular level. Specifically the research focused on the role of the 70-kDa heat shock protein(s) from chloroplasts (ct-HSP70) in the assembly of chlorophyll-protein complexes.

ct-HSP70:

Molecular chaperones are a class of cellular proteins that facilitate transport, folding and assembly of other proteins. HSP70 comprises a major family of such chaperone proteins. It has been shown that HSP70, or its bacterial analog DnaK, keep a partially unfolded conformation of the proteins they bind, to allow translocation-competence across, or into, the ER, mitochondria and thylakoid membranes (1).

Recently, the involvement of a member of this class of chaperones, the chloroplast HSP70 (ct-HSP70) in the integration of pLHCP into the thylakoid membranes was demonstrated (2).

pLHCP, the apoprotein of the major light-harvesting complex of photosystem II, is synthesized in the cytoplasm and imported into the chloroplast in its precursor form. Following import, or incubation with stroma, pLHCP was found to form a soluble intermediate (of about 120KDa) with stromal factor(s) (3). The latter maintains the protein's solubility and its competence for membrane insertion. The interaction with HSP70 did not seem to be a part of this 120KDa complex.

In the present study several methods were used to characterize the interactions between ct-HSP70 and photosynthetic proteins such as pLHCP, the oxygen evolving 33kDa and others. .

In the first year of funding, the set-up of the experimental systems was achieved (see first annual report). In the second year, major efforts in

both labs were devoted to characterize the interactions of HSP70 on the molecular level. In the third year we further investigated the nature of these interactions and gained further insight into these mechanisms.

References:

1. Hohfeld, J.&Hartl, FU.(1994) Current Opinion in Cell Biology 6: 499-509.
2. Yalovsky, S. et al (1992) Proc. Natl. Acad. Sci. USA 89, 5616-5619.
3. Payan, L. A. & Cline, K. (1991) J. Cell Biol. 112, 603-613.

E2. Methodology:

* pLHCP - production & labeling:

pLHCP cloned in pDS vector, was overexpressed in E. coli in the presence of radioactive sulfate. The labeled inclusion bodies were isolated by low speed centrifugation. After washing the inclusion bodies, pLHCP was solubilized from them with 8M urea. The solubilized protein was dialyzed and incubated with the chloroplast's stromal fraction or with proteins isolated from it.

* Detection of pLHCP-ct-HSP70 complex formation:

pLHCP was incubated with stroma at 25⁰C for 15 minutes. The formation of a chaperon-pLHCP complex was then assayed by glycerol gradient centrifugation, native gel electrophoresis and gel filtration chromatography.

* Characterization of ion effects on the integration of pLHCP into the thylakoid membranes:

Labeled pLHCP was incubated with increased concentration of cations, like MgCl₂ or NaCl, in order to characterize their effect on the integration of the protein into the thylakoid membrane. The purified stromal fraction and/or isolated ct-HSP70 were used as control in these experiments in order to compare the effects of salts to those obtained with chaperons.

* Isolation of complexes containing ct-HSP70:

Isolated stromal fractions were fractionated on a DEAE-cellulose column. The fractions containing ct-HSP70 were precipitated with 70% ammonium sulfate and the pellet was dissolved in a minimum volume (about 2ml) of Tricine buffer. It was then separated by gel filtration chromatography and fractions containing ct-HSP70 were further purified on an Agarose-ATP column.

E3. Results and discussion:

* Contributions of the Israeli investigator

Research performed in Prof. Nechushtai's laboratory at
The Hebrew University of Jerusalem

In Jerusalem, major efforts were devoted to the developments of the experimental system that will allow to study the role of the ct-HSP70 in the organization and assembly of photosynthetic complexes in the chloroplast thylakoid membranes. Most of the efforts were dedicated to the characterization on the molecular level of the ct-HSP70 mode of action, as well to characterize additional stromal proteins containing HSP70.

1. Characterization of ct-HSP70-pLHCP interactions detected on non-denaturing PAGE:

Overproduced pLHCP, radio-labeled and/or "cold", were incubated with stroma and/or purified ct-HSP70 for 30 minutes at room temperature and then centrifuged (2 min at 15,000 rpm). The polypeptides were then separated by electrophoresis on 6% acrylamide Deriphat non-denaturing gels for 30-60 min at 100 V. Following the electrophoresis, the gels were electro-transferred to nitrocellulose filters which were immunolotted with antibodies raised against the ct-HSP70; HSP60 or pLHCP.

The results obtained indicated that the complex formed between pLHCP and ct-HSP70 is not very stable, an additional incubation and separation

steps were introduced. The pLHCP solubilized at 6M G-HCl was deleted (20 times) with the chloroplast stromal fraction (9 mg protein/ml) in the presence [+Mg-ATP] or absence [-Mg-ATP] of 10mM Mg-ATP. Following a 15 min incubation at room temperature, the samples were layered on 3-10% sucrose gradient containing 50mM Tricine-NaOH pH 8.0 and centrifuged for 14 hours in a SW-50 rotor at 45,000 rpm. Ten fractions (1-10) were collected (labeled from top to bottom of the tube) and a sample of 40 microliters was examined by non-denaturing Deriphat gel electrophoresis. The gels were electrophorized, electroblotted to nitrocellulose and immunodecorated with antibodies raised against pea ct-HSP70 and/or spinach LHCP (Fig. E3.1).

Fig E3.1 The detection of a ct-HSP70-pLHCP complex following sucrose gradient centrifugation. pLHCP expression, solubilization and dilution with the isolated stromal fraction (9 mg protein/ml) was performed, in the presence [+] or absence [-] of 10 mM Mg-ATP, as described in Fig. 1. Following a 15 min incubation at 25°C the samples were centrifuged on 10-30% sucrose gradients as described in the text. Following the electrophoresis on a Deriphat non-denaturing gel, the fractions were analyzed by western blotting to determine the presence of ct-HSP70 and/or pLHCP.

2. The effect of different cations on the integration of pLHCP in the thylakoid membranes:

We further characterized the requirements for ct-HSP70 -mediated insertion of pLHCP into the thylakoid membranes. We used the *in thylakoids* system developed by the PIs in which *in vitro* produced pLHCP is incubated with isolated thylakoid membranes.

The overexpressed pLHCP inclusion bodies were solubilized with 6M GHCl for overnight. The solubilized material (supernatant of a 2 minute centrifugation at 15,000 rpm) was then extensively dialyzed to reduce the denaturing agent concentration so that the purified precursor could be used to study its assembly and organization within the thylakoid membranes. The effects of cations on the requirement for the presence of stroma or purified ct-HSP70 at the dialysis stage (renaturation) and during the insertion process was tested. Fig.E32 indicates that 50 mM Mg^{+2} , can replace the need for the presence of the stromal fraction or purified ct-HSP70 . The presence of the high concentration of cations is essential during the dialysis step.

To analyze whether the replacement of the chaperone by high concentrations of cations is specific to Mg^{+2} , similar reactions were performed with other divalent cations such as Mn^{+2} or Ca^{+2} . Fig.E33 indicates that it is a general phenomenon and that other cations can replace the ct-HSP70 as well.

We believe that the preliminary results obtained in this part of the study are of important to the understanding of the mechanism of the chaperone-mediated insertion of pLHCP. The fact that high concentrations of different divalent ions (above 25mM) can replace the chaperone strongly suggests that the interaction between the two proteins is electrostatic in nature. The chaperone may prevent intra and inter-molecular interactions in pLHCP that induce its folding and/or aggregation. It was previously shown that the competent conformation for insertion of pLHCP into the thylakoids the protein is a partially unfolded one. The results here strongly favor the idea that the latter is maintained by electrostatic interactions with the chaperones.

Fig.E32: Insertion of pLHCP into isolated thylakoids can occur in the presence of high concentrations of cations without the stromal fraction or purified ct-HSP70 . Insertion of pLHCP into thylakoids membranes isolated from pea was performed with total stromal extract [S] or purified ct-HSP70 [H] or Tricine Buffer [B] - 0.1 M Tricine- NaOH pH 8.0. The presence of S/H/B was kept during dialysis and insertion steps. The overexpressed pLHCP was solubilized overnight at 6M GHCl and following 2 min centrifugation the solubilized material was diluted (X20) into stromal extract (3mg protein/ml); ct-HSP70 (0.2 mg protein/ml) or 0.1 M Tricine-buffer. MgCl_2 (50mM) was added to the dialyzing drop and dialysis preceded 30 minutes at 4°C against Tricine-buffer that contained (Right Panel) or did not contain 50 mM MgCl_2 (Left Panel). Following dialysis the samples were added to thylakoid membranes containing 40 μg of chlorophyll. In addition, a typical insertion system contained 10mM Mg-ATP . Insertion was performed at 25°C for 30 min. The thylakoids were pelleted and washed with 2 M NaBr . A photograph of SDS-PAGE gel is presented.

FigE33 The insertion of pLHCP into isolated barley thylakoids in the presence of high concentrations of divalent cations. Production, solubilization and dilution of pLHCP were performed as described in Fig 3. Different concentrations of cations, as specified in the figure, were added to the dialyzing drop and dialysis preceded 30 minutes at 4 degree C against Tricine-buffer containing the same cation concentration as in the dialyzing drop. Following dialysis the samples were added to an insertion system containing thylakoid membranes isolated from barley (40 μ g of chlorophyll per reaction) and 10mM Mg-ATP. The thylakoids were treated as described in FigE3.2.

The results presented show that the complexes between pLHCP and ct-HSP70 do not form or are very un-stable in the presence of Mg-ATP and thus can not be detected by the non-denaturing gel system used. Moreover, the presence of Mg-ATP generates a faster migrating form of ct-HSP70. This may be the monomeric form of the chaperone that does not interact with the denatured pLHCP substrate. The results also show that when the pLHCP was incubated with the total stromal fraction, a slowly migrating form was recognized by all antibodies used (anti-HSP60, anti-ct-HSP70, anti-pLHCP). This form may represent the pLHCP or the pLHCP-ct-HSP70 complex which may interact with HSP60.

3. Characterization of ct-HSP70 containing complexes present in the chloroplast stromal fractions:

Due to these observations, major efforts were allocated to analyze whether the stromal fraction contains other protein complexes having the HSP70 as a component. Moreover, the involvement of these in the integration of pLHCP into the thylakoid membranes was also analyzed. The major findings indicated that:

1. The ct-HSP70 present in the stromal fraction as a free protein and part of a complex

Fig E3.4. The detection of HSP70 complexes in the stromal fraction. Stromal protein(s) (lanes 1&2) and pure ct-HSP70 (lanes 3&4) were incubated for 15 minutes at 25°C with 10mM Mg-ATP (lane 1 & 3) or buffer (lane 2 & 4) and then separated on 7% acrylamide native gels. The gels were prepared according to Schagger except the omission of SDS and β -Mercaptoethanol. Following electrophoresis the gel's protein were transferred to nitrocellulose. The latter was immunoblotted with antibodies raised against Pea ct-HSP70 and [125 I]-Protein A.

2. pLHCP-stromal protein complexes can be separated on Sephacryl S-300 gel filtration column

Fig E3.5. pLHCP complexes separated by gel filtration on Sephacryl S-300.

Reaction solution of pLHCP incubated with stromal fraction was applied on Sephacryl S-300 gel filtration column (60ml) and eluted with 50mM Tricine-NaOH (pH 8.0), 50mM KCl, 5mM MgCl₂, 3mM Mg-ATP and 1mM PMSF at a rate of 10ml per hour. Fractions (1ml each) were collected and their radioactivity measured.

All the data clearly indicated that other stromal protein-complexes contain the ct-HSP70. So our second main goal was their isolation and characterization.

4. Isolation of ct-HSP70 containing complexes from the chloroplast stromal fraction

Since the analysis of the stromal fraction indicated that additional complexes beside the pure ct-HSP70 exist, a procedure to isolate these other complexes was developed:

The chloroplast stromal fraction was isolated from Swiss chard and applied to a DEAE-Cellulose column equilibrated with 0.1 M Tricine-NaOH pH 8.0. Following a wash with the same buffer containing 50mM NaCl, fractions were eluted with a gradient of 50-250mM NaCl in 0.1M Tricine-NaOH pH 8.0. The ct-HSP70 containing fractions were pooled and subjected to Ammonium sulfate precipitation at steps of 30/40/50 and 70%. Following a 20 minutes incubation in each salt concentration, the solution was centrifuged for 15 minutes at 40,000 X g. The supernatant and pellet of each precipitation were analyzed on 10% SDS-PAGE and it was found that the pellet of the 70% $(\text{NH}_4)_2\text{SO}_4$. This pellet was solubilized at a minimum volume of 0.1M Tricine-NaOH pH 8.0 and loaded on a size exclusion (S-300) column. The HSP70 containing fractions eluted from the column were pooled and applied on an ATP-Agarose affinity column. The column was then washed with buffer containing 100mM NaCl and eluted with 5mM Na-ATP. The proteins present in the different fractions along the different steps of purification were analyzed on SDS-PAGE (Fig E3.6).

Fig E.3.6 ct-HSP70 complexes purified from the stromal fraction. Stromal protein(s) (lane 1) were applied to DEAE-cellulose. The eluted fractions (lane 2) were precipitated with 70% $(\text{NH}_4)_2\text{SO}_4$ (lane 3) and applied to ATP-Agarose column. Following elution with 5mM Na-ATP (lane 4), the fractions were chromatographed on a size exclusion column (lane 5). Purified ct-HSP70 was run as control (lane 6) Lane 7 contained molecular weight markers. The different fractions were analyzed on 10% SDS-PAGE [LEFT PANEL] which was transferred to nitrocellulose and immunodecorated with antibodies raised against Pea ct-HSP70 and $[^{125}\text{I}]$ -Protein A.

The complex isolated resembled the bacterial DnaK-J complex. To determine whether this complex facilitates the integration of pLHCP into the thylakoid membranes, an insertion reaction was performed. Labeled pLHCP was solubilized at 8M urea, diluted 20 times with 0.1M Tricine-NaOH pH 8.0 and diazayed for 30 minutes against the Tricine buffer in the presence of a sample from the different fractions of the isolation steps. The solubilized pLHCP was then incubated with isolated thylakoids in the presence of 10mM methionine, 10mM Mg-ATP and a second aliquot of the different purification steps. The insertion reactions were performed for 30 minutes at 25°C. The thylakoids were pelleted and washed with 0.1M NaOH to verify the properly inserted protein. Following two additional washes with Tricine buffer, the membranes were analyzed on SDS-PAGE (Fig E.3.7).

Fig E.3.7. Insertion of pLHCP into isolated thylakoids in the presence of different ct-HSP70 containing complexes. The insertion reaction was performed in the presence of total stromal fraction (lane 1); The DEAE-cellulose eluted fractions (lane 2); The pellet of 70% $(\text{NH}_4)_2\text{SO}_4$ precipitation (lane 3); The eluted fraction from the ATP-Agarose column. (lane 4). Labeled pLHCP (tp) was run as control. The different reactions were analyzed on 10% SDS-PAGE.

1. Introduction

from the PI, Israeli investigator

2. Contributions of the Israeli Investigator

from the Israeli investigator

3. Contributions of the US investigator

3.1. Molecular cloning and expression of cDNAs for ct-HSP70

During the first year, cDNA clones for chloroplast ct-HSP70s were isolated from a cDNA library using oligonucleotide probes. These clones were characterized and partial sequences were obtained. At this time, we received a spinach cDNA clone containing the complete open reading frame for ct-HSP70. We used this clone for expression of HSP70 protein in *E. coli*.

Our strategy for overexpression of HSP70 involved production of this protein as a fusion protein containing a polyhistidine tag and a specific site for a protease to cleave it. The nickel-binding property of the protein can be used to purify the protein in a single step on an nickel column and the protease specificity can be used to cleave the purified fusion protein to yield complete HSP70. This strategy avoids potential contamination with the native DnaK protein and does not use protein properties, such as ATP affinity, for purification. Using appropriate oligonucleotides, the 5' region of the cDNA clone was amplified, digested with NdeI and XhoI restriction endonucleases. NdeI site was introduced during PCR while the XhoI site is a unique site in the original clone (Fig. 3.1). The remaining part of the gene was obtained by restriction digestion of the original clone by XhoI and EcoRI. These two fragments were cloned together in an expression vector to yield a clone containing the complete coding region for the mature ct-HSP70 (Fig. 3.2).

Fig. 3.1. Restriction map of a Spinach cDNA clone encoding a ct-HSP70.

Fig. 3.2 Scheme for generating an appropriate clone for overexpression of ct-HSP70

Initially the overexpression was performed according to the standard protocol in the pET expression system manual. The pET15H plasmid containing HSP70 cDNA under T7 promoter was introduced into BL21 (DE3) strain. The presence of plasmids in the colonies was confirmed by plasmid preparation and restriction digestion. The resulting strain was used for optimization of overexpression of HSP70. The HSP70 in the cellular proteins was detected by polyacrylamide gel electrophoresis and western blotting. The solubility of the overexpressed proteins was examined by cell fractionation. Western blotting with an anti-HSP70 antibody and enhanced chemiluminescence reagents was used to confirm the identity of the protein and to quantify the relative abundance by laser densitometry of X-ray film.

Table 3.1 shows the proportion of soluble protein was greater at the early stage of induction while the overall yield of soluble protein was more at the maximal level of induction. Since our experiments require soluble overexpressed protein, we attempted to optimize induction conditions and strain background to obtain maximal level of soluble overexpressed protein (Table 3.2). Unfortunately, most overexpressed protein accumulated into inclusion bodies. We attempted to scale up the overproduction using a 16 L fermentor. Condition were again optimized for overexpression of soluble protein; however, maximal yield of soluble proteins ranged from 110-245 µg purified protein per 16L batch of cells. *Therefore overexpression of the soluble HSP70 protein turned out to be tremendous work with little success.*

Table 3.1. Time course of induction

Time after induction	% Protein in pellet	% soluble protein	Relative level ¹
0	0	0	0
1	94	6	9
2	92	8	22
3	96	4	37
6	97	3	65
9	98	2	89
16	98	2	100
36	98	2	93

¹ As % of the maximum yield (7.2 mg HSP70 protein per g wet weight of the cells)

Table 3.2. Expression of ct-HSP70 under different induction conditions in two *E. coli* strains.

Conditions of induction			% soluble HSP70 protein	
Temperature (°C)	Time (h)	IPTG (mM)	BL21 (DE3)	BL21 (DE3) pLysS
37	3	1	5	4
37	16	0.1	2	4
37	16	1	2	3
37	16	10	3	3
25	16	1	5	4
42	16	1	3	4

3.2. An in vivo system to study assembly of the photosynthetic apparatus

During the second year of funding, we also initiated a project to develop an in vivo system for studying assembly of photosynthetic proteins. Although not a specific objective of the original proposal, this project is in accordance with the overall goal of the original project: to study biogenesis of the photosynthetic apparatus. The in vivo study extended the project to make productive utilization of BARD funds.

Summary:

We studied role of the presequence in targeting of the precursor of PsbO (prePsbO), the 33-kDa Mn-stabilizing protein of PSII, to thylakoids of the cyanobacterium *Synechocystis* sp. PCC 6803. We generated a recipient mutant strain that lacks the *psbO* gene. We also constructed a recombinant DNA to introduce mutated *psbO* into the recipient strain. The PsbO-less cyanobacterial strain is sensitive to calcium depletion from the medium, thus providing a suitable selectable marker for generating and characterizing assembly and targeting mutants of this protein. Site-directed mutations in the presequence of PsbO were generated using mutagenic primers for PCR. Characterization of photosynthetic activities and membrane localization of these mutants revealed that some of the mutations led to improper targeting or defective processing. Therefore, the presequence of prePsbO contains information for protein translocation, processing enzyme specificity and for targeting specificity for distinguishing between thylakoid and cell membranes.

Details can be found the progress report of the second year.

3.3. Degradation of the photosynthetic apparatus

During the third year of funding, we initiated yet another project to develop a system for studying degradation of photosynthetic proteins. We believe that this diversion in the project was necessary to remain competitive in the rapidly progressing field of membrane protein biogenesis. The in vivo study adds a novel dimension to the project and makes maximum and efficient utilization of BARD funds.

Summary:

Photosystem I (PSI) is a multisubunit pigment-protein complex that functions as a light-driven plastocyanin-ferredoxin oxidoreductase in thylakoid membranes of cyanobacterial and higher plants. The availability of PsaD-less (ADC4), PsaE-less (AEK2), and PsaD-PsaE-less (DE) strains of the cyanobacterium *Synechocystis* sp. PCC 6803 and of the immunological probes against most of PSI subunits allowed us to examine stress-induced alterations in the accumulation of PSI proteins *in vivo*. When the wild-type cells were grown at 38°C under a light intensity of $21 \mu\text{mol m}^{-2} \text{s}^{-1}$ for 4 days, there was no detectable loss of PSI subunits in the photosynthetic membranes. In contrast, the DE strain exhibited rapid and selective decrease in the accumulation of PSI subunits under the same conditions. Western blot analyses using the isolated photosynthetic membranes in the DE strain showed that steady state level of PsaB, PsaC, and PsaL rapidly declined with concomitant accumulation of a series of proteolytic intermediates retaining the C-terminal epitope of PsaB. The level of PsaF and PsaI, however, remained steady at 38°C in the DE strain. Level of PsaB in the DE strain grown at 38°C in the presence of protein synthesis inhibitors declined more rapidly than in wild type. These results suggested that the enhanced protein degradation in the DE strain may account for the rapid loss of PSI subunits. When the DE cells were grown at 38°C under darkness, there was a steady accumulation of PsaB, PsaC, PsaL, and PsaK in the photosynthetic membranes, indicating a light-regulated *in vivo* proteolysis was involved in degrading some of the PSI subunits. Interestingly, accumulation of PsaC, PsaL, and PsaK in the DE strain responded differently to the block of electron transfer between the PSII complex and the plastoquinone pool by 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) under light at 38°C. Accumulation of PsaC, but not PsaL and PsaK, was responsive to the addition of DCMU, demonstrating that there were different mechanisms regulating the *in vivo* degradation of PSI subunits. The role of PsaD and PsaE in regulating the *in vivo* stability of PSI was further examined by growing the wild type, ADC4, AEK2, and DE strains at 38°C under light and subsequent analysis in the accumulation of PsaB, PsaC, and PsaF. The absence of PsaD but not PsaE led to a more enhanced decline in levels of PsaB and PsaC. However, a synergistic effect due to absence of PsaD and PsaE, which are required for optimal ferredoxin- or flavodoxin-mediated NADP^+ photoreduction, was notably evident in decreasing level of PsaB and PsaC. Overall, our data demonstrate that the *in vivo* proteolysis of PSI subunits in the light- and high temperature-sensitive DE strain is regulated by multiple mechanisms.

Results:

Damage to PSI leads to a decrease in the steady-state levels of PSI proteins.

- When grown at 37°C, the level of PsaB significantly decreases in the DE strain, but not in the wild type, with a concomitant accumulation of PsaB fragments (Fig. 3.3). Under these conditions, there was an accumulation of high mass protein species, that was immunoreactive with anti-PsaB antibody, perhaps representing a tagged protein destined for degradation or a cross-linked aggregation caused by reactive groups. Thus PSI complexes in the DE mutant strain are susceptible to high temperature stress, perhaps due to the lack of peripheral

polypeptides. The mutant PSI complex is functional in charge separation, but cannot reduce ferredoxin, thus likely to produce elevated levels of oxygen radicals. In some cyanobacterial mutants, PsaB is more accessible *in vitro* to exogenous proteases but is not degraded more rapidly *in vivo*. Therefore, an increased accessibility to proteases does not necessarily increase turnover rate, indicating the presence of a tagging mechanism for damaged proteins. This mechanism could involve initial cleavage of specific regions of PsaB to expose highly degradable N-termini.

- When the membranes from the strains lacking PsaD *OR* PsaE were examined, there was not a significant decrease in PsaB level after exposure to high temperature (data not shown). Therefore, PSI core is susceptible to rapid degradation only when *BOTH* PsaD and PsaE are missing.

There are multiple distinct mechanisms for degradation of different PSI subunits.

- Accumulation of PSI subunits in the photosynthetic membranes, such as PsaA-PsaB, PsaD, PsaF, PsaL, and PsaI, remains steady when the wild-type cells are grown at 37° and 42°C for 4 days (Fig. 3.3). In contrast, steady level of PsaB, PsaC and PsaL sharply declined when the DE cells are grown at 37°C for 4 days. Interestingly, there is no detectable change in the PsaF and PsaI level, demonstrating that their degradation is independent of the stability of the PSI core.
- We examined the role of high temperature, light, and PSII-mediated electron transport in degradation of PSI proteins of the DE cells (data not shown). Light, but not the PSII-mediated linear electron flow, was required for the reduced level of the PsaB, PsaL and PsaF proteins. In contrast, stability of PsaC at high temperature is increased by the addition of DCMU. Again, PSI proteins show differences in the factors regulating their steady-state accumulation in the membranes. *In vivo* PsaB fragments can be detected in membranes.

Some PsaB degradation intermediates are associated with, but not integrated in membranes.

- Several PsaB fragments reactive to PsaA-PsaB antibodies accumulate in thylakoids of the DE strain grown at 37°C. Since these fragments are further degraded during extended incubation, they represent intermediates that transiently accumulate during degradation. *Additional intermediates may be present, but not detectable due to the lack of suitable antibody probes.*
- Chaotropes (such as NaI) are routinely used to distinguish peripheral proteins from the integral membrane-proteins. A C-terminal degradation-intermediate of PsaB can be extracted from membranes by 3 M NaI (Fig. 3.4). If a similar PsaB fragment is generated *in vitro* by treating membranes of the DE strain with proteases, it cannot be removed by NaI (data not shown). This fragment is expected to contain at least four hydrophobic domains (Fig. 2). Therefore, cleavage of PsaB does not automatically result in the removal of its fragments from membranes. During intracellular PsaB degradation, an active machinery removes the PsaB degradation intermediate from the lipid bilayer.

Fig. 3.3. High-temperature stress causes degradation of PSI subunits in the DE strain of *Synechocystis*. The wild-type and DE strains, grown at 20°C prior to stationary phase under light intensity of $21 \mu\text{mol m}^{-2} \text{s}^{-1}$ (labeled 20*), are subjected to temperature treatments for 4 days. PSI subunits in the photosynthetic membranes containing 8 μg of chlorophyll (Chl) are resolved. The separated proteins are blotted to Immobilon-P membranes and probed with antibodies. Immunoreaction is visualized by enhanced chemiluminescence.

Fig. 3.4. Removal of PsaB degradation intermediates by NaI. The photosynthetic membranes of the wild-type and DE strains are exposed to 0, 1, 2, and 3 M NaI for 30 min on ice, followed by removal of NaI. Proteins in the photosynthetic membranes containing 5 μg of Chl are resolved, blotted, and immunodetected as in Fig. 4 with an exception that anti-PsaB antibody is used.

Fig. 3.5. Levels of PsaB in the DE strain in the presence of protein synthesis inhibitors. The wild-type and DE strains are grown in the presence of protein synthesis inhibitors, streptomycin and tetracycline, at a final concentration of 80 $\mu\text{g/ml}$ each at 37°C. Cells are harvested at 6, 12, and 24 h after addition of inhibitors. Proteins in the membranes (8 μg Chl) are resolved by PAGE and probed with anti-PsaAB antibody.

PsaB degradation rate is enhanced after shifting the DE cells to high temperature.

- We studied PsaB levels in the membranes from cells grown in the presence of protein synthesis inhibitors to determine the role of enhanced degradation rates in lowering PsaB levels in the DE cells that have been grown at 37°C (Fig. 3.5). There was a significant decrease in the PsaB levels, with concomitant increase in the degradation intermediates and in the high-mass species.

Soluble protease(s) and other factor(s) are required for complete degradation of PsaB.

- When cyanobacterial cytoplasm or chloroplast stroma was included during incubation, PsaA-PsaB were degraded to fragments that could not be detected by anti-PsaA-PsaB or anti-PsaB antibodies (Fig. 3.6). Addition of apyrase inhibits complete degradation of PsaB, demonstrating requirement of ATP (Fig. 7). Preliminary experiments with protease inhibitors suggest that the soluble protease that degrades PSI may be a serine-protease (data not shown). Interestingly, Clp is a serine protease.

A membrane-associated protease can degrade PsaB to membrane-embedded fragments.

- When thylakoids were incubated at 37°C for different duration, PsaA-PsaB were degraded to small fragments. 25 and 22 kDa C-terminal PsaB fragments were most prominent. These fragments remained stable for at least 72 h at 37°C. Treatment of membranes with 3 M NaI or high temperature (60°C for 30 min) or lowering incubation temperature (4°C) inhibited this proteolysis. In contrast, addition of apyrase or ATP to the reaction did not affect the partial proteolysis (data not shown). The 25 and 22 kDa products were not removed by chaotropes (data not shown).

Long incubation periods (up to 24 h) are required as PSI has a half life of several days. We use sterile conditions and routinely add protein synthesis inhibitors to inhibit microbial growth.

Fig. 3.6. In vitro reconstitution of PSI degradation. Wild-type membranes were incubated in 10mM Tris (pH 8.0). Reaction conditions, including other additions are listed in the figure. The membranes were solubilized, proteins separated by PAGE and PsaB was detected by Western blotting. Arrows indicate ~22 kDa PsaB fragments.

4. Collaboration:

1. Visits of the Israeli investigators

Person	Dates	Purpose
Rachel Nechushtai	Fall 1995	Joint experiments; Graduate student training
	Summer 1996	Evaluation of research progress and new plans
Limor Minai	Fall 1995	Joint experiments
Michal Cohen	Fall 1995	Joint experiments
Zengyong He	Fall 1996	Joint experiments

2. Visits of the US investigators

Person	Dates	Purpose
Parag R. Chitnis	Summer 1995	Discussion of current status and planning of new directions Joint experiments; Training of Israeli graduate students

3. Joint Publications

Five publications

5. Publications:

Cohen Y, Nelson N, Chitnis PR, Nechushtai R (1995) Integration of cyanobacterial PsdD into spinach photosystem I requires the authentic C-terminal domain. *Photosynth. Res.* 44:157-164.

Nechushtai R, Cohen Y, Chitnis PR (1995) Assembly of Chlorophyll-protein Complexes *Photosynth. Res.* 44:165-181.

Chitnis PR, Xu Q, Chitnis VP, Nechushtai R (1995) Function and organization of photosystem I polypeptides. *Photosynth. Res.* 44:23-40.

Minai L, Cohen Y, Chitnis PR, Nechushtai R (1996) The precursor of PsdD assembles into the photosystem I complex in two steps. *Proc. Natl. Acad. Sci. USA* 93:6338-6342

Fan L, Sarada Devi K, Chitnis VP, Chitnis PR (1996) Molecular genetics of cyanobacteria: New Avenues in biotechnology. *J. Scientif. Industr. Res.* 55:555-563

Chitnis PR (1997) Biogenesis of chloroplast proteins In: Raghavendra AS (ed) *Photosynthesis: A comprehensive Treatise*. Cambridge University Press. In Press

Ramiah S, Chitnis VP, Nechushtai R, Chitnis PR (1997) Presequence requirements for targeting of cyanobacterial prePsbO to thylakoid lumen. Submitted

Xu Q, Chitnis VP, Fan Lu, Chitnis PR (1997) The absence of PsdD and PsdE in a cyanobacterial mutant strain enhances degradation of photosystem I at high temperature. submitted.

F. DESCRIPTION OF THE COOPERATION

Dr. Rachel Nechushtai and two of her students (Limor Minai and Michal Cohen) stayed in Dr. Chitnis' laboratory in Kansas State for the first summer of the granting period. During this visit joint experiments were performed and the planning of the second year experiments was conducted. In addition, Dr. Nechushtai and Dr. Chitnis participated in the Mid-West Photosynthesis Conference where they jointly presented results emerging at the first year of funding.

In the second year of funding, Dr. Chitnis visited the laboratory in Jerusalem for and joint experiments and discussions were conducted. In summer 1994, both PIs attended the Gordon Research Conference on Biochemical Aspects of Photosynthesis where they presented data obtained in this project. In addition to the mutual visits, close contacts were kept between the PIs. On weekly basis we exchanged e. mails; telephone calls and faxes. We also summarized the findings in several manuscripts that were jointly written (see section I).

G. EVALUATION OF THE RESEARCH ACHIEVEMENT

The research described was a fruitful and enjoyable collaborative work between our two groups. We did not achieve all the original objectives but we did make significant and valuable progress in the characterization of the role of ct-HSP70 in the integration and assembly of pLHCP into the chloroplast thylakoid membranes. Hence objective 1 - which in the original proposal was Studying the role of ct-HSP70 in the assembly of pigment-protein complexes - was achieved.

We also made considerable advancement in the achievement of objective 2 of the original proposal - Mutational dissection of the interaction between ct-HSP70 and LHCP. By using deletion mutants of LHCP we identify that the N-terminus of the mature LHCP is required for these interactions.

Objective 3 of the original proposal the Molecular cloning, expression and mutagenesis of ct-HSP70 - was only partially achieved. We did isolate

and characterize some preliminary clones, however we did not complete the overexpression and planned mutagenesis studies.

H. CONCLUSIONS

The data obtained clearly indicate that the ct-HSP70 is involved in the assembly of pLHCP in the thylakoid membranes. The two major findings that resulted from the present study are:

1. ct-HSP70 is present in the stromal fraction as individual protein and as a part of at least one complex. Partial purification of one of these complexes indicated strong similarities to the bacterial DnaJ and K chaperone complex.
2. pLHCP forms two complexes with stromal proteins. One had the apparent molecular weight of about 120KDa, and the other was of 220-250KDa. ct-HSP70 is present in the heavier complex.

I. LIST OF PUBLICATIONS RESULTING FROM THE RESEARCH PROJECT

1. Y. Cohen, S. Yalovsky and R. Nechushtai. Integration and assembly of photosynthetic protein complexes in chloroplast thylakoid membranes. A refereed review; *Biochem. Biophys. Acta.* 1241, 1-30 (1995).
2. Y. Cohen, N. Nelson, P.R. Chitnis and R. Nechushtai. The carboxy terminal region of the spinach Psad subunit contains information for its specific assembly into plant thylakoids. *Photosynthesis Research.*, 44, 157-164 (1995).
3. P.R. Chitnis, Q. Xu, V.P. Chitnis and R. Nechushtai. Function and organization of photosystem I polypeptides. A refereed minireview; *Photosynthesis Research.*, 44, 23-40 (1995).
4. R. Nechushtai, Y. Cohen, and P.R. Chitnis. Assembly of the chlorophyll-protein complexes. A refereed minireview; to be published in *Photosynthesis Research*, 44, 165-181 (1995).
5. L. Minai Y. Cohen, P.R. Chitnis, D. Michaeli, N. Nelson and R. Nechushtai. The assembly of the precursor of photosystem I subunit II (Psad) is a two steps process. To be submitted to *Proc. Natl. Acad. Sci. USA.* (1995)

6. R. Nechushtai, A. Eden, Y. Cohen, and J. Klein. Introduction to photosystem I - reaction center function, composition and structure; a review article to be published in: The light reactions; a book in the new series Advances in photosynthesis. (C.F. Yocum and D. Ort eds.) Kluwer Academic Publishers, Dordrecht, The Netherlands. (1995).

7. L. Minai and R. Nechushtai. The assembly of PsaD, a peripheral subunit of photosystem I, into the thylakoid membranes. Advances in Research in Photosynthesis, to be submitted (1995).