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DnaK3, One of the Three DnaK Proteins of Cyanobacterium *Synechococcus* sp. PCC7942, Is Quantitatively Detected in the Thylakoid Membrane

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Subcellular localization of three DnaK proteins of cyanobacterium *Synechococcus* sp. PCC7942 was determined. DnaK1 and DnaK2 proteins were detected mainly in the cytosolic fraction. On the other hand, the DnaK3 protein occurred in large amounts in the thylakoid membrane fraction. Furthermore, DnaK3 was found to be located on the surface of the thylakoid membrane on the cytosol side. Subcellular localization of chimeric and truncated DnaK3 proteins was also determined, and it was suggested that the region a.a. 381 to a.a. 597 of DnaK3 protein, which is considered to correspond to the peptide-binding domain, was required for the association with the thylakoid membrane.

DnaK proteins of prokaryotic cells belong to the Hsp70 protein family which is one of the highly conserved ubiquitous groups of heat shock proteins.

In most of the eukaryotes, Hsp70 proteins encoded by multigene families, occur in various cellular compartments, including the cytoplasm, nuclei, mitochondria, chloroplasts, and endoplasmic reticulum (3, 4). These observations suggest that a chaperon function is required within multiple cellular compartments. In *Escherichia coli,* there are conflicting reports concerning the localization of the DnaK protein. Immunological studies of the DnaK protein indicate that the majority of the DnaK molecules are located in the cytoplasm, with the possibility that a subpopulation of DnaK is membrane associated (5). In minicells infected with lambda phages containing the *dnaK* gene, the DnaK protein was predominantly found in the membrane fraction (6). Moreover, it was suggested that DnaK belongs to a specific group of cytoplasmic proteins released by osmotic shock. This group of proteins is possibly located at Bayer's adhesion sites where the inner and outer membranes are contiguous (7).

Cyanobacteria are prokaryotic cells which have developed an oxygen-producing photosynthetic system similar to that of chloroplasts of higher plants. They show the typical structure of cell envelopes consisting of outer and inner cytoplasmic membranes, as well as thylakoid membranes in the cytoplasm.

We previously identified three *dnaK* homologues (*dnaK1*, *dnaK2*, and *dnaK3*) in the genome of the unicellular cyanobacterium *Synechococcus* sp. PCC7942 (8, 9). DnaK3 is peculiar among them, since it has a long C-terminal non-conserved region. Our preliminary experiments showed that three *dnaK* genes responded separately to the heat shock treatment, suggesting the possible functional differentiation of these three DnaK proteins. It is interesting to determine whether multiple DnaK proteins in the same compartment play different roles. In this regard, the examination of the subcellular localization (membrane association) of proteins may enable to elucidate their function, especially in the case of cyanobacteria which developed a subcellular organelle-like structure, the thylakoid membrane. In this study, we subfractionated the cyano-

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bacterial cells and examined the localization of three DnaK proteins. The DnaK3 protein was abundantly detected in the thylakoid membrane fraction. To determine the region required for the thylakoid membrane association, we expressed chimeric and truncated DnaK3 proteins in cyanobacterial cells and examined their subcellular localization.

MATERIALS AND METHODS

Strains and culture conditions. Synechococcus sp. PCC7942 (R2-SPc) strain which cured its indigenous plasmid pUH24 (10) was supplied by T. Endo (Nagoya University, Nagoya). Cells were grown photoautotrophically at 30°C in BG-11 medium (11) under bubbling with air and illumination.

Subcellular fractionation. Cells were disrupted by passage twice through a French pressure cell at 66 MPa (700 kg/cm^2) and fractions of cell walls, cytoplasmic membranes and thylakoid membranes were separated by flotation centrifugation on a discontinuous sucrose density gradient as described by Murata and Omata (12). The soluble cytosolic fraction was prepared by centrifugation of the crude cell extracts for 1 hour at 100,000 × g. Fractionation was confirmed by two methods: 1) recording of absorption spectra for each fraction in a wavelength range from 250 to 800 nm, 2) western blot analyses using antisera against proteins whose localization had already been examined (see text).

Western blot analysis. Protein concentration of each fraction was measured by the Lowry method (13) and the fractions were suspended in SDS sample buffer (50 mM Tris-HCl, pH 6.8, 100 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue, 10% glycerol). Membrane and wall fractions were incubated at 37°C for 5 min, and the cytosolic fraction was heated at 95°C for 5 min, then subjected to 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), as described by Laemmli. (14). Proteins were transferred to an Immobilon poly-vinylidene difluoride membrane (Millipore) using a semi-dry blotting system (Biocraft). For the detection of each DnaK protein, mouse antisera specific to each DnaK protein were raised using polypeptides from the non-conserved C-terminus region of DnaK proteins (Nimura et al., unpublished). For the detection of human c-Myc epitope tag, anti c-Myc monoclonal antibody (9E10, Berkeley antibody company) was used. These antisera were used as primary antisera and alkali phosphatase conjugated anti-mouse IgG (H+L) antiserum (goat, BioRad) was used as the secondary antibody. Nitro blue tetrazolium (NBT, Sigma) and 5-Bromo-4-chloro-3-indolyl phosphate (BCIP, Sigma) were used for color development (15). Fluorescent substrate AttoPhos (Boehringer Mannheim) was also used for the detection and the signals were scanned using FluorImager (Molecular Dynamics).

Alkali and urea treatment of the thylakoid membranes. Thylakoid membranes (10 μg protein) were suspended in each 20 μl of 10 mM TES-NaOH (pH 7.0), 20 mM Hepes-NaOH (pH 8.0), 0.2N Na_2CO_3 (pH 11.0), or 6 M urea and incubated on ice for 30 min. Membranes were collected by centrifugation at 100,000 × g for 30 min at 4°C. Aliquots of membrane and soluble fraction were analyzed for the DnaK3 protein by western blotting.

Protease treatment of the thylakoid membranes. Thylakoid membranes (30 μg protein) were suspended in 60 μl of 10 mM TES-NaOH (pH 7.0), or 10mM TES-NaOH (pH 7.0) containing 100 μg/ml trypsin and incubated on ice for 30 min. To end the protease digestion, soybean trypsin inhibitor was added to a final concentration 200 μg/ml and the samples were incubated for 5 min on ice. Membranes were precipitated with trichloroacetic acid (TCA) and aliquots were analyzed for the DnaK3 protein by western blotting.

Construction of plasmids for expression of chimeric genes. Plasmid pNS1 is a derivative of pTZ18R(TOYOBO) containing a spectinomycin resistance cassette of pHP45Ω (28) in the middle of the *Synechococcus* fragment designated as 'neutral site'(29) which allows homologous recombination between the transforming plasmid DNA and the recipient cyanobacterial chromosome to take place. Construction of the fusion proteins used in this study is shown in Fig. 4. DNA fragments encoding each component of the fusion proteins were amplified by using the polymerase chain reaction (PCR) and were recombined. All the recombinant fragments were once cloned into pTrc99A/X (Pharmacia) to provide a promoter for fused genes. All the fragments containing *lacI^q, trc* promoter and fused genes were isolated, and except one for DK1C3, they were recloned into pNS1 between the separated neutral site segments and next to the spectinomycin-resistant cassette. A fragment for DK1C3 was cloned into pUC303, a cyanobacterium-*E. coli* shuttle vector (10). These plasmids were introduced into either *Synechococcus* PCC7942 DK1KM (Δ*dnaK1*, unpublished) or wild type cells. Transformants were selected and cultured on BG11 medium containing 10 μg/ml streptomycin (for pUC303 derivatives), or 40 μg/ml spectinomycin (for pNS1 derivatives) as needed. Expression from the trc promoter was induced by the addition of 2 mM isopropyl-β-*D*-thiogalactopyranoside (IPTG).

RESULTS

Subcellular fractionation of Synechococcus sp. PCC7942 cells. To determine the localization of the three DnaK proteins in the *Synechococcus* cells, we fractionated the cells into subcellular fractions: cell walls, cytoplasmic membranes, thylakoid membranes and cytosol. We first measured the light absorption spectra of each membrane fraction (data not shown) and observed

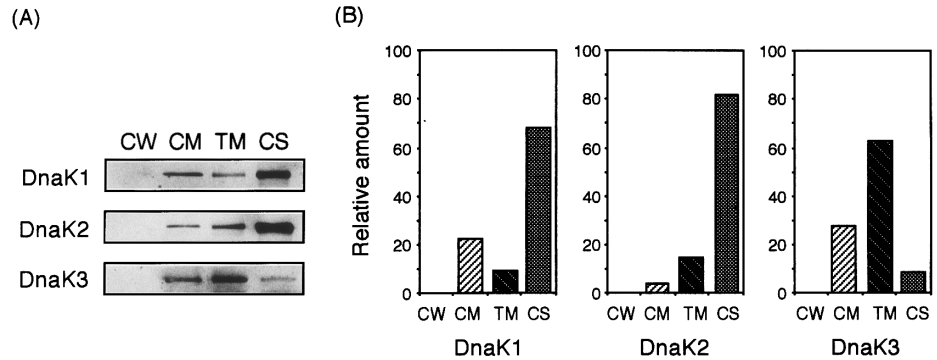

FIG. 1. Subcellular localization of three DnaK proteins in *Synechococcus* sp. PCC7942. (A) Cells were subfractionated into cell walls (CW), cytoplasmic membranes (CM), thylakoid membranes (TM), and cytosol (CS) as described under Materials and Methods. Aliquots (10 μg proteins) were analyzed for the distribution of the DnaK proteins by western blotting. (B) Densitometric quantification of (A). Total amounts of each DnaK protein corresponded 100.

the main absorption peaks at 419, 438, 495 and 678 nm in the thylakoid membrane fraction presumably due to chlorophyll *a* and carotenoids as described by Murata et al. (18). In the cytoplasmic membrane fraction, there were main peaks at 437, 456 and 488 nm and a minor peak at 673 nm probably due to the carotenoids contained in the cytoplasmic membrane. These peaks as well as overall patterns of spectra were very similar to those reported previously (12, 18). We also performed western blot analysis using antisera against SecA protein (located in the cytosol, cytoplasmic membrane, and thylakoid membrane, 16), SecY protein (located in both cytoplasmic and thylakoid membranes, 19), and NrtA protein (located only in the cytoplasmic membrane, designated as 45 kDa protein in ref. 20), confirming that there was no cross-contamination of any other fractions.

Subcellular localization of each DnaK protein. Western blot analyses with each DnaK-specific antiserum were carried out for subcellular fractions (Fig. 1). The results showed that the three DnaK proteins were located in three subcellular fractions: cytosol, cytoplasmic membrane, and thylakoid membrane. The DnaK1 and DnaK2 proteins were found mainly in the cytosol and a small amount in both cytoplasmic and thylakoid membranes. On the other hand, the major band for DnaK3 was clearly seen in the thylakoid membrane fraction and only a light band was detected in the cytosol fraction, in contrast to the localization of DnaK1 and DnaK2.

The results shown in Fig. 1 did not reflect directly the relative distribution of each protein in the subcellular fractions, since an equal amount of proteins (10 mg) was loaded on a gel. The relative amount of proteins contained in each subcellular fraction to total cell proteins was roughly estimated to be: cytosol, 75%; thylakoid membrane, 15%; cytoplasmic membrane, 5%; and cell wall, less than 5% (data not shown). Taking these ratios into account, the distribution of each DnaK protein in the thylakoid membrane was as follows: DnaK1, 3%; DnaK2, 3%; and DnaK3, 50%. Although we observed a slight proteolytic activity in the cytosol fraction resulting in a decrease of the intensity during storage, the distribution of DnaK3 in the thylakoid membrane was remarkably high compared with that of DnaK1 or DnaK2.

Thylakoid membrane-associated DnaK3 is a peripheral membrane protein. We further determined whether DnaK3 is associated with the thylakoid membrane as a peripheral membrane protein or as an integral membrane protein by alkali or urea treatment (16, 17). When the thylakoid membrane was extracted with 20 mM Hepes-NaOH at pH 8.0 or 0.2 M Na_2CO_3 at pH 11, a substantial amount of DnaK3 protein in the thylakoid membrane was recovered in the soluble fraction (Fig. 2, lanes 3, 5) and after treatment with 6 M urea, all the DnaK3

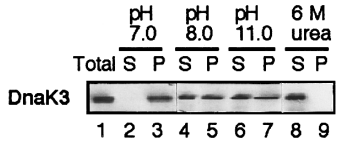

FIG. 2. Extractability of the thylakoid membrane-bound DnaK3 protein by alkali or urea treatment. Thylakoid membranes (10 μg) were treated with 10 mM TES-NaOH (pH 7.0, lanes 2, 3) or 20 mM HEPES-NaOH (pH 8.0, lanes 4, 5) or 0.2 M Na_2CO_3 (pH 11, lanes 6, 7) or 6 M urea (lanes 8, 9). Peripheral membrane proteins were recovered in the supernatant (sup, lanes 2, 4, 6, 8) and membrane-integrated proteins were precipitated as membrane pellets (ppt, lanes 3, 5, 7, 9).

protein was recovered in the soluble fraction (Fig. 2, lane 7). On the other hand, all the DnaK3 protein was still associated with the thylakoid membranes when washed with 10 mM TES-NaOH at pH 7.0 (Fig. 2, lane 2). These results indicate that DnaK3 is a peripheral membrane protein. Since DnaK is known to bind and dissociate from its substrate in an ATP-dependent manner, we also determined the effect of the presence of Mg-ATP. When the thylakoid membrane was treated with 10 mM TES-NaOH (pH 7.0) containing 5 mM Mg-ATP, all the DnaK3 protein in the thylakoid membrane was still recovered in the membrane pellet (data not shown).

We also found that the membrane-associated DnaK3 was sensitive to protease (Fig. 3B, lane 2). Since some proteins were not affected by the protease treatment (Fig. 3A, lane 2), it is suggested that the thylakoid membrane associated DnaK3 protein is located on the surface of the thylakoid membrane on the cytosol side.

Peptide-binding domain of DnaK3 is required for the association with the thylakoid membrane. DnaK3, which is a 84 kDa protein consisting of 749 amino acids (a.a.), is about 100 a.a. longer than most of the Hsp70 members. Secondary structure prediction of the C-terminal region of Hsp70 proteins shows that they commonly display an extensive a-helical region at

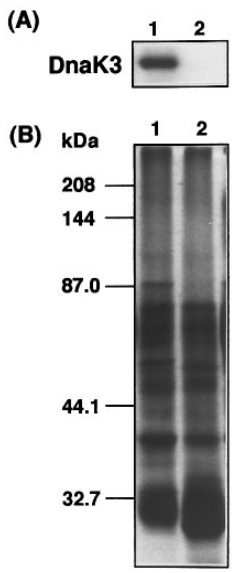

FIG. 3. Protease sensitivity of the thylakoid membrane-bound DnaK3 protein. Thylakoid membranes (30 μg) were treated with (lane 2) or without (lane 1) 100 μg/ml of trypsin as described under Materials and Methods. (A) Coomassie blue stained gel. (B) Western blot.

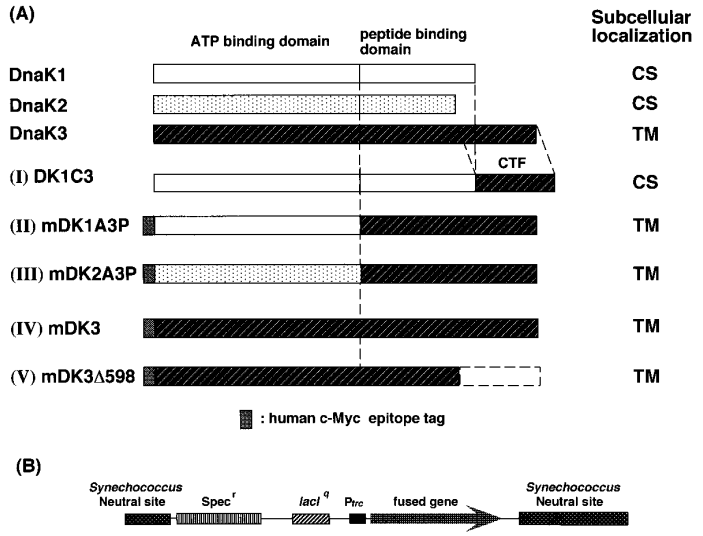

FIG. 4. Construction of fusion protein and subcellular localization. (A) Schematic drawing of each Dnak and fusion proteins (I–V). Components of each fusion were, (I) DK1C3: entire DnaK1/carboxyl terminal fragment of DnaK3 (CTF, a.a. 599–749), (II) mDK1A3P: human c-Myc epitope tag (EQKLISEEDL)/ATP binding domain of DnaK1 (a.a. 1–384)/a.a. 381–749 of DnaK3, (III) mDK2A3P: c-Myc/ATP binding domain of DnaK2 (a.a. 1–379)/a.a. 381–749 of DnaK3, (IV) mDK3: c-Myc/entire DnaK3, and (V) mDK3Δ598: c-Myc/a.a.1–598 of DnaK3 (deletion of CTF). The results of western blottings (Fig. 5) are summarized on the right-hand side. CS indicates that the proteins were mainly located in the cytosol as was DnaK1 or DnaK2. TM indicates that these were located in large amounts in the thylakoid membrane as was DnaK3. (B) Construction of expression systems of fusion proteins except the DK1C3.

amino acid 500 to the end. The C-terminal region of the DnaK3 protein also fitted this prediction up to ca 600 a.a. and further extended sequence of this protein is predicted to contain alternating aperiodic structures. We designated this extension as CTF for C-terminal fragment (Fig. 4). To identify the region responsible for the association with the thylakoid membrane, we expressed chimeric and truncated DnaK3 proteins in *Synechococcus* cells and determined the subcellular localization of each protein. Fig. 4. shows the construction of fusion proteins used in this study. These chimeric genes except for Dk1C3 were integrated into the chromosome at the neutral site because the genes, when in the plasmid, caused recombinations with intact genes on the chromosome and gave rise to a complex interpretation of subcellular localization. The results of western blots are shown in Fig. 5 and a summary is included in Fig. 4. These results indicate that CTF does not play a major role in the association with the thylakoid membrane. On the other hand, whole C-terminal region (a.a. 381-749) was sufficient for the association with the thylakoid membrane to take place. Moreover the deletion of CTF did not affect the DnaK3 localization in the thylakoid membrane. Therefore, the region required for the association with the thylakoid membrane occurs between a.a. 381 to 598 in DnaK3, which presumably corresponds to the peptide binding domain of this protein.

DISCUSSION

Three DnaK proteins of *Synechococcus* sp. PCC7942 were located in the subcellular fractions according to two distinct patterns: DnaK1 and DnaK2 were mainly located in the cytosolic fraction and partly in the membrane fractions, and DnaK3 was located in the thylakoid membrane. Although most of the members of known Hsp70 proteins are located in the cytosolic

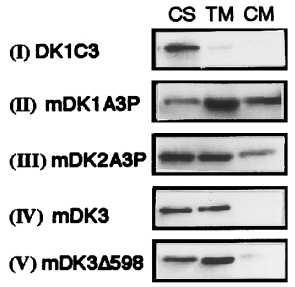

FIG. 5. Subcellular localization of DnaK1, DnaK2, and DnaK3 fusion proteins. Fusion proteins indicated in Fig. 4 were expressed in the wild type *Synechococcus* PCC7942 (mDK2A3P, mDK3, and mDK3Δ598) or strain DK1KM(Δ*dnaK1* of PCC7942, DK1C3 and mDk1A3P). Cells were subfractionated and western blot analysis was carried out using anti DnaK3 or anti c-Myc antiserum for the cytosol (CS), thylakoid membrane (TM), and cytoplasmic membrane fraction. 10 μg proteins were loaded on each lane.

fraction, some have been reported to be associated with membrane fractions and DnaK3 reported in this paper is the first example in which such proteins were associated with the thylakoid membrane.

Hsp70 proteins consist of two domains, highly conserved N-terminal ATP-binding domain (ca. 380 to 390 amino acids) and less conserved C-terminal peptide-binding domain (21). Among the three DnaK proteins, about 500 a.a. residues from the N-terminus including the ATP-binding domain are also highly conserved. Moreover DnaK2 and DnaK3, which are closely related to each other, contain commonly several motifs specific to Hsp70 proteins of chloroplasts (9). In the chloroplasts, three Hsp70 homologues have been immunologically detected (22). Two of them were found in soluble proteins in the stroma (22, 23) and are considered to be involved in several chaperon functions, including the integration of proteins into the thylakoid membrane (24), the translocation of proteins across the envelope membranes, or in the folding and the assembly of proteins (25, 26). The other one was associated with the outer envelope membrane (22, 27). It was suggested that this protein played a role in protein import into chloroplasts through the association with import intermediates (30). No Hsp70 homologues were detected in the thylakoid membrane of the chloroplast (22).

Apparently DnaK3 does not contain a thylakoid-targeting sequence nor hydrophobic region which could be a membrane-spanning domain. These facts indicate that DnaK3 is associated with the thylakoid membrane on the cytosol side. This type of interaction suggests the presence of a receptor protein for DnaK3 in the thylakoid membrane. Our study using chimeric and truncated DnaK3 protein indicated that the region required for the association with the thylakoid membrane was not CTF but the region between a.a. 381 and a.a. 598. Since this region varies considerably among the Hsp70 proteins and is known to be a substrate-binding domain, it is possible that this region provides a functional specificity to DnaK3 in the thylakoid membrane association. DnaK3 may recognize some receptor on the thylakoid membrane in the same way as DnaK recognizes its substrate and binds to it. The function of CTF remains unclear. Our preliminary work suggests that the deletion of CTF does not affect the normal growth of the cell (data not shown). However, since one of the three DnaK proteins of cyanobacterium *Synechocystis* sp. PCC6803 also has a C-terminal extension homologous to CTF including the conserved GWDDDDDXWF sequence (data base accession no. D29968), this region may be endowed with a specific function.

Strict protein sorting between the cytoplasmic and the thylakoid membranes has been proposed as a mechanism due to the difference in the protein composition between these two membranes. The SecA and the SecY proteins which are components of the protein translocation

machinery across the membrane, have been reported to be located in both membranes (16, 19). Therefore other factor(s) that facilitate protein sorting to a specific membrane may be involved. It is intriguing to note that a chaperon like DnaK3 is such a factor leading proteins to cytoplasmic or thylakoid membranes.

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