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Salinity-Stress-Induced Proteins in Two Nitrogen-Fixing *Anabaena* Strains Differentially Tolerant to Salt

SHREE KUMAR APTE* AND ARVIND A. BHAGWAT†

Molecular Biology & Agriculture Division, Bhabha Atomic Research Centre, Trombay, Bombay 400085, India

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Salinity altered the protein synthesis patterns in two cyanobacterial strains: *Anabaena torulosa*, a salt-tolerant brackish water strain, and *Anabaena* sp. strain L-31, a salt-sensitive freshwater strain. The cyanobacterial response to salinity was very rapid, varied with time, and was found to be correlated with the external salt (NaCl) concentration during stress. Salinity induced three prominent types of modification. First, the synthesis of several proteins was inhibited, especially in the salt-sensitive strain; second, the synthesis of certain proteins was significantly enhanced; and third, synthesis of a specific set of proteins was induced de novo by salinity stress. Proteins which were selectively synthesized or induced de novo during salt stress, tentatively called the salt-stress proteins, were confined to an isoelectric pI range of 5.8 to 7.5 and were distributed in a molecular mass range of 12 to 155 kilodaltons. These salt-stress proteins were unique to each *Anabaena* strain, and their expression was apparently regulated coordinately during exposure to salt stress. In *Anabaena* sp. strain L-31, most of the salt-stress-induced proteins were transient in nature and were located mainly in the cytoplasm. In *A. torulosa*, salt-stress-induced proteins were evenly distributed in the membrane and cytoplasmic fractions and were persistent, being synthesized at high rates throughout the period of salinity stress. These initial studies reveal that salinity-induced modification of protein synthesis, as has been demonstrated in higher plant species, also occurs in cyanobacteria and that at least some of the proteins preferentially synthesized during salt stress may be important to cyanobacterial osmotic adaptation.

The molecular basis of microbial and plant resistance to environmental stress is not fully understood. Regulation of gene expression during temperature, or anaerobic stress has been examined in several microorganisms (3, 6, 18, 29), plants (9, 12, 21, 25), and animals (25). Salinity is an important deterrent to agriculture in many areas of the world, but investigations on its molecular effects are few.

Diazotrophic cyanobacteria (blue-green algae) are a unique group of photoautotrophic nitrogen-fixing organisms with an oxygenic mode of photosynthesis. In general, cyanobacteria exhibit considerable tolerance to salt and osmotic stress (11, 29). Although the molecular basis of the mechanisms involved in cyanobacterial salt tolerance is not fully understood, some concepts have emerged in recent years. Prominent among these mechanisms are (i) curtailment of Na^+ influx and prevention of intracellular Na^+ accumulation, which reduce the need to pump out excess Na^+ and conserve energy, resulting in greater salt tolerance (1, 2) (this mechanism, which is inherent in salt-tolerant strains [2], was recently shown to be also induced by environmental factors which inhibit Na^+ influx, for example, high pH [1] or the presence of combined nitrogen [B. R. Reddy, S. K. Apte, and J. Thomas, Plant Physiol., in press]); (ii) accumulation of internal osmoticum in the form of inorganic ions, such as K^+ (17, 24, 28), or organic solutes, such as glucopyranosylglycerol (7), sucrose (4), trehalose (23), or glycine betaine (22); and (iii) metabolic adjustments to tune the cellular activities to function at higher internal osmoticum (5, 28). Obviously, all these mechanisms imply modification(s) of the synthesis and/or activity of cell proteins to facilitate osmotic adaptation. Such modifications of proteins during salt-induced stress have not been investigated in cyanobacteria so far.

In this paper, we report the effect of salinity-induced stress on protein synthesis in nitrogen-fixing cultures of two *Anabaena* strains from either fresh water (*Anabaena* sp. strain L-31, salt sensitive) or brackish water (*Anabaena torulosa*, salt tolerant). The results show that exposure to salt (NaCl) stress alters the protein profiles and induces the synthesis of a new set of salt-specific proteins in both cyanobacterial strains.

MATERIALS AND METHODS

Organisms. Axenic cultures of two filamentous, heterocyst-forming nitrogen-fixing cyanobacteria isolated in this laboratory, *A. torulosa* (an akinete-forming, brackish water form [10]) and *Anabaena* sp. strain L-31 (a nonsporulating freshwater form [27]), were used. The two *Anabaena* strains differed considerably in their sensitivities to salt; in N-free agar medium, the 50% lethal dose of salt was 170 mM for *A. torulosa* and 55 mM for *Anabaena* sp. strain L-31 (1).

Growth conditions. Fivefold-diluted cyanophycean medium (CM/5) (8) free of combined nitrogen and containing 1 mM Na^+ at pH 7.0 was used for growth and maintenance of all the cultures. Culture medium pH was modified, when desired, by the addition of 1 M HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)–LiOH of suitable pH to CM/5 at a final HEPES concentration of 5 mM. When required, nitrate was added (as KNO_3) at 10 mM and ammonium was added (as NH_4Cl) at 3 mM to medium buffered to pH 7.0 with 5 mM HEPES. Liquid cultures were grown photoautotrophically at 30°C under constant illumination (2.5 mW/cm^2) and agitation (150 rpm). During growth in N-free medium without salt, the mean generation times of *A. torulosa* and *Anabaena* sp. strain L-31 were 18 and 17 h, respectively. *A. torulosa* did not form a akinetes under the

* Corresponding author.

† Present address: Nitrogen Fixation and Soybean Genetics Laboratory, Beltsville Agricultural Research Center, Agricultural Research Service, U.S. Department of Agriculture, Beltsville, MD 20705.

growth conditions used. Growth was assessed on the basis of the chlorophyll *a* content (16) or the content of soluble proteins (15).

Salinity-stress conditions. Cultures in the mid-exponential phase of growth (3 days) were harvested, suspended in fresh CM/5 (supplemented as required) at a chlorophyll *a* density of 10 μg/ml, and grown overnight before the addition of salt. Salt was added as NaCl (from a 5 M stock solution) at a final concentration of 55 mM for *Anabaena* sp. strain L-31 and 120 mM for *A. torulosa*, unless specified otherwise. Control (without salt) and salt-stressed cultures were incubated under illumination (2.5 mW/cm^2) in an orbital incubator shaker (30°C, 150 rpm) for 30 min, unless specified otherwise.

In vivo radiolabeling of proteins with [^{35}S]methionine. Cyanobacterial cell suspensions were pulse-labeled with [^{35}S]methionine (specific activity, > 30 TBq/mmol) added at 1.48 MBq/ml (*A. torulosa*) or 2.3 MBq/ml (*Anabaena* sp. strain L-31) during the last 5 min of exposure to salt stress. Cell suspensions were incubated under the growth conditions wherein the uptake of [^{35}S]methionine was linear for at least 20 min in both *Anabaena* spp. Radiolabeling was terminated after 5 min by transfer of cells to ice and rapid (1 min) centrifugation in a microcentrifuge. Cells were washed three times with CM/5 and finally washed with distilled water. For separation of membrane and cytoplasmic fractions, cells were lysed by freezing them in liquid nitrogen (1 h) and subsequently thawing them (on ice) and by dilution with an equal volume of CM/5. A subsequent 15-min centrifugation in a microcentrifuge yielded an intensely blue supernatant (treated as the cytoplasmic fraction) and a green pellet (treated as the crude membrane preparation).

Preparation of protein samples for electrophoresis. For preparation of sodium dodecyl sulfate (SDS)-extracted proteins, membranes or cytoplasmic fractions were suspended in CM/5. An equal volume of extraction buffer (Tris hydrochloride [pH 6.8], 125 mM; 2-mercaptoethanol, 5% [wt/vol]; SDS, 2% [wt/vol]; glycerol, 20% [wt/vol]; sodium azide, 20 mM; phenylmethylsulfonylfluoride, 1 mM; ethylene glycol-bis[β-aminoethyl ether]-*N,N,N',N'*-tetraacetic acid [EGTA], 20 mM; bromophenol blue, 0.002% [wt/vol] was added and mixed thoroughly. Samples were boiled for 5 min and then returned to ice and centrifuged for 15 min. Supernatants were used for electrophoresis. For two-dimensional electrophoresis, SDS-extracted proteins (prepared as described above) were supplemented with urea (9.5 M) and Nonidet P-40 (1%, wt/vol), mixed, and then clarified by centrifugation in a microcentrifuge and used.

Radioactivity incorporated into proteins was estimated by spotting 1-μl portions onto Whatman GF/C filter paper circles and immersing them in chilled 10% trichloroacetic acid (TCA). The filters were washed with chilled 5% TCA and chilled ethanol and air dried. Radioactivity was determined by transferring filters to 2,5-(5-bis tetrabutyl-2-benzoxazolyl) thiophene (BBOT) (0.4% [wt/vol] in toluene-methanol, 1:1) and counting in a Packard Tri-Carb liquid scintillation spectrometer. For proper comparison, equal amounts of radioactivity (based on TCA-precipitable counts) were loaded onto gels.

PAGE. For one-dimensional separation, proteins were electrophoresed on 5 to 14% polyacrylamide–SDS linear gradient slab gels (0.75-mm thickness) overlaid with a stacking gel. The gradient gel was prepared by mixing 9 ml each of freshly prepared solutions A and B through a gradient mixer. Solution A consisted of 0.375 M Tris hydrochloride (pH 8.8) and the following components (on a weight-to-volume basis): sucrose, 20%; acrylamide, 14%; bisacrylamide, 0.35%; SDS, 0.1%; and ammonium peroxydisulfate (APS), 0.035%. Solution B contained 0.375 M Tris hydrochloride (pH 8.8) and the following components (on a weight-to-volume basis): acrylamide, 5%; bisacrylamide, 0.135%; SDS, 0.1%; and APS, 0.035%. TEMED (*N,N,N',N'*-tetramethylethylenediamine) was added, to both solutions A and B, at 0.54 μl/ml just prior to gradient formation. The stacking gel contained 0.125 M Tris hydrochloride (pH 6.8) and the following components (on a weight-to-volume basis): acrylamide, 3.75%; bisacrylamide, 0.135%; SDS, 0.1%; APS, 0.075%; and TEMED, 0.75 μl/ml. Electrophoresis buffer contained 20 mM Tris-glycine (pH 8.3) and SDS (0.1%, wt/vol). Polyacrylamide gel electrophoresis (PAGE) was usually performed at 40 V for 16 h followed by 300 V for 1 h.

For two-dimensional separation, proteins were first subjected to isoelectric focusing (ampholine range, pH 3.5 to 10.0), followed by SDS-PAGE (10% polyacrylamide–SDS gel with a 3.75% polyacrylamide stacking gel [13,19]).

Autoradiography and visualization of proteins. High- and low-molecular-weight calibration kits (Sigma Chemical Co., St. Louis, Mo.) were used as molecular weight markers on all the gels. After electrophoresis, gels were fixed, stained with Coomassie brilliant blue-R250, and destained for visualization of molecular weight standards. The gels were then dried in a gel drier (Hoefer Scientific Instruments, San Francisco, Calif.) at 80°C for 2 h, cooled, and exposed to X-ray films (INDU, Bombay, India) for an appropriate period of time at room temperature. Typically, a 20-h exposure was adequate for visualization of most of the proteins when the radioactivity was added at 8×10^5 cpm per lane (for one-dimensional gels) or 8×10^6 cpm per isoelectric focusing gel (for two-dimensional gels).

RESULTS

Preliminary experiments established inherent differences in the uptake of methionine by the two *Anabaena* strains; *A. torulosa* displayed a methionine uptake nearly 1.6-fold higher than that of *Anabaena* sp. strain L-31 (data not shown). This difference was compensated for partly by adding more radiotracer methionine for radiolabeling *Anabaena* sp. strain L-31 proteins (2.2 MBq/ml) than for radiolabeling *A. torulosa* proteins (1.48 MBq/ml) in vivo. Under our experimental conditions, the rate of uptake of [^{35}S]methionine was linear for at least 20 min in both *Anabaena* spp. (data not shown) and 5 min was chosen as an appropriate time for radiolabeling proteins in all the experiments.

At concentrations higher than the 50% lethal dose of salt (i.e., 55 mM for *Anabaena* sp. strain L-31 and 170 mM for *A. torulosa*), salt inhibited the uptake of methionine in both *Anabaena* strains (Table 1). Lower NaCl concentrations were not inhibitory, and (unless specified otherwise) 55 mM (*Anabaena* sp. strain L-31) and 120 mM (*A. torulosa*) NaCl were generally applied as salinity stress in all the experiments. These salt concentrations were found to be adequate to create stressful conditions (i.e., inhibited growth and nitrogen fixation), did not affect methionine uptake or incorporation, and were, therefore, suitable for proper comparison of control and salt-stressed cultures on a qualitative as well as a quantitative basis.

Exposure of both *Anabaena* strains to salinity stress produced marked changes in their protein synthesis patterns. Three types of alterations were noted. (i) Synthesis of certain proteins declined significantly. (ii) Specific synthesis

TABLE 1. Uptake of [^{35}S]methionine and protein synthesis in *Anabaena* spp. exposed to salinity stress

Treatment	NaCl concn (mM)	[^{35}S]methionine uptake or incorporation (cpm/μg, 10^3) by[a]:			
		Anabaena sp. strain L-31		*A. torulosa*	
		Uptake[b]	Incorporation[c]	Uptake[b]	Incorporation[c]
Control	0	16.02	2.82	18.29	3.06
NaCl	10	16.30 (101)	2.88 (102)	18.61 (102)	3.06 (100)
	30	16.25 (101)	2.93 (104)	18.42 (101)	3.15 (103)
	55	16.11 (100)	2.78 (99)	18.33 (100)	3.30 (108)
	90	14.20 (89)	2.50 (89)	18.01 (98)	3.22 (105)
	120	13.36 (83)	2.06 (73)	18.03 (99)	3.01 (98)
	150	12.37 (77)	1.88 (67)	16.52 (90)	2.91 (95)
	170	10.85 (68)	1.72 (61)	15.17 (83)	2.72 (89)
	200	9.85 (62)	1.44 (51)	14.26 (78)	2.42 (79)

[a] Values in parentheses are percentages of controls.
[b] Total radioactivity in SDS-extracted cells was regarded as [^{35}S]methionine uptake.
[c] Methionine incorporation (protein synthesis) was estimated from the TCA-precipitable counts in SDS-extracted cells.

of certain other proteins was remarkably enhanced. (iii) Synthesis of a set of specific proteins was induced de novo. The kinetics of salinity-induced cyanobacterial responses, their distribution in time and cellular space, and their dependence on salt concentration are described in the legends to Fig. 1 to 3. Only the reproducible and prominent differences were taken into account.

The time course of salinity-induced modifications of protein synthesis in the two *Anabaena* spp. is shown in Fig. 1. It should be mentioned here that the control (unstressed) cultures also showed changes in protein profiles with time. This was because these cultures were heterocyst-differentiating cultures, and the protein profiles showed morphogenesis-dependent changes. At each time interval, the protein profile of a salt-stressed culture was therefore compared with its own respective control at that time point to distinguish the salinity-induced changes from those associated with heterocyst differentiation. The response to salt stress was quite rapid; most of the discernible changes occurred within 10 min of exposure to salt. In *Anabaena* sp. strain L-31, synthesis of a set of proteins (apparent molecular masses, 208, 158, 47, 40, and 19.5 kilodaltons [kDa]) significantly declined after 30 min of exposure to 55 mM NaCl (Fig. 1a). Another polypeptide (56 kDa), synthesized at high rates by control cultures at 2 h, was detected at very low levels in the salt-stressed cultures. In comparison, in *A. torulosa* salinity slowly inhibited the synthesis of several proteins (apparent molecular masses, 310, 280, 160, 143, 127, 115, 103, 89, 88, 84, 66, and 51 kDa) in a time-dependent manner. In this strain, synthesis of only 3 proteins was inhibited within 10 min, that of 6 proteins was inhibited after 30 min, and that of 10 proteins was inhibited after 2 h, while after 6 h synthesis of a total of 12 proteins was inhibited under exposure to 120 mM NaCl (Fig. 1b).

Salinity specifically enhanced the synthesis of a set of proteins (Fig. 1b, dotted arrows) and also induced de novo synthesis of another set of proteins (Fig. 1b, solid arrows) in both *Anabaena* strains. These proteins were considered likely to be important in osmotic adaptation and were tentatively called the salt-stress proteins (SSP). In *Anabaena* sp. strain L-31 (Fig. 1a), nine SSP (molecular masses, 82, 41, 38.5, 35, 25, 23, 21, 20, and 19 kDa) were induced within 10 min of salt stress; their synthesis increased up to 30 min but

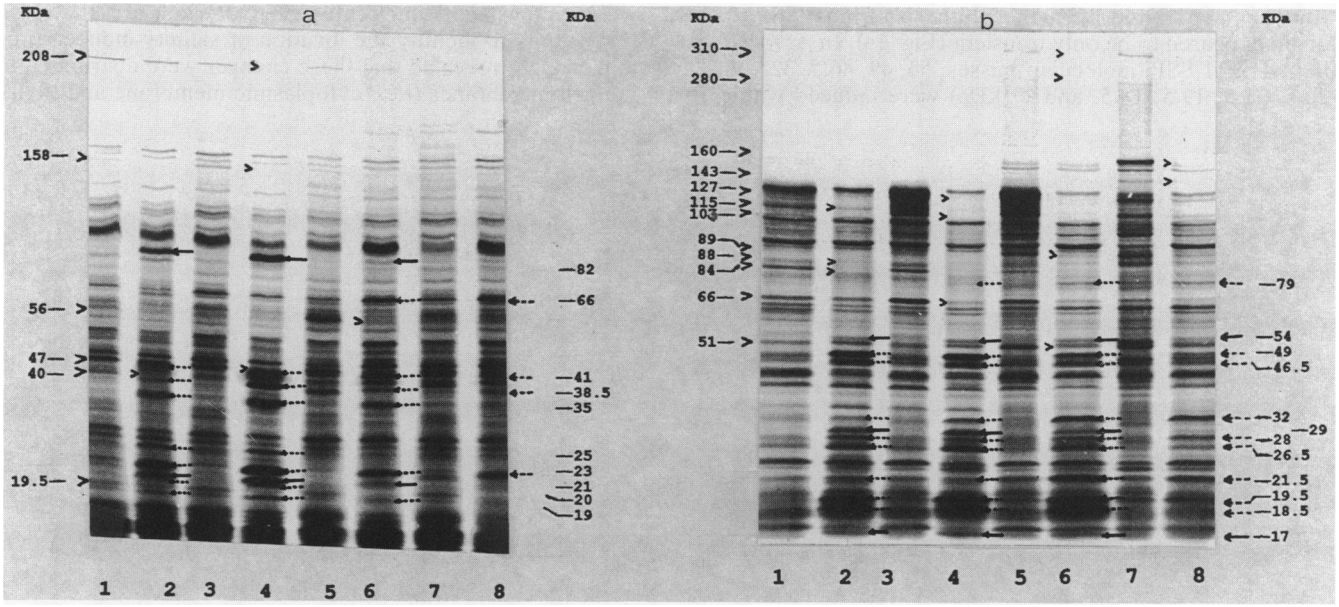

FIG. 1. Time course of salinity-induced modifications of protein synthesis in *Anabaena* sp. strain L-31 (a) and *Anabaena torulosa* (b). Cyanobacteria were exposed to either 55 mM NaCl (a) or 120 mM NaCl (b). At time intervals of 10 min (lanes 1 and 2), 30 min (lanes 3 and 4), 2 h (lanes 5 and 6), and 6 h (lanes 7 and 8), 1-ml portions of cells were radiolabeled with [^{35}S]methionine at either 2.20 MBq/ml (a) or 1.48 MBq/ml (b) for 5 min. Proteins were extracted and resolved by SDS-PAGE on a 5 to 14% linear gradient gel followed by autoradiography. At each time point, salt-treated cells (lanes 2, 4, 6, and 8) were compared with untreated control cells (lanes 1, 3, 5, and 7). Proteins whose synthesis declined are shown by solid arrowheads (>) to the left of lanes. Proteins which were preferentially synthesized during salt stress are shown to the right of lanes. Dotted arrows (<--) indicate proteins whose synthesis was selectively enhanced, and solid arrows (<—) denote proteins whose synthesis was induced de novo during salt stress. The numbers indicate the apparent molecular masses (in kilodaltons) of different proteins.

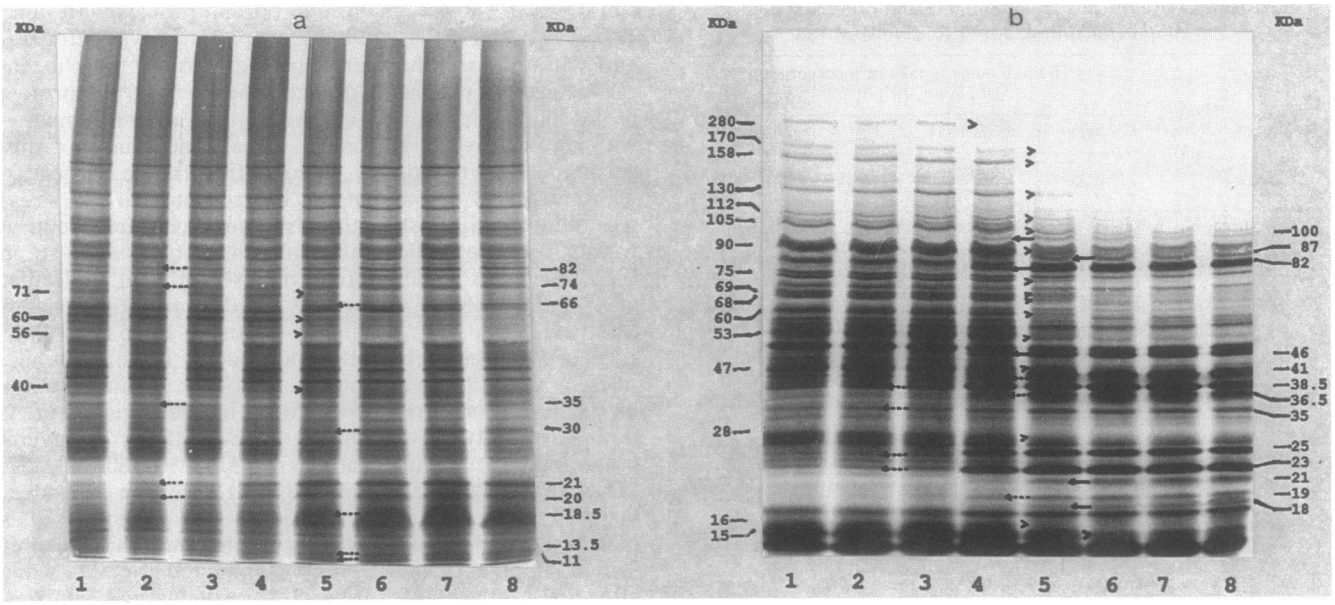

FIG. 2. Effect of external salt (NaCl) concentrations on protein synthesis in the membrane (a) and cytoplasmic fraction (b) of *Anabaena* sp. strain L-31. Cyanobacterial suspensions were exposed to the following concentrations (millimolar) of NaCl: none (lane 1), 2 (lane 2), 5 (lane 3), 10 (lane 4), 20 (lane 5), 40 (lane 6), 60 (lane 7), and 80 (lane 8). Salt stress was applied for 25 min and was followed by radiolabeling with 2.2 MBq of [^{35}S]methionine per ml for 5 min in the presence of salt. Cells were washed, membrane and cytoplasmic fractions were separated, and proteins were extracted and resolved by SDS-PAGE on a 5 to 14% linear gradient gel, followed by autoradiography. Other details are given in the legend to Fig. 1.

declined thereafter. Another SSP (66 kDa) was observed after 2 h of exposure to salt. After 6 h of salt stress, only 4 of the initially observed 10 SSP continued to be synthesized at much lower rates in this salt-sensitive strain. Thus, in *Anabaena* sp. strain L-31, the synthesis of most SSP (6 out of 10) appeared to be only transient (Fig. 1a). In *A. torulosa*, a total of 11 SSP (molecular masses, 54, 49, 46.5, 32, 29, 28, 26.5, 21.5, 19.5, 18.5, and 17 kDa) were induced within 10 min and 1 more SSP (79 kDa) was induced after 30 min of exposure to 120 mM NaCl (Fig. 1b). In contrast to *Anabaena* sp. strain L-31, in salt-tolerant *A. torulosa* almost all of the SSP (except one, of 29 kDa) were found to be persistent and were synthesized at high rates even after 6 h of salt stress.

Attempts to identify the location of salinity-induced protein changes revealed that these changes were distributed in the cell membranes (i.e., cytoplasmic membrane and thyla-

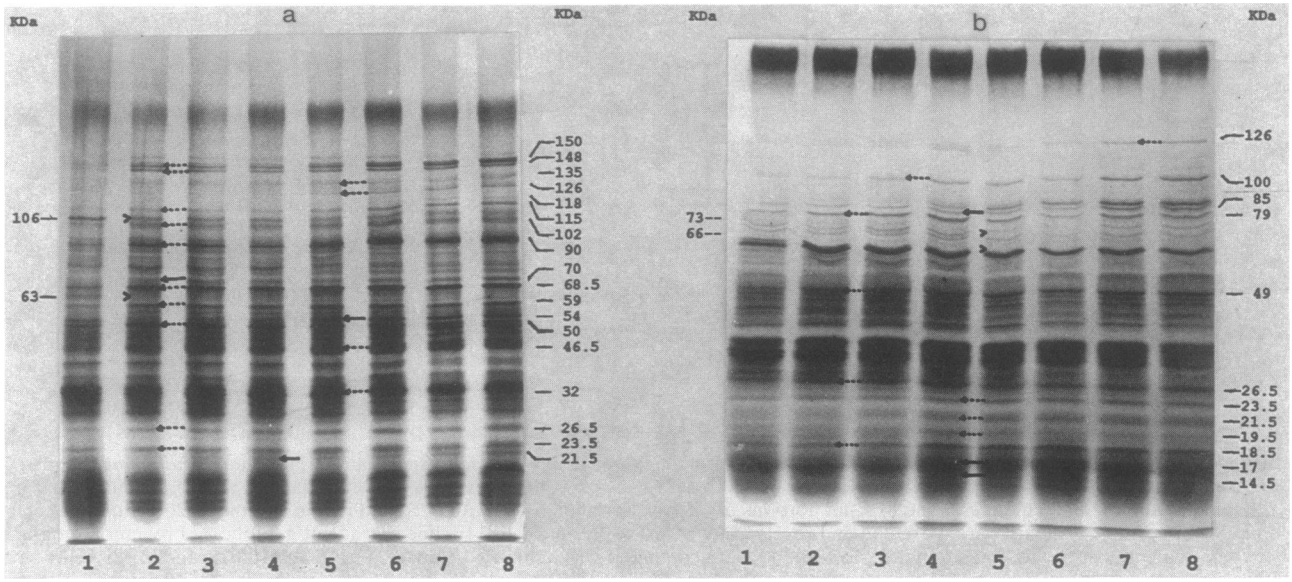

FIG. 3. Effect of external salt (NaCl) concentrations on protein synthesis in the membrane (a) and cytoplasmic fractions (b) of *A. torulosa*. Salt was applied at following concentrations (millimolar): none (lane 1), 5 (lane 2), 10 (lane 3), 20 (lane 4), 40 (lane 5), 60 (lane 6), 90 (lane 7), and 120 (lane 8). Other details are given in the legends to Fig. 1 and 2.

koids) as well as in the cytoplasm. The efficacy of the fractionation of membrane and cytoplasmic fractions was tested by assaying for the specific markers glucose 6-phosphate dehydrogenase (a soluble cytoplasmic enzyme) and chlorophyll *a* (a membrane-bound pigment). Cross-contamination of glucose 6-phosphate dehydrogenase in the membrane was nil, while the cytoplasmic fractions sometimes contained 3 to 6% of the chlorophyll *a* found in the membrane fraction as compared on a per-milligram-of-protein basis. Thus, the partial fractionation was considered satisfactory and adequate to determine the cellular location of these proteins.

In *Anabaena* sp. strain L-31, 6 of 10 SSP shown in Fig. 1a were located mainly in the cytoplasm, 2 were located mainly in the membrane, and 2 were found both in the membrane and in the cytoplasmic fractions (Fig. 2a and b). In *A. torulosa*, of the SSP shown in Fig. 1b five SSP were found in the cytoplasm, three were found in the membrane, and two were found in both fractions (Fig. 3a and b). Separation of membrane and cytoplasmic fractions helped in the identification of four new SSP (30, 18.5, 13.5, and 11 kDa) in the membrane and five new SSP (100, 87, 46, 36.5, and 18 kDa) in the cytoplasmic fractions of *Anabaena* sp. strain L-31 (Fig. 2). In *A. torulosa* also (Fig. 3), 13 new SSP (150, 148, 135, 126, 118, 115, 102, 90, 70, 68.5, 59, 50, and 23.5 kDa) were identified in the membrane and 4 new SSP (126, 100, 85, and 14.5 kDa) were identified in the cytoplasm. These additional SSP could not be detected in the crude extracts of whole filaments (Fig. 1). In each *Anabaena* strain, only two SSP were found in both the membrane and cytoplasmic fractions. The various proteins whose synthesis was inhibited or induced by salinity appeared to be unique to each *Anabaena* strain.

Salinity-altered protein profiles in the membrane and cytoplasmic fractions of both *Anabaena* spp. were found to be dependent on the concentration of NaCl applied during salt stress (Fig. 2 and 3). Irrespective of their sensitivities to salt, both *Anabaena* strains synthesized SSP at very low concentrations of NaCl. In *Anabaena* sp. strain L-31 (Fig. 2a and b), SSP were observed at 2 mM NaCl and their synthesis increased with increasing salt concentrations up to 20 to 40 mM. Induction and enhanced synthesis of all the SSP and repression of proteins were complete in this salt-sensitive strain at 20 mM NaCl, and no additional effects on protein profiles were observed at higher salt concentrations. In *A. torulosa* (Fig. 3a and b), few SSP could be detected at 5 mM; the synthesis of other SSP was dependent on salt concentration, and all the SSP were observed only at NaCl concentrations above 90 mM in both the membrane and cytoplasmic fractions.

To assess whether the salinity-induced modifications of protein synthesis resulted from the external salinity or from intracellularly accumulated NaCl during salt stress, experiments were performed with cyanobacteria grown under physiological conditions known to inhibit Na^+ influx. The presence of 10 mM NO_3^-, 3 mM NH_4^+, and alkaline external pH had earlier been shown to inhibit Na^+ influx (1, 2) and to enhance the salt tolerance of both *Anabaena* sp. strain L-31 and *A. torulosa* (1). Although the protein profiles of cyanobacteria grown with nitrate or ammonium or at pH 8.0 were different from those of nitrogen-fixing cyanobacteria grown at pH 7.0, when exposed to salt the synthesis of SSP was found to be completely identical (data not included) to that shown in Fig. 1 to 3, irrespective of the differences in growth conditions used prior to the application of salt stress. Thus, the modifications of protein synthesis patterns observed were a consequence of the external, not the intracellular, NaCl concentrations.

Addition of specific inhibitors of transcription (rifampin, 50 μg/ml) or translation (chloramphenicol, 100 μg/ml) inhibited the salinity-induced synthesis of SSP in both *Anabaena* strains (data not shown).

Two-dimensional separation (isoelectric focusing followed by SDS-PAGE) of proteins synthesized by control and salt-stressed cultures of both *Anabaena* spp. confirmed all the results shown in Fig. 1 to 3 (data not shown). Partial to complete loss of a total of 30 peptides in *Anabaena* sp. strain L-31 and of 17 peptides in *A. torulosa* and synthesis of 28 SSP in *Anabaena* sp. strain L-31 and of 24 SSP in *A. torulosa* could be clearly identified by two-dimensional separations. Most of the SSP were confined to a narrow isoelectric pI range of 5.8 to 7.5 in both *Anabaena* strains (data not shown) and to a molecular mass range of 18 to 82 kDa in *Anabaena* sp. strain L-31 and 12 to 155 kDa in *A. torulosa*. Interestingly, none of the proteins whose synthesis was modified by salt appeared to be common to both *Anabaena* spp., each of which exhibited a unique protein synthesis response to salinity stress.

DISCUSSION

Cyanobacteria are generally considerably tolerant to salt stress, and the occurrence of halophilic or marine strains is by no means rare among this group of photosynthetic procaryotes (11, 17, 22, 26, 28). In the absence of suitable cyanobacterial mutants, molecular analysis of genotypes that show significant variation in salt sensitivity seems to be a potentially fruitful approach for analysis of salt tolerance. This approach has been employed in the present study to elucidate molecular changes accompanying osmotic adaptation in nitrogen-fixing cyanobacteria.

Exposure to salinity results in a qualitative and quantitative regulation of individual proteins in the two *Anabaena* strains irrespective of their abilities to resist salt stress. Synthesis of a wide spectrum of proteins is either curtailed or enhanced, and in addition, synthesis of a specific set of proteins is coordinately induced de novo (Fig. 1 to 3). The response to salt is very rapid, varies with the duration of exposure to salinity (Fig. 1), and is dependent on the concentration of NaCl (Fig. 2 and 3). The proteins preferentially synthesized during salt stress have been tentatively called salt stress proteins (SSP) and are found distributed both in the cell membrane and in the cytoplasm (Fig. 2 and 3).

Although the general nature of the response to salt is similar in the two *Anabaena* strains, the individual proteins whose synthesis is affected by salt appear to be unique to each strain. On the basis of the apparent molecular masses, pI (data not shown), time of induction, concentration of salt required for induction, and their cellular location, no protein appears to be common to the two strains investigated (Fig. 1 to 3). The regulation of protein synthesis by salinity thus appears to be specific to each genotype.

In general, the responses of the two strains to salinity are in agreement with their respective sensitivities to salt stress. In salt-sensitive *Anabaena* sp. strain L-31, salinity inhibits the synthesis of a large number of polypeptides (30, compared with 17 in *A. torulosa*). Also, the response to salinity is saturated at relatively low salt concentrations (20 mM) (Fig. 2) and is complete within 30 min (Fig. 1). In contrast, in salt-tolerant *A. torulosa* salinity-mediated modifications of proteins progress in a time- and concentration-dependent

manner (Fig. 1 and 3) and are completed only after 6 h of exposure to 90 mM NaCl or higher concentrations. Also, the induction of most SSP in *Anabaena* sp. strain L-31 is only transient, while in *A. torulosa* almost all of the SSP persist throughout the period of salinity stress. The kinetics of SSP induction (Fig. 1 to 3) are indicative of a slow, gradual osmotic adaptation in *A. torulosa* but not in *Anabaena* sp. strain L-31.

The cyanobacterial response to salinity stress seems to involve some properties in common with the salinity-induced protein modifications observed in higher plants (9, 21) and is also reminiscent of the heat shock response typical of eucaryotes (3, 12, 25) as well as prokaryotes (18, 25, 29), including cyanobacteria (6). One major difference between the cyanobacterial response and that of plants, however, is that the stress proteins (both heat shock proteins and SSP) of plants are predominantly of low molecular mass (12, 21), while cyanobacterial SSP (Fig. 1 to 3) and heat shock proteins (6) appear to be distributed over a wide range of molecular masses (10 to >100 kDa). Nevertheless, such a wide occurrence of these analogous phenomena suggests that SSP or heat shock protein induction must be important for cellular adaptation to changes in environmental temperature and salinity.

The data available at present suggest that the expression of SSP may be regulated at the level of transcription in cyanobacteria. In both the salt-sensitive strain and the salt-tolerant strain, salinity exerted its influence externally; i.e., an actual transport of NaCl into cells was not essential to elicit modifications of protein synthesis. This strongly suggests that the sensor of salt or osmotic stress is located on the outer cell membrane and that this sensor may trigger a salt-stress-specific regulator to bring about the apparently synchronous expression of SSP.

Mechanisms important to osmotic adaptation in several organisms, including cyanobacteria, include Na^+ pumps for active Na^+ extrusion, transport systems for K^+ and osmotically active organic molecules, enzymes responsible for intracellular synthesis of organic osmotica, and qualitative and quantitative modifications of metabolic pathways. Most of these mechanisms have been shown to be induced by salt or osmotic stress (4, 5, 7, 14, 17, 20, 22–24, 28). It is obvious that while some of these mechanisms would operate in the cell membrane others would have to be developed in the cytoplasm during osmotic stress. Indeed, in salt-tolerant *A. torulosa* the induced SSP are found evenly distributed in the cell membrane (Fig. 3a), as well as in the cytoplasm (Fig. 3b). Induction of not just a few but several SSP is in agreement with the fact that salt tolerance is probably a polygenic trait (14, 21). Failure to synthesize appropriate membrane proteins, the transient nature of most of its prominent SSP, and the predominant inhibition of several vital proteins may partly account for the salt sensitivity of *Anabaena* sp. strain L-31.

The biological significance of the SSP in cyanobacteria is unknown at present, as are the identities and functions of the individual SSP. The rapidity and the magnitude of the response of cyanobacterial proteins to salinity and its dependence on the salt concentration indicate the involvement of the proteins in the ongoing osmotic adjustments. Modifications of proteins with increasing salt concentrations or increasing durations of exposure to salinity and the persistence of SSP in *A. torulosa* strongly favor a role for at least some of the SSP in cyanobacterial salt tolerance.

LITERATURE CITED

1. **Apte, S. K., B. R. Reddy, and J. Thomas.** 1987. Relationship between sodium influx and salt tolerance of nitrogen-fixing cyanobacteria. Appl. Environ. Microbiol. **53**:1934–1939.
2. **Apte, S. K., and J. Thomas.** 1986. Membrane electrogenesis and sodium transport in filamentous nitrogen-fixing cyanobacteria. Eur. J. Biochem. **154**:395–401.
3. **Berg, G. R., W. E. Inniss, and J. Heikkila.** 1987. Stress proteins and thermotolerance in psychrotrophic yeasts from arctic environments. Can. J. Microbiol. **33**:383–389.
4. **Blumwald, E., R. J. Mehlhorn, and L. Packer.** 1983. Studies of osmoregulation in salt adaptation of cyanobacteria with ESR spin probe techniques. Proc. Natl. Acad. Sci. USA **80**:2599–2602.
5. **Blumwald, E., and E. Tel-Or.** 1983. Salt adaptation of the cyanobacterium *Synechococcus* 6311 growing in continuous culture (turbidostat). Plant Physiol. **74**:183–185.
6. **Borbely, G., G. Suranyi, A. Korcz, and Z. Palfi.** 1985. Effect of heat shock on protein synthesis in the cyanobacterium *Synechococcus* sp. strain PCC 6301. J. Bacteriol. **161**:1125–1130.
7. **Borowitzka, L. J., S. Demmerle, M. A. Mackay, and R. S. Norton.** 1980. Carbon-13 nuclear magnetic resonance study of osmoregulation in a blue-green alga. Science **210**:650–651.
8. **David, K. A. V., and J. Thomas.** 1979. Extracellular peptides of *Anabaena* L-31: evidence for their role in regulation of heterocyst formation. J. Biosci. **1**:447–455.
9. **Ericson, M. C., and S. H. Alfinito.** 1984. Proteins produced during salt stress in tobacco cell culture. Plant Physiol. **74**:506–509.
10. **Fernandes, T., and J. Thomas.** 1982. Control of sporulation in the filamentous cyanobacterium *Anabaena torulosa*. J. Biosci. **4**:85–94.
11. **Fogg, G. E., W. D. P. Stewart, P. Fay, and A. E. Walsby.** 1973. The blue-green algae. Academic Press, Inc. (London), Ltd., London.
12. **Kimpel, J. A., and J. L. Key.** 1985. Heat shock in plants. Trends Biochem. Sci. **85**:353–357.
13. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
14. **Le Rudulier, D., A. R. Strom, A. M. Dandekar, L. T. Smith, and R. C. Valentine.** 1984. Molecular biology of osmoregulation. Science **224**:1064–1068.
15. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
16. **Mackinney, G.** 1941. Absorption of light by chlorophyll solutions. J. Biol. Chem. **140**:315–322.
17. **Miller, D. M., J. H. Jones, J. H. Yopp, D. R. Tindall, and W. D. Schmid.** 1976. Ion metabolism in a halophilic blue-green alga *Aphanothece halophytica*. Arch. Microbiol. **111**:145–149.
18. **Neidhardt, F. C., R. A. Van Bogelen, and E. T. Lau.** 1983. Molecular cloning and expression of a gene that controls the high temperature regulon of *Escherichia coli*. J. Bacteriol. **153**:597–603.
19. **O'Farrell, P. H.** 1975. High resolution two dimensional electrophoresis of proteins. J. Biol. Chem. **250**:4007–4021.
20. **Ostrem, J. A., S. W. Olson, J. M. Schmitt, and H. J. Bohnert.** 1987. Salt stress increases the level of translatable mRNA for phosphoenolpyruvate carboxylase in *Mesembryanthemum crystallinum*. Plant Physiol. **84**:1270–1275.
21. **Ramagopal, S.** 1987. Salinity stress induced tissue specific proteins in barley seedlings. Plant Physiol. **84**:324–331.
22. **Reed, R. H., J. A. Chudek, R. Foster, and W. D. P. Stewart.** 1984. Osmotic adjustments in cyanobacteria from hypersaline environments. Arch. Microbiol. **138**:333–337.
23. **Reed, R. H., D. L. Richardson, S. R. C. Warr, and W. D. P. Stewart.** 1984. Carbohydrate accumulation and osmotic stress in cyanobacteria. J. Gen. Microbiol. **130**:1–4.
24. **Reed, R. H., and W. D. P. Stewart.** 1985. Evidence for turgor sensitive K^+ influx in cyanobacteria *Anabaena variabilis* ATCC 29413 and *Synechocystis* PCC 6714. Biochim. Biophys. Acta **812**:155–162.

25. **Schlessinger, M. J., M. Ashburner, and A. Tissieres.** 1982. Heat shock: from bacteria to man. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
26. **Stacey, G. C., C. Van Baalen, and F. R. Tabita.** 1977. Isolation and characterisation of a marine *Anabaena* sp. capable of rapid growth in molecular nitrogen. Arch. Microbiol. **114**:197–201.
27. **Thomas, J.** 1970. Absence of the pigments of photosystem II of photosynthesis in heterocysts of a blue-green alga. Nature (London) **228**:181–183.
28. **Thomas, J., and S. K. Apte.** 1984. Sodium requirement and metabolism in nitrogen-fixing cyanobacteria. J. Biosci. **6**:771–794.
29. **Yamamori, T., and T. Yura.** 1982. Genetic control of heat shock protein synthesis and its bearing on growth and thermal tolerance in *Escherichia coli* K-12. Proc. Natl. Acad. Sci. USA **79**:860–864.