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Comparative Analysis of Proteins Induced by Heat Shock, Salinity, and Osmotic Stress in the Nitrogen-Fixing Cyanobacterium *Anabaena* sp. Strain L-31

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Heat, salinity, or osmotic stress influenced protein synthesis in nitrogen-fixing *Anabaena* sp. strain L-31. Salinity and osmotic stresses were identical and specifically induced 15 polypeptides. Four polypeptides were unique to heat shock, and four other polypeptides were induced under every stress. The results demonstrate a commonality and a stress specificity of protein synthesis regulation.

Environmental-stress-induced modifications of protein synthesis have been observed in microbes, plants, and animals (3, 7, 10, 12–14). However, the mechanisms which govern gene expression during stress and the biological significance of the stress-induced proteins are not well understood. A few cases are known in which exposure to a certain stress has been found to induce protein responses typical of another stress (3, 6) or even tolerance to another stress (9). This indicates that a certain commonality exists between responses to different stresses, and yet, individual stresses also induce a unique response.

Heterocystous cyanobacteria are photosynthetic procaryotes capable of diazotrophy. They are thought to have originated during the mesozoic era ($>3 \times 10^9$ years ago) and as a group are known to have survived a wide spectrum of environmental stresses such as heat and cold shock, anaerobiosis and oxygeny, photooxidation, nitrogen starvation, salinity, and osmotic stress (8). Cyanobacteria thus appear to be a suitable system for analysis of adaptive mechanisms developed in response to changing environmental conditions. Synthesis of heat shock proteins has been demonstrated in a unicellular cyanobacterium, *Synechococcus* sp. strain PCC 6301 (4). We have recently also identified salinity-induced alterations of cyanobacterial proteins in two *Anabaena* strains (1). We report here the comparative analysis of the effects of heat shock, osmotic shock, and salinity (with NaCl and KCl) stress on protein synthesis in a filamentous, heterocystous, nitrogen-fixing cyanobacterium, *Anabaena* sp. strain L-31, of freshwater origin.

Axenic cultures of *Anabaena* sp. strain L-31 (15) grown in fivefold-diluted cyanophycean medium (CM/5 [5]) were harvested in the mid-exponential phase of growth (3 days) and were used for stress induction experiments at a chlorophyll *a* density of 10 μg/ml (1). Heat treatment involved exposure to 45°C. Osmotic stress was applied as 20 to 60 mM sucrose, while salinity stress was applied as 20 to 60 mM NaCl or KCl. All the stress conditions elicited a protein synthesis response without affecting methionine uptake and incorporation (1; data not shown). Stressed and control cultures were incubated under illumination (2.5 mW/cm^2) in an orbital incubator shaker (30 or 45°C, 150 rpm) for 30 min. The cultures were pulse-labeled with [^{35}S]methionine (specific activity, >30 TBq/mmol) added at 2.3 MBq/ml during the last 5 min of exposure to each stress. Preparation of total-cellular-protein samples, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and two-dimensional electrophoresis (isoelectric focusing followed by SDS-PAGE) were performed as previously described (1, 11). Only the reproducible and prominent differences were taken into account.

Exposure to heat, salinity, or osmotic stress resulted in alterations in cyanobacterial-protein syntheses (Fig. 1). Three prominent types of modifications were noted: (i) synthesis of several proteins declined, (ii) synthesis of certain other proteins was selectively enhanced, and (iii) synthesis of a new set of proteins was induced de novo. Some of these responses were observed under all stress conditions (tentatively called the common-stress proteins), while others were found to be specific to either heat (heat shock proteins) or salinity/osmotic stress (osmotic-stress proteins).

Two-dimensional separation of stress-modulated proteins (Fig. 2) further resolved protein synthesis responses and identified additional polypeptides which could not be visualized by SDS-PAGE (Fig. 1). The various polypeptides repressed or induced under each stress condition are listed in Table 1. Among the proteins that were inhibited, four (290, 155, 96, and 52 kilodaltons [kDa]) were common to all the stresses and five (two of 56 kDa and three of 42 kDa) were repressed only during heat shock, while four (65, 47, 26, and 18.5 kDa) were repressed only during salinity/osmotic stress. Among the proteins which were preferentially synthesized or induced de novo, 4 (82, two of 23, and 19 kDa) were discernible under all the stresses and 15 (41.5, two of 41, 38.5, 25, two of 24, two of 23, 21, 20, 19, 18.5, and 18 kDa) were specifically induced only under salinity/osmotic stress, while induction of 4 proteins (92, 75, 65, and 32 kDa) were restricted to heat shock. The responses of protein synthesis to salt stress and osmotic stress were identical, indicating that the salinity stress-induced proteins previously reported (1) were all a result of osmotic stress per se and that the effect of ionic stress, if any, was negligible. This also explains our earlier observation that the cyanobacterial salinity stress-induced proteins could be induced externally

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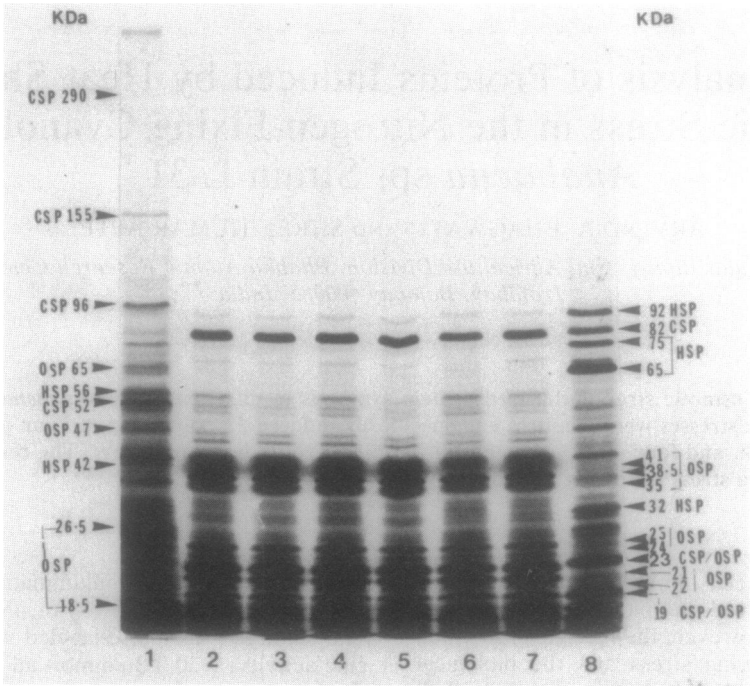

FIG. 1. Stress-induced modifications of protein synthesis in *Anabaena* sp. strain L-31. The cells were exposed to NaCl (lane 2, 20 mM; lane 3, 60 mM), KCl (lane 4, 20 mM; lane 5, 60 mM), sucrose (lane 6, 20 mM; lane 7, 60 mM), or heat shock (lane 8, 45°C) for 30 min. Untreated cells served as a control (lane 1). Portions of cells (1 ml) were radiolabeled with 2.3 MBq of [^{35}S]methionine per ml for the last 5 min under each stress. Total cellular proteins were extracted, added at equal counts per minute per lane, and resolved by SDS-PAGE on a 5 to 14% linear gradient gel followed by autoradiography as previously described (1). Proteins whose synthesis declined are named on the left. Proteins which were preferentially synthesized or induced de novo are named on the right. The numbers indicate apparent molecular masses of different proteins in kilodaltons. HSP, Heat shock proteins; CSP, common-stress proteins; OSP, osmotic-stress proteins.

TABLE 1. Analysis of the stress-induced proteins in *Anabaena* sp. strain L-31

Stress specificity of proteins	Characteristics of polypeptides:			
	Repressed		Induced or enhanced	
	Mass (kDa)	pI	Mass (kDa)	pI
Common	290	6.3	82[a]	6.3
	155	6.5	23	6.3, 6.6
	96	6.5	19	7.1
	52	5.6		
Osmotic-salinity	65	6.8	41.5[a]	6.6
	47	6.9	41[a]	5.8, 6.4[a]
	26.5	6.2	38.5	5.8
	18.5	5.8	35[a]	5.9
			25	6.5
			24[a]	6.3, 6.9[a]
			23[a]	6.0[a], 6.9[a]
			21[a]	6.4
			20	6.2
			19	6.5
			18.5	6.4
			18[a]	6.0
Heat shock	56	6.5, 7.5	92[a]	6.7
	42	6.5, 6.7, 6.9	75	6.3
			65	6.6
			32[a]	6.3

[a] Peptides induced de novo.

and why the intracellular presence of salt was not essential to evoke their induction.

The induction of several osmotic-stress proteins and the lack of induction of any salinity stress-specific proteins appear to correlate well with the considerable tolerance of *Anabaena* sp. strain L-31 to osmotic stress (concentrations required to inhibit growth by 50% were 55 mM NaCl [2] and >300 mM sucrose; T. A. Fernandes and S. K. Apte, unpublished results). This implies that at least some of the osmotic-stress proteins may be involved in osmotic adaptation in this cyanobacterium. The results also indicate that *Anabaena* sp. strain L-31 has no mechanisms analogous to osmotic-stress protein modulation to combat the ionic stress during exposure to salinity.

Inhibitors of transcription (rifampin, 50 μg/ml) and translation (chloramphenicol, 100 μg/ml) prevented induction of all the stress-induced proteins (data not shown). It appears that the expression of stress-induced proteins may therefore be regulated at the level of transcription. The present data also imply two levels of gene regulation during exposure to stress. A primary regulation appears to be common to several stresses and brings about coordinate expression of the common stress proteins. A second level of regulation may be more specific to individual stresses and may bring about expression of proteins unique to each stress condition, such as heat shock proteins and osmotic-stress proteins.

The commonality of the proteins repressed or induced in response to such divergent stresses as heat, osmosis, or salinity indicates that these proteins have an important role in the maintenance of vital cellular functions. Alternatively,

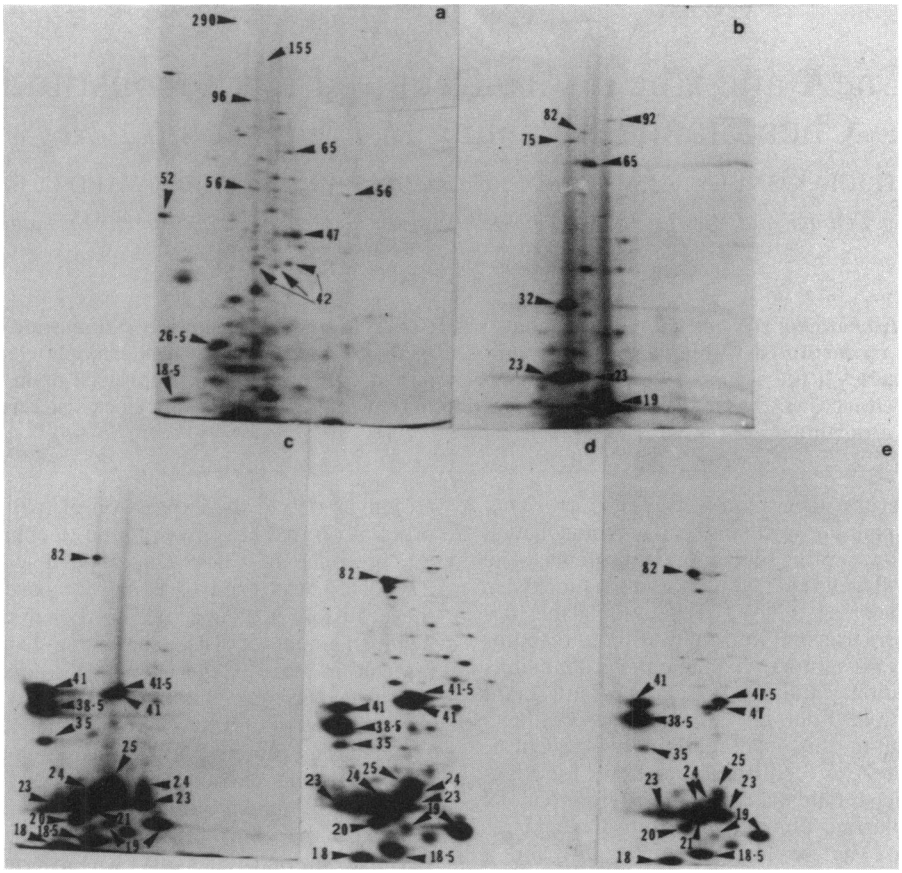

FIG. 2. Two-dimensional resolution of total cellular proteins synthesized by *Anabaena* sp. strain L-31 grown in the absence of stress (a); exposed to heat shock at 45°C (b); or exposed to 60 mM NaCl (c), 60 mM KCl (d), or 60 mM sucrose (e). Cyanobacterial proteins were radiolabeled in vivo with 2.3 MBq of [^{35}S]methionine per ml added for the last 5 min under each stress condition. Proteins were first subjected to isoelectric focusing (Ampholine; pH range, 3.5 to 10.0) and then resolved on the second dimension by SDS-PAGE (10% gel) followed by autoradiography as previously described (1, 11). The final pH range attained in the first dimension (isoelectric-focused gels) was 5.5 to 8.0. (a) Polypeptides in unstressed control culture which declined in response to various stresses; (b through e) polypeptides induced under various stress conditions. The numbers represent apparent molecular masses of proteins in kilodaltons.

they could be abnormal proteins synthesized in response to stress (16).

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