Metabolic Thermotolerance: Magnetic Resonance Detected Protection of Glutamate Synthase†

T. M. Logan, P. Zhong, and D. G. Lynn*

Department of Chemistry, University of Chicago, 5735 Ellis Avenue, Chicago, Illinois 60637

Received August 30, 1991; Revised Manuscript Received March 19, 1992

ABSTRACT: Metabolism in maize meristem cultures exposed to different heat treatments has been analyzed by ^{13}C-NMR spectroscopy of tissue extracts. The effects of a 40 °C permissive stress were compared with a 45 °C lethal stress, and the metabolism of glutamate and glutamine were markedly altered by both temperatures. Changes in the incorporation of labeled precursors, alterations due to the in vivo application of enzyme inhibitors, and differences in the activity of enzymes in cell free extracts have confirmed that glutamate synthase (GluS) is partially inactivated by the lethal thermal exposure. This enzyme is quantitatively protected by the induction of thermotolerance. The time dependence for the protection correlates with the appearance of a set of late-arising heat shock proteins (hsps). The function of these late-arising proteins is not yet known, but only one of them, a 67-kDa protein, is spatially correlated with GluS protection. Therefore, the quantitative protection of a key metabolic enzyme has been correlated with the in vivo function of a specific hsp.

Organisms must orchestrate an intricate and highly interdependent series of metabolic reactions to ensure viability. In any such network or series the weakest step defines the limits on the viability of the entire network. In the evolution of a metabolic network, a more durable enzyme catalyst may be in the process of forming or inherent physical limitations on the chemical transformation itself may have forced a separate protective mechanism to have developed. The transcriptionally controlled stress responses appear to represent such a separate protective strategy.

The universal biological response to elevated temperatures is the rapid synthesis of a set of so-called heat shock proteins, or hsps[1] (Lindquist, 1986; Lindquist & Craig, 1988; Vierling, 1991). While there is considerable variability in the number and molecular weight distributions and in the temperature required for inducing hsp synthesis among different organisms, the hsps are among the most highly conserved proteins in nature and undoubtedly serve common critical functions (Craig & Gross, 1991; Pechan, 1991; Gething & Sambrook, 1992). While the heat shock response has been intensely studied for the last decade, still little is known regarding the specific chemical and biochemical functions of these proteins.

A link between the presence of abnormal or denatured proteins and hsp synthesis has been established. Osmotic shock (Bonham-Smith et al., 1987), ethanol, abscissic acid (Heikkila, 1984), amino acid analogues and puromycin treatments (Hightower, 1980; Goff & Goldberg, 1985), and even the injection of denatured proteins (Anathan et al., 1986) elicit some subset of hsp synthesis. In vitro, these hsps bind to denatured protein aggregates (Pelham, 1986; Palleros et al., 1991), bind to nascent polypeptide chains on the ribosome (Lindquist & Craig, 1988; Rothman, 1989), facilitate translocation of proteins across membranes (Deshaies et al., 1988; Chirico et al., 1988), refold and assemble translocated proteins (Hemmingsen et al., 1988; Prasad & Hallberg, 1989; Ellis, 1990), and bind to and stabilize organellar membranes (Ignolia & Craig, 1982; Vierling, 1991). The hsps, then, are induced by denatured proteins and interact with denaturing protein domains. The proteins which require such stabilization in vivo during such heat shocks are, however, unknown.

Leenders et al. (1974) demonstrated a link between respiratory metabolism and the heat shock response of *Drosophila.* Application of respiratory inhibitors such as azide, salicylate, or rotenone or anaerobic conditions resulted in a stimulation of the same chromosomal regions as did heat shock. Chou et al. (1989) have shown that thermotolerated soybeans contained a higher fraction of mitochondria which remained coupled at elevated temperatures in in vitro oxygen consumption assays. Localization of hsps to the chloroplast (Amir-Shapira et al., 1990; Marshall et al., 1990; Vierling et al., 1986) or to the mitochondria (Nieto-Sotelo, et al., 1990) may function to protect specific metabolic reactions compartmentalized to those organelles. In addition, principle metabolic enzymes including enolase (Iida & Yahara, 1985), phosphoglycerate kinase, and GAPDH (Piper et al., 1988) have been shown to be heat shock proteins.

Therefore, it appears that the heat shock response has evolved to protect biological reactions, including the reactions of primary metabolism, against thermal stress. The question as to which reactions require such protection remains. This report details the use of ^{13}C-NMR methods (London, 1988; Cohen, 1989) to assign one of these metabolic weak links in maize meristem cultures to glutamate synthase (GluS), the primary enzyme of glutamate/glutamine metabolism.

METHODS AND MATERIALS

Tissue Culturing and Sampling Preparation. Maize seeds (*Zea mays*, var MX085A flats; OLDS Seed Co., Madison WI 53707) were surface sterilized by immersion in 1:1 bleach: H_2O for 20 min. The seeds were exhaustively rinsed with 10 volumes of sterilized water and hydrated for 4 h at room temperature. The seeds were planted on moistened sterile trays, wrapped in foil, and germinated at ambient temperature (25 ± 1 °C). After three days, the terminal 5–7 mm of the root tip was removed and approximately 150 root tips were placed

† This work was supported by the CIBA–GEIGY Corporation, The Midwest Plant Biotechnology Consortium, and Dow Chemical Co.

* To whom correspondence should be addressed.

[1] Abbreviations: GluS, glutamate synthase; GDH, glutamate dehydrogenase; GS, glutamine synthetase; hsp, heat shock protein; Glu-γ, glutamate C-4; Gln-γ, glutamine C-4; gdw, gram dry weight.

0006-2960/92/0431-7256$03.00/0

in sterile 125-mL Erlenmeyer flasks containing 7 mL of White's medium (White, 1943) with 1 mM NaOAc as a carbon source. The cultures were shaken (70 rpm) in the dark for 24 h before an additional milliliter of medium was added which contained sufficient sodium acetate, either at natural abundance or 99% [2-^{13}C]acetate, to give a final 10 mM acetate concentration. The cultures were maintained under these conditions for 45 min at the indicated temperatures. Following excision, plant tissues mount a wound response which includes altered respiration and increased metabolic fluxes through specific pathways (Uritani & Asahi, 1980). Culturing the roots for 24 h prior to metabolite analysis allowed sufficient time for the wound response to maximize and decline.

Application of Temperature Stresses. The cultured corn root tips were exposed to either 40 °C or 45 °C heat shock for 45 min coincident with the acetate feeding in a temperature-controlled (±1 °C) shaken water bath. The culturing roots were covered with foil during the heat shocks to maintain etiolated conditions. Following the methods of Lin et al. (1984), thermotolerance was induced by exposing the root tip cultures to a 10-min, 45 °C stress in the water bath shaker followed by an immediate return to 24 °C. These cultures were maintained for up to 4 h before the additional 1 mL of media was added to give the final 10 mM acetate concentration and they were exposed to the desired temperature treatment for 45 min.

Tissue Extraction and NMR Sample Preparation. ^{13}C-NMR samples were collected from a minimum of six replicate flasks, corresponding to about 1000 separate meristematic tips. The root tissues were collected with a minimum of handling (Wallace et al., 1984), rinsed with H_2O, and quickly frozen with liquid N_2. The frozen roots were lyophilized to dryness and combined into two separate samples, and the dry weights were obtained. The dried samples, ranging from 150 to 300 mg, were ground to a fine powder with a mortar and pestle and extracted overnight at 4 °C with 5% $HClO_4$ (approximately 10 mL). Insoluble material was removed by vacuum filtration through Whatmann No. 1 filter paper, and the filtrate was neutralized to pH 7.0 with 3 N KOH. The precipitate that formed during the neutralization was removed by vacuum filtration, and the filtrate was lyophilized to dryness. The lyophilized powder was suspended in a minimum of cold H_2O (<5 mL), and any insoluble material was removed by centrifugation in a desk-top centrifuge. The supernatant was lyophilized to dryness again, and this powder was dissolved in 0.7 mL of 5 mM Na_4EDTA in D_2O (99.9%) containing 2 μL/mL *p*-dioxane (Baker) as internal standard. Any insoluble material was removed by centrifugation as above prior to NMR analysis. The extraction gives ≥70% recovery of the metabolites as measured by the recovery of added standards.

^{13}C-NMR Spectroscopy and Signal Intensity. All ^{13}C-NMR spectra were collected on a GE 300 Omega NMR spectrometer operating at 75.7 MHz for carbon. A 20-kHz spectral window was digitized by 32K complex points, which were apodized by a 1.5-Hz decreasing exponential line-broadening factor prior to Fourier transformation to improve signal-to noise. An on-resonance 81° excitation pulse was used for excitation and a 4.18 s relaxation delay was given to provide a total time of 5.82 s between pulses. A total of 8000–10 000 scans were signal averaged per spectrum, and the total acquisition time was approximately 13 h. Continuous proton decoupling with Waltz modulation was applied throughout the experiment. Carbon chemical shifts were referenced to added *p*-dioxane at δ = 67.4 ppm.

The lineshape of the signals in the ^{13}C-NMR spectra (Figure 1) was sufficient to allow assignments based upon one- and two-dimensional NMR techniques, and the assignments were generally consistent with those made previously (London, 1988). Unassigned resonances could be determined by the initial assignment of the proton spectrum of the mixture with a combination of COSY and HOHAHA experiments followed by correlating the protons with the carbon spectrum using indirect detection (HMQC) experiments (Logan, 1991). These assignments were confirmed either by incorporation of ^{13}C-enriched acetate into specific carbons of various metabolites or by addition of a known standard (Table I).

Quantification of the metabolite levels in natural abundance experiments required conversion of the measured resonance intensity to concentration. The lineshapes of the metabolite carbons following apodization (Figure 1, insert) made peak heights the most reliable measure of peak intensity (Armitage et al., 1974). A recent article quantifying the accuracy of the use of peak heights has appeared (Bain et al., 1991). Each measured value was corrected for differences in resonance offset (effective excitation angle), T_1 relaxation rates and nuclear Overhauser enhancements (nOe) using eq 1 (Christensen et al., 1974), where I_{obs} is the observed intensity, I_{corr} is the corrected intensity, t is the time between pulses, T_1 is the longitudinal relaxation time constant, θ is the effective excitation angle, and $(\eta + 1)$ is the Overhauser enhancement factor. Effective excitation angles, θ, were determined as $\tan^{-1}(\gamma B_1/\Delta\omega)$, where γB_1 is the effective field produced by the excitation pulse (13.5 kHz) and $\Delta\omega$ is the resonance offset. Carbon T_1's were measured using the standard Freeman–Hill inversion–recovery experiment (Freeman & Hill, 1970) and the nOe was measured by gated decoupling (Freeman et al., 1972). T_1 and nOe measurements were made using 15-s relaxation delays to allow for complete relaxation (Harris & Newman, 1976), and the carrier frequency was moved upfield to approximately 60 ppm to ensure complete inversion of all resonances. The corrected intensity was converted to a concentration by comparing the corrected intensity of a particular resonance X (normalized to the sample dry weight) with that of dioxane, present at a fixed concentration, as shown in eq 2. Under these conditions, the standard deviation in the concentration from four replicate measurements varied between 15% and 24%, depending on the metabolite. This variability was larger than found in related studies (Cohen et al., 1981), due primarily to the comparison of samples from different root cultures, but was sufficiently small to identify the major changes. The largest variation in metabolite concentrations was seen in the 24 °C cultures, and those data are given in Table I.

$$I_{X(corr)} = \frac{I_{X(obs)}\{\exp(t/T_1) - \cos\theta\}}{\sin\theta\{\exp(t/T_1) - 1\}}\frac{1}{(\eta + 1)} \quad (1)$$

$$\mu\text{mol of X} = \frac{I_{X(corr)}}{I_{diox(corr)}/4} \times 16.4\ \mu\text{mol of dioxane} \quad (2)$$

Enzyme Extraction and GluS/GDH Assays. Frozen roots were prepared as described above and thawed in ice-cold extraction buffer consisting of 50 mM Tricine (pH 7.6), 500 mM sucrose, 10 mM Na_4EDTA, 12.5 mM 2-mercaptoethanol, and 1 mM phenylmethane sulfonyl fluoride (PMSF). The thawed tissue was ground to homogeneity in a chilled mortar and pestle. The homogenate was filtered through four layers of cheesecloth equilibrated in the extraction buffer minus the PMSF. The filtrate was centrifuged for 20 min at 10000*g*. The pellet was discarded and the supernatant brought to 35% saturation with ammonium sulfate. This solution was cen-

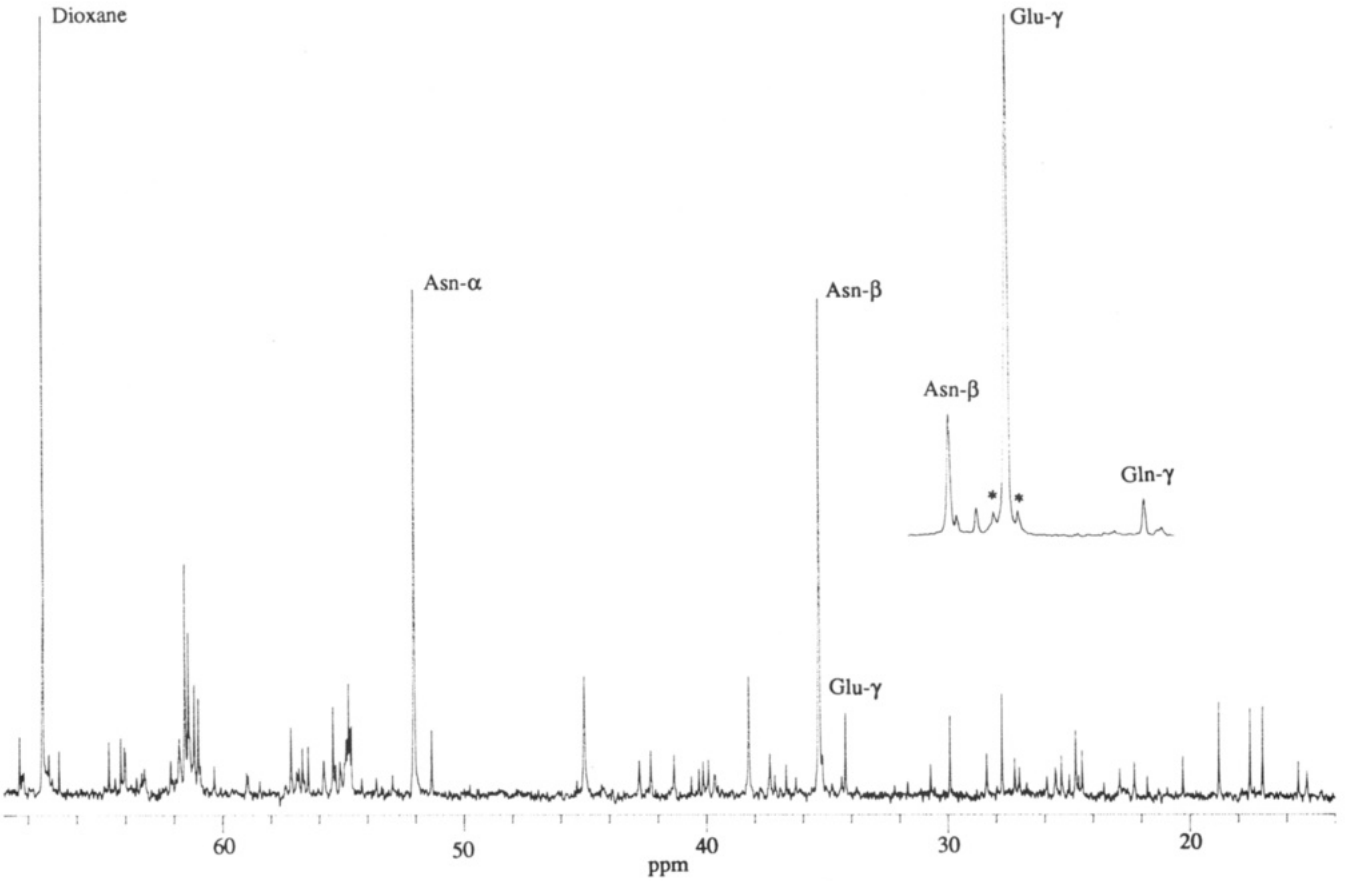

FIGURE 1: ^{13}C-NMR spectrum of the $HClO_4$ extract of the meristem cultures fed 10 mM sodium acetate for 45 min at 24 °C. The spectral region shown covers 14–70 ppm, and resonances assigned to the internal standard dioxane, glutamate-γ(Glu), and asparagine-β (Asn) are indicated. The insert shows the expansion between 31 and 36 ppm following culture with [2-^{13}C]acetate; the glutamine-γ (Gln), glutamate-γ(Glu), asparagine-β (Asn), and the ^{13}C satellites (*) of the glutamate carbon are indicated.

Table I: Quantitation of Metabolite Concentrations of Meristem Cultures at 24 °C by ^{13}C-NMR

metabolite carbon	δ	μmol/gdw (±SD)	θ_{eff}	T_1 (s)	(η + 1)
Ala-α	51.3	28 ± 6	66	2.1 ± 0.3	2.5 ± 0.1
Ala-β	17.0	33 ± 5	58	1.9 ± 1.1	3.2 ± 0.4
Asn-α	52.1	220 ± 37	66	1.2 ± 0.1	2.5 ± 0.2
Asn-β	35.3	190 ± 46	62	0.7 ± 0.2	2.8 ± 0.2
citrate-CH_2	45.1	49 ± 7	65	0.3 ± 0.1	2.9 ± 0.1
Gaba-α	35.2	14 ± 2	62	1.3 ± 0.1	3.0 ± 0.2
Glu-α	55.4	30 ± 4	67	0.9 ± 0.2	2.8 ± 0.1
Glu-β	27.7	37 ± 5	60	0.6 ± 0.1	2.9 ± 0.1
Glu-γ	34.2	29 ± 4	62	0.7 ± 0.1	2.8 ± 0.1
Gln-γ	31.7	3 ± 0.6	61	0.8 ± 0.2	2.9 ± 0.1
Mal-β	42.8	21 ± 3	64	0.6 ± 0.1	2.5 ± 0.5
succ-CH_2	34.8	5 ± 1	62	1.5 ± 0.1	2.8 ± 0.3
Val-β	29.9	32 ± 7	61	1.3 ± 0.4	2.7 ± 0.2
dioxane	67.4	100	71	3.0 ± 0.2	2.7 ± 0.2

trifuged as above and the pellet discarded. The supernatant was brought to 60% saturation with ammonium sulfate and centrifuged, and the pellet was retained. The pellet was suspended in a minimal amount of extraction buffer and passed through a Sephadex G-50 column (2.5 × 8 cm) equilibrated in the extraction buffer minus the tosyl fluoride. The fractions with high absorbance at 280 nm were pooled, and the total protein concentration was determined using the BCA method (Pierce Chemical Co., Rockford, IL).

The activities of GluS and GDH were assayed in this crude enzyme preparation by monitoring the change in absorbance of NADH at 340 nm as described by Miflin and Lea (1975). The assay buffer was the same as the extraction buffer, minus the PMSF. the assay system for either enzyme contained 5 mM α-ketoglutarate, 160 μM NADH, and 5 mM specified nitrogen source. NH_4^+ was used as the nitrogen source to assay for GDH activity. Glutamine was used as the nitrogen source to assay for GluS activity. The rate of NADH consumption was corrected for any background NADH consumption in the presence of the enzyme preparation (~0.14 mg of protein), and the enzyme assay was initiated by the addition of α-ketoglutarate.

In Vivo Protein Labeling. Cultured root tips, five roots for each sample, were prepared as above; 100 mCi/mL [^{35}S]methionine (>1000 mCi/mmol; Amersham, Inc., Arlington Hts, IL) and 10 mM NaOAc were added for 45 min at the desired temperature. At the end of the labeling period, the labeled methionine was removed, and the tissue was rinsed with 10 mM unlabeled methionine at ambient temperature for 2 min and then with distilled H_2O before the roots were frozen in liquid N_2. The protein extraction buffer consisted of 200 mM Tris (pH 7.4), 12 mM 2-mercaptoethanol, 5% SDS, 10 mM sodium ascorbate, and 2 wt % poly(vinylpyrrolidone). The roots were ground in a mortar and pestle, and insoluble material was removed by centrifugation in a desk-top centrifuge. All operations were performed at 4 °C. Proteins were precipitated with 5% (vol) trichloroacetic acid and pelleted by centrifugation. The pellet was rinsed with 1:1 Et_2O:EtOH to remove any remaining trichloroacetic acid prior to dissolution in the cracking buffer of Laemmli (1970). Total protein was determined with the BCA assay (Pierce Chemical, Co.), modified for assaying in the presence of high mercaptol (Smith et al., 1985). The amount of label in the acid-precipitated protein fraction was determined by scintillation counting. The protein samples were separated in one dimension on a 12% polyacrylamide gel (Laemmli, 1970) loaded to give approximately equal amounts of radioactivity per lane. The gels were dried and visualized autoradiographically.

Table II: Metabolite Pool Size (and Enhancements) following 45-min Heat Treatments

metabolite carbon	δ	amounts (μmol/gdw) at 24 °C	40 °C	45 °C
Ala-β	17.8	33 (2.4)	87 (1.3)	85 (1.3)
Asn-β	35.3	186 (1.2)	160 (1.2)	170 (1.0)
Gaba-α	35.2	14 (2.3)	22 (3.6)	23 (2.1)
Glu-γ	34.2	29 (37)	55 (29)	23 (34)
Gln-γ	31.7	3 (18)	ND (≥18)	5 (33)
succ-CH_2	34.8	5 (7.8)	12 (9.9)	9 (11)

Isolation of the Plastid-Enriched Fraction. Plastid isolation followed essentially the methods of Emes and England (1986). Approximately 5 g of frozen roots was thawed and homogenized gently at 4 °C with a mortar and pestle in one volume of extraction buffer containing 50 mM Tricine–NaOH (pH 7.9), 330 mM sorbitol, 1 mM EDTA, 1 mM $MgCl_2$, and 0.1% bovine albumin. The extract was filtered through six layers of cheesecloth and centrifuged at 200*g* for 1 min to remove intact cells, nuclei, and cell debris. The resulting supernatant was underlaid with one volume of 50 mM Tricine–NaOH, pH 7.9, containing 330 mM sorbitol, 0.1% bovine albumin, and 10% (v/v) freshly dialyzed Percoll and spun at 4000*g* for 5 min. The resulting pellet was gently resuspended in a small volume of extract buffer and centrifuged again at 4000*g* for 5 min. This fraction was positive for GluS (plastids) and negative for fumarase (mitochondria) and alcohol dehydrogenase (cytosol).

RESULTS

Root meristems represent a persistent embryonic tissue of rapidly dividing cells maintaining root elongation. These tissues must exhibit sufficient metabolic and genetic plasticity to take advantage of conditions dictated by the environment of the plant. Organ cultures of these tissues should display the same resilience and plasticity and were therefore well suited for study.

Samples for the ^{13}C-NMR experiments prepared from extracts of freshly excised root tips proved to be extremely viscous, and the ^{13}C-NMR spectra were dominated by sugar resonances. To minimize the levels of internal sugars in the root tip extracts, the meristems were cultured for 24 h in a minimal growth medium (White, 1943) containing 1 mM sodium acetate as the sole carbon source. The cultures were viable for extended periods, and after 24 h the carbons of glucose were of equal intensity with the other major metabolites. [^{35}S]Methionine labeling showed the heat shock response (hsp, see below) in the presence of added 10 mM acetate to be no different from that of cells cultured in the presence of 5% sucrose (Lanks et al., 1986).

Heat-Induced Metabolite Distributions. Several maize varieties were screened in qualitative growth assays, and the MX-085 variety was found to be highly responsive to temperature changes and to thermotolerance induction. Both the intact seedlings and the meristem cultures were viable following 45 min at 40 °C, but an exposure of 45 °C for the same period was lethal. The lethality of the 45 °C stress was alleviated in thermotolerated seedlings. Therefore, this variety and these temperature treatments were chosen for the course of this study.

Heat shocks of 40 °C and 45 °C resulted in altered in vivo concentrations of several metabolites; the major ones are shown in Table II. Asparagine is listed as the metabolite present in highest concentration, although little change appears to have occurred at the indicated time point. Gaba, succinate, and

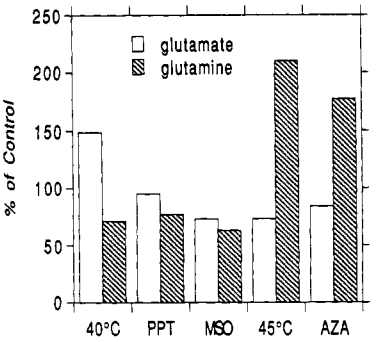

FIGURE 2: The glutamate and glutamine signal intensities expressed as percentage of the 24 °C control. [2-^{13}C]Acetate (10 mM) was cultured together with either 100 μM methionine sulfoximine (MSO), 100 μM phosphinothricin (PPT), or 100 μM azaserine (AZA) for 45 min at 24 °C.

alanine showed marked increases in concentrations at both temperatures consistent with previous observations of heat-induced accumulations of amino acids in cowpea suspension cultures (Mayer et al., 1990). The concentration of glutamate and glutamine, however, exhibited different responses to the 40 °C and 45 °C heat shocks. The concentration of glutamate increased from 29 μmol/gdw at 24 °C to 55 μmol/gdw at the permissive 40 °C treatment but did not change from control levels at the lethal 45 °C (23 μmol/gdw). The concentration of glutamine dropped below the level of detection at 40 °C (<2 μmol/gdw) but accumulated to 5 μmol/gdw in response to the 45 °C stress.

Replacement of the 10 mM sodium acetate with [2-^{13}C]acetate during the 45-min heat shocks amplified changes in pathways deriving carbons from the TCA cycle. These data are presented in Table II as the enhancements (determined as the resonance intensity ratio of the enriched to the natural abundance resonance) of the specified carbons. As expected, citrate, succinate, and malate showed large enhancements following a 45-min exposure to labeled acetate, but the most enhanced carbons under all temperature treatments were the γ-carbons of glutamate and glutamine. Such enrichment in glutamate and glutamine has been observed previously in other systems (London, 1988, and references therein) and has been generally attributed to an active flux of label from the TCA cycle through the glutamine synthetase–glutamate synthase (GS–GluS) pathway (Stewart et al., 1980). The major changes in the relative enhancement between the 40 °C and 45 °C treatments involved the metabolites of the GS–GluS pathway. Gaba-α and Gln-γ enhancements (the enhancement of Gln-γ was not available at 40 °C, but estimates from the minimal detectable concentration suggest an enhancement of ≥18) and, to a lesser extent, succinate-CH_2 and Glu-γ were different between the permissive and lethal stresses (Table II). Since all four of these metabolites are related via the GS–GluS pathway (Magalhaes et al., 1990) and since the largest changes between 40 °C and 45 °C occurred in the concentration of glutamate and glutamine and in the enrichment of glutamine, we predicted that the 45 °C treatment directly altered the functioning of this pathway.

To test this prediction, the root tip cultures were exposed to several enzyme inhibitors at 24 °C and these results were compared with the heat shocks. Because of the difficulty of detecting glutamine at natural abundance, Figure 2 shows the intensity of the γ-carbons of glutamate and glutamine following a 45-min exposure in the presence of 10 mM [2-^{13}C]acetate. While both glutamine synthase inhibitors, phosphinothricin (PPT; Leason et al., 1982) and methionine sulfoximine (MSO; Leason et al., 1982; Stewart & Rhodes, 1976) reduced the intensity of both the glutamate γ-and glu-

Table III: Pool Sizes (and Enhancements) of Metabolites following the Induction of Thermotolerance

	amounts (μmol/gdw) at time after thermotolerance					
	0 h		3 h		4 h	
metabolite	24 °C	45 °C	24 °C	45 °C	24 °C	45 °C
Ala-β	33 (2.4)	85 (1.3)	66 (1.4)	136 (1.0)	66 (2.0)	58 (2.3)
Asn-β	186 (1.2)	170 (1.0)	120 (1.3)	73 (2.3)	140 (1.0)	160 (1.3)
Gaba-α	14 (2.3)	23 (2.1)	20 (1.1)	36 (2.4)	22 (2.3)	18 (3.8)
Glu-γ	29 (37)	23 (34)	57 (18)	68 (22)	35 (28)	30 (29)
Gln-γ	3 (20)	5 (30)	5 (20)	7 (20)	5 (20)	6 (40)
succ-CH_2	5 (8)	9 (10)	10 (10)	25 (6)	11 (10)	16 (10)

tamine γ-carbons, they did not mimic the effects seen at 45 °C as did the treatment with the glutamate synthase inhibitor azaserine (AZA; Buchanan, 1973; Oaks & Johnson, 1973).

Both the labeled and unlabeled metabolite pools contribute to the observed resonance intensity in Figure 2, and the ^{13}C-enriched experiments alone cannot distinguish between them. In the azaserine treatments, the natural abundance glutamine concentrations could be measured from cultures with natural abundance acetate. This increase in the intensity of glutamine in azaserine-treated roots was found to result from an increase in concentration (8 μmol/gdw) with a constant level of ^{13}C enhancement (20), whereas the increase at 45 °C was due to both an increase in concentration and ^{13}C enhancement. The differences in the enhancements obtained in these two experiments may reflect different rates of GluS inhibition by the two stresses and also may arise from differences in the rates of other reactions involving glutamate and glutamine due solely to different temperatures. Nevertheless, the results were consistent with the loss of GluS as primarily responsible for the observed glutamate and glutamine changes by the lethal thermal treatment.

Induction of Thermotolerance. As reported for other maize varieties (Lin et al., 1984), exposing MX-085 to 45 °C for 10 min followed by a return to room temperature could induce thermotolerance to the 45-min heat stress. Similar thermotolerance experiments have been shown to decrease the susceptibility of many organisms to lethal heat shocks (Lindquist, 1986). Experiments were performed to measure metabolite concentrations and enhancements in the presence and absence of the challenge 45 °C heat shock at various times following thermotolerance induction. These data, Table III, were analyzed in terms of the ability to maintain the synthesis of glutamate and glutamine and to prevent accumulation of label in the glutamine γ-carbon. After 3 h of thermotolerance, glutamine concentrations increased from 5 μmol/gdw at 24 °C to 7 μmol/gdw in the presence of the challenge heat shock. However, this accumulation of glutamine occurred with a similar increase in glutamate concentration and with no change in the enhancement of the glutamine γ-carbon, a fundamentally different response to the 45 °C heat stress than was observed in nonthermotolerated root tips. In 4-h-thermotolerated roots, the glutamine concentration increased in response to the 45 °C stress, but this increase occurred with increased enhancement and was not coupled to an increase in glutamate concentrations, similar to the response observed in nonthermotolerated roots. On the basis of these data, it was proposed that GluS activity was protected in 3-h-thermotolerated roots, but this protection was much less evident after 4 h of thermotolerance.

Analysis of Cell-Free Extracts. To confirm the proposed loss of GluS activity following the 45 °C heat shock, GluS activity was assayed in cell-free extracts (Miflin & Lea, 1975; Singhal et al., 1989). GluS activity extracted from control corn root tips gave an NADH-dependent initial reaction rate of 8.9 ± 0.4 nmol/(min·mg, of protein) using glutamine as a nitrogen source, essentially the same value reported for *Pisum* (Miflin & Lea, 1975). As shown in Table IV, exposure to 45 °C for 45 min reduced extractable GluS activity to 55% of control. A 40 °C heat shock did not alter the extractable activity, but AZA treatment dropped GluS activity by 55%. The same treatments had no effect on extractable glutamate dehydrogenase (GDH) activity.

Table IV: Analysis of the Extractable Enzyme Levels from Meristem Cultures after Various Temperature and Inhibitor Treatments

	enzymatic activity (% of control)	
treatment	GluS	GDH
45 °C, 45 min	55 ± 12	100 ± 10
40 °C, 45 min	100 ± 10	100 ± 8
2 h of thermotolerance (2 h TT)	45	–
3 h of thermotolerance (3 h TT)	100 ± 10	85 ± 10
4 h of thermotolerance (4 h TT)	70	
control + cycloheximide (10 μg/mL)	100 ● 10	
3 h of TT + cycloheximide (10 μg/mL)	55 ± 5	
10 mM azaserine for 45 min	45 ± 5	100

The NMR data showed protection of glutamate and glutamine metabolism in 3-h-thermotolerated root tips. At least two mechanisms for this protection were anticipated. Either the protection of GluS activity or the elevation of GDH activity to compensate for the loss of GluS activity (Munoz-Blanco & Cardenhas, 1989) would result in the observed metabolite distributions. Assays of the 3-h-thermotolerated root extracts following exposure to a 45 °C heat shock found the GluS activity no different from that of the 24 °C control (Table IV). The activity of GDH was found to drop slightly over the thermotolerance induction period.

Protein Synthesis and GluS Protection. The enzyme preparations from both control and the 3-h-thermotolerated tissues showed equivalent in vitro thermal stability (data not shown), eliminating the possibility either that GluS protection involved the expression of a thermally stable GluS or that the enzyme was covalently modified. In order to evaluate the role of protein synthesis in this protection, the cultures were exposed to cycloheximide (McAlister & Finkelstein, 1980) for the final 90 min of the 3-h-thermotolerance induction period. This exposure had no effect on GluS activity in control roots but completely prevented the protection of GluS during the development of thermotolerance (Table IV). Therefore, protein synthesis was required for the protection of extractable GluS activity by thermotolerance.

To better understand the nature of the protein synthesis in GluS protection, newly synthesized proteins were labeled by [^{35}S]methionine incorporation at various times following thermotolerance induction. As shown in Figure 2, a 45-min exposure to [^{35}S]methionine at 40 °C labeled proteins of M_r 81 000, 70 000, 64 000, 57 000, 23 000, and 18 000, typical molecular weights for maize hsps (Baszczynski et al., 1982; Cooper & Ho, 1983). A 45-min exposure to 45 °C resulted in greatly diminished hsp synthesis and reduced total label

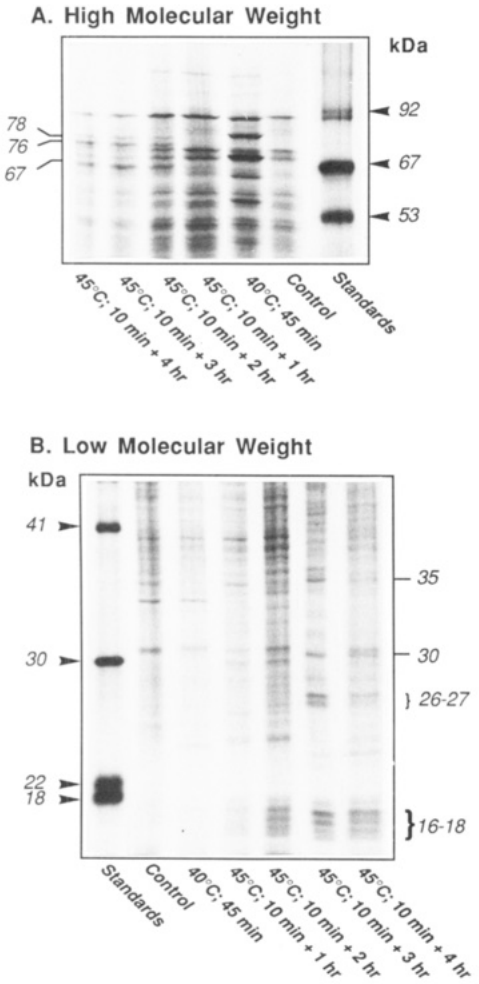

FIGURE 3: Autoradiograms of the SDS–PAGE analyses (12% gels) of protein synthesis patterns during various temperature treatments. Methionine exposure occurred over the last 45 min of each assay condition: (A) high molecular weight proteins; (B) low molecular weight proteins.

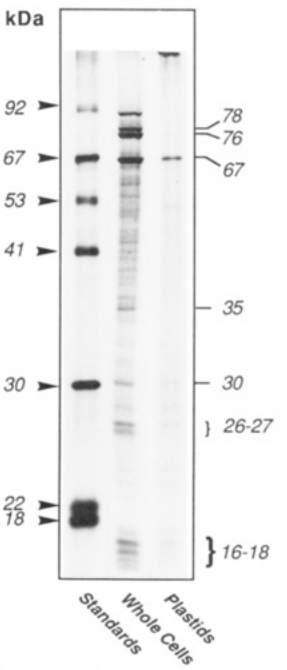

FIGURE 4: Autoradiograms of the SDS–PAGE analyses (12% gels) of proteins either from whole cells or from the isolated plastids.

incorporation into the acid-precipitable protein (data not shown).

Exposing the cultures to 45 °C for 10 min followed by a 1-h [^{35}S]methionine labeling period at room temperature gave an hsp pattern similar to that of the 40 °C induction. However, labeling during the second hour at room temperature showed that the original 40 °C hsp pattern had begun to subside, and a new set of proteins had appeared. These new proteins, M_r 78 000, 76 000, 67 000, 35 000, 27 000–26 000, and 16 000–18 000, were not detected in control or in the 40 °C heat-shocked tissues. These late-arising hsps were most intensely labeled in the third hour following the 45 °C thermotolerance pulse and began to subside in the fourth hour (Figure 3). The late-arising hsps were therefore temporally correlated with thermotolerance and the protection of GluS.

Since NADH–GluS is localized to the plastids (Stewart et al., 1980), the plastid-enriched fraction following [^{35}S]methionine labeling was purified from a total cellular lysate. The autoradiogram of the SDS–PAGE chromatogram of this fraction is shown in Figure 4. Of the hsps temporally correlated with GluS protection, a specific subset, M_r 67 000 and more weakly the proteins at M_r 16 000–18 000, became associated with this organelle. The association of these hsps with the plastid occurred in the absence of further heat shocks in contrast to results obtained with soybeans (Lin et al., 1984).

Hsp70 homologues have been detected in chloroplasts and mitochondria (Amir-Shapira et al., 1990; Marshall et al., 1990) and have been shown to autophosphorylate in a manner analogous with the 67-kDa DnaK from *Escherichia coli*. As in the case of maize chloroplasts (Amir-Shapira et al., 1990), there was a 70-kDa protein that was phosphorylated in the plastids in both control and thermotolerated tissues, but the phosphorylation was not dependent on high Mg^{2+} or Ca^{2+} (data not shown). The late-arising hsp67 in the plastids was not phosphorylated under these conditions, nor did it cross-react with antibodies raised against a *Drosophila* hsp 70 (Kurtz et al., 1986). These antibodies did cross-react with hsp70s from the maize root tips and from *Arabidopsis* (Wu et al., 1988).

DISCUSSION

A search for the function of the heat shock proteins and their role in the acquisition of thermotolerance has been ongoing since their discovery. The current perception that these proteins can specifically identify denaturing protein domains is both appealing in its simplicity and baffling with regard to its mechanistic and molecular details. Increasingly, attention has focused on the characteristics of the denatured protein substrate and the mechanism of renaturation. However, questions regarding which specific metabolic steps require protection by the hsps for thermotolerance and the effectiveness of such a strategy have largely been ignored. These questions cannot be addressed until more is known about the effects of hyperthermia on the intricate series of reactions that define a living cell.

Metabolism in intact tissue was monitored in extracts using ^{13}C-NMR spectroscopy. Changes in metabolite concentrations and in [2-^{13}C]acetate incorporation showed glutamine and glutamate metabolism to be highly responsive to altered temperatures. On the basis of the observed metabolite distributions, 40 °C heat shock appeared to increase the activity of GluS, while the activity of this enzyme appeared to be diminished at 45 °C. Enzyme inhibitors applied to intact root tips supported a loss of GluS activity at the higher temperature. An alternative explanation for the accumulation of glutamine, however, would be a loss of interorganelle transport systems as the synthesis of glutamate and glutamine occur in different cellular compartments. Heat shock has been proposed to alter the integrity of cell membranes (Patterson

& Graham, 1987) leading to "leaky" organelles and possibly to diminished metabolite transport capacity. Assays for GluS activity in enzyme preparations obtained from 45 °C heat shocked roots showed a loss of 45% of the activity extractable from control tissues. Although diminished plastid membrane integrity might indirectly reduce GluS activity, the fact that the azaserine treatment resulted in metabolite distributions and enzyme activity similar to those obtained from 45 °C heat shocked tissues strongly suggests a heat-induced denaturation of GluS.

Glutamate synthase was previously shown to be a relatively fragile enzyme (Stewart et al., 1980; Singhal et al., 1989), but at least the thermal instability is not due to a physical limitation of the reaction since a far more temperature-insensitive GluS has been identified in thermophilic bacteria (Schmidt & Jervis, 1982). In these meristem tissues, NADH–GluS is the only enzyme which contributes significantly to net glutamate synthesis (Magalhaes et al., 1990) and therefore plays an essential role in the biosynthesis of amino and nucleic acids. An additional ferredoxin-dependent GluS is expressed in green tissues. Mutants lacking this enzyme in *Arabidopsis* are not viable under normal photorespiratory conditions (Somerville & Ogren, 1980, 1982) but display a normal phenotype under conditions that suppress photorespiration (1% CO_2 in air). The observation that both the 45 °C and azaserine treatments were lethal and reduced GluS activity to the same extent suggests that the loss of the ability to synthesize amino acids at 45 °C could contribute to the heat-induced cell death. In that regard, Saunders and Kon (1991) have shown that certain minimal concentrations of glutamate and glutamine are critical for hsp production.

Short hyperthermal exposures thermotolerate the meristems to the lethal 45 °C treatment as measured both by the NMR experiments and by seedling growth. There is a strict time dependence to this protection, with some degree of protection evident from 2 to 4 h after thermotolerance induction, but 3 h is optimal for protection of GluS activity. Metabolite changes during the course of thermotolerance induction indicate that this is a highly dynamic process.

Further evidence for the dynamic nature of the thermotolerance induction period is the appearance of a distinct set of hsps hours after thermotolerance induction. These proteins were not present at detectable levels in 40 °C heat shocked roots, nor were they present 1 h after thermotolerance induction. The time dependence for the maximal expression of these proteins coincided with optimal protection of GluS. The role of the hsps in the acquisition of thermotolerance is not well understood. It has been assumed that the hsps function to protect the cells against high temperature stress (Pelham, 1986), and this proposal has received some direct experimental support. Cycloheximide treatment following a severe heat shock was found to inhibit the cells ability to recover (McAlister & Finckelstein, 1980), and Lindquist and co-workers have shown that the deletion of one hsp protein from yeast prevents thermotolerance acquisition (Parsell et al., 1991). On the other hand, plant cells have been thermotolerated against severe heat stress without the synthesis of hsps (Bonham-Smith et al., 1987). Presumably, the synthesis of other stress proteins or changes in metabolite levels (Nash et al., 1982; Rathnam, 1978) stabilized the cells against heat-induced damage in the absence of hsps. Exposing the cultured maize root tips to cycloheximide during the last 90 min of the 3-h thermotolerance induction period allowed the synthesis of the initial hsps but prevented the synthesis of the late-arising hsps. Assays of GluS activity showed no protection of this activity in the cycloheximide-treated root tips, establishing a requirement for the synthesis of a specific set of proteins for maintaining GluS activity.

One of these late-arising proteins, hsp67, becomes associated with the plastid, the organelle in which GluS is located. Many hsps become associated with specific organelles in heat shocked tissues, but this association was found to require elevated temperatures (Lin et al., 1984; Howarth, 1990). The localization of hsp67 to the plastid occurs in the absence of further heat shocks. Because of its molecular weight, it was initially assumed that hsp67 was a member of the hsp70 family of proteins.

The hsp70s are a diverse group of genetically and functionally distinct proteins which can renature denatured proteins (Dubois et al., 1991; Gaitanaris et al., 1990) and can function as molecular chaperones (Ellis & Hemmingsen, 1989; Kang et al., 1990). hsp70s generally bind ATP and show weak ATPase activity while the organellar-encoded/-localized hsp70s have been shown to autophosphorylate (Amir-Shapira et al., 1990). The hsp67 associated with the plastid in 3-h-themotolerated root tips does not autophosphorylate in vitro, nor does it cross-react with antibodies raised against yeast hsp70. The hsp70 present in 40 °C heat shocked corn root tips did cross-react with these antibodies as well as autophosphorylate. Further characterization of hsp67 including the nature of its association with the plastid and its protective mechanism are continuing. Nevertheless, the identification of hsp67 and its role in protecting GluS activity represent the first example of a critical metabolic conversion which is protected in vivo by a specific hsp.

These experiments identified glutamate metabolism as a central functional loss in thermal stress and pinpointed GluS as a metabolic weak spot. Neither the nature of the thermal inactivation nor the mechanism of the thermotolerance protection is understood at this point, but it is clear that the hsp strategy quantitatively protects the enzyme during the stress. The analysis of metabolic alterations will become increasingly important with the extensive use of recombinant DNA technology. Whether transgenic organisms are developed for toxin resistance (Mazur & Falco, 1989), for metabolic engineering (Bailey, 1991; Stephanopoulos & Vallino, 1991), or simply for gene function assignment (Lagrimini et al., 1990; Liang et al., 1989), methods that enable characterization of the alterations in the homeostatic state of the cell will be critical. It has been possible to mathematically model well-defined metabolic pathways such as the TCA cycle (Walsh & Koshland, 1984; Chance, et al., 1983) with magnetic resonance experiments, and with the radical improvements in sensitivity offered by inverse detection (Bax et al., 1989) and editing (Wollborn et al., 1990) experiments it should be possible to analyze much larger sections of the metabolic network.

ACKNOWLEDGMENT

We thank Dr. P. Zhu for the initial work on the ^{13}C-NMR assignments and development of the meristem culture conditions and Professor Susan Lindquist and John Taulien for providing the hsp70 antibodies.

REFERENCES

Amir-Shapira, D., Leustek, T., Dalie, B., Weissbach, H., & Brot, N. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 1749–1752.

Ananthan, J., Goldberg, A. L., & Voellmy, R. (1986) *Science 232*, 522–524.

Armitage, I. M., Huber, H., Live, D. H., Pearson, H., & Roberts, J. D. (1974) *J. Magn. Reson. 15*, 142–149.

Bailey, J. E. (1991) *Science 252*, 1668–1675.

Bain, A. D., Fahie, B. J., Kozluk, T., & Leigh, M. J. (1991) *Can. J. Chem. 69*, 1189–1192.
Bax, A., Sparks, S. W., & Torchia, D. A. (1989) *Methods Enzymol. 176*, 134–150.
Baszczynski, C. L., Walden, D. B., & Atkinson, B. G. (1982) *Can. J. Biochem., 60*, 2229–2246.
Bonham-Smith, P. C., Kapoor, M., & Bewley, J. D. (1987) *Plant Physiol. 85*, 575–580.
Buchanan, J. (1973) *Methods Enzymol. 39*, 91–183.
Chance, E. M., Seeholzer, S. H., Kobayashi, K., & Williamson, J. R. (1983) *J. Biol. Chem. 258*, 13785–13794.
Chirico, W. J., Waters, M. G., & Blobel, G. (1988) *Nature 332*, 805–810.
Chou, M., Chen, Y.-M., & Lin, C.-Y. (1989) *Plant Physiol. 89*, 617–621.
Christensen, A., Grant, D. M., Schulman, E. M., & Walling, C. (1974) *J. Phys. Chem. 78*, 1973–1977.
Cohen, S. M. (1989) *Methods Enzymol. 177*, 417–434.
Cohen, S. M., Rognstad, R., Schulman, R. G., & Katz, J. (1981) *J. Biol. Chem. 256*, 3428–3432.
Cooper, P., & Ho, T.-H. D. (1983) *Plant Physiol. 71*, 215–222.
Craig, E. A., & Gross, C. A. (1991) *Trends Biochem. Sci. 16*, 135–40.
Deshaies, R. J., Koch, B. D., Werner-Washburne, M., Craig, E. A., & Scheckman, R. (1988) *Nature 332*, 800–805.
Dubois, M. F., Hovanessian, A. G., & Bensaude, O. (1991) *J. Biol. Chem. 266*, 9707–9711.
Ellis, R. J. (1990) *Science 250*, 954–959.
Ellis, R. J., & Hemmingsen, S. M. (1989) *Trends Biochem. Sci. 14*, 339–342.
Emes, M. F., & England, S. (1986) *Planta 168*, 161–166.
Freeman, R., & Hill, H. D. W. (1970) *J. Chem. Phys. 53*, 4103–4105.
Freeman, R., Hill, H. D. W., & Kaptein, R. (1972) *J. Magn. Reson. 7*, 327–329.
Gaitanaris, G. A., Papvassiliou, A. G., Rubock, P., Silverstein, S. J., & Gottesman, M. E. (1990) *Cell 61*, 1013–1020.
Gething, M.-J., & Sambrook, J. (1992) *Nature 355*, 33–45.
Goff, S. A., & Goldberg, A. L. (1985) *Cell 41*, 587–595.
Harris, R. K., & Newman, R. H. (1976) *J. Magn. Reson. 24*, 449–456.
Heikkila, J. J., Pap, J. E. T., Schults, G. H., & Bewley, J. D. (1984) *Plant Physiol. 76*, 270–274.
Hemmingsen, S. M., Woolford, C., van der Vies, S. M., Tilly, K., Dennis, D. T., Georgopolous, C. P., Hendrix, R. W., & Ellis, R. J. (1988) *Nature 333*, 330–334.
Hightower, L. (1980) *J. Cell. Physiol. 102*, 407–427.
Ignolia, T. D., & Craig, E. A. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 2360–2361.
Iida, H., & Yahara, I. (1985) *Nature 315*, 688–690.
Kang, P.-J., Ostermann, J., Shilling, J., Neupert, W., Craig, E. A., & Pfanner, N. (1990) *Nature 348*, 137–143.
Kurtz, S., Rossi, J., Petko, L., & Lindquist, S. (1986) *Science 231*, 1154–1157.
Laemmli, U. K. (1970) *Nature 227*, 680–685.
Lagrimini, L. M., Bradford, S., & Rothstein, S. (1990) *Plant Cell 2*, 7–18.
Lanks, K. W., Shah, V., & Chin, N. W. (1986) *Cancer Res. 46*, 1382–1387.
Leason, M., Cunliffe, D., Parkin, D., Lea, P. J., & Miflin, B. J. (1982) *Phytochemistry 21*, 855–857.
Liang, X., Dron, M., Schmid, J., Dixon, R. A., & Lamb, C. J. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 9284–8.
Lindquist, S. (1986) *Annu. Rev. Biochem. 55*, 1151–1191.
Lindquist, S., & Craig, E. M. (1988) *Annu. Rev. Genet. 22*, 631–677.
Lin, C. Y., Roberts, J. K., & Key, J. L. (1984) *Plant Physiol. 74*, 152–160.
Logan, T. M. (1991) Ph.D. Thesis, The University of Chicago.
London, R. E. (1988) *Prog. Nucl. Magn. Reson. Spectrosc. 20*, 337–383.
Magalhaes, J. R., Ju, G. C., Rich, P. J., & Rhodes, D. (1990) *Plant Physiol. 94*, 647–656.
Marshall, J. S., DeRocher, A. E., Keegstra, K., & Vierling, E. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 374–378.
Mayer, R. R., Cherry, J., & Rhodes, D. (1990) *Plant Physiol. 94*, 796–810.
Mazur, B. J., & Falco, S. C. (1989) *Annu. Rev. Plant Physiol. Mol. Biol. 40*, 441–470.
McAlister, L., & Finkelstein, D. B. (1980) *J. Bacteriol. 143*, 603–612.
Miflin, B. J., & Lea, P. J. (1975) *Biochem. J. 149*, 403–409.
Muñoz-Blanco, J., & Cardenas, J. (1989) *Plant, Cell Environ. 12*, 173–182.
Nieto-Sotelo, J., Vierling, E., & Ho, T.-H. D. (1990) *Plant Physiol. 93*, 1321–1328.
Oaks, A. G., & Johnson, F. J. (1973) *Can. J. Bot. 51*, 91–95.
Palleros, D. R., Welch, W. J., & Fink, A. L. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 5719–5723.
Parsell, D. A., Sanchez, Y., Stitzel, J. D., & Lindquist, S. (1991) *Nature 353*, 270–273.
Patterson, B. D., & Graham, D. (1987) in *The Biochemistry of Plants: A Comprehensive Treatise* (Davies, D. D., Ed.) Vol. 12, pp 153–199, Academic Press, New York.
Pechan, P. M. (1991) *FEBS Lett. 280*, 1–4.
Pelham, H. R. B. (1986) *Cell 46*, 959–961.
Piper, P. W., Curran, B., Davies, M. W., Hirst, K., Lockheart, A., & Sewart, K. (1988) *Mol. Microbiol. 2*, 353–361.
Prasad, T. K., & Hallberg, R. L. (1989) *Plant Mol. Biol. 12*, 609–618.
Rothman, J. E. (1989) *Cell 59*, 591–601.
Saunders, M. M., & Kon, C. (1991) *J. Cell. Physiol. 146*, 180–190.
Schmidt, C. N. G., & Jervis, L. (1982) *J. Gen. Microbiol. 128*, 1713–1718.
Singhal, R. K., Krishnan, I. S., & Dua, R. D. (1989) *Biochemistry 28*, 7928–7935.
Smith, P. K., Krohn, R. I., Hermanson, G. T., Mullia, H. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J., & Klenk, D. C. (1985) *Anal. Biochem. 150*, 76–85.
Somerville, C. R., & Ogren, W. L. (1980) *Nature 286*, 257–259.
Somerville, C. R., & Ogren, W. L. (1982) *Trends Biochem. Sci. 7*, 171–174.
Stephanopoulos, G., & Vallino, J. J. (1991) *Science 252*, 1675–1681.
Stewart, G. R., & Rhodes, D. (1976) *FEBS Lett. 64*, 296–299.
Stewart, G. R., Mann, A. F., & Fentem, P. A. (1980) in *The Biochemistry of Plants: A Comprehensive Treatise* (Davies, D. D., Ed.) Vol. 5, pp 153–328, Academic Press, New York.
Uritani, I., & Asahi, T. (1980) in *The Biochemistry of Plants: A Comprehensive Treatise* (Davies, D. D., Ed.) Vol. 2, pp 463–487, Academic Press, New York.
Vierling, E. (1991) *Annu. Rev. Plant Physiol. Mol. Biol. 42*, 579–620.
Vierling, E, Mishkind, M. L., Schmidt, G. W., & Key, J. L. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 361–365.
Walsh, K., & Koshland, D. E., Jr. (1984) *J. Biol. Chem. 259*, 9646–9654.
White, P. R. (1943) *A Handbook of Plant Tissue Culture*, Jacques Cattell, Landcaster, PA.
Wollborn, U., Domke, T., & Leibfritz, D. (1990) *J. Magn. Reson. 90*, 575–579.
Wu, C. H., Caspar, T., Browse, J., Lindquist, S., & Somerville, C. R. (1988) *Plant Physiol. 88*, 731–740.

Registry No. GluS, 65589-88-0; GDH, 9001-46-1; Glu, 56-86-0; Gln, 56-85-9; Gaba, 56-12-2; Ala, 56-41-7; Asn, 70-47-3; succinate, 110-15-6; citrate, 77-92-9; malate, 97-67-6.