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단 신

열충격단백질 HSP104에 의한 아미노산 대사의 조절

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Regulation of Amino-acid Metabolisms by HSP104 Protein

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Induction of HSP104 by a mild heat treatment is crucial for yeast cells to withstand exposures to a subsequent severe heat stress.[1] It is expressed at a basal level at a normal temperature (25 °C) and is very strongly induced at a moderate temperature (37 °C). It is also induced by a variety of other stresses, including ethanol, arsenate, and cadmium etc.[2]

A great deal of attention have been drawn to investigate a molecular basis of HSP104 function in providing thermotolerance. It was demonstrated either by electron microscopy of whole cells or by *in vitro* luciferase assay that HSP104 does not function as a molecular chaperone, but instead helps cells resolubilize any aggregated proteins resulting from severe heat shocks.[3] Interestingly, the basal level of HSP104 is higher in yeast cells grown in acetate and galactose than in cells grown in glucose.[4] Recently another possible role of HSP 104 protein on the respiration pathway in yeast was clearly demonstrated by metabolic studies carried out by ^{13}C-NMR spectroscopy as well as by measurement of O_2 consumption.[5]

HSP104 protein is highly homologous throughout diverse organisms and contains two essential ATP binding sites around Lys^{218} and Lys^{620}.[6] A series of site directed mutagenesis studies have confirmed that these two ATP binding sites in HSP104 protein are crucial not only for providing thermotolerance[6] but also for resolubilizing heat-damaged protein aggregates.[3] In corns, a 70 kDa HSP which provides thermotolerance to cells partially protects glutamate synthase, a fundamental metabolic regulator of amino acid biosynthesis via the TCA (tricarboxylic acid) cycle in mitochondria.[7] Thus a question has arised whether these two ATP binding sites of HSP 104 protein are required for regulation of respiration pathway as well as amino acid metabolisms. In pursuit of monitoring changes in cell metabolisms, yeast strains transformed with several hsp104 gene derivatives altered in ATP binding sites were examined for the intracellular concentrations of amino acids. Contents of some amino acids were measured by an HPLC method coupled with post-column sodium hypochlorite-*o*-phthalaldehyde (NaOCl-OPA) reaction.

EXPERIMENTAL

Strains. *S. cerevisiae* W304 (Mata *can1 his3 leu2 trp1 ura3 ade2 hsp104::LEU2*) deleted for the chromosomal *HSP104* gene was used as a parental strain for construction of mutants.[1] Strains carry a centromere-containing plasmid pRS316 with the wild type *hsp104* gene (A494), the vector alone (A 558) or the mutated *hsp104* gene (A726).[6] The mutations of HSP104 product are performed with changes of both from Lys^{218} to Thr^{218} and from Lys^{620} to Thr^{620}. They were grown at 25 °C in a minimal medium which lacks in Uracil to maintain

the plasmids and contains yeast nitrogen base without amino acids (Difco) and acetate as a carbon source.

Preparation of Cell Metabolites. Cells were grown in 1 L minimal medium to an early log phase ($\sim 5\times 10^6$ cells/ml) and harvested by centrifugation. Collected cells were resuspended in 200 ml medium (SAc) containing sodium acetate at a concentration of 0.5 g/liter. After 30 min incubation at different temperatures, cells were collected by rapid filtration and extracted by adding 2 ml cold 6% perchloric acid ($HClO_4$). After vortexing followed by 1 hr precipitation on ice, unbroken cells were removed and supernatant was neutralized by 3M KOH. After another centrifugation, supernatant was frozen immediately with liquid nitrogen and lyophilized.

Reagents for HPLC. Amino acid standards were purchased from Sigma. A mixture of protein hydrolyzate amino acids were obtained at a level of 2.5 moles/ml from Pierce. An amino pak (SCX) column with a size of 120×4.6 mm was purchased from Shisheido (Japan). Solutions used for elution of samples are prepared as follows; Solution A (75.9 mM trisodium citrate dehydrate, 160 ml/l ethanol, 12.9 ml/l HCl, 100 μl/l n-carplic acid), Solution B (56.9 mM trisodium citrate dehydrate, 35.1 g/l NaCl, 9.5 g/l Borax, 0.6 g/l NaOH, 100 μl/l n-carplic acid) and Solution C (8 g/l NaOH, 100 μl/l n-carplic acid). OPA reagent was prepared by dissolving 200 mg of OPA in 1 ml of ethanol and mixing into 1 L of 0.3 M borate buffer (pH 10.4), to which 0.5 ml 2-mercaptoethanol and 2 ml of Brij 35 were then added.[8] The sodium hypochlorite reagent was obtained by adding 1 ml of sodium hypochlorite (4-6%) to 1 L borate buffer (pH 10.4).

Analysis of Amino Acids by Post-Column OPA Reactions. Intracellular contents of each amino acid was examined by an HPLC method coupled with post-column sodium hypochlorite-*o*-phthalaldehyde (NaOCl-OPA) reaction.[8] The OPA method was attempted since it is five to ten times more sensitive than ninhydrin reaction.[9] 20 μl sample dissolved in 0.1 mM HCl was injected into Amino pak (SCX) column attached to the HPLC system. The system consists of Perkin-Elmer 410 pump, Rheodyne 7725i, Waters temperature control module (Milipore), Waters 470 fluorescence detector (Milipore) and Dischrom[plus] computer program (Donam, Korea). The elution was performed at 60 °C with 60% Solution A and 40% Solution B for 25 min, 100% Solution B for 25 min and 100% Solution C for 2 min at a flow rate of 0.4 ml/min.

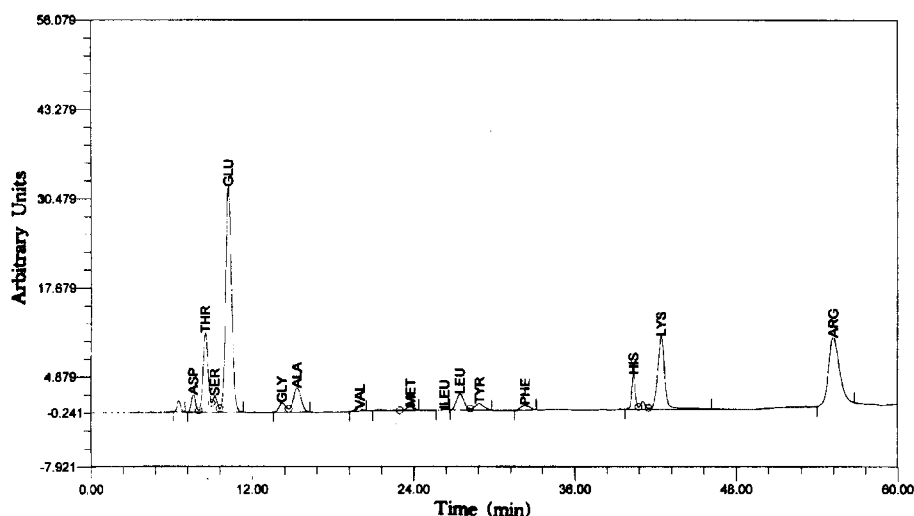

Fig. 1. HPLC trace of each amino acid. Using an Amino pak (SCX) column with a size of 120×4.6 mm, each amino acid was separated before derivatization with OPA.

The amino acid fractions eluted through the column were derivatized by sodium hypochlorite reagent followed by OPA reagent. The derivatized amino acids were detected by fluorescence spectroscopy with excitation at 340 nm and emission at 450 nm.

Enzymatic Analysis of Glutamate Concentration. Glutamate concentration was measured by a conventional enzymatic method. Cell extracts were assayed for quantification of glutamate by monitoring conversion of α-ketoglutarate to glutamate by glutamate dehydrogenase.[10]

RESULTS AND DISCUSSION

Separation of Each Amino Acid by HPLC. Since we found that HSP104 protein plays a crucial role on regulation of TCA cycle in the mitochondria,[5] we attempted to measure the contents of amino acids whose biosynthesis and degradation are carried out branched out from TCA cycle. Strains used in this study carry an empty vector as a negative control (A558), the mutated *hsp104* gene in the two ATP binding sites (A726), and the wild type *hsp104* gene (A494).[6] Total cell metabolites from the log-phase grown A 494, A558 and A726 cells were obtained by 6% perchloric acid extraction followed by neutralization. Each amino acid was separated by a conventional HPLC method in conjunction with post-column sodium hypochlorite-OPA reactions.[8] A typical separation of our sample is shown in *Fig.* 1. The analysis was sensitive enough to detect each amino acid at a fmole level and took only 60 min for each run. The contents of individual amino acid vary depending on the type of strains and the presence of a mild heat treatment. All the strains contain every heat shock proteins so that they can perform normal heat shock responses. They differ only in the state of HSP proteins, with A494 showing thermotolerance (functional) and A558 and A726 showing no thermotolerance (unfunctional). When cells were cultured at 25 °C without any heat treatment, A558 having no copy of *hsp 104* gene showed the highest levels of all amino acids. This result is consistent with the ^{13}C-NMR data, in which HSP104 exhibited a negative role in controlling the TCA cycle.[5] The fact that A726 contains amino acids at relatively low levels as in A494 implies that metabolisms of these amino acids are totally independent of the presence of functional HSP104 or the two ATP binding sites. Depending on the changes of amino acid contents

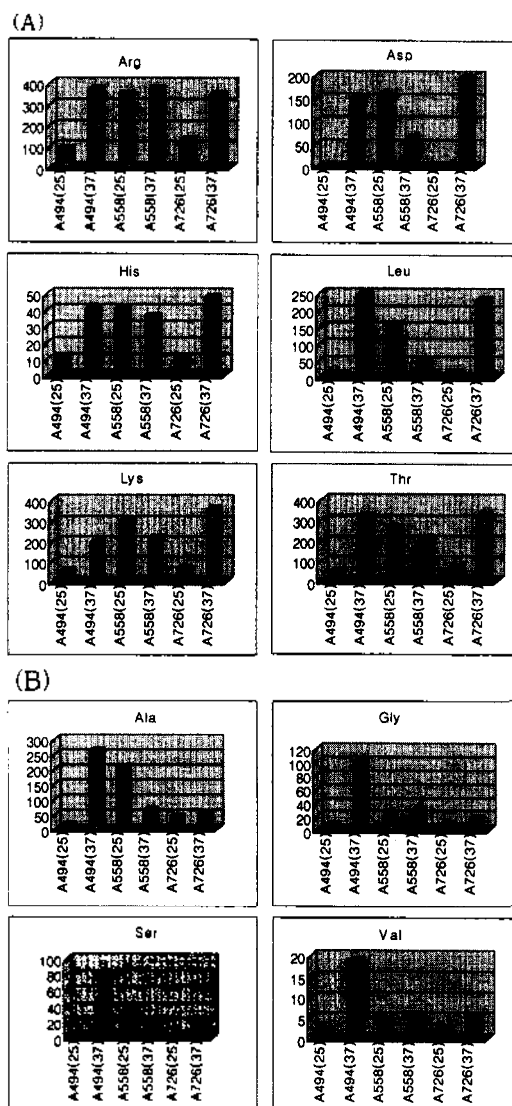

Fig. 2. Concentration of each amino acid was compared in each strain after incubated in acetate at 25 °C and 37 °C. The concentration is reported with a unit of fmole/number of cells in (A) for Arg, Asp, His, Leu, Lys and Thr (B) for Ala, Gly, Ser and Val.

upon raising incubation temperature to 37 °C, each amino acid can be categorized into two groups. In the first case shown for Arg, Asp, His, Leu, Lys and Thr, the increase of their levels were detected at 37 °C only in A494 and A726 (*Fig*. 2A). The ATP binding sites of HSP104 protein do not appear to be involved in their biosynthesis. Instead the overall HSP104 itself seems to be required for regulating the metabolic response of these amino acids. In contrast, A558 dose not exhibit any significant enhancement of these amino acids resulting from heat treatments as expected. The second case is shown for Ala, Gly, Ser and Val, in which only A494 showed the increase in their levels upon incubation at 37 °C whereas either A 558 or A726 displayed decrease or no change, respectively (*Fig*. 2B). In this case the functional HSP protein resulting from the presence of two normal ATP binding sites is absolutely required for the efficient metabolism.

Taken all these results together, HSP104 appears to be directly involved in regulation of amino acid metabolism, especially for Ala, Gly, Ser and Val. However, the presence of functional HSP104 protein is not required for the metabolism of other amino acids such as Arg, Asp, His, Leu, Lys and Thr.

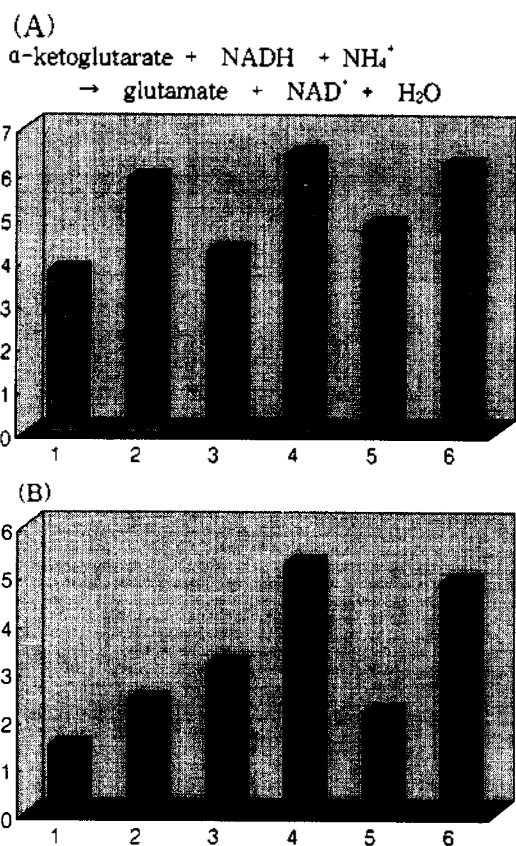

Fig. 3. Relative concentration of glutamate by an enzymatic method (A) and an OPA-post column HPLC method (B). The levels of glutamate in A494 (lane 1 and 2), A558 (lane 3 and 4) and A726 (lane 5 and 6) were compared after incubation either at 25 °C (lane 1, 3 and 5) or 37 °C (lane 2, 4 and 6).

Determination of Glutamate Concentration. Branched from the TCA cycle, glutamate is rapidly formed from α-ketoglutarate. Therefore the level of glutamate in the cell extract can directly reflect the amount of α-ketoglutarate in the TCA cycle, thereby indicating the turnover rate of metabolites in the TCA cycle. In the previous study with ^{13}C-NMR spectroscopy, glutamate is present inside cells at a higher concentration in the wild type than in hsp104 deleted mutant.[5] The conventional enzymatic analysis shows that our strains displayed the intrinsic difference in the relative ratio of glutamate levels at 25 °C depending on the state of HSP104 protein (*Fig*. 3A). Both A558 and A726 with unfunctional HSP 104 proteins have higher levels than A494 by 1.5 fold, suggesting that the functional HSP104 protein regulates the turnover of TCA cycle rather negatively. This result was also confirmed with the HPLC data shown in *Fig*. 3B. The level of Glu increased up to 2 fold as the incubation temperature goes up to 37 °C for all the strains. Therefore this result indicates that enhancement of Glu levels at a higher temperature is caused by other heat shock responses regardless of the presence of functional HSP104 protein.

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REFERENCES

1. Sanchez, Y.; Lindquist, S. L. *Science* **1990**, *248*, 1112.
2. Parsell, D. A.; Taulien, J.; Lindquist, S. *Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences* **1993**, *339*, 279.
3. Parsell, D. A.; Kowal, A. S.; Singer, M. A.; Lindquist, S. *Nature* **1994**, *372*, 475.
4. Sanchez, Y.; Taulien, J.; Borkovich, A.; Lindquist, S. *EMBO J.* **1992**, *11*, 2357.
5. Lee, K.; Kang, S.; Lindquist, S. *Bull. Korean Chem. Soc.* **1998**, *19*, 295.
6. Parsell, D. A.; Sanchez, Y.; Stitzel, J. D.; Lindquist, S. *Nature* **1991**, *353*, 270.
7. Logan, T. M.; Zhong, P.; Lynn, D. G. *Biochemistry* **1992**, *31*, 7256.
8. Dong, M. W.; Gant, J. R. *J. Chromatography*, **1985**, *327*, 17.
9. Benson, J. R.; Hare, P. E. *Proc. Natl. Acad. Sci. U.S.A.* **1975**, *72*, 619.
10. Bergmeyer, H.U.; Graβl, M. *Methods of Enzymatic Analysis*; VCH Publishers: Deerfield Beach, U.S.A., 1985; p. 20.