Proteomic analysis of heat-stable proteins in *Escherichia coli*

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Some proteins of *E. coli* are stable at temperatures significantly higher than 49°C, the maximum temperature at which the organism can grow. The heat stability of such proteins would be a property which is inherent to their structures, or it might be acquired by evolution for their specialized functions. In this study, we describe the identification of 17 heat-stable proteins from *E. coli*. Approximately one-third of these proteins were recognized as having functions in the protection of other proteins against denaturation. These included chaperonin (GroEL and GroES), molecular chaperones (DnaK and FkpA) and peptidyl prolyl isomerases (trigger factor and FkpA). Another common feature was that five of these proteins (GroEL, GroES, Ahpc, RibH and ferritin) have been shown to form a macromolecular structure. These results indicated that the heat stability of certain proteins may have evolved for their specialized functions, allowing them to cope with harsh environments, including high temperatures. [BMB reports 2008; 41(2): 108-111]

INTRODUCTION

The growth and multiplication of organisms occurs within a narrow range of physico-chemical conditions. Temperature is one of such environmental factors that dramatically influence growth. *E. coli* is capable of growth in a temperature range from 7.5°C to 49°C, with an optimum at approximately 37°C (1). The growth rate of this organism drops precipitously as the temperature rises above 46°C, with no growth at all observed at 50°C (2). The exact cause of death at high temperatures has yet to be clearly determined, but it is likely to involve the denaturation and the loss of function of essential protein(s).

In general, the temperature range of the function of a protein is closely associated with the preferred habitat of the particular organism from which the protein originates. For example, proteins from thermophilic bacteria function effectively at high temperatures, but those from psychrophiles generally operate only at low temperatures, losing their functions even at

moderate temperatures (3). Proteins from mesophiles tend to function well at temperatures between these two extremes.

It appears self-evident that an organism would gain no advantage from maintaining protein stability at temperatures higher than its survival temperature. However, some proteins from mesophilic bacteria have been determined to be heat-stable (4, 5). This heat stability of certain proteins from mesophilic bacteria may be a property intrinsic to their structures, or may be a property acquired by evolution for their special functions, including protection against the denaturation of other proteins at high temperatures. Therefore, it would be interesting to determine which proteins are heat-stable, and what their common features are.

In this study, we describe the identification of 17 heat-stable proteins from *E. coli*. Approximately one-third of these proteins were recognized as having functions in the protection of other proteins against denaturation. Another common feature was that five of these proteins have been shown to form a macromolecular structure. These results indicated that the heat stability of certain proteins may have evolved for their specialized functions, allowing them to cope with harsh environments, including high temperatures.

RESULTS AND DISCUSSION

Heat treatment of *E. coli* proteins

In order to detect highly temperature-resistant proteins, soluble proteins after heat treatment at various temperatures were analyzed by SDS-PAGE. Sonic extracts were prepared and incubated for 10 min at various temperatures from 60°C to 100°C, at 5°C intervals. After centrifugation, proteins in the supernatants and pellets were analyzed by 12% SDS PAGE. As is shown in Fig. 1, the majority of protein species were precipitated by heat treatment at 60°C (Fig. 1B. lane 2), which was approximately 10 degrees higher than the highest temperature at which the organism could grow (1), but a considerable number of proteins remain in solution (Fig. 1A. lane 2). The protein species in the supernatant were progressively reduced as the temperature increased and the number of soluble protein species was minimal at temperatures between 80-90°C (Fig. 1). Interestingly, some proteins were precipitated only at very narrow temperature ranges. For example, a protein indicated by the arrowhead (a) in Fig. 1A was in the supernatant at 65°C, but the majority of the protein was precipitated at 75°C. It is also intriguing that certain proteins reappeared in
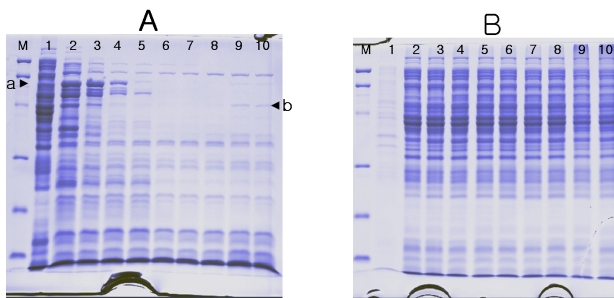
Fig. 1. Soluble proteins after heat treatments. *E. coli* BL21 was cultivated in LB medium, harvested by centrifugation and resuspended in a 1/10 culture volume of phosphate-buffered saline. The cells were broken by sonication and the supernatant was collected following centrifugation. The supernatant was incubated for 20 min at various temperatures and centrifuged for 20 min at 12,000 rpm. The pellets were resuspended in SDS-PAGE sample buffer with a volume identical to the supernatant, such that the relative partitioning of proteins between the supernatant and pellet could be compared. Proteins were analyzed by 12% SDS-PAGE. Panel A, proteins from supernatants. Panel B, proteins from pellets. Lane 1, No heat treatment. Samples were treated at following temperatures; lane 1, $0^{o}C$; 2, $60^{o}C$; 3, $65^{o}C$; 4, $70^{o}C$; 5, $75^{o}C$; 6, $80^{o}C$; 7, $85^{o}C$; 8, $90^{o}C$; 9, $95^{o}C$; 10, $100^{o}C$. Arrowheads indicated by a or b indicated a protein precipitated at a narrow range of temperature or a protein that was re-solubilized at high temperatures. See text for details.

Fig. 2. Two-dimensional gel electrophoresis of heat stable proteins. Proteins extracted from *E. coli* BL21 were treated for 10 min at $85^{o}C$ and the soluble proteins were collected by centrifugation. 1 hour of further clearing by ultracentrifugation at 100,000 g was conducted prior to analysis by two-dimensional gel electrophoresis. The protein spots were identified by peptide mass fingerprinting.

the supernatant as the temperature reached $95^{o}C$ or higher. One of these proteins is indicated with an arrowhead (b) in Fig. 1A. Nevertheless, the results presented in Fig. 1 demonstrated that protein species in the soluble fraction were minimal at $85^{o}C$. This temperature was selected for further analysis and for the identification of heat-stable proteins in *E. coli*.

Identifications of heat-stable proteins in *E. coli*

The soluble fraction obtained as the result of 10 min of treatment at $85^{o}C$ was cleared further by one hour of ultracentrifugation at 100,000 × g and the supernatant was analyzed by two-dimensional gel electrophoresis as described in Materials and Methods. Protein spots visualized by silver staining were identified by peptide mass fingerprinting with a MALDI-TOF mass spectrometer. We were able to identify 17 soluble proteins from proteins treated for 10 minutes at $85^{o}C$. The results are provided in Fig. 2 and Table 1.

Features of heat-stable proteins in *E. coli*

It is interesting that, among 17 heat-stable proteins, six were proteins that function in protein folding. They were components of chaperonin (GroEL and GroES), molecular chaperones (DnaK and FkpA), and peptidyl prolyl isomerases (trigger factor and FkpA) (6). These three classes are the principal proteins operating both in the protection of protein denaturation and in the folding of newly synthesized proteins. The common denominator of this group may be their capacity to interact with many proteins. For example, it was determined that GroEL in-
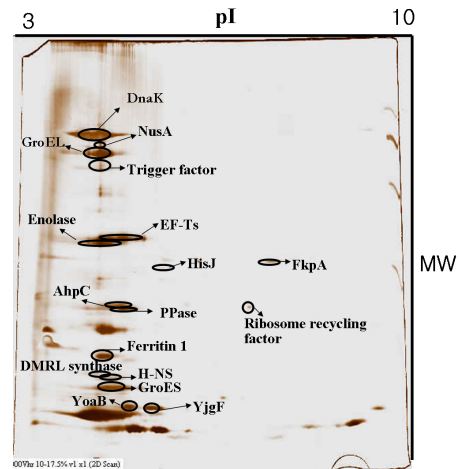
teracted with approximately 300 newly translated polypeptides (7). It will be interesting to see whether the heat stability characteristic of GroEL is relevant to its capability to interact with such a variety of proteins.

Another common feature of proteins listed in Table 1 is that five of these proteins, i.e. GroEL, GroES, Ahpc, RibH and ferritin, can form a supramolecular structure. It was determined that GroEL formed an 800 kDa cylinder from two back-to-back heptameric rings of 57 kDa subunits (8). It was recently determined that the AhpC from *H. pylori* formed a macromolecular structure under oxidative conditions and functioned as a molecular chaperone (9), although it was undetermined whether or not *E. coli* AhpC could form such a macromolecular structure. The product of *ribH*, lumazine synthase, was an icosahedral capsid comprised of 60 subunits with a mass of approximately 1 MDa, as evidenced by hydrodynamic studies and the results of electron microscopy (10) It is intriguing that a good portion of heat stable proteins can form a supramolecular structure for their functions.

Two proteins in our heat-stable protein list, pyrophosphatase (Ppa) (5) and histidine binding protein (4), the *hisJ* product, was determined to be heat stable, and this property was exploited for their purification.

The YjgF protein is a member of a class of proteins of unknown function which evidence a striking degree of conservation across a broad range of organisms, from bacteria to humans. It was determined that the mammalian homologue was quite resilient, indicating that they were soluble in perchloric acid (11). However, its heat stability has yet to be characterized.

Enolase, a glycolytic enzyme, has been identified as a component of the RNA degradosome in *E. coli*, and has been

Table 1. Heat-stable proteins from *E. coli* BL21

Protein	Accession No.	MW	p*I*	MALDI (SC:%)
Chaperone Hsp70 (Dnak)	P04475	69116	4.8	55
60 kDa chaperonin (GroEL)	P06139	57329	4.8	30
N utilization substance protein A (NusA)	P03003	54871	4.5	34
Trigger factor	P22257	48193	4.8	29
Enolase	P08324	45655	5.3	28
Elongation factor Ts (EF-Ts)	P02997	30423	5.2	25
FKBP-type peptidydyl-prolyl cis-trans isomerase (FkpA)	P45523	28882	8.4	29
Histidine-binding periplasmic protein (HBP)	P39182	28484	5.5	54
Alkyl hydroperoxide reductase C22	P26427	20762	5.0	49
Ribosome recycling factor	P16174	20639	6.4	38
Inorganic pyrophosphatase (PPase)	P17288	19704	5.0	46
Bacterioferritin 1	P23887	19424	4.8	31
6,7-dimethyl-8-ribityl lumazine synthase (DMRL synthase)	P25540	16157	5.2	45
DNA binding protein H-NS	P08936	15540	5.4	29
Hypothetical protein YjgF	P39330	13612	5.4	50
Hypothetical protein YoaB	P76258	12493	5.0	33
10kDa chaperonin (groES)	P05380	10370	5.1	24

shown to perform a crucial function in the regulation of RNA degradation (12). It also involved tRNA transport to the mitochondria in yeast (13). It remains to be determined whether this newly discovered function is related in any way to the heat-stability of enolase.

The property that keeps proteins in solution following heat treatment can be interpreted in three ways (Fig. 3). First, the protein structure is so rigid and stable that the applied heat is not sufficiently strong to break its folded structure (pathway A in Fig. 3). Proteins from thermophilic organisms may be members of this class. Second, the protein was indeed unfolded as the result of heat treatment, but the unfolded protein remained soluble in water throughout the process (pathway B). Third, the protein was unfolded as the result of heat treatment, but the unfolded protein remained soluble in water at 85°C, and refolded to its normal structure when the temperature again dropped (pathway C in Fig. 3). Proteins belonging to the second or the third class would have no stretch of hydrophobic amino acids, and would be composed principally of hydrophilic amino acids. The hydrophobicity plot constructed by the Kyte-Doolittle method with a window size of 7 showed that all 17 proteins we identified as heat-stable were indeed hydrophilic, without a stretch of more than 7 amino acids with a hydrophobicity index of greater than one. However, many proteins are hydrophilic but are still precipitated by heat treatment. Therefore, hydrophilicity alone is not sufficient to explain the solubility observed following heat treatment. Comparisons of protein structures at different temperatures by instrumental analysis, such as circular dichronism, may shed some light onto the mechanism underlying the heat stability of these proteins.

As an attempt to investigate energetics and dynamics of protein conformation on a proteomic scale, Park et al. challenged the *E. coli* proteome with extensive proteolysis (14). They
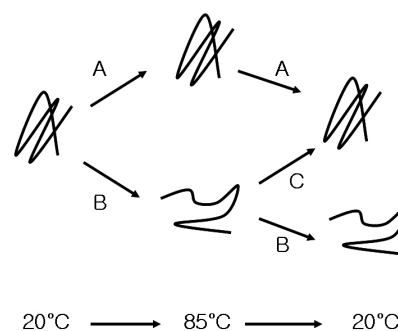

20°C ⟶ 85°C ⟶ 20°C

Fig. 3. Schematic representation of the pathways to survive heat-treatment. Protein structure would be so stable that the applied heat is not strong enough to break its folded structure (pathway A), or it was indeed unfolded as the result of heat treatment, but the unfolded protein remains soluble throughout the process (pathway B) or folds back to its normal structure when the temperature dropped (pathway C).

identified 22 survivors from digestion with trypsin and 34 survivors from digestion with thermolysin. It was suggested that many of these proteins might have evolved extreme proteolytic resistance because of their critical roles under stressed conditions. Their stress-related proteins have functions associated with starvation and oxidative stress, but ours have functions associated with unfolded protein stress. Nineteen out of their 40 identified survivors were periplasmic binding proteins, but in our list only two (FKBP-type peptidydyl-prolyl cis-trans isomerase and histidine-binding periplasmic protein) were periplasmic proteins. In fact, only a single protein, bacterioferritin, was common in their proteolysis-resistant proteins and our heat-stable proteins. It seems that thermal stability of proteins and the property of proteolytic resistance are not related and might have evolved independently for their relevent functions.

In this study, we describe 17 *E. coli* proteins which were not precipitated by 10 minutes of heat treatment at 85°C. Five of these proteins have been shown to form a supramomolecular structure. In this case, heat stability may be associated with this special structure. Interestingly, six heat-stable proteins were identified as chaperonins, molecular chaperones, or peptidyl prolyl isomerases. These classes of proteins are the principal proteins functioning both in the protection of proteins against denaturation or in the folding of denatured and newly synthesized proteins. It appears that the heat stability of these proteins may have evolved for their special functions, which allow them to cope with harsh environments.

MATERIALS AND METHODS

Heat treatment and SDS-PAGE analysis
E. coli BL 21(DE3) was cultivated at 37°C with good aeration until optical density of about 0.8. Cells were collected by centifugation and washed with phosphate-buffered saline (PBS). Cells were resuspended in PBS (5 ml per 1 gram of cell paste) and broken by sonication. Soluble proteins were collected by centrifugation (12,000 rpm, for 10 min). The protein extract was incubated for 10 min at various temperatures from 60°C to 100°C, at 5°C intervals, and centrifuged for 20 min at 12,000 rpm. Proteins in the supernatants and pellets were analyzed through 12% SDS-PAGE.

Two-dimensional gel electrophoresis and protein identification
The soluble fraction obtained as the result of 10 min treatment at 85°C was cleared further by one hour of ultracentrifugation at 100,000 x g. The supernatant was analyzed by two-dimensional gel electrophoresis as described previously (15).

Isoelectric focusing (IEF) was conducted using an IPGphor system (Amersham Pharmacia Biotech). The protein samples were mixed with a rehydration buffer (8 M urea, 2% CHAPS, 0.5% IPG buffer, 20 mM DTT) and applied to 13-cm Immobiline Drystrips, 3-10NL (Amersham Pharmacia Biotech) by in-gel rehydration. The proteins were focused for a total of 30,000 Vh. IPG strips were reduced, alkylated, and applied on top of 10-17% polyacrylamide gradient gels. The gels were stained with silver nitrate.

Protein spots were identified by peptide mass fingerprinting as described previously (16). For this, the protein spots were excised, carbamidomethylated, and digested with trypsin (Promega, sequencing grade). Peptide mass maps of the tryptic peptides were generated by MALDI-TOF MS using a Voyager-DE STR (PerSeptive Biosystems). Monoisotopic peptide masses were analyzed using the MS-Fit public software tool (http://prospector. ucsf.edu).

Acknowledgements

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