Overexpression of L-Isoaspartate O-Methyltransferase in Escherichia coli Increases Heat Shock Survival by a Mechanism Independent of Methyltransferase Activity*

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Over time and under stressing conditions proteins are susceptible to a variety of spontaneous covalent modifications. One of the more commonly occurring types of protein damage is deamidation; the conversion of asparagines into aspartyls and isoaspartyls. The physiological significance of isoaspartyl formation is emphasized by the presence of the conserved enzyme L-isoaspartyl O-methyltransferase (PIMT), whose physiological function appears to be in preventing the accumulation of deamidated proteins. Seemingly consistent with a repair function, overexpression of PIMT in Drosophila melanogaster extends lifespan under conditions expected to contribute to protein damage. Based on structural information and sequence homology we have created mutants of residues proposed to be involved in co-factor binding in Escherichia coli PIMT. Both mutants retain S-adenosyl L-methionine binding capabilities but demonstrate dramatically reduced kinetic capabilities, perhaps suggestive of catalytic roles beyond co-factor binding. As anticipated, overexpression of the wild type enzyme in E. coli results in bacteria with increased tolerance to thermal stress. Surprisingly, even greater levels of heat tolerance were observed with overexpression of the inactive PIMT mutants. The increased survival capabilities observed with overexpression of PIMT in E. coli, and possibly in Drosophila, are not due to increased isoaspartyl repair capabilities but rather a temperature-independent induction of the heat shock system as a result of overexpression of a misfolding-prone protein. An alternate hypothesis as to the physiological substrate and function of L-isoaspartyl methyltransferase is proposed.

Proteins are susceptible to a variety of spontaneous, covalent modifications that have the potential for disruption of both structure and biological activity. The formation of isoaspartyl residues, through either the deamidation of asparagines or isomerization of aspartates, are among the most rapidly occurring types of damage that afflict proteins under physiological conditions (1).

The metamorphosis of asparagine and aspartate residues is initiated by the nucleophilic attack of the neighboring peptide nitrogen on the side chain carbonyl, resulting in the cyclization of the side chain with the main chain to the formation of a succinimide ring (Fig. 1) (2). Succinimides are unstable intermediates that undergo rapid, nonenzymatic hydrolysis to a mixture of aspartyl and isoaspartyl residues. Isoaspartyls typically account for two-thirds of the emerging products and with the introduction of an extra carbon into the main chain have the greater potential for disruption of protein structure and biological activity (2). The precise ratio of the aspartyl and isoaspartyl products is determined by the accessibility of the succinimide carbonyls to attacking water or hydroxide molecules; attack at the main chain carbonyl produces isoaspartyls, whereas attack at the side chain carbonyl produces aspartyls. The α-carbon of a succinimide is also prone to racemization, resulting in the D-configurations of these amino acids, although at low yields (3).

The physiological significance of isoaspartyl formation is emphasized by the presence of an enzyme, L-isoaspartyl O-methyltransferase (PIMT) whose function appears to be in limiting the accumulation of isoaspartyl-containing proteins within the cell. PIMT is a highly conserved enzyme found in a wide variety of organisms, including plants (4), insects (5), bacteria (6), and mammals (7), that has the ability to specifically recognize and methylate isoaspartyl residues in a variety of peptide and protein contexts (1, 2). PIMT catalyzes the transfer of a methyl group from S-adenosyl-L-methionine (AdoMet) to the α-carboxyl of the L-isoaspartyl group, methylation stimulates reformation of the succinimide, and subsequent hydrolysis converts a fraction of the offending residues to aspartyls. The overall reaction therefore represents true repair when the isoaspartyl results from an isomerized aspartyl and only partial repair of a deamidated asparagine. Because only a fraction of the isoaspartyls are converted to aspartyls with each round of methyltransferase activity, complete repair requires numerous rounds of cycling utilizing an energetically expensive methyl group donor, making the “repair” reaction quite inefficient.

Genetic studies of the physiological role of PIMT in lower organisms have not been clearly supportive of a repair function. In Caenorhabditis elegans, mutants having a disruption of the gene show poor Dauer stage survival, but substrate for the enzyme was found primarily in peptide fragments in both the...
wild type and PIMT-deficient organisms, suggesting that damaged proteins are cleared by proteolysis rather than repair (8). An Escherichia coli strain deficient in PIMT did not exhibit greater susceptibility to conditions anticipated to promote deamidation but did demonstrate increased sensitivity to changing conditions in the stationary phase (9).

Genetic studies in higher organisms are more supportive of a repair function for PIMT. Mice lacking PIMT show an accumulation of damaged proteins, particularly in the brain; display abnormal brain activity; and suffer fatal seizures at an early age (10). Consistent with the hypothesis that the accumulation of isoaspartyl-containing proteins can be limiting to lifespan, overexpression of PIMT in Drosophila melanogaster extends the lifespan by 32–39% under conditions that accelerate the rates of deamidation, suggesting that PIMT activity is limiting in these situations (11).

The ability of overexpression of a protein to extend lifespan is rare but not unprecedented. In particular, overexpression of enzymes that function in protein repair have been shown to prolong lifespan, supportive of the hypothesis that senescence results from a decline in cellular repair processes (12). Examples include the limiting of the accumulation of potentially damaging agents, as with superoxide dismutase (13), as well as general protein repair systems, such as the heat shock proteins (14). The ability for overexpression of these proteins to increase longevity is presumably due to an increased capacity to deal with damaged or misfolded proteins that limit the lifespan of the organism.

Recently, crystallographic structures of PIMT have been determined from human (15), Thermotoga maritima (16), and Pyrococcus furiosus (17). All three show high similarity in their amino acid sequences as well as three-dimensional folds, which are characteristic of the AdoMet-dependent methyltransferase superfamily. Complexes of PIMT with AdoMet, as well as isoaspartyl-containing substrate, provide structural insights into the workings of this enzyme.

A particularly interesting feature of co-factor binding in PIMT is the occurrence of two highly conserved, negatively charged residues buried in the interior of the protein within the AdoMet-binding pocket. These residues are Glu108 and Glu123 in P. furiosus, Asp80 and Asp109 in human, and Glu108 and Glu108 in T. maritima. The side chain carboxyl of the first acidic residue interacts with the α-carbon amino group of AdoMet, and the side chain carboxyl of the second acidic residue hydrogen bonds with the hydroxyls of the ribose. Similar interactions between protein and co-factor have been reported for other AdoMet-dependent methyltransferases (18–20) as well as S-adenosyl-homocysteine hydrolase (21).

Although the precise role of these acidic residues has not been determined, given their conservation and atypical positioning, it seems likely that they play an important role in the reaction cycle. The electrostatic contribution of these residues might be toward the energetics of substrate and co-factor binding or perhaps playing a more direct role in catalysis. Notably, prior to the reaction, the isoaspartyl substrate has a negatively charged carboxyl group, and the AdoMet co-factor has a positively charged sulfonium; upon completion of the reaction, both of these charged groups are neutralized. Based on sequence alignments, the corresponding residues in E. coli PIMT are Glu81 and Glu104 (Fig. 2). In this investigation we examine the role of Glu81 and Glu104 in the catalytic mechanism of E. coli PIMT, as well as the ability of PIMT overexpression in E. coli to protect cells in conditions where the accumulation of damaged proteins may limit survival.

**EXPERIMENTAL PROCEDURES**

Cloning and Purification of E. coli l-Isopropyl O-Methyltransferase—Two oligonucleotide primers were designed based on the E. coli pcm gene sequence (22) to allow for amplification of the full-length gene from E. coli genomic DNA. The 5′ primer (5′-CGGCCGGCATATGTTGA-GCAGGCGC-3′) overlaps the ATG initiation codon (underlined) of the pcm gene and was designed to include a NdeI restriction site (shown in bold type). The 3′ primer (5′-GGGATCCCTAGTTAAGCCGACGC-3′) incorporates a BamHI restriction site (shown in bold type). PCR amplification using a single colony of E. coli as template for a total of 25 cycles resulted in a single product of the appropriate size as detected by agarose gel electrophoresis.

The PCR product was purified and ligated into the pET7 vector using the pET Perfectly Blunt cloning kit (Novagen). The resulting plasmid DNA was digested with NdeI and BamHI, and the insert DNA was ligated into the pET15b plasmid under the control of the T7 promoter with the incorporation of an N-terminal His₆ tag. The complete sequence of the gene and correct incorporation into the plasmid was confirmed by DNA sequencing. Site-directed mutagenesis was performed using QuickChange site-directed mutagenesis with mutations being confirmed by DNA sequencing.

Overexpression of all proteins was carried out in Tuner (DE3) pLysS cells. At mid-log phase growth in 1.5 liters of Terrific Broth (100 µg/ml ampicillin, 34 µg/ml chloramphenicol) at 37 °C, the cultures were induced with 0.5 mM isopropylthiogalactoside and grown for an additional 3 h. The cells were harvested and sonicated as previously described (23).

Crude lysate was diluted 2-fold in buffer B (50 mM sodium phosphate, 300 mM NaCl, 10 mM imidazole, pH 8.0) and applied to a nickel column equilibrated with buffer B. The column was washed with three column volumes of buffer B, followed by three column volumes of buffer W (50 mM sodium phosphate, 300 mM NaCl, 250 mM imidazole, pH 8.0) and 20-ml fractions were collected. Protein-containing fractions were determined by SDS-PAGE, and purity was estimated to be greater than 95%. PIMT-containing fractions were pooled, and myoglobin, a carrier protein for PIMT, was added to a final concentration of 0.5 mg/ml. This mixture was dialyzed overnight against 20 mM potassium phosphate (pH 7.5), 2 mM EDTA, and 1 mM DTT. For PIMT samples for thermal denaturation investigation.

**FIG. 1.** Formation and hydrolysis of a succinimide from asparagine.
PIMTs were analyzed immediately after purification without dialysis or the addition of carrier protein. Methyltransferase Assays—Methyltransferase activities were performed based on a modified protocol (24). Each reaction mixture contained 1 μCi of (methyl-[3H])S-adenosyl-L-methionine, 20 μM S-adenosyl-L-methionine, 50 mM sodium phosphate (pH 6.8), 5 mM EGTA, 0–0.5 mM methyl-accepting substrate, either 1 μg of wild type PIMT or 30 μg of the PIMT mutants, in a final volume of 50 μl. The substrate, HpRv-2, was generated by incubating E. coli HpRv (5 mg/ml 50 mM Tris, pH 8.0) at 80 °C for 3 h to promote deamidation of both Asn13 and Asn18 (25). Treated and untreated HPrs were visualized by isoelectric focusing to ensure that complete deamidation of both positions had occurred. This isoaspartyl substrate has been previously characterized with respect to their methyl group accepting abilities from the human PIMT (26).

Methyltransferase reactions were incubated at 30 °C for 2 h and then placed on ice for 10 min to slow the PIMT reaction and condense any methanol vapor. The tubes were centrifuged for 10 s at 4 °C in a microcentrifuge and returned to ice. 50 μl of Stop solution (0.4 mM CAPS, pH 10, 5% SDS, 2.2% methanol) was added and mixed thoroughly by pipetting. The tubes were placed within a scintillation vial containing 3 ml of Beckman ReadySafe scintillation mixture. The samples were run at a flow rate of 1 ml/min, and absorbance was measured at 254 nm. All of the column runs were preceded by a 20-min equilibration in buffer A. Elution profiles for AdoMet and S-adenosyl L-homocysteine standards, each at a concentration of 4.0 mM, were determined; S-adenosyl L-homocysteine eluted at 15.4 min, and AdoMet eluted at 3.12 min.

Parallel experiments were also performed under non-denaturing conditions in which the protein samples were not treated with perchloric acid. For these investigations, the protein component was removed from the solution by centrifugation through a Millipore Ultrafree-MC centrifugal filter device with a molecular weight cut-off 10,000. SDS-PAGE confirmed retention of the protein by the spin column.

Stress Survival Tests—Stress survival tests were based on a modified protocol (9). Resistance to heat shock was measured using cultures that were grown to mid-log phase in LB broth (100 μg/ml ampicillin, 34 μg/ml chloramphenicol), induced with 0.5 mM isopropylthiogalactoside, and allowed to grow for 3 h. The cells from these cultures were pelleted by centrifugation and resuspended to one-tenth their original density in 0.85% NaCl. In 1.5-ml Eppendorf tubes, aliquots of 50 μl were heated to 60 °C for 10 min in a thermal cycler; control samples were left on ice for this period. Viable counts were performed by making serial dilutions in 0.85% NaCl and plating them on LB agar (100 μg/ml ampicillin, 34 μg/ml chloramphenicol) and incubated at 16 h at 37 °C to determine the number of surviving bacteria; these results were then compared as the percentages of surviving colonies of the samples that were not heat-shocked.

Stability of the E. coli Isoaspartyl O-Methyltransferase—To assess the stability of the wild type and mutant proteins, thermal denaturation profiles were measured with a heating rate of 0.5 °C/min on a Gilford 600 spectrophotometer. The absorbance was continuously monitored at 293 nm over a temperature range of 25–90 °C. The proteins were in Buffer E of the purification protocol.

Western Blot Analysis of Hsp70 Induction—One hour following isopropylthigalactoside induction, 5 μl of culture was pelleted by centrifugation. For Western analysis, equivalent amounts of protein were resolved by SDS-PAGE. Following electrophoresis, the protein samples were transblotted onto polyvinylidene difluoride membranes (PolyScree; PerkinElmer Life Sciences). The membranes were blocked with 5% molecular grade fat-free skim milk powder in phosphate-buffered saline containing 0.02% (v/v) Tween 20. Primary and secondary washes were carried out using this sample buffer. Primary antibodies to Hsp70 were obtained from Stressgen (Victoria, Canada). The antibody SC-7298 manufactured by Santa Cruz Biotechnologies was used to probe for protein levels of Hsc70. Protein levels were assessed by Western analysis.

An enhanced chemiluminescent detection system (PerkinElmer Life Sciences) was used to detect antigen-antibody complex.

Fig. 2. Sequence alignment of L-isoaspartyl methyltransferases from E. coli, T. maritima, Homo sapiens, and P. furiosus. The sequences for H. sapiens, T. maritima, and P. furiosus PIMT were from the Protein Data Bank. The sequence of E. coli PIMT was taken from a published report (26). The sequences were aligned using ClustalW (35). Highly conserved sequences are indicated in dark gray, and moderately conserved sequences are in light gray.
Methyltransferase Activity and Heat Shock Protection

E81A and E104A PIMT Have Limited Methyltransferase Activity—The catalytic capabilities of wild type and mutant E. coli PIMTs were determined. Using fully deamidated E. coli HPr as an isoaspartyl substrate, E. coli wild type PIMT had a $V_{\text{max}}$ of 2.55 nmol/min/mg and a $K_m$ of 160 $\mu$M, comparable with the kinetic parameters previously determined for the same substrate using human PIMT. The E81A PIMT mutant has a $V_{\text{max}}$ of 0.1 nmol/min/mg and a $K_m$ of 220 $\mu$M for the isoaspartyl substrate, corresponding to a $V_{\text{max}}$ of $\sim$4% that of the wild type enzyme with a small increase in $K_m$ (Table I).

Purified E104A PIMT failed to demonstrate any detectable methyltransferase activity. Similarly, assays performed on the E104A crude extract prior to purification also failed to demonstrate any methyltransferase activity above background.

E81A and E104A E. coli PIMT Retain Co-factor Binding Ability—To determine whether the kinetic consequences of these mutations were due to an inability to bind the AdoMet co-factor, HPLC analysis for co-factor co-purification was performed. PIMT has a remarkably high affinity for the co-factor, HPLC analysis for co-factor co-purification was performed on PIMT samples under nondenaturing conditions. Without disruption of protein structure, AdoMet remained effectively chelated and could not be detected in the supernatants of either the wild type PIMT or the E81A mutant. For the E104A mutant, however, AdoMet is present in the supernatant, although in smaller quantities than with the denatured sample (Fig. 4), suggesting that this mutant has reduced affinity for the co-factor or a propensity for spontaneous unfolding. Another as yet unidentified peak eluted at 1.79 min. This peak was not detected in any other runs. Given the previous reports of a specific interaction between adenosine and PIMT (17), we are investigating the possibility of the co-purification of adenosine specifically by this inactive mutant and not the other forms of PIMT.

Methyltransferase Activity and Heat Shock Protection—It has been reported that in Drosophila increased expression of PIMT can prolong lifespan under conditions in which the formation of damaged proteins may limit survival (11). We examined the ability of overexpression of wild type, partially active, and completely inactive PIMTs to influence survival capabilities of heat-shocked bacteria.

RESULTS

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| PIMT     | $V_{\text{max}}$ | $K_m$ | $T_m$ | Soluble protein yield* |
|----------|-----------------|------|-------|------------------------|
| Wild type| 25.5            | 160  | 41.5  | 100                    |
| E81A     | 0.1             | 220  | 42.1  | 65–75                  |
| E104A    | 39.8            | 15   | 15–25 |                        |

* Percentage of soluble yield is based on comparison of purification efficiencies over two large scale purifications performed under identical conditions.

Fig. 3. HPLC analysis of co-factor binding by wild type and mutant isoaspartyl methyltransferases. Protein samples (0.1 mg) were incubated in 6% perchloric acid for 30 min at room temperature and then centrifuged for 10 min. A, S-adenosylmethionine standard; B, wild type PIMT; C, E81A PIMT; D, E104A PIMT.

There is a considerable increase in the survival rates of bacteria overexpressing the wild type enzyme as compared with those harboring empty expression vector or expressing an unrelated protein such as the PhoB response regulator from Salmonella typhimurium. However, overexpression of the partially active E81A mutant resulted in comparable increases in survival capabilities as the wild type enzyme. Surprisingly, overexpression of the inactive E104A mutant had significantly higher survival rates than cells enriched with the wild type enzyme (Table II). These results indicate that the survival capabilities bestowed by PIMT overexpression, at least in this circumstance, are independent of PIMT repair function and are likely due to some secondary characteristic of the protein.

Stability of the Wild Type and Mutant Isoaspartyl Methyltransferases—We considered the possibility that the increased survival capabilities were being mediated through the induction of a heat shock response as a consequence of overexpression of an unstable protein. The overexpression of any unstable or misfolding-prone protein has the potential for induction of a heat shock response, regardless of the physiological role of the
E. coli PIMT is known to be a rather unstable enzyme (22), and the substitution of glutamate with alanine may further contribute to this instability. Through thermal denaturation curves, wild type E. coli PIMT has a melting temperature of 41.5 °C. The E81A mutant is very comparable with a melting temperature of 42.1 °C, and the E104A showed slightly reduced stability, with a melting temperature of 39.8 °C (Table I). Although these relative stabilities would appear to correlate with the observed survival phenotypes, the stability of a folded protein is not necessarily predictive of the potential for misfolding following translation. The effects of the mutations on misfolding are more accurately reflected by the yields of soluble protein obtained from identical overexpression trials.

The expression levels of mutant and wild type PIMTs also support the hypothesis of misfolding of a fraction of the mutant proteins. Over numerous purifications the yields of soluble protein for the mutant PIMTs were consistently less than for the wild type. Typically the yield of the E85A mutant was 65–75% that of the wild type, and that of the E104A mutant was only 15–25% (Table I). The effects of the mutations on the propensity to misfold is not necessarily a consequence of changes in stability of the correctly folded protein.

Induction of Heat Shock Response by Methyltransferase Overexpression—The major signals for induction of the heat shock response are elevated temperature and the presence of unfolded or misfolded proteins within the cell. In E. coli, the overexpression of either the E81A mutant or wild type PIMT results in a slightly greater than 2-fold increase in levels of inducible Hsp70 relative to identical induction of cells harboring the empty expression vector. Cells overexpressing the E104A PIMT mutant had the largest effect on the heat shock system with an ~3-fold increase in Hsp70 expression (Fig. 5). These levels of induction correlate with the observed survival phenotypes, with moderate increases in heat shock induction and survival phenotype with wild type and E81A overexpression and more dramatic increases with overexpression of the E104A mutant. For all of these cells levels of Hsc70, the cognate form of Hsp70 remained constant, confirming equivalence of protein load.

The temperature-independent induction of the heat shock response as a result of overexpression of large quantities of misfolding-prone proteins seems sufficient to induce a expression of the bacterial heat shock system. The initiation of the heat shock response prior to heat shock would be anticipated to offer a survival advantage to these bacteria upon the shift to a high temperature environment.

**DISCUSSION**

Proteins are susceptible to a variety of spontaneous covalent modifications that may result in alterations to both structure and function. Asparagines are particularly labile with the potential to undergo deamidation through cyclization of the side chain carbonyl with the amide nitrogen of the n + 1 peptide.
The catalytic mechanism of PIMT appears to operate through an ordered mechanism with the binding of AdoMet preceding binding of the isoaspartyl substrate (30). The ability of the E81A to co-purify AdoMet, coupled with the minimal changes in $K_m$ for the isoaspartyl substrate, would seem to indicate that neither the binding of the co-factor nor the substrate have been significantly affected as a result of the mutation, suggesting that Glu$^{104}$ may have additional roles beyond co-factor binding, although it is unclear what role this may be. It seems unlikely that this residue is involved in methyl group transfer, because this portion of the reaction is already energetically favorable. Additionally, unlike some other AdoMet-dependent methyltransferases, PIMT is believed to function without a covalent intermediate or the aid of metal ions, because the oxygen of the $\alpha$-carboxyl of isoaspartyl is a sufficiently strong nucleophile for direct attack on AdoMet (16).

Although the structural mechanisms underlying the catalytic capabilities of PIMT are being elucidated, the physiological significance of these reactions, as well as the in vitro substrate for the enzyme, remains mysterious. Despite being the focus of extensive published investigations since its initial characterization 20 years ago, a precise physiological role for the enzyme has not been defined. It has generally been assumed that the enzyme functions in preventing the accumulation of isoaspartyls that emerge as a consequence of protein aging, but there are numerous inconsistencies with this function.

PIMT is quite inefficient in the traditionally assigned role of repair of isoaspartyl residues emerging from deamidation. In considering the isoaspartyls that emerge from deamidation, conversion to aspartyls does not represent complete repair, being unable restore the original asparagine residue. The complete conversion of a population of isoaspartyls to aspartyls requires repeated cycling through the succinimide intermediate, with each round requiring the expenditure of an energetically expensive methyl group. Although prolonged in vitro incubation of deamidated proteins with PIMT results in the conversion of isoaspartyl to aspartyls, the process is often unable to restore full biological activity (31).

The repair function for PIMT has not been validated by in vivo experiments, particularly with lower organisms. An E. coli strain deficient in PIMT did not exhibit greater susceptibility to conditions that promote deamidation (9). In C. elegans, substrate for the enzyme was found primarily in peptide fragments in both the wild type and PIMT-deficient organisms, suggesting that damaged proteins are cleared by proteolysis rather than repair (8). These inconsistencies have led to alternate theories as to the physiological function of the enzyme, including an involvement in neurotransmitter metabolism (32), in the development of the central nervous system (33), or as an intramolecular clock (34). However, a recent report that overexpression of PIMT extends the lifespan of Drosophila under conditions anticipated to promote deamidation is extremely significant in supporting the traditional assigned role of the enzyme in the repair of damaged proteins (11).

To confirm that accumulation of isoaspartyl residues may become limiting to the survival of an organism under certain conditions and that PIMT overexpression bestows increased survival capabilities through increased capability to deal with these aberrant residues, we examined the ability of overexpression of both active and inactive forms of PIMT to influence bacterial survival to temperature stress. With overexpression of wild type PIMT, we observed a significant increase in the heat shock survival rates as compared with cells containing an empty expression vector. However, a comparable increase in survival capabilities is observed with overexpression of the
kinetically compromised E81A mutant, and surprisingly, bacteria overexpressing the inactive E104A mutant had the highest survival capabilities, indicating that the increased survival is independent of PIMT repair function. The increased survival was therefore postulated to emerge as a consequence of induction of the heat shock system as a consequence of overexpression of large quantities of unstable or misfolding-prone protein. Western blot analysis confirmed the induction of the E. coli DnaK chaperone protein in a manner that correlated with the observed increase in survival capabilities.

The major signals for induction of the heat shock response are elevated temperatures and the presence of unfolded protein within the cell. In E. coli, overexpression of thermolabile proteins may result in the induction of the DnaK chaperone system, the prokaryotic homolog of the Hsp70 family. The DnaK system has dual functions both in limiting protein aggregation, and as a consequence bestowing thermal tolerance, and in functioning as the primary stress-sensing and transducing system of the E. coli heat shock response (35). The sequestration of the DnaK chaperone system through binding to misfolded proteins is thought to be a direct determinant of the modulation of heat shock gene expression. Small changes in levels of DnaK (<1.5-fold of wild type) have significant impact on the levels and activity of heat shock activation at intermediate temperatures (36).

In Drosophila, overexpression of PIMT only resulted in extension of lifespan at 29 °C, with normal lifespan observed at 25 °C (11). This suggests that either isoaspartyl formation only becomes limiting to survival at elevated temperatures where higher rates of deamidation would be anticipated or that an induction of the heat shock system is at least partially responsible for the phenotype. The authors report that 29 °C is just below the threshold for induction of the Drosophila heat shock system but do mention the possibility that PIMT works in concert with the heat shock system toward the observed survival phenotype. Although our results support the involvement of the heat shock system in the Drosophila model, they suggest that the induction is a nonspecific consequence of the inherent instability of the PIMT protein, independent of methyltransferase activity. The physiological role of the isoaspartyl methyltransferase enzyme as well as its in vivo substrate, therefore, remain unclear.

An alternate hypothesis for PIMT function emerges from the hypothesis that succinimide formation may represent a deliberate physiological mechanism for the recycling of modified aspartyls, most notably those that are modified by phosphorylation. As demonstrated in H15D HPr as well as the chemotaxis response regulator CheY, the presence of an activated aspartyl residue can prompt rapid formation of a succinimide, resulting in the removal of the modifying group. We have previously reported that phosphoryaspartate groups have the potential to instigate rapid formation of a succinimide ring as a consequence of the superior leaving group and that this rearrangement could possibly represent a physiological mechanism of autophosphatase activity as observed in both the response regulator proteins of two-component systems as well as the P-type ATPases. Hydrolysis of the succinimide to an aspartyl results in recycling of the active dephosphorylated enzyme. Isoaspartyl formation would likely be limited by protein design (37), representing either a strategy for the reversible desensitization of a population of proteins or perhaps occurring as an inadvertent consequence of the rearrangement chemistry. In this scenario 1-isoaspartyl methyltransferase functions largely as a regulatory enzyme rather than a repair enzyme, possibly representing a previously unsuspected mechanism of modulation of enzyme activity.

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