The Chemical Chaperone Proline Relieves the Thermosensitivity of a dnaK Deletion Mutant at 42°C

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Received 18 June 2004/Accepted 19 July 2004

Since, like other osmolytes, proline can act as a protein stabilizer, we investigated the thermoprotectant properties of proline in vitro and in vivo. In vivo, elevated proline pools in Escherichia coli (obtained by altering the feedback inhibition by proline of γ-glutamyl kinase, the first enzyme of the proline biosynthesis pathway) restore the viability of a dnaK-deficient mutant at 42°C, suggesting that proline can act as a thermoprotectant for E. coli cells. Furthermore, analysis of aggregated proteins in the dnaK-deficient strain at 42°C by twodimensional gel electrophoresis shows that high proline pools reduce the protein aggregation defect of the dnaK-deficient strain. In vitro, like other “chemical chaperones,” and like the DnaK chaperone, proline protects citrate synthase against thermodenaturation and stimulates citrate synthase renaturation after urea denaturation. These results show that a protein aggregation defect can be compensated for by a single mutation in an amino acid biosynthetic pathway and that an ubiquitously producible chemical chaperone can compensate for a defect in one of the major chaperones involved in protein folding and aggregation.

Acclimation to osmotic stress involves intracellular accumulation (up to 0.5 M), by synthesis or uptake, of small organic molecules known as osmolytes, such as glycine betaine, proline, or trehalose (5, 6, 14). They serve as stabilizers of proteins and cell components against the denaturing effect of ionic strength. Furthermore, some of these osmolytes behave as “chemical chaperones” by promoting the correct refolding of proteins in vitro and in the cell and by protecting native proteins from heat denaturation (1, 4, 9, 13, 19).

Heat shock induces the massive production of heat shock proteins. In Escherichia coli, IbpA/IbpB (small heat shock proteins), DnaK/Hsp70, GroEL/Hsp60, HtpG/Hsp90, and ClpB/Hsp100 are the major chaperones that cooperate in preventing protein aggregation during heat stress and in promoting protein refolding after the stress (12). The DnaK system (DnaK/DnaJ/GrpE) appears to be the most effective chaperone system in preventing protein aggregation in vivo (15), and this activity explains the temperature-sensitive phenotype of ΔdnaK mutant cells (16). The major role of the DnaK system in preventing protein aggregation in vivo is supported by the lack of strong protein aggregation phenotypes of mutants lacking functions of other chaperones and by the inability of overproduction of GroEL/GroES, ClpB, HtpG, or IbpA/IbpB to restore the growth of ΔdnaK mutant cells at 42°C (15).

In enteric bacteria, proline can be transported from the medium by the ProP and ProU transporters and is catabolized to glutamate by the putA gene product (14, 22). Proline transport, however, does not result, even under osmotic stress conditions, in high intracellular proline pools (14). Proline is endogenously synthesized from glutamate by the proA, proB, and proC gene products, which are synthesized at similar levels regardless of the availability of proline (7). Proline synthesis is negatively regulated through feedback inhibition of the first biosynthetic enzyme of the pathway, the proB gene product γ-glutamyl kinase (2). Proline-overproducing mutants, affected in the proB gene, have been isolated from Salmonella enterica serovar Typhimurium (5); these mutants possess a γ-glutamyl kinase which is resistant to feedback inhibition by proline and have acquired osmotolerance. The most osmotolerant strain, carrying the proline-overproducing mutation proB74, has, depending on the osmolarity of the growth medium, a 30- to 400-fold-higher proline level than the parental strain. The proB74 mutation has been cloned in E. coli and has been shown to confer osmotolerance to the cells, a consequence of an increased proline pool (8).

In the present study, we show that proline overproduction (in an E. coli strain producing a desensitized γ-glutamyl kinase) can restore the viability of a dnaK-deficient mutant at 42°C and partially reverses its protein aggregation defect in vivo; we also compare the effects of proline and DnaK on citrate synthase folding and aggregation in vitro.

Suppression of the thermosensitive phenotype of a dnaK deletion mutant at 42°C by increased proline pools. The dnaK deletion mutant GW 4813 (ΔdnaK52::Cm′, derived from strain AB1157) is deficient for growth and viability at 42°C (16), probably as a consequence of protein denaturation and aggregation (15). The dnaK mutant was transformed with plasmid pDU117, carrying proB74, the γ-glutamyl kinase gene impaired in the allosteric repression by proline, or with plasmid pDU417, carrying the wild-type γ-glutamyl kinase gene (both plasmids also contain the proA gene, coding for γ-glutamyl phosphate reductase, the second enzyme of the proline biosynthetic pathway); they were constructed as described previ-
Carpenter et al. (8) and introduced into the dnaK deletion mutant GW4813 by electroporation.

We first checked that the presence of a desensitized γ-glutamylphosphoribosyl transferase in the dnaK mutant expressing the proB74 gene (analysis of free proline pools was carried out on a Beckman amino acid analyzer as described previously [5]) on bacteria grown in Luria-Bertani medium, towards the end of the exponential phase). An intracellular proline pool of 295 nmol/mg of cell protein was measured in the proline overproducer, versus 12 nmol/mg of cell protein in the control strain (295 nmol of proline/mg of cell protein corresponds to a proline pool of around 90 mM, assuming an intracellular volume of 3.5 μl/mg of cell protein (17).

The colony-forming abilities of the two strains at 30 and 42°C were compared. At 30°C, the colony-forming abilities of the two strains were similar (the dnaK proB74 strain formed smaller colonies than the dnaK proB+ control strain) (not shown). At 42°C, the dnaK proB+ strain displayed less than 0.2% plating efficiency (that at 30°C); in contrast, the dnaK proB74 strain displayed 52% plating efficiency (of that at 30°C) (not shown). These results suggest that a high intracellular proline pool can partially compensate for the deficiency of the dnaK mutant at 42°C, probably by reducing its known protein aggregation defect at elevated temperatures (see below).

Effects of proline pool on protein aggregation in the dnaK mutant. We investigated the extent of heat-induced protein aggregation in the wild-type strain AB1157 and in the isogenic dnaK-deficient mutant transformed either with plasmid pDU117, carrying proB74, the γ-glutamylphosphoribosyl transferase gene impaired in the allosteric repression by proline, or with plasmid pDU117 carrying the wild-type γ-glutamylphosphoribosyl transferase gene. Cells were grown at 30°C and then subjected to heat shock treatment for 60 min at 42°C (within the time course of the experiment, none of the strains showed growth defects as judged by increases in turbidity of the cultures). After lysis of cells, the insoluble cell fraction was isolated by centrifugation at 15,000 × g as described previously (15), and the pellets were analyzed by two-dimensional gel electrophoresis (10).

Heat shock treatment caused an increased protein aggregation in the dnaK-deficient strain transformed with the wild-type proB gene (Fig. 1B, displaying approximately 30 major spots and 60 minor spots) compared with the wild-type strain (Fig. 1A, displaying approximately 10 major spots and 20 minor spots) (similar increased protein aggregation was observed in the dnaK-deficient strain without any plasmid [reference 15 and data not shown]). In contrast, the dnaK-deficient strain transformed with the proB74-producing plasmid was much less prone to protein aggregation (Fig. 1C, showing approximately 10 major spots and 40 minor spots) than the dnaK deficient strain transformed with the control proB+ plasmid. The anti-aggregative effect of proline overproduction did not seem to be related to the size or pI of the proteins. The aggregation of most aggregated proteins was dramatically reduced by high proline pools, in accordance with a general effect of chemical chaperones on protein aggregation resulting from a modification of the water-protein interactions (1). However, the pattern of aggregated proteins in the dnaK-deficient strain overproducing proline was more similar to the pattern of the dnaK-deficient strain than to that of the wild-type strain, suggesting that the DnaK deficiency is still apparent but attenuated. The results described in this section suggest that high proline pools can significantly reduce the protein aggregation defect at 42°C resulting from dnaK deficiency.

Proline protects citrate synthase from denaturation and irreversible aggregation during thermal stress. Citrate synthase loses its native conformation and undergoes aggregation during incubation at 43°C (3, 18). Molecular chaperones (DnaK, GroEL, and small heat shock proteins) and chemical chaperones (glycine betaine and choline) protect citrate syn-
We investigated whether proline also protects citrate synthase (from porcine heart \( \Sigma \)) against thermal aggregation in vitro. The native enzyme was incubated at 43°C in the absence or in the presence of proline or DnaK. Citrate synthase aggregation was monitored by measuring the absorbance at 650 nm as described previously (18). Proline concentrations of between 150 and 500 mM efficiently reduced citrate synthase aggregation (Fig. 2A). Under similar conditions, 5 \( \mu \text{M} \) DnaK completely suppressed citrate synthase aggregation (Fig. 2A) (18). Thus, like a classical chaperone, although at a much higher concentration (a characteristic of other chemical chaperones), proline efficiently protects citrate synthase against thermal aggregation (4).

Proline also stabilizes the enzymatic activity of citrate synthase during thermal stress. Citrate synthase inactivation at 45°C followed first-order kinetics, with a half-life ranging from 10 min in the absence of proline to 35 min in the presence of 500 mM proline (Fig. 2B). Thus, like glycine betaine and choline (4), proline stabilizes the activity of citrate synthase during thermal stress.

Proline increases the amount of correctly folded citrate synthase. Chaperones not only protect proteins against thermal stress but also stimulate protein renaturation. We investigated whether proline can stimulate the folding of citrate synthase, a protein whose refolding is facilitated by several chaperones, such as GroEL, DnaK, Hsp90, and small heat shock proteins (3, 18). Citrate synthase was unfolded in the presence of urea and allowed to refold upon dilution of the denaturant in the absence or in the presence of proline or of the DnaK/DnaJ/GrpE/ATP chaperone machine. Under our experimental conditions, the refolding yield of 0.1 \( \mu \text{M} \) citrate synthase was increased from 7% in the absence of proline to 12% in the presence of 200 mM proline and to 17% in the presence of 500 mM proline (Fig. 3). Under similar conditions, the maximal renaturation of citrate synthase in the presence of DnaK/DnaJ/GrpE/ATP was 33%, which was obtained in the presence of 1 \( \mu \text{M} \) DnaK, 0.3 \( \mu \text{M} \) DnaJ, and 0.3 \( \mu \text{M} \) GrpE (Fig. 3). Thus, proline, like classical chaperones, stimulates the renaturation of an unfolded protein, although at much higher concentrations and with a lower efficiency.
Proline as an antiaggregation agent. Our results suggest that proline, in addition to being an osmoprotectant, is involved in thermoprotection both in vitro and in vivo. In vitro, proline protects citrate synthase against thermodenaturation and stimulates its renaturation, like protein chaperones, although at much higher concentrations. High concentrations of chemical chaperones (100 mM to 1 M), which are easily attained under several stress conditions, are usually required for their effect during osmotic or thermal stress (4, 5, 8, 9, 19).

Proline restores the viability of the dnaK deletion mutant at 42°C and decreases its protein aggregation defect. This supports the hypothesis that the temperature sensitivity of dnaK-deficient mutants is due to a protein aggregation defect (15). It has been recently reported that aggregation of a single enzyme (homoserine transsuccinylase, the first enzyme of the methionine biosynthesis pathway) limits growth of *E. coli* at elevated temperatures (11). Such protection is interesting, since protein chaperones, including GroEL/GroES, ClpB, HtpG, and IbpAB, do not significantly prevent the protein aggregation defect of the dnaK-deficient strain (15).

The present study shows that proline, one of the so-called chemical chaperones (19), can partially assume the function of a bona fide chaperone, the dnaK gene product, and emphasizes the role played by small molecules such as polyols (13, 21), trimethylamines (19, 21), and amino acids (21) in the protection of cells against stress. Finally, this study suggests that thermoprotection both in vitro and in cells under combined salt and heat stresses. J. Bacteriol. 170:2374–2378.


