Thermoprotection by glycine betaine and choline

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Glycine betaine is mostly known as an osmoprotectant. It is involved in the osmotic adaptation of eukaryotic and bacterial cells, and accumulates up to 1 M inside cells subjected to an osmotic upshock. Since, like other osmolytes, it can act as a protein stabilizer, its thermoprotectant properties were investigated. In vitro, like protein chaperones such as DnaK, glycine betaine and choline protect citrate synthase against thermodenaturation, and stimulate its renaturation after urea denaturation. In vivo, the internal concentration of glycine betaine is neither increased nor decreased after heat shock (this contrasts with a massive increase after osmotic upshock). However, even in exponential-phase bacteria grown in usual minimal salts media, the internal glycine betaine concentration attains levels (around 50 mM) which can protect proteins against thermodenaturation in vitro. Furthermore, glycine betaine and choline restore the viability of a dnaK deletion mutant at 42 °C, suggesting that glycine betaine not only acts as a thermoprotectant in vitro, but also acts as a thermoprotectant for Escherichia coli cells in vivo.

Keywords: glycine betaine, choline, osmoprotectant, thermoprotectant, heat-shock protein DnaK

INTRODUCTION

The osmoprotectant glycine betaine is accumulated in Escherichia coli cells to molar concentrations at high osmolarity, usually by uptake from the extracellular environment, although certain strains can also synthesize betaine from choline (Conska & Epstein, 1996). The addition of low concentrations of glycine betaine to the growth medium (e.g. 1 mM) raises the upper limit of external osmolarity at which growth occurs (Chambers & Cunin, 1985), and increases the growth rate of osmotically stressed E. coli and Salmonella typhimurium (Perroud & Le Rudelier, 1985). Two osmotically regulated permeases, ProP (Milner et al., 1988) and ProU (Stirling et al., 1989), mediate uptake of glycine betaine in E. coli (Gowrishankar, 1986; Stirling et al., 1989). E. coli can also synthesize glycine betaine from exogenous choline (Strom et al., 1986), which is accumulated by the BetT and ProU systems, and is converted to betaine dehydrogenase and betainealdehyde dehydrogenase, the betA and betB gene products, respectively. The protective effects of glycine betaine against salt stress can also be demonstrated in vitro: betaine can restore the activity of malate dehydrogenase inhibited by 0-3 M NaCl (Pollard & Wyn Jones, 1979). Furthermore, betaine and other trimethylamines display more general stabilizing effects on macromolecules, increasing their melting temperature, and counteracting the perturbation of enzyme structure and function by urea (Hand & Somero, 1982; Yancey & Somero, 1979; Yancey et al., 1982). During heat shock, cellular proteins are prone to aggregation. Cells produce specific proteins such as the major chaperones GroEL/GroES, DnaK/DnaJ/GroP and the proteases Lon and Clp, which help to cope with the accumulation of heat-denatured proteins (Georgopoulos et al., 1994; Hendrick & Hartl, 1993). Chaperones interact with heat-denatured proteins, prevent their aggregation, and catalyse their renaturation (Georgopoulos et al., 1994; Hendrick & Hartl, 1993). In the present study, we show that glycine betaine and choline, at relatively low concentrations, protect citrate synthase and β-galactosidase against thermodenaturation in vitro, and stimulate citrate synthase renaturation after urea denaturation. Glycine betaine and its precursor choline also restore the viability of a
DNAK deletion mutant at 42 °C. These results suggest that glycine betaine, in addition to its role as an osmoprotectant, is involved in the thermoprotection of bacteria.

METHODS

Bacterial strains. E. coli K-12 strains C600 (leuB6 thyB-1 thi-1 supE44) and 3080 (tsx-23 relA1 spoT1 thi-1) were used for glycine betaine and choline transport, respectively. The DNAK deletion mutant GW4813 (ΔdnaK52::Cm') and its parental strain AB1157 (F thr-1 ara-14 leuB6 Δ(gpt-proA)62 lacY1 tsx-33 qsr supE44 galK21 rac hisG4 rpsL31 kdgK51 xyl-5 mtl-1 argE3 thi-1) were both tested for their colony-forming ability at 30 and 42 °C.

Thermal aggregation of citrate synthase. The native enzyme (80 µM) was diluted 100-fold in 40 mM HEPES, 50 mM KCl, 10 mM (NH₄)₂SO₄, 2 mM potassium acetate, pH 8.0 at 43 °C in the absence or in the presence of glycine betaine or choline. Citrate synthase aggregation was monitored by measuring the A₄₅₀ as described by Richarme & Caldas (1997).

Thermal inactivation of citrate synthase and β-galactosidase. Citrate synthase was diluted to a final concentration of 80 nM at 49 °C in the absence or presence of glycine betaine or choline. Citrate synthase activity was determined by measuring the β-galactosidase activity of the reaction mixture.

Refolding of citrate synthase. Denaturation and renaturation reactions were carried out at 20 °C. Renaturation was initiated by pouring the renaturation solvent onto the unfolded protein, under vortex agitation, in Eppendorf polyethylene tubes. Citrate synthase was denatured at a concentration of 10 µM in 8 M urea, 50 mM Tris/HCl, 2 mM EDTTA, 20 mM dithiothreitol, pH 8.0 for 50 min. Renaturation was initiated by a 100-fold dilution in 40 mM HEPES, 50 mM KCl, 10 mM (NH₄)₂SO₄, 2 mM potassium acetate, pH 8.0. The enzymic activity of citrate synthase was measured as described by Richarme & Caldas (1997). DNAK was prepared as described by Richarme & Caldas (1997).

Transport measurements. For glycine betaine (May et al., 1986) and choline uptake (Lanfald & Strom, 1986), cells were grown in minimal medium M63 (Miller, 1972), supplemented with 0.4% glycerol or 0.4% glucose, respectively, as carbon source, and with the required amino acids added at 50 µg ml⁻¹. Bacteria were harvested in the exponential phase of growth, washed once with the culture medium and diluted in the same medium supplemented with 300 mM NaCl to an OD₆₀₀ of 2. The cell suspension was equilibrated at 22 °C for 10 min, unless otherwise indicated. Transport was performed aerobiically (200 r.p.m.), and was initiated by mixing 2 ml cells with [³¹C]glycine betaine [synthesized as described by Ikuta et al. (1977), and used at 10 mCi mmol⁻¹ (370 MBq mmol⁻¹) at the concentrations indicated in the text] or [³¹H]choline (obtained from Amersham, and used at 50 mCi mmol⁻¹ (1850 MBq mmol⁻¹) at the concentration indicated in the text). Samples (200 µl) were removed, filtered through Millipore filters, washed with 3 x 1 ml transport medium and their radioactivity measured. To measure the effect of temperature shift-up on the rate of glycine betaine or choline uptake, exponential-phase cultures of E. coli were transferred from 30 to 42 °C, and transport activities were measured at 22 °C at several times before and after the temperature shift-up. Glycine betaine uptake was measured at 2 µM glycine betaine, at which there is a major contribution of ProU, and at 200 µM glycine betaine, at which both ProU and ProP are effective. Choline uptake was measured at 10 µM and 200 µM choline.

Colonization ability of the dnaK mutant. The colonization ability of the DNAK deletion strain GW4813 was studied by plating 100 µl of a dilution of exponentially growing cells in M63 medium supplemented with 0.4% glucose, the required amino acids at 50 µg ml⁻¹ and 1 mM glycine betaine or choline, as indicated, containing 200 bacteria (assuming 6 x 10⁸ bacteria ml⁻¹ at OD₆₀₀ = 1; Stock et al., 1977), onto minimal medium agar plates (M63 medium supplemented with 0.4% glucose, the required amino acids at 50 µg ml⁻¹, and 1 mM glycine betaine or choline, as indicated).

Materials. Citrate synthase (from porcine heart) was obtained from Sigma. All other chemicals were from Sigma and were reagent grade.

RESULTS AND DISCUSSION

Glycine betaine and choline protect citrate synthase from irreversible aggregation during thermal stress

We investigated the function of glycine betaine and choline under heat-shock conditions in vitro. Citrate synthase loses its native conformation and undergoes aggregation during incubation at 43 °C (Buchner et al., 1991; Richarme & Caldas, 1997). Molecular chaperones (DNAK, GroEL and small heat-shock proteins) reduce or suppress citrate synthase aggregation (Buchner et al., 1991; Richarme & Caldas, 1997). Low glycine betaine concentrations, around 50 mM (as compared with the molar concentration attained after an osmotic upshock) efficiently reduced citrate synthase aggregation (Fig. 1). Higher glycine betaine concentrations completely suppressed citrate synthase aggregation (not shown, and Richarme & Caldas, 1997). In similar conditions, choline, at concentrations between 50 and 200 µM, efficiently protected citrate synthase against thermal aggregation (Fig. 1b). Trehalose was much less efficient than glycine betaine and choline for the thermoprotection of citrate synthase, since concentrations lower than 300 mM did not significantly affect aggregation (not shown).

Glycine betaine and choline stabilize the enzymic activities of citrate synthase and β-galactosidase during thermal stress

The velocity of citrate synthase inactivation at 49 °C followed first-order kinetics, with a halftime ranging from 6 min in the absence of thermoprotectant, to 11 min in the presence of 300 mM glycine betaine and 21 min in the presence of 300 mM choline (Fig. 2a). We also tested the ability of glycine betaine to protect the activity of the E. coli β-galactosidase against thermodenaturation. As observed by Moses & Sharp (1970), the thermal inactivation of β-galactosidase at 56 °C followed
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Fig. 1. Thermal aggregation of citrate synthase in the presence of glycine betaine or choline. The kinetics of citrate synthase aggregation was determined by light scattering at 650 nm. Native citrate synthase was diluted to a final concentration of 0.8 µM at 42 °C, as described in Methods: (a) in the absence (●), or in the presence of 15 mM (○), 50 mM (□), 80 mM (△), 200 mM (×) or 500 mM (+) glycine betaine; (b) in the absence (●) or in the presence of 50 mM (□), 200 mM (×) or 500 mM (+) choline.

biphasic kinetics. Both phases of thermodenaturation were protected by 300 mM glycine betaine. The $t_{1/2}$ of the second phase rose from 3 min in the absence of glycine betaine to 7 min in its presence (Fig. 2b).

Glycine betaine and choline increase the amount of correctly folded citrate synthase

Since chaperones not only protect proteins against thermal stress, but also stimulate protein renaturation, we investigated whether glycine betaine and choline can stimulate the folding of proteins. Citrate synthase, whose refolding is facilitated by several chaperones such as GroEL, DnaK, Hsp90 and small heat-shock proteins (Buchner et al., 1991; Richarme & Caldas, 1997), was chosen as substrate for this reaction. It was unfolded in the presence of 8 M urea, and allowed to refold upon dilution of the denaturant, in the absence or presence of several glycine betaine or choline concentrations (citrate synthase refolding in the presence of DnaK was studied in parallel). Under our experimental conditions, the refolding yield of 0.1 µM citrate synthase was increased from 9% in the absence of thermoprotectant, to 17% in the presence of 100 mM glycine betaine and 25% in the presence of 100 µM choline (Fig. 3). In the presence of 300 mM glycine betaine or choline, citrate synthase renaturation rose to 29% and 35%, respectively. Thus, both glycine betaine and choline stimulate the renaturation of an unfolded protein. In similar conditions, the maximal renaturation of citrate synthase in the presence of DnaK was 33%, and it was obtained in the presence of 3 µM DnaK (not shown). The glycine betaine concentration which stimulates the reactivation of an unfolded protein can be easily attained in the bacterial cytoplasm in many physiological conditions, especially during stationary phase or osmotic stress, by accumulation of either glycine betaine or choline (Conska & Epstein, 1996; Hengge-Aronis, 1996; Perroud & Le Rudelier, 1985, Lanfald & Strom, 1986).
Glycine betaine and choline pools before and after heat shock

A temperature shift-up did not significantly affect the rate of glycine betaine and choline uptake (transport activities were measured at 2 µM or 200 µM glycine betaine and 10 or 200 µM choline, as described in Methods; not shown). This contrasts with the stimulation of glycine betaine uptake after a hyperosmotic upshock (Perroud & Le Rudelier, 1985). In our experiments, the plateau of glycine betaine concentration was obtained after 2-5 h (not shown), and corresponded to an internal glycine betaine concentration of 42 mM, assuming an internal volume of 3-5 µl per mg cell protein (Perroud & Le Rudelier, 1985; Stock et al., 1977). Similar glycine betaine pools were found when accumulation occurred at 30 °C or 42 °C (not shown). Similar glycine betaine pools (around 50 mM) were also observed at 30 °C and 42 °C, after the accumulation of 200 µM choline (not shown) (it has been previously shown that choline is transported and quantitatively oxidized into glycine betaine by the BetT, BetA, BetB proteins: Lanfald & Strom, 1986). Thus, although heat shock does not stimulate glycine betaine or choline accumulation, it does not reduce them either, and the internal glycine betaine concentration, independently of the heat shock procedure, attains 50 mM in bacteria grown in usual minimal salts media in the presence of 200 µM glycine betaine or choline. This concentration is in the same range as those which inhibit citrate synthase aggregation \textit{in vitro} (see above). We also measured the glycine betaine pools of the \textit{dnaK} deletion mutant described below, in the presence of 200 µM glycine betaine or 200 µM choline. We found intracellular glycine betaine pools of 35 µM and 58 µM, respectively, suggesting that this strain is not impaired in choline transport and metabolism. The higher glycine betaine pool obtained in the presence of external choline might explain the higher survival of the \textit{dnaK} strain at 42 °C in the presence of choline, as compared to its survival in the presence of glycine betaine (see below). However, the physiological state of bacteria in liquid medium is different from that of bacterial colonies on agar plates.

Suppression of the thermosensitive phenotype of a \textit{dnaK} deletion mutant by glycine betaine and choline

The \textit{dnaK} deletion mutant GW4813 is deficient for growth and viability at 42 °C (Paek & Walker, 1987), probably as a consequence of protein aggregation. Samples (100 µl) of a dilution of the \textit{dnaK} mutant were plated on M63-glucose agar plates, in the absence or presence of 1 mM glycine betaine or choline, at 30 and 42 °C. The colony-forming abilities of the \textit{dnaK} mutant at 30 and 42 °C were compared. The colony-forming ability of the mutant at 42 °C rose from 0% (of that at 30 °C) in the absence of additives, to 11% and 29% (mean value of three experiments) in the presence of 1 mM glycine betaine and choline respectively (Fig. 4) (glycine betaine and choline had no effect on the colony-forming ability of the parental strain; not shown). Since choline is quantitatively metabolized into glycine
thermotolerance. A possible explanation is that glycine betaine partially restores the viability and growth deficiency of dnaK deletion mutants at 42 °C, suppressing the known protein aggregation defect at elevated temperatures. When bacteria were grown at 30 °C in the presence of 1 mM glycine betaine or choline, before plating at 42 °C (in the presence of 1 mM glycine betaine or choline), the colony-forming ability of the dnaK deletion mutant rose to 29% and 42%, respectively (data not shown).

We measured the dependence of the colony-forming ability of the dnaK deletion mutant on the external concentrations of glycine betaine and choline (in the experimental conditions of Fig. 4). Half-maximal stimulation occurred at 2 μM glycine betaine (not shown) and 6 μM choline (Fig. 5). These concentrations are similar to the $K_m$ of the ProU and BetT transport systems for glycine betaine and choline, respectively (May et al., 1986; Lamark et al., 1991; Boch et al., 1994).

We also checked whether incubation of the dnaK strain at 42 °C in liquid medium containing glycine betaine improves its survival at 30 °C on agar plates without glycine betaine. The dnaK mutant was incubated for 90 min at 42 °C, in minimal medium, in the absence or presence of 1 mM glycine betaine. Bacteria were then washed with culture medium without glycine betaine, and subsequently plated on agar plates without glycine betaine at 30 °C. The survival rate of the dnaK strain rose from 15% in the absence of glycine betaine to 55% in its presence, and the colonies were larger in the presence of glycine betaine (data not shown).

FIG. 5. Dependence on choline concentration of the colony-forming ability of the dnaK mutant. This experiment was done as described in the legend to Fig. 4, at different choline concentrations.

**Implications**

Our results suggest that glycine betaine and its precursor choline are involved in bacterial thermoprotection. *In vitro*, glycine betaine and choline protect citrate synthase against thermodenaturation and stimulate its renaturation, with an efficiency similar to that of protein chaperones, although at much higher concentrations. However, the glycine betaine concentrations which protect citrate synthase against thermodenaturation *in vitro* (around 50 mM) are similar to those found in bacteria grown in usual minimal media (this study). Although the protection of proteins by osmolytes *in vitro* often requires high concentrations of the latter (Yancey et al., 1982; Arakawa & Timasheff, 1985), the protective effect of trimethylamine against the cold lability of phosphofructokinase shows a concentration threshold of around 50 mM (Hand & Somero, 1982), similar to that observed in our work. The amount of glycine betaine accumulated in our study (48 nmol per mg cell protein after 30 min) is not significantly different from that obtained by others in similar conditions (35 nmol per mg cell protein after 30 min: Perroud & Le Rudelier, 1985). The amounts of glycine betaine accumulated after an osmotic upshock and during the stationary phase are severalfold higher (Conska & Epstein, 1996; Hengge-Aronis, 1996; Perroud & Le Rudelier, 1985), and the thermoprotectant effect of glycine betaine should thus be even more effective in these physiological conditions. Although glycine betaine and choline are not synthesized endogenously, and have to be taken up from the external medium, they are often present in the natural environment of enterobacteria (Conska & Epstein, 1996). Our results show that glycine betaine and choline partially restore the viability and growth deficiency of a dnaK deletion mutant at 42 °C. Suppressors of dnaK null mutants that grow at 42 °C have been isolated (Paek & Walker, 1987), suggesting that cells do not have an absolute requirement for the DnaK function for growth at 42 °C. The present study shows that one of the so-called chemical chaperones (Talzelt et al., 1996), glycine betaine, can partially assume the function of the dnaK gene product, and emphasizes the role played by small molecules such as polyols (Hengge-Aronis et al., 1991; Singer & Lindquist, 1998; Yancey et al., 1982), trimethylamines (Talzelt et al., 1996; Yancey et al., 1982) and amino acids (Yancey et al., 1982) in the protection of cells against stress.

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