Chemical Chaperones Regulate Molecular Chaperones *in Vitro* and in Cells under Combined Salt and Heat Stresses*

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Salt and heat stresses, which are often combined in nature, induce complementing defense mechanisms. Organisms adapt to high external salinity by accumulating small organic compounds known as osmolytes, which equilibrate cellular osmotic pressure. Osmolytes can also act as "chemical chaperones" by increasing the stability of native proteins and assisting refolding of unfolded polypeptides. Adaptation to heat stress depends on the expression of heat-shock proteins, many of which are molecular chaperones, that prevent protein aggregation, disassemble protein aggregates, and assist protein refolding. We show here that *Escherichia coli* cells preadapted to high salinity contain increased levels of glycine betaine that prevent protein aggregation under thermal stress. After heat shock, the aggregated proteins, which escaped protection, were disaggregated in salt-adapted cells as efficiently as in low salt. Here we address the effects of four common osmolytes on chaperone activity *in vitro*. Systematic dose responses of glycine betaine, glycerol, proline, and trehalose revealed a regulatory effect on the folding activities of individual and combinations of chaperones GroEL, DnaK, and ClpB. With the exception of trehalose, low physiological concentrations of proline, glycerol, and especially glycine betaine activated the molecular chaperones, likely by assisting local folding in chaperone-bound polypeptides and stabilizing the native end product of the reaction. High osmolyte concentrations, especially trehalose, strongly inhibited DnaK-dependent chaperone networks, such as DnaK+GroEL and DnaK+ClpB, likely because high viscosity affects dynamic interactions between chaperones and folding substrates and stabilizes protein aggregates. Thus, during combined salt and heat stresses, cells can specifically control protein stability and chaperone-mediated disaggregation and refolding by modulating the intracellular levels of different osmolytes.

Salt Stress—Mechanisms of acclimation to osmotic stress involve intracellular accumulation, by synthesis or uptake, of small organic solutes known as osmolytes, which equilibrate cellular osmotic pressure (1). *In vitro*, osmolytes such as glycine betaine (subsequently referred to as betaine), proline, trehalose, and glycerol may also protect native proteins from heat

denaturation and favor the formation of native protein oligomers (2–8). Some osmolytes behave as "chemical chaperones" by promoting the correct refolding of unfolded proteins *in vitro* and in the cell (5, 6, 9–11). The nature and cellular concentrations of osmolytes depend on the organism, osmolyte availability in the medium, and the type, severity, and duration of the osmotic stress. Hence, betaine is a universal compatible solute in various prokaryotes including *Escherichia coli*, animals, algae, and salt-tolerant plants (2, 12). It is a most effective osmoprotectant in many bacteria (13), which allows growth in hyperosmotic minimal medium (4, 14). When free betaine or proline is available, each may accumulate in the cell up to 0.7 and 0.4 M, respectively (15–18). In the absence of other osmolytes, salt-stressed *E. coli* cells may also accumulate molar concentrations of glycerol and up to 0.4 M trehalose (19–22).

Heat Stress—The massive production of heat-shock proteins (HSPs)[1] during various types of stresses, especially heat shock, provides various mechanisms that protect and recover stress-damaged proteins in the cell (23). In *E. coli*, proteins IbpA/B (small HSPs), GroEL (HSP60), DnaK (HSP70), and ClpB (HSP100) (eukaryotic chaperones in parentheses) are major components of a sophisticated network of "holding" and "folding" chaperones that cooperate in preventing protein aggregation during heat stress and promoting protein disaggregation and refolding after the stress (24, 25). Hence, small HSP-bound denatured polypeptides can be sequentially refolded by the DnaK and then by the GroEL chaperones (26). Similarly, stable protein aggregates can be efficiently resolubilized and refolded by the sequential action of ClpB and DnaK (27–30).

Although combined heat and salt stresses are frequent in nature, little is known about how osmolytes control protein stability and chaperone-mediated protein refolding in the cell. Trehalose accumulation in yeast suppresses protein aggregation during heat shock but also interferes with chaperone-assisted protein refolding *in vivo* and *in vitro* (31). Osmolytes promote the *in vitro* refolding by GroEL of a mutant enzyme, which cannot be refolded either with the chaperone or osmolytes alone (11). This indicates that in addition to their activity as chemical chaperones that directly controls protein stability, osmolytes may also indirectly regulate protein homeostasis in the cell by controlling the activity of molecular chaperones.

Here we show that adaptation of *E. coli* cells to a mild salt stress inhibits protein aggregation during heat stress but does not interfere with protein disaggregation after heat stress. *In vitro*, dose-response analysis shows qualitative and quantitative differences between osmolytes regulating protein folding by specific activation or inhibition of complex chaperone networks.

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[1] The abbreviations used are: HSP, heat-shock protein; MDH, malate dehydrogenase; LS, GroEL+GroES; KJE, DnaK+DnaJ+GrpE.

MATERIALS AND METHODS

Proteins—Chaperones were purified according to previously published procedures: DnaK, DnaJ, and GrpE were purified as described in Ref. 32, ClpB was purified as described in Ref. 33, and GroEL and GroES were purified as described in Ref. 34. Rabbit muscle pyruvate kinase, L-proline, D(+)trehalose, and betaine were obtained from Sigma, mitochondrial malate dehydrogenase (MDH) from pig heart was obtained from Roche Diagnostics. Chaperone concentrations were determined using the Bio-Rad Bradford assay, with bovine serum albumin as standard. Protein concentrations were expressed in protomers.

Salt Stress and Heat Shock of E. coli Cells—E. coli cells (DH5α) were grown in LB medium (1% Bacto-tryptone, 0.5% Bacto-yeast extract, and 0.17 M NaCl) supplemented with additional NaCl to the indicated concentrations. At A_{600} = 1.2, cells were heat-shocked at 47 °C for up to 15 min in the presence of 50 μg/ml streptomycin to inhibit growth and protein synthesis. Recovery was then carried out at 30 °C for 10 min. At the indicated times, aliquots were removed and frozen in liquid N$_2$.

Quantitative Evaluation of Protein Aggregates in E. coli Cells—The method for the isolation of a cellular fraction enriched with *de novo* heat-aggregated proteins was based on a modified protocol of Tomoyasu *et al.* (35). Frozen bacterial pellets from 1.5-ml aliquots were lysed in 1 mg/ml lysozyme on ice for 3 min and then sonicated (Heat Systems-Ultrasonics disrupter W-385). Aggregated proteins were separated from the insoluble cell membranes and debris by two washes with 200 μl of 2% (v/v) Nonidet P-40. Nonidet P-40-insoluble pellets were resuspended in 50 μl of guanidine-HCl (6 M). Protein content was measured by the Bio-Rad Bradford reagent. Results in Fig. 1 are the average of four experiments. Aggregated proteins were expressed as a fraction (percentage) of total proteins in the cell.

NMR Spectroscopy—^{1}H NMR spectra were acquired at the ^{1}H proton frequency of 600.13 MHz on a 600 MHz Bruker Avance DMX spectrometer (The Wolfson Center for Applied Structural Biology, The Hebrew University of Jerusalem, Jerusalem, Israel). The carrier frequency was set to a signal-free area of the spectrum, and the spectrum was referenced to the residual water signal at 4.75 ppm at 300 K. The relative concentrations of betaine and trehalose in the samples were estimated using reference samples of pure compound under identical acquisition conditions. The normalized relative peak intensity of the ammonium methyl peaks of betaine and the methine protons at position 4 on the glucopyranosyl and glucopyranoside moieties of trehalose was compared. The comparison between the samples was done in the same manner. Integrating and normalizing the aforementioned peaks determined the relative amounts of betaine and trehalose.

Protection of Native MDH—0.5 μM MDH was denatured at 44 °C in folding buffer containing increasing concentrations of osmolytes. The extent of protection (*P*) was expressed as a percentage using the following formula: $P = 100 - ((V_a/V_o) \times 100)$, where V_o and V_a are the inactivation rates without and with osmolytes, respectively.

MDH Denaturation and Refolding—Several forms of denatured MDH were used. 1) MDH unfolded by urea for osmolyte-mediated refolding was used: 50 μM MDH was chemically denatured in 5 M urea and 10 mM dithiothreitol for 35 min at 37 °C. Refolding without chaperones was initiated at 37 °C on a 100-fold dilution into the refolding buffer containing 100 mM Tris-HCl, pH 7.5, 20 mM MgAc$_2$, 150 mM KCl, 10 mM dithiothreitol, and increasing concentrations of osmolytes. 2) Heat-denatured MDH prebound to chaperones was used: 0.5 μM MDH was denatured at 47 °C for 30 min in folding buffer containing 3 mM phosphoenol pyruvate and chaperones (3.5 μM GroEL, 6 μM GroES, or 3.5 μM DnaK, 0.7 μM DnaJ, and 0.35 μM GrpE). Osmolytes and 20 μg/ml pyruvate kinase were added at 25 °C, and refolding was initiated with 3 mM ATP. 3) Aggregated MDH was used: 0.7 μM MDH was heat-inactivated and aggregated at 47 °C in the absence of chaperone as described in Ref. 27. Disaggregation and refolding were initiated at 25 °C in the presence of added chaperones (1 μM DnaK, 0.2 μM DnaJ, 0.1 μM GrpE, and 0.5 μM ClpB), 20 μg/ml pyruvate kinase and osmolytes, and 3 mM ATP. The MDH activity was measured as described in Ref. 36.

Viscosity—The viscosity of solutions containing different concentrations of osmolytes was determined at 25 °C, 30 °C, 37 °C, and 44 °C using a capillary viscometer (Cannon-Manning semi-micro viscometer). Results were expressed in time units (minutes) needed for 1 ml of solution to flow-through the capillary (AU). Time units are proportional to viscosity units.

RESULTS

Salt Stress Inhibits Heat-induced Protein Aggregation in Vivo—We examined the effect of increasing concentrations of
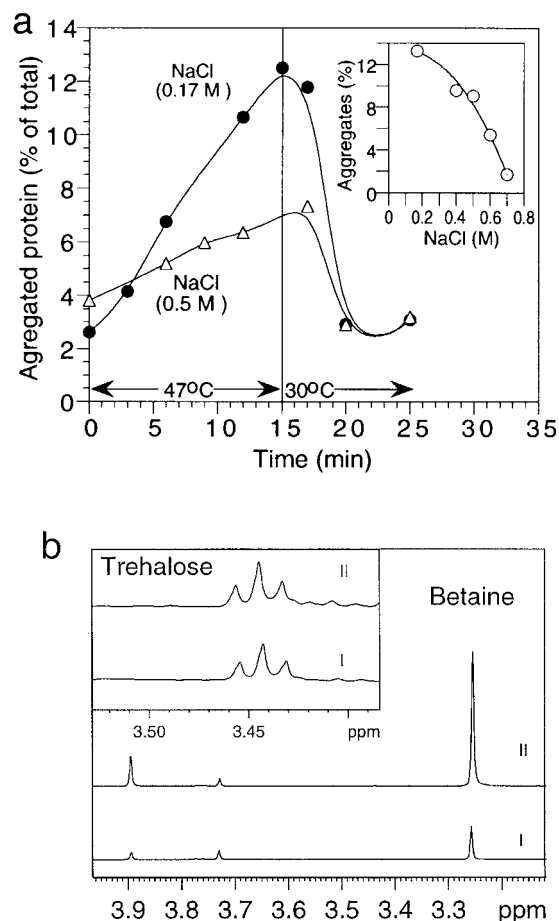

FIG. 1. **Salt adaptation affects heat-induced protein aggregation in the cell.** *a,* time-dependent protein aggregation at 47 °C and disaggregation at 30 °C in *E. coli* cells preadapted to 0.17 or 0.5 M NaCl in LB medium. *Inset,* aggregated proteins formed after a 15-min incubation at 47 °C in *E. coli* cells preadapted to increasing NaCl concentrations in the medium. *b,* ^{1}H NMR spectra of extracts from *E. coli* cells grown in LB medium with 0.17 (I) and 0.5 M (II) NaCl. Specific peaks for betaine and trehalose are indicated.

salt in rich growth medium (LB medium) on protein aggregation and disaggregation in *E. coli* cells during and after heat stress. Protein aggregates were measured using a modified protocol by Tomoyasu *et al.* (35). Less than 3% of the total proteins were found to be insoluble aggregates in cells grown at 30 °C in standard LB medium containing 0.17 M NaCl. This aggregated fraction was dramatically increased to 13% during a 15-min treatment at 47 °C (Fig. 1*a*). Noticeably, as the NaCl concentration increased, the heat-induced protein aggregation in salt-adapted cells decreased (Fig. 1*a, inset*). Hence, osmolyte accumulation in the salt-adapted cells correlates with increasingly effective protection of labile proteins against thermal denaturation and aggregation.

Betaine Accumulates in the Salt-adapted E. coli Cells—Understanding the nature and composition of the osmolytes that accumulate in salt-adapted *E. coli* cells is essential to understanding the mechanism by which osmotic stress controls protein stability and refolding during heat shock. NMR spectroscopy revealed that betaine is the main osmolyte present in *E. coli* cells grown in standard LB medium containing 0.17 M NaCl. Trehalose was also present, albeit at concentrations 4–5 times lower than that of betaine (Fig. 1*b*). We found that in cells grown in LB medium with 0.5 M NaCl, the betaine concentration was 4 times higher, whereas the trehalose concentration was only 1.2 times higher than that in cells grown in 0.17 M NaCl (Fig. 1*b*).
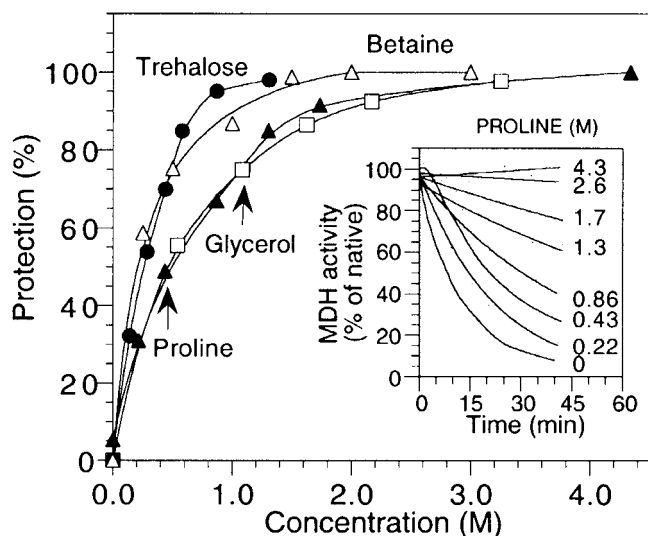
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FIG. 2. **Osmolytes protect native MDH from thermal inactivation.** MDH (0.5 μM) was incubated at 44 °C in the presence of increasing concentrations of betaine, trehalose, proline, or glycerol. Inactivation rates were derived from exponential curves at each osmolyte concentration. Protection was calculated from the inactivation rates as described in "Materials and Methods." *Inset,* representative MDH inactivation curves in the presence of increasing concentrations of proline.

FIG. 3. **Osmolytes affect GroEL-mediated refolding.** MDH (0.5 μM) was heat-denatured at 47 °C in the presence of GroEL (3.5 μM) as described in Ref. 42. In the presence of supplemented betaine, trehalose, proline, or glycerol, refolding was initiated at 25 °C by the addition of ATP (3 mM), with (*top part*) or without (*bottom part*) added GroES (6.0 μM).

Osmolytes Protect Native Proteins in Vitro—To address the thermoprotective nature of various osmolytes, the activity of a model protein, mitochondrial MDH, was examined *in vitro* under mild heat-denaturing conditions in the presence of betaine and other representative categories of osmolytes: proline (amino acids), trehalose (sugars), and glycerol (polyols). Physiological concentrations of all osmolytes examined significantly stabilized the native MDH at 44 °C: Without osmolytes, native MDH was irreversibly inactivated at a rate of 6.3%/min. This rate of thermal inactivation was halved in the presence of 0.2 M betaine, 0.26 M trehalose, 0.44 M proline, and 0.48 M glycerol (Fig. 2). Noticeably, 1 M trehalose, 1.5 M betaine, 2 M proline, or 2 M glycerol fully protected native MDH over a 40-min period at 44 °C (Fig. 2).

Osmolytes Regulate Chaperone Activity—The insoluble protein aggregates that formed in the cell during heat shock became rapidly resolubilized within 5 min of recovery at 30 °C, regardless of the salt concentration in the growth medium (Fig. 1*a*). This suggests that, unlike the case of trehalose-enriched trehalase mutants from yeast (31), the thermoprotective betaine that accumulates in *E. coli* (Fig. 1*b*) does not interfere with protein disaggregation after stress. We therefore addressed the specific effects of various osmolytes on the activity of simple and complex chaperone networks, using *in vitro* refolding assays.

When heat-denatured MDH was prebound to GroEL and then supplemented after the stress with ATP, LS, and increasing concentrations of osmolytes, specific effects were observed. Physiological concentrations of up to 1 M proline or trehalose did not affect chaperone-mediated refolding of MDH. Betaine and glycerol activated LS chaperone by 30–40% (Fig. 3), but high, nonphysiological concentrations of betaine or proline inhibited the reaction. Interestingly, a significant refolding was also observed without GroES in the presence of specific osmolytes. Whereas betaine did not affect this ATP- and GroEL-dependent but GroES-independent activity, refolding rates with 0.56 M trehalose, 1.2 M proline, or 3.8 M glycerol were 8, 6, and 12 times faster, respectively, than the refolding rates without osmolytes (Fig. 3). Whereas the mechanism by which osmolytes can functionally substitute for GroES remains to be
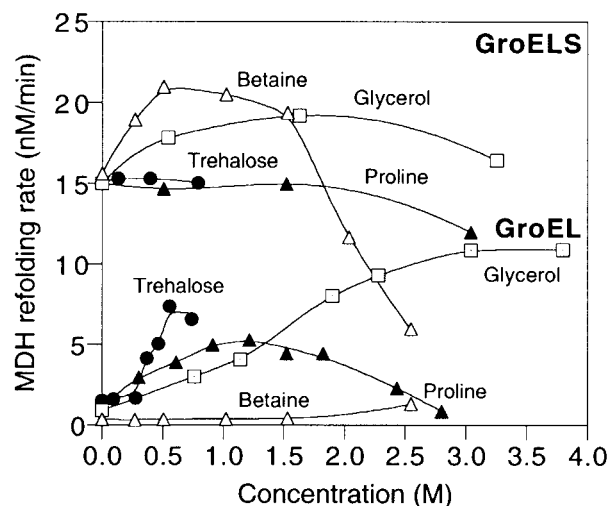
elucidated, this clearly demonstrates that different osmolytes have different effects on chaperonin activity. This is in contrast with the protection mechanism, which appears similar for all the osmolytes tested.

Similar specific activatory and inhibitory effects by the different osmolytes were also observed in the case of the DnaK+DnaJ+GrpE (KJE) chaperone system. Low concentrations of trehalose readily inhibited the refolding rates of KJE-bound heat-denatured MDH, whereas betaine, proline, and glycerol activated the chaperone by 30–50% (Fig. 4*a*). Optimal rates were observed with 0.6 M betaine, 1 M proline, and 1.5 M glycerol. Higher, nonphysiological concentrations of either osmolyte inhibited this chaperone.

We tested the effect of osmolytes on more complex networks of molecular chaperones. The refolding activity of KJE-bound MDH can be activated by supplemented GroELS, which alleviates KJE from slow and inefficient folding of late MDH-folding intermediates (37). In the presence of increasing osmolyte concentrations, this KJE+LS bichaperone network was, as in the case of KJE alone, activated by low concentrations of betaine, proline, and glycerol, but not trehalose. Noticeably, betaine was the most effective activator of KJE+LS-mediated refolding (Fig. 4*b*), as in the case of LS alone (Fig. 3) High osmolyte concentrations, in a range that was significantly lower than that for the individual KJE or LS chaperone systems (Figs. 3 and 4*a*), inhibited this complex reaction.

Another bichaperone network composed of ClpB and KJE has the ability to disaggregate and refold stable protein aggregates (27, 28). The disaggregation activity of ClpB+KJE was more sensitive to low trehalose than KJE alone. Low proline and glycerol concentrations did not activate, and inhibition occurred at concentrations 2–3-fold lower than those in the case of KJE alone (Fig. 4, *a* and *c*). The finding that more complex chaperone networks are inhibited, whereas individual chaperones are not, suggests that the shuttling of folding intermediates between chaperones is particularly sensitive to nonphysiologically high concentrations of osmolytes.

Remarkably, contrary to the other osmolytes, physiological concentrations of betaine strongly activated the ClpB+KJE system. Hence, disaggregation was activated 2.5-fold by 0.5 M betaine, whereas as little as 70 mM trehalose inhibited this reaction by 50% (Fig. 4*c*). This suggests that, by a mechanism
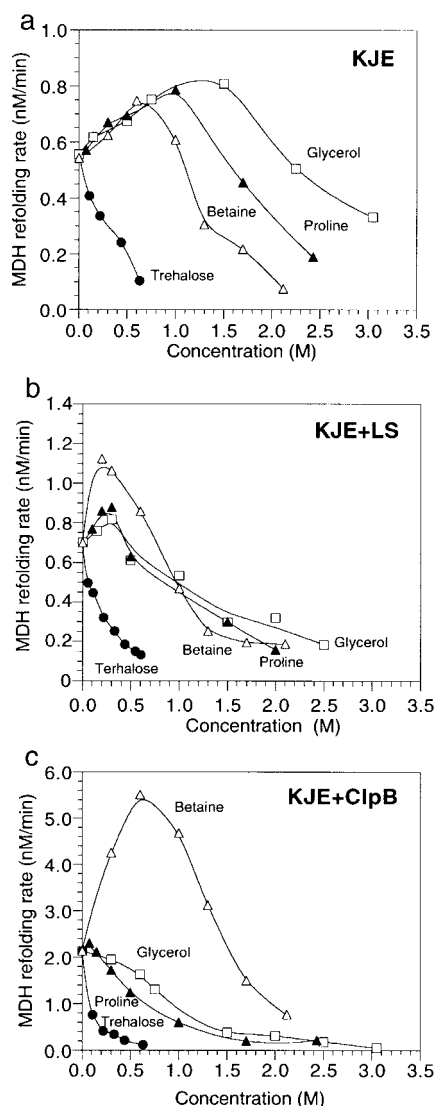
FIG. 4. **Osmolytes affect KJE-mediated refolding.** MDH (0.5 μM) was heat-denatured at 47 °C in the presence of KJE chaperones (1.0, 0.2, and 0.1 μM, respectively). Refolding at 25 °C was performed in the presence of increasing concentrations of betaine, trehalose, proline, or glycerol, without (*a*) or with (*b*) GroEL (3.5 μM) and GroES (6.0 μM). *c,* MDH (0.7 μM) was heat-denatured in the absence of chaperones. The refolding was performed at 25 °C in the presence of supplemented KJE (1.0, 0.2, and 0.1 μM, respectively) and ClpB (0.5 μM) and increasing concentrations of betaine, trehalose, proline, or glycerol.

yet to be elucidated, betaine can specifically activate protein disaggregation by ClpB and KJE chaperones, and trehalose can specifically inhibit protein disaggregation by ClpB and KJE chaperones.

Osmolytes Promote Refolding of Extensively Unfolded Proteins—One possible mechanism by which osmolytes can activate chaperone-mediated refolding is by promoting the local correct refolding of segments within the chaperone-bound polypeptides (37). This possibility was addressed by measuring the ability of various osmolytes to promote the correct refolding of urea-unfolded MDH. After dilution in a chaperoneless solution, urea-unfolded MDH was not reactivated at 37 °C. However, when urea-unfolded MDH was diluted in the presence of betaine, glycerol, proline, or trehalose, it was efficiently reactivated at rates and yields that generally increased with concentration, with the exception of betaine, which inhibited refolding at concentrations above 3 M (Fig. 5). Yields were highest with glycerol (70%) > proline (40%) > trehalose (28%) > betaine (19%) (Fig. 5*b, inset*), implying that the nature of the
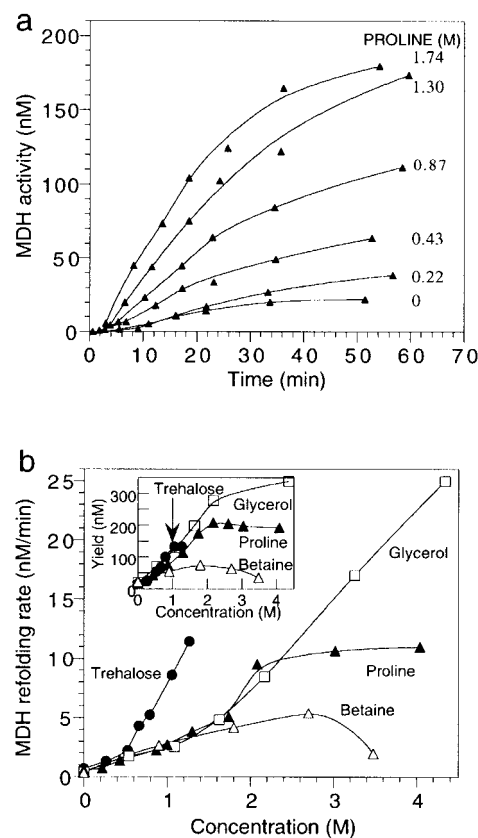

FIG. 5. **Osmolytes chaperone refolding of urea-unfolded MDH.** *a,* MDH (50 μM) was denatured in 5 M urea and 10 mM dithiothreitol and then diluted 100 times into folding buffer containing increasing concentrations of proline at 37 °C. Refolding rates (*b*) and yields (*b, inset*) are shown, as in *a,* with increasing concentrations of betaine, proline, trehalose, or glycerol.

osmolyte controls the partitioning and commitment of the unfolded species to the proper refolding or improper misfolding pathways.

The Role of Viscosity in Osmolyte Protection and Protein Folding—Osmolytes all have high viscosity indexes. We therefore addressed the possible contribution of viscosity to osmolyte-mediated thermal protection, promotion of refolding, and regulation of molecular chaperone activity. The viscosity of refolding solutions containing increasing osmolyte concentrations was measured at various temperatures: 25 °C (Fig. 6*a*), 30 °C, 37 °C, and 44 °C (data not shown). Hence, at 25 °C, 0.9 M trehalose was 5.4 times more viscous than equimolar proline, glycerol, or betaine (Fig. 6*a*). When the rates of KJE-, KJE+LS-, and ClpB+KJE-mediated refolding were expressed as a function of viscosity, the general patterns of activation and inhibition were preserved, although some differences among osmolytes became less pronounced (Fig. 6, *b*−*d*). In addition, no direct correlation was found between osmolyte viscosity and the ability of the osmolyte to promote correct refolding of urea-unfolded MDH (Fig. 6*e*). In contrast, when expressed as function of viscosity, differences in the protective nature of osmolytes were strongly reduced (Fig. 6*f*), indicating that viscosity is a major component in the mechanism of osmolyte-induced thermoprotection.

DISCUSSION

Cellular accumulation of osmolytes has been involved in the adaptation of organisms to salt, desiccation, and osmotic stresses in general (2). In parallel, accumulation of heat-shock proteins, especially molecular chaperones, has been involved in the adaptation to thermal stress (23). Although heat and salt

FIG. 6. **Effects of osmolyte viscosity.** *a,* viscosity at 25 °C of refolding buffers containing increasing concentrations of betaine, trehalose, proline, or glycerol. Rates of MDH refolding by KJE (*b*), KJE+LS (*c*), and ClpB+KJE (*d*), as in Fig. 4, expressed as a function of the measured viscosity at 25 °C. *e,* rates of spontaneous MDH refolding (as in Fig. 3*b*), expressed as a function of the measured viscosity at 37 °C. *f,* protection of MDH against heat inactivation (as in Fig. 2), expressed as a function of the measured viscosity at 44 °C.
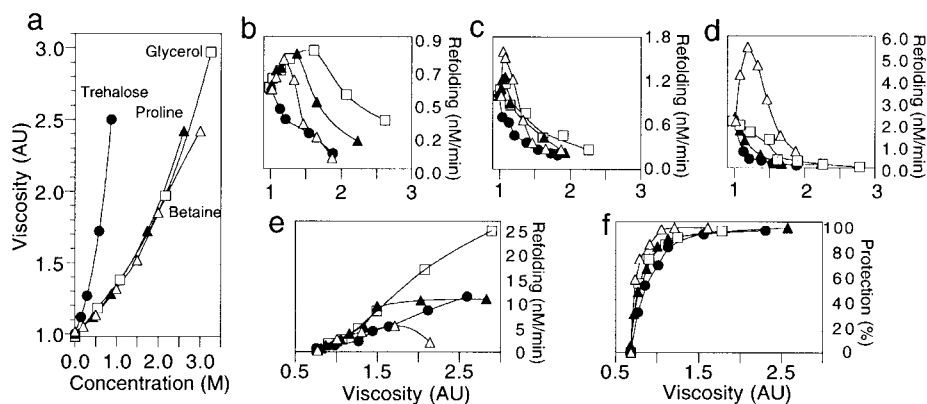
stresses often occur concomitantly in nature, a possible cross-talk between the two defense mechanisms has not been addressed in detail. Here we investigated the effect of osmolyte accumulation on the aggregation and disaggregation of proteins in *E. coli* cells and *in vitro*. We found that preadaptation of *E. coli* cells to increasingly high concentrations of salt in the medium decreased the tendency of soluble proteins to form insoluble aggregates during heat shock. NMR analysis revealed that betaine was the major osmolyte that accumulated in *E. coli* cells grown in rich medium (standard LB medium containing 0.17 M NaCl), and the amounts of betaine strongly increased in salt-adapted cells. Intracellular betaine can originate from the medium or be synthesized from choline. Both betaine and choline are common components of rich media (1). Although trehalose is also present in the medium, it is mostly degraded in the periplasm as a carbon source. Only the trehalose that is endogenously synthesized can have an osmoprotective function (1). In addition, betaine uptake is strongly preferred by the cell over trehalose synthesis (18). These findings reinforce our observation that betaine is the main osmolyte that accumulates in the *E. coli* cells grown in rich medium during salt stress.

The correlation between thermal protection and osmolyte accumulation in whole cells was confirmed *in vitro*. Physiological concentrations (<1 M) of betaine, trehalose, proline, or glycerol all fully protected MDH from thermal denaturation at 44 °C. The possible mechanism by which osmolytes may protect native proteins implies the existence of a thermodynamic force, the "osmophobic effect," which makes the interaction between osmolytes and peptide backbone unfavorable (38). The exclusion of osmolytes from protein cores, hydration shells, and crevasses on the protein surface increase protein solvation, leading to stabilization and protection of native proteins (39, 40). Vapor pressure osmometry showed that betaine is significantly more excluded from bovine serum albumin than proline, which in turn is excluded more than trehalose and glycerol (41). In contrast, we found that betaine and trehalose protect native MDH equally well and that both are more effective protectants than glycerol and proline. This indicates that osmolyte exclusion and osmolyte protection do not necessarily correlate. In contrast, in the case of all four osmolytes tested here, we found a good correlation between protection and osmolyte viscosity. This suggests that protection results primarily from the slowing down by the viscous solution of heat-induced movements in labile proteins that would otherwise lead to denaturation and aggregation.

Protection of proteins during heat shock by trehalose in yeast cells correlates with inhibition of HSP104-mediated protein disaggregation (31). However, this is not a general feature of osmolytes because we observed efficient protein disaggregation, likely by the ClpB+KJE chaperones (25, 27), despite a

significant accumulation of betaine in salt-adapted *E. coli* cells (Fig. 1). Consequently, we addressed the specific effects of betaine, proline, glycerol, and trehalose on the protein disaggregation activity of the ClpB+KJE chaperone network *in vitro*, as compared with the protein refolding activity of individual and combined chaperones KJE, LS, and KJE+LS.

Possible Mechanisms of Chaperone Activation

With the exception of trehalose, which inhibited KJE-dependent reactions at all concentrations, physiological concentrations (up to 1 M) of betaine, proline, and glycerol activated KJE and LS chaperones. Noticeably, betaine was a potent activator of protein disaggregation by ClpB+KJE. The mechanism for such activation may involve 1) promotion of spontaneous local refolding within the chaperone-bound polypeptides, 2) stabilization of the end product, 3) stabilization of chaperone structure, and 4) specific activation of the chaperones.

Promotion of Spontaneous Refolding—As expected from "chemical chaperones," all the osmolytes tested but betaine efficiently promoted the correct refolding of urea-unfolded MDH *in vitro*. However, completely unfolded polypeptides are unlikely to form in the cell, especially during thermal stress, because partially unfolded proteins are highly unstable species that readily seek alternative stable structures by association with chaperones (42) or by aggregation (43). The osmolyte concentrations that optimally refolded urea-MDH were non-physiologically high. Moreover, the same trehalose concentrations that strongly inhibited KJE promoted refolding of urea-unfolded MDH, suggesting that the two effects are not connected. Conversely, the same betaine concentrations that strongly activated ClpB+KJE did not promote refolding. Nevertheless, a minor contribution to local refolding by low concentrations of specific osmolytes still remains possible.

Stabilization of the End Product and of Chaperones—Protection by osmolytes occurs at the same low physiological concentrations that also activate chaperones. By preventing inactivation of the end product of the folding reaction, osmolyte protection could produce a net activation of the chaperone reactions. Moreover, osmolytes may stabilize the molecular chaperones themselves. However, the fact that low physiological concentrations of betaine strongly activate chaperone-mediated disaggregation under conditions where glycerol, proline, and trehalose strongly inhibit chaperone-mediated disaggregation suggests that betaine has a specific activatory interaction with ClpB and/or DnaK, whose mechanism is under investigation.

Possible Mechanisms of Chaperone Inhibition

Nonphysiologically high concentrations of glycerol, proline, and betaine inhibited chaperone activity. Noticeably, trehalose was a very potent inhibitor of all the reactions that depended

on KJE, especially protein disaggregation, even at physiological concentrations. Inhibition may result from increased viscosity, which can limit dynamic interactions between chaperones, co-chaperones, and folding substrates. This is suggested by the observation that the sensitivity to high concentration osmolytes increases with the complexity of the chaperone network. The simplest system with co-chaperone GroES, chaperone GroEL, and a prebound MDH was the least inhibited by high osmolyte concentrations. In contrast, multichaperone networks such as KJE+LS or ClpB+KJE were inhibited by physiological concentrations of osmolytes. High osmolyte concentrations may also stabilize protein aggregates, like native proteins, resulting in the inhibition of chaperone unfolding activity. Low physiological concentrations of trehalose strongly inhibited chaperone-mediated disaggregation under conditions where other osmolytes activated the reaction. It is therefore possible that trehalose can specifically interact with ClpB or DnaK.

The mechanisms by which trehalose specifically inhibits chaperone-mediated disaggregation and betaine specifically activates chaperone-mediated disaggregation remain to be elucidated. However, it is significant that protein disaggregation was very efficient in salt-adapted *E. coli* cells, which accumulated high levels of betaine but not of trehalose. This suggests that an organism grown in hyperosmotic nutrient-rich media can optimally protect its native proteins from heat damage and, at the same time, keep its network of molecular chaperones optimally active. However, under poor and unfavorable growth conditions and under prolonged and acute heat and osmotic stresses, trehalose accumulation may be a better option because it is also a powerful protecting agent of membranes (44). Thus, the timing of heat stress in relation to osmotic stress and the availability of osmolytes from the medium both determine the cellular composition and concentration of osmolytes in salt-adapted cells and hence the extent of thermal damage in proteins and the efficiency of protein recovery after heat stress.

Molecular Crowding

In addition to osmolyte accumulation, osmotic stress increases the confinement and crowding of the cellular macromolecules (16, 45). Crowding enhances interactions between proteins in general and between unstable folding intermediates in particular and thus favors protein aggregation (45–47). However, increased protein-protein interactions may equally favor binding of unstable folding intermediates to molecular chaperones and their stabilization as soluble refolding-competent species (46, 47) and may favor stabilization of native proteins. Thus, increased molecular crowding, by low concentration of osmolytes, may increase the overall stability of cellular proteins and encourage proper protein refolding, whereas under extreme stress conditions, crowding may favor protein aggregation (45).

In conclusion, complex interactions between specific chemical the molecular chaperones can potentiate the ability of cells to control protein stability, protein aggregation, disaggregation, and refolding during and after combined osmotic and heat stresses.

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