Biochemistry. In the article "Ligation-anchored PCR: A simple amplification technique with single-sided specificity" by Anthony B. Troutt, Michael G. McHeyzer-Williams, Bali Pulendran, and G. J. V. Nossal, which appeared in number 20, October 15, 1992, of Proc. Natl. Acad. Sci. USA (89, 9823–9825), the authors inadvertently omitted reference to a prior publication (1), which describes a formally equivalent strategy. This occurred despite two separate Medline searches by Troutt et al. using as keywords T4 RNA ligase, anchored (and) PCR, single-sided (and) PCR, and ligation (and) PCR. The authors also searched the corresponding sets using polymerase-chain-reaction and searched all fields (e.g., title, abstract). Dumas Milne Edwards et al. (1) confirm that their paper does not appear in the Medline database under those keywords. However, their paper is listed with the keywords oligodeoxynucleotide, polymerase chain reaction, and single-stranded. The failure to find the prior paper highlights the difficulty of detecting pertinent articles by computer-assisted searching of the current biomedical literature and emphasizes the need for careful attention to the choice of keywords used in such searches. The Nossal laboratory offers an unreserved apology to the Mallet laboratory and to the readers of these Proceedings for this unfortunate error of omission, which was quite unintentional.


Biochemistry. In the article "Substoichiometric amounts of the molecular chaperones GroEL and GroES prevent thermal denaturation and aggregation of mammalian mitochondrial malate dehydrogenase in vitro" by Dallas J. Hartman, Brian P. Surin, Nicholas E. Dixon, Nicholas J. Hoogenraad, and Peter B. Høj, which appeared in number 6, March 15, 1993, of Proc. Natl. Acad. Sci. USA (90, 2276–2280), the authors wish to make a correction. On p. 2277, column 1, line 19, the concentration of NADH should be 188 μM, not 4.7 μM.

Medical Sciences. In the article "Tyrosine phosphorylation is a mandatory proximal step in radiation-induced activation of the protein kinase C signaling pathway in human B-lymphocyte precursors" by Fatih M. Uckun, Gary L. Schieven, Lisa M. Tuel-Abilgren, Ilker Dibirkik, Dorothea E. Myers, Jeffrey A. Ledbetter, and Chang W. Song, which appeared in number 1, January 1, 1993 of Proc. Natl. Acad. Sci. USA (90, 252–256), the authors wish to make a correction. Herbimycin A was obtained from GIBCO/BRL, not Sigma (p. 252, column 1, 11 lines above the abbreviations footnote).
Substoichiometric amounts of the molecular chaperones GroEL and GroES prevent thermal denaturation and aggregation of mammalian mitochondrial malate dehydrogenase in vitro

(Escherichia coli/enzyme reactivation/protein refolding/ATP)

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ABSTRACT The molecular chaperones GroEL and GroES were produced at very high levels in Escherichia coli, purified, and shown to protect pig mitochondrial malate dehydrogenase (MDH) against thermal inactivation in vitro. The apparent rate of MDH inactivation at 37°C was reduced by a factor of at least 5 in a process which required only GroEL, GroES, and ATP. GroEL alone did not protect MDH against thermal inactivation but kept the denatured protein soluble and thereby prevented its aggregation. Reactivation of this soluble and inactive form of MDH could be achieved by addition of GroES even after 120 days of storage at −20°C. Protection could be extended for more than 24 hr at 37°C and was observed at molar ratios of chaperones to MDH as low as 1:4, suggesting that GroEL and GroES perform multiple turnovers in the absence of auxiliary chaperones. The availability of these chaperones in large quantities combined with the apparent promiscuity of GroEL binding shows great potential for stabilization of many proteins for which thermostable variants are not available. We speculate that GroEL and GroES perform similar protective roles in vivo and thereby increase the half-life of proteins which otherwise might aggregate under physiological conditions.

Although many isolated polypeptides can fold in vitro in a spontaneous process dictated solely by their primary structures, recent studies suggest that folding of proteins in vivo requires ubiquitous class of accessory components, termed molecular chaperones (1). A large number of molecular chaperones have now been identified in a variety of organisms and many have been shown to be identical to highly conserved heat shock proteins (HSPs) (e.g., refs. 2–5). Of the major classes of HSPs most is known about the HSP70 and HSP60 family members. A number of recent studies have addressed the role of the Escherichia coli HSP70 (DnaK) and HSP60 (GroEL) proteins in protein folding (6–8). Thus Langer et al. (7) employed five purified bacterial HSPs (DnaK, DnaJ, GrpE, GroEL, and GroES) to develop a model system in which their interaction with progressively more complex folding intermediates results in the folding of a chemically denatured polypeptide into a functional tertiary structure. The data suggest that DnaK and DnaJ bind to nascent polypeptides as they emerge from ribosomes. In a reaction dependent upon GrpE, the partially folded polypeptide is passed onto GroEL and its co-chaperone GroES. After a series of ATP-dependent reactions involving binding and release, the polypeptide assumes its fully folded and functional state.

Much less is known about the functions molecular chaperones perform on existing cellular proteins. Pelham (9) suggested that HSP70 homologues may bind to proteins and prevent their aggregation. Support for this hypothesis has been provided by elegant in vivo experiments with DnaK, DnaJ, and GrpE mutants (10) and also by in vitro experiments in which a 60-fold molar excess of DnaK was shown to protect E. coli RNA polymerase from thermal inactivation (11). DnaK binds to and recognizes relatively linear polypeptide conformations, whereas GroEL binds substrates with a higher degree of secondary structure (7, 12, 13). Since the denatured (D0) state of proteins obtained under mild physiological conditions appear to be highly compact with extensive secondary structure (14), we speculated that proteins undergoing continuous intracellular denaturation as a result of "cellular aging" would present suitable substrates for GroEL. Here we show that substoichiometric amounts of GroEL, in a process completely dependent upon GroES and ATP, protects mitochondrial malate dehydrogenase (MDH) from thermal inactivation. GroEL alone does not prevent activity loss but efficiently protects MDH from thermal aggregation. Attempts to protect MDH from activity loss with DnaK and DnaJ were not successful. Based on this in vitro model system, it is suggested that the chaperones GroEL and GroES may play a major role in protein stabilization and rescue under physiological conditions. A dual role for the GroEL and GroES chaperons in protein rescue and in de novo protein synthesis may explain why they are essential for growth under all conditions tested to date (3), whereas the lack of DnaK is lethal only at elevated, nonphysiological temperatures (15).

MATERIALS AND METHODS

Overproduction of GroE Proteins in E. coli. A 2.3-kb HindIII–EcoRI fragment encoding the groE genes (16) was excised from the plasmid pOF39 (gift of Costa Georgopoulos), filled in with the Klenow fragment of E. coli DNA polymerase I, and ligated into the Smal I site of the expression vector pCE30 (17) to produce the plasmid pBS559, in which the expression of the groE genes is under control of the bacteriophage P_R and Pl promoters. E. coli AN1459 cells containing pBS559 were grown in 2-liter flasks in 800 ml of Luria broth medium (18) supplemented with thymine (25 μg/ml), glucose (12.5 mM), and ampicillin (100 μg/ml) until an OD of 0.5 was reached. At this time the cI857 repressor was inactivated by raising the temperature to 42°C, and the cultures were incubated for 4 hr at this temperature before the

Abbreviations: BSA, bovine serum albumin; DTT, 1,4-dithiothreitol; HSP, heat shock protein; MDH, malate dehydrogenase.

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cells were collected by centrifugation. After suspension in 0.05 M Tris-HCl, pH 7.5/10% (wt/vol) sucrose to give an OD595 of 200, cells were frozen in liquid nitrogen and stored at −70°C. Cells were lysed by the addition of lysozyme and Brij-58 essentially as described (19) and centrifuged at 30,500 g for 1 hr. The resulting supernatant (1155 mg of protein) was fractionated with PolyMin P (0.35%, wt/vol), and the resuspended protein pellet was subjected to standard chromatographic procedures (details available upon request) to yield about 210 mg of GroEL and about 15 mg of GroES. This preparation of GroEL and GroES is functionally intact and has been used for the reconstitution of chemically denatured ornithine carbamoyltransferase (5).

Additional Analytical Techniques. Pig heart mitochondrial MDH (Boehringer Mannheim) was assayed at 37°C at pH 6.8 in buffers containing 94.3 mM potassium phosphate, 0.2% bovine serum albumin (BSA), 9.4 mM trans-1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid, 645 µM oxaloacetic acid, 1 mM 1,4-dithiothreitol (DTT), and 4.7 µM NADH, by continuously monitoring the decrease in A340. SDS/12.5% PAGE with Tris/glycine buffer was carried out as described (20) and protein was determined by quantitative amino acid analysis and/or Coomassie brilliant blue staining with BSA as a standard (21).

RESULTS

GroEL and GroES Protect MDH from Thermal Inactivation in an ATP-Dependent Manner. The mitochondrial citric acid cycle enzyme MDH is a homodimer of nuclear-encoded 35-kDa subunits which share 59% sequence identity with their E. coli counterparts (22). MDH readily loses activity at 37°C and exhibits a first-order loss of activity with τ1/2 = 2 min at 51°C (23). When incubated for 6 hr at 37°C with GroEL, GroES, and ATP (Fig. 1A and B), MDH was relatively stable exhibiting a first-order loss of activity with a decay constant of 1.5 × 10⁻³ min⁻¹. By contrast, the omission of either GroEL, GroES, or ATP resulted in loss of stabilization as indicated by an ≈6-fold increase in the decay constant (Fig. 1 A and B). Although there were small variations between experiments, comparison of a large number of independent experiments did not disclose any significant stabilizing effect of GroEL, GroES, or ATP alone. The increased stability of MDH in the presence of all three components could not be attributed to an increase in protein concentration, as this was kept comparable in all tubes by the addition of BSA (Fig. 1 A and B). Furthermore, incubation of MDH in the presence of excess BSA did not result in any increase in the stability of MDH (data not shown).

Although the inactivation of MDH in the presence of chaperones followed a first-order reaction for about 6 hr, extended incubation times revealed a biphasic decay resulting in larger than expected residual activity after 25 hr at 37°C (Fig. 2). Thus, 58% residual activity was detected after 6 hr of incubation in the presence of a 4- to 5-fold molar excess of GroEL and GroES, whereas 45% residual activity was detected after 25 hr of incubation. The reason for this repeatable phenomenon is not clear but could be related to the dimeric nature of MDH. Substitution of GTP (Fig. 2) or the nonhydrolyzable ATP analogue adenosine 5’-[β,γ-imido]triphosphate (data not shown) for ATP completely abolished stabilization. In separate experiments, we tried to protect MDH against thermal inactivation by using the chaperones DnaJ and DnaK at 3- and 7-fold molar excess, respectively, but protection was not observed, even in the presence of ATP (data not shown).

Substoichiometric Amounts of GroEL and GroES Are Sufficient for MDH Stabilization. To determine the minimum molar ratio of GroEL and GroES to MDH required for stabilization, MDH was incubated with ATP at 37°C in the presence of decreasing molar ratios of GroEL and GroES and assayed for residual activity at the indicated time points (Fig. 1C). Molar ratios of GroEL and GroES to MDH as low as 1:4 were sufficient to support a large degree of stabilization (see apparent inactivation rates in the legend to Fig. 1C). This is, to our

![Graph showing the effects of GroEL and GroES on MDH activity](image-url)

Fig. 1. Requirements for chaperonin-mediated protection of MDH against thermal inactivation. (A and B) MDH (89 nM, 1.25 µg) was incubated at 37°C in 38.4 mM Tris-HCl, pH 7.6/1.8% (vol/vol) glycerol/34 mM NaCl/7.7 mM KCl/5.4 mM MgCl₂/1.5 mM DTT. Reaction vessels, prequenched with BSA, also contained GroEL (349 nM, 59 µg), GroES (557 nM, 7.8 µg), ATP (1.5 mM), and/or BSA (4.5 µM, 60 µg) as indicated. All vessels had identical buffer compositions and displayed nearly identical MDH activity at time zero (defined as 100% activity). Residual enzyme activity in 20-µl aliquots was measured at appropriate time intervals, and apparent rate constants for inactivation (kapp) were determined by semilogarithmic plots: (A) GroEL + GroES + ATP (kapp = 1.5 × 10⁻⁵ min⁻¹); (B) BSA + ATP (kapp = 8.1 × 10⁻⁵ min⁻¹); GroEL + GroES + ATP (kapp = 9.8 × 10⁻⁵ min⁻¹); BSA + ATP (kapp = 11.5 × 10⁻⁵ min⁻¹); GroES + ATP + BSA (kapp = 9.4 × 10⁻⁵ min⁻¹); GroEL + ATP (kapp = 9.4 × 10⁻⁵ min⁻¹). (C) MDH (89 nM, 1.25 µg) was incubated at 37°C in 40.2 mM Tris-HCl, pH 7.6/1.4% glycerol/28 mM NaCl/8 mM KCl/5.6 mM MgCl₂/1.6 mM DTT/1.6 mM ATP. Reaction vessels prequenched with BSA were fortified with GroEL and GroES at the indicated molar ratios of chaperones to MDH (the ratio of GroEL to GroES was kept constant at 1:1). Residual enzyme activity in 20-µl aliquots was measured at the time intervals shown and apparent rate constants for inactivation (kapp) were determined by semilogarithmic plots: ratios of GroEL/ES to MDH were 3:1 (kapp = 2.0 × 10⁻⁵ min⁻¹), 1:2 (kapp = 1.9 × 10⁻⁵ min⁻¹), 1:4 (kapp = 3.2 × 10⁻⁵ min⁻¹), and 1:20 (kapp = 9.8 × 10⁻⁵ min⁻¹); MDH without GroEL/ES was the control (kapp = 11.1 × 10⁻⁵ min⁻¹). One hundred percent activity is defined as above. Molecular mass values of 840 kDa for GroEL (14-mer), 70 kDa for GroES (7-mer), and 70 kDa for MDH (dimer) were used to calculate the concentrations shown.

knowledge, the first demonstration that substoichiometric amounts of chaperones can provide stabilization (11, 24).

GroEL Alone Protects MDH from Aggregation But Does Not Prevent Its Inactivation. During heat shock many newly synthesized and mature proteins are rendered enzymatically inactive as a result of insolubilization (25). To determine whether the thermal inactivation of MDH was accompanied by aggregation, MDH was incubated at 37°C in the absence of chaperones and its aggregation state was determined by low-speed centrifugation and subsequent SDS/PAGE analysis of the resulting pellets and supernatants (Fig. 3 A and B). At zero time MDH was detectable only in the supernatant (Fig. 3A, lanes 6 and 14); however, after incubation for 5.7 hr (>2% residual activity) MDH was barely detectable in the supernatant (Fig. 3B, lane 6) but prominent in the pelleted material (Fig. 3B, lane 14). In contrast, when GroEL and GroES were present (ratio of GroEL and GroES to MDH, 0.8) aggregation of MDH was not observed (Fig. 3B, lane 10). Although GroES is required for retention of MDH activity (see Fig. 1), it is not required to prevent aggregation of MDH (Fig. 3B, lanes 5 and 13). Stabilization presumably involves continuous GroES-dependent refolding of MDH previously captured in an inactive form on GroEL. Consistent with this model, we have found that MDH denatured in 6 M guanidinium chloride can be refolded only in the simultaneous presence of GroEL, GroES, and ATP (data not shown). MDH incubated in the presence of lysozyme (Fig. 3B, lanes 7 and 15) was found exclusively in the pellet after 5.7 hr of incubation, whereas BSA appeared to retard the aggregation slightly (Fig. 3B, lanes 5 and 12). GroES in the presence of BSA did not appear to retard aggregation (Fig. 3B, lanes 11 and 12).

Since all experiments shown in Fig. 3 A and B were performed in the presence of ATP, we next sought to determine whether GroEL alone could protect MDH from thermal aggregation. MDH was therefore incubated at 37°C with GroEL in the presence or absence of ATP and GTP, and the degree of aggregation was determined as described above (Fig. 3C). After 5.7 hr the MDH was found exclusively in the supernatants regardless of the presence or absence of ATP or GTP. GroEL can thus keep an inactivated enzyme soluble for prolonged periods without continuous expenditure of energy. The solubility of MDH in the presence of GroEL and absence of GroES is almost certainly due to complex formation between GroEL and denatured MDH. This point was illustrated by incubating MDH at 37°C in the presence of GroEL but absence of GroES exactly as described in the legend to Fig. 3. After 6 hr the sample had about 3% residual activity and was frozen at −20°C for 120 days. After thawing the sample was fortified with an excess of GroES and a time-dependent reactivation of MDH was observed, to the extent that 40% of the original MDH activity was recovered. By contrast, when MDH inactivated in the presence of DnAK was fortified with GroEL and GroES, no MDH activity was regained. It appears that GroEL sequesters inactive yet refoldable MDH and that this complex formation must take place before MDH aggregation has occurred.

**DISCUSSION**

The free energies of stabilization ($\Delta G_{stabil} = G_{native} - G_{denatured}$) of globular proteins cluster within the narrow range of
20–80 kJ/mol, irrespective of protein size and structure (14, 26). The relative instability of globular proteins reflects the biological requirement for protein turnover and for a delicate balance between rigidity and flexibility. Rigidity on the one hand ensures a well-defined protein structure and thereby specificity of interaction with substrates, while flexibility is a requirement for catalysis, allosteric transformations, and receptor activation. A costly consequence of evolutionary optimization of protein structure and function at the expense of stability is that many proteins undergo continuous denaturation under physiological conditions (14). This raises the question as to how cells have evolved to cope with this problem. Pelham (9) suggested that HSPs would bind thermally damaged proteins and thereby prevent their aggregation. Although much evidence points to a role of HSPs in protection of cells against heat stress (27), and heat shock gene expression is required for refolding of denatured proteins (10), little evidence has accumulated to demonstrate a direct role of the HSPs in protecting already folded polypeptides against thermal denaturation and aggregation (11, 24, 28).

In this paper we have demonstrated that the chaperones GroEL and GroES in the presence of ATP prevent thermal inactivation of MDH very effectively for at least 24 hr. Both GroEL and GroES are required. An excess of alternative proteins of differing size and isoelectric point such as BSA and lysozyme afford no protection. The ATP requirement is also strict, as neither GTP nor adenosine 5′-β,γ-imidodiphosphate can substitute for ATP. The obvious interpretation of our data is that the chaperonins recognize and bind to early unfolding intermediates and subsequently promote their refolding. A consequence of this ongoing chaperone activity is that the concentration of unfolded MDH is kept below the critical aggregation concentration whereby irreversible activity loss due to precipitate formation is prevented. It is important to note that prevention of aggregation is not a sufficient criterion for thermal protection against inactivation. This point is clearly demonstrated when MDH is incubated with GroEL in the absence of GroES and ATP (see Figs. 1 and 3). Although precipitate formation is completely inhibited, protection against thermal inactivation was not observed. This finding is in agreement with an elegant study on α-glucosidase from yeast (28) but at variance with a recent study by Mendoza et al. (24), who reported that GroEL alone could retard the thermal inactivation of rhodanese activity for at least 2 hr. The reason for this discrepancy is not clear. Since rhodanese requires GroES and ATP for refolding (6), the phenomenon reported by Mendoza et al. most likely relies exclusively on sequestering denatured polypeptides rather than on continuous cycles of unfolding and refolding.

A crucial feature of the stabilizing system is its ability to operate when substoichiometric amounts of the chaperones are employed. This leads to the important conclusion that the GroEL/GroES chaperones alone can function in a catalytic sense by carrying out multiple rounds of protein folding in the absence of auxiliary chaperones. Multiple cycles of GroEL/GroES action have been reported in only one previous study (7), in which unfolded rhodanese was supplied as a complex with DnaK and DnaJ in the presence of GrpE.

Is the in vitro action of the GroEL/GroES system likely to operate in vivo? By several criteria this now seems likely. (i) GroEL binds most nonnative (but not native) E. coli proteins tightly, with dissociation constants suggested to be in the subnanomolar range (29). Further, the ability of GroEL to bind, with positive cooperativity, more than two unfolded polypeptides is perfectly suited to sharply lowering the concentration of free denatured polypeptides and thereby minimizing the risk of aggregate formation (30). (ii) Under physiological and/or mildly stressing conditions the denatured state of most proteins appears to be highly compact with extensive secondary structure (14, 31), a requirement for binding to GroEL (7, 12). (iii) At 37°C E. coli cells contain one GroEL oligomer (14 protomers) per 1500 polypeptides (32). Since on the average only 1 of every 10^4–10^5 cellular protein molecules is assumed to be in a denatured state under physiological conditions (14), the concentration of GroEL appears high enough for this chaperone to undertake a general stabilizing function in the cell. (iv) Overexpression of GroEL and GroES has been shown to suppress temperature-sensitive mutations in several enzymes (33) and to increase the in vivo protein half-life 10-fold in the case of human procollagenase synthesized in E. coli (34). All these lines of evidence suggest a general role of the GroEL/ES system for stabilization of proteins in vivo, and the results reported here show that stabilization can be mediated directly by these two chaperones in the absence of auxiliary factors.

Skowyra et al. (11) demonstrated that a 60-fold molar excess of DnaK afforded significant protection to E. coli RNA polymerase from thermal inactivation. Using a 7-fold molar excess of DnaK we were unable to extend this observation to MDH even in the presence of ATP and DnaJ (data not shown). Although the difference may be related to the lower amount of DnaK used in our experiments, an alternative explanation is that the rather mild heating conditions used here to cause loss of MDH activity may not have been severe enough to present a sufficiently unfolded substrate for DnaK (7). Such a scenario may explain why GroEL and GroES are essential for growth at all temperatures (3), whereas the lack of DnaK is lethal only at elevated temperatures (15).

Recently, Langer et al. (7) demonstrated the role of bacterial HSPs in the folding of denatured proteins and suggested that DnaK and DnaJ bind to nascent polypeptides emerging from the ribosomes. In a process dependent upon GrpE the partially folded polypeptide with considerable secondary structure is passed from the DnaK/DnaJ complex on to GroEL/ES for final guidance into a native state. Our results now suggest an extension of this model to include the stabilizing function of GroEL/ES on native proteins which, due to their marginal stability, continually undergo thermal denaturation under physiological conditions (Fig. 4). Our data and previous reports (24, 28, 36) support an important implication of the model—namely, that GroEL can sequester proteins in an inactive yet soluble and refoldable state. This could have important regulatory implications. DnaK, for example, sequesters the heat shock gene-specific RNA polymerase subunit α2 in a dormant state until heat shock signals its release via a mechanism which may involve DnaK phosphorylation (37, 38). This is believed to be the central mechanism for the regulation of the heat shock response in E. coli. In this regard it is interesting that GroEL is also phosphorylatable (39) and involved in the transcriptional regulation of the Vibrio fischeri luminescence genes in E. coli due to its requirement for a functional LuxR transcriptional activator (40).

The sequestration of partially unfolded polypeptides on the surface of GroEL does not necessarily lead to stabilization in a cellular context. In the absence of auxiliary components such as GroES the sequestered polypeptide may be an easily accessible target for proteases and thus subject to increased protein turnover, a critical feature of cellular adaptation to changes in their environment. This aspect of GroEL function has been addressed by in vitro studies which showed that newly imported mitochondrial proteins are extremely sensitive to protease degradation when associated with the mitochondrial GroEL homologue Hsp60 (41) and by in vivo studies which demonstrated that mutations in the heat shock genes dnaK, dnaJ, grpE, and groEL result in defective proteolysis of some abnormal proteins (42).
Fig. 4. A hypothetical model for the dual role of GroEL and GroES in folding of newly synthesized proteins and rescue of existing proteins undergoing thermal denaturation at physiological temperatures. This model incorporates the findings in this report into a scheme previously devised by Langer et al. (7) for the chaperone-mediated folding pathway of de novo synthesized proteins. The order of DnaK and DnaJ action shown in this model is tentative; binding of DnaJ may precede binding of DnaK (compare refs. 7 and 35).

While much more work is required to define the relative importance of chaperones for folding of de novo synthesized proteins, for repair or degradation of denatured proteins, and for modulation of protein activity in vivo, the stabilizing function in vitro has obvious practical implications for the use of enzymes in diagnostics, medicine, and science. The ability to use substoichiometric amounts of overexpressed and readily purified chaperones, combined with the apparent promiscuity of GroEL binding (29), shows great potential for stabilization of many proteins for which thermostable variants are not available.

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