A Comparison of the GroE Chaperonin Requirements for Sequentially and Structurally Homologous Malate Dehydrogenases: The importance of folding kinetics and solution environment.

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Running title: Refolding homologous dehydrogenases with Chaperonins

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ABSTRACT:

*Escherichia coli* malate dehydrogenase (EcMDH) and its eukaryotic counterpart, porcine mitochondrial malate dehydrogenase (PmMDH) are highly homologous proteins with significant sequence identity (60%) and virtually identical, native structural folds. Despite this homology, EcMDH folds rapidly and efficiently *in vitro* and does not seem to interact with GroE chaperonins at physiological temperatures (37°C), while PmMDH folds much slower than EcMDH and requires these chaperonins to fold to the native state at 37°C. Double-jump experiments indicate that the slow folding behavior of PmMDH is not limited by proline isomerization. Although the folding enhancer glycerol (<5M) does not alter the renaturation kinetics of EcMDH, it dramatically accelerates the spontaneous renaturation of PmMDH at all temperatures tested. Kinetic analysis of PmMDH renaturation with increasing glycerol concentrations suggests that this osmolyte increases the on-pathway kinetics of the monomer folding to assembly competent forms. Other osmolytes such as TMAO, sucrose, and betaine also reactivate PmMDH at non-permissive temperatures (37°C). Glycerol-jump experiments with preformed GroEL-PmMDH complexes indicate that the shift between stringent (requires ATP and GroES) and relaxed (only requires ATP) complex conformations are rapid (< 3-5 sec). The similarity in irreversible misfolding kinetics of PmMDH measured with glycerol or the activated chaperonin complex (GroEL-GroES-ATP) suggests that these folding aids may influence the same step in the PmMDH folding reaction. Moreover, the interactions between glycerol-induced PmMDH folding intermediates and GroEL-GroES-ATP are diminished. Our results support the notion that the protein folding kinetics of sequentially and structurally homologous proteins, rather than the structural fold, dictate the GroE chaperonin requirement.
**Introduction**

GroEL is a complex, allosteric, protein folding machine whose function is controlled by associations with nucleotides, the co-chaperonin, GroES, and substrate polypeptides (1-3). As with all allosteric proteins, ligand binding influences the structural constraints within the system, which in turn ultimately induce shifts between various functional states. Although detailed information is available on the structures of GroEL, with and without bound nucleotide, and of one GroEL-GroES complex, the exact mechanism(s) explaining chaperonin-assisted folding of substrate proteins remain(s) unclear. Differences in binding and conditions for productive release of substrate proteins are routinely observed, but the structural and energetic basis of these differences is not understood at the molecular level. Although molten globule folding intermediates have been suggested to be preferred substrates for chaperonins, the structures of these intermediate populations have broad distributions thus making it difficult to identify specific transient conformations that interact with the GroE chaperonins.

*In vitro* and *in vivo* studies have shown that many proteins can interact with and fold from the chaperonin system, but the chaperonin requirements are highly variable. For instance, some proteins require the full complement of GroEL, GroES, and ATP in order to fold at physiological temperatures, while under the same conditions, other proteins have folded to high yields with only GroEL and ATP. It has been observed that a number of structurally homologous isozymes show differences in their chaperonin requirements. In an effort to explain the origins of these differences, Clarke and coworkers have compared the chaperonin requirements for the structurally homologous cytoplasmic and mitochondrial malate dehydrogenases. They have found that the increase in chaperonin requirements of the mitochondrial form is correlated with an increase in its global hydrophobicity (4). In another study, Martinez and coworkers have suggested that shifts in chaperonin requirements between the mitochondrial and the cytoplasmic forms of aspartate transaminase are correlated with shifts in the global pI of the substrate (5). Frieden and coworkers, on the other hand, have suggested that the differences in the chaperonin interactions for structurally homologous murine and *E. coli* dihydrofolate reductases (DHFR) may be due to additional extensions of omega loops present on the murine DHFR (6).

Although these correlations have been proposed to explain the variability in chaperonin requirements for folding isozymes, none of the correlations are generally applicable. For example, although the increased hydrophobicity of mMDH over cMDH has been proposed to explain its stringent chaperonin requirements (i.e. requiring GroEL, GroES, and ATP) (4), this correlation does not hold for the cytoplasmic and mitochondrial isoforms of aspartate transaminase (5). Likewise, the correlation of chaperonin stringency with the basic pI of
mitochondrial isozymes does not hold, because rhodanese, a highly stringent mitochondrial chaperonin substrate, has a slightly acid pI. In addition, the potential chaperonin substrate proteins identified from high affinity chaperonin-protein complexes isolated from *E. coli* cell extracts have a wide range of global pI values (7). Furthermore, the role that specific folding motifs play in dictating interactions between mammalian DHFR and the chaperonin has been recently questioned (8).

Rather than identifying correlations between chaperonin stringency and primary sequences or native tertiary and quaternary structures of homologous proteins, it appears that the folding kinetics and the lifetime of the folding intermediates (9,10) may be more reasonable properties that ultimately define the chaperonin requirements. In more recent work, Clark and Frieden provide strong evidence that kinetic factors may indeed explain the differences in chaperonin requirements for the homologous *E. coli* and mammalian DHFR isozymes (11,12). Specifically, an intermediate population of mammalian DHFR, existing as a partially folded species, can interact with the chaperonin before refolding to the native conformation (11,12). Unfortunately, these studies have only examined the interaction of the homologous DHFR substrates with the nucleotide-free high-affinity form of GroEL, a species that may have very transient lifetimes in vivo. In addition, the recent observation that the activated GroE species (i.e. GroEL, GroES and ATP) may actively unfold bound substrates suggests that the high affinity form of GroEL represents an oversimplification of chaperonin-substrate interaction (13). Thus, if we are to understand the dynamic nature of the interactions between chaperonins and their protein substrates, we must examine systems where *E. coli* substrates and their homologous mitochondrial counterparts interact with more physiologically relevant, activated chaperonin species.

In this current work, we have compared the folding of a highly homologous pair of malate dehydrogenase enzymes, *Escherichia coli* (EcMDH) and porcine mitochondria (PmMDH), in the absence and presence of the GroE chaperonins. The correlative studies of Hartl and coworkers indicate that intrinsic GroEL polypeptide substrates contain predominantly αβ tertiary folds containing two or more domains. PmMDH has this same type of fold and has been suggested to be a model *in vitro* substrate of the bacterial GroE chaperonins (7). The homologous PmMDH and EcMDH proteins in our study fold within cellular environments that contain group I chaperonins (GroEL/GroES in *E. coli* and Hsp60/Hsp10 in mitochondria). In addition, these two isozymes are more highly related to each other than any other homologous proteins used to study chaperonin interactions.

In the absence of chaperonins, *E. coli* MDH has been found to reactivate much more rapidly than PmMDH at 25°C (14). In this study, we have examined the refolding of EcMDH at a physiological temperature of 37°C to determine if EcMDH will now require chaperonins to aid in its folding. Although PmMDH and
EcMDH are highly similar with respect to sequence and structure (~60% sequence identity, ~80% sequence similarity), we find no evidence that EcMDH binds to or even interacts with the activated chaperonin complex (GroEL-GroES-ATP). Since the renaturation rate and probably the folding speed of EcMDH are more rapid, an increase in the folding of PmMDH should also diminish the chaperonin requirement.

Numerous investigators have found that including polyols in the refolding buffer can lead to increased protein refolding rates (15,16). We have found that the inclusion of the osmolyte, glycerol, in the refolding buffer leads to an enormous increase in both yield and reactivation rate of PmMDH at all temperatures tested. Under these conditions, the initial interactions between the refolding PmMDH subunits and the activated GroE chaperonin complex are no longer detectable. These studies suggest that the folding kinetics of the protein, rather than its particular structural fold, dictate the observed variability in chaperonin interactions with structurally homologous isozymes.

**Methods and Materials**

**The GroE Molecular Chaperonins, GroEL and GroES**

The *Escherichia coli* chaperonins, GroEL and GroES, were isolated from the lysate of cells containing the appropriate overexpression plasmid (gifts from Dr. Edward Eisenstein and Dr. George Lorimer, respectively). GroEL and GroES were purified as described by Voziyan and Fisher (17) and Eisenstein et al. (18). Since GroEL and GroES do not contain tryptophan residues, the removal of tryptophan containing contaminants, as assayed by second derivative analysis of the absorption spectra and tryptophan fluorescence were used as a criterion for purity of the chaperonin preparations as well as by silver stained SDS-polyacrylamide gel electrophoresis (19).

**Anti-GroEL Antibodies**

Polyclonal IgG antibodies reactive to GroEL were purified from the sera of rabbits immunized with purified GroEL (20). A 1:1 solution of sera and the provided binding buffer at a physiological pH (pH = 7.5) was equilibrated with a protein A column (Pierce Chemical Company). The protein A column was then washed with the 3-5 column volumes of binding buffer. The IgG was eluted with an acidic elution buffer (pH = 2.8) and the fractions were collected. The IgG fractions were dialyzed thoroughly against 50 mM Tris-HCl, pH 7.5, and 0.5 mM (Na)₂EDTA, and concentrated using Amicon Centricon-30 ultrafiltration units.
**Malate Dehydrogenase (MDH)**

Porcine mitochondrial malate dehydrogenase (PmMDH) and *Escherichia coli* malate dehydrogenase (EcMDH) were purchased from Sigma Chemical Company. The purity of each protein was examined by SDS-polyacrylamide gel electrophoresis followed by a silver staining procedure to resolve the protein bands. No foreign protein bands for PmMDH or EcMDH were observed.

**Denaturation and Renaturation of MDH**

5 µM of PmMDH or EcMDH were first denatured in standard buffer (50 mM triethanolamine hydrochloride (TEA), 20 mM MgCl₂, 50 mM KCl, and 10 mM dithiothreitol (DTT), pH 7.5 at 37°C) containing 6 M guanidine hydrochloride (GdnHCl) or 8 M urea for at least 1-2 hours at 0°C on ice, unless otherwise stated. Under these conditions, both isozymes were completely unfolded as accessed by the complete loss of activity. Complete unfolding of secondary and tertiary structures were confirmed by both tyrosine fluorescence and near UV circular dichroism spectroscopy (data not shown). Renaturation of a small aliquot of concentrated MDH was initiated by a rapid 100-fold dilution of standard buffer alone or with various combinations of 1 µM GroEL, 2 µM GroES, 5 mM ATP, and different concentrations of glycerol. The final MDH subunit concentration was 0.1 µM, unless otherwise stated. ATP was added to the refolding solution containing the GroE chaperonins 30 - 60 seconds before the initiation of protein folding.

**MDH Enzymatic Activity Assay**

The enzymatic activity of MDH was determined at 37°C using a substrate analog, ketomalonic acid (1 mM) and 0.2 mM NADH under standard buffer conditions, and following the rate of oxidation of NADH at 340nm on a SLM Aminco 3000 spectrophotometer (21,22). The use of ketomalonic acid as a competitive substrate for MDH eliminates the use of the natural substrate oxaloacetate, which undergoes significant decarboxylation to pyruvate at room temperature (22). The final MDH subunit concentration in the assay mixture was between 0.05 and 0.09 µM. At these MDH concentrations, for both EcMDH and PmMDH, the absorbance decline of NADH was linear within the time range of the data acquisition (1-3 min).

**90° Light Scattering**

A SLM 8000S fluorescence spectrophotometer with both the excitation and the emission wavelengths set at 360 nm was used to measure the formation of protein aggregates during the spontaneous renaturation of PmMDH with or without 35% glycerol in standard buffer conditions at 37°C. The bandpass for the emission monochromator was set at 5 nm. The final PmMDH subunit concentration in these experiments was 1 µM.
Proline Isomerization “Double Jump” Experiment

The “double jump” experiment was designed to detect the effect of proline isomerization as it pertains to the renaturation kinetics of proteins (23,24). The term, “double jump”, refers to the change of environment of a protein during the transition (i) from native to denaturing solution conditions, and (ii) from denaturing back to native solution conditions. Native PmMDH was denatured at 20°C for various times from 30 seconds to 6 hours before a rapid dilution with standard buffer at 20°C was performed to initiate spontaneous folding. In these experiments, PmMDH was completely unfolded after 30 sec as determined by tyrosine fluorescence. The enzymatic activity of PmMDH and EcMDH was used to monitor protein renaturation.

Commitment to the Native State

The commitment experiments were originally designed to examine the efficiency of the release of protein substrates in either a native state or committed to fold to the native state from chaperonin-substrate complexes (25). This methodology was used to determine whether PmMDH folding intermediates could interact with various components of the GroE chaperonin-mediated protein folding mechanism (i.e. GroEL, GroES, and ATP) in the presence and absence of 35% glycerol. In the presence and absence of 35% glycerol, PmMDH renaturation was initiated either spontaneously, with GroEL alone, with GroEL and ATP, or with GroEL, GroES, and ATP at 37°C in standard buffer conditions. Polyclonal anti-GroEL antibodies were added to aliquots of renaturing protein at 15 seconds or nearly 2 hours after the initiation of protein refolding. The final concentration of solution components was as follows: PmMDH monomers, 0.1 µM; GroEL, 1 µM; GroES, 2 µM; ATP, 5 mM; anti-GroEL antibodies, 50 µM. After the addition of anti-GroEL, the immunoprecipitants were rapidly removed by spinning the mixture in a microcentrifuge for 20-30 seconds. The supernatant was extracted, incubated at 37°C, and analyzed for enzymatic activity 2 hours after the initiation of refolding.

Irreversible Misfolding Kinetics

To measure irreversible misfolding rates, either the nucleotide-activated chaperonin complex (GroEL-GroES-ATP) or 35% glycerol were added to samples of refolding PmMDH at different times ranging from 0 to 60 seconds after spontaneous folding was initiated in standard buffer at 37°C according to the protocol outlined in (33). After the folding reaction took place for 4 - 6 hours to reach maximum refolding yields, the enzymatic activity of each sample was determined. The final concentration of the solution components was as follows: PmMDH monomers, 0.1 µM; GroEL, 1 µM; GroES, 2 µM; ATP, 5 mM.

Glycerol Jump Experiments

PmMDH renaturation was initiated at 0% or 35% glycerol in standard buffer with GroEL at 37°C. GroEL bound to the refolding protein and arrested its renaturation. 10 seconds or 60 minutes after folding was
initiated, the glycerol concentration was rapidly changed to either 35% or 3.5%, respectively, with and without ATP. The final concentration of the solution components was as follows: PmMDH monomers, 0.1 µM; GroEL, 1 µM; ATP, 5 mM. The renaturation of dimeric PmMDH was monitored by the recovery of enzymatic activity.

**Curve Fitting**

The renaturation data for PmMDH and EcMDH were fit to two different models. PmMDH renaturation was modeled from the folding reaction scheme of PmMDH as determined by Ranson et al. (21). For PmMDH renaturation, a numerical analysis of the data was performed with a software program developed by Micromath Inc, Micromath Scientist for Windows, version 2.01. The protein folding model and representative rate constants for the PmMDH folding scheme were as follows:

\[
\begin{align*}
4U & \rightleftharpoons 4M \rightleftharpoons 2N \\
2I_{agg} & \rightleftharpoons Agg
\end{align*}
\]

Renaturation of PmMDH

The rate constants associated with M → U, N → M, and Agg → I_{agg} were assumed to be negligible. For EcMDH, the renaturation data showed better fits to a single exponential rise (rather than a double exponential rise) using a non-linear least squares procedure. However, the renaturation profiles of EcMDH observed when GroEL alone was present were best fit to a double exponential rise. The best fits were determined based on reduced $\chi^2$ values and residuals.

**Results**

*Comparison of the sequence and structure of EcMDH and PmMDH.* A comparison of the amino acid sequences between *E. coli* and porcine mitochondrial malate dehydrogenase reveals a large degree of sequence identity and similarity, 58.2% and 80.3%, respectively (Figure 1A). Functionally significant residues show even larger degrees of conservation. For example, the subunit interfaces of the dimeric structures are 74% identical (26). Hydropathy index (27) measurements show that the mammalian mitochondrial form of malate dehydrogenase is more hydrophobic than the mammalian cytoplasmic form (0.145 vs. -0.035 respectively). However, the hydropathy index of EcMDH (0.194) is more similar to PmMDH, reflecting their high sequence identity.
identity and similarity. On the other hand, the calculated theoretical pI of the EcMDH (5.6) is similar to cytoplasmic MDH (5.91).

A comparison of the three dimensional, peptide backbone of the EcMDH and PmMDH monomers reveals that these two proteins have identical folds with very little variation (Figure 1B). In contrast to the differences observed with the EcDHFR and mDHFR structures, there are no extra loop structures present in the PmMDH when compared to the EcMDH (26,28). Indeed, Banaszak and coworkers have used the partially refined structure of the porcine isoform to provide the initial phases for solving the structure of the *E.coli* enzyme (26).

Refolding reactions of EcMDH with PmMDH at physiological temperatures in the absence and presence of chaperonins. Despite the high degree of sequence and structural similarity, the bacterial form folds much faster than its mitochondrial counterpart. The refolding kinetics and chaperonin requirements for EcMDH and PmMDH are compared under the same solution conditions at refolding temperatures of 37°C. Although both of these proteins can potentially interact with a group I chaperonin family (i.e. GroE or Hsp60) in their respective host organisms, it is clear that the EcMDH, under the defined solution conditions, does not need the GroE chaperonin system during refolding (Figure 2A). The renaturation kinetics are virtually identical for spontaneous refolding (refolding without chaperonins) or folding with GroEL when either ATP or ATP and GroES are present. All three kinetic profiles showed optimal fits to a single exponential rise function (0.35 ± 0.01 sec \(^{-1}\)). When ATP is absent, the high-affinity nucleotide-free GroEL oligomer slows down the renaturation of EcMDH and the kinetic profile now fits a double exponential rise (\(k_1 = 0.0074\) sec \(^{-1}\) and \(k_2 = 0.00037\) sec \(^{-1}\)). In all cases, the activity yields of refolded protein are high (80-100% regain of original activity).

In contrast, under exactly the same solution conditions, PmMDH absolutely requires the complete chaperonin system, GroEL-GroES-ATP to refold and reactivate (Figure 2B). No reactivation is observed when refolding is initiated without the chaperonins present or when GroEL or GroEL and ATP are present. The chaperonin requirements of PmMDH agree with previous studies (4,29). In addition to the differences in the chaperonin requirements, the refolding yields of PmMDH are substantially lower (~60%) when compared to the recoveries observed with EcMDH (~90-100%). It is clear that the folding mechanisms for these two proteins under these solution conditions are different.
Proline isomerization and folding of PmMDH. Comparative folding studies between homologous prokaryotic and eukaryotic aspartate transaminase performed by Widmann and Christen (2000) indicate that the slower folding rate of the mitochondrial enzyme is due, in part, to differences in proline isomerization (14). A sequence comparison between EcMDH and PmMDH reveals that there are more proline residues present within the mitochondrial primary sequence than in its bacterial homologue (21 vs. 13 prolines, respectively). To test the possibility that these additional proline residues are responsible for the slower folding rate of PmMDH, we performed double jump experiments (23,24). Both EcMDH and PmMDH homologues undergo very rapid denaturation when incubated in 6 M GdnHCl. This unfolding reaction is complete within 30 sec as characterized by a complete loss of native near UV CD and tyrosine fluorescence signals for both EcMDH and PmMDH (data not shown). For comparative purposes, the double-jump experiments are performed at 20°C where the renaturation of PmMDH can be observed with ~50% recovery. At the shortest times of incubation with a denaturant (30 sec), there is only a slight increase in the refolding rate (at t = 30 sec, \( k_1 = 3.0 \times 10^{-4} \text{ sec}^{-1} \) and \( k_2 = 9.06 \times 10^3 \text{ M}^{-1} \text{sec}^{-1} \)) as compared to the refolding rates observed following a typical long term denaturation (t = 2 hr, \( k_1 = 4.9 \times 10^{-3} \text{ sec}^{-1} \) and \( k_2 = 4.0 \times 10^3 \text{ M}^{-1} \text{sec}^{-1} \)) (Figure 3). Although there is a slight increase in \( k_1 \) when the PmMDH denaturation time is decreased to 30 sec, these kinetic data indicate that it is unlikely that proline isomerization contributes significantly to the differences in folding rates between PmMDH and EcMDH.

Effect of glycerol on the refolding of EcMDH and PmMDH. A number of investigators have suggested that increased solution viscosities can influence the observed folding rates by slowing the intramolecular chain collapse or domain pairing (reviewed in 30), associative kinetics, and monomer folding. However, these same solutes used to increase solution viscosity can also influence the solvation states of proteins, usually by stabilizing the native fold and thus, affecting folding transition states. As a result, the opposite effect of enhanced protein folding rates can also occur. A number of studies indicate that the protein folding kinetics are accelerated when various polyol osmolytes are present (15,16). Since both MDH isozymes can refold and assemble at 20°C, we decided to examine what effect an increasing polyol (glycerol) concentration may have on the refolding and assembly reactions of both EcMDH and PmMDH. Polyols such as glycerol do not affect the activities of these enzymes and are considered to be “compatible” solutes (31). Indeed, we found that the specific activities of both dehydrogenases are virtually identical in the presence or absence of glycerol (data not shown).
At glycerol concentrations of up to 35% (4M), the refolding and reassembly rates of EcMDH at 20°C are virtually unaffected (Figure 4A). In stark contrast, the reassembly and reactivation of PmMDH is dramatically increased under the same conditions (Figure 4B). The renaturation profile is influenced by the concentration of glycerol present in the renaturation mixture (Figure 5A, Table 1). The optimal concentration range for glycerol-enhanced renaturation rates and yields are from 20 to 35% glycerol. At higher glycerol concentrations (> 50%), the reactivation rates are slower, but the overall yields are higher than those observed in the absence of glycerol (Figure 5A). Furthermore, including glycerol in the renaturation mixture at “non-permissive” physiological temperatures of 37°C also accelerates the refolding and assembly reaction with the refolding yields as high as ~80% (Figure 5B, Table 1). Under these temperature conditions, which are non-permissive in the absence of glycerol, the optimal renaturation rates are also observed around glycerol concentrations of about 35% (Figure 5B).

_change in the chaperonin requirements for PmMDH folding in the presence of glycerol_. When glycerol is added to the renaturation solution, the requirement for GroES is relaxed. Denatured PmMDH can now be substantially reactivated in 35% glycerol with GroEL and ATP instead of just GroEL, GroES, and ATP. (Figure 6). It is important to note that the high affinity chaperonin (GroEL alone) can still completely arrest the renaturation of PmMDH (Figure 6). This observation indicates that, in the presence of glycerol, the folding intermediates of PmMDH still have a high affinity for GroEL and can form tight, arrested complexes with GroEL. But more importantly, the spontaneous folding and the GroEL-GroES-ATP reactivation profiles are virtually identical in the presence of glycerol (Figure 6, Table 1). Curiously, the folding and assembly of PmMDH in the presence of the GroE chaperonins is faster with glycerol than without it (Table 1).

Given this observation, we wanted to test if the PmMDH folding intermediates, generated in the presence of glycerol, will even interact with the activated chaperonin complex (GroEL,GroES, and ATP). In order to observe this initial interaction, a rapid immunoprecipitation technique with anti-GroEL antibodies is employed. A similar protocol is used to measure the rates at which a renaturing protein commits to fold to the native state in the presence of chaperonins (25). These experiments have shown that the addition of a large excess of GroEL antibody within 5-15 sec after initiating the chaperonin-mediated folding reaction is sufficient to completely precipitate the chaperonin-substrate complex from the solution (24; Fisher, unpublished observations).

As expected, the immunoprecipitation reactions at 15 sec completely removed any recoverable PmMDH activity from the supernatant when glycerol is absent from renaturation solutions containing the GroE chaperonins (Figure 7). In the absence of glycerol, the precipitation of activated GroEL complexes after 2
hours shows the expected amount of recovered PmMDH activity. In contrast, in the presence of activated GroE and glycerol, antibodies failed to remove all of the PmMDH activity from the renaturation solution. Nearly 40% of the recovered PmMDH is retained in the supernatant upon the immediate (within 15 sec) and rapid removal of the chaperonin by immunoprecipitation, while in control experiments, the addition of anti-GroEL antibodies containing a spontaneous refolding mixture in glycerol shows a similar amount PmMDH activity recovery (Figure 7). When the high affinity form of GroEL is used instead of the activated chaperonin complex in the presence of glycerol, very little PmMDH activity is recovered in the supernatant after GroEL immunoprecipitation. This indicates that the PmMDH monomers still binds to the chaperonin in the presence of glycerol and refolding buffer. The addition of ATP and GroES to the precipitated GroEL-PmMDH complexes results in substantial release and refolding of the protein substrate (Low, Smith and Fisher unpublished results) indicating that the immunoprecipitated complex is still active. Taken together, our immunoprecipitation experiments indicate that the interaction between the folding intermediates of PmMDH and the activated chaperonin is substantially decreased when PmMDH refolds in the presence of 35% glycerol.

Glycerol prevents deleterious aggregation of PmMDH. Besides aiding in the reactivation of PmMDH, glycerol also prevents deleterious aggregation of PmMDH (Figure 8). At non-permissive temperatures (37°C), 90° light scattering measurements show that the addition of 35% glycerol to the refolding mixture of PmMDH prevents any detectable large-scale aggregation. Regardless of the mechanism of aggregation prevention, glycerol allows PmMDH folding intermediates to reactivate to a high yield (~80%). These high levels of reactivation are observed even at MDH concentrations as high as 1 µM. Horowitz and coworkers have also shown that glycerol can also prevent large-scale rhodanese aggregation (32).

Glycerol jump experiments with GroEL-PmMDH complexes. Since the chaperonin requirements for PmMDH become less stringent when glycerol is present, this raised the following question. Will a GroEL-PmMDH complex formed in the presence of glycerol retain a non-stringent chaperonin requirement if this system is rapidly switched from a glycerol containing solution to one without glycerol? Starting with an arrested PmMDH-GroEL complex, a jump from low (0%) to high glycerol (35%) solution concentrations shows that PmMDH is released from GroEL as soon as ATP is added and it refolds to an active state (Figure 9). In fact, if the jump solution contains ATP, the reactivation kinetics is identical to the situation where ATP is added to a GroEL-PmMDH complex that is formed under high glycerol conditions. However, if the chaperonin-PmMDH complex is formed in the presence of 35% glycerol and the solution conditions are rapidly jumped to
lower glycerol concentrations (3.5%) in the presence of ATP, reactivation of PmMDH is no longer observed (Figure 9). Only when GroES is included in the refolding mixture at low glycerol concentrations can PmMDH once again reactivate (data not shown). These experiments indicate that the glycerol dependent shift in chaperonin stringency is less than 3-5 sec (typical manual mixing times).

Irreversible misfolding experiments with glycerol and chaperonins. During spontaneous refolding at 37°C, refolding subunits of PmMDH preferentially partition to an irreversibly misfolded species. Clarke and coworkers have shown that the folding competence of the PmMDH folding intermediate decays at a particular rate, the reaction is second order, and that this rate can be easily measured (21,32). Rate measurements for PmMDH decay are accomplished by initiating the refolding reaction and then adding the activated chaperonin system (GroEL- GroES-ATP) at variable delay times (0-60 sec). For each time point, the maximum rescued activity is measured and the irreversible misfolding kinetics is determined from the declining reactivation amplitude. Previous experimental results showed that there can be significant differences in the irreversible misfolding kinetic profiles of some proteins when the nature of the rescuing chaperonin was switched from the high affinity (GroEL alone) form to the fully activated (GroEL-GroES-ATP) chaperonin complex (33). These previous results suggested that a broader population of folding intermediates partitions onto a high affinity (nucleotide free) GroEL chaperonin. Since glycerol or activated GroE (as well as GroEL alone) inhibit PmMDH misfolding, we compared the irreversible misfolding kinetics of PmMDH when either glycerol or the activated chaperonin complexes were added to rescue folding intermediates. We wanted to test if glycerol, like the proposed action of the Hsp104 molecular chaperone class, could reverse or disrupt aggregates. Our data shows that the irreversible misfolding kinetics and amplitude profiles at set PmMDH concentrations are identical when either 35% glycerol or the activated chaperonins are used to rescue the transient folding intermediates (Figure 10).

Concentration dependence of PmMDH renaturation in glycerol. The kinetics and concentration dependencies of the reassembly and reactivation of both the porcine cytoplasmic and mitochondrial MDH isozymes were originally studied by Jaenicke and coworkers (34). These investigators found that the reactivation of cytoplasmic MDH follows first order kinetics and are independent of concentration. In contrast, the mitochondrial isozyme (PmMDH) reactivation is found to be concentration dependent. One model used to fit the data assumes that the monomer rapidly assembles into an inactive dimer that undergoes a unimolecular reaction before an active dimer is formed, a bi-unimolecular mechanism (34). Since there is an enhanced
renaturation of PmMDH in glycerol, we set up experiments to determine if the molecular mechanism of PmMDH assembly changes in the presence of this osmolyte to be independent of protein concentration.

The easiest way to determine if glycerol affects the mechanism of the kinetic processes is to carry out the renaturation of PmMDH in 35% glycerol at two different concentrations (Figure 11). We found that there is a noticeable concentration dependent increase in the renaturation kinetic profiles of PmMDH in the presence of glycerol. These results suggest that the mechanism of this reaction still depends on a second order process.

The effects of other folding additives on PmMDH renaturation. To determine if other osmolytes may also enhance the renaturation reaction of PmMDH, other natural osmolytes such as trimethylamine-N-oxide (TMAO), betaine, and sucrose are added to the renaturation mixture at 37°C (Table 2). At 37°C, where PmMDH renaturation is non-permissive, all of the osmolytes tested facilitate substantial folding of PmMDH (>30%). Betaine and glycerol are similar in their ability to enhance renaturation of PmMDH. However, the polyol osmolytes (sucrose and glycerol) are the only additives that are able to change the chaperonin stringency for PmMDH folding from GroEL, GroES, and ATP to GroEL and ATP. No PmMDH reactivation is observed when either betaine or TMAO are present with GroEL and ATP alone (Table 2). With these latter two osmolytes, reactivation from GroEL-PmMDH is only observed when GroES and ATP are added together (last column Table 2).

Discussion

It has been repeatedly demonstrated that homologs in protein folds do not translate into similar protein interactions with the GroE chaperonins (4-6). Data collected by Frieden and Clark strongly suggest that the differences in folding kinetics of dihydrofolate reductase isozymes (DHFR) determine of chaperonin interactions (35,36). Based on structural differences these investigators also suggested that additional unstructured loop regions in the protein determined chaperonin interactions (6). However, Horwich and coworkers suggest that these unstructured regions may be irrelevant to the interaction between GroEL and DHFR, and it is the folding core that is responsible for the kinetic control of chaperonin-substrate interaction (8). Regardless of the exact mechanism of interaction, these experiments with DHFR homologs indicate that folding speed may be a crucial parameter governing chaperonin-protein substrate interactions.

It is important to note that DHFR falls into the Class II substrates category for chaperonin interaction (37). These substrates do not require the complete contingent of the chaperonins (i.e. GroEL and GroES) to
fold (38). The homologous malate dehydrogenase isozymes, EcMDH and PmMDH, used in our work naturally fold in the presence of group I chaperonin (Cpn60/Cpn10) systems in vivo. Since PmMDH absolutely requires the entire set of GroE chaperonins and ATP to mediate its folding under non-permissive in vitro conditions, this in vitro substrate is classified as a potential Class III substrate (37,38). Based on the identities of a broad set of chaperonin substrates isolated by co-immunoprecipitation experiments from *E. coli* cell extracts, Hartl and coworkers suggested that the αβ fold, with a particular reference to the αβ fold of porcine mitochondrial malate dehydrogenase, correlate with potential chaperonin substrate identification (7). Our experiments have shown that, unlike its structurally homologous porcine mitochondrial counterpart, *E. coli* MDH folds very rapidly without the assistance of the GroE chaperonins and does not appear to interact with any of the physiologically relevant activated forms of the GroE chaperonins. Our results strongly suggest that structural folds cannot be used alone to identify potential class III chaperonin substrates. Based on our results however, we do predict that a substantial portion of the potential *Escherichia coli* chaperonin substrates identified by Houry et al., (7) should have slower folding/assembly kinetics than the other *E. coli* proteins that do not require chaperonin assistance to fold.

Widmann and Christen have found that the folding rates of three different oligomeric *E. coli* proteins are much faster than their mitochondrial homologues. These investigators have found that the mitochondrial aspartate aminotransferase folds slower than its *E. coli* counterpart because of cis-trans proline isomerization. Furthermore, these investigators also note that the EcMDH folds faster than PmMDH at 25°C. Unlike mitochondrial aspartate aminotransferase, our results suggest that proline isomerization is not the rate limiting step for renaturation of PmMDH. In addition, if proline isomerization was the rate limiting step, one would also predict that isomerization will still retard the folding rate even when folding enhancers such as glycerol are added to the refolding mixture. If so, the renaturation rates for the short pulsed denatured PmMDH (i.e. short incubation times in the denaturant) with glycerol could be comparable to those rates observed with EcMDH renaturation. However, contrary to this expectation, variable denaturation times of PmMDH did not result in disparate renaturation kinetics in the presence of glycerol (Data not shown).

Our results indicate that the renaturation of porcine mitochondrial malate dehydrogenase, PmMDH, is enhanced by glycerol addition at all temperatures tested and that the chaperonin requirements diminish in its presence (39). 90⁰ light scattering experiments have confirmed that the addition of glycerol inhibits the large-scale off-pathway aggregation reaction. We initially thought that the aggregation prevention may be sufficient to facilitate the rapid renaturation of PmMDH. It has been shown in silico that a small addition of urea at non-denaturing concentrations may prevent early aggregation of some proteins (40). To test if early observed
aggregation is the main reason for slow PmMDH refolding, we added a small amount of non-denaturing urea to
during spontaneous folding of PmMDH. As expected, urea inhibits the accumulation of large aggregates of
malate dehydrogenase (data not shown). However, unlike glycerol, low urea concentrations could not influence
the spontaneous reactivation profiles of PmMDH at any of the temperatures tested. Urea also destabilizes the
renaturation of PmMDH in the presence of glycerol and reflects the opposing effects that osmolytes and
denaturants have on protein stability (41). Thus, it appears that osmolytes may be acting at a different step in
the folding reaction (i.e. on pathway reaction landscape) to facilitate renaturation.

One interesting result came from our comparisons of the irreversible misfolding kinetics of MDH with
35% glycerol or activated GroE. By delaying the addition of the activated chaperonin complex or glycerol after
initiating a PmMDH reactivation, we could obtain comparative irreversible kinetic profiles prior to chaperonin
or glycerol dependent rescue and could determine the time it takes for PmMDH to partition into an irreversible
misfolded conformation. If glycerol, like the Hsp100 classes, could affect or disrupt the aggregation reaction
prior to the commitment to irreversible misfolding, a delayed addition of glycerol should yield a different
kinetic decay profile (i.e. slower decline in activity than that observed with the chaperonin). Contrary to this
prediction, the irreversible misfolding kinetics for both systems was identical. The observed similarity in rates
may indicate that glycerol and the chaperonin are rescuing the same intermediate populations from misfolding.

Some aspects of the glycerol-assisted folding of PmMDH do mirror the chaperonin-assisted folding
reaction. For both systems, glycerol accelerates the apparent PmMDH reactivation and large-scale aggregation
is prevented. If the Ranson kinetic scheme for PmMDH refolding (21) truly reflects the molecular events, the
fits to our data indicates that the collapse or accumulation of folded monomers to assembly competent forms is
accelerated or enhanced when glycerol is present (Table 1). Apparently, this glycerol dependent collapse may
be more substantial than the increase in assembly competent monomers observed when chaperonin is used to
facilitating folding (Table 1). However, we must be cognizant of the fact that we are only examining
the renaturation profiles and that the molecular events preceding the association and renaturation (i.e. the
folding reaction toward an assembly competent PmMDH monomer) cannot be directly determined by the
analysis presented here. The PmMDH reactivation reflects the final step in the formation of the native active
dimer (21,34). With glycerol present, the chaperonin-dependent PmMDH reactivation occurs with ATP alone
instead of with ATP and GroES combined. Glycerol-jump experiments demonstrate that the GroEL-PmMDH
complex relaxes very rapidly when the folding conditions are quickly shifted between non-permissive to
permissive folding environments. It appears that glycerol inhibits off-pathway large-scale aggregation and its
presence influences the folding of the bound intermediate. The effects of glycerol are certainly not limited to
the substrate itself. Curiously, glycerol appears to affect the chaperonin itself by stabilizing this oligomer against denaturation. Titrations show that the hydrophobic dye binding reactions involving ANS approaches saturation at lower ANS concentrations (Voziyan and Fisher, preliminary data).

We have observed that various osmolytes also accelerate PmMDH reactivation with or without chaperonins. In addition, with glycerol present, the interaction between activated chaperonin and refolding PmMDH subunits cannot be detected by rapid immunoprecipitation. Given the observed influence of glycerol on spontaneous PmMDH folding, we tend to favor the explanation that osmolytes, particularly glycerol, primarily influences the folding of the PmMDH subunits. Indeed, other investigators have provided evidence suggesting that osmolytes may enhance folding because these compounds may decrease the activation energy barrier for folding, leading to a more rapid polypeptide collapse (15,42).

Even though the molecular basis behind the glycerol-induced enhancement of the PmMDH renaturation rates cannot be determined by the data presented here, it is clear that this enhancement leads to a reduction in the chaperonin requirements. The accumulated literature data supports the notion that some polyols such as sucrose and glycerol can stabilize proteins by shifting the equilibrium toward more compact or collapsed states, perhaps explaining the observed increase in rates of the apparent folding reactions (15,16). Likewise, a reduction in the population of transient expanded states may also diminish the available species that lead to off-pathway aggregation (43). Consequently, this decline in the population of transient expanded states may explain why the activated chaperonin no longer interacts very well with the refolding PmMDH monomers.

Although the exact mechanism governing this accelerated folding are not clear, polyols may decrease the volume of the transient state intermediate (15). By measuring specific transfer free energies of amino acid and peptide backbone between aqueous and osmolyte containing solutions, Bolen and coworkers have uncovered a possible molecular explanation for osmolyte-induced stabilization (44,45). Experimental evidence suggests that the denatured and native states of proteins are both destabilized by osmolytes. However, Bolen’s thermodynamic data indicates that the destabilization of the denatured state far exceeds the destabilization free energy of the native state because the peptide backbone solubility decreases in osmolyte solutions. This disparity in destabilization results in a net increase in the free energy of stabilization (44,45). If we extend this property to the energy levels of folding intermediates and transition states, an acceleration in folding can result primarily from a destabilization of the intermediate state, a stabilization (or smaller destabilization) of the more native and compact transition states or osmolytes may affect both states. The bottom line is that any subsequent decrease in the activation energy barriers (the folding landscapes) will result in faster folding rates (46,47). With glycerol present, faster folding of the PmMDH intermediate towards an assembly competent,
native-like monomer may result from any decrease in the activation energy differences between folded intermediates and transition states. Using the kinetic model presented by Clarke and coworkers (21,22), our analysis suggests that there may be an increase in the microscopic rate constant for the collapse toward an assembly competent monomer by at least 1 to 4 orders of magnitude (Table 1). Unfortunately, the analysis performed herein does not provide any information about the energy levels of the transition states. However, any resulting increase in the concentration of assembly competent monomers will naturally lead to an increase in apparent rate of the bimolecular assembly reaction and may bypass the need for any chaperonin assistance.

The effect of glycerol on the entire reaction mechanism also shows that the off-pathway kinetics have changed. Clarke and coworkers have suggested that the chaperonins influence the partitioning reactions of off-pathway intermediates (21,22). The fit of our data to the model predicts that the aggregation reaction will also decrease by an order of magnitude (see k5, Table 1). The slower aggregation reaction coupled with the rapid collapse to assembly competent monomers correlates with the observed loss in large aggregates formation during spontaneous refolding (Figure 8). The effect of glycerol on renaturation of PmMDH may not be specific because we also observe an accelerated and/or enhanced folding (37°C) of PmMDH by other osmolytes such as betaine, sucrose, and trimethyl amine N-oxide (TMAO). Curiously, betaine and TMAO do not change the chaperonin requirements when refolding PmMDH binds to the high affinity chaperonin (Table 2). Here again is another situation where the GroES requirement is not dictated by “permissivity” of the folding conditions (17, 39). Since various osmolytes show differential interactions with proteins and change the chaperonin requirements (48-50), our future studies will examine how betaine and TMAO effect PmMDH folding kinetics.

In summary, our experimental results support the notion that the folding rates, rather than the structural identity or folding class of chaperonin substrates, are the key determinants that dictate interactions between transient folding intermediates populations and chaperonins (9,10). The folding intermediate structures of homologous proteins that require chaperonins to fold are predicted to have longer lifetimes and/or slower on-pathway folding rates. In addition, our studies indicate that some osmolytes will profoundly influence the chaperonin requirements and that osmolytes certainly may influence the folding kinetics and stabilization of on-pathway protein folding intermediates.
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Table 1. Kinetic fits to the Spontaneous PmMDH Renaturation profile with Various Concentrations of Glycerol using Ranson Model (Ranson et al., 1995).

at 20°C.

|          | 0% glycerol | 7% glycerol | 20% glycerol | 35% glycerol |
|----------|-------------|-------------|--------------|--------------|
| $k_1$    | 2.41E-04    | 5.96E-04    | 1.28E-03     | 8.84E-04     |
| $k_2$    | 1.39E+04    | 4.04E+04    | 8.46E+04     | 7.68E+05     |
| $k_3$    | 1.17E+05    | 8.11E+04    | 5.68E+04     | 8.19E+04     |
| $k_4$    | 4.56E-04    | 2.34E-04    | 1.14E-04     | 1.46E-04     |
| $k_5$    | 9.15E+03    | 1.02E+04    | 7.30E+03     | 2.33E+03     |

|          | 50% glycerol | 60% glycerol | 70% glycerol |
|----------|-------------|-------------|--------------|
| $k_1$    | 4.07E-04    | 8.15E-04    | 1.27E-04     |
| $k_2$    | 4.24E+05    | 8.04E+04    | 6.22E+04     |
| $k_3$    | 2.77E+04    | 3.95E+05    | 1.07E+04     |
| $k_4$    | 4.57E-04    | 2.78E-04    | 4.40E-05     |
| $k_5$    | 1.32E+04    | 2.00E+03    | 2.09E+03     |

at 37°C (no chaperonins)

|          | 0% glycerol | 20% glycerol | 35% glycerol | 50% glycerol |
|----------|-------------|--------------|--------------|--------------|
| $k_1$    | 9.68E-04    | 1.18E-02     | 3.94E+00     | 8.21E+00     |
| $k_2$    | 3.84E+06    | 1.50E+07     | 1.59E+05     | 4.82E+04     |
| $k_3$    | 7.81E+06    | 3.84E+07     | 3.46E+08     | 2.36E+08     |
| $k_4$    | 2.76E-04    | 8.08E-03     | 1.98E-03     | 1.18E-03     |
| $k_5$    | 3.53E+05    | 2.02E+04     | 4.89E+04     | 2.94E+04     |

With chaperonins and 35% glycerol at 37°C.

| Spontaneous, 35% glycerol | GroEL and ATP, 35% glycerol | GroEL, GroES, & ATP, 35% glycerol | GroEL, GroES ATP, no glycerol |
|---------------------------|-----------------------------|-----------------------------------|--------------------------------|
| $k_1$                     | 4.60E+00                    | 9.97E+00                          | 3.45E-02                       |
| $k_2$                     | 1.49E+05                    | 1.82E+04                          | 1.10E+06                       |
| $k_3$                     | 3.34E+08                    | 2.81E+08                          | 1.96E+07                       |
| $k_4$                     | 1.64E-03                    | 1.83E-03                          | 5.50E-03                       |
| $k_5$                     | 3.18E+04                    | 7.28E+04                          | 1.39E+05                       |
Table 2: Renaturation of PmMDH with other osmolytes.

The data show that other commonly used osmolytes will facilitate the renaturation of PmMDH at non-permissive folding temperatures (37°C). The folding conditions were the same as described in the Materials and Methods section.

| additive (conc.)     | percent original activity recovered* |
|----------------------|-------------------------------------|
|                      | no GroEL | + GroEL and 5 mM ATP | +GroEL,GroES and 5 mM ATP |
| no additive          | 0        | 6 ± 3                | 60 ± 13                   |
| Glycerol (4M)        | 60 ± 12  | 73 ± 12              | 75 ± 13                   |
| Sucrose (1M)         | 45 ± 3   | 59                   | 71 ± 15                   |
| Betaine (1M)         | 78 ± 30  | 0                    | 91 ± 10                   |
| TMAO**(1M)           | 36 ± 20  | 0                    | 95 ± 10                   |

* At least 3 different series were measured with three replicates per series.

** TMAO – trimethylamine N-Oxide.
Figure legends

Figure 1. A) The sequence comparisons show that these isozymes exhibit 58.2% sequence identity and 80.3% sequence similarity. The top sequence and bottom sequences shown are *E. coli* malate dehydrogenase, EcMDH, and porcine mitochondrial malate dehydrogenase, PmMDH, respectively. B) PmMDH and EcMDH are structurally homologous (shown here as monomers). These structures were generated using RasMol v.2.6-UCB (Glaxo Wellcome Ltd.) with PDB files 1mld.pdb (PmMDH) and 2cmd.pdb (EcMDH).

Figure 2. Comparison of refolding EcMDH and PmMDH in the absence and presence of chaperonins at 37°C. For both isozymes, folding is initiated by a 100 fold dilution of concentrated MDH (5 µM) into refolding buffer. The refolding buffer solutions for both sets of experiments contain 50 mM triethanolaime hydrochloride, 20 mM MgCl₂, 50 mM KCl, and 10 mM Dithiothreitol, pH 7.5 at 37°C. The final monomeric concentrations of MDH are 0.1 µM. The chaperonin concentrations are 1 µM GroEL oligomer, 2 µM GroES oligomer, and 5 mM ATP. In Panel A, the refolding of EcMDH is examined in the absence of GroE chaperonins (●), in the presence of GroEL alone (■), in the presence of GroEL and ATP (▲) and in the presence of GroEL, GroES and ATP (▼). The spontaneous refolding of EcMDH was essentially as rapid (0.032 sec⁻¹) as the refolding profiles with GroEL-ATP (0.048 sec⁻¹) and GroEL-GroES-ATP (0.032 sec⁻¹) per a single exponential function. The slower renaturation rate (fitted to a double exponential function, k₁ = 0.0073 sec⁻¹ and k₂ = 0.00037 sec⁻¹) is only observed with GroEL alone. In panel B, the same conditions and symbols are illustrated as outlined in panel A. Here, the refolding of PmMDH is only observed when GroEL, GroES and ATP are present. The kinetic parameters resulting in the best fit to the renaturation profiles of PmMDH in the absence and presence of chaperonins are listed in Table 1.

Figure 3. Double-Jump experiments to determine if proline *cis-trans* isomerization is a major rate limiting step for PmMDH renaturation. The spontaneous renaturation rates and yield of active PmMDH are compared under various denaturation times. Denaturations of PmMDH are performed for 30 sec (●) and 2 hours (O). Although there is a slight enhancement of the renaturation rate, this rate is still considerably slower than the renaturation profile of active EcMDH (■) even after this isozyme is denatured for 2 hours.
Figure 4. Spontaneous renaturation of EcMDH and PmMDH with (●) and without (▲) 35% glycerol at 20°C. The spontaneous renaturation rate profiles and yields of EcMDH (Panel A) and PmMDH (Panel B) are performed at monomeric concentrations of 0.1 µM. The renaturation profiles demonstrate that the renaturation of PmMDH yields and rates are significantly enhanced with glycerol while EcMDH shows little change in the renaturation profiles (single exponential fits – k = 0.06 sec⁻¹ for 0% glycerol (▲) vs. 0.02 sec⁻¹ for 35% glycerol (●)). The kinetic parameters resulting in the best fit to the renaturation profiles of PmMDH are listed in Table 1.

Figure 5. The Spontaneous Renaturation of PmMDH with Various Concentrations of Glycerol. 0.1 µM PmMDH subunits are renatured in the presence of various concentrations of glycerol (v/v). Panel A) The spontaneous renaturation of PmMDH with different concentrations of glycerol permissive temperatures: 0% glycerol (Ο), 7% glycerol (■), 20% (▲) 35% (●), 50% (● ■■ ● ■■ ), 60% (■● ● ● ■●), and 70% (巨额) glycerol. At 20°C, the fastest renaturation profile is found at 20% glycerol. The highest yield is found when 35% glycerol is present. Panel B) The spontaneous renaturation of PmMDH with different concentrations of glycerol at non-permissive temperatures (37°C): 0% glycerol (Ο), 20% glycerol (●), 35% glycerol (■), and 50% glycerol (▲). PmMDH renaturation rates increase as the glycerol concentration is increased from 0 to 35%(v/v). The spontaneous recovery rates show a slight decrease as the glycerol concentration is raised from 35% to 50%. The kinetic constants are listed in Table 1.

Figure 6. The GroE Chaperonin Stringency for PmMDH Renaturation Is Diminished in the Presence of 35% Glycerol at 37°C. The refolding buffer is composed of 50 mM TEA, 50 mM KCl, 20 mM MgCl₂, and 10 mM DTT at pH 7.5. The final PmMDH subunit concentration is 0.1 µM. The chaperonin concentrations are 1 µM GroEL oligomer, 2 µM GroES oligomer, and 5 mM ATP. Renaturation profiles in the absence of chaperonins (●), the presence of GroEL (■), the presence of GroEL and ATP (▲) and in the presence of GroEL,GroES and ATP (巨额). Interestingly, in the presence of glycerol, the renaturation kinetics of spontaneous folding and folding with GroEL, GroES, and ATP are almost identical, and the renaturation of PmMDH in the presence of GroEL is severely arrested. The kinetic constants are listed in Table 1.
Figure 7. PmMDH Commitment to the Active State in the Presence of Different Combinations of the GroE Chaperonin Folding System with (open bar) and without (solid bar) 35% Glycerol. The PmMDH subunit concentration in all experiments is 0.1 µM. In the absence of glycerol, PmMDH rapidly combines with GroEL, GroEL and ATP, or the full complement of GroEL, GroES, and ATP and is removed from the folding medium. After two hours, only the full complement (last panel) shows significant activity. In the presence of glycerol, GroEL alone arrests PmMDH renaturation (second panel). Although PmMDH folds with GroEL and ATP (see third panel), substantial amounts of PmMDH still immunoprecipitates upon the immediate addition of anti-GroEL antibodies. In contrast, with glycerol present, almost equivalent amounts of PmMDH commit to reactivation in the absence (first panel) and presence (last panel) of the activated chaperonin complex (GroEL, GroES, ATP) when the immunoprecipitation is carried out at within 15sec and 2 hour after initiating renaturation.

Figure 8. The spontaneous renaturation of PmMDH at 37°C monitored by 90° Light Scattering. The formation of large protein aggregates during the spontaneous renaturation of PmMDH can be prevented when 35% glycerol is present in the refolding solution. The final PmMDH subunit concentration is 1µM (subunit).

Figure 9. The Final Renaturation Environment Determines the Stringency of the GroE Chaperonins for PmMDH at 37°C. The kinetic profiles represent the recovery of PmMDH activity from GroEL-PmMDH complexes after a 10 sec allowance of complex formation before either a 0 to 35% (●) or 35% to 3.5% (▼) glycerol jump is initiated. Similar profiles are obtained if the glycerol jump is performed on the GroEL-PmMDH complex after the complex is formed and incubated in the starting solution for 1 hr. The final yields for the successful jumps (35% glycerol final concentration) are similar for short and long delays. Only when the final renaturation environment contains 35% glycerol will the GroE chaperonin stringency requirements for renaturing PmMDH diminish from the full supplement of GroEL, GroES, and ATP to only GroEL and ATP.

Figure 10. The Kinetics of Irreversible Misfolding of PmMDH with 35% Glycerol and GroEL-GroES-(ATP)7 at 37°C. The irreversible misfolding kinetics of PmMDH with 35% glycerol (●) and with the nucleotide-activated chaperonin complex, GroEL-GroES-(ATP)7 (■), are essentially identical. Both sets of data can be
fit to a double exponential decay function with virtually identical rate constants for $k_1 = 0.48 \pm 0.6 \text{ sec}^{-1}$ and $k_2 = 0.06 \pm 0.008 \text{ sec}^{-1}$ or $0.01 \pm 0.010 \text{ sec}^{-1}$ for activated chaperonin and 35% glycerol, respectively. The final oligomer concentrations of GroEL and GroES were 1 $\mu$M and 2 $\mu$M. The ATP and PmMDH concentrations were 5 mM and 0.1 $\mu$M (monomer) respectively.

**Figure 11.** In the presence of 35% glycerol, PmMDH shows concentration-dependence renaturation rates. At final PmMDH subunit concentrations of 0.1 $\mu$M ($\Delta$) and 0.025 $\mu$M (O), the renaturation profile is clearly more rapid as the initial PmMDH concentration increases.
Figure 1. Tieman et al.,

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Sequence Identity = 58.2%  Sequence Similarity = 80.3%

MKVAVLGAAGGQAALALLKTQLPGSGEISLYDIAPVTGVAIVDLHPTAVKIKGFSGEDAT-
AKVAVLGAAGGQAALALLKTQLPGSGEISLYDIAPVTGVAIVDLHPTAVKIKGFSGEDAT-
ALEGADVVLISAGVARKPGRSDLFVNNAGIVKNIVQVQAKTPCIGIITNPNTTVAIAYEV
KLKCVDVVPAGVPRKPGMTDNLFNTNATIVATTAACAGCPPAMICIIISPVNTIPITAEV
LKKAVYIDKNLFGVTILDIISSNFVAELKGQGEVEFVIPGSSGTVILPLLSQI-VGVSFT
FKEHVNPKKEVGVLIDEKAAEFAVDELKGLDVARVSFYGVGAQKTIIPQCTHIPQ
QEVADLTKRIQAGTeVEAAGGQSAALSMCSAARFGLSVRLAGQGVECATVEGEQYAR
DQLSTLTGRIQEAGTEVKAGAGCSATLSMAYAGREFVFLYDAKNGKEGVECSFYKXSQETDCP
FFSQPLLKGKNGVEERSIGTLSAFEQNALEGMLDTHKIALGEFV--K  312 a.a.
YFRRLKLGKIGKSNFPQEEKMIAEAIPELMASIKKGEFVKNMK  314 a.a.

: conserved substitution,  | identical amino acid
Figure 2. Tieman et al.,

Fraction of Recovered MDH Activity

Time (minutes)

A

B

0 15 30 45 60 75 90

0.0 0.2 0.4 0.6 0.8 1.0

0 15 30 45 60 75 90

0.0 0.2 0.4 0.6 0.8 1.0

AB
Figure 3. Tieman et al.,
Figure 4. Tieman et al.
Figure 5. Tieman et al.,

Fraction of recovered PmMDH activity

Time (Hours)

0.0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8

0 1 2 3 4 21 22

A
Figure 6. Tieman et al.,
Figure 7. Tieman et al.,
Figure 8. Tieman et al.,
Figure 9. Tieman et al.,

![Graph showing Fraction of Recovered MDH Activity over Time (minutes)](image-url)
Figure 10. Tieman et al.,  

![Graph showing the fraction of recovered MDH activity over time.](image-url)
Figure 11. Tieman et al.,
A comparison of the GroE chaperonin requirements for sequentially and structurally homologous malate dehydrogenase: The importance of folding kinetics and solution environment

Bryan C. Tieman, Mary F. Johnston and Mark T. Fisher

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