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DOI:

[10.1016/j.bbapap.2018.05.015](https://doi.org/10.1016/j.bbapap.2018.05.015)

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Document Version

Peer reviewed version

Citation for published version (Harvard):

Jain, N, Knowles, TJ, Lund, PA & Chaudhuri, TK 2018, 'Minichaperone (GroEL191-345) mediated folding of MalZ proceeds by binding and release of native and functional intermediates', *Biochimica et Biophysica Acta*, vol. 1866, no. 9, pp. 941-951. <https://doi.org/10.1016/j.bbapap.2018.05.015>

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Publisher Rights Statement:

First published in *Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics*
<https://doi.org/10.1016/j.bbapap.2018.05.015>

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Minichaperone (GroEL191-345) mediated folding of MalZ proceeds by binding and release of native and functional intermediates

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ABSTRACT

It has long been known that isolated apical domains of GroEL (known as “minichaperone”) have the ability to bind and assist the folding of a wide variety of client proteins without GroES and ATP, but only little attention has been paid so far to the mechanism by which these much simpler chaperones act. Here, in order to probe into the mechanism of minichaperone consisting of residues 191-345 of the GroEL apical domain action in detail, we have examined the fate of a large, multidomain and highly aggregation prone maltodextrin glucosidase (MalZ) during minichaperone-mediated folding reactions. The key objective was to identify whether this MalZ is free in solution, or whether it represents protein that is still bound to, or cycling on and off, the minichaperone. When interference molecule like ‘GroES’ (trap all the hydrophobic binding sites present on the minichaperone molecule) was introduced during refolding process, we found that production of the native state of MalZ was strongly inhibited. We also observed the same findings with a trap mutant form of GroEL, which can stably capture MalZ that is released from minichaperone in a predominantly non-native conformation during refolding process in solution, but does not release it. Tryptophan and ANS fluorescence measurements indicated that refolded MalZ has the same structure as the native MalZ, but that its structure when bound to minichaperone is different. Surface plasmon resonance measurements provide an estimate for the equilibrium dissociation constant K_D for the MalZ-minichaperone complex of $0.21 \pm 0.04 \mu\text{M}$, which are significantly higher than for most GroEL clients. This showed that minichaperone interacts loosely with MalZ to allow the protein to change its conformation and fold while bound during the refolding process. These observations suggest that the minichaperone works by carrying out repeated cycles of binding aggregation-prone protein MalZ in a relatively compact conformation and in a partially folded but active state, and releasing them to attempt to fold in solution.

KEYWORDS:

Protein folding, Minichaperone assisted protein folding, folding intermediates, Maltodextrin glucosidase, Surface Plasmon Resonance

1. Introduction

When folding, small single domain proteins generally fold rapidly into their final conformations via a reversible two-state mechanism (1-6), although studies at the level of single molecules are starting to reveal multiple pathways even in these apparently simple cases (7). The situation with larger multi-domain proteins is more complex, and details of the pathways by which they fold are still under intensive study using a wide range of methods (8, 9). Folding of these proteins can be adversely affected by their propensity to misfold, due to incorrect interactions between hydrophobic side chains of their domains during protein folding causing the formation of misfolded kinetically trapped structures or non-functional aggregates (10-12). This problem is even more acute *in vivo*, due to excluded volume effects that can increase the likelihood of incorrect associations occurring between nascent or partially folded proteins (13). Cells circumvent this problem in part by the expression of molecular chaperones, which act by binding to nascent polypeptides or by providing protected environments where proteins can fold without interacting with other aggregation-prone intermediates (14-17)

The chaperonin GroEL is a complex molecular machine, consisting of 14 identical 57-kDa subunits arranged in two identical heptameric hollow cylindrical rings (18). It generally requires the action of the co-chaperonin GroES, and is probably the most extensively studied chaperone system. GroEL assists protein folding by a cycle of binding and release of its clients during which they are sequestered in the cavity in the centre of one of the heptameric rings, which is capped transiently by the heptameric GroES complex during the folding cycle. The cycle is mediated by the binding of the client protein and by the binding and hydrolysis of ATP, and during the course of the cycle the complex undergoes very significant structural rearrangements which are critical for its action (reviewed in 19-22). Each GroEL protomer has three domains: an equatorial domain which provides contacts for

transmission of allosteric information between the two rings and also is the site for ATP binding, an apical domain which binds client proteins and the cochaperonin GroES, and an intermediate domain which acts as a hinge allowing the rigid body movements of the reaction cycle. An intact GroEL complex is required for the full activity of the protein in vivo (23, 24). Despite intense study, several aspects of its function remain controversial, including whether it acts passively in provision of a protected folding space or actively in assisting the folding process, by disruption of misfolded intermediates (25-29).

Although full activity of GroEL requires the complete double ring structure, reduced forms can also show some activity. For example, a decrease in the tightness of the interaction between GroES and GroEL enables single ring forms of GroEL to support *E. coli* growth at normal temperatures and to mediate protein refolding in vitro (30-33). Monomeric fragments of the apical domain alone, referred to as “minichaperones”, show polypeptide binding and protein refolding activity even though they lack the allosteric properties and central cavity of wild type GroEL (34). Minichaperones have a conformation very similar to the corresponding region in the intact full-length protein and bind peptides in regions corresponding to the same position in intact multimeric GroEL (35, 36). Minichaperones can chaperone the refolding in vitro of human interferon gamma, cyclophilin A and rhodanese and can also unfold barnase by transiently binding to folding intermediates with exposed hydrophobic residues (34, 37). Minichaperones cannot support viable growth on their own in the absence of intact GroEL, but can partially suppress the temperature sensitivity of some *groEL* mutants, showing they do have some activity in vivo (38).

Molecular dynamics simulations have led to the proposal of two hypotheses to explain the mechanism of minichaperones. One proposes that the process of repeatedly binding and release polypeptides and that this is effective in assisting protein folding; this is referred to as the transient binding and release, or TBR, mechanism. The TBR model suggests that chaperones transiently and repeatedly bind proteins which are kinetically trapped in a partially folded state, disrupt their structure, and release them in a different state. These rounds of binding and releasing allow client proteins to find pathways either into the native state (which is no longer recognized by the chaperone), or into an unfolded state (which can undergo further processing by the chaperone) (39). The other is that folding by a

cageless chaperone-like minichaperone can only be accomplished if the protein binds loosely enough to the chaperone that it can change its configuration and fold while bound to the chaperone (40). To date, these two hypotheses have not been assessed experimentally.

Here, we report the results of a study on the interactions between *Escherichia coli* maltodextrin glucosidase (MalZ) and the minichaperone GroEL (191-345) that addresses this issue. MalZ is a 69 kDa monomeric enzyme that catalyses the degradation of maltodextrin to maltose by eliminating one glucose residue from the reducing end at each time (41); the biochemical assay for this is simple which allows easy monitoring of MalZ refolding from the denatured state. Its structure is known (42), and as it has 22 tryptophan residues, spectroscopic studies can be done at low concentrations. MalZ folding in vivo is significantly enhanced by co-expression of GroEL and GroES, and it has been shown to fold through a trans-only mechanism on GroEL, as it is too large to fit in the GroEL cavity under GroES (43). We show here using a range of methods that GroEL (191-345) can enhance the thermostability of MalZ by forming a binary complex with it, and enhances its refolding when present in significant molar excess.

2. Materials and Methods

2.1. Reagents and materials

Luria broth (LB), Luria agar (LA) and antibiotics were obtained from HiMedia (Mumbai, India). Standard molecular weight markers and isopropyl-d-thiogalactoside (IPTG) were obtained from Bangalore Genei (Bangalore, India). p-Nitrophenyl-d-maltoside (pNPM) was obtained from Merck Biosciences (Darmstadt, Germany). Gel extraction and plasmid purification kits were purchased from QIAGEN (Valencia, CA, USA). DNA ligase and other restriction enzymes were obtained from New England Biolabs (Ipswich, MA, USA). All other reagents were from Sigma-Aldrich (St. Louis, MO, USA) unless noted. Double distilled or Milli-Q (Merck Millipore) water was used throughout.

2.2. Strains and plasmids

DH5 α , BL21, and BL21-DE3 *Escherichia coli* strains were used respectively for cloning, expression, and purification of MalZ and minichaperone. The plasmid pCS19MalZ contains the *malZ* gene fused to a hexahistidine tag, and was a generous gift from W. Boos (University of Konstanz, Germany) (43). The plasmid expressing minichaperone (pET15b-miniCh) was a generous gift from Prof. Hideki Taguchi (Tokyo Institute of Technology, Yokohama, Japan). The plasmid pET23A expressing BirA biotin ligase (pET23a-BirA) was a generous gift from Dr. Mahboob Salim (University of Birmingham, U.K.).

2.3. Expression and purification of minichaperone and MalZ

Escherichia coli BL21 (DE3) cells were transformed with pET15b-miniCh and pCS19MalZ for overexpression of minichaperone and MalZ, respectively. A single colony was inoculated in 5ml LB and incubated at 37°C in a shaking incubator at 220 RPM. When the OD600 reached 0.8, the culture was transferred into 1 litre LB medium and grown under similar conditions. Cultures were induced with 0.1mM IPTG when OD600 of 0.6 was reached; these were then further grown for 12h. The culture was harvested by centrifugation and washing with 20mM NaPO₄, 500mM NaCl (buffer A), followed by resuspension in 30 ml buffer A. Cells were lysed by sonication, and centrifuged at 25,000 g for 60 min in a Sorvall RC6 plus centrifuge to yield a soluble extract. The supernatant was collected and passed through a 0.45 μ m filter before chromatography using an AKTA FPLC system (GE Healthcare and Life Sciences). Both MalZ and minichaperone were purified by passing the supernatant through a Ni-NTA chelating column, equilibrated in buffer A containing 1 mM DTT. The proteins were eluted using a linear gradient of 0–500 mM imidazole. Minichaperone and MalZ eluted at 300 mM and 250 mM imidazole respectively. Fractions containing proteins were identified by SDS-PAGE, and those having greater than 99% purity were pooled.

The concentration of MalZ and minichaperone were determined spectrophotometrically at 280nm with extinction coefficients, estimated from the amino acid sequences using the ProtParam tool of ExPASy (<http://web.expasy.org/protparam/>), of 152665 M⁻¹ cm⁻¹ and 8480 M⁻¹ cm⁻¹

respectively.

2.4. MalZ assay

The enzymatic activity of MalZ was determined as described (41). Briefly, MalZ activity was measured by the rate of hydrolysis of p-nitrophenyl acetate by monitoring the increase in the absorbance at 405 nm as a function of time. Activity assays were carried out at 25°C.

2.5. Minichaperone-assisted refolding of GdnHCl-denatured MalZ

All denaturation and renaturation experiments were done in 20 mM NaPO₄, pH 7.4, 100 mM NaCl (RF buffer) at room temperature. MalZ is fully denatured in 2 M GdnHCl (44). MalZ (30µm) was denatured for 2h in the presence of 2 M GdnHCl. Renaturation experiments were initiated by diluting the sample 1: 100 in the absence and presence of various concentrations of minichaperone. Renaturation was expressed as a percentage of the activity of the same mass of native enzyme incubated under the same conditions. For spontaneous refolding, the same refolding buffer was used. To control for possible macromolecular crowding effects, both lysozyme and BSA were used in separate control experiments at the same concentration as minichaperone (45). The effect of GroES was assayed by diluting chemically denatured MalZ into refolding buffer in the presence of minichaperone. The MalZ was allowed to refold for different time duration (20-300 min) followed by addition of GroES to the refolding mixture. GroES and minichaperone concentrations were calculated with respect to monomeric molecular weight. The effect of adding a GroEL trap was assayed by allowing MalZ to refold in the presence of minichaperone, and then removing aliquots at different time points, adding GroEL G337S/I349E in a 1:1 molar ratio to MalZ, and determining MalZ activity after further 10 minutes incubation.

2.6. Time course of thermal inactivation and reactivation of MalZ

The time course of temperature induced inactivation of MalZ was measured by incubating a 0.5 µM solution of the enzyme at the desired temperature (45, 50 or 55°C) in RF buffer in the absence or presence of minichaperone and periodically

removing samples and assaying them for enzymatic activity at 25°C. For reactivation experiments, MalZ was thermally denatured at the desired temperature until it lost its activity, after which samples were cooled down to 25°C. Aliquots (50µl) were taken at the different times following this and MalZ activity was assayed.

2.7. Aggregation Kinetics

The kinetics of heat-induced aggregation of MalZ were measured by light scattering in a Cary Eclipse fluorescence spectrophotometer equipped with a temperature controller and thermo probes using a quartz cuvette of path length 1 cm. 0.5 µM MalZ was heated at 50°C in RF buffer alone or in the presence of different molar ratios of minichaperone in RF buffer. Aggregation was monitored by light scattering at 500 nm with excitation and emission slits 5 and 2.5 nm, respectively. Solutions were constantly stirred to avoid formation of bubbles.

2.8. Purification of tagged minichaperone for SPR

A *birA* tag was added to the 3'-end of the minichaperone gene so that the minichaperone could be immobilized on a CN5 streptavidin-coated chip for the SPR experiments. To do this, a *PsiI* site was introduced into pET-miniGro using an Agilent Quick-change mutagenesis kit with primers 1 and 2 (SUP Table T1) which introduce a TTA before the stop codon in the minichaperone gene to make a *PsiI* site (TTATAA). The resulting plasmid was called Mut pET-miniGro. The sequence encoding BirA was PCR amplified from pET23a (BirA) using primers 3 and 4 (SUP Table T1) and the ThermoScientific Phusion High Fidelity PCR kit. Primers were designed in such a way that PCR products share 15 bp of homology with the ends of linearized Mut pET-miniGro following digestion at the *PsiI* site, with the remaining primer sequence complementary to the BirA tag. PCR fragments were purified using the Qiaquick PCR purification kit (Qiagen, UK) and the BirA tag was then integrated into the vector by incubating PCR-amplified birA with *PsiI*-cut Mut pET-miniGro with the In-fusion HD cloning kit. Plasmids containing fragments in the correct orientation were identified by DNA sequencing, and the resultant construct was named BirA/pET-miniGro (Fig. S2). The presence of the BirA tag in the minichaperone protein was confirmed by SDS-PAGE followed by Western blotting.

2.9. Surface Plasmon Resonance

A Biacore 3000 (Sweden) surface plasmon resonance-based biosensor was used to measure affinity constants for the interactions between proteins. CM5 research grade sensor chips and P20 surfactant were from Biacore. N-Hydroxysuccinimide, N-ethyl-N9- (3-diethylaminopropyl) carbodiimide, and ethanolamine coupling reagents (Biacore) were used to immobilize 5,000 RU of the streptavidin to the sensor surface using a standard amine-coupling procedure. Minichaperone protein containing the BirA tag (100 μ M) was biotinylated using succinimidyl-6-(biotinamido) hexanoate (NHS-LC biotin, 500 μ M) according to the manufacturer's instructions. Biotinylated minichaperone protein was coupled to the chips to ~800-1000 response units. The flow cell temperature was 25°C. The running buffer was RF buffer plus 0.005% P20 surfactant (Biacore). MalZ was denatured in 2M GdnHCl for 60 min confirmed through the loss of activity and loss of intrinsic fluorescence of MalZ. Chemically denatured MalZ protein was injected over the minichaperone coupled chips and streptavidin control chips with a flow rate of 10 μ l/min. Binding surfaces were regenerated by washing with 0.5 M EDTA.

To determine the dissociation constant (K_D), the concentration of denatured MalZ injected onto the sensor chip was varied from 0.05 to 0.5 μ M. In all sensograms shown, the signals obtained from streptavidin control chips were subtracted from the signals obtained from the chips with coupled minichaperone. The K_D value was determined from the plot of the equilibrium binding responses as a function of the concentrations of MalZ, using steady-state affinity program of BIA evaluation Software (Biacore, Sweden).

2.10. Thermofluor assay

Thermofluor experiments were performed on Stratagene MxPro QPCR system. Solutions of MalZ and minichaperone separately were made by mixing with 1X sypro orange dye and were loaded to the wells of a 96-well thin-wall PCR plate (Bio-Rad). The plates were sealed with Optical-Quality Sealing Tape (Bio-Rad) and heated from 20 to 90 °C in increments of 1°C/min. The changes of fluorescence in the wells of the plate were detected with a charge-coupled device (CCD) camera. The excitation and emission wavelengths were 490 and 575 nm respectively. The concentration of MalZ and minichaperone were used 2 μ M and 8 μ M respectively.

2.11. Intrinsic and extrinsic fluorescence measurements

Fluorescence spectra were recorded in a Cary Eclipse fluorescence spectrophotometer (U.S.A.) equipped with temperature controller and thermo probes. The excitation wavelength was 295nm. Excitation and emission slits were set at 5 nm. Fluorescence of ANS (8-anilino-1-naphthalene sulfonic acid) was used to probe the accessibility of hydrophobic surfaces on the protein. Fluorescence emission signals of ANS in the presence and absence of 0.5 μ M MalZ were collected in the range between 400 and 600 nm with excitation wavelength 375nm.

2.12. MALDI-TOF mass spectrometry

The molecular mass of minichaperone was confirmed by MALDI-TOF mass spectrometry (ABI Sciex 5800 TOF). Super DHB (2, 5-dihydroxybenzoic acid) matrix was used. Minichaperone of concentration 20 μ M was put to the two spot sites on a 384-well stainless steel target plate and the samples were air-dried for 10 min. The matrix–organic solvent mixture (1 μ L) was then added to the spot sites and air-dried at room temperature. The minichaperone sample was spotted in duplicate for reproducibility. Once the samples were completely dry, they were introduced into the mass spectrometer and then the spectrum was recorded.

2.13. Size exclusion chromatography

Native minichaperone (100 μ M) and native MalZ (20 μ M) were passed through a prepacked size exclusion column (Superdex-200, 10/300) pre-equilibrated with RF buffer connected to an AKTA FPLC system. A flow rate of 0.5 ml/min was maintained throughout. The MalZ/minichaperone complex was obtained by incubating MalZ and minichaperone (in a molar ratio of 1:5) at 50°C for 60 minutes, until complete loss of the MalZ activity. The mixture was then cooled to 25°C and centrifuged at 14,000 rpm for 10 minutes to remove any aggregate. The supernatant was collected and applied to the column. Samples were injected into the column using a 50 μ l loop. Three fractions from each of the two peaks were collected and run on a 12% SDS gel followed by Coomassie blue staining. The data was analysed using inbuilt Unicorn software (GE Health care, USA) and the area under the peak corresponding to MalZ and minichaperone was calculated in accordance with manufacturer's instructions.

3. Results

3.1. Molecular mass determination

The oligomerization state of minichaperone was determined by MALDI-TOF. Three peaks were seen (Fig. S1), suggesting that the protein exists in solution in mixed oligomeric states including monomers, dimers and trimers (molecular weight 18.834 kDa, 37.556 kDa and 57.313 kDa, respectively). In the experiments below, the molecular mass of minichaperone was always calculated as a monomer.

3.2. Minichaperone can reactivate chemically-inactivated MalZ

To determine the ability of minichaperone to reactivate chemically denatured MalZ, MalZ was unfolded in 2 M GdnHCl and then diluted 100-fold into refolding buffer in the presence or absence of different concentrations of minichaperone. Aliquots from each refolding mixture were withdrawn after 20 minutes and their MalZ activity was measured. As shown in Fig. 1A, the spontaneous refolding of MalZ in the absence of any chaperone was ~5%. The percentage of recovery of MalZ activity ranged from 7% (at 5-fold molar excess of minichaperone) to 45% (at 100-fold molar excess of minichaperone). In Fig. 1B, the model is representing the spontaneous and minichaperone assisted folding of MalZ. Increasing the concentration of minichaperone beyond 30 μ M (100-fold molar excess) had no further effect on refolding yield. Renaturation of MalZ was not affected by a 30-fold molar excess of either lysozyme or BSA, showing the renaturation is not simply caused by the presence of an excess of another protein with hydrophobic surfaces.

Analysis of the time course of MalZ reactivation in the presence of 100 fold molar excess of minichaperone showed an initially rapid increase in MalZ activity, which decreased to effectively zero at twenty minutes, after which no further change took place up to 120 minutes (Fig. 2). Thereafter, a gradual decrease in activity was observed until it reached ~35% final residual activity after 180 min. This pattern of initial increase and subsequent decrease in activity is consistent with multiple rounds of binding and release with MalZ being folded into a form that is active but not fully

folded, and which can undergo aggregation during prolonged incubation with minichaperone.

To measure the K_D between minichaperone and denatured MalZ, we used surface plasmon resonance (SPR) with a sensor chip coated with immobilized minichaperone. When 2 M GdnHCl–denatured MalZ was injected onto this sensor chip, an increase in response units (RU) was detected (Fig. 3). No increase of RU was detected when native state MalZ was injected, showing that minichaperone does not bind to native MalZ but binds to one or more of its folding intermediates (Fig. 3A). The values of RU as a function of the concentration of denatured MalZ are shown in Fig. 3B. These data were fitted to a hyperbolic curve according to a single binding affinity model using in-built nedit software. The value of the dissociation constant, K_D , was estimated to be $0.21 \pm 0.04 \mu\text{M}$. Association and dissociation of denatured MalZ were too fast to accurately determine the kinetic constants k_{on} and k_{off} .

3.3. Using GroES and a GroEL-trap as a probe suggests multiple rounds of binding and release of MalZ

After 20 minutes of refolding in the presence of minichaperone, MalZ regains ca. 45% of its activity, and, as discussed above, SPR showed that the binding of MalZ to minichaperone is relatively weak. We wished to know whether this MalZ is free in solution, or whether it represents protein that is still bound to, or cycling on and off, the minichaperone. As the minichaperone is part of the GroEL apical domain and contains the hydrophobic sites to which GroES binds (46), we investigated the use of GroES as a competitor to probe the interaction between MalZ and minichaperone. Different amounts of GroES were added to the folding reaction after 20 minutes, at which point the MalZ has regained its maximal level of activity. The results (Fig. 4A) showed that addition of GroES caused a concentration-dependent drop in MalZ activity, reaching a maximum at a two-fold molar excess of GroES, after which no further effect was seen. At this concentration of GroES, only 15% of MalZ activity could be recovered. We interpret this as showing that approximately two thirds of the measured activity of MalZ at 20 minutes is due to protein that is still interacting with the minichaperone and is still aggregation-prone. We propose that when GroES is added, some of this bound MalZ protein is displaced into the solution, where it either

misfolds or aggregates. If this hypothesis is correct, then we can predict that the addition of a molar excess GroES to the refolding mix at the start of the reaction will remove the effect of the minichaperone, since all the sites needed to bind the MalZ client protein will be occupied by higher affinity GroES. As a result, only 5% refolding activity would be achieved, equivalent to spontaneous refolding. We confirmed that this was indeed the case (Fig. 4b). This figure also shows that GroES alone does not interfere with the spontaneous refolding of MalZ in the absence of minichaperone. This hypothesis was further tested using spectroscopic methods, described below.

Our observation (Fig. 2) that MalZ activity in the refolding reaction gradually declined after 20 minutes to a final stable level of 35% is consistent with a TBR model where bound MalZ continues to cycle on and off the minichaperone and as it does so some folds into the stable native state and some is lost to aggregation. By this model, the minichaperone is exerting its effect predominantly by providing hydrophobic surfaces to which unstable intermediates can transiently bind, thus reducing the concentration of those intermediates in the folding solution. If this model is correct, we would predict that over time the effect of GroES on the refolding reaction would decrease, since less MalZ would be bound by the minichaperone and hence be able to be competed off by GroES binding, and more would be fully folded in solution. We tested this by adding GroES to a minichaperone/MalZ refolding reaction at different times after initiating the reaction and confirmed that the effect of GroES did indeed decrease, until at 180 minutes it had no effect on yield at all (Fig. 5).

Although this result is consistent with the TBR model, it does not rule out the possibility that folding takes place entirely on the surface of the minichaperone, and that when used as a probe GroES displaces the MalZ into the solution due to its higher affinity for the sites to which MalZ binds. To distinguish these two possibilities, we used a trap variant of GroEL (GroEL G337S/I349E (47)), which can stably capture non-native MalZ in solution, but does not release it. We first showed that the presence of the GroEL trap alone has no impact on spontaneous refolding of MalZ (Fig. 6A). We then allowed MalZ to fold in the presence of minichaperone, removed aliquots at different time points, added the GroEL-trap in a 1:1 molar ratio with MalZ, and determined MalZ activity after further 10 minutes incubation. The results (Fig. 6B) showed that for the first 100 minutes of the reaction, the addition of GroEL-trap

reduced MalZ activity to ~5%, equivalent to spontaneous refolding. When GroEL-trap was added later, the reduction was reduced, and after 180 minutes, no effect on the yield was seen. This is more consistent with the TBR model, with the trap binding to and preventing further refolding of MalZ that is cycling on and off the minichaperone, and with the appearance of an intermediate (or fully folded) form of the protein that does not bind to GroEL trap at approximately 100 minutes.

3.4. Fluorescence analysis of intermediates in minichaperone-assisted refolding

To get a more detailed insight into the conformations of MalZ intermediates formed during minichaperone-assisted refolding in both the absence and presence of GroES, MalZ was further characterized by intrinsic and extrinsic fluorescence spectroscopy.

Fluorescence can be used to specifically examine MalZ because minichaperone lacks tryptophan residues, whereas MalZ from *E. coli* contains 22 tryptophan residues (41). Fig. 7A shows the fluorescence spectra of different conformations of MalZ. Native MalZ exhibited an emission maximum at 341 nm (curve a), whereas in 2 M GdnHCl, in which MalZ fully unfolds (44), the emission maximum was shifted to 356 nm (curve f). This corresponds to the fluorescence maximum of tryptophan in aqueous solution, the red shift of fluorescence λ_{max} reflecting transfer of Trp residues to a more polar environment. However, the fluorescence spectrum of MalZ refolded for 20 minutes from a GdnHCl-denatured state in the presence of minichaperone exhibited λ_{max} at 346 nm (curve b). The blue shift of λ_{max} from the MalZ unfolded state suggests that at least some of the tryptophan residues have returned to a native state environment. These results provide further supporting evidence for the hypothesis that MalZ bound to minichaperone is neither native-like nor completely unfolded but more in a partially folded intermediate. Curve e shows the spectrum when GroES was added after 20 minutes to the sample refolded in the presence of minichaperone. This reduced the fluorescence intensity of MalZ by 53% (compared to fluorescent intensity of MalZ refolded in the presence of minichaperone; curve b), with a slight blue shift (λ_{max} =344 nm). This is consistent with the model proposed above that GroES displaces bound MalZ intermediate from minichaperone and prevents further interactions; the loss in fluorescence intensity

and the blue shifted spectrum is consistent with the release of more compact MalZ along with the aggregation of loosely folded intermediates. We further observed that when the fluorescence spectrum of MalZ refolded in the presence of minichaperone was recorded with or without GroES after 180 min of refolding, the λ_{max} shifted to 342 nm (curves c and d), similar to that of native MalZ. This indicates recovery of substantial tertiary structure following prolonged incubation with minichaperone, consistent with the results above. The fluorescence intensity is somewhat reduced, presumably due to loss of aggregated protein during refolding.

The compactness of MalZ at different stages of refolding under different conditions was further analysed by using the external fluorescent probe 1-anilino-naphthalene-8-sulphonate (ANS), which is widely used to report the different states that occur during protein folding, with the results shown in Fig. 7B. In aqueous solution, ANS has very weak fluorescence intensity with λ_{max} of 525. As shown in Fig. 7B (bar 1), the ANS fluorescence intensity of native MalZ was also very low, as expected since hydrophobic side chains are largely buried in native globular proteins. Both minichaperone (bar 2) and GroES (bar 3) show moderate fluorescence, consistent with the exposure of some hydrophobic residues in the native states of these proteins. The ANS fluorescence of the GroES and minichaperone mixture (bar 6) was much less than the sum of their individual fluorescences. This suggests that they are interacting in a way that leads to burial of some of the hydrophobic residues, consistent with the proposed interaction of the GroES with the binding site on the minichaperone. Denatured MalZ exhibited almost the same fluorescence intensity as the native form, consistent with our earlier studies (44 and data not shown). However, denatured MalZ when refolded for 20 minutes in the presence of minichaperone exhibited high ANS fluorescence (bar 5). This gain in the ANS fluorescence intensity, along with blue shifting of the spectrum, points towards generation of exposed hydrophobic regions in the MalZ/minichaperone complex during refolding. To probe the effect of adding GroES to the MalZ-minichaperone mix, the ANS fluorescence of minichaperone and GroES was monitored. The ANS fluorescence of MalZ that has been refolded for 20 minutes in the presence of minichaperone followed by addition of GroES (bar 7) is higher than the fluorescence of GroES and minichaperone alone (bar 6). However, after 180 minutes of refolding in the presence of minichaperone without or with the addition of GroES, native-like

ANS fluorescence was observed (bars 8 and 9 respectively). This is consistent with the earlier data suggesting that MalZ acquires a structurally compact and native-like conformation after 180 minutes of refolding in the presence of minichaperone.

3.5. Minichaperone reduces thermal inactivation and enhances reactivation of MalZ

A further test of the role of minichaperone was undertaken by studying its impact on thermal inactivation of MalZ. We examined the fluorescence spectrum of MalZ after heating to 50°C, and found it had a λ_{max} of 346 nm, intermediate between that of native and chemically denatured MalZ maximum. This is consistent with the protein retaining some structure at this temperature. By determining the melting temperature (T_m) for both proteins, we found minichaperone to be more thermally stable than MalZ, consistent with earlier reports (48) (Fig. S3). We then examined the ability of minichaperone to protect MalZ against thermal inactivation at different temperatures. We measured the ability of minichaperone to protect MalZ against thermal aggregation at 50°C at a range of molar ratios (Fig. S4) by using light scattering to detect the presence of aggregates. The smaller molar excess of minichaperone which is required to protect MalZ against thermal denaturation relative to chemical denaturation may be due to the increase in hydrophobic interactions with temperature. The rates of thermal inactivation of MalZ activity were then determined in the presence and absence of minichaperone at 45°C, 50°C and 55°C. The results are shown in Fig. 8. A single-exponential equation was found to fit well to the data points (regression coefficient 0.98), showing that the inactivation follows first-order kinetics and is strongly temperature dependent. The first order rate constants for the inactivation reactions were estimated and are shown in Table 1. We concluded that the presence of minichaperone substantially reduced the rate of thermal inactivation of MalZ, particularly at 45°C.

If MalZ was cooled from 45°C to 25°C, almost full recovery of activity of MalZ was seen on incubating the sample for five minutes, even in the absence of minichaperone. However, reactivation after incubation at 50°C and 55°C was very low or absent. We examined whether the presence of minichaperone had an effect

on these results. As shown in Fig. S5, if MalZ enzyme that had been thermally inactivated in the presence of minichaperone was shifted from 50°C to 25°C a high recovery (about 65%) of activity of MalZ was obtained. A recovery of less than 10% was observed in the absence of minichaperone or in the presence of lysozyme (inset, Fig. S5). No MalZ reactivation was detected on shifting the samples from 55°C to 25°C followed by prolonged incubation, even in the presence of minichaperone. The protective effect of minichaperone was observed only if minichaperone was present during the initial high temperature incubation; addition at the time of temperature shift to 25°C did not result in MalZ reactivation (data not shown). This is consistent with formation of a minichaperone/MalZ complex during the thermal unfolding of MalZ at 50°C, which keeps MalZ in a reactivable form. This gave us the opportunity to use size exclusion chromatography to determine the nature of the species involved.

When native minichaperone was mixed with native MalZ at 25°C in a 5:1 molar ratio and run on a size exclusion column both proteins eluted as separate peaks at 17 ml and 15 ml respectively (Fig. 9A), consistent with a lack of measurable interaction between the proteins in their native states. When MalZ was heated alone at 50°C until its activity was reduced to zero, cooled to 25°C, and centrifuged before application to the column, no peak was observed, consistent with the formation of high molecular weight insoluble aggregates under these conditions (Fig. 9C). In contrast, when minichaperone was heated alone for 60 min and analyzed, no change in retention time was observed, confirming that minichaperone does not become insoluble on heating (Fig. 9B). We then incubated minichaperone and MalZ together in a 5:1 molar ratio at 50°C for 60 min, cooled them to 25°C, centrifuged them at high speed and applied them to the column. Two peaks were obtained (Fig. 9D) at the position of MalZ and minichaperone. Different fractions corresponding to each peak were collected and run on a 12% SDS-PAGE to analyze the species present. Only MalZ was seen in the earlier peak (Lanes 1-3, Figure 9E), but minichaperone plus a small amount of MalZ protein was seen in the later peak (Lanes 4-6, Fig. 9E), indicating that some of the MalZ was bound with minichaperone in a complex. It thus appears that some of the thermally denatured MalZ proteins regained their native conformation with the help of minichaperone and were released from the complex, while some are still attached to the minichaperone. A higher

elution time of MalZ/minichaperone complex (Fig. 9D) can be attributed to the interaction of a hydrophobic complex of MalZ/minichaperone with that of the size exclusion column matrix (49).

4. Discussion

There is debate about whether minichaperones mediate folding through a single round of association followed by folding on the surface of the minichaperone and eventual release of native forms, or through cycles of binding and release in which only a fraction of released molecules reaches the native form in any cycle (39, 40). While studying the molecular organization of minichaperone, we found that minichaperone exists in solution in a polydispersed state, and we detected mixed oligomeric species (monomer, dimer and trimer; Fig. S1). Our results show that when allowed to refold in the presence of minichaperone, MalZ denatured in 2 M GdnHCl exhibits a functional intermediate state, part of which eventually undergoes irreversible inactivation, but much of which can be folded to an active form if an excess of minichaperone is present. The residual activity was significantly higher (35%) than that of spontaneous refolding (5%) or folding in the presence of excess lysozyme or BSA (both proteins with significant surface hydrophobicity), affirming the chaperoning effect of minichaperone. Studies using GroES as a competitor at different time intervals during MalZ refolding suggest that during the early stage of refolding, some fraction of denatured MalZ is partially folded into an enzymatically active conformation (i.e., a functional intermediate), but that this intermediate is still aggregation prone and may be still cycling on and off the minichaperone. Such functional intermediates have been reported in the refolding pathways of malate synthase G (50). The GroEL-trap experiments confirm that the intermediate spends at least some time in free solution. Our experimental data support the Transient Binding and Release (TBR) model of chaperone assistance by minichaperone with a MalZ protein, as proposed in simulations (39) and both the ANS and tryptophan fluorescent data are fully consistent with the TBR model. It shows that the apical domain of GroEL alone weakly, transiently and repeatedly binds and releases the client protein, supporting its refolding in an ATP-independent process. Other in vitro studies with purified client proteins like rhodanese, RUBISCO, and malate

dehydrogenase also suggest that cycles of binding and release are linked to the chaperonin-assisted production of the native state (51-53). The refolding reaction to one or a collection of functional intermediates that are enzymatically active is relatively rapid, taking place in the first twenty minutes of the experiments, and is abolished by the presence of either GroES or GroEL-trap. This shows that the binding to the minichaperone reduces non-productive aggregation (possibly by lowering the concentration of the MalZ in free solution) but also that cycling of MalZ on and off the minichaperone must be occurring in this time. Hence, due to the cycle of binding and release, the protein concentration is reduced in the bulk solvent and as a result, the probability of aggregation formation decreases. Because of this, there is an enhancement in the yield of protein in the native state. Similar observations have been reported with regard to GroEL/ES- assisted folding pathways (54). The fluorescence studies of the intermediate are consistent with this interpretation. Similar intermediates states have been proposed in the case of chaperonin-assisted folding of DHFR and rhodanese (55). MalZ reached a state that is either fully folded, or at least no longer aggregation prone or able to be bound by GroEL-trap, after 180 minutes of refolding in the presence of the minichaperone.

The precise mechanism of the minichaperone is still not known. It could be inducing the MalZ to form an active intermediate that structurally favours non-local interactions with minimum energetic barriers, thus helping in attainment of native structure, and this could still take place while the protein is bound. However, its role may be more of a kinetic partitioning one, where unfavourable events that could lead to aggregation (which will require collision of partially folded intermediates and hence will be concentration dependent) are reduced purely by most of the protein being bound to the minichaperone at any given time, even though it is also cycling on and off. Binding of the intermediate is clearly demonstrated by the SPR experiments, as well as by the SEC of a minichaperone-MalZ complex that was seen to be present when minichaperone assisted in the refolding of thermally denatured MalZ. We note that the binding of MalZ to the minichaperone is weaker than that of other proteins to GroEL. For example, the binding constant of the complex between GroEL and reduced form of alpha albumin (rLA) has previously been measured by SPR, and in this case the K_D was estimated to be 1.06 nM (56). The binding constant of complexes between GroEL and clients like non-native lactate dehydrogenase, and a

peptide derived from β -lactamase which were measured by SPR and for which K_D values were estimated to be 9 nM and 10-100 nM respectively (57-58). This is consistent with the model which states that weak binding may be necessary to allow cageless chaperones to release their protein clients without the aid of ATP (40).

Like minichaperone, monomeric GroEL (one subunit of GroEL that is unable to form a central cavity) also possesses chaperone like activity and helps in prevention of aggregation and folding of chemically denatured client protein in the absence of GroES and ATP (59). The present study showed that a yield of 45% requires 100-fold molar excess of minichaperone, whereas up to 50% MalZ reactivation has been observed in the presence of only 1-fold molar excess of the complete GroEL system (43) and 25% MalZ activity regained in the presence of 14 fold molar excess of monomeric GroEL (59). This is consistent with the reported need for a large excess of cageless chaperone molecules to be present for them to be effective (24, 60). This may be necessary to reduce the likelihood that multiple proteins will bind to the same chaperone, which could lead to aggregation, or may simply be a reflection of the weak binding and the need to reduce the overall level of protein in free solution. The much lower stoichiometry required for minichaperone to refold thermally denatured MalZ is likely to be a reflection of incomplete denaturation of the heated MalZ; it may for example be the case that early folding intermediates produced by chemical denaturation are much more prone to aggregation and hence need a much high concentration of minichaperone to refold. We note from our thermal data that residual activity of MalZ decreases to zero at 45°C (Fig.8A), whereas full protein melting was only seen at 50°C (Fig. S3). This is likely to be because MalZ was heated from 20 to 90°C in increments of 1°C/min to determine the melting temperature, in contrast to the experiment shown in Figure 8A, where MalZ was heated at 45°C for a long time (Fig.8A), suggesting a slow loss of full structure occurs even at 45°C. The need for minichaperone to be present for recovery from thermal denaturation of MalZ, when misfolding and/or aggregation are most likely to occur, has also been observed with firefly luciferase and citrate synthase (61, 62). The failure of minichaperone to significantly protect MalZ heated to 55°C is reminiscent of the situation with small HSPs, which fail to reactivate citrate synthase denatured at higher temperatures (63).

Our present data provide a detailed insight into the action of minichaperone on one specific client, including some insight into the conformations that this client adopts during refolding, and in general support the TBR model for minichaperone assisted folding, though the possibility that some folding takes place on the chaperone surface itself certainly cannot be ruled out. However, we still do not understand completely the precise way in which minichaperone facilitate the folding of proteins. More work needs to be done to study the thermodynamic and kinetics of the folding of MalZ in the presence of minichaperone to gain further insights into the functioning of minichaperone as a chaperone. With the current sets of experiments it was not possible to determine how many cycles are needed before a non-aggregation prone intermediate is produced, although the GroEL-trap experiments showed that this was a slow process. Additional experiments using FRET or stopped-flow kinetic analysis are needed to investigate this. Taking into consideration the present studies, and further careful investigation on the changing conformational properties of a large protein along the minichaperone assisted folding pathway in the near future, this type of study can open the window to explore interactions between folding intermediates and minichaperone for elucidating the chaperone-assisted folding pathways of multidomain proteins.

It is tempting to speculate that molecular chaperones may have evolved from proteins analogous to the minichaperone described here, using ATP-independent cycling on and off the chaperone to protect the client from misfolding, and only later acquired the ATP-dependence seen in full length chaperones such as GroEL and DnaK.

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Acknowledgments

NJ gratefully acknowledges financial support from Boeheringer Ingelheim Fonds to facilitate my experiments (SPR and thermofluor) at the University of Birmingham, United Kingdom. NJ acknowledges joint Council of Scientific and Industrial Research-University of Grant Commission, Govt. of India for doctoral fellowship award no. 20-6/2008/ 2009. The authors acknowledge Indian Institute of Technology Delhi and the University of Birmingham for infrastructural support. TK acknowledges support from the Biotechnology and Biological Sciences Research Council, UK (grant number BB/L00335X/1).

Author contributions

NJ performed all the experiments. NJ, TKC, PL and TK designed the experiments. NJ, TK, PL, and TKC analyzed the data, and TKC, NJ and PL wrote the manuscript.

Competing financial interests

The authors declare no competing financial interests.

ABBREVIATIONS

SPR- Surface Plasmon Resonance; TBR- Transient binding and release; ANS- 8-anilino-1-naphthalene sulfonate; GdnHCl- Guanidine hydrochloride; T_m - Temperature of melting; MalZ- Maltodextrin Glucosidase.

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Table 1. First-order rate constants for thermal inactivation of MalZ in absence and presence of minichaperone. MalZ was at 0.5 μ m and minichaperone was at 2.5 μ m.

Temp (°C)	Rate constant (min ⁻¹)	
	Without minichaperone	With minichaperone
45	0.038 ± 0.004	0.004 ± 0.0003
50	0.25 ± 0.03	0.10 ± 0.015
55	0.56 ± 0.04	0.12 ± 0.01

SUP Table T1. Primers used for *birA* gene amplification and vector modification

Primers	Sequences (5'-3')
1.	GCAATCCAGGGCCGTT TT ATAAGGATCCGGCTGC
2.	GCAGCCGGATCCT TT ATAAACGGCCCTGGATTGC
3.	GCAATCCAGGGCCGTT TT <u>AGGTGGTGGTCTGAACGAT</u>
4.	GCAGCCGGATCCTT <u>ACCATTTCGATTTTCTGAGC</u>

The introduced TTA IS shown in bold letters. In primer 3 and 4, the coding sequence for the *birA* gene is underlined

FIGURE LEGENDS

Fig. 1. *In vitro* refolding of GdnHCl denatured MalZ in the absence or presence of minichaperone. (A) MalZ activity after 20 min of refolding is shown relative to the same amount of native MalZ (N-MalZ) (bar 1). Refolding was initiated by diluting 2 M GdnHCl denatured MalZ into the RF buffer under the following conditions: SR_f MalZ, RF buffer alone; MalZ+ lyso(1:30), buffer containing 30 molar excess of lysozyme; MalZ+BSA (1:30), buffer containing 30 molar excess of BSA; MalZ + mini (1:X), refolding in presence of X-fold molar excess of minichaperone. Numbers above bars show percentage of recovered MalZ activity. Final concentration of MalZ was 0.3 μM. Error bars represent standard deviations calculated from three separate experiments. (B) Model represents the refolding of chemically denatured MalZ in the presence or absence of minichaperone. Denatured MalZ was allowed to refold in the presence of minichaperone for the initial 20 minutes, a transient interaction between

MalZ and minichaperone occurred. Spontaneously refolded MalZ undergoes extensive aggregation with only 5% recovery of the activity

Fig. 2. Time course of reactivation of MalZ with minichaperone. MalZ unfolded in 2 M GdnHCl was diluted into a refolding buffer containing a molar excess of minichaperone. At the indicated time intervals, aliquots from the refolding mixture were withdrawn and MalZ activity was measured. The final concentration of MalZ and minichaperone used were 0.3 μ M and 30 μ M respectively. Error bars represent SD from three separate experiments

Fig. 3. (A). Sensorgrams showing the binding of folding intermediate (solid line) and native (broken line) MalZ to immobilized minichaperone. Injections were done at time zero for 60 s. (B) Relationship between the equilibrium binding response and concentration of denatured MalZ. The solid line shows the fitted curve for a single binding site affinity model using BIA evaluation program.

Fig. 4. Effect of GroES on MalZ activity during refolding in the presence of minichaperone. (A) Effect of increasing GroES concentration on the refolding of GdnHCl-denatured MalZ in the presence of minichaperone. MalZ was refolded in the presence of minichaperone as described. After 20 min, GroES was added at different molar ratios to the refolding mixtures, as indicated, and the enzyme activity was measured. (B) GroES-mediated refolding of MalZ in the absence or presence of minichaperone. MalZ unfolded in 2 M GdnHCl was diluted into a refolding buffer in the presence of minichaperone alone (bar 1), minichaperone and 2-fold molar excess of GroES over minichaperone (bar 2), or the same amount of GroES alone (bar 3). Refolding was allowed to proceed for 20 min, and the mixture was assayed for MalZ activity. Protein concentrations and error bars are as in Figure 2.

Fig. 5. Time course of reactivation of MalZ with minichaperone in the absence or presence of GroES. MalZ unfolded in 2 M GdnHCl was diluted into a refolding buffer containing minichaperone. At the indicated time intervals, aliquots from the refolding mixture were withdrawn and measured for enzymatic activity. GroES was added in a 2:1 ratio to minichaperone at different times after initiating the refolding reactions. At the indicated time intervals, aliquots from the refolding mixture were withdrawn and measured for enzymatic activity. Downward arrows show the measured level of MalZ activity after GroES addition. Protein concentrations and error bars are as in Figure 2.

Fig. 6. Impact of GroEL G337S/I349E trap on refolding of MalZ in presence and absence of minichaperone. A) Effect of GroEL G337S/I349E on refolding of MalZ. MalZ unfolded in 2 M GdnHCl was diluted into a refolding buffer containing GroEL G337S/I349E. Aliquots from the refolding mixture were withdrawn at indicated time intervals and MalZ activity was determined. B) Minichaperone-mediated refolding of MalZ in the presence of GroEL-G337S/I349E. MalZ was unfolded and refolded as in panel A, with minichaperone present in the refolding buffer. GroEL G337S/I349E was added in a 1:1 molar ratio to MalZ at different times after initiation of refolding as

shown, and MalZ activity was measured 10 minutes after this. Protein concentrations and error bars are as in Figure 2.

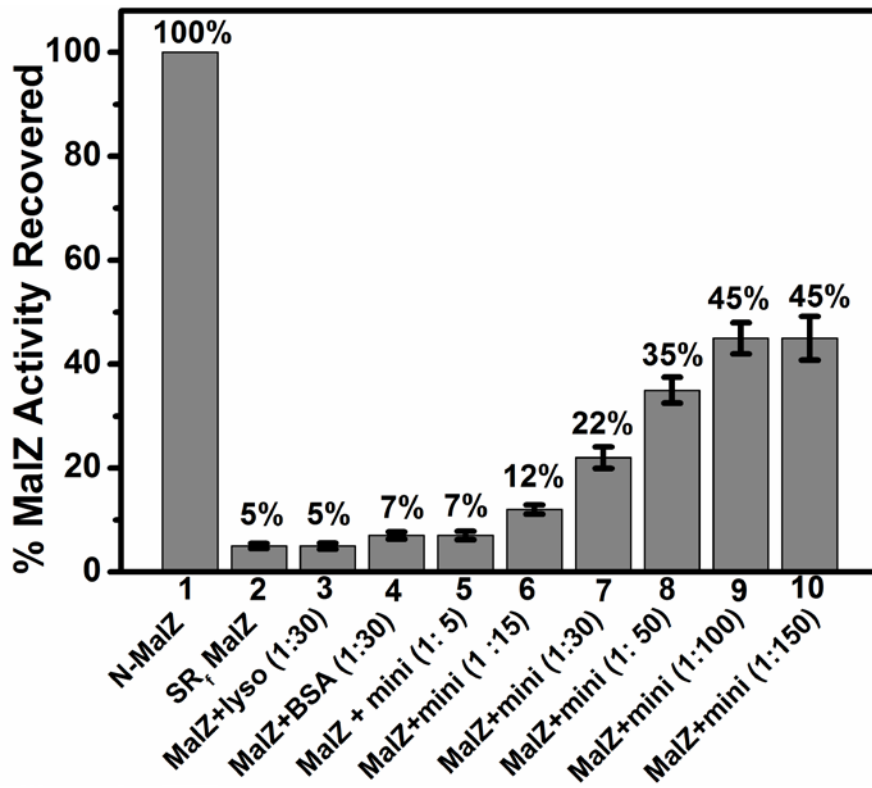
Fig. 7. Monitoring conformational changes of MalZ during refolding with minichaperone in the absence or presence of GroES. MalZ unfolded in 2 M GdnHCl was diluted into a refolding buffer containing minichaperone and was allowed to refold for 20 min or 180 min. GroES was then added. (A) Tryptophan fluorescence spectra of different conformation of MalZ. Fluorescence spectra of the MalZ under different conditions. a: Native MalZ; b: + minichaperone at 20 min; c: + minichaperone at 180 min; d: + minichaperone with GroES added at 180 min; e: + minichaperone with GroES added at 20 min; f: MalZ denatured in 2M GdnHCl. (B) ANS fluorescence of different conformations of MalZ, minichaperone, and GroES. 1: Native MalZ; 2: minichaperone alone; 3: GroES alone; 4: Native MalZ + minichaperone after subtraction of fluorescence of minichaperone alone; 5: MalZ + minichaperone at 20 min; 6: minichaperone and GroES mix; 7: minichaperone + MalZ with GroES added at 20 min; 8: minichaperone + MalZ at 180 min; 9: minichaperone + MalZ with GroES added at 180 min. Fluorescence was recorded after incubation with 50 μ M ANS for 5 min at 25°C. Error bars represent SD calculated from three separate experiments.

Fig. 8. Residual MalZ activity following thermal denaturation in the absence (red line) and presence of 2.5 μ M mini-chaperone (pink line). Panel (A), incubation at 45°C. Panel (B), incubation at 50°C. Panel (C), incubation at 55°C. Samples were withdrawn at the times shown and the residual activity of MalZ was determined at 25°C. The continuous lines are the fits to the data, generated using Graphpad Prism software. Error bars represent SD calculated from three separate experiments. The final concentration of MalZ was 0.5 μ M.

Fig. 9. Reactivation of thermally denatured MalZ in presence of minichaperone investigated by Size Exclusion Chromatography. The traces show (A) Native minichaperone (N-mini) and MalZ (N-MalZ) elution peaks; (B) minichaperone heated at 50°C (T_{H_T} - mini) for 60 min; (C) thermally refolded MalZ in absence of minichaperone heated at 50°C for 60 min; and (D) thermally refolded MalZ in presence of minichaperone (D). Gel filtration profiles were monitored by absorbance measurements at 280 nm. mAU = milli absorbance units. (E) SDS-PAGE gel showing the MalZ-minichaperone binary complex. Lane 1-6: fractions as shown in chromatogram (D). The numbers appearing on each of the two peaks represent the three fractions constituting the peak. Thus the intensity of MalZ bands in SDS gel represents the eluted protein distributed in three fractions. Lane 7: Molecular weight markers in kDa.

Fig 1

A.



B.

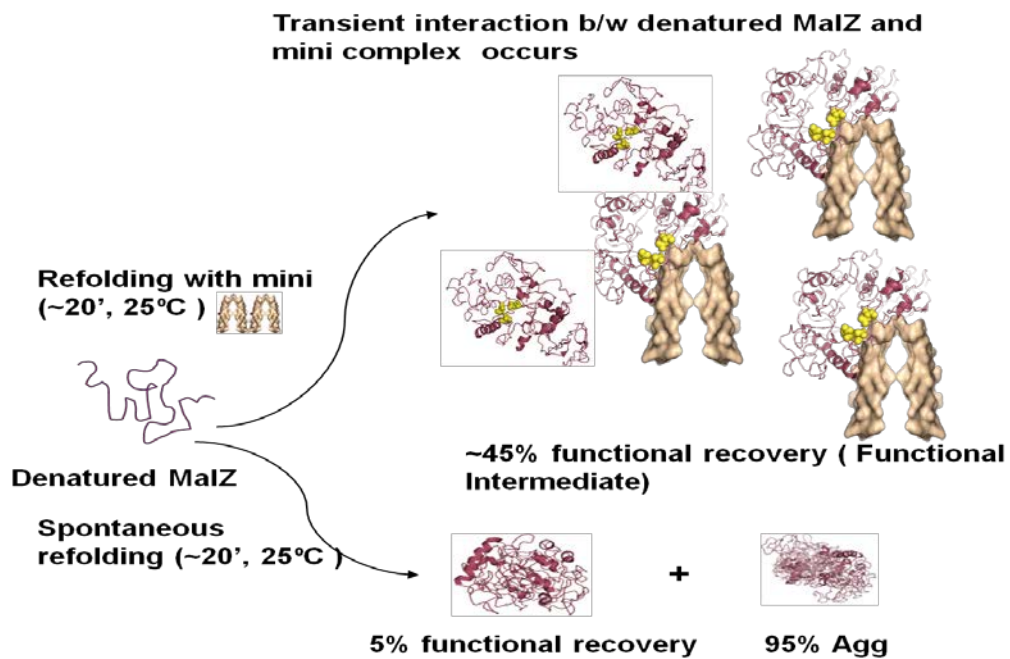


Fig. 2.

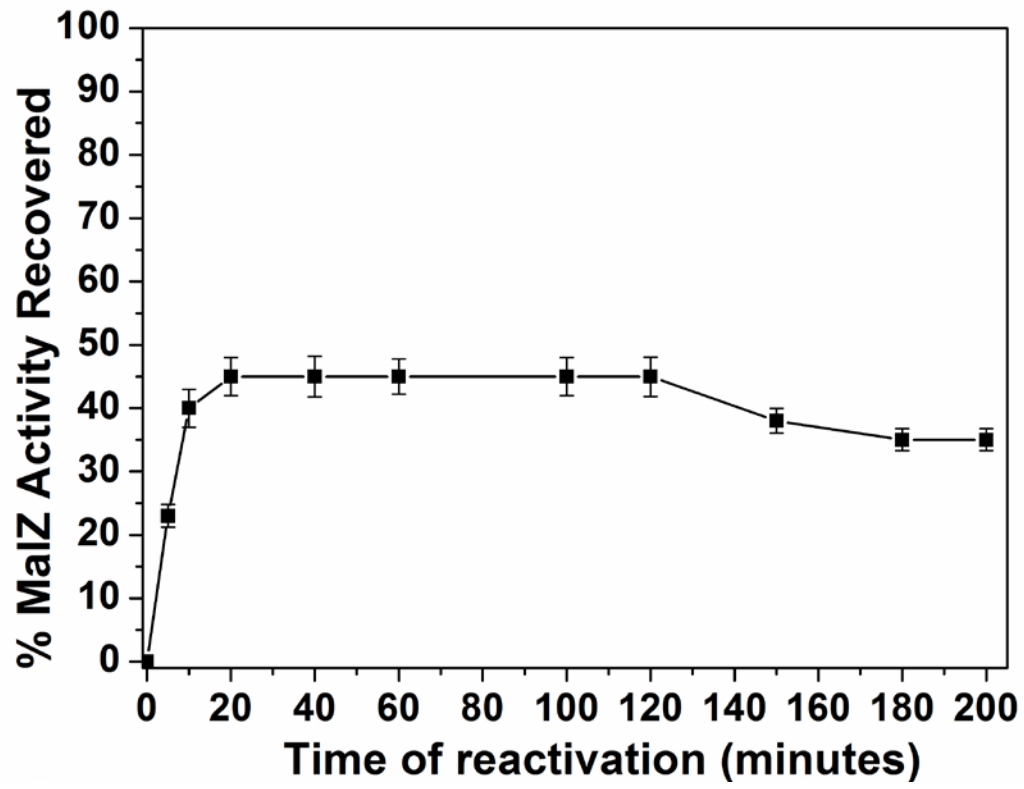
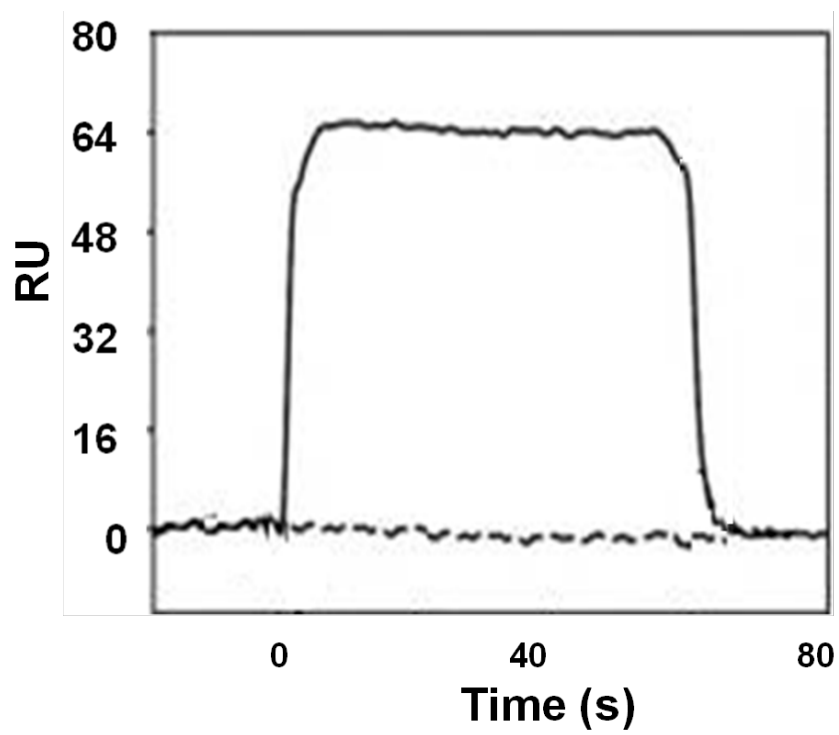
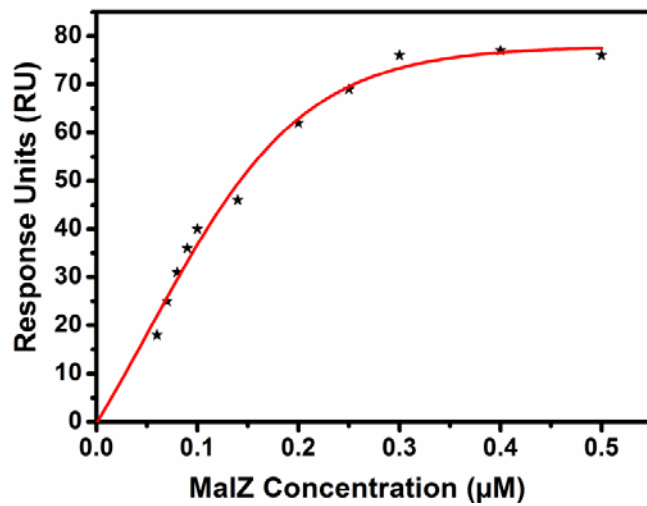


Fig. 3.

A.



B.



#

Reduced Chi-Sqr	6.5
Adj. R Square	0.99

Fig. 4.

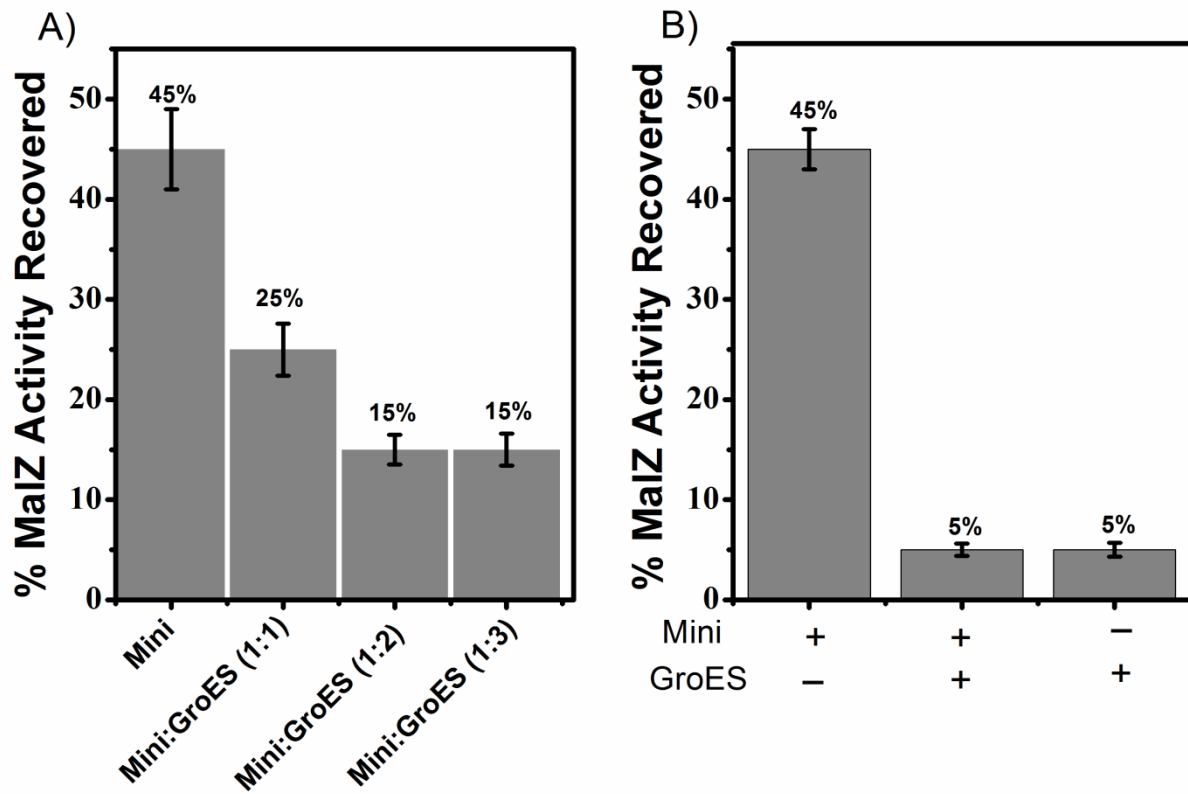


Fig. 5.

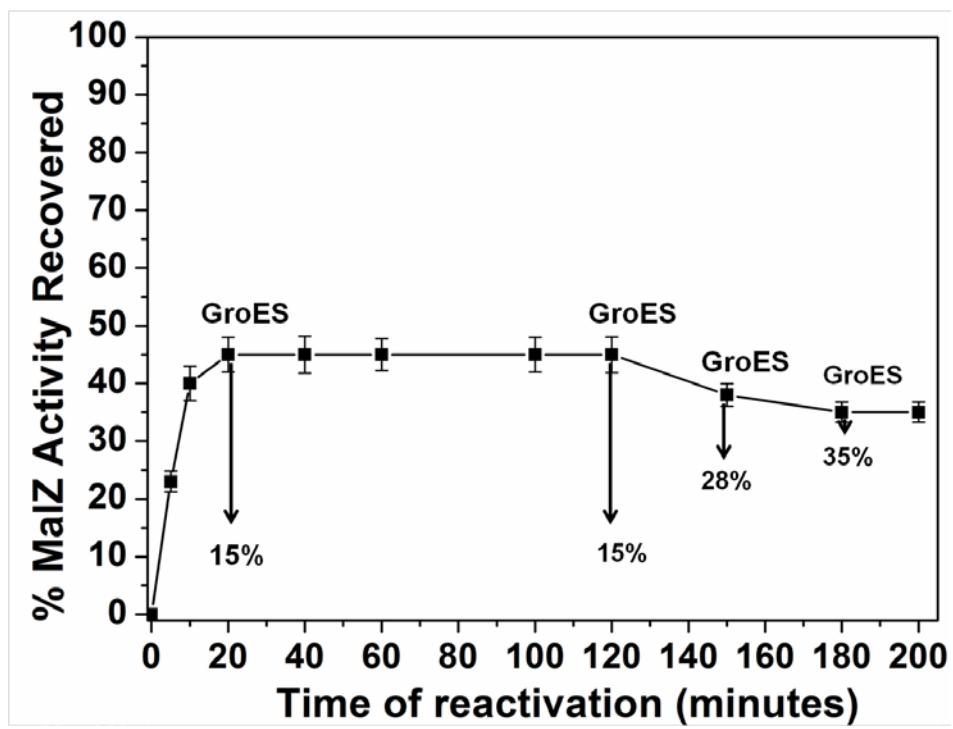


Fig. 6.

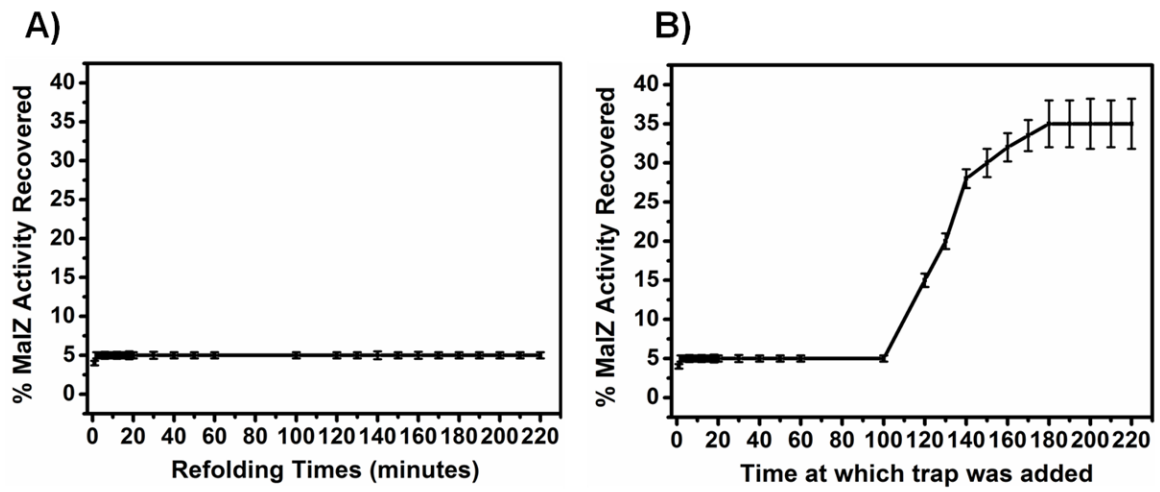


Fig. 7.

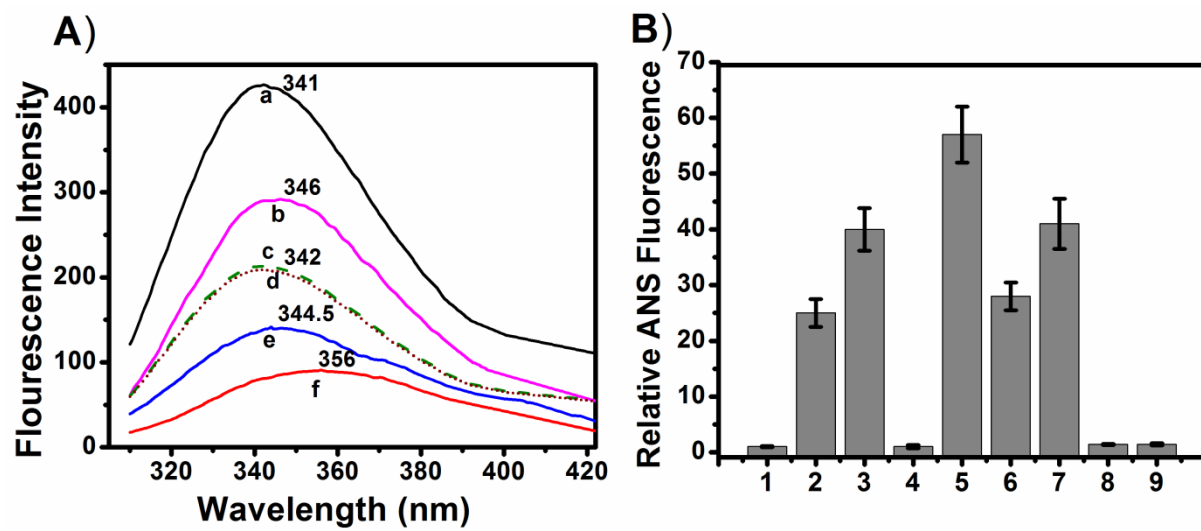


Fig. 8.

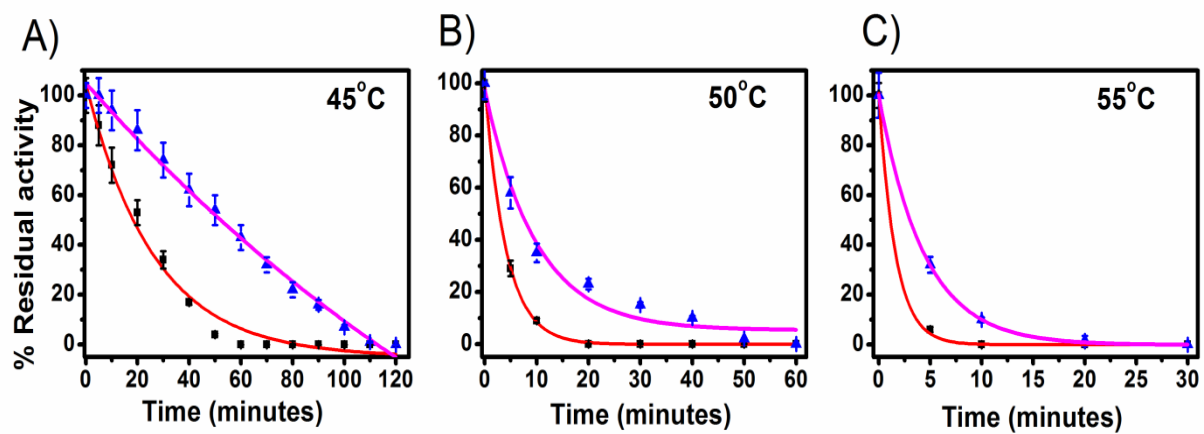
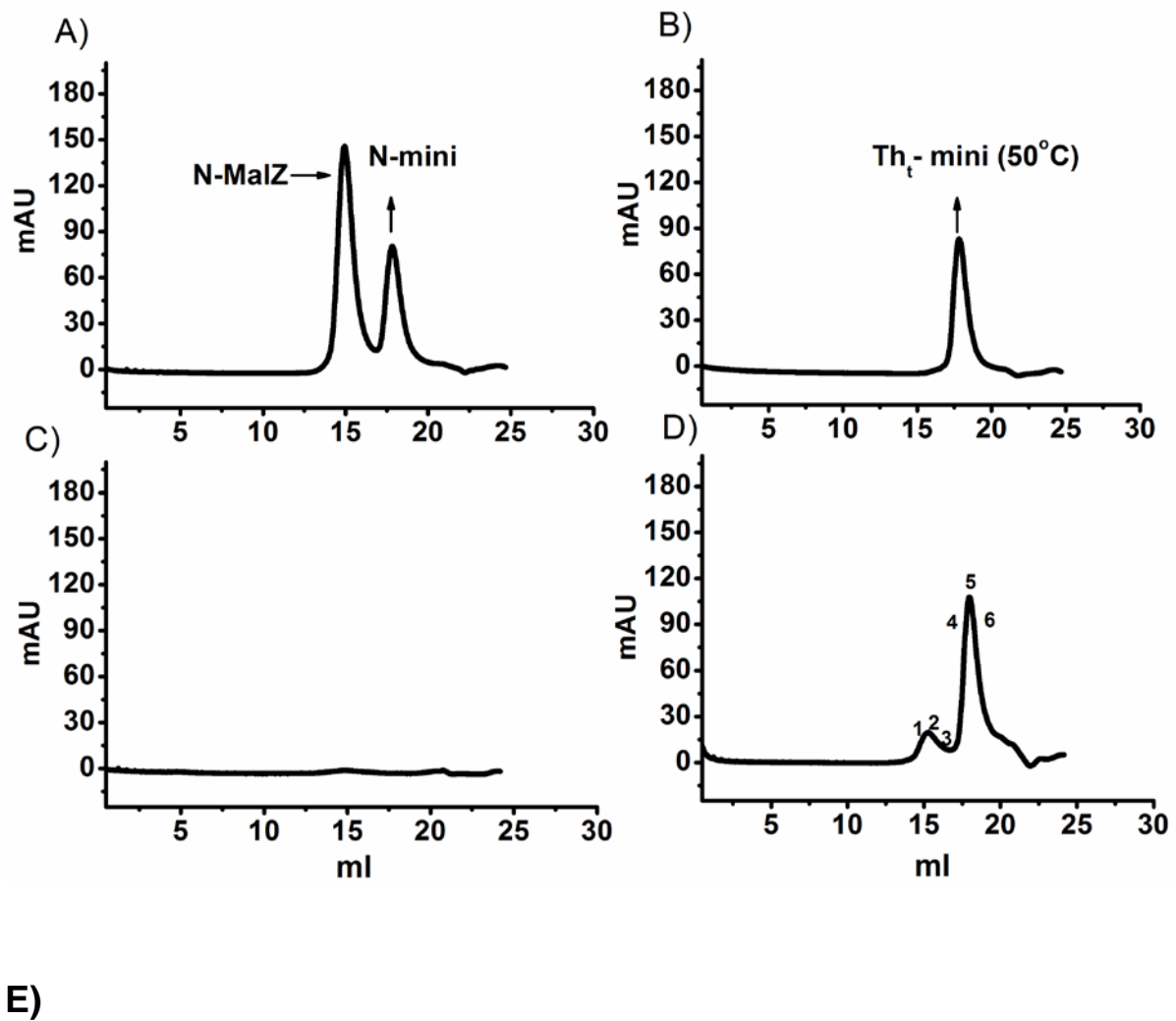
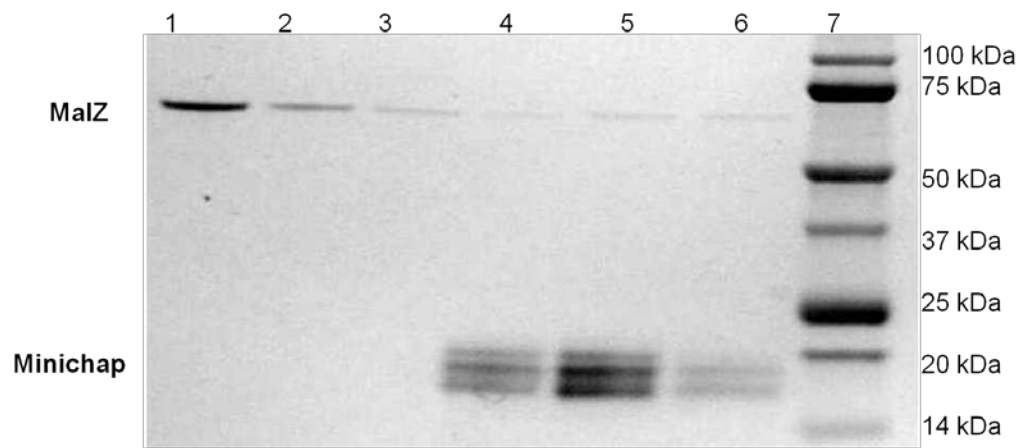


Fig. 9.





Supplementary Figures

Fig. S1

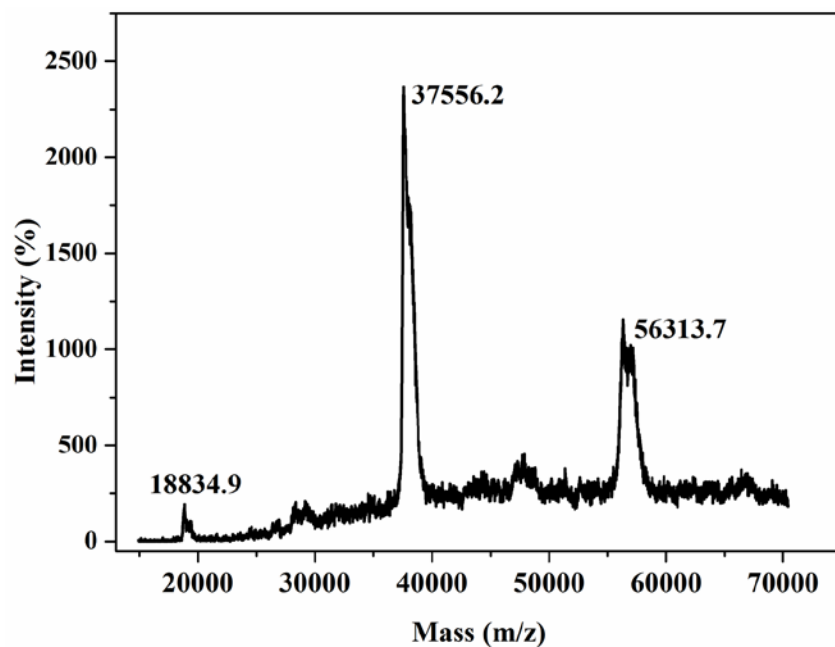


Fig.S1. Detection of oligomeric forms of minichaperone. MALDI-TOF MS of minichaperone (20 μ M) demonstrating oligomerization of minichaperone showing monomer, dimer and trimer with molecular masses of 18834.9, 37556.2 and 56313.7 Da, respectively.

Fig. S2

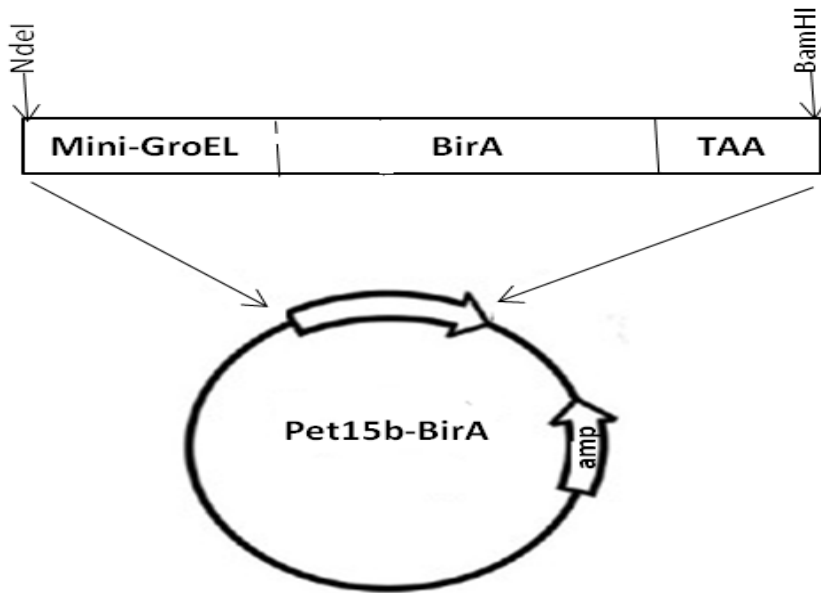


Fig. S2.representation of Pet 15b containing BirA gene and minichaperone gene.

Fig. S3.

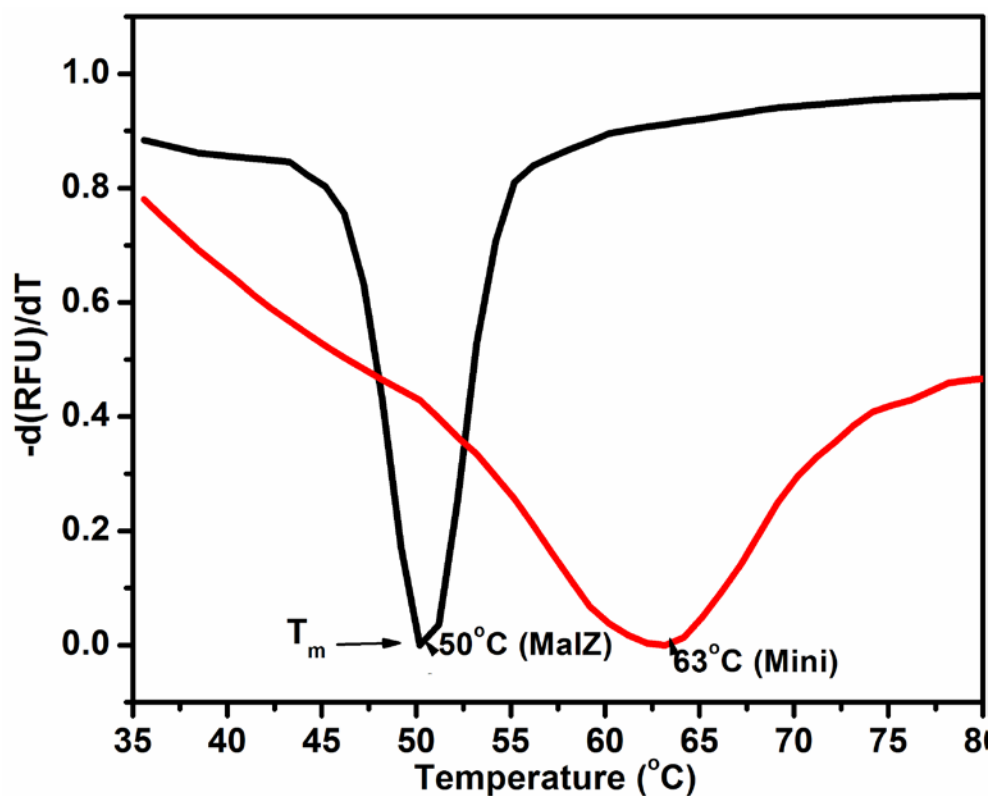


Fig. S3. Melting curves for MalZ (black curve) and minichaperone (red curve), determined by thermofluor. Here, T_m was identified by plotting the first derivative of the fluorescence emission, $-d(\text{RFU})/dT$, against temperature. T_m is given by the lowest part of the curve.

Fig. S4.

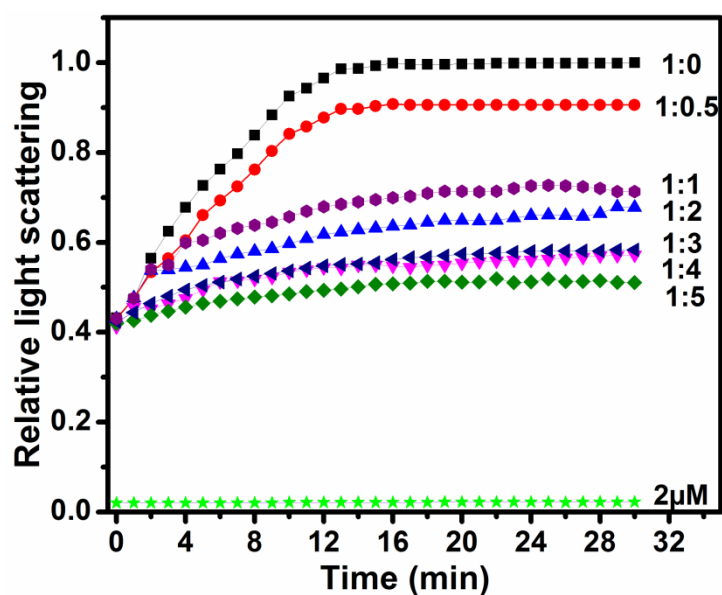


Fig. S4. Prevention of thermal aggregation of MalZ by minichaperone. 0.5µM MalZ was incubated at 50°C in the absence or presence of increasing amounts of minichaperone as indicated. The molar ratio of MalZ and minichaperone is indicated against each aggregation trace. Aggregation kinetics was monitored by light scattering at 500nm and normalized to the value of MalZ aggregation in the absence of minichaperone. The green star trace shows that minichaperone alone does not aggregate at 50°C.

Fig. S5.

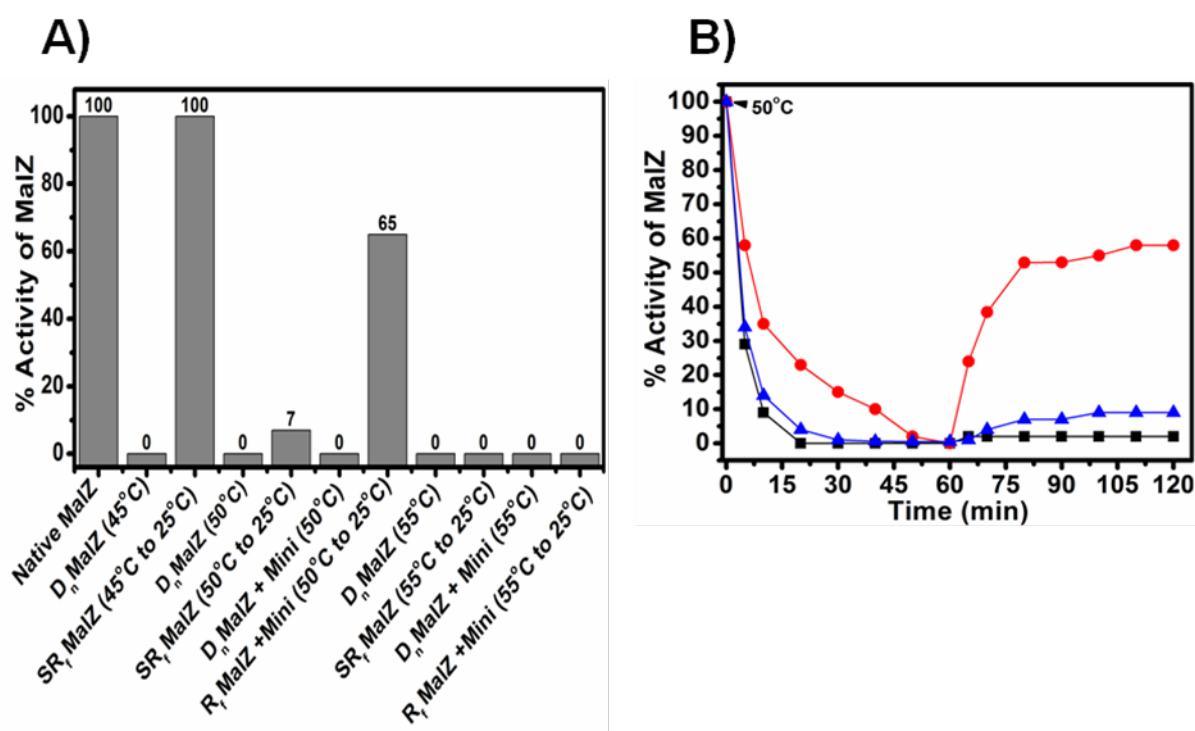


Fig. S5. Reactivation of thermally inactivated MalZ. A) 0.5 μ m MalZ was subjected to thermal denaturation at three different temperatures (as shown in different bars) in the absence or presence of 2.5 μ M minichaperone until its activity was reduced to zero. Samples were then cooled down to 25° C and reactivation of MalZ was measured. The extent of regain of activity is expressed as a percentage of the activity of the same amount of native MalZ. Here D_n , SR_f and R_f represents denatured, spontaneously refolded and refolded MalZ respectively. B) Time course of reactivation of MalZ at 25°C in the absence or presence of lysozyme and minichaperone. In this experiment, MalZ was denatured at 50°C (indicated by arrow) for 60 min in the absence (■) or presence of 2.5 μ M lysozyme (▲) or 2.5 μ M minichaperone (●, Red color). After 60 min, samples were then cooled to 25°C and reactivation of MalZ was measured over time.

