Thermal inactivation and chaperonin-mediated renaturation of mitochondrial aspartate aminotransferase

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Mitochondrial aspartate aminotransferase is inactivated irreversibly on heating. The inactivated protein aggregates, but aggregation is prevented by the presence of the chaperonin 60 from *Escherichia coli* (GroEL). The chaperonin increases the rate of thermal inactivation in the temperature range 55–65 °C but not at lower temperatures. It has previously been shown [Twomey and Doonan (1997) *Biochim. Biophys. Acta* **1342**, 37–44] that the enzyme switches to a modified, but catalytically active, conformation at approx. 55–60 °C and the present results show that this conformation is recognized by and binds to GroEL. The

thermally inactivated protein can be released from GroEL in an active form by the addition of chaperonin 10 from *E. coli* (GroES)/ATP, showing that inactivation is not the result of irreversible chemical changes. These results suggest that the irreversibility of thermal inactivation is due to the formation of an altered conformation with a high kinetic barrier to refolding rather than to any covalent changes. In the absence of chaperonin the unfolded molecules aggregate but this is a consequence, rather than the cause, of irreversible inactivation.

INTRODUCTION

Protein folding and unfolding are topics of intense study not only because of the inherent interest of the subject but also because of the practical implications of the stability of proteins for their use in commercial applications (reviewed in [1–4]). Perturbation of the native three-dimensional structure of a protein can be effected by the addition of chemical agents such as urea or guanidinium chloride, in which case the process is usually reversible provided that experimental conditions are carefully controlled. In contrast, exposure to high temperature frequently leads to an irreversible loss of native structure for reasons that are by no means well understood [5]. It is widely held that these two processes are interconnected in that irreversible thermal unfolding involves a reversible unfolding process followed by irreversible modifications that might be chemical or conformational in nature [5,6]. If this is indeed true, strategies can be devised for the thermostabilization of proteins based on suppressing the initial reversible unfolding step [5,7].

Our interest in these issues concerns the enzyme aspartate aminotransferase (EC 2.6.1.1). This is a homodimeric, pyridoxal 5′-phosphate-dependent, enzyme that exists in at least two distinct forms in higher eukaryotes, one located in the cytosol and the other in the mitochondria [8]. The cytosolic and mitochondrial isoenzymes are structurally related, with approx. 48% identity of amino acid sequence [9], but they exhibit considerable differences in stability in respect of both response to denaturing agents [10,11] and sensitivity to thermal denaturation [12]. The latter study has shown that relative rates of irreversible thermal inactivation of the isoenzymes are determined by the nature of the transition states for inactivation rather by intermolecular interactions in the native proteins. The activation enthalpy for inactivation of the mitochondrial isoenzyme is greater than that of the cytosolic form in spite of the fact that the mitochondrial isoenzyme is the less conformationally stable of the two; this is clearly inconsistent with a model in which the

initial step of inactivation is the reversible unfolding of the native structure.

To further our understanding of the thermal inactivation of aspartate aminotransferase we decided to investigate the extent to which aggregation contributes to irreversibility, with the mitochondrial isoenzyme as the model. One obvious approach to this was to attempt to sequester the inactivated protein with the chaperonin 60 from *Escherichia coli* (GroEL) followed by reactivation with the chaperonin 10 from *E. coli* (GroES) (see [13] for a recent review of the GroEL/GroES system). It has already been shown that the presence of GroEL/GroES can profoundly influence the recovery of activity during renaturation of mitochondrial aspartate aminotransferase that had been denatured by treatment with guanidinium chloride [14,15]; this is presumably by decreasing off-pathway aggregation processes. The unfolded state of the protein after heat inactivation is, however, likely to be very different from that resulting from chemical denaturation and hence it was considered to be of interest to compare the ability of GroEL to interact with these different denatured forms of the protein.

EXPERIMENTAL

Proteins

Mitochondrial aspartate aminotransferase was purified from pig hearts as described by Barra et al. [16]. A yield of 120 mg was obtained from six hearts and the specific activity was 230 units/mg. The protein was pure as judged by SDS/PAGE [15% (w/v) gel]. Protein concentrations were determined by taking $A_{280}^{1\%}$ as 14.0 [16]. The protein was stored at –20 °C in 85%–satd. $(\text{NH}_4)_2\text{SO}_4$.

GroEL and GroES were purified from *Escherichia coli* strain OFB748 which was a generous gift from Dr. Olivier Fayet (Laboratoire de Microbiologie Genetique Moleculaire CNRS, Toulouse, France). This strain harbours a multicopy plasmid containing genes for both proteins [17]. Cells were grown in a

Abbreviations used: GroEL, the chaperonin 60 from *Escherichia coli*; GroES, the chaperonin 10 from *Escherichia coli*.

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medium containing 1 % (w/v) tryptone, 0.5 % yeast extract, 1 % (w/v) NaCl and 50 μ g/ml ampicillin at 42 °C for 5 h. The cells (10 g wet weight) were suspended in 30 ml of 50 mM Tris/HCl buffer, pH 7.5, containing 2 mM EDTA and 2 mM 2-mercaptoethanol, and were ruptured by sonication (six 15 s bursts at an amplitude of 18 μ m with an MSE Soniprep 150). Cell debris was removed by centrifugation at 12000 *g* for 1 h; 3 ml aliquots of the supernatant were used for subsequent purification of GroEL and GroES.

GroEL was purified from cell extracts by size exclusion chromatography with Sephacryl S300 followed by ion-exchange chromatography with Q-Sepharose, essentially as described by Buchner et al. [18]. The yield was 10 mg of pure protein from 10 g wet weight of cells. Purity was established by SDS/PAGE [15 % (w/v) gel]. GroES was purified from fractions emerging later than GroEL after chromatography on Sephacryl S300, appropriate fractions being located by SDS/PAGE analysis. Fractions were combined, dialysed against 50 mM Tris/HCl buffer, pH 7.5, containing 2 mM EDTA and 2 mM 2-mercaptoethanol, and applied to a column (3 cm $\times$ 15 cm) of DEAE-cellulose DE52 equilibrated in the same buffer. After the column had been washed with 500 ml of equilibration buffer GroES was eluted with a linear gradient (total volume 500 ml) of 0–1 M NaCl in the same Tris buffer. Fractions containing GroES were pooled and dialysed against 50 mM sodium acetate buffer, pH 6.0. After dialysis the pH was decreased to 4.5 by the addition of dilute HCl and the sample was applied to a MonoS HR FPLC column equilibrated in 50 mM sodium acetate buffer, pH 4.5. GroES was eluted with a linear gradient of 0–1 M NaCl in the same buffer. The protein was pure as judged by SDS/PAGE [20 % (w/v) gel] and the yield was 2–3 mg from 10 g wet weight of cells.

Concentrations of solutions of GroEL were determined by absorbance measurements, taking $A_{276}^{1\%}$ as 2.50 [19], and by using the bicinchoninic acid method [20] with BSA as standard; a kit for this assay was obtained from Pierce (Rockford, IL, U.S.A.). Both methods were used for initial measurements with new preparations of GroEL but, provided that they gave consistent results, the absorbance method was subsequently used for routine purposes with that batch of protein. Concentrations of solutions of GroES were determined by absorbance measurements, taking $A_{276}^{1\%}$ as 1.42 as estimated from the content of aromatic amino acids in the protein [21]. The proteins were stored at –20 °C in 50 % (v/v) glycerol.

Enzyme assays

Aspartate aminotransferase activity was measured at 25 °C with the linked assay method of Karmen [22]. The assay solution (3 ml) contained 0.05 M sodium phosphate buffer, pH 6.0, 3.3 mM L-aspartate, 3.3 mM 2-oxoglutarate, 80 μ M NADH and 5 μ g of malate dehydrogenase (Sigma). Reaction was started by the addition of enzyme; progress of the reaction was followed by the measurement of absorbance change at 340 nm. Activities are expressed in units of μ mol/min.

Rates of thermal inactivation

Mitochondrial aspartate aminotransferase was transferred to 50 mM Tris/HCl buffer, pH 8.0, by exhaustive dialysis and was incubated at the desired temperature in closed tubes. Aliquots were removed at appropriate intervals and assayed for residual enzymic activity at 25 °C by the method described above.

Light scattering and fluorescence

Light scattering measurements were made with an Aminco-Bowman series 2 luminescence spectrometer equipped with a temperature-controlled cell holder and a magnetic stirrer. The excitation and emission wavelengths were both set at 360 nm and slit widths were 4 nm. The same equipment was used to record fluorescence spectra with the excitation wavelength at 290 nm and slit widths of 8 nm.

GroEL-mediated refolding

Mitochondrial aspartate aminotransferase in 50 mM Tris/HCl buffer, pH 8.0, was partly inactivated by being heated at an appropriate temperature in the presence of GroEL for a duration such that approx. 80 % of the initial activity was lost. The incubation tube was then held at the desired temperature for reactivation for 1 min, after which an equal volume of GroES solution in the same Tris buffer containing 20 mM KCl and 20 mM MgCl_2 was added, as were pyridoxal 5'-phosphate, dithiothreitol and ATP to final concentrations of 0.1, 3 and 10 mM respectively. Samples were removed for assay at suitable time intervals.

RESULTS

Thermal inactivation of mitochondrial aspartate aminotransferase

The rates of thermal inactivation of mitochondrial aspartate aminotransferase in 50 mM Tris/HCl buffer at a nominal pH of 8.0 were determined at 55, 60 and 65 °C; no attempt was made to allow for changes in pH of the buffer as a function of temperature because it is known that the rate of inactivation of the enzyme is essentially independent of pH in the range 7.5–9.0 [12]. Results are shown in Figure 1 as plots of $\ln(A_0/A_t)$ as a function of time, where A_0 and A_t are the activities at zero time and time t respectively. The plots were linear (regression coefficients in the range 0.946–0.975), showing that the inactivation was a first-order process. This was further confirmed by the observation that, at 65 °C, the rate of inactivation was independent of enzyme concentration over the range 0.005–0.090 mg/ml (results not shown). The first-order rate

Figure 1 First-order plots of the thermal inactivation of mitochondrial aspartate aminotransferase at pH 8.0

The enzyme concentration used was 54 nM. Incubation temperatures were 55 °C (○), 60 °C (□) and 65 °C (●). Samples were withdrawn at the times shown and the residual activity (A_t) was determined at 25 °C.

Figure 2 Suppression of aggregation of the enzyme during thermal inactivation by addition of GroEL

Enzyme (54 nM) was incubated at 65 °C in the absence of GroEL (○) or in its presence at concentrations of 20 nM (□), 40 nM (△), 60 nM (●) and 108 nM (■). Light scattering was monitored at 360 nm.

constants for the inactivation reactions shown in Figure 1 were 0.0033, 0.0098 and 0.0197 min⁻¹ at 55, 60 and 65 °C respectively; the corresponding half-lives were 210, 71 and 35 min respectively. The inactivation was essentially irreversible as shown by the fact that, at any degree of inactivation, transfer to 25 °C followed by prolonged incubation in the presence of cofactor did not result in a measurable recovery of activity.

Aggregation as a consequence of thermal inactivation and its prevention by GroEL

During thermal inactivation, visible aggregates of the enzyme were formed; the increase in turbidity was more rapid at higher temperature. To investigate this further, thermal inactivation of the enzyme (concentration 54 nM dimer) was performed at 65 °C in the presence of various amounts of GroEL from 20 to 108 nM tetradecamer. The process was monitored by light-scattering measurements with the results shown in Figure 2. At sub-stoichiometric ratios of GroEL to enzyme, aggregation was delayed compared with the control in the absence of chaperonin, whereas at a 1:1 ratio of GroEL to mitochondrial aspartate aminotransferase monomers the aggregation was completely suppressed. Hence it seemed that each GroEL tetradecamer was able to recognize and sequester one monomer of the thermally inactivated enzyme. In a control experiment (results not shown) it was established that GroEL itself was stable under the conditions used here.

Fluorescence spectra

Mitochondrial aspartate aminotransferase (54 nM) was incubated at 65 °C, pH 8.0, in the presence of GroEL (108 nM) for 45 min and then cooled to 25 °C. The fluorescence spectrum of the complex was recorded in the range 300–400 nm and with an excitation wavelength of 290 nm. The spectrum of GroEL alone was also determined under the same conditions and the values obtained were subtracted from those of the complex to yield the difference spectrum shown in Figure 3; the contribution of GroEL to the fluorescence of the complex was very small because the chaperonin does not contain tryptophan [23]. Also shown in

Figure 3 Fluorescence spectra of native mitochondrial aspartate aminotransferase and of the enzyme thermally inactivated in the presence of GroEL

The spectra shown are those of the complex formed between mitochondrial aspartate aminotransferase and GroEL after being heated for 45 min at 65 °C (□) and of the native enzyme at the same concentration (○). The excitation wavelength was 290 nm.

Figure 3 is the spectrum of the native enzyme at the same concentration as in the complex.

Effect of GroEL on rate of thermal inactivation

Thermal inactivation was performed over a range of temperatures in the presence of stoichiometric quantities of GroEL. It was found that the reaction continued to be first order but that the rate of inactivation was increased by the presence of the chaperonin, the extent of the rate enhancement increasing with increasing temperature. Typical logarithmic plots for the reactions with and without added GroEL at 60 °C are shown in Figure 4. The derived values of the rate constants at the various

Figure 4 Effect of GroEL on the rate of thermal inactivation of mitochondrial aspartate aminotransferase

Thermal inactivation was performed at 60 °C, pH 8.0, and at an enzyme concentration of 54 nM either in the absence of GroEL (□) or in the presence of 108 nM GroEL (○).

Table 1 First-order rate constants for thermal inactivation of mitochondrial aspartate aminotransferase in the presence or absence of GroEL

The enzyme concentration was 54 nM (of the dimer) and the GroEL concentration was 108 nM. Results are means $\pm$ S.D. ($n = 4$ or 5).

Temperature ($^{\circ}\text{C}$)	Rate constant (min^{-1})	
	— GroEL	+ GroEL
55	0.0033 ± 0.0003	0.0054 ± 0.0004
58	0.0057 ± 0.0005	0.0130 ± 0.0010
60	0.0106 ± 0.0010	0.0601 ± 0.0050
65	0.0228 ± 0.0022	0.1422 ± 0.0080

Figure 5 Reactivation by GroES of mitochondrial aspartate aminotransferase thermally inactivated in the presence of GroEL

Enzyme (54 nM) was partly inactivated at 65 $^{\circ}\text{C}$ for 10 min in the presence of GroEL (108 nM) and then cooled to 25 $^{\circ}\text{C}$. The percentage activity regained compared with that of the enzyme before inactivation was determined on incubation in the presence of 10 mM ATP, 10 mM MgCl_2 , 10 mM KCl and 200 nM GroES (○), the same system without GroES (□), and without both GroES and ATP (●). All reactivation systems contained 3 mM dithiothreitol and 0.1 mM pyridoxal 5'-phosphate.

temperatures used are given in Table 1, where each value is the mean of four or five independent determinations. The extent of rate enhancement increased with temperature from 1.6-fold at 55 $^{\circ}\text{C}$ to 6.2-fold at 65 $^{\circ}\text{C}$. At temperatures below 55 $^{\circ}\text{C}$ GroEL had no effect on the rate of thermal inactivation; similarly, prolonged incubation of the enzyme with GroEL at 25 $^{\circ}\text{C}$ did not lead to any loss of activity.

Reactivation of enzyme inactivated in the presence of GroEL

Mitochondrial aspartate aminotransferase was partly (approx. 80%) inactivated in the presence of stoichiometric quantities of GroEL for 10 min at 65 $^{\circ}\text{C}$; the sample was then cooled to 25 $^{\circ}\text{C}$. Prolonged incubation of the inactivated protein with cofactor plus dithiothreitol in either the absence or the presence of ATP failed to produce any reactivation (Figure 5); that is, the activity remained at the residual value obtained at the end of the inactivation period. However, when GroES was included in the system a rapid recovery of approx. 14% of the original activity of the sample occurred (Figure 5) and the recovery increased to 24% after about 90 min; that is, the final activity was approx.

Table 2 Extent of reactivation as a function of GroES concentration

Experimental conditions were as described in the legend to Figure 5. Reactivation is given as the percentage of activity recovered compared with that of the enzyme before inactivation.

GroES (nM)	Reactivation (%)
60	10.0
108	13.8
150	16.8
200	24.0

Figure 6 Release of reactivated mitochondrial aspartate aminotransferase from the GroEL/GroES complex by the addition of ATP

The enzyme was inactivated in the presence of GroEL as described in the legend to Figure 5 and then cooled to 25 $^{\circ}\text{C}$. The recovery of activity was measured after the addition of 10 mM MgCl_2 , 10 mM KCl and 200 nM GroES in the absence of ATP (□) or when ATP (10 mM) was added (arrow) after 40 min of incubation (○).

44% of that of the original sample. The extent of reactivation depended on the concentration of GroES (Table 2); maximum reactivation required a molar ratio of GroES/GroEL of approx. 2:1.

The fast initial phase of the reactivation was decreased to approx. 10% when the process was performed at 0 $^{\circ}\text{C}$, whereas at 37 $^{\circ}\text{C}$ full reactivation was achieved within the 1 min allowed for temperature equilibration before the first sample was taken for assay; the final extent of reactivation was the same in all cases (results not shown in detail). It was also shown that the complex between GroEL and inactivated mitochondrial aspartate aminotransferase was stable on incubation at 0 or 25 $^{\circ}\text{C}$ for 24 h; no recovery of activity occurred during that period but subsequent addition of GroES/ATP at 25 $^{\circ}\text{C}$ resulted in the recovery of 22% and 18% respectively of the original activity.

The need for both ATP and GroES in the reactivation system is confirmed by the results in Figure 6. Inactivation in the presence of GroEL followed by incubation at 25 $^{\circ}\text{C}$ in the presence of GroES alone did not lead to any recovery of activity. In contrast, when ATP was added after 40 min to the sample incubated in the presence of GroES, a rapid reactivation occurred up to a limit of approx. 22% of the original activity. That the reactivation process depends on hydrolysis of the ATP, rather than simply on binding, was demonstrated by experiments in

which ATP was replaced by the non-hydrolysable analogue 5'-adenylylimidodiphosphate, which is known to bind to GroEL [24]; less than 2% reactivation was observed (results not shown). Similarly, neither GTP nor GDP was able to substitute for ATP.

DISCUSSION

The initial objective of this work was to investigate the nature of the processes occurring in the irreversible thermal inactivation of mitochondrial aspartate aminotransferase. It was first necessary to establish the conditions under which the inactivation could be conveniently monitored and it was found that, at pH 8.0 in a Tris/HCl buffer, thermal inactivation occurred over an appropriate time scale in the temperature range 55–65 °C. The results in Figure 1 show that the inactivation process is a first-order reaction in agreement with previous work in which thermal inactivation of both the cytosolic and the mitochondrial aspartate aminotransferases was studied over a wide temperature range [12]; it is not possible to compare the actual rates of inactivation obtained here with those reported previously because a different buffer was used in the present work. A second central feature reported in the previous work and confirmed here is that the inactivation is irreversible even at the earliest stages of the process.

One obvious explanation for irreversibility would be that the inactivated molecules aggregate immediately, and indeed protein aggregates became visible as inactivation proceeded; however, the fact that the process is first-order makes it unlikely that aggregation is the primary rate-limiting event. The likely causes of the irreversibility of inactivation are therefore either chemical modification of the protein or change to a conformation with a high kinetic barrier to reversibility. In the former case irreversibility is inevitable, whereas in the second it might be avoided if the unfolded protein could be trapped in a reversibly unfolded state.

To investigate the latter possibility, inactivation was performed in the presence of the chaperonin GroEL, and light scattering measurements were used to monitor the suppression of the aggregation process. The results showed (Figure 2) that, at a 1:1 ratio of GroEL tetradecamer to enzyme monomer, aggregation was completely prevented. At sub-stoichiometric ratios of GroEL to enzyme, aggregation still occurred but was delayed compared with a control in the absence of added chaperonin. These results suggest that the GroEL molecule is able to sequester one monomer of heat-inactivated mitochondrial aspartate aminotransferase, whereas the unbound remainder aggregates more slowly.

To establish the stoichiometry of binding it was necessary to be confident of the concentrations of GroEL solutions. These were routinely determined from absorbance measurements, difficulties with which have previously been reported [25]. In the present work, however, concentrations of highly purified GroEL preparations determined by using absorbance methods and by using the bicinchoninic acid method [20] were found to be in good agreement, thus demonstrating the reliability of the former.

Evidence for binding of the inactivated enzyme to GroEL is provided by the fluorescence spectra in Figure 3. When mitochondrial aspartate aminotransferase was inactivated in the presence of GroEL, the fluorescence increased in intensity and the λ_{max} value of the emission shifted to a higher value than that of the native enzyme. This is very similar to the behaviour observed with dihydrofolate reductase and with rhodanese, and is suggestive of the adoption of a molten globule conformation by the bound protein [26].

Several studies of the potential effect of GroEL on the

inactivation of native proteins have been reported. For example, Holl-Neugebauer et al. [27] found that GroEL had no effect on the kinetics of thermal inactivation of α -glucosidase, consistent with the view that the chaperonin does not interact with the native states of proteins. In contrast, Viitanen et al. [28] and Laminet et al. [29] showed that native human dihydrofolate reductase, in the absence of substrates, and pre- β -lactamase respectively slowly lose activity and bind to the chaperonin on incubation with GroEL. The latter observations were taken to show that these proteins are unstable relative to inactive, less folded, states that bind to GroEL; that is, inactivation is promoted by thermodynamic partitioning between native and unfolded conformations. With barnase, GroEL acts to catalyse the unfolding of the native molecule but also exerts thermodynamic partitioning effects [30].

In the present work it was found that GroEL increased the rate of inactivation of the enzyme in the temperature range 55–65 °C (see Figure 4 and Table 1) but not at lower temperatures. At 25 °C the enzyme did not lose activity on prolonged incubation with the chaperonin. The latter observation presumably means that at ambient temperature there is no kinetically accessible inactive conformation to which GroEL can bind. The observation of the catalysis of unfolding at high temperatures is more interesting and can be explained on the basis of the fact that, at temperatures greater than 55–60 °C, the enzyme adopts a modified, but still catalytically active, conformation [12]; the same behaviour has been reported for the aspartate aminotransferase from *Sulfolobus solfataricus* [31]. We therefore conclude that this modified conformation of the enzyme is recognized by GroEL, binds to it and subsequently unfolds in the rate-determining step to produce an inactive conformation. These results strongly support the view that GroEL can act as an 'unfoldase' by binding misfolded or partly unfolded proteins, promoting their unfolding, and allowing subsequent refolding [32].

Given that the monomeric form of aspartate aminotransferase is catalytically inactive [33], the formal possibility arises that binding of GroEL to the high-temperature conformation of the enzyme leads to inactivation by promoting monomer formation rather than by catalysing unfolding; that is, the rate-determining step of inactivation might be different in the presence and in the absence of the chaperonin. However, the results in Figure 3 show that the conformations of the native enzyme and of the inactivated enzyme bound to GroEL are very different, which argues against simple monomer formation being the cause of inactivation in the presence of GroEL.

It was important to establish that mitochondrial aspartate aminotransferase thermally inactivated in the presence of GroEL was capable of refolding. That this was so is shown by the results in Figures 5 and 6. In these experiments the enzyme was not fully inactivated; this was to minimize the possibility of irreversible covalent changes occurring on prolonged heating. Release and recovery of activity required the presence of both GroES and ATP; no reactivation could be obtained in the absence of either of these components. The enzyme/GroEL complex was stable for at least 24 h; that is, no reactivation occurred during this time and the extent of reactivation achieved subsequently by the addition of ATP/GroES was the same as for samples reactivated soon after inactivation. Interestingly, maximum reactivation required the presence of an approx. 2-fold molar excess of GroES heptamer compared with GroEL tetradecamer, although there is no reason to believe that binding of GroES to both ends of GroEL is required for refolding [13].

Recovery of 12–14% of the initial catalytic activity occurred in a rapid burst that was complete within the approx. 1 min that

elapsed after the addition of GroES/ATP and before the first activity measurement. This burst was decreased only slightly if reactivation was performed at 0 °C. This is consistent with the estimated 15–30 s required for the release of GroES from the GroEL/GroES/protein ternary complex [34–36] and hence the time available for a round of refolding. The reason for the slow component in the refolding curves is not clear. It might be that some protein was released from the chaperonin in a folding-competent but not fully refolded form and that final achievement of the native conformation for this protein was a slow process. Alternatively (or in addition) it might be that some isomerization of proline residues was required in a fraction of the refolded molecules (mitochondrial aspartate aminotransferase contains two *cis*-proline residues per monomer [37]). Equally the reason for the failure to achieve complete reactivation is not clear but the level achieved was comparable to that obtained in other work (see, for example, [27]); it could be that under the conditions used the formation of active dimers from monomers released from GroEL is not efficient. The important point is that the protein could be reactivated, at least in part, showing that thermal inactivation was not a result of irreversible chemical changes. Thus the results obtained in this work suggest that the irreversibility of thermal inactivation of mitochondrial aspartate aminotransferase is a result of conformational changes with a high kinetic barrier to reversal and that, in the absence of a chaperone, the unfolded protein then aggregates.

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