Binding of a burst-phase intermediate formed in the folding of denatured D-glyceraldehyde-3-phosphate dehydrogenase by chaperonin 60 and 8-anilino-1-naphthalenesulphonic acid

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Upon dilution, D-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) that has been fully inactivated, but only partially unfolded, in dilute guanidine hydrochloride (GuHCl) recovers activity completely. The fully unfolded enzyme, however, is re-activated only to a limited extent after dilution, and refolds rapidly in a burst phase to a partially folded intermediate characterized by increases in both the emission intensity of intrinsic fluorescence and binding to 8-anilino-1-naphthalenesulphonic acid (ANS). This intermediate aggregates with a time lag of a few minutes, and the aggregation can be suppressed completely by chaperonin 60 (GroEL). Stoichiometric analysis of the suppression of GAPDH re-activation by GroEL suggests that the tetradecameric GroEL binds to a dimeric GAPDH folding intermediate. This intermediate can be re-activated by

ATP or ATP/chaperonin 10 (GroES) to an extent considerably greater than that obtained on spontaneous re-activation of the fully denatured enzyme upon dilution. Probing with a fluorescent derivative of NAD⁺ shows that this folding intermediate does not have a native conformation at the active site. The similar profiles of the effects of GroEL and ANS on the re-activation of GAPDH denatured by different concentrations of GuHCl suggest that GroEL and ANS recognize and bind to the same folding intermediate, which is similar to the relatively stable, partially unfolded, state of the enzyme denatured in 0.5–1.0 M GuHCl. However, the complexes of the intermediate with GroEL or ANS appear to be different, in that GroEL, but not ANS, suppresses aggregation and assists folding in the presence of ATP.

INTRODUCTION

D-Glyceraldehyde-3-phosphate dehydrogenase (GAPDH; EC-1.2.1.12), a key enzyme in glycolysis, is a homotetramer, and has been used as a model for studies on the unfolding, refolding, dissociation and association of oligomeric proteins [1–4]. The inactivation, conformational changes and aggregation of GAPDH during denaturation have been compared [2,5]. For the refolding of GAPDH, most previous reports have focused on the effects of external factors on the refolding yield, which is very low under most conditions for spontaneous refolding [6]. Kinetic studies on the recovery of activity of denatured GAPDH indicated that dimerization of the dimers is the rate-limiting step in the process of re-activation [7]. ATP was used to induce the dissociation of GAPDH at low temperature to obtain structurally intact subunits for studies on re-association and re-activation [8]. More recently, using GAPDH from the hyperthermophilic eubacterium (*Thermotoga maritima*), Jaenicke's group characterized a 'cold intermediate' that represented an 'assembled molten globule', but this intermediate is native-like and exhibits a reversible and highly co-operative conformational transition to the unfolded state [9,10]. GAPDH was also used to examine the possible chaperone-like activity of protein disulphide isomerase, independent of its isomerase activity [11,12].

In the present paper, we report that, upon dilution, the extended chain of guanidine hydrochloride (GuHCl)-denatured GAPDH collapses in a burst phase to form an intermediate,

which is similar to the relatively stable unfolding intermediate of the enzyme denatured in 0.5–1.0 M GuHCl and which binds to either chaperonin 60 (GroEL) or 8-anilino-1-naphthalenesulphonic acid (ANS), suppressing its re-activation. The stable complex with GroEL can be re-activated in the presence of either ATP or ATP/chaperonin 10 (GroES) to a level considerably greater than that obtained on spontaneous re-activation of the denatured enzyme.

EXPERIMENTAL

Materials

D-Glyceraldehyde 3-phosphate was prepared from its diethyl-acetal monobarium salt (Sigma) by the method provided. BSA (98–99% albumin; Fraction V), ANS, GuHCl and NAD⁺ were from Sigma. Dithiothreitol (DTT) was from Promega, ATP from Boehringer and iodoacetic acid from Merck. All other reagents were local products of analytical grade.

Preparation and determination of proteins

The multicopy plasmid pGroESL, containing the coding sequences of GroEL and GroES, was a gift from Dr. A. A. Gatenby, Central Research and Development, E. I. DuPont de

Abbreviations used: ANS, 8-anilino-1-naphthalenesulphonic acid; DTT, dithiothreitol; GAPDH, D-glyceraldehyde-3-phosphate dehydrogenase; GroEL, chaperonin 60; GroES, chaperonin 10; GuHCl, guanidine hydrochloride.

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Nemours and Co. (Wilmington, DE, U.S.A.) [13], and was over-expressed in *Escherichia coli* HB101. The expression product was purified as described by Landry and Gierash [14]. Further purification of the GroES fraction was carried out by hydroxyapatite chromatography using a 0–0.2 M phosphate gradient to remove the co-expressed chloramphenicol acetylase. The GroEL fraction was treated with ATP according to Schmidt et al. [15] to remove endogenously bound polypeptides.

GroEL and GroES thus purified each showed one band on SDS/PAGE, with the expected subunit masses of 60 kDa and 10 kDa respectively. The purified GroEL preparation has very low intrinsic fluorescence, especially at an excitation wavelength of 295 nm, allowing fluorimetric determination of GAPDH binding. The preparation and assay of activity of rabbit muscle GAPDH were as described by Liang et al. [2]. The preparation and irradiation of carboxymethylated GAPDH were according to Ho and Tsou [16]. The concentrations of GroEL, GroES and irradiated carboxymethylated GAPDH were determined by the method of Bradford [17], with BSA as a standard. The concentrations of GAPDH and BSA were determined by measuring the absorbance at 280 nm, with absorbance coefficients ($A_{1\text{cm}}^{0.1\%}$) of 1.00 [18] and 0.66 respectively. Unless otherwise specified, GroEL, GroES and GAPDH are considered as being a tetradecamer, heptamer and monomer (as the denatured enzyme is presumably fully dissociated) respectively in the calculations of concentrations and molar ratios. In all experiments, 0.1 M potassium phosphate buffer containing 1 mM EDTA, pH 7.5, was employed.

Denaturation of GAPDH

The denaturation of GAPDH was carried out by incubation of the enzyme (69 μM) in 4.0 M GuHCl with 1 mM DTT for at least 4 h at 4 °C. For some refolding experiments in the presence of ANS, 0.14 mM enzyme was denatured in GuHCl at the desired concentrations with 1 mM DTT overnight at 4 °C.

Refolding of GAPDH

The refolding of GuHCl-denatured GAPDH was initiated by 100- or 50-fold dilution into the refolding buffer (25 °C) containing 5 mM NAD^+ and 5 mM DTT, with or without GroEL or ANS as specified. Aliquots containing 1–2 μg of GAPDH were taken after different time intervals for activity measurements. The re-activation yield was defined as a percentage of the activity of native GAPDH treated in the same way but in the absence of GuHCl. GroEL-assisted re-activation was initiated at 25 °C by adding MgATP or MgATP/GroES 30 min after dilution of denatured GAPDH in the presence of GroEL, to allow the binding between GroEL and GAPDH folding intermediates to reach equilibrium.

The aggregation of GAPDH during refolding was monitored continuously at 25 °C by 90° light scattering at 488 nm. The change in the intrinsic fluorescence of denatured GAPDH upon dilution was followed at 25 °C by monitoring the emission intensity at 335 nm, with excitation at 295 nm. In this case NAD^+ was omitted, since it quenches significantly the intrinsic fluorescence of GAPDH [16]. The fluorescence intensity at 410 nm of denatured and irradiated carboxymethylated GAPDH during refolding was measured at different times, with excitation at 325 nm. The ANS fluorescence spectra of all samples were measured with excitation at 365 nm. All determinations of light scattering and fluorescence were performed in a Hitachi F4010 spectrofluorimeter.

RESULTS

Effect of GroEL concentration on the aggregation and re-activation of GuHCl-denatured GAPDH

Upon dilution of denatured GAPDH, the enzyme aggregated markedly (as monitored by light scattering) after a lag period of a few minutes. Both the rate and the extent of aggregation decreased with increasing concentrations of GroEL in the dilution buffer, and aggregation was fully suppressed at a molar ratio of GroEL/GAPDH of 0.38 (Figure 1A).

Figure 1(B) shows the re-activation of denatured GAPDH after dilution to concentrations of 0.11 μM , 0.69 μM and 2.78 μM in the presence of GroEL. The spontaneous re-activation of GAPDH at all concentrations decreased with an increasing concentration of GroEL, and was fully suppressed at a ratio of GroEL/GAPDH of 0.5. The suppression of both the spontaneous re-activation and the aggregation of GAPDH during refolding in the presence of GroEL indicates the formation of a stable complex between GroEL and a folding intermediate of GAPDH [19,20].

Thus the re-activation of GAPDH decreased with increasing GroEL concentration and became fully suppressed at a molar ratio of 0.5, suggesting the binding of one tetradecameric GroEL molecule to one dimer or two monomers of GAPDH, i.e. two tetradecameric GroEL molecules to one GAPDH tetramer. Careful inspection of Figure 1(B) shows that the binding of one GroEL per tetrameric GAPDH (i.e. GroEL/GAPDH = 0.25) suppressed more than 50 % of the re-activation. This recalls the stochastic treatment of activity change during the chemical modification of proteins [21] that has since been successfully applied to ascertain the number of essential groups involved in protein folding [22]. This treatment relates the fractional activity remaining (a) to the number of side-chain groups modified (m) and the number of essential groups (i) as:

$$a^{1/i} = x = 1 - m/2$$

where x is the fraction of groups remaining unmodified [21]. In the present case, the number of groups modified (m) is the number of GroEL tetradecamers bound to a GAPDH tetramer out of a total number of two, and the number of essential groups (i) corresponds to the number of GroEL binding sites per GAPDH tetramer that are essential for the refolding of GAPDH. Treatment of the re-activation data for GAPDH using this method to correlate the stoichiometry of GroEL binding with the folding of GAPDH, by plotting $a^{1/i}$ against m , gives the Tsou plot [21] shown in the inset of Figure 1(B), where a is the relative re-activation yield corresponding to the fractional activity remaining in the original treatment by Tsou [21] and m is the ratio of GroEL tetradecamer bound to GAPDH tetramer, and corresponds to the number of groups modified. Only the plot with $i = 2$ gives a straight line, indicating that re-assembly to form the native tetramer requires both dimers in the free and unbound state.

Effect of GroEL on the intrinsic fluorescence of GAPDH during refolding

The intrinsic fluorescence spectra of native and GuHCl-denatured GAPDH and of a GroEL-bound GAPDH folding intermediate are shown in Figure 2(A). GroEL has no tryptophan residues [23,24], and the purified GroEL preparation shows very low intrinsic fluorescence emission at an excitation wavelength of 295 nm. Both the intensity and the emission maximum of the GroEL-bound GAPDH folding intermediate are between those of the native and the fully denatured enzymes, indicating a

Figure 1 Effect of GroEL concentration on the refolding of GuHCl-denatured GAPDH

(A) Aggregation of GAPDH (0.69 μM) during refolding monitored by light scattering in the presence of GroEL with molar ratios as indicated. (B) Re-activation of GAPDH after dilution to 0.11 μM ($\circ$), 0.69 μM ($\square$) and 2.78 μM ($\triangle$) in the presence of GroEL. The molar ratios of GroEL/GAPDH are considered as tetradecamer and monomer respectively as indicated, and re-activation was assayed 3.5 h after dilution. The inset shows the Tsou plot of the re-activation data for all three GAPDH concentrations, where m is the ratio of GroEL tetradecamer to GAPDH tetramer and a is the relative re-activation of GAPDH in the presence of GroEL as compared with the results for spontaneous re-activation.

Figure 2 Intrinsic fluorescence of GAPDH with excitation at 295 nm under different conditions

(A) Native (curve 1), GroEL-bound folding intermediate (curve 2) and GuHCl-denatured (curve 3) GAPDH. (B) Changes in fluorescence intensity at 335 nm (curves 1 and 2) and aggregation (curves 3 and 4) of denatured GAPDH upon dilution to 0.69 μM in the absence (curves 1 and 3) and presence (curves 2 and 4) of GroEL at a molar ratio of 0.76.

Table 1 Fluorescence of ANS bound to GAPDH under different conditions

ANS (50 μM) was added to native GAPDH, GroEL or fully denatured GAPDH immediately before the fluorescence intensity at 480 nm was measured. For the refolding product of GAPDH in the presence or absence of GroEL, ANS was added 35 min after dilution. In all experiments, final concentrations of 0.69 μM GAPDH and 0.52 μM GroEL were used.

Sample	Fluorescence intensity (arbitrary units)
GADPH	
Native	2.1
Denatured	0
Refolding product	66.1
GroEL-bound intermediate	434.6
GroEL	193.7

conformation intermediate between those of the denatured and the native enzymes. GroEL has no effect on the intrinsic fluorescence of native GAPDH.

The change in the intrinsic fluorescence of denatured GAPDH upon dilution (Figure 2B) shows an initial burst in the first 15 s, indicating rapid initial folding that leads to the formation of an early folding intermediate. This initial increase in emission intensity is then followed by a slow decline, suggesting further conformational changes to the enzyme. Compared with the fluorescence change, the course of aggregation is much slower, with a lag phase in the first few minutes followed by a gradual increase to approach completion in about 1 h, suggesting that the early folding intermediates aggregate by incorrect association. The presence of GroEL at a molar ratio of 0.76 suppresses aggregation completely, but does not affect the initial burst in fluorescence emission. The decreased emission corresponds to the fluorescence of the folding intermediate bound to GroEL (Figure 2A).

ANS fluorescence of GroEL-bound GAPDH

As shown in Table 1, the fluorescence intensity of ANS at 480 nm showed very little increase in the presence of either native or denatured GAPDH, indicating that neither binds ANS signifi-

Figure 3 Changes in ANS fluorescence intensity at 480 nm upon dilution of denatured GAPDH

Refolding of denatured GAPDH ($0.69 \mu\text{M}$) was carried out in the presence of $50 \mu\text{M}$ ANS without (curve 1) and with (curve 2) $0.52 \mu\text{M}$ GroEL. In the latter case, the fluorescence of ANS bound to GroEL was corrected for.

cantly. The fluorescence intensity of ANS showed some increase in the presence of GroEL or the spontaneous folding products of denatured GAPDH. However, the folding intermediate of GAPDH complexed with GroEL showed by far the greatest increase in ANS emission compared with that of ANS with GroEL, native GAPDH, denatured GAPDH or its spontaneously refolded products. It appears that GroEL stabilizes the folding intermediate of GAPDH in a state having an exposed hydrophobic region that is absent from both the native and the fully denatured enzymes. These results are similar to those reported for rhodanese and dihydrofolate reductase [25].

Figure 3 shows the change in ANS fluorescence during the refolding of GAPDH. On dilution of denatured GAPDH in a folding buffer containing $50 \mu\text{M}$ ANS, there is a rapid initial burst of fluorescence at 480 nm, indicating the rapid formation of a folding intermediate capable of binding ANS. The presence of GroEL in the dilution mixture had no effect on the appearance of the burst phase of ANS emission. Careful comparison of Figure 3 with Figure 2(B) shows that the initial increase in intrinsic fluorescence is more rapid than the corresponding increase in ANS fluorescence for the refolding of either the denatured enzyme itself or the denatured enzyme in the presence of GroEL. These results suggest either that the binding of the folding intermediate to ANS is a little slower than its formation, or that the folding intermediate formed upon dilution isomerizes in a slightly slower reaction to a form that binds ANS.

Effect of ANS on the re-activation and aggregation of denatured GAPDH

With increasing concentrations of ANS in the folding buffer, the re-activation of GuHCl-denatured GAPDH at $2.78 \mu\text{M}$ decreased and its aggregation increased markedly (Figure 4). Re-activation was suppressed completely by ANS at concentrations higher than $150 \mu\text{M}$. During refolding in the presence of ANS, the appearance of ANS fluorescence greatly preceded aggregation (results not shown). It appears that the binding of ANS to the early folding intermediate shields an important hydrophobic region that is required for correct assembly and re-activation, and probably the lack of correct assembly leads to incorrect association and markedly increased aggregation.

Figure 4 Effect of ANS concentration on the re-activation (○) and aggregation (□) of GuHCl-denatured GAPDH upon dilution

Refolding of GAPDH at a final concentration of $2.78 \mu\text{M}$ was carried out in the presence of different concentrations of ANS.

Effects of GroEL and ANS on the re-activation of GAPDH denatured by different concentrations of GuHCl

As shown in Figure 5, both the intrinsic fluorescence intensity and the emission maximum of GAPDH change in two stages with increasing concentration of GuHCl, similar to that reported by Xie and Tsou [26] and Ju and Tsou [27] except for the increase in fluorescence intensity in the first stage, which is different from that reported by Ju and Tsou [27] but in agreement with that observed by He et al. [28]. The fluorescence at 480 nm of ANS bound to GAPDH also changes in two stages with the increase in GuHCl concentration, with an initial increase from 0.2 M to 0.5 M followed by a region of relatively little change and then a decrease to the original level from 0.9 M to 1.5 M GuHCl. These ranges of GuHCl concentration affecting ANS binding are similar to those required for the two-stage changes in its intrinsic fluorescence. The conformation of the partially denatured GAPDH at $0.5\text{--}0.9 \text{ M}$ GuHCl seems to be in a relatively stable intermediate state, with changes in the micro-environment of tryptophan residues and exposure of hydrophobic regions, as

Figure 5 Intrinsic and bound ANS fluorescence of GAPDH in GuHCl solutions of different concentrations

GAPDH ($2.78 \mu\text{M}$) was treated with different concentrations of GuHCl overnight at 4°C before the intensity at 335 nm (○) and the maximum emission wavelength (□) of intrinsic fluorescence were measured. The emission intensity of bound ANS at 480 nm (△) was measured after incubation of the enzyme denatured as above with $10 \mu\text{M}$ ANS for 10 min.

Figure 6 Effects of GroEL and ANS on the re-activation of GAPDH denatured by various concentrations of GuHCl

Re-activation of 2.78 μM GAPDH denatured overnight by different concentrations of GuHCl as indicated was carried out in the absence (○) or presence of 200 μM ANS (□) or 2.08 μM GroEL without (△) or with (●) addition of 5 mM magnesium acetate and 1 mM ATP. The activities recovered were assayed 3.5 h after dilution.

indicated by increases in both intrinsic fluorescence and ANS binding (Figure 5).

The spontaneous re-activation yield of denatured GAPDH depends to a large extent on the initial concentration of GuHCl used for denaturation, and therefore on the initial conformation of the enzyme for refolding (Figure 6). A low concentration of GuHCl (0.4 M) is sufficient for complete inactivation of the enzyme, but leads to very little conformation change as detected by commonly employed methods [27]. The recovery of activity of 2.78 μM GAPDH denatured by 0.4 M GuHCl can be close to 100%. However with increasing GuHCl concentrations, the re-activation yield decreases until, at an initial GuHCl concentration of 1.2 M, no recovery of activity can be observed, probably due to irreversible aggregation of the enzyme [2]. With further increases in GuHCl concentration, the spontaneous re-activation on dilution increases slightly to about 5%. It is interesting to note that the presence of GroEL at a molar ratio of 0.76, of ANS at 200 μM or of GroEL with ATP has little effect on the spontaneous

re-activation yield of GAPDH denatured in GuHCl at a concentration of less than 1.2 M. However, when GAPDH was denatured by GuHCl at concentrations greater than 1.6 M, at which the enzyme shows low spontaneous re-activation, the presence of either GroEL or ANS completely suppressed re-activation. Furthermore, with both GroEL and ATP in the refolding mixture, GAPDH denatured by 1.1–1.3 M GuHCl showed no re-activation upon dilution, but the recovery of GAPDH activity increased markedly with increasing GuHCl concentrations above 1.4 M.

GroEL-assisted re-activation of denatured GAPDH

As shown in Figure 7(A), GroEL completely suppressed the re-activation of GAPDH, most probably by forming a stable complex with the folding intermediate. Re-activation could be initiated by the addition of MgATP alone, or to a greater extent by MgATP and GroES together. In the presence of MgATP, re-activation approached 28%, increasing to 50% with GroES; however, like spontaneous re-activation, it is a slow process, taking about 3.5 h for completion.

On UV irradiation, modified holo-GAPDH with thiol groups carboxymethylated forms a fluorescent NAD⁺ derivative at the active site with an emission maximum at 410 nm [29]. The active-site conformation of the modified enzyme has to be in the native state for this derivative to show its fluorescent properties [26]. As shown in Figure 7(B), denatured GAPDH carrying this fluorophore showed no fluorescence at 410 nm. During spontaneous folding of the denatured modified enzyme upon dilution, the increase in fluorescence at 410 nm paralleled the recovery of activity of the denatured unmodified GAPDH (Figure 7A), indicating recovery of the native active-site conformation. In the presence of GroEL, no increase in fluorescence at 410 nm accompanied binding of folding intermediates of GAPDH carrying the fluorophore, indicating that the active site of the GroEL-bound intermediate was in a non-native state. However, the addition of either MgATP alone or MgATP/GroES brought about the immediate appearance of fluorescence at 410 nm of the modified enzyme, and the increase in emission paralleled the recovery of activity of the denatured unmodified enzyme, indicating the formation of the native conformation at the active site

Figure 7 Refolding of denatured GAPDH and of GAPDH carrying the active-site fluorophore

(A) Re-activation of denatured GAPDH (0.69 μM). (B) Relative fluorescence intensity at 410 nm of denatured GAPDH (0.69 μM) carrying the active-site fluorophore during folding compared with that of the modified enzyme. Refolding was carried out by dilution in the absence (○) or presence of 0.52 μM GroEL (●, ▲, ■) with addition of 5 mM magnesium acetate and 1 mM ATP without (▲) or with (■) 0.52 μM GroES added at the time indicated by the arrow.

upon the dissociation of the folding intermediate from the GroEL complex by MgATP or MgATP/GroES.

Todd and Lorimer [30] reported that low concentrations of GuHCl had a profound effect on the activity/structure of the chaperonins at low K^+ concentrations (1 mM), but that the ATPase activity of GroEL changed only slightly at high concentrations of K^+ (100 mM). Similarly, in the present study, in 0.1 M potassium phosphate buffer, concentrations of residual GuHCl in the range 13–120 mM had no effect on the level of recovery of activity of denatured GAPDH in the presence of GroEL/ATP (results not shown).

DISCUSSION

During the unfolding of GAPDH in GuHCl, both the changes in intensity and emission maximum of its intrinsic fluorescence [26,27] and the dissociation/association behaviour of the tetrameric enzyme, as shown by light-scattering measurements [2], take place in two stages with increasing GuHCl concentration. It is now shown that the first stage is accompanied by an increase in ANS binding, indicating exposure of a hydrophobic region that is destroyed in the second stage of unfolding, as shown by a decrease in ANS fluorescence to the level of that of free unbound ANS. It is likely that exposure of the hydrophobic region in the intermediate stage is responsible for the association and aggregation of the partially unfolded GAPDH [2]. Upon dilution, the initial folding of the fully denatured GAPDH (unfolded monomer) takes place very rapidly, as indicated by a burst-phase increase in its intrinsic fluorescence within a few seconds; this is followed, after a time lag of a few minutes, by an increase in light scattering that indicates aggregation of the partly folded species. It appears likely that a rapid initial collapse of the extended chain of the denatured enzyme leads to the formation of an early intermediate that is prone to aggregation.

GroEL recognizes and binds tightly to the folding intermediate of GAPDH to form a stable complex with a stoichiometry of (probably) one tetradecameric GroEL molecule to one GAPDH dimer under saturating conditions (Figure 1B). The dimensions of the cavity of the GroEL molecule were suggested by Braig et al. [31], according to the crystal structure, to be approx. $47 \text{ \AA} \times 146 \text{ \AA}$, and might accommodate a protein of 147, 100 or 50–60 kDa depending on assumptions made for different folding states of the protein. The molecular mass of the GAPDH dimer (72 kDa) is similar to the above range. Different proteins fold differently and have different specific volumes. The shape, conformation and size of the GAPDH folding intermediates bound to GroEL are unclear. Furthermore, the conformation of the GroEL cavity is presumably not rigid, and could also change upon binding to its substrate. Recently it has been reported by Zahn et al. [32] that the isolated monomeric polypeptide binding fragments of GroEL exhibit high chaperone activity *in vitro* and are active without the allosteric properties or the central cavity, which has been proposed to act as a 'folding cage' *in vivo*. It is not impossible that GAPDH folding intermediates *in vitro* bind GroEL at the surface of the rather flexible apical domain and only partly within the cavity. Further work to examine these possibilities is in progress. The formation of the complex of GroEL with the GAPDH folding intermediate suppresses both the re-activation and the aggregation of GAPDH during folding. The conformation of the folding intermediate of GAPDH in the complex is different from those of both the native and the fully denatured enzymes, and is in a partly folded state. This folding intermediate is stabilized by binding to GroEL, and is the only species to bind ANS, indicating the exposure of a hydrophobic region in the folding intermediate. For the enzyme carrying the

fluorescent NAD^+ derivative at the active site, only the enzyme in the native state shows marked fluorescence emission at 410 nm, indicating conformational integrity at the active site [26]. Under identical denaturation and refolding conditions used for the unmodified enzyme, neither the denatured enzyme nor the GroEL-bound intermediate carrying the fluorescent NAD^+ derivative displayed fluorescence emission at 410 nm, indicating the non-native state of the active-site conformation.

As GuHCl at a concentration of 0.9 M inactivates the enzyme completely, with a perturbed conformation at the active site but no noticeable changes in the global conformation of the enzyme molecule [26,27], it appears likely that the molecule denatured by GuHCl concentrations below 0.9 M, although completely inactivated does not unfold sufficiently to produce, on dilution, folding intermediates that could interact with either GroEL or ANS. GAPDH denatured by 1.1–1.3 M GuHCl aggregates irreversibly [2] and does not dissociate upon dilution to bind GroEL, as no re-activation of GAPDH occurs in the presence of ATP. However, GAPDH denatured in GuHCl at concentrations greater than 1.4 M is completely unfolded and monomeric [2], and on dilution goes through an intermediate stage that is recognized by and binds to either GroEL or ANS. The binding suppresses the spontaneous re-activation of GAPDH completely, and dissociation from GroEL in the presence of ATP results in partial refolding to form the native molecules. The interactions with GroEL of folding intermediates of lactate dehydrogenase at different states of denaturation caused by 2–4 M GuHCl were analysed by Badcoe et al. [33].

ANS has been used to characterize conformational changes during protein folding [34–38]. In the present study, the presence of ANS in the refolding buffer lowered the re-activation of GAPDH but increased its aggregation. It appears that ANS binding interferes with correct folding of the folding intermediate, but does not prevent the incorrect interactions leading to aggregation. In addition to being a hydrophobic probe, other reactions of ANS have been reported, e.g. its stimulatory effect on the catalytic activity of cytochrome *P*-450 [39], its inhibitory effect on RNA polymerase [40] and its denaturing effect on DnaK [41].

The close similarity of the effects of GuHCl concentration on the intrinsic fluorescence and ANS binding of GAPDH suggests that the folding intermediate is similar to the relatively stable partially unfolded state of the enzyme denatured in 0.5–1.0 M GuHCl, and might represent the partially unfolded and aggregation-prone dimer suggested by Liang et al. [2]. This is also the GroEL binding species, which is formed in the burst phase after dilution of the denatured enzyme, is stabilized against both re-activation and further aggregation, and is re-activatable when assisted by GroEL and ATP.

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