ELSEVIER

FEBS Letters 339 (1994) 45–49

FEBS 13630

FEBS LETTERS

Generation of a stable folding intermediate which can be rescued by the chaperonins GroEL and GroES

Dadna Peralta, Dallas J. Hartman, Nicholas J. Hoogenraad, Peter B. Høj*

Department of Biochemistry, La Trobe University, Bundoora, Victoria 3083, Australia

Received 17 December 1993

Abstract

Pig heart mitochondrial malate dehydrogenase was chemically denatured in guanidine HCl. Upon 50-fold dilution of the denaturant spontaneous refolding could be observed in the temperature range 12–32°C. At 36°C spontaneous refolding was not observed but a stable folding intermediate that is fairly resistant to aggregation was formed. This intermediate is readily refolded by the chaperonins GroEL and GroES and may prove useful in future attempts to describe several aspects of chaperonin action at physiological temperatures.

Key words: Malate dehydrogenase; Aggregation; Denaturation

1. Introduction

Although many proteins can fold and assemble spontaneously in vitro, it has become apparent in recent years that folding in vivo often requires the action of ubiquitous helper proteins called molecular chaperones (reviewed in [1]). The chaperonins comprise a class of molecular chaperones defined by their sequence homology with the *E. coli* proteins GroEL and GroES. GroEL is a tetradecamer comprised of 14 identical 60 kDa subunits arranged as two stacked rings with a seven fold axis of symmetry [2,3] and a central cylindrical cavity in which substrate polypeptides are apparently bound [4,5]. GroES is a heptamer comprised of 7 identical 10 kDa subunits [6,7] which, in the presence of MgATP, binds to one end of the GroEL 14-mer and thereby attenuates its resident ATPase activity [4]. Biochemical [8–10] and genetic [11] evidence indicate that GroEL function is frequently dependent upon a physical interaction with GroES for efficient chaperone action.

A significant number of in vitro refolding experiments have been employed in an attempt to dissect the mechanistic details of chaperonin action. The collective evidence obtained so far indicates that chaperones do not increase the rate constant of refolding per se but merely, via a 'molecular sponge action', enhance the yield of folded protein by kinetic partioning of an uncommitted polypeptide in favour of productive folding pathways over aggregation [12,13]. Detailed studies with rhodanese [13], ribulose 1,5-bisphosphate carboxylase-oxygenase [8] and citrate synthase [14] have further shown that chaperone-substrate interaction preferably should take place at an early stage during the protein refolding reaction as aggregates cannot be rescued once formed.

Although a broad understanding of chaperonin action has been achieved, the mechanistic details and substrate specificities still remain unknown. A major stumbling block is the lack of well defined and stable chaperonin substrates which are hard to obtain due to the propensity of denatured proteins to undergo either spontaneous refolding or aggregation extremely quickly following dilution of a denaturant. Here we describe conditions enabling the formation of a denatured state of malate dehydrogenase (MDH) that does not appear to aggregate quickly and constitutes a substrate for chaperonin mediated refolding. This stable folding intermediate may prove useful in future attempts to dissect the nature of chaperonin action and for a description of structural features of their substrates.

2. Materials and methods

E. coli GroEL and GroES were purified and pig heart mitochondrial MDH (Boehringer Mannheim) assayed with 188 μM NADH as previously described [15]. Concentrations of MDH were calculated for the monomer using a molecular mass value of 35 kDa. The protein concentrations of GroEL-14mer and GroES-7mer were determined spectrophotometrically using extincition co-

* Corresponding author. Fax: (61) (3) 479 2467.

Abbreviation: MDH, malate dehydrogenase.

SSDI 0014-5793(94)00014-M

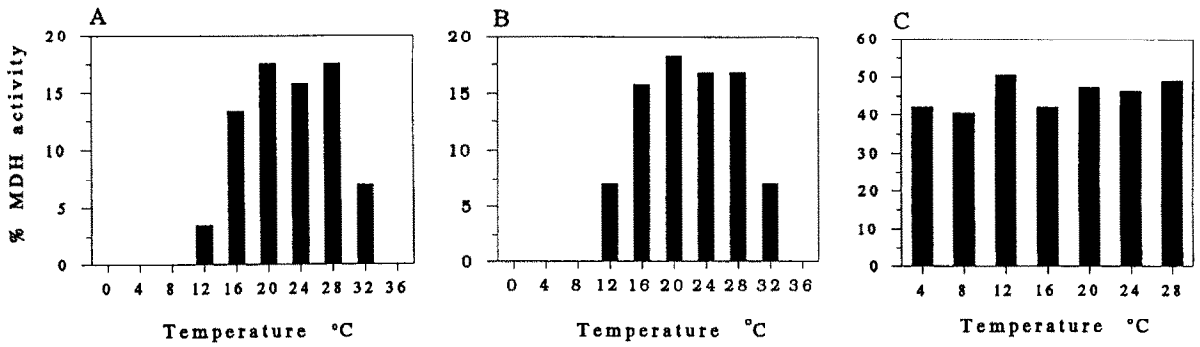

Fig. 1A and B. MDH, denatured in 7.1 M guanidine HCl, was diluted 50-fold into 50 mM Tris-HCl pH 7.6, 7 mM $MgCl_2$, 10 mM KCl and 1 mM DTT to give a final MDH protomer concentration of 178 nM. Reactions were incubated at the indicated temperatures and aliquots assayed for MDH activity after 125 min (A) and 185 min (B). One hundred percent activity is defined as the activity displayed by an equal amount of MDH that had not been denatured before dilution but was otherwise treated identically. C. MDH, denatured in 7.1 M guanidine HCl, was diluted 50-fold (final protomer concentration of 178 nM) into 50 mM Tris-HCl pH 7.6, 7 mM $MgCl_2$, 10 mM KCl, 1 mM DTT, 1.6 mM ATP, 357 nM GroEL and 331 nM GroES, at the indicated temperatures. Immediately after dilution, the reaction mixtures were incubated at 36°C for 30 min and then assayed for MDH activity. One hundred percent activity is defined as above.

efficient values quoted by Price et al. [16] and Jackson et al. [17], respectively. MDH was chemically denatured by incubation at 0°C for at least 1 h in 50 mM Tris pH 7.6 and 4.8 mM DTT containing guainidine HCl (concentration stated in figure legends).

3. Results

The spontaneous refolding of chemically denatured MDH at 20°C has been thoroughly studied [18]. Since temperature is an important variable in the in vitro refolding of many proteins, we determined the temperature range at which MDH was competent for refolding. MDH, denatured by incubation in 7.1 M guanidine HCl, was diluted into folding buffer (final MDH monomer concentration of 178 nM) and incubated at temperatures ranging from 0 to 36°C. Following 125 min and 185 min of incubation, aliquots were removed and assayed for MDH activity (Fig. 1A and B). Varying degrees of MDH reconstitution occurred at incubation temperatures between 12 and 32°C, with a maximal yield of only 20%, even following incubation for as long as 45 h (data not shown). The most efficient reconstitution occurred in the narrower temperature range of 16 to 28°C.

Recently, the *E. coli* chaperonins GroEL and GroES were reported to mediate the refolding of chemically denatured mitochondrial MDH at 20°C [12]. We sought to determine if GroEL and GroES could mediate the refolding of MDH at 36°C, a temperature at which spontaneous refolding does not occur (Fig. 1A and B). Indeed, refolding yields reaching 50% were achieved when GroEL, GroES and ATP were included in the refolding buffer (Fig. 2). Omission of GroES or ATP completely abolished refolding, however, the subsequent addition of the missing component resulted in a time dependent increase in the refolding yield, reaching approximately 35%. ATP hydrolysis was necessary for refolding since substituting ATP with the non-hydrolysable analogue AMP-PNP completely abolished refolding.

Since aggregation competes kinetically with refolding, conditions which decrease hydrophobic interactions and thereby aggregation, such as low temperatures, are expected to enhance the refolding yield in vitro [19]. We therefore sought to determine if MDH refolding in vitro could be enhanced by first diluting denatured MDH into the complete chaperonin system preincubated at low non-physiological temperatures to trap GroEL-MDH folding intermediates, before raising the temperature to 36°C. When MDH was diluted into the chaperonin system preincubated at temperatures ranging from 4 to 28°C before the temperature upshift to 36°C, no significant correlation between the dilution temperature and the refolding yield was observed (Fig. 1C). The most logical explanation for the lack of variability in the yield of native MDH as a function of dilution temperature is that virtually all denatured MDH binds to GroEL before aggregation can occur. Such a scenario is most likely to operate if binding to GroEL is instantaneous, as suggested for the barnase-GroEL complex formation [20], but alternatively, it can be ensured if longlived folding intermediates with a low propensity to aggregate are formed and survive until their encounter with the chaperones. To address the latter possibility, i.e. the formation of longlived intermediates, MDH was diluted into folding buffer and incubated for 10 min at 36°C before addition of GroEL and GroES (Fig. 3A).

Immediately prior to GroEL and GroES addition, no MDH refolding was observed, however, a time dependent increase in the yield of refolding was observed immediately following chaperonin administration. Omission of either GroEL, GroES or ATP from the refolding mixture abolished reconstitution (data not shown). The yield of MDH activity was about 42% of that obtained when GroEL and GroES were present from the time of dilution. The time between dilution of the denaturant and

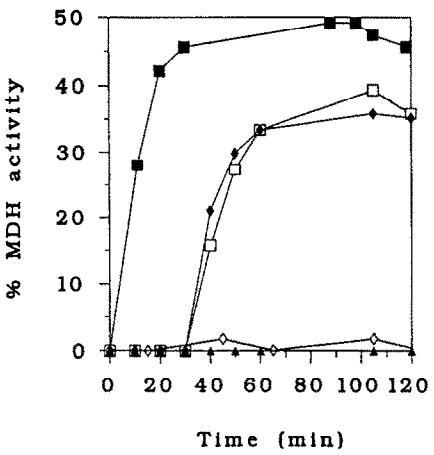

Fig. 2. Refolding of MDH with the *E. coli* chaperonins GroEL and GroES. MDH, denatured by incubation in 7.1 M guanidine HCl, was diluted 50-fold (final protomer concentration of 178 nM) into 50 mM Tris-HCl pH 7.6, 7 mM $MgCl_2$, 10 mM KCl and 1 mM DTT containing: 357 nM GroEL, 331 nM GroES and 1.6 mM ATP (■), 357 nM GroEL and 331 nM GroES (□), 357 nM GroEL and 1.6 mM AMP-PNP (▲), 357 nM GroEL and 1.6 mM ATP (◆), or alternatively buffer alone (◇), and incubated at 36°C. After 30 min the reaction mixtures containing GroEL and ATP (◆) and GroEL and AMP-PNP (▲) were supplemented with 473 nM GroES, while the reaction mixture containing GroEL and GroES (□) was supplemented with 1.6 mM ATP. One hundred percent activity is defined as in Fig. 1.

addition of GroEL and GroES could be extended to at least 40 min, although the refolding yield did tend to decrease slightly as the time between dilution and chaperonin addition increased (Fig. 3A).

To investigate whether the folding intermediate generated at 36°C was prone to aggregation, we diluted increasing amounts of MDH from denaturant and incubated the mixture at 36°C for 10 min before the addition of chaperonins (Fig. 3B). Increasing the concentration of MDH 3-fold (534 nM) in comparison to the initial experiments (178 nM), did not decrease the percentage yield of MDH activity regained. Indeed, a slight increase in the yield was observed, probably as a result of enhanced dimerisation of folded MDH monomers at the higher protein concentrations.

We next sought to determine whether the folding intermediate generated upon dilution of MDH from denaturant at 36°C was still competent for spontaneous refolding at 20°C, a temperature at which spontaneous refolding can occur (see Fig. 1A and 1B). Denatured MDH was diluted into folding buffer and incubated at 36°C for 10 min before being shifted to 20°C. Although it appeared slower, the amount of MDH activity regained by spontaneous folding at 20°C after the temperature shift was not significantly different from that obtained when, in a seperate experiment, GroEL and GroES, were added 10 min after MDH dilution and incubation continued at 36°C (data not shown).

4. Discussion

The spontaneous refolding of chemically denatured mitochondrial MDH was studied over the temperature range 0–36°C. Refolding was observed at temperatures between 12 and 32°C but the recovery of MDH activity never exceeded approximately 20%. The inability to observe spontaneous refolding of MDH at temperatures above 32°C is most likely due to the increase in hydrophobic interactions at high temperatures which can lead to aggregation events, or alternatively, rapid denaturation. For this reason, in vitro refolding experiments are often performed at low non-physiological temperatures. In vivo, a group of proteins called molecular chaperones are believed to modulate protein-protein interactions and thereby prevent aggregation events thus promoting the efficiency of protein refolding (reviewed in [1]). Much of our current knowledge about chaperone action has been gathered from in vitro studies with the *E. coli* chaperones GroEL and GroES which can ensure refolding of numerous denatured proteins at temperatures at which spontaneous refolding does not occur. We have shown that chemically denatured MDH can be refolded in an ATP dependent manner by GroEL and GroES at 36°C, a temperature at which activity is not regained in the absence of chaperonins. The level of refolding in the presence of the chaperonins approached 50% in as little as 30 min. These initial results are in complete agreement with a recent study published by Miller et al. [12] who investigated the modulation by GroEL and GroES of MDH refolding at 20°C, a temperature at which spontaneous folding does occur. A minor but important practical difference between the two studies is that we readily observed chaperonin mediated folding in Tris buffers supplemented with KCl while Miller et al. [12] reported that chaperonin action did not take place in Tris-buffers but readily in sodium phosphate buffers without the need for exogenous K^+ supplement.

The question as to whether chaperonins can act as true folding catalysts, or merely as passive molecular sponges by inhibiting protein aggregation, has yet to be determined, as has the exact stoichiometry of ATP-hydrolysis associated with the chaperonin-mediated refolding process. Since aggregation competes kinetically with protein folding, particularly at high protein concentrations and physiologically relevant temperatures, technical difficulties arise when trying to answer these important questions by performing refolding experiments with substoichiometric levels of chaperonins. There is therefore a need to find conditions under which stable folding intermediates can be generated in relatively large quantities. A step towards this goal was taken by van der Vies et al. [21] who, in an elegant study of *Rhodospirillum rubrum* RUBISCO, defined and spectroscopically characterised a folding intermediate (I_1-state) which could be formed and refold spontaneously to the native state at tempera-

tures below about 20°C. Folding of the I_1-state was arrested by GroEL.

Here we describe the formation of a folding intermediate of MDH that is not prone to aggregation but which still represents a competent substrate for the chaperonin refolding apparatus. What could the conformational state of such an intermediate be? Since extensive secondary structures are formed within milliseconds of the initiation of protein folding in vitro (reviewed in [22]) and since the denatured states of proteins exhibit significant amounts of persistent residual structure [23] it is likely that the inactive folding intermediate defined here possesses significant residual structure which somehow has been arrested in the process of folding. This arrest in the folding process is dependent upon temperature, as decreasing the incubation temperature of the intermediate to 20°C results in recovery of activity. Recently, Langer et al. [24] studied the chaperonin mediated refolding of rhodanese and showed that DnaK and DnaJ recognised the folding polypeptide as an extended chain which was prevented from aggregation before GrpE dependent transfer of a more compact intermediate onto GroEL and GroES. The data we presented in this study suggests that some polypeptides may not rely on a full complement of chaperones to ensure 'delayed' folding by the GroEL/GroES system and may at least in part explain why *E. coli* DnaK [25] and DnaJ [26], but not GroEL and GroES [27], mutants are viable, albeit severely impaired in their growth.

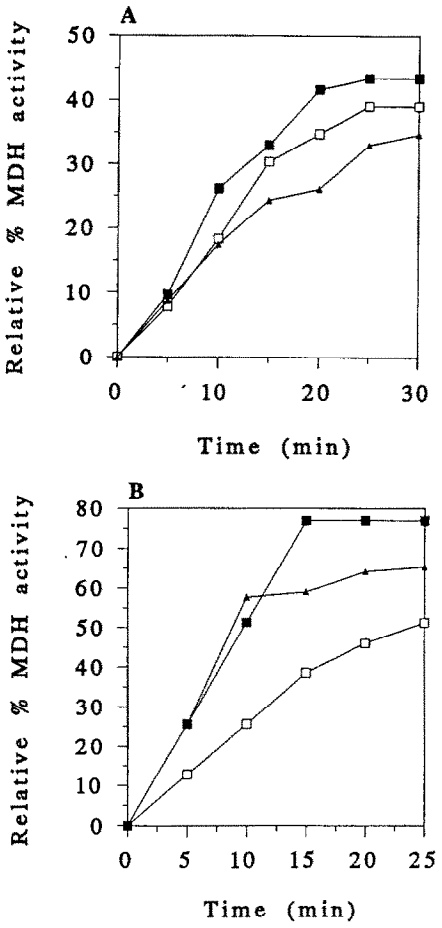

Fig. 3. Formation of a stable MDH folding intermediate which is still competent for interaction with GroEL. A. MDH, denatured by incubation in 7.1 M guanidine HCl, was diluted 50-fold (final protomer concentration of 178 nM) into 50 mM Tris-HCl pH 7.6, 7 mM $MgCl_2$, 10 mM KCl, 1.9 mM ATP and 1 mM DTT at 36°C. After 10 min (■), 20 min (□) or 40 min (▲), the reactions were supplemented with 401 nM GroEL and 417 nM GroES. At the indicated time points after the addition of GroEL and GroES, aliquots were removed and assayed for MDH activity. One hundred percent relative activity is defined as the percentage of activity displayed by an equal amount of MDH reconstituted when GroEL, GroES and ATP were present at the point of MDH dilution (compare with Fig. 2). B. MDH, denatured by incubation in 6.1 M guanidine HCl (final protomer concentrations of 178 nM (□), 356 nM (■) and 534 nM (▲), was diluted 50-fold into 50 mM Tris-HCl pH 7.6, 7 mM $MgCl_2$, 10 mM KCl, 1.9 mM ATP and 1 mM DTT and incubated at 36°C. After 10 min, reactions were supplemented with 399 nM GroEL and 622 nM GroES (□), 704 nM GroEL and 1,098 nM GroES (■) and 987 nM GroEL and 1,540 nM GroES (▲). At the indicated time points after the addition of GroEL and GroES, aliquots containing equal amounts of MDH were removed and assayed for activity. One hundred percent relative activity is defined as above but using 178 nM MDH, 399 nM GroEL and 622 nM GroES. No activity was detected prior to the addition of chaperonins.

In conclusion, we have described conditions that enable the formation of relatively high concentrations of an inactive folding intermediate of MDH that is not prone to aggregation and is a competent substrate for chaperonin mediated refolding. In future experiments the isolation and characterisation of larger amounts of this intermediate may provide further insights into the nature of this process. This task is further facilitated since MDH is available in large quantities from commercial sources, is easy to assay and has a known 3D structure [28].

Acknowledgements: D.P. and D.J.H. contributed equally. We thank Lin Chen for purifying GroEL and GroES used in these studies. This work was supported in part by grants from the National Health and Medical Research Council of Australia to P.B.H. and N.J.H.

References

[1] Gething, M.-J. and Sambrook, J. (1992) Nature 355, 33–45.
[2] Hendrix, R.W. (1979) J. Mol. Biol. 129, 375–392.
[3] Hohn, T., Hohn, B., Engel, A. and Wurtz, M. (1979) J. Mol. Biol. 129, 359–373.
[4] Langer, T, Pfeifer, G., Martin, J., Baumeister, W. and Hartl, F.-U. (1992) EMBO J. 11, 4757–4765.
[5] Braig, K., Simon, M., Furuya, F., Hainfeld, J.F. and Horwich, A.L. (1993) Proc. Natl. Acad. Sci. USA 90, 3978–3982.
[6] Chandrasekhar, G.N., Tilly, K., Woolford, C., Hendrix, R. and Georgopoulos, C. (1986) J. Biol. Chem. 261, 12414–12419.
[7] Georgopoulos, C., Ang, D., Liberek, K. and Zylicz, M. (1990) in: Stress Proteins in Biology and Medicine (Morimoto, R.I., Tissières, A. and Georgopoulos, C., Eds.) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 191–221.

[8] Goloubinoff, P., Christeller, J.T., Gatenby, A.A. and Lorimer, G.H. (1989) Nature 342, 884–889.
[9] Martin, J., Langer, T., Boteva, R., Schramel, A., Horwich, A.L. and Hartl, F.- U. (1991) Nature 352, 36–42.
[10] Viitanen, P.V., Lubben, T.H., Reed, J., Goloubinoff, P., O'Keefe, D.P. and Lorimer, G.H. (1990) Biochemistry 29, 5665–5671.
[11] Tilly, K. and Georgopoulos, C. (1982) J. Bacteriol. 149, 1082–1088.
[12] Miller, A.D., Maghlaoui, K., Albanese, G., Kleinjan, D.A. and Smith, C. (1993) Biochem. J. 291, 139–144.
[13] Mendoza, J.A., Rogers, E., Lorimer, G.H. and Horowitz, P.M. (1991) J. Biol. Chem. 266, 13044–13049.
[14] Buchner, J., Schmidt, M., Fuchs, M., Jaenicke, R., Rudolph, R., Schmid, F.X. and Kiefhaber, T. (1991) Biochemistry 30, 1586–1591.
[15] Hartman, D.J., Surin, B.P., Dixon, N.E., Hoogenraad, N.J. and Høj, P.B. (1993) Proc. Natl. Acad. Sci. USA 90, 2276–2280.
[16] Price, N.C., Kelly, S.M., Thomson, G.J., Coggins, J.R., Wood, S. and auf der Mauer, A. (1993) Biochim. Biophys. Acta 1161, 52–58.
[17] Jackson, G.S., Staniforth, R.A., Halsall, D.J., Atkinson, T., Holbrook, J.J., Clarke, A.R. and Burston, S.G. (1993) Biochemistry 32, 2554–2563.
[18] Jaenicke, R., Rudolph, R. and Heider, I. (1979) Biochemistry 18, 1217–1222.
[19] Mendoza, J.A., Lorimer, G.H. and Horowitz, P.M. (1991) J. Biol. Chem. 266, 16973–16976.
[20] Gray, T.E. and Fersht, A.R. (1993) J. Mol. Biol. 232, 1197–1207.
[21] van der Vies, S.M., Viitanen, P.V., Gatenby, A.A., Lorimer, G.H. and Jaenicke, R. (1992) Biochemistry 31, 3635–3644.
[22] Matthews, C.R. (1993) Annu. Rev. Biochem. 62, 653–683.
[23] Shortle, D. (1993) Curr. Opin. Struct. Biol. 3, 66–74.
[24] Langer, T., Lu, C., Echols, H., Flanagan, J., Hayer, M.K. and Hartl, F.-U. (1992) Nature 356, 683–689.
[25] Paek, K.-H. and Walker, G.C. (1987) J. Bacteriol. 169, 283–290.
[26] Sell, S.M., Eisen, C., Ang, D., Zylicz, M. and Georgopoulos, C. (1990) J. Bacteriol. 172, 4827–4835.
[27] Fayet, O., Ziegelhoffer, T. and Georgopoulos, C. (1989) J. Bacteriol. 171, 1379–1385.
[28] Roderick, S.L. and Banaszak, L.J. (1986) J. Biol. Chem. 261, 9461–9464.