The reassembling process of the nonameric *Mycobacterium tuberculosis* small heat-shock protein Hsp16.3 occurs via a stepwise mechanism

Xiuguang FENG*^{†1}, Sufang HUANG*^{†1}, Xinmiao FU*[†], Abuduaini ABULIMITI*[†] and Zengyi CHANG*^{†2}

*Department of Biological Science and Biotechnology, Tsinghua University, Beijing 100084, People's Republic of China, and [†]Protein Science Laboratory of the Education Ministry, Beijing 100084, People's Republic of China

Conditions are reported under which the reassembled intermediates of the heat-shock protein Hsp16.3 after being denatured in 8 M urea were detected by mainly using urea-gradient PAGE (with modifications) and urea-denaturing pore-gradient PAGE. Hsp16.3 is the small heat-shock protein from *Mycobacterium tuberculosis*, which exists as a specific nonamer and was proposed to form a trimer-of-trimers structure. The refolding and reassembling of this protein was achieved rapidly by dilution or dialysis, suggesting an effectively spontaneous recovery of quat-

ernary structure. Data presented in this report demonstrate that the *in vitro* reassembling process of Hsp16.3 protein occurs through a spontaneous and effective stepwise mechanism. Modified urea-gradient PAGE may provide a general method for studying the reassembling processes of other oligomeric proteins.

Key words: electrophoresis, oligomeric protein, reassembling, urea denaturing, urea-gradient PAGE.

INTRODUCTION

Many proteins exist as oligomers. One of the outstanding questions is how these oligomers are formed. To understand the structure and functional relationships of proteins [1,2], it is necessary to understand the mechanisms governing the folding and assembling of open peptides into oligomers of various sizes. This is a problem of wide interest and draws considerable attention at present. The study of protein folding and subunit recognition is crucial in understanding such mechanisms, as well as for the elucidation of rules that govern protein folding.

Although many studies have been carried out for understanding the folding process of proteins [3–7], nothing much has been done to elucidate the assembly process of the oligomeric proteins except that the chaperonin GroEL was found to refold and reassemble in the presence of ammonium sulphate [8] or under macromolecular crowding conditions [9] after being completely denatured by 8 M urea. Similarly, the co-chaperonin GroES was also found to be able to refold and self-reassemble effectively [10]. However, the reassembling mechanisms for both GroEL and GroES were not closely investigated [7–10].

A member of the small heat-shock protein (sHsp) family, the *Mycobacterium tuberculosis* Hsp16.3, exists as a nonamer, and acts as molecular chaperone *in vitro* [11]. Formation of oligomeric structures of specific or variable sizes has been found to be a general feature for members of the sHsp family. For instance, Hsp16.5, the sHsp from *Methanococcus jannaschii*, consists of 24 subunits [12]; both Hsp18.1 and Hsp17.7 from pea consist of 12 subunits [13].

To understand how such oligomeric proteins are formed, the reassembling process of purified recombinant Hsp16.3 proteins was examined, mainly using urea-gradient and urea-containing pore-gradient PAGE. Urea-gradient PAGE, in the presence of a linear gradient of urea that affects protein stability across the gel, was generally used to examine the folding/unfolding transition of various proteins [14]. This method was adapted here to detect

reassembling intermediates of Hsp16.3 protein. The results reported here demonstrated strongly that the *in vitro* reassembling of Hsp16.3 protein occurs via a spontaneous and effective stepwise process.

MATERIALS AND METHODS

Materials

Poly(ethyleneimine)(NH) ion exchange resin was obtained from J.T. Baker (Phillipsburg, NJ, U.S.A.). Tris, urea and other substances used for electrophoresis were all obtained from Sigma. AgNO₃ and other chemical reagents were from the Beijing Chemical Reagents Company.

Protein overexpression and purification

Wild-type protein was overexpressed in pET-Hsp16.3-transformed BL21(DE3) cells with L122A mutant protein overexpressed in JM109(DE3) cells. Protein overexpression was performed without isopropyl β -D-thiogalactoside induction. The proteins were purified to almost homogeneity using DEAE cellulose and poly(ethyleneimine)(NH) ion-exchange chromatography by the methods described previously by Chang and co-workers [11]. The proteins were dried to a powder form at 0 °C and stored at –20 °C. All protein samples were dissolved in 25 mM sodium phosphate (pH 7.0) when used. Protein concentrations were determined by measuring the absorption at 280 nm (molar absorption coefficient being 0.2 mg/cm² for Hsp16.3).

Urea-gradient PAGE

Urea-gradient PAGE was carried out as described by Goldenberg and Creighton [14]. The 0–8 M urea-gradient gel was made by mixing two acrylamide solutions having 0 and 8 M urea. As the gel viscosity would differ with different amounts of urea present,

Abbreviation used: Hsp, heat-shock protein.

¹ These authors have contributed equally to this work.

² To whom correspondence should be addressed (e-mail changzy@tsinghua.edu.cn).

a compensating inverse gradient of polyacrylamide concentration was applied in the slab, whereby 7.5 and 5.5% acrylamide was present in the two solutions having 0 and 8 M urea, respectively. The degree of compensation was considered appropriate when Bromophenol Blue dye ran at the same mobility across the urea-gradient PAGE gel. The 1-mm-thick gel was 12.5 × 10 cm. The gel, having a 0–8 M urea gradient from top to bottom, was rotated by 90° before it was loaded into the electrophoresis tank. As a result of this rotation, electrophoresis was performed perpendicular to the urea gradient in non-SDS Tris/glycine electrophoresis buffer.

Urea-containing pore-gradient PAGE

Urea-containing pore-gradient PAGE was performed as described by Ausubel and co-workers [15], except that urea was added to a certain concentration (6 M for the results presented in Figure 5) before the gel was prepared and without pouring a stacking gel on the top part. The electrophoresis was performed in non-SDS Tris/glycine electrophoresis buffer.

Circular dichroism

CD measurements of Hsp16.3 were performed on a Jasco J-751 spectropolarimeter. The light path for all measurements was 1 mm. The near-UV-CD spectra were measured in the range 250–300 nm with 4 mg/ml proteins in 25 mM sodium phosphate, pH 7.0.

Size-exclusion chromatography

This was performed using a Superose 6 HR10/30 column (Amersham Pharmacia Biotech). All samples were directly loaded at 100 µl sample volume and eluted at a flow rate of 0.3 ml/min. No dialysis was performed for the 8 M urea-denatured samples before loading to the size-exclusion column.

RESULTS

The unfolded Hsp16.3 protein effectively recovers its oligomeric structure upon the elimination of the denaturants

The capacity of the Hsp16.3 protein to recover its oligomeric structure after being completely denatured with high concentrations of chemical denaturants was examined by UV-CD spectrophotometry and size-exclusion chromatography. The top two curves in Figure 1(a) represent the near-UV-CD spectra of the untreated (native) and refolded Hsp16.3 proteins respectively, where the overlapping feature suggests an almost complete recovery of the protein tertiary structure after the urea was dialysed. The bottom curve in Figure 1(a) represents the near-UV-CD spectrum of Hsp16.3 protein in the presence of 8 M urea, where the appearance of a negative ellipticity at ≈ 280 nm suggests a dramatic disturbance of the tertiary structure.

Size-exclusion chromatography results shown in Figure 1(b) also demonstrated clearly that the untreated and refolded Hsp16.3 proteins were eluted at almost the same position, suggesting a complete recovery of the oligomeric size after going through such a denaturation–renaturation process.

No reassembled intermediates could be detected from the 8 M urea-denatured wild-type Hsp16.3 using urea-gradient PAGE

Only one major band was observed after the wild-type Hsp16.3 protein was unfolded in the presence of 8 M urea and loaded directly for examination using urea-gradient PAGE (Figure 2b). This band has the same shape as that of the untreated Hsp16.3

Figure 1 Examination of the structural reversibility of Hsp16.3 after being unfolded in the presence of 8 M urea

(a) Analysis of the tertiary structure by near-UV-CD. The curves represent the UV-CD spectra obtained from wild-type Hsp16.3 that were untreated (■), in the presence of 8 M urea (▲) and dialysed after being pretreated in 8 M urea for 16 h (△). Each curve represents the average of 12 scans from a sample containing 4 mg/ml purified protein. (b) Analysis of the size of the recovered oligomers by size-exclusion chromatography. A Superose 6 HR10/30 column was used. The elution curves represent those of the wild-type Hsp16.3 protein samples that are untreated (Curve 1) or loaded directly to the column without being dialysed after being treated with 8 M urea (Curve 2). Curves 1 and 2 almost completely overlap.

protein (Figure 2a). These results suggest that the Hsp16.3 protein treated by 8 M urea rapidly refolded/reassembled at the loading stage of gel electrophoresis, with no other reassembled intermediates present when the electrophoresis began. The reassembled intermediates, if present, should have been detected on such gel electrophoresis as shown below.

Nine reassembled intermediates were detected from the 8 M urea-denatured L122A mutant Hsp16.3 using urea-gradient PAGE

Our previous studies have shown that the structural stability of L122A mutant Hsp16.3 protein is significantly lower than that of the wild-type protein [16]. Therefore, the reassembling process of this mutant Hsp16.3 protein is expected to be less efficient than that of the wild-type protein. Indeed, results presented in Figure 3(b) show clearly that nine bands were detected when the 8 M denatured L122A mutant Hsp16.3 protein was directly loaded on to a urea-gradient gel for electrophoresis separation. The fact that exactly nine bands were detected here strongly suggests that each of the nine bands represents one reassembled intermediate for the mutant Hsp16.3 protein, respectively. This conclusion is also supported by results shown below (Figure 5).

When the untreated L122A mutant Hsp16.3 protein was subjected to the same procedure of electrophoresis, only one

Figure 2 Examination of the unfolding/unassembling process of the wild-type Hsp16.3 proteins using the 0–8 M urea-gradient PAGE

Protein samples, either untreated (**a**) or treated in the presence of 8 M urea at 25 °C (**b**), were loaded directly into the running buffer of the 0–8 M urea-gradient polyacrylamide gel. The electrophoresis was performed with constant current of 12 mA at 25 °C until the Bromophenol Blue reaches the bottom of the gel.

major band, together with two minor bands, was detected (Figure 3a). No such minor bands were detected when the wild-type Hsp16.3 was subjected to the same procedure of electrophoresis (Figure 2a), suggesting that the two minor bands represent two disassociated forms of the mutant protein. Another notable feature of the behaviour of the mutant protein is the disappearance of the transition of the protein band easily seen at approx. 3 M urea for the wild-type protein (Figures 2a and 3a). This demonstrates that a certain structural transformation occurring in the wild-type protein no longer occurred in the mutant protein.

Occasionally, faint bands between the marked major bands (e.g. between bands 5 and 6 when numbered from the top in Figure 3b) were observed. However, such faint bands were never observed when the wild-type protein was examined (see Figure 4). These faint bands may represent certain transitory conformational states between the two forms represented by the two neighbouring major bands. Nevertheless, the actual meaning of such faint bands in the reassembling process needs further investigation.

Nine reassembled intermediates were also detected from the 8 M urea-denatured wild-type Hsp16.3 using modified urea-gradient PAGE

The successful detection of nine reassembled intermediates (Figure 3b) suggested that similar reassembled intermediates may also be present for the wild-type protein, but failed to be detected due to a higher efficiency of the reassembling process (Figure 2b). Therefore, to locate such intermediates for the wild-type protein, the reassembling process, which most likely occurs at the loading

Figure 3 Urea-gradient PAGE attempting to detect refolding/reassembling intermediates of the L122A mutant Hsp16.3 proteins

The L122A mutant Hsp16.3 proteins were untreated (**a**) or treated (**b**) with 8 M urea for 20 h at 25 °C and then loaded directly into the running buffer of the 0–8 M urea-gradient PAGE. The horizontal arrows indicate the positions of the nine forms of mutant Hsp16.3 present in the refolding/reassembling process. The electrophoresis was performed with a constant current of 12 mA at 25 °C until the Bromophenol Blue reached the bottom of the gel.

stage of the urea-gradient PAGE, would have to be slowed down. For this purpose, the unfolded wild-type protein was directly loaded to the bottom of a pre-added layer of 4 M urea. This operation was expected to avoid the immediate refolding/reassembly of the wild-type protein due to the immediate dilution of the sample caused by the running buffer. Indeed, at least five evenly distributed bands were clearly detected for the wild-type protein under such an operation (Figure 4a). These bands demonstrate that the reassembled intermediates of the wild-type protein do exist as we expected.

To slow down further the reassembling process of the wild-type protein, the whole gel electrophoresis was performed at higher temperatures. Here, it is assumed that the reassembling process would become less effective at higher temperatures. Indeed, as shown in Figure 4(b), when the gel electrophoresis was performed at 37 °C, seven bands were observed by Coomassie Blue staining and nine bands by silver staining. When the operation temperature was increased further to 45 °C, nine bands were detected merely by Coomassie Blue staining (Figure 4c). The band marked by an arrow below was faintly but clearly visible on the stained gel, but hardly visible in Figure 4.

The reassembled intermediates of wild-type Hsp16.3 protein have accumulating sizes

To provide solid evidence that each of the protein bands observed in the urea-gradient gels described above (Figures 3 and 4) indeed represents a reassembled intermediate with different sizes, pore-gradient gel electrophoresis was performed by having a certain concentration of urea in the gel (such a method is thus named 'urea-containing pore-gradient gel electrophoresis'). When examined using such pore-gradient gel electrophoresis containing 6 M urea, nine evenly distributed bands were clearly

Figure 4 Urea-gradient PAGE to detect refolding/reassembling intermediates of the wild-type Hsp16.3 protein

The protein samples were pretreated with 8 M urea for 20 h at 25 °C, and the same quantity of samples were loaded on to the 0–8 M urea-gradient polyacrylamide gel. In (a), a 4 M urea layer was pre-added in the well before the treated sample was directly loaded and the electrophoresis was performed at 25 °C. In (b), the unfolded Hsp16.3 protein sample was mixed with a non-reducing loading buffer containing 8 M urea, loaded and covered with running buffer very gently to avoid any stirring of the loaded sample. The electrophoresis was performed at 37 °C. The gel was cut into two parts after the electrophoresis and stained by silver or Coomassie Blue as indicated. In (c), the loading operation was similar to that for (b), except that the electrophoresis was run at 45 °C. The positions of all detected forms of wild-type Hsp16.3 present in the refolding/reassembling process are indicated with horizontal arrows, respectively. Note that when the protein amount is in excess, silver staining gives a transparent band, instead of a brown one (appearing as a colourless band, instead of black in the Figure).

observed (Figure 5) for the refolding/reassembling wild-type Hsp16.3 protein. A pore-gradient gel of 5–15% acrylamide, having a good linear gradient, was used for the separation of the refolding/reassembling Hsp16.3. Similar results were also obtained using pore-gradient polyacrylamide gels having 5–25% acrylamide (data not shown). The presence of 6 M urea in the gel was chosen based on the observation that the various reassembled intermediates could be maintained from 4 to 8 M urea concentration (with an average of 6 M) for the wild-type Hsp16.3 as shown in Figure 4.

DISCUSSION

Urea, a commonly used denaturant, was used here to slow down protein refolding and reassembling processes [2], which allowed

Figure 5 Urea-containing pore-gradient PAGE for attempting to distinguish the sizes of the refolding/reassembling intermediates of the wild-type Hsp16.3 proteins

The unfolded Hsp16.3 protein sample was mixed with a non-reducing loading buffer containing 8 M urea, loaded and covered with running buffer very gently to avoid any stirring of the loaded sample. The electrophoresis was performed at 37 °C and the electrophoresis was continued for a further period of ≈ 3 h after the Bromophenol Blue reaches the bottom of the gel. The horizontal arrows indicate the position of the nine forms of wild-type Hsp16.3 protein.

the reassembled intermediates of Hsp16.3 to be detected by using gel electrophoresis separations. These *in vitro* reassembling studies revealed the following. (1) The unfolded wild-type Hsp16.3 polypeptide chains are able to refold and reassemble spontaneously into their oligomeric structure when urea was eliminated (as shown in Figures 1 and 2). This observation strongly suggests that the amino acid sequence of Hsp16.3 contains all the information needed for this protein refolding, as well as the formation of the oligomers. (2) No reassembled intermediates could be detected within the dead time of operation for the regular urea-gradient PAGE (Figure 2b), which suggests that the reassembling of Hsp16.3 is almost complete within minutes. (3) Although the reassembling process of Hsp16.3 becomes less effective, the oligomer formed eventually is still a nonamer when leucine-122 is replaced with an alanine residue [16]. Such a mutant protein allowed a ‘slow snapshot’ of the reassembling process of the wild-type Hsp16.3 protein ‘caught’ by the urea-gradient PAGE (Figure 3b). (4) Indeed, a ‘slow snapshot’ for the reassembling process of the wild-type Hsp16.3 protein could also be ‘taken’ by modulating the electrophoresis conditions.

In light of these observations, we strongly believe that the reassembling of Hsp16.3 protein occurs via a stepwise process as summarized in Figure 6. Such a ‘stepwise model’ might be described as follows. Initially, the unfolded Hsp16.3 polypeptide chains (as represented by U in Figure 6) may rapidly fold into some kind of ordered monomeric structure ([M]). Subsequently, two monomers collide to form a dimer ([D]), after which one monomer is added at each step of collision, until a nonamer ([N]) is formed. The continuously increasing density of the nine bands in Figures 3(b)–5, which are taken to represent the nine forms of the reassembling Hsp16.3 protein, does indeed provide additional support to the stepwise model proposed here. Occasionally, an extra faint band was observed having the highest mobility when the wild-type protein was examined using the urea-containing pore-gradient PAGE. This band might represent the unfolded state of one subunit of the Hsp16.3 protein.

Urea-gradient gel electrophoresis has been utilized conventionally to examine the conformational transformation during protein unfolding and refolding [2]. Our report represents the

Figure 6 The stepwise model describing the *in vitro* reassembling process of the Hsp16.3 nonamers

U, unfolded protein; [M], monomer with a certain degree of ordered structure; [D], dimer; [T], trimer; [N], nonamers after the pile-up stage (before the conformational readjustment stage); N, nonamer after the conformational readjustment. Labels in square brackets illustrate unstable intermediates.

first attempt to study the reassembling process of oligomeric proteins by using this technique. The successful detection of the reassembled oligomeric intermediates in the urea-gradient gel, especially in the region of the gel where a high concentration of urea is supposed to be present (Figures 3 and 4), is difficult to understand by following the traditional way of thinking. The most likely explanations that we can offer are the following: either the subunit interaction of the reassembled oligomeric intermediates was still present, although being weakened to a certain degree, within the relatively narrow time window when the electrophoresis occurred (approx. 2 h), or alternatively, the high concentrations of urea assumed to be present in the gel may act in a dramatically different way towards proteins from those present in free solutions. Of course, we cannot rule out that both of these, or other effects yet to be considered, played a role in the urea-gradient PAGE described here.

Previous studies have shown that Hsp16.3 protein forms a three-globule structure, which might exist as trimer-of-trimers [11,17]. Furthermore, Hsp25, the sHsp from mouse having a hexadecameric structure, was found to have a concentration-dependent equilibrium with the tetrameric and dimeric forms of the protein in solutions [18]. It was thus believed that the tetramers (assembled from dimers) represent the basic building blocks of Hsp25 oligomers. A logical expectation from these observations would be that the nonamers of Hsp16.3 are assembled from preformed relatively stable trimers. Nevertheless, the data presented here, with either the wild-type or the L122A mutant Hsp16.3 proteins, did not support the enrichment of such trimers during the reassembling processes (Figures 3b–5). To account for these apparent contradictions, we propose that some kind of post-assembly conformational readjustment occurs to generate the trimer-of-trimers structure following the pile-up of nine subunits.

Another obvious phenomenon is the lack of conformational transformations, as marked by the disappearance of transitions of the protein bands (at approx. 2 M urea), when the L122A mutant Hsp16.3 protein was examined using the urea-gradient gel electrophoresis (Figure 3). Results presented in Figures 2, 4(a) and 4(b) show clearly that the mobility of wild-type Hsp16.3 decreased gradually when the urea concentration increased from 1 to 3 M, and remained almost unchanged when urea con-

centration was increased further. The decrease of protein mobility accompanying an increase of urea concentration indicates an opening of the protein conformation. On the contrary, results presented in Figure 3 show that the L122A mutant protein has an electrophoretic mobility similar to that of the wild-type protein in the presence of urea at 3 M or higher concentrations. The mobility change accompanying the increased concentration of urea from 1 to 3 M for the wild-type protein was apparently not observed for the L122A mutant protein. These results suggested that the reassembling pathway of the L122A mutant protein might differ from that of the wild-type protein, even though the basic reassembling mechanisms are conserved (with the oligomer eventually formed still being a nonamer [16]). Interestingly, when the wild-type and L122A mutant proteins were mixed, unfolded by 8 M urea and then dialysed completely, no nonameric hybrids having both the mutant and wild-type subunits were formed during the reassembling process (Huang, S. F. and Chang, Z., unpublished results). This provided additional evidence to suggest that the actual details of the reassembling process of L122A mutant protein, as well as the conformational status of the nonamers eventually formed, are somehow different from those of the wild-type protein.

Furthermore, a similar lack of conformational transformation was also observed when the wild-type protein was examined by using the urea-gradient gel electrophoresis performed at a high temperature, sharing a similar band pattern as that of the L122A mutant protein run at room temperature (Figure 4c). These two observations suggest strongly that the wild-type protein, when examined at room temperature, reached a more compact structure (thus having a higher mobility on the gel), most likely through a conformational readjustment, after the pile-up step of the reassembling process. Apparently, such a conformational readjustment did not occur in the L122 mutant protein, leading to the formation of a structureless compact (thus having a lower mobility) than that of the wild-type protein. This analysis is highly consistent with our previous observations, where the L122A mutant protein was revealed to have a significantly lower mobility than that of the wild-type protein when examined by non-denaturing native PAGE [16]. A similar explanation may also be true for the wild-type protein when examined using a gel preformed at a higher temperature (Figure 4c). Whether such a conformational readjustment leads to the formation of the trimer-of-trimers structure, as in Figure 6 ([N] to N), needs to be further studied.

If such a stepwise model proposed to explain the *in vitro* reassembling of Hsp16.3 also reflects what happens *in vivo* for the assembly of this protein, then one likely advantage would be that this mechanism allows the hydrophobic surface, believed to be required for the chaperone activity [19–21], to be formed by conformational readjustment after the assembling process. The preassembling appearance of such hydrophobic surfaces may generate harmful effects for protein folding and assembling.

The α -crystallin (also a sHsp family member) and co-chaperonin GroES were also found to be able to refold and reassemble successfully *in vitro* after being denatured and renatured by dilution or dialysing away the 8 M urea [5,10]. Chemically synthesized GroES not only folds and assembles properly but also shows complete activity in refolding rubisco in the presence of GroEL [22]. The concept of 'self-chaperoning', introduced to explain this spontaneous refolding and reassembling, seems to be supported by our observations reported here. It would be interesting to find out whether a similar stepwise mechanism also exists during the reassembling of the 'self-chaperoning' oligomeric proteins such as α -crystallin and GroES.

This work was supported by National Key Basic Research Foundation of China (grant no. G1999075607) and the National Science Foundation for Outstanding Young Scientists (grant no. 39725008). We thank Dr Qilong Mao, Dr Wangwang Jiao, Dr Xiaoyou Chen, Professor Xicheng Wang and Ms Xiaolan Ding at the Department of Biological Science and Biotechnology, Tsinghua University, for technical help and discussions.

REFERENCES

- Richards, F. M. (1992) In *Folded and Unfolded Proteins – An Introduction in Protein Folding* (Creighton, T. E., ed.), W. H. Freeman, New York
- Creighton, T. E. (1986) Detection of folding intermediates using urea-gradient electrophoresis. *Methods Enzymol.* **131**, 156–172
- Wildegger, G. and Kiefhaber, T. (1997) Three-state model for lysozyme folding: triangular folding mechanism with an energetically trapped intermediate. *J. Mol. Biol.* **270**, 294–304
- Watson, J. T., Yang, Y. and Wu, J. (2001) Capture and identification of folding intermediates of cystinyl proteins by cyanilation and mass spectrometry. *J. Mol. Graph. Model* **19**, 119–128
- Raman, B., Ramakrishna, T. and Mohan Rao, C. H. (1995) Rapid refolding studies on the chaperone-like α -crystallin effect of α -crystallin on refolding of α -crystallin and α -crystallin. *J. Biol. Chem.* **270**, 19888–19892
- Raman, B., Ramakrishna, T. and Mohan Rao, C. H. (1997) Effect of the chaperone-like α -crystallin on the refolding of lysozyme and ribonuclease A. *FEBS Lett.* **416**, 369–372
- Ybarra, J. and Horowitz, P. M. (1995) Inactive GroEL monomers can be isolated and reassembled to functional tetradecamers that contain few bound peptides. *J. Biol. Chem.* **270**, 22962–22967
- Ybarra, J. and Horowitz, P. Z. (1995) Refolding and reassembly of active chaperonin GroEL after denaturation. *J. Biol. Chem.* **270**, 22113–22115
- Galan, G., Sot, B., Llorcai, O., Carrascosai, J. L., Valpuestai, J. M. and Muga, A. (2001) Excluded volume effects on the refolding and assembly of an oligomeric protein GroEL. *J. Biol. Chem.* **276**, 957–964
- Seale, J. W., Gorovits, B. M., Ybarra, J. and Horowitz, P. M. (1996) Reversible oligomerization and denaturation of the chaperonin GroES. *Biochemistry* **35**, 4079–4083
- Chang, Z., Primm, T. P., Jakana, J., Lee, I. H., Serysheva, I., Chiu, W., Gilbert, H. F. and Quiocho, F. A. (1996) *Mycobacterium tuberculosis* 16-kDa antigen (Hsp16.3) functions as an oligomeric structure *in vitro* to suppress thermal aggregation. *J. Biol. Chem.* **271**, 7218–7223
- Kim, K. K., Kim, R. and Kim, S. H. (1998) Crystal structure of a small heat-shock protein. *Nature (London)* **394**, 595–599
- Shearstone, J. R. and Baneyx, F. (1999) Biochemical characterization of the small heat shock protein IbpB from *Escherichia coli*. *J. Biol. Chem.* **274**, 9937–9945
- Goldenberg, D. P. and Creighton, T. E. (1984) Gel electrophoresis in studies of protein conformation and folding. *Anal. Biochem.* **138**, 1–18
- Ausubel, F., Brent, R., Kingdom, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. and Struhl, K. (1997) Electrophoretic separation of proteins. *Analysis of Proteins*. In *Current Protocols in Molecular Biology* (Gallagher, S. R., ed.), pp. 10.2.18–10.2.21, Wiley, New York
- Dai, H., Mao, Q., Yang, H., Huang, S. and Chang, Z. (2000) Probing the roles of the only universally conserved leucine residue (Leu122) in the oligomerization and chaperone-like activity of *Mycobacterium tuberculosis* small heat shock protein Hsp16.3. *J. Protein Chem.* **4**, 319–326
- Berengian, A. R., Parfenova, M. and McHaourab, H. S. (1999) Site-directed spin labeling study of subunit interactions in the α -crystallin domain of small heat-shock proteins. Comparison of the oligomer symmetry in α A-crystallin, HSP 27, and HSP 16.3. *J. Biol. Chem.* **274**, 6305–6314
- Ehrnsperger, M., Lilie, H., Gaestel, H. and Buchner, J. (1999) The dynamics of Hsp25 quaternary structure: structure and function of different oligomeric species. *J. Biol. Chem.* **274**, 14867–14874
- Raman, B. and Rao, C. M. (1994) Chaperone-like activity and quaternary structure of α -crystallin. *J. Biol. Chem.* **269**, 27264–27268
- Raman, B., Ramakrishna, T. and Rao, C. M. (1995) Temperature dependent chaperone-like activity of α -crystallin. *FEBS Lett.* **365**, 133–136
- Das, K. P. and Surewicz, W. K. (1995) Temperature-induced exposure of hydrophobic surfaces and its effect on the chaperone activity of α -crystallin. *FEBS Lett.* **369**, 321–325
- Mascagni, P., Tonolo, M., Ball, H., Lim, M., Ellis, R. J. and Coates, A. (1991) Chemical synthesis of 10 kDa chaperonin. Biological activity suggests chaperonins do not require other molecular chaperones. *FEBS Lett.* **286**, 201–203

Received 29 October 2001/8 December 2001; accepted 21 January 2002