Biochemistry (Moscow), Vol. 66, No. 8, 2001, pp. 904-908. Translated from Biokhimiya, Vol. 66, No. 8, 2001, pp. 1111-1116.

Electrostatic Interactions Play a Critical Role in *Mycobacterium tuberculosis* Hsp16.3 Binding of Substrate Proteins

Q. Mao, D. Ke, and Z. Chang*

Department of Biological Science and Biotechnology and State Key Laboratory of Biomembranes and Membrane Biotechnology, Tsinghua University, Beijing 100084, P. R. China; fax: 86-10-62785505; E-mail: changzy@mail.tsinghua.edu.cn

Received January 13, 2001

Abstract—*Mycobacterium tuberculosis* Hsp16.3, a member of a small heat shock protein family, has chaperone-like activity *in vitro* and suppresses thermally or chemically induced aggregation of proteins. The nature of the interactions between Hsp16.3 and the denatured substrate proteins was investigated. A dramatic enhancement of chaperone-like activity of Hsp16.3 upon increasing temperature was accompanied by decreased ANS-detectable surface hydrophobicity. Hsp16.3 exhibited significantly enhanced chaperone-like activity after preincubation at 100°C with almost unchanged surface hydrophobicity. The interaction between Hsp16.3 and dithiothreitol-treated insulin B chains was markedly weakened in the presence of NaCl but greatly enhanced by the addition of a low-polarity alcohol, accompanied by significantly increased and decreased surface hydrophobicity, respectively. A working model for Hsp16.3 binding to its substrate proteins is proposed.

Key words: small heat shock protein, heat pretreatment, chaperone-like activity, surface hydrophobicity

All organisms express heat shock (or, more precisely, stress-induced) proteins to protect themselves under stress conditions (e.g., high temperature, low oxygen concentration, and the presence of toxic chemicals). Five major families of such protective so-called heat shock proteins have been identified: Hsp60, Hsp70, Hsp90, Hsp104, and small heat shock proteins (sHsps) [1, 2].

The sHsps, with monomers of relatively low molecular weight, usually form oligomers [2]. One distinguishing feature of this family of heat shock proteins is the low sequence similarity between different members [2]. Recent studies indicated that sHsps could function as molecular chaperones *in vitro*, being able to suppress thermal and chemical-induced aggregation of various substrate proteins [3-6]. The structure determined for the first small heat shock protein (Hsp16.5) from *Methanococcus jannaschii*, a hyperthermophilic Archaeon, revealed hollow spherical and octahedral symmetric oligomer containing 24 subunits [7].

Hsp16.3 from *Mycobacterium tuberculosis* was originally identified as an immunodominant antigen [8] and was later found to be the major membrane protein [9]. This protein was maximally synthesized and became the predominant protein in the stationary phase with strong association with cell envelope thickening [10, 11]. The deduced amino acid sequence of Hsp16.3 suggested that it belongs to the sHsp family [12]. Gene knock-out studies indicated that Hsp16.3 is important for the growth of *M. tuberculosis* in the macrophage host cells [13]. Our previous studies indicated that Hsp16.3 forms a trimer-of-trimers oligomeric structure and functions as a molecular chaperone [5, 6].

Although it is generally believed that hydrophobic interaction is the major driving force for sHsps to bind to their partially denatured substrate proteins, there have been also reports showing that other forces may also play important roles in the binding process (for a review, see [2]). It is still poorly understood where the oligomeric sHsp molecules bind the wide spectrum of denatured substrate proteins and what forces drive such binding. Even less is known about the way in which the bound substrate proteins are released from the sHsp proteins (e.g., for refolding back to their native conformations).

In this study, the nature of the force utilized by Hsp16.3 to bind its substrate proteins was investigated. The lack of correlation between the level of chaperone-like activity and the amount of ANS-detectable hydrophobic surfaces, as well as the weakening effect of salt and strengthening effect of organic reagents on substrate binding were observed. These data suggest that non-hydrophobic, most likely electrostatic forces play a critical role in Hsp16.3 binding to DTT-treated insulin B chains. The significance of these observations for under-

Abbreviations: ANS) anilinonaphthalene sulfonate; DTT) dithiothreitol.

* To whom correspondence should be addressed.

0006-2979/01/6608-0904$25.00 ©2001 MAIK "Nauka/Interperiodica"

standing the mechanism of action of small heat shock proteins and molecular chaperones in general is discussed.

MATERIALS AND METHODS

Materials. The Hsp16.3 gene was inserted into pET-9d (Novagen) expression vector and transformed into BL21(DE3) (F^- *ompT* r_B^- mB^- (*DE3*)) for protein expression. The recombinant Hsp16.3 was purified almost to homogeneity according to previously reported methods [5, 6]. Insulin and DTT were both obtained from Sigma (USA). All other reagents were of an analytical grade.

Heat pretreatment. Hsp16.3, dissolved in 50 mM sodium phosphate buffer (pH 7.0), was heat-treated at 100°C for 15 min and then cooled on ice before measurements.

Chaperone-like activity assay. The chaperone-like activity of Hsp16.3 was measured at different temperatures as reported before [6]. The aggregation of DTT-induced insulin B chains (60 μM) was initiated by the addition of 20 mM DTT and monitored at 360 nm using a UV-Vis 8500 spectrophotometer (Shanghai Tianmei). The reaction buffer was 50 mM sodium phosphate (pH 7.0).

Fluorescence measurements. Fluorescence spectra were measured with a Hitachi F-4000 fluorescence spectrophotometer (Japan) equipped with a constant temperature cell holder. The excitation and emission bandpasses were both set at 5 nm. For ANS fluorescence spectra, the excitation monochromator was set at 390 nm and the emission monochromator was scanned from 400 to 660 nm. The final concentrations for Hsp16.3 and ANS were 6.1 and 100 μM, respectively.

RESULTS

Hsp16.3 exhibited greatly enhanced chaperone-like activity at elevated temperatures or after heat pretreatment. As a member of the small heat shock protein family, Hsp16.3 was able to suppress the aggregation of non-native substrate proteins. In the presence of DTT, the disulfide bond between the A and B chains of insulin is broken, leading to aggregation of insulin B chains. Therefore, insulin has been widely used as a substrate protein to measure the chaperone-like activity of molecular chaperones. When measured at 25°C, Hsp16.3 exhibited slight chaperone-like activity (Fig. 1), but the activity was dramatically enhanced by increasing the temperature from 25 to 45°C, indicating that Hsp16.3 binds the non-native substrate proteins more effectively at higher temperatures. It is evident that Hsp16.3 preheated at 100°C for 15 min exhibited a much higher level of chaperone-like activity than the untreated protein (Fig. 1).

The dramatically enhanced chaperone-like activity of Hsp16.3 is not accompanied by an increase in surface hydrophobicity. It is conventionally believed that small heat shock proteins interact with their substrate proteins via hydrophobic interaction [2]. It has been reported that increased chaperone activity of α-crystallin at elevated temperatures correlates with an increased amount of hydrophobic surface [14]. Are the dramatically enhanced chaperone-like activities of Hsp16.3 detected upon increased measuring temperatures or after being preheated accompanied by exposure of hydrophobic surfaces? The amount of hydrophobic surface of the untreated and preheated Hsp16.3 as a function of temperature was thus estimated using ANS as the probe. The results, shown in Fig. 2, failed to demonstrate a correlation between the chaperone-like activity of Hsp16.3 and exposure of hydrophobic surfaces. The emission maximum of protein-bound ANS is near 480 nm (Fig. 2a). It is obvious that the ANS-binding capacity of Hsp16.3 dropped quickly when the temperature was raised from 25 to 60°C (Fig. 2b), indicating much lower surface hydrophobicities of Hsp16.3 at higher temperatures. However, both the untreated and the 100°C-preheated Hsp16.3 proteins exhibited almost the same ANS-binding spectra at 37°C (curves *1* and *2* in Fig. 2a) and other temperatures (data not shown), suggesting that their surface hydrophobicities are almost identical.

The binding interaction between Hsp16.3 and DTT-treated insulin B chains is greatly weakened in the presence of salt. The lack of correlation between the level of chaperone-like activities and the amount of ANS-detectable hydrophobic surfaces of Hsp16.3 described above

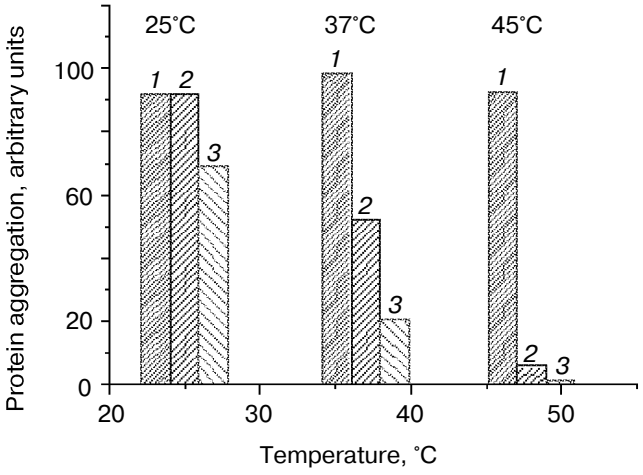

Fig. 1. Suppression of the aggregation of DTT-treated insulin by Hsp16.3 at 25, 37, and 45°C. Bar *1*) the aggregation of insulin alone; bar *2*) in the presence of Hsp16.3 (18.3 μM); bar *3*) in the presence of Hsp16.3 preheated at 100°C. The insulin was incubated at various temperatures in the presence of DTT with or without Hsp16.3 for 1 h, and the aggregation was measured at 360 nm.

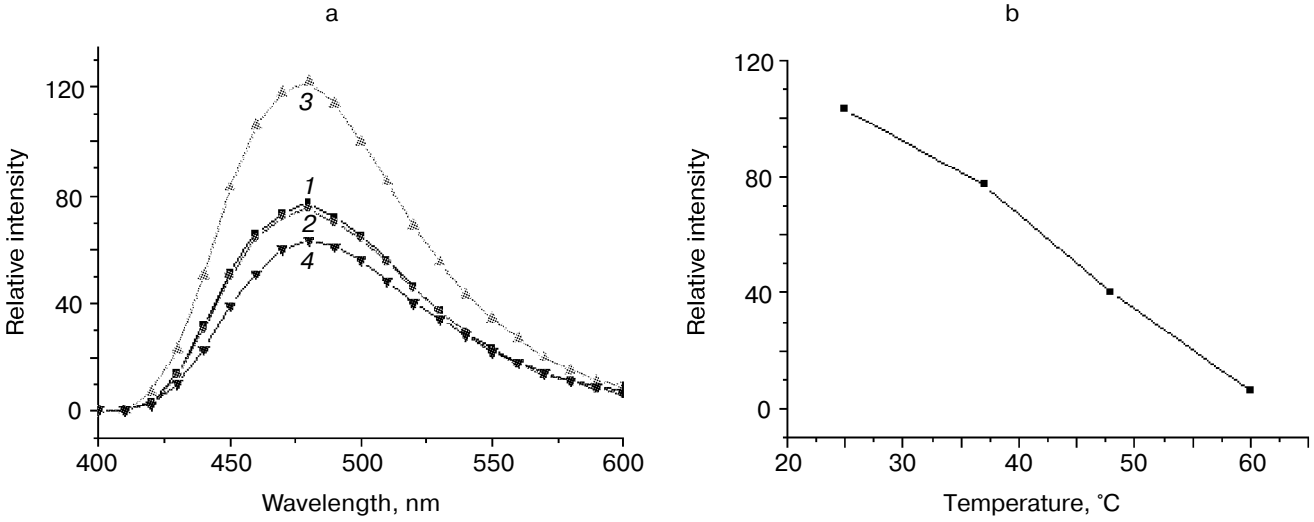

Fig. 2. Surface hydrophobicity of Hsp16.3. a) ANS-binding fluorescence spectra of Hsp16.3 at 37°C: *1*) untreated Hsp16.3; *2*) 100°C-preheated Hsp16.3; *3*) in the presence of 1.0 M NaCl; *4*) in the presence of 2% isopropanol. b) Temperature-dependent decrease in the surface hydrophobicity of Hsp16.3.

prompted us to further investigate the nature of the forces Hsp16.3 uses to interact with its substrate proteins. To examine whether charge–charge interaction plays any role for Hsp16.3 to bind to its substrate proteins, the chaperone-like activities were measured in the absence and presence of NaCl.

The results shown in Fig. 3 clearly indicate that the capacity of Hsp16.3 to suppress aggregation of DTT-treated insulin B chains at 32°C was completely inhibited in the presence of 1.0 M NaCl (curves *3* and *4*). Inhibition of the chaperone-like activity of Hsp16.3 by NaCl was also observed at other temperatures (data not shown). It was observed that the same concentration of NaCl is able to greatly promote the exposure of hydrophobic surfaces of Hsp16.3 (Fig. 2a, curves *1* and *3*). However, it was also observed that when NaCl was added to the reaction mixture in which Hsp16.3–substrate complex had already formed, no increase of aggregation was observed (data not shown), suggesting that the addition of NaCl is unable to dissociate the Hsp16.3–substrate complexes.

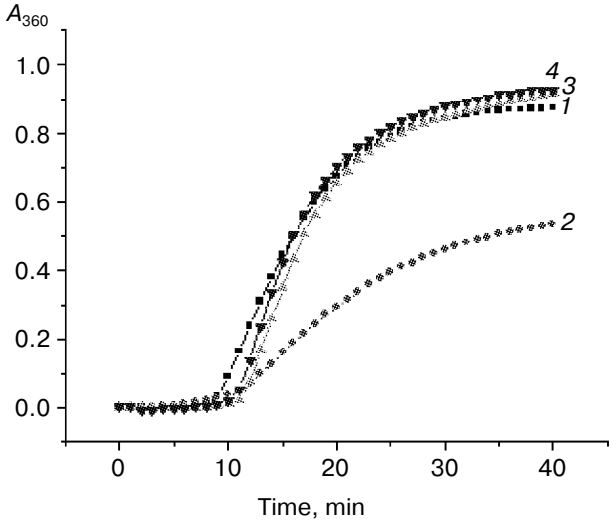

Fig. 3. Effect of NaCl on the chaperone-like activity of Hsp16.3. The aggregation of insulin B chains (60 μM) initiated by the addition of DTT (20 mM) was used to detect the chaperone-like activity of Hsp16.3 (18.3 μM) at 32°C. *1*) Insulin alone; *2*) in the presence of Hsp16.3; *3*) in the presence of NaCl (1.0 M); *4*) in the presence of both NaCl and Hsp16.3.

The chaperone-like activity of Hsp16.3 is enhanced to a different degree in the presence of different alcohols. The nature of the force used by Hsp16.3 to interact with its substrate proteins was further investigated using various alcohols. Isopropanol, a low-polarity organic solvent, dramatically enhanced the binding between Hsp16.3 and DTT-treated insulin B chains. The results shown in Fig. 4a indicate that Hsp16.3 (6.1 μM) suppressed the aggregation of DTT-treated insulin B chains by nearly 90% in the presence of 2% isopropanol (curve *4*), while less than 10% of the aggregation was inhibited in the absence of isopropanol (curve *3*). However, ANS fluorescence measurement showed a significant decrease in the amount of hydrophobic surface on Hsp16.3 in the presence of 2% isopropanol (Fig. 2a, curves *1* and *4*). It should be noted that the presence of 2% isopropanol did not affect the aggregation of DTT-treated insulin B chains under the measuring conditions (Fig. 4a, curve *2*).

The effects of a few other alcohols, including methanol, ethanol, propanol, 1,2-propanediol, and glycerol were also examined. The results shown in Fig. 4b

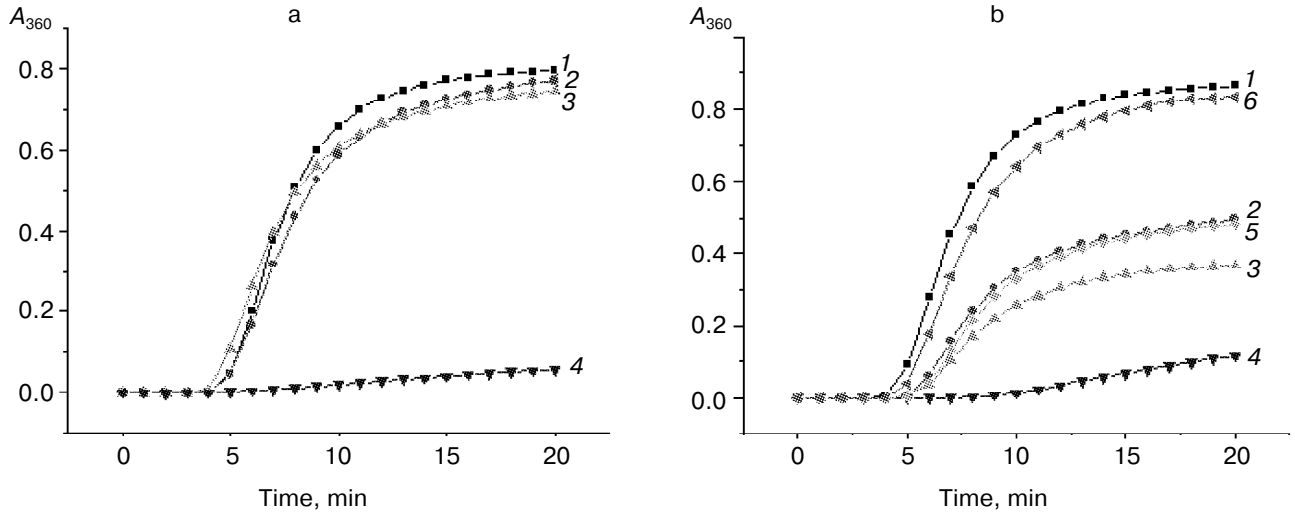

Fig. 4. Effect of alcohols on the chaperone-like activity of Hsp16.3. The aggregation of insulin B chains (60 μM) initiated by the addition of DTT (20 mM) was used to detect the chaperone-like activity of Hsp16.3 (6.1 μM) at 37°C. a: *1*) insulin alone; *2*) in the presence of isopropanol (2%); *3*) in the presence of Hsp16.3; *4*) in the presence of both isopropanol and Hsp16.3; b: *1*) insulin alone; *2*) plus Hsp16.3 and methanol (2%); *3*) plus Hsp16.3 and ethanol (2%); *4*) plus Hsp16.3 and propanol (2%); *5*) plus Hsp16.3 and 1,2-propanediol (2%); *6*) plus Hsp16.3 and glycerol (2%).

clearly demonstrate that the level of enhancement of the chaperone-like activity of Hsp16.3 is higher for the less polar alcohol (i.e., having more carbon atoms or fewer hydroxyl groups). It should be pointed out that the addition of each organic alcohol alone (at a concentration of 2%) had little effect on the aggregation of DTT-induced insulin B chains (data not shown).

DISCUSSION

Heat shock proteins, including sHsps, generally recognize, bind, and eventually release non-native proteins to function as molecular chaperones in cells. Only the binding activities are usually measured when small heat shock proteins are tested for their chaperone-like activities in *in vitro* studies. How does each sHsp recognize the various denatured substrate proteins? What is the nature of the force for the binding process? These critical issues for understanding the mechanism of action of molecular chaperones are still largely unresolved.

Hydrophobic interaction has long been considered to be of great importance in understanding protein–protein interaction [15]. It is a general belief that small heat shock proteins interact with their substrate proteins hydrophobically [2]. We previously reported an inconsistency between the maxima of the ANS-detectable hydrophobic exposure and chaperone-like activity for Hsp16.3 [6]. This study, representing a systematic investigation of this phenomenon, strongly suggests that an electrostatic interaction rather than a hydrophobic interaction plays a critical role in Hsp16.3 recognition and binding of DTT-treated insulin B chains. Evidence leading to this conclusion came mainly from the following four observations. First, Hsp16.3 exhibited much higher chaperone-like activity after heat pretreatment at 100°C for 15 min, but almost unchanged ANS-detectable surface hydrophobicity. Second, the significantly enhanced chaperone-like activity of Hsp16.3 at elevated temperatures was actually accompanied by a dramatically decreased fluorescence intensity of the bound ANS (thus exposed hydrophobic surfaces). Third, the chaperone-like activity of Hsp16.3 was greatly inhibited in the presence of salt (NaCl), while the ANS-detectable hydrophobic surface was greatly enhanced. Finally, the chaperone-like activity of Hsp16.3 was strongly enhanced upon addition of low-polarity organic reagents, which was also accompanied by a decrease of the exposed hydrophobic surfaces. It is generally believed that salt is able to enhance hydrophobic and weaken electrostatic interactions between protein molecules, while organic reagents exhibit their effects in the opposite way.

The conclusion that hydrophobic interaction plays the critical role for sHsps binding to their substrate proteins mainly came from studies on α-crystallin. It has been reported that α-crystallin exhibited increased chaperone-like activity when the temperature was elevated, accompanied with a corresponding increase of exposed hydrophobic surfaces [14, 16-19]. The enhancement of the chaperone-like activity was thus proposed to result from the exposure of hydrophobic surfaces.

Similarly to our conclusion, Reddy et al. [20] also proposed that the exposure of hydrophobic surface is not the sole determinant of the chaperone activity of α-crys-

tallin. This conclusion was mainly based on the following observations. First, it was noted that αA-crystallin and αB-crystallin homooligomers exhibited apparently different level of chaperone-like activities at 37°C, although their bis-ANS-detectable hydrophobic surfaces were very similar in size [20]. In addition, αB-crystallin showed no significant change in chaperone-like activity upon preincubation at temperatures up to 60°C, even though a decrease in hydrophobic surface was detected [20]. Interestingly, Reddy et al. have also noted that αB-crystallin exhibited lower chaperone-like activity and stronger surface hydrophobicity at lower temperatures than at higher temperatures, which is consistent with our observations reported here.

We also observed that NaCl failed to exhibit any effect once the Hsp16.3–substrate complex is formed. This suggests that the hydrophobic interaction still plays an important role in forming the Hsp16.3–substrate complex. In light of all these observations, we propose the following working model to illustrate the way Hsp16.3 binds to its substrate proteins (represented by DTT-treated insulin B chains). Hsp16.3 has no or very low chaperone-like activity at room temperature. Under heat shock conditions the Hsp16.3, with dramatically increased chaperone activity, binds denatured target proteins in two successive steps: Hsp16.3 initially attracts (recognizes) the denatured substrate proteins via a charge–charge interaction to form a relatively loose Hsp16.3–substrate complex, which then converts to a tighter complex mainly through hydrophobic interactions. This model shares certain similarity with that proposed by Plater et al. to explain how αB-crystallin interacts with its substrate proteins [21].

The fact that the chaperone-like activity of Hsp16.3 increases dramatically at heat shock temperatures makes great sense in physiological terms. Obviously, cells would need an immediate defending mechanism to protect their proteins from being harmed (denatured) when the environmental temperature is suddenly increased. The dramatically enhanced chaperone activity of small (and other) heat shock proteins at higher temperature would provide such an immediate protection mechanism. Haslbeck et al. also speculated that the heat-induced dissociation and activation of Hsp26 (from *Saccharomyces cerevisiae*) represents an early and simple mechanism for its functional activation [22]. In contrast, the induction of heat shock proteins by turning on gene transcription would be too slow to provide such immediate protection, but it may serve as a second defense line for protecting cells.

This work was supported by the National Science Foundation in China (grant No. 39700025) and the National Science Foundation for Outstanding Young Scientists in China (grant No. 39725008 to Prof. Z. Chang). We would like to thank Ms. X. Ding at the Department of Biological Science and Biotechnology at Tsinghua University for her technical help.

REFERENCES

1. Morimoto, R. I., Tisscleves, A., and Georgeopovlos, C. (eds.) (1994) *The Biology of Heat Shock Proteins and Molecular Chaperones*, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
2. Ehrnsperger, M., Buchner, J., and Gastel, M. (1998) in *Molecular Chaperones in the Life Cycle of Proteins: Structure, Function and Mode of Action* (Fink, A. L., and Goto, Y., eds.) Marcel Dekker, Inc., New York, pp. 533-575.
3. Horwitz, J. (1992) *Proc. Natl. Acad. Sci. USA*, **89**, 10449-10453.
4. Merck, K. B., Groenen, P. J., Voorter, C. E., de Harrd-Hoekman, W. A., Horwitz, J., Bloemendal, H., and de Jong, W. W. (1993) *J. Biol. Chem.*, **268**, 1046-1052.
5. Chang, Z., Primm, T. P., Jakana, J., Lee, I. H., Serysheva, I., Chiu, W., Gilbert, H. F., and Quiocho, F. A. (1996) *J. Biol. Chem.*, **271**, 7218-7223.
6. Yang, H., Huang, S., Dai, H., Gong, Y., Zheng, C., and Chang, Z. (1999) *Protein Sci.*, **8**, 174-179.
7. Kim, K. K., Kim, R., and Kim, S. H. (1998) *Nature*, **394**, 595-599.
8. Engers, H. D., Houba, V., Bennedsen, J., Buchanan, T. M., Chaparas, S. D., Kadival, G., Closs, O., David, J. R., can Embden, J. D. A., Godal, T., Mustafa, S. A., Ivanyi, J., Young, D. B., Kaufmann, S. H. E., Khomenko, A. G., Kolk, A. H. J., Kubin, M., Louis, J. A., Minden, P., Shinnick, T. M., Trnka, L., and Young, R. A. (1986) *Infect. Immun.*, **51**, 718-720.
9. Lee, B. Y., Hefta, S. A., and Brennan, P. J. (1992) *Infect. Immun.*, **60**, 2066-2074.
10. Yuan, Y., Crane, D. D., and Barry, C. E. (1996) *J. Bacteriol.*, **178**, 4484-4492.
11. Cunningham, A. F., and Spreadbury (1998) *J. Bacteriol.*, **180**, 801-808.
12. Verbon, A., Hartskeerl, R. A., Schuitema, A., Kolk, A. H. J., Young, D. B., and Lathigra, R. (1992) *J. Bacteriol.*, **174**, 1352-1359.
13. Yuan, Y., Crane, D. D., Simpson, R. M., Zhu, Y., Hickey, M. J., Sherman, D. R., and Barry, C. E., III. (1998) *Proc. Natl. Acad. Sci. USA*, **95**, 9578-9583.
14. Das, K. P., and Surewicz, W. K. (1995) *FEBS Lett.*, **369**, 321-325.
15. Jones, S., and Thornton, J. M. (1996) *Proc. Natl. Acad. Sci. USA*, **93**, 13-20.
16. Raman, B., and Rao, C. M. (1994) *J. Biol. Chem.*, **269**, 27264-27268.
17. Raman, B., Ramakrishna, T., and Rao, C. M. (1995) *FEBS Lett.*, **365**, 133-136.
18. Rao, C. M., Raman, B., Ramakrishna, T., Rajaraman, K., Ghosh, D., Datta, S. A., Trivedi, V. D., and Sukhaswami, M. B. (1998) *Int. J. Biol. Macromol.*, **22**, 271-281.
19. Datta, S. A., and Rao, C. M. (1999) *J. Biol. Chem.*, **274**, 34773-34778.
20. Reddy, G. B., Das, K. P., Petrash, J. M., and Surewicz, W. K. (2000) *J. Biol. Chem.*, **275**, 4565-4570.
21. Plater, M. L., Goode, D., and Crabbe, M. J. C. (1996) *J. Biol. Chem.*, **271**, 28558-28566.
22. Haslbeck, M., Walke, S., Stromer, T., Ehrnsperger, M., White, H. E., Chen, S., Saibil, H. R., and Buchner, J. (1999) *EMBO J.*, **23**, 674-675.