FEBS 26791 FEBS Letters 532 (2002) 309–312


# Properties of the chaperonin complex from the halophilic archaeon *Haloferax volcanii*

Andrew T. Large[1], Eszter Kovacs[1], Peter A. Lund*

*School of Biosciences, University of Birmingham, Birmingham B15 2TT, UK*

Received 11 September 2002; revised 1 November 2002; accepted 4 November 2002

First published online 14 November 2002

Edited by Gunnar von Heijne

**Abstract** **The halophilic archaeon *Haloferax volcanii* has three genes encoding type II chaperonins, named *cct1*, *cct2* and *cct3*. We show here that the three CCT proteins are all expressed but not to the same level. All three proteins are further induced on heat shock. The CCT proteins were purified by ammonium sulphate precipitation, sucrose gradient centrifugation and hydrophobic interaction chromatography. This procedure yields a high molecular mass complex (or complexes). The complex has ATPase activity, which is magnesium dependent, low salt-sensitive and stable to at least 75°C. Activity requires high levels of potassium ions and was reduced in the presence of an increasing concentration of sodium ions.**


*Key words:* Chaperonin; Heat shock; Archaeon; Halophile

## 1. Introduction

Chaperonins are a highly conserved and nearly ubiquitous class of molecular chaperone. Their main functions are mediating the correct folding and assembly of selected polypeptides during cell growth, and limiting damage caused by stresses such as heat shock [1–3]. They can be divided into two subclasses by sequence homology: the group I or 'GroE-type' chaperonins found in eubacteria and eukaryotic organelles, and the group II or 'TCP-1-type' chaperonins found in archaea and in the cytosol of eukaryotes [4–6]. Both assemble into a double-ring oligomer structure with a central cavity which can bind non-native proteins and releases them in an ATP-dependent fashion, facilitating their folding to the native state [5,7,8]. The group I chaperonins form a double-toroid oligomer containing seven subunits in each ring and require a co-chaperonin (typified by the *Escherichia coli* protein GroES) for substrate folding, while the group II chaperonins form eight- or nine-membered rings and are independent of a GroES co-chaperonin, the function of which is instead probably fulfilled by a helical protusion of the apical domain [9–11]. Another significant difference between the two chaperonin groups is that archaea and eukaryotes mostly encode multiple group II chaperonin genes, whereas most eubacteria encode a single groEL homologue [12,13]. The highest level of multimerisation is observed in the case of the TCP-1 complex of the eukaryotic cytosol which has eight different subunits [14], all of which are essential in yeast [12,15]. The binding of actin (one of the few identified substrates of the TCP-1 complex) is subunit-specific and geometry dependent [16], suggesting that, to some degree, TCP-1 may have co-evolved with its substrates leading to specialisation of the subunits [9,13].

Archaea constitute a separate phylogenetic domain distinct from eukaryotes and eubacteria [17]. The number of group II chaperonin genes found in archaea varies from one to three [18]. The thermosome of the crenarchaeote *Thermoplasma acidophilum* is the most intensively studied of the archaeal group II chaperonins, and has two eight-membered rings with two alternating subunits (α and β) in a stoichiometry of 1:1 [19]. Thermococcus strain KS-1 also has two genes but in this case the ratio of α to β varies depending on growth conditions [20]. the *Sulfolobus shibatae* and *Sulfolobus solfataricus* thermosome has two nine-membered rings [21,22], and the presence of three genes has been confirmed [18] although the subunit composition of the complex or complexes is unknown at present. Phylogenetic analysis suggests that archaeal chaperonin genes are constantly being both duplicated and lost, and are unlikely to have evolved functions which are subunit-specific [13,23]. However, this remains to be tested as archaea are generally hard to manipulate genetically. An exception is *Haloferax volcanii*, a halophilic and moderately thermophilic euryarchaeote.

*H. volcanii* was originally reported to synthesise five major heat-shock proteins with apparent molecular masses of 98, 91, 85, 79 and 21 kDa by sodium dodecylsulphate–polyacrylamide gel electrophoresis (SDS–PAGE) [24]. In subsequent experiments, the three *cct* genes were all shown to be heat shock inducible [25,26]. The *cct1* and *cct2* genes were identified and sequenced (accession number AF010470 and AF010469, respectively), whilst *cct3* was only partially sequenced [25–27]. For a review of stress-response genes and proteins in the archaea see [28].

We are characterising CCT proteins and genes in this organism, both as a general model for group II chaperonin function and because the high internal salt concentration in these organisms poses interesting problems for protein folding that the chaperonins may help to overcome. Here we report the purification and analysis of these proteins, the first characterisation of a CCT protein complex from a halophile.

## 2. Materials and methods

### 2.1. Bacterial strains, plasmids, cosmids and culture conditions

The *E. coli* strain DH5α was grown in LB supplemented with

*Corresponding author. Fax: (44)-121-414 5925.
*E-mail address:* p.a.lund@bham.ac.uk (P.A. Lund).

[1] These authors contributed equally to the work presented.

0014-5793/02/$22.00 
PII: S0014-5793(02)03685-2

ampicillin (100 μg/ml) or kanamycin (50 μg/ml) to select for pUC19.1-based plasmids or cosmids, respectively. The *H. volcanii* strain DS70 was grown aerobically in modified growth medium containing 23% total salt at 37°C as described in [29]. Vector pUC19.1, a modified version of the pUC19 vector containing an extra unique *Mlu*I restriction site between the *Bam*HI and *Xba*I sites, and cosmids A199, 268 and 452 from the cosmid library of *H. volcanii* DS2 strain [27] harbouring the *cct1*, *cct2* and *cct3* genes [25,26], were kind gifts of Professor Charles Daniels.

*2.2. DNA sequencing*

The *cct3* gene was sequenced using the Big Dye chain terminator method according to the manufacturer's instructions (Perkin Elmer, Warrington, UK). The 3.9 kb *Mlu*I fragment of cosmid 452 (containing the entire *cct3* gene) was subcloned into pUC19.1. Universal primers were used to determine the sequences from both ends of the fragment. Subsequently, internal primers were designed for both strands to complete the sequence. Sequencing reactions were done on the Perkin Elmer Biosystems 877 robotic system.

*2.3. Analysis of H. volcanii heat stress proteins*

Three ml aliquots of mid-log phase *H. volcanii* cultures grown at 37°C were transferred to a 60°C (for heat shock) or 37°C (as a control) shaking water bath and $^{14}$C-amino acid mixture (Amersham Biosciences, UK) was added to 11.1 kBq/ml final specific activity. Cultures were incubated for 0.5, 1, 2 and 3 h, respectively, and then cooled on ice. Cells were harvested by centrifugation (13 000 × *g*, 5 min) from 1 ml of cultures and solubilised in 100 μl in one- or two-dimension sample buffer and subjected to gel electrophoresis.

One-dimensional SDS–PAGE was carried out on 8% gels according to Laemmli [30], analysing 10 μl of samples prepared as described above. Two-dimensional gel electrophoresis using 50 μl of sample was done as described [31], using Ampholine 3/10 (Pharmacia, Uppsala, Sweden) for isoelectric focusing, and 8% SDS–PAGE for the second dimension. Gels were stained with Coomassie brilliant blue R-250 or, in the case of radioactively labelled samples, gels were stained, dried onto Whatman paper and exposed to Kodak Biomax MR film. Autoradiograms were scanned and quantified using QuantityOne (Bio-Rad, UK).

*2.4. Immunological identification of the archaeal chaperonins*

Following electrophoresis, polypeptides were transferred to Immobilon P membrane (Millipore). The membranes were then probed with an antiserum against the α-subunit of the *T. acidophilum* thermosome (a generous gift of Gundula Bosch and Wolfgang Baumeister, Martinsried, Germany), at 1:1000 dilution. Horseradish peroxidase-conjugated goat anti-rabbit IgG (Sigma) was used in 1:10 000 dilution as a secondary antibody. The immunoblots were developed with Boehringer ECL reagents and analysed using QuantityOne (Bio-Rad, UK).

*2.5. Purification of CCT from H. volcanii cells*

Cells from 1 l of mid-log phase *H. volcanii* culture grown at 37°C were pelleted (8000 × *g*, 15 min, 4°C). Cell paste was resuspended in a minimum volume of isolation buffer (50 mM Tris–HCl, pH 7.5, 2 M NaCl, 2 mM EDTA) containing 50 μg/ml bacterial protease inhibitor cocktail (Sigma Chemicals, Poole, UK) and put on ice. The suspension was sonicated three times for 1 min with 2 min cooling intervals in between, centrifuged at 40 000 × *g* for 20 min, and the supernatant brought to 65% saturation of ammonium sulphate by the slow addition of finely powdered solid with stirring at room temperature. This step was crucial in removing membrane fragments that otherwise became major contaminants in subsequent steps. Stirring was continued for 30 min and precipitated protein removed by centrifugation at 40 000 × *g* for 40 min. The extract was dialysed against the isolation buffer and concentrated by ultrafiltration using a 100 000 kDa cut-off membrane (Millipore). Concentrated protein solution (5 ml) was loaded onto a sucrose gradient consisting of 2 ml each of 0.3, 0.5 and 0.7 M sucrose on a 2 ml 36% Iodixanol (Axis-Shield, Oslo, Norway) cushion in isolation buffer and centrifuged at 200 000 × *g* (gmax) for 19 h in a Beckman SW-40Ti swing-out ultracentrifuge rotor. One ml fractions were collected and their protein content was analysed by SDS–PAGE after precipitation of the proteins with 10% w/v trichloroacetic acid and re-solubilising in one-dimensional gel electrophoresis sample buffer [30]. Peak fractions were dialysed overnight against 2.5 M NaCl, 20 mM TES pH 7.5 to remove sucrose and loaded onto a 10 ml octyl–Sepharose column (Sigma) equilibrated with the same buffer. The column was then washed with 10 ml of 2 M NaCl, 20 mM TES pH 7.5 and the protein eluted with a 30 ml gradient of 2 M NaCl, 0% glycerol to 1 M NaCl, 50% glycerol (all with 20 mM TES pH 7.5). Peak fractions were identified as above by one-dimensional SDS–PAGE. The protein was dialysed for several days against 3 M KCl, 20 mM TES pH 7.5, concentrated by ultrafiltration and used for ATPase assays.

*2.6. ATPase activity of CCT protein*

Purified CCT protein (~0.5 μg) was incubated with 1 mM ATP (magnesium salt) to which 1 μCi [γ-$^{32}$P]ATP (Perkin Elmer Life Sciences) was added per assay in a 50 μl volume. Typically, the assay mixture contained 2.9 or 3.1 M KCl, 20 mM TES pH 7.5, 50 mM $MgCl_2$. At 10 min intervals 10 μl aliquots were removed and added to 700 μl of 30 mg/ml charcoal suspension in 40 mM HCl on ice. The suspension was vortexed and centrifuged to sediment the charcoal. 500 μl of supernatant was removed and added to 5 ml of scintillation fluid. An aliquot of the assay mixture was also added to scintillation fluid to estimate the total counts in each assay. Counts per minute were measured by Cerenkov counting in a Packard Tri-Carb 2700TR counter. All assays were performed in triplicate, with suitable controls included to allow for spontaneous ATP degradation. Rates were determined by linear regression.

*2.7. Other methods*

Protein concentration was determined according to Bradford [32] using bovine serum albumin as a standard. CCT1 and CCT3 proteins were separated by 2-D electrophoresis and blotted onto an Immobilon P membrane; N-terminal sequence analysis of the proteins was carried out by AltaBioscience, University of Birmingham, UK, using a Perkin-Elmer (Warrington, UK) Biosystems 473a automated sequencer.

## 3. Results and discussion

*3.1. Identification of H. volcanii chaperonins*

We have completed the sequencing of the *cct3* gene (accession number AF298660). Sequence analysis of the cct3 gene shows an open reading frame of 1572 bp, which is predicted to encode a 55.3 kDa protein of 524 amino acids with a calculated p*I* of 4.26. The high acidity of the CCT3 protein is typical for proteins from halophilic archaea [33]. This compares with the molecular masses of CCT1 and CCT2 at 58.9 and 59.3 kDa, and p*I*s of 3.9 and 4.1, respectively. CCT1 shares 57% identity and 72% similarity with CCT2, but is less homologous to CCT3. The lowest homology is found between CCT2 and CCT3 (45%/64% identity/similarity). An intriguing feature is the presence of a long GGM 'tail' at the C-terminus of the CCT1 and CCT2 proteins but not CCT3. This feature is highly conserved in bacterial GroEL homologues but is absent from all eukaryotic and many archaeal CCT proteins. Its significance is unknown, although it is largely dispensable under most growth conditions for *E. coli* GroEL protein [34,35].

A heterologous antibody raised against the α-subunit of the *T. acidophilum* thermosome recognised three proteins in Western blots of cell extracts from *H. volcanii*, with apparent molecular masses of 95.4, 86.3 and 76.2 kDa (not shown). Although these values are significantly different from the predicted values for the three CCT proteins, it is known that halophilic acidic proteins migrate slower in SDS–PAGE gels [36]. The N-terminal sequences of CCT1 and CCT2 are identical for the first 13 amino acids, so we first determined which protein was CCT2 by over-expressing *cct2* in *E. coli* under the control of the lac promoter in pUC19.1. The position of the protein expressed in *E. coli* was identical on Western blots to that of the middle of the three CCT proteins seen in *H. volcanii* (not shown). CCT1 and CCT3 have two differences in their first five amino acids and were readily distinguished by N-terminal sequencing of protein spots isolated from 2-D gels.

The spots or bands corresponding to the three proteins are shown in Fig. 1a.

Estimation of the relative levels of the three proteins by scanning of autoradiograms, Western blots and Coomassie-stained gels showed an excess of both CCT1 and CCT2 over CCT3 by between three- and five-fold. The same result was seen with the purified complex, which suggests that there was no selective loss of subunits during purification.

Using radiolabelling we confirmed that all three CCT proteins showed a significant increase in the level of expression following heat shock at 60°C (Fig. 1b). Quantitative analysis of the radiolabelled gels showed that although the overall levels of each of the three proteins increased, their relative ratios stayed approximately the same (not shown).

### 3.2. Purification and analysis of the CCT oligomer

The CCT protein oligomer was purified by a combination of selective ammonium sulphate precipitation, sucrose gradient sedimentation and hydrophobic interaction chromatography, maintaining high salt throughout. The advantage of the sucrose gradient step is that only CCT protein present in assembled complexes is sedimented sufficiently to be collected in the peak fractions. A sucrose gradient was also used to confirm that the CCT obtained after octyl–Sepharose chromatography was assembled as a large oligomer (not shown). We estimate the purity of the final preparation to be >95% (Fig. 2). At this stage, we do not know whether the three CCT proteins assemble into a single unique complex or a mixture of complexes. More detailed experiments using physical methods are currently under way to address this question.

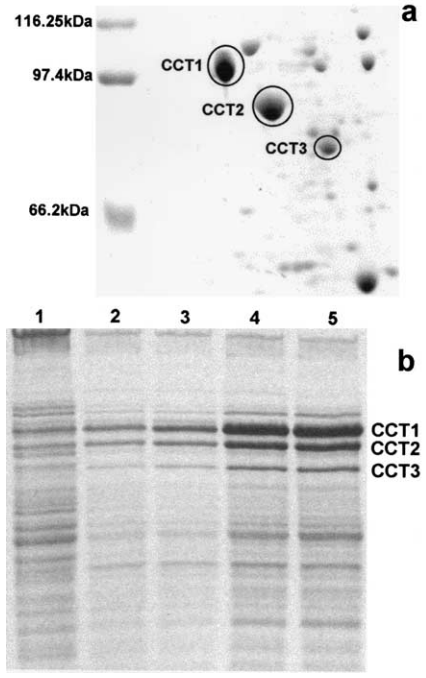


Fig. 1. Identification of CCT proteins and heat shock induction of CCT proteins. a: Coomassie-stained two-dimensional gel of *H. volcanii* proteins (from non-heat-shocked cells) as used for N-terminal sequence determination, showing the three spots corresponding to the three CCT proteins. b: Autoradiogram of one-dimensional 8% SDS–PAGE gel. Lane 1: 3 h labelling of control (37°C), lane 2: 30 min at 60°C, lane 3: 1 h at 60°C, lane 4: 2 h at 60°C, lane 5: 3 h at 60°C. In both cases the identity of the spots or bands was confirmed using Western blotting, data not shown.

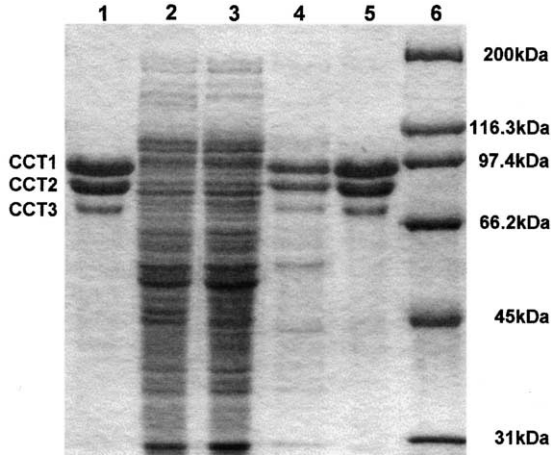


Fig. 2. SDS–PAGE of CCT proteins during purification. Lane 1: final CCT preparation, lane 2: crude cell lysate supernatant, lane 3: post-ammonium sulphate fractionation, lane 4: post-sucrose gradient centrifugation, lane 5: final CCT preparation, lane 6: marker proteins.

All chaperonins tested to date have a low ATPase activity. Consistent with this, the purified CCT complex was found to possess a low magnesium-dependent ATPase activity. Activity in the absence of added magnesium ions and using the sodium salt of ATP was virtually undetectable (<5% of that in the presence of magnesium ions). The activity was stable and linear for more than 30 min at 75°C although at 85°C the rate was linear for only the first 20 min indicating that the protein was unstable at this temperature. High thermal stability is a common feature of chaperonins in general, even from mesophiles, and as *H. volcanii* grows optimally at around 45°C, the high thermal stability of the CCT complex was expected. The mean activity of three preparations of CCT complex at 55°C was 0.15 μmol ATP/min/mg protein, which is broadly comparable to values for other archaeal CCT complexes.

The ATPase activity rose with increasing potassium ion concentration and was still increasing at 3.2 M (the maximum practical concentration using our assay method) (Fig. 3a,b). However, no activity could be detected in a buffer with high levels of sodium ions but no potassium ions (data not shown). Further analysis showed that the ATPase activity was sensitive to increasing concentrations of sodium ions even in the presence of excess potassium ions and maintaining equal ionic strength (Fig. 3c). The high potassium ion concentration required for optimum activity is as predicted as halophilic archaea accumulate high internal concentrations of potassium ions (up to 5 M potassium concentration in the cell) to buffer against the osmotic effects of high sodium ions in the environments where these organism are found. However, the strong inhibition by sodium ions is puzzling given that the intracellular sodium concentration may also reach 1 M [37].

The role of chaperones in halophilic archaea is a particularly interesting one because of the internal environment in these organisms, where water is tightly bound to the ions, and proteins have had to evolve special mechanisms (chiefly the presence of large numbers of surface acidic residues) to be able to stay in solution. The CCT proteins are induced by low salt as well as by heat [25] and another related halophilic archaeon (*Haloarcula marismortui*) produces at least one other large molecular mass complex when stressed by low salt con-

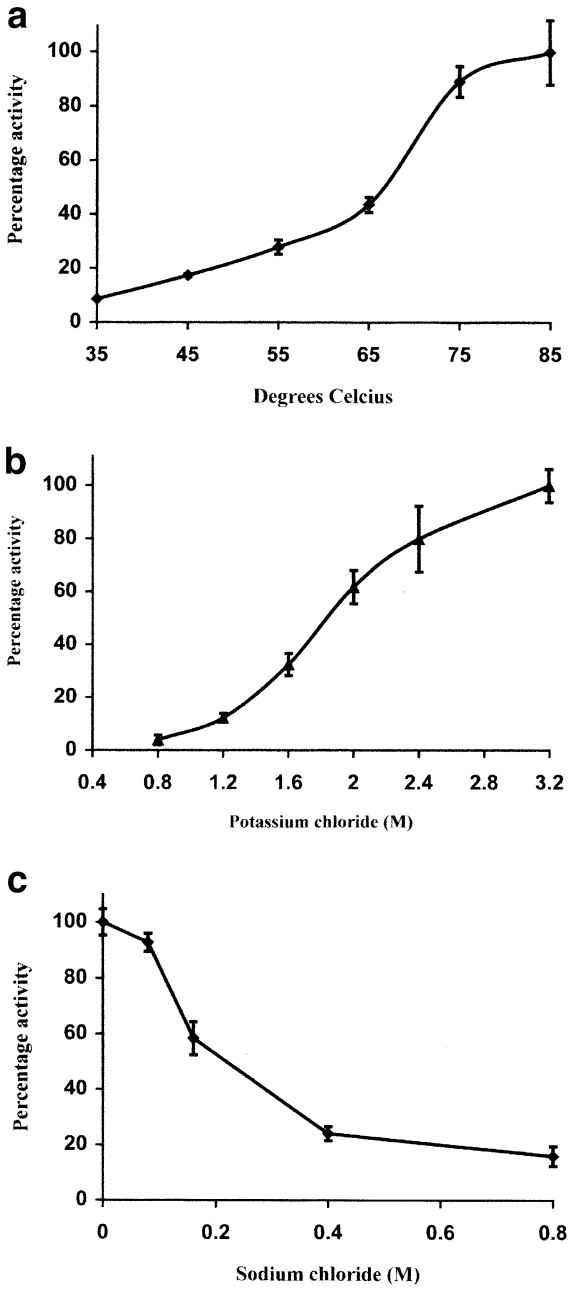


Fig. 3. a: ATPase activity with varying temperature at 2.9 M KCl. Activity was measured over 30 min except at 85°C where rates were linear for only 20 min. 100% = 0.458 nmol ATP hydrolysed per min per μg of protein. b: ATPase activity with varying KCl concentration at 55°C. 100% = 0.0865 nmol ATP hydrolysed per min per μg of protein. c: ATPase activity with varying sodium concentration. Assays were performed as described in the text with 3.1 M total salt (KCl+NaCl, ionic strength constant) at 55°C. 100% = 0.177 nmol ATP hydrolysed per min per μg of protein.

ditions [38]. We are currently further exploring the role of the CCT complex in vivo and in vitro.

*Acknowledgements:* We are grateful to Mike Dyall-Smith for much help with techniques, provision of strains, and general discussion, Charles Daniels for provision of cosmids and plasmids, and Hardip Kaur for Western blot analysis of CCT2 expression in *E. coli*. Funding was provided by NATO and the Biotechnology and Biological Sciences Research Council.

## References

[1] Georgopoulos, C. and Welch, W.J. (1993) Annu. Rev. Cell Biol. 9, 601–634.
[2] Hartl, F.U. (1996) Nature 381, 571–580.
[3] Hartl, F.U. and Hayer-Hartl, M. (2002) Science 295, 1852–1858.
[4] Ellis, R.J. (1992) Nature 358, 191–192.
[5] Horwich, A.L., Weber-Ban, E.U. and Finley, D. (1999) Proc. Natl. Acad. Sci. USA 96, 11033–11040.
[6] Carrascosa, J.L., Llorca, O. and Valpuesta, J.M. (2001) Micron 32, 43–50.
[7] Richardson, A., Landry, S.J. and Georgopoulos, C. (1998) Trends Biochem. Sci. 23, 138–143.
[8] Sigler, P.B., Xu, Z.H., Rye, H.S., Burston, S.G., Fenton, W.A. and Horwich, A.L. (1998) Annu. Rev. Biochem. 67, 581–608.
[9] Willison, K.R. and Horwich, A.L. (1996) in: The Chaperonins (Ellis, R.J., Ed.), pp. 107–135, Academic Press, New York.
[10] Gutsche, I., Essen, L.O. and Baumeister, F. (1999) J. Mol. Biol. 293, 295–312.
[11] Yoshida, T., Kawaguchi, R., Taguchi, H., Yoshida, M., Yasunaga, T., Wakabayashi, T., Yohda, M. and Maruyama, T. (2002) J. Mol. Biol. 315, 73–85.
[12] Stoldt, V., Rademacher, F., Kehren, V., Ernst, J.F., Pearce, D.A. and Sherman, F. (1996) Yeast 12, 523–529.
[13] Archibald, J.M., Blouin, C. and Doolittle, W.F. (2001) J. Struct. Biol. 135, 157–169.
[14] Kubota, H., Hynes, G. and Willison, K. (1995) Eur. J. Biochem. 230, 3–16.
[15] Lin, P. and Sherman, F. (1997) Proc. Natl. Acad. Sci. USA 94, 10780–10785.
[16] Llorca, O., McCormack, E.A., Hynes, G., Grantham, J., Cordell, J., Carrascosa, J.L., Willison, K.R., Fernandez, J.J. and Valpuesta, J.M. (1999) Nature 402, 693–696.
[17] Woese, C.R., Kandler, O. and Wheelis, M.L. (1990) Proc. Natl. Acad. Sci. USA 87, 4576–4579.
[18] Archibald, J.M., Logsdon, J.M. and Doolittle, W.F. (1999) Curr. Biol. 9, 1053–1056.
[19] Nitsch, M., Klumpp, M., Lupas, A. and Baumeister, W. (1997) J. Mol. Biol. 267, 142–149.
[20] Yoshida, T., Ideno, A., Hiyamuta, S., Yohda, M. and Maruyama, T. (2001) Mol. Microbiol. 39, 1406–1413.
[21] Trent, J.D., Nimmesgern, E., Wall, J.S., Hartl, F.U. and Horwich, A.L. (1991) Nature 354, 490–493.
[22] Knapp, S., Schmidtkrey, I., Hebert, H., Bergman, T., Jornvall, H. and Ladenstein, R. (1994) J. Mol. Biol. 242, 397–407.
[23] Archibald, J.M. and Roger, A.J. (2002) J. Mol. Biol. 316, 1041–1050.
[24] Daniels, C.J., McKee, A.H.Z. and Doolittle, W.F. (1984) EMBO J. 3, 745–749.
[25] Kuo, Y.P., Thompson, D.K., StJean, A., Charlebois, R.L. and Daniels, C.J. (1997) J. Bacteriol. 179, 6318–6324.
[26] Thompson, D.K. and Daniels, C.J. (1998) Mol. Microbiol. 27, 541–551.
[27] Charlebois, R.L., Schalkwyk, L.C., Hofman, J.D. and Doolittle, W.F. (1991) J. Mol. Biol. 222, 509–524.
[28] Macario, A.J.L., Lange, M., Ahring, B.K. and Macario, E.C. (1999) Microbiol. Mol. Biol. Rev. 63, 923–967.
[29] Dyall-Smith, M. (2001) http://www.microbiol.unimelb.edu.au/micro/staff/mds/HaloHandbook/HaloHB.web/Halohandbook4.5.pdf.
[30] Laemmli, U.K. (1970) Nature 227, 680–685.
[31] O'Farrell, P.H. (1975) J. Biol. Chem. 250, 4007–4021.
[32] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[33] Danson, M.J. and Hough, D.W. (1998) Trends Microbiol. 6, 307–314.
[34] Burnett, B.P., Horwich, A.L. and Low, K.B. (1994) J. Bacteriol. 176, 6980–6985.
[35] McLennan, N.F., McAteer, S. and Masters, M. (1994) Mol. Microbiol. 14, 309–321.
[36] Bonete, M.J., Pire, C., Llorca, F.I. and Camacho, M.L. (1996) FEBS Lett. 383, 227–229.
[37] Kushner, D.J. (1988) Can. J. Microbiol. 34, 482–486.
[38] Franzetti, B., Schoehn, G., Ebel, C., Gagnon, J., Ruigrok, R.W.H. and Zaccai, G. (2001) J. Biol. Chem. 276, 29906–29914.