Volume 336, number 2, 363–367 FEBS 13448 December 1993

Truncated GroEL monomer has the ability to promote folding of rhodanese without GroES and ATP

Yoshihide Makino, Hideki Taguchi, Masasuke Yoshida*

Research Laboratory of Resources Utilization, R-1, Tokyo Institute of Technology Nagatsuta 4259, Yokohama 227, Japan

Received 8 November 1993

Similar to chaperonins from other sources, intact chaperonin from *Escherichia coli* (GroEL) exists as a tetradecamer, and the ability to promote folding of other proteins has been considered to be dependent on this oligomeric structure. However, the peptide fragments of GroEL of molecular size 34–50 kDa, which are produced by limited proteolysis of monomeric GroEL and are unable to assemble into an oligomer, retain the ability to promote folding of rhodanese even though the yield of productive folding is lower than the intact GroEL/GroES/ATP system. This promotion by truncated GroEL obeys rapid kinetics and does not require GroES and ATP.

Chaperonin; GroEL; GroES; Protein folding; Rhodanese

1. INTRODUCTION

The *Escherichia coli* heat shock protein, GroEL, and its homologs in other organisms belong to a chaperonin (or HSP60) family of molecular chaperones [1–4]. They are well conserved in amino acid sequences, ubiquitous in various cells, and indispensable for living cells [3–6]. Chaperonin captures labile folding intermediates of other proteins to suppress their aggregation and, with aid of ATP and the 10 kDa protein (cpn10, *E. coli* GroES), the intermediates are slowly released to continue productive folding [5,7,8]. Intact GroEL is a tetradecamer ($GroEL_{14}$) of the 57.3 kDa protein ($GroEL_m$), and $GroEL_m$ are arranged as two layers of a heptamer ring in $GroEL_{14}$ [9–14]. It has been considered that the activity of $GroEL_{14}$ to promote protein folding is dependent on this 'double doughnut' structure. However, we report here that the truncated $GroEL_m$, which cannot assemble into an oligomer, acts as a molecular chaperone.

2. EXPERIMENTAL

2.1. *Proteins*

Bovine mitochondrial rhodanese (thiosulfate sulfurtransferase, 33 kDa monomer enzyme) type II, lysozyme, bovine serum albumin, and immobilized thermolysin were purchased from Sigma. Bovine pancreas RNaseA was from Boehringer-Mannheim. $GroEL_{14}$ and GroES were purified from lysates of *E. coli* cells bearing the multicopy plasmid, pACYC 184 carrying *groES–groEL* genes which was a kind gift from Dr. K. Ito [15]. Cell lysate was applied to a DEAE-Sephacel column and was eluted with 0–0.5 M NaCl gradient in 25 mM Tris-Cl buffer (pH 7.5) containing 1 mM EDTA and 1 mM dithiothreitol. The same buffer was used throughout following procedures. Fractions containing $GroEL_{14}$ and those containing GroES were individually concentrated with ammonium sulfate precipitation. Then $GroEL_{14}$ was purified with a Sepharose CL-6B column. GroES was purified with successive three column chromatographies; an Ether Toyopearl column (30–0% ammonium sulfate gradient), a Sepharose CL-6B column, and a DEAE-Sephacel column (0–0.5 M NaCl gradient). Purified $GroEL_{14}$ and GroES were stored as 65% ammonium sulfate precipitation at 4°C.

*Corresponding author. Fax: (81) (45) 922 5179.

Abbreviations: GroELm, monomer of GroEL molecule; GroEL14, tetradecamer of GroEL molecules; rhodanese, thiosulfate sulfurtransferase; SDS, sodium dodecylsulfate.

2.2. *Preparation and limited proteolysis of $GroEL_m$, and fractionation of peptide fragments*

The purified $GroEL_{14}$ was denatured in 6 M guanidine HCl and 5 mM dithiothreitol and, after a 30 min incubation on ice, the solution was applied to a Sephadex G-50 column equilibrated with 25 mM Tris-Cl buffer (pH 7.5) containing 1 mM EDTA and 1 mM dithiothreitol to remove guanidine HCl. The resultant solution contained $GroEL_m$ as a major component as described later. To the solution of $GroEL_m$ (7 mg/ml), $CaCl_2$ was added at a final concentration 5 mM, and proteolysis was initiated by addition of 0.7 U of immobilized thermolysin. After a 60 min incubation at 25°C, the reaction was terminated by removing immobilized thermolysin by centrifugation and successive filtration through a nitrocellulose filter (0.2 μm). Then the mixture of digested $GroEL_m$ was applied to gel permeation HPLC on a Tosoh G3000SW column (21.5 mm × 30 cm) equilibrated with 25 mM Tris-Cl (pH 6.8) and 100 mM Na_2SO_4. The column was eluted with the same buffer at a flow rate of 2 ml/min, and products of limited proteolysis were fractionated according to the molecular size.

2.3. *Rhodanese folding assay*

Bovine mitochondrial rhodanese (0.25 mg/ml) was denatured in 50 mM potassium phosphate buffer (pH 7.8), 5 mM dithiothreitol and 6 M guanidine HCl at 25°C [16]. Denatured rhodanese was diluted 25-fold into the dilution buffer and pre-incubated at 30°C containing 50 mM potassium phosphate (pH 7.8), 50 mM $Na_2S_2O_3$, 5 mM dithiothreitol, and indicated component(s). After incubation at 30°C for 30 min or indicated periods, reactivated rhodanese activity was determined according to Sörbo [17].

2.4. *Peptide sequencing and other methods*

Proteins were analyzed by polyacrylamide gel electrophoresis either on a 7.5% polyacrylamide gel without SDS or on a 13% polyacrylamide gel in the presence of SDS [18]. Gels were stained with Coomassie brilliant blue R-250. When necessary, protein bands in the gel were blotted to a polyvinylidene difluoride membrane (Bio-Rad Corp.), stained with Coomassie brilliant blue R-250, and membrane pieces of blotted peptides were analyzed with a peptide sequenator (Tosoh). Protein concentration was determined by the method of Bradford with bovine serum albumin as a standard [19] except for RNaseA and lysozyme, for which concentrations were determined from absorbance at 280 nm.

3. RESULTS

3.1. *Preparation of proteolytic fragments of GroEL$_m$*

When guanidine HCl-denatured GroEL was applied to a gel filtration column to remove guanidine HCl, $GroEL_m$ and a small amount of $GroEL_{14}$ were regenerated (Fig. 1a, lane 2). We tried to remove $GroEL_{14}$ from the solution with Centricon 100 (100 kDa cut-off) but a small amount of $GroEL_{14}$ was always found in the $GroEL_m$ fraction. Although it was reported that the assembly of $GroEL_m$ into $GroEL_{14}$ was dependent on ATP [20], a small amount of $GroEL_{14}$ is spontaneously formed from $GroEL_m$ in the absence of ATP under our experimental conditions. For this reason, the $GroEL_m$ preparations used in this paper contained small amounts of $GroEL_{14}$. In an attempt to prevent the formation of $GroEL_{14}$, $GroEL_m$ was subjected to limited proteolysis by immobilized thermolysin. After 60 min incubation with thermolysin, the undigested $GroEL_m$ almost disappeared and several peptide fragments were produced (Fig. 1b, lane 3). Analysis of NH_2-terminal amino acid sequences of digested peptides revealed the presence of several thermolysin-sensitive regions in the primary structure of $GroEL_m$. The regions are Ile^{49}–Val^{56}, and Ile^{144}–Ile^{150}. The regions around Val^{56}, Ile^{120}, and Ile^{134} appear also to be susceptible to thermolysin. These regions are most likely unstructured loop regions in the tertiary structure of $GroEL_m$. It is obvious from the estimated size of each peptide band that the COOH-terminal side of $GroEL_m$ is also cleaved by thermolysin. The 34 kDa fragment, for example, can be tentatively assigned to peptide Ile^{150}–Leu^{456}. The 34 kDa fragment is the major product after long incubation (1 h) with thermolysin, indicating the presence of a rigid core structure of $GroEL_m$ (Fig. 1b, lane 3). Further digestion by thermolysin did not produce a relatively stable peptide fragment but produced small peptides (< ~ 5 kDa) which were not analyzable by SDS-PAGE (data not shown). Probably $GroEL_{14}$ is more resistant to proteolysis and trace amounts of $GroEL_{14}$ still remained in the digested mixture (Fig. 1a, lane 3) [21].

The mixture was applied to gel permeation HPLC, and three major peak fractions were collected (Fig. 2). Peak A was eluted almost at void volume of the column, and contained oligomeric GroEL and aggregates of various peptides (Fig. 2, inset). Peaks B and C were mixtures of several fragments of $GroEL_m$ of different molecular sizes; the former (fraction B) contained peptide fragments of molecular sizes ~ 50 – ~ 40 kDa (Fig. 1b, lane 4), and the latter (fraction C) contained mainly the 34 kDa fragments (Fig. 1b, lane 5). The ATPase activities of fractions B and C were 6% and <1 % of that of the $GroEL_{14}$, respectively. Fractions B and C were free from $GroEL_{14}$ (Fig. 1a, lanes 4,5), and peptides contained in these fractions were unable to form $GroEL_{14}$ even in the presence of GroES and ATP (data not shown). These results are consistent with the report that a mutation at the NH_2-terminal region of GroEL molecule results in destabilization of oligomeric structure of $GroEL_{14}$ [22,23].

3.2. *Promotion of folding of rhodanese by fractions B and C*

The effect of fractions B and C on protein folding was measured using rhodanese as a substrate protein; guanidine HCl-denatured rhodanese was diluted into the dilution buffer containing fraction B or C or other components and reactivation of rhodanese was assayed as a measure of productive folding [16,22,23]. As shown in

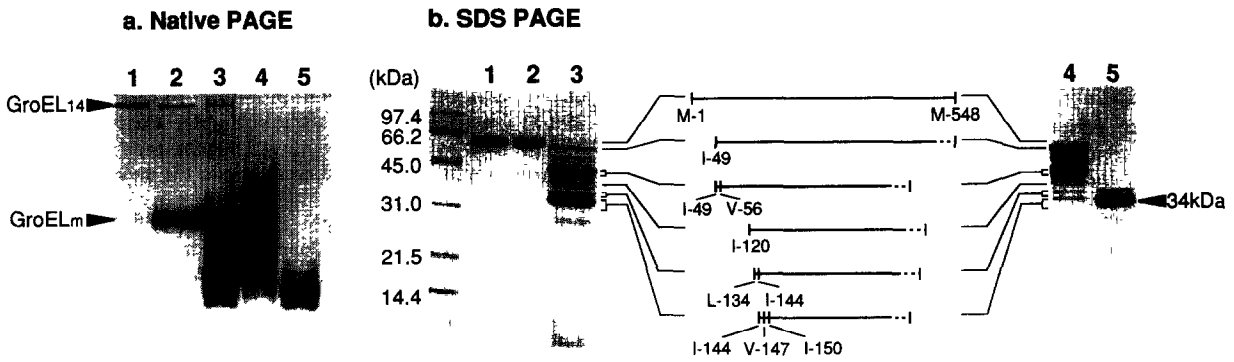

Fig. 1. Polyacrylamide gel electrophoresis of $GroEL_{14}$, $GroEL_m$, and thermolysin-digested $GroEL_m$ (a) in the absence of or (b) in the presence of SDS. Concentrations of polyacrylamide were (a) 7.5% and (b) 13%. Lanes: 1, intact $GroEL_{14}$; 2, $GroEL_m$; 3, $GroEL_m$ subjected to limited proteolysis by thermolysin; 4,5, fractions B and C, respectively, of gel permeation HPLC (see Fig. 2). In panel b between lanes 3 and 4, a schematic diagram of the distribution of thermolysin-cleaved sites in the primary structure of GroEL was inserted.

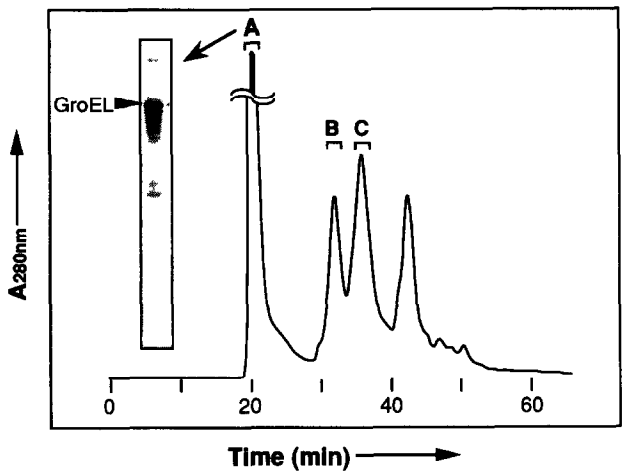

Fig. 2. Fractionation of peptide fragments produced by limited proteolysis of $GroEL_m$ with gel permeation HPLC on a G3000SW column. Elution was monitored by absorbance at 280 nm. Fractions A, B, and C are indicated. Analysis of proteins contained in fraction A with 13% polyacrylamide gel electrophoresis in the presence of SDS is shown in the figure. Analysis of fractions B and C are shown in Fig. 1.

Fig. 3a, the folding yield of rhodanese in the presence of fraction B or C was almost 2-fold (~40%) of that of spontaneous folding (~20%). The mixture of the small peptides (< ~5 kDa) produced by rather extensive proteolysis of $GroEL_m$ with immobilized thermolysin did not promote folding (data not shown). The presence of lysozyme or RNaseA, which were expected to exert a non-specific protein effect on the folding, did not improve the yield of spontaneous folding. The $GroEL_m$, as well as intact $GroEL_{14}$ (± GroES), inhibited spontaneous folding of rhodanese. Since it has been known that folding of rhodanese is arrested by $GroEL_{14}$ when either GroES or ATP is absent [16,22,23], the inhibition by $GroEL_m$ can be explained by the arrested folding by $GroEL_{14}$ contained in our $GroEL_m$ preparation. When $GroEL_{14}$, GroES and ATP were present in the dilution buffer, nearly 100% of the rhodanese activity was recovered. In contrast to $GroEL_{14}$, the promotion of folding of rhodanese by fractions B and C did not require the presence of GroES and ATP (Fig. 3b).

3.3. *The fraction B- and C-promoted folding leveled off rapidly*

As shown in Fig. 4, the time courses of promoted folding of rhodanese by the fraction B and C were clearly different from that by the complete system ($GroEL_{14}$ + GroES + ATP). The folding promoted by the complete system proceeded slowly and the final yield was achieved after 30 min. On the contrary, the folding promoted by fractions B and C reached maximum yield within 5 min of dilution. Spontaneous folding also leveled off rapidly, irrespective of the presence of lysozyme or RNaseA in the dilution buffer.

3.4. *The effect of concentrations of fractions B and C on the folding yield*

The yield of productive folding of rhodanese increased as concentrations of fractions B and C in the dilution buffer increased until they reached 0.2–0.4 mg/ml, that is, peptide fragments present were approximately 20- to 40-fold molar excess over rhodanese (Fig. 5). Lysozyme or RNaseA did not show a significant effect on the yield of folding even at the highest concentrations tested.

4. DISCUSSION

The results reported here suggest that truncated $GroEL_m$ possesses the ability to promote protein folding. The smallest fragment that seems to have chaperone activity in our experiments is the 34 kDa fragment. This fragment lacks 149 NH_2-terminal residues and ~93 COOH-terminal residues. Interestingly, the putative

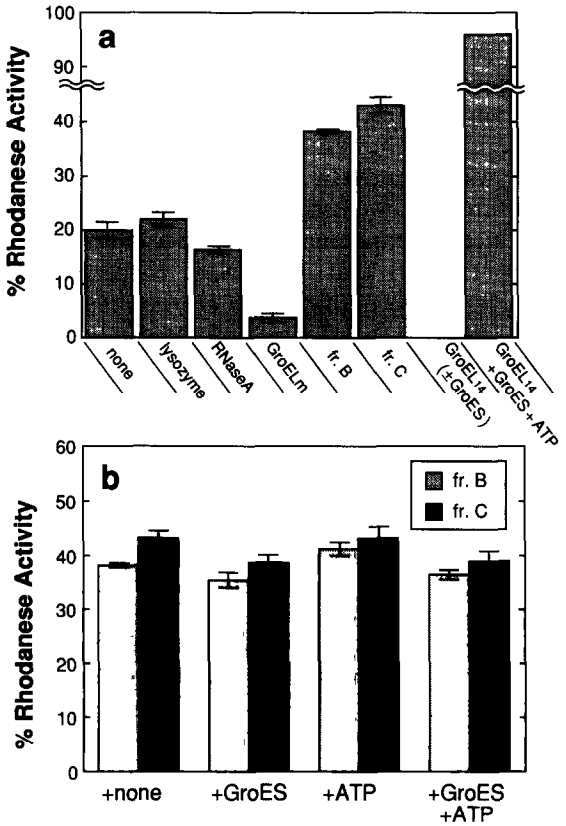

Fig. 3. (a) Effect of fractions B, C, and other proteins on the reactivation yield of rhodanese. Denatured rhodanese was diluted into the buffer containing indicated component(s). Final concentrations of protein components were 10 μg/ml rhodanese, 40 μg/ml GroES, and 0.5 mg/ml of other proteins. When the addition of ATP was indicated, 1 mM ATP and 1 mM $MgCl_2$ were included. After 30 min of incubation at 30°C, an aliquot was withdrawn and reactivated rhodanese activity was measured. More details are described in the text. (b) Effect of GroES and ATP on the yield of the rhodanese reactivation promoted by fraction B and C. Experimental conditions are the same as in panel a. The same experiments were repeated twice except the samples containing $GroEL_{14}$ (single experiment) and the averaged values are shown. Bars indicate the value of each experiment.

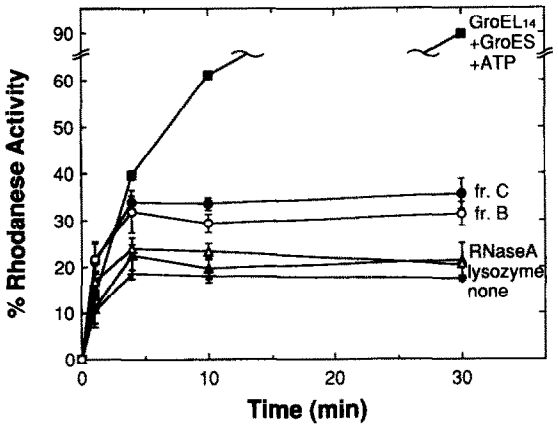

Fig. 4. Time-course of reactivation of rhodanese in the presence of indicated component(s). Reactivated rhodanese activity was measured at indicated time. Other conditions are the same as described in legend of Fig. 3. The same experiments were repeated five times except $GroEL_{14}$ + GroES+ATP (single experiment), and error ranges (standard deviation) were indicated by bars.

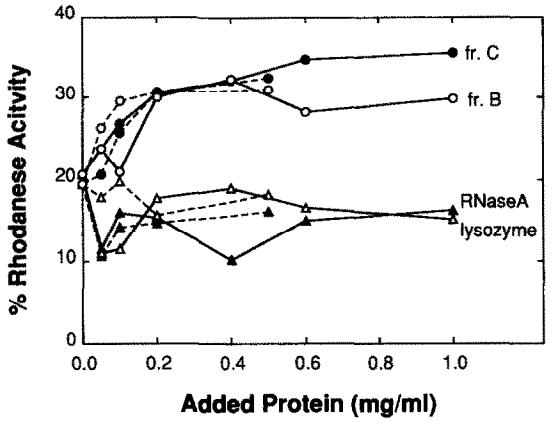

Fig. 5. Effect of concentrations of indicated proteins on the reactivation yield of rhodanese. The final concentration of rhodanese was 10 μg/ml. After 30 min incubation at 30°C, rhodanese activity was measured. Other conditions are the same as described in legend of Fig. 3. Experiments were repeated twice and shown individually as solid and dashed lines.

shortest functional domain of chaperonin suggested from complementation of *E. coli ams* (altered mRNA stability) mutant [24,25] is contained in the 34 kDa fragment. Although the result of the intact (not digested) $GroEL_m$ was obscured by the presence of spontaneously formed $GroEL_{14}$, we assume that intact $GroEL_m$ also has this kind of chaperone activity since our preliminary experiment shows that monomeric chaperonin-60 (a $GroEL_m$ homolog) purified from *Thermus thermophilus*, which is free from the oligomeric form, can promote the folding of rhodanese. Thus, the oligomeric arrangement of GroEL molecules is not an absolute requirement for primitive chaperone activity. This kind of ATP-independent chaperone activity was reported for small heat shock proteins and Hsp90 [26,27].

It should be noted, however, that the mode of promotion by truncated $GroEL_m$ is very different from that by $GroEL_{14}$; the yield of productive folding is low, folding is rapidly saturated with time, and it is not affected by GroES and ATP. The reason for these differences is not yet known but one possibility is that truncated $GroEL_m$ binds folding intermediates only transiently and promotes productive folding for only a part of the folding intermediates. It might also be related to the critical question; why is $GroEL_m$ organized into such a large complex as a tetradecamer? Probably oligomerization of $GroEL_m$ is necessary for efficient, rapid capturing of all folding intermediates which tend to aggregate in a highly concentration-dependent manner. When a binding site for the folding intermediate residing on each $GroEL_m$ in the $GroEL_{14}$ is arranged as a heptamer ring, the affinity to folding intermediate could be cooperatively strengthened due to the physical proximity of binding sites. Then, the tightly bound folding intermediate is released from $GroEL_{14}$ in a regulated manner by GroES and ATP so as to keep the concentration of the free folding intermediate low in the medium and to attain the best yield of productive folding. This speculation is, of course, based on several unproved assumptions, for example that a single folding intermediate molecule has several regions recognizable by $GroEL_m$ (otherwise, cooperative binding to $GroEL_{14}$ will not occur), and further study is needed to examine this speculation.

Acknowledgements: We thank Dr. K. Ito, Kyoto University, for providing us a plasmid pACYC184-groESL.

REFERENCES

[1] Hemmingsen, S.M., Woolford, C., van der Vies, S.M., Tilly, K., Dennis, D.T., Georgopoulos, C.P., Hendrix, R.W. and Ellis, R.J. (1988) Nature 333, 330–334.
[2] Ellis, J. (1987) Nature 328, 378–379.
[3] Ellis, R.J. and van der Vies, S.M. (1991) Annu. Rev. Biochem. 60, 321–347.
[4] Gething, M.-J. and Sambrook, J. (1992) Nature 355, 33–45.
[5] Hartl, F.U., Martin, J. and Neupert, W. (1992) Annu. Rev. Biophys. Biomol. Struct. 21, 293–322.
[6] Georgopoulos, C. (1992) Trends Biochem. Sci. 17, 295–299.
[7] Zeilstra-Ryalls, J., Fayet, O. and Georgopoulos, C. (1991) Annu. Rev. Microbiol. 45, 301–325.
[8] Lorimer, G.H. (1992) Curr. Opin. Struct. Biol. 2, 26–34.
[9] Hohn, T., Hohn, B., Engel, A., Wurtz, M. and Smith, P.R. (1979) J. Mol. Biol. 129, 359–373.
[10] Hendrix, R.W. (1979) J. Mol. Biol. 129, 375–392.
[11] Pushkin, A.V., Tsuprun, V.L., Solovjeva, N.A., Shubin, V.V., Evstigneeva, Z.G. and Kretovich, W.L. (1982) Biochim. Biophys. Acta 704, 379–384.
[12] Viitanen, P.V., Lorimer, G.H., Seetharam, R., Gupta, R.S., Oppenheim, J., Thomas, J.O. and Cowan, N.J. (1992) J. Biol. Chem. 267, 695–698.
[13] Ishii, N., Taguchi, H., Sumi, M. and Yoshida, M. (1992) FEBS Lett. 299, 169–174.
[14] Langer, T., Pfeifer, G., Martin, J., Baumeister, W. and Hartl, F.-U. (1992) EMBO J. 11, 4757–4765.
[15] Ito, K. and Akiyama, Y. (1991) Mol. Microbiol. 5, 2243–2253.

[16] Martin, J., Langer, T., Boteva, R., Schramel, A., Horwich, A.L. and Hartl, F.-U. (1991) Nature 352, 36–42.
[17] Sörbo, B.H. (1953) Acta Chem. Scand. 7, 1129–1136.
[18] Laemmli, U.K. (1970) Nature 227, 680–685.
[19] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[20] Lissin, N.M., Venyaminov, S.Y. and Girshovich, A.S. (1990) Nature 348, 339–342.
[21] Price, N.C., Kelly, S.M., Thomson, G.J., Coggins, J.R., Wood, S. and Mauer, A.a.d. (1993) Biochim. Biophys. Acta 1161, 52–58.
[22] Mendoza, J.A., Rogers, E., Lorimer, G.H. and Horowitz, P.M. (1991) J. Biol. Chem. 266, 13044–13049.
[23] Mendoza, J.A., Lorimer, G.H. and Horowitz, P.M. (1991) J. Biol. Chem. 266, 16973–16976.
[24] Chanda, P.K., Ono, M., Kuwano, M. and Kung, H.-F. (1985) J. Bacteriol. 161, 446–449.
[25] Alconada, A. and Cuezva, J.M. (1993) Trends Biochem. Sci. 18, 81–82.
[26] Jakob, U., Gaestel, M., Engel, K. and Buchner, J. (1993) J. Biol. Chem. 268, 1517–1520.
[27] Wiech, H., Buchner, J., Zimmermann, R. and Jakob, U. (1992) Nature 358, 169–170.