Characteristics of super αA-crystallin, a product of in vitro exon shuffling

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Received 20 July 2000; accepted 28 July 2000

Edited by Jesus Avila

Abstract αA-Crystallin, a small heat shock protein with chaperone-like activity, forms dynamic multimeric complexes. Recently we described the spontaneous generation of a mutant protein (super αA-crystallin) by exon duplication arisen via exon shuffling confirming a classic hypothesis by Gilbert [Nature 271 (1978) 501]. Comparison of super αA-crystallin, which is viable in a mouse skeletal muscle cell line, with normal αA-crystallin shows that it has diminished thermostability, increased exposure of hydrophobic patches, a larger complex size and lost its chaperone activity. However, super αA-crystallin subunits exchange as readily between complexes as does normal αA-crystallin. These data indicate that chaperone-like activity may vanish independent of subunit hydrophobicity and exchangeability.

Key words: Mutant αA-crystallin; Small heat shock protein; Exon shuffling

1. Introduction

Among the seven known human small heat shock proteins (sHSPs) [1,2], a special position is taken by α-crystallin [3]. This protein was first discovered as a major structural eye lens constituent and occurs as large globular complexes of up to 800 kDa, composed of two related subunits of 20 kDa, αA and αB [4]. While αB occurs in many tissues outside the lens, notably in heart and striated muscle, αA is essentially restricted to the lens [5–7]. Like other members of the sHSP family, α-crystallins display in vitro chaperone-like activity, suppressing the aggregation of denaturing proteins [8]. The most conspicuous in vivo feature of α-crystallins is their ability to confer thermotolerance upon overexpression in different cell types [9].

With the discovery of super αA – a product of exon shuffling in a mouse skeletal cell line transfected with the hamster αA gene [10] – we now have access to a new αA-crystallin with a large insertion. The gene encoding super αA has arisen by illegitimate recombination at two CCCAT similarities in the normal hamster αA gene. As a consequence, a tandem-duplicated exon 3 is present in the mature mRNA, resulting in a 41-residue repeat in the translated protein. Super αA, expressed at levels similar to the wild-type, shows normal in vivo stability and cytoplasmic distribution, and does not disturb cell growth. Size exclusion chromatography shows that it is incorporated into normal α-crystallin complexes. The duplication of 41 residues thus does not interfere with the in vivo viability of super αA. Surprisingly the gross structural intrusion can be accommodated by the evolutionary highly conserved αA-crystallin molecule, that has not even accepted a single amino acid replacement in most rodent lineages for at least 60 million years [11]. Since super αA is a viable protein, the study of the structural and functional properties of this new αA may shed light on the formation and stability of normal α-crystallin complexes and its chaperone-like activity. With this in mind we examined the morphology and size of the super αA homomultimer, its behavior during the chaperone assay, heat stability, exposure of hydrophobic patches and its ability to interchange subunits with normal αA homomultimer complexes. The latter two parameters are considered to be important determinants of chaperoning capacity [12,13].

2. Materials and methods

2.1. Plasmid construction

Total RNA was isolated from C2C12 cells expressing hamster αA and super αA. cDNA was made by the 5′–3′ rapid amplification of cDNA ends kit of Boehringer Mannheim [10]. After PCR using specific primers, with an *Nde*1 restriction site at the ATG and a *Bam*H1 restriction site at the stop codon, the obtained cDNAs were ligated into a pGem-T vector (Promega), cut out of this vector with proper restriction enzymes and ligated into the prokaryotic expression vector pET3a.

2.2. Expression and purification of recombinant super αA

The expression vectors were transformed in the host *Escherichia coli* Bl21(DE3)plysS. Induction and cell lysis were essentially as described by Merck et al. [14]. Hamster αA was purified from the water-soluble fraction of a cell lysate. This fraction was dialyzed against DEAE starting buffer (50 mM NaCl, 1 mM EDTA, 20 mM Tris–HCl, pH 7.5 and applied onto a DEAE-Sepharose column (Pharmacia-LKB). The mobile phase was a gradient from 50 mM to 500 mM NaCl in 1 mM EDTA, 20 mM Tris–HCl, pH 7.5 at a flow rate of 3 ml/min. Fractions of αA were pooled, dialyzed against demineralized water and lyophilized.

Super αA was purified from the water-insoluble cell lysate fraction using a DE52 anion exchange column under denaturing conditions. This fraction was directly stirred in DE52 starting buffer (6 M urea, 0.02% β-mercaptoethanol (vol./vol.), 5 mM Tris–HCl, pH 8.0) at 4°C (2 h), centrifuged (15 000 × *g*, 30 min), dialyzed against DE52 starting buffer and separated on a DE52 column. The mobile phase was a gradient from 5 mM to 0.1 M Tris–HCl, pH 8.0 in 6 M urea and 0.02% β-mercaptoethanol (vol./vol.), at a flow rate of 0.2 ml/min. DE52 fractions of super αA were pooled, dialyzed against water and lyophilized.

2.3. Reconstitution

For structural and functional studies αA and super αA were refolded under identical conditions. Lyophilized protein was dissolved in denaturing buffer, 20 mM Na_2SO_4 and 20 mM NaP_i at pH 6.90 (buffer P) containing 6 M urea and 0.02% β-mercaptoethanol (vol./vol.), incubated on ice for 1 h and diluted with buffer P containing 1 M urea and 0.02% β-mercaptoethanol (vol./vol.). Subsequently, the

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0014-5793/00/$20.00
PII: S0014-5793(00)01908-6

solution was dialyzed against buffer P and used in the following experiments.

2.4. Gel permeation

An LKB Bromma HPLC system was used in combination with a Superose 6HR 10/30 prepacked size exclusion column (Pharmacia LKB) for analysis of aggregate sizes. Samples contained 500 μg of super αA and bovine αA (gift of Martinus van Boekel) and 100 μg of hamster αA, in 1 ml of buffer P. The mobile phase was buffer P at a flow rate of 0.5 ml/min. Absorbance was monitored at 225 nm. Purified recombinant hamster αA, super αA and purified bovine αA were dissolved in buffer P containing 6 M urea and 0.02% β-mercaptoethanol (vol./vol.) and reconstituted as written above.

2.5. Electron microscopy

Lyophilized, purified recombinant hamster αA, super αA and purified bovine αA, after reconstitution were applied onto a copper grid coated with formvar and carbon. After 5 min excess liquid was removed using filter paper. The proteins were counterstained with 1% uranyl acetate (thrice 30 s). Dried grids were examined in a Jeol 1210 electron microscope at 80 kV.

2.6. Chaperone-like activity

Chaperone-like activity was determined essentially as described by Farahbakhsh et al. [15]. Various amounts of super αA and recombinant rat αB (a gift of Bas Kokke) were pre-incubated with 248 μg bovine pancreas insulin (3 min at 40°C). Denaturation of insulin was initiated by adding 20 μl of 1 M dithiothreitol and light scattering was measured for 15 min at 360 nm using a Perkin-Elmer Lambda 2 UV–vis spectrophotometer equipped with a thermostated circulating water-bath at 37°C and a thermocouple. The total volume was 1 ml. All solutions were in buffer P.

2.7. ANS fluorescence

Hydrophobicity by means of 1-anilinonaphthalene-8-sulfonic acid (ANS) binding was measured essentially as described by Smulders et al. [16]. The hydrophobicity of, super αA, αA (rat) and αB (bovine, a gift of Gerard Stege), was determined, 75 μg protein of each was used. The excitation wavelength was set to 380 nm with a bandpass of 10 nm. The emission spectra were recorded at 25°C over a range of 420–520 nm with a 10 nm bandpass using a Shimadzu RF-5301 PC spectrofluorometer with a thermostated circulating water-bath. All conditions were chosen to minimize inner-filter effects [17].

2.8. Thermostability

Light scattering was measured from 20 to 70°C at 360 nm using a Perkin-Elmer spectrophotometer (see above). 700 μg of protein, hamster αA and super αA, were used; the total volume was 1 ml. All solutions were in buffer P.

2.9. Subunit exchange

Fluorescence resonance energy transfer was employed to determine subunit exchange between multimeric super αA and multimeric hamster αA complexes over a time period of 110 min at different temperatures, essentially as described by Bova et al. [18]. The emission intensity at 415 nm of the sample excited at 335 nm was recorded using a Shimadzu RF-5301 PC spectrofluorometer as described above. Hamster αA was labeled with 4-acetamido-4′-((iodoacetyl)amino)stilbene-2,2′-disulfonic acid (AIAS) as an energy donor and super αA was labeled with lucifer yellow iodoacetamide (LYI), which is an energy acceptor. In a control experiment rat αA was labeled with the energy donor or with the energy acceptor. The differently labeled complexes were mixed.

3. Results

3.1. Expression and purification of recombinant super αA

To obtain the recombinant hamster αA and super αA, total RNA was extracted from C2C12 cells producing these proteins [10]. cDNAs were constructed via PCR with primers for the hamster αA gene. Since the super αA gene contains additionally only a duplicated exon 3 the same primer set could be used for both cDNAs, which were cloned into a pET3a prokaryotic expression vector and transfected into *E. coli*. Water-soluble and -insoluble fractions of cell lysates were analyzed for the presence of the recombinant proteins. As expected hamster αA protein was found in the water-soluble fraction, the super αA protein appeared in the water-insoluble fraction. Proteins were purified as described in Section 2.

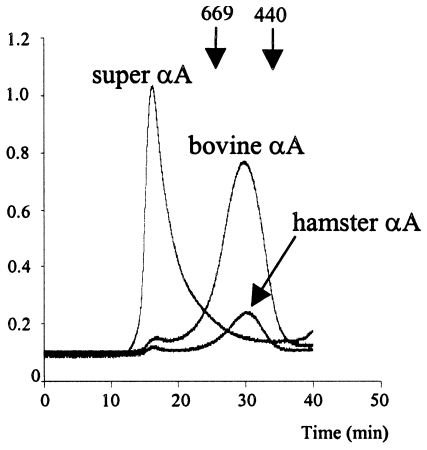

Fig. 1. Size exclusion chromatography. Bovine and hamster αA elute at the same column volume, whereas super αA elutes much earlier, reflecting a larger complex size. Elution times for molecular weight markers (in kDa) are indicated by arrows.

3.2. Gel permeation analysis

To check the nature of the complexes of bovine αA, recombinant hamster αA and super αA (all refolded) were subjected to size exclusion chromatography. Recombinant hamster αA and reconstituted bovine αA homomultimers appeared to have masses of about 650 kDa. Super αA displayed a higher molecular mass as demonstrated by the smaller elution volume, directly after the void volume of the column (Fig. 1). The size of super αA was calculated to be at least 3000 kDa as extrapolated from hamster αA (molecular mass obtained from size exclusion chromatography used in combination with particle diameter derived from the electron micrograph to determine the volume). Since the monomeric super αA has a molecular weight of 25 kDa [10], the larger multimeric size of super αA is only to a minor extent due to this bigger mass, but must rather be due to a higher amount of the approximately 40 subunits of normal αA.

3.3. Electron microscopy

Electron microscopical analysis reveals that bovine αA (not shown) and recombinant hamster αA (Fig. 2A) form more or less globular particles. However, the super αA particles appear not only significantly larger than normal αA (compare Fig. 2A and B) they also have the tendency to form rod-like particles. The width of the rods is identical with the diameter of the globular super αA particles. Therefore, it cannot be excluded that a particle appearing globular is actually seen from the top of a rod-like structure. Differences in size observed for super αA, might be explained by the fact that the first peak in the elution profile is tailing.

3.4. Chaperone-like activity

Super αA chaperone-like activity was tested by means of the insulin assay in which reduction of the interchain disulfide

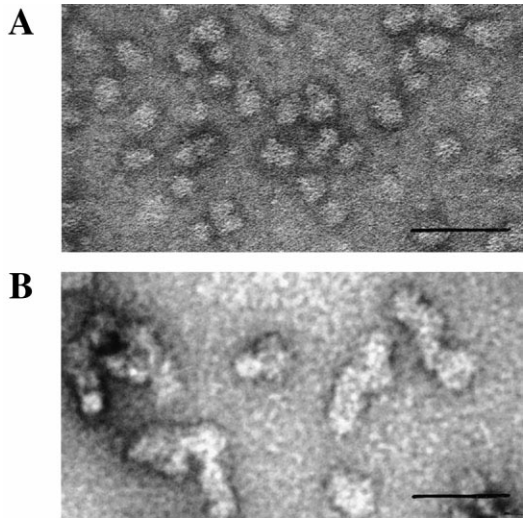

Fig. 2. Electron micrographs of recombinant hamster αA (A) and recombinant super αA (B). Bar = 50 nm. Hamster αA complexes appear globular; super αA is more rod-like.

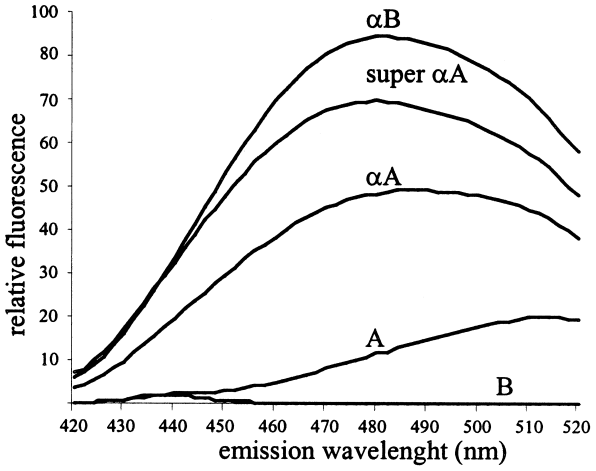

Fig. 4. ANS fluorescence spectroscopy of recombinant rat αA, super αA and bovine αB. Note that the buffer containing protein (pattern B) and the buffer containing ANS (pattern A) show negligible fluorescence.

bonds by DTT results in precipitation of aggregated B-chains, whereas the A-chains remain in solution. Fig. 3 shows the inability of super αA to prevent the aggregation of insulin. At equal masses of super αA to insulin (100%) even a higher relative absorption is seen than with insulin alone. The positive control, αB-crystallin, at 100% fully prevents insulin from aggregation. This means that also super αA tends to aggregate, whereas normal αA at 100% shows a good protection although to a lesser extent than αB [19].

3.5. ANS fluorescence

Structural changes in α-crystallin that induce exposure of hydrophobic surfaces can increase chaperone-like activity [12]. To determine whether the extra exon 3 affects the hydrophobicity of super αA, we have assessed this protein for its ability to bind the fluorescent probe ANS at its exposed hydrophobic patches. Fig. 4 shows that super αA has an intermediate ability to bind ANS compared to αB and αA. Obviously the fluorescence intensity of the protein in buffer without ANS or buffer containing just ANS can be neglected. As super αA has better ANS-binding than αA, surface hydrophobicity cannot explain the observed differences in chaperone-like activity.

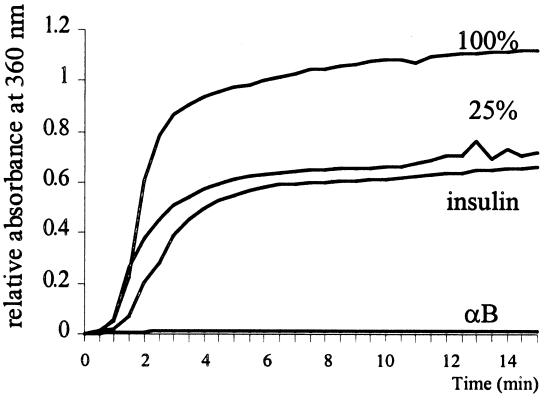

Fig. 3. Ability of increasing amounts of recombinant super αA to prevent DDT-induced aggregation of insulin. Aggregation of insulin is given as a function of time in the absence and presence of super αA. The super αA to insulin mass ratios (weight/weight) are indicated as percentages. As a positive control recombinant αB was used at a 100% mass ratio. Super αA itself is not denatured by DTT (not shown).

3.6. Thermostability

To determine whether the thermostability of super αA is conserved, recombinant reconstituted hamster αA and super αA were heated and the absorption was monitored at 360 nm as a function of temperature. Fig. 5 shows that super αA rapidly starts to precipitate at 45°C, whereas wild-type hamster αA remains in solution until at least 70°C.

3.7. Subunit exchange

Subunit exchange as a measure of complex flexibility was analyzed by the fluorescence energy transfer (FRET) assay by mixing two labeled homomultimer complexes, one of super αA labeled with the energy acceptor (LYI) and another of hamster αA, labeled with the energy donor (AIAS). Time-dependent changes in emission intensity due to subunit exchange were determined. Fig. 6 shows that super αA can perfectly

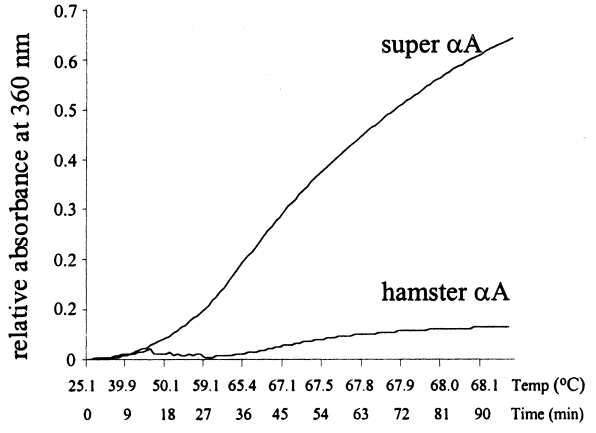

Fig. 5. Thermostability of recombinant hamster αA and super αA. Aggregation is given as a function of time and temperature.

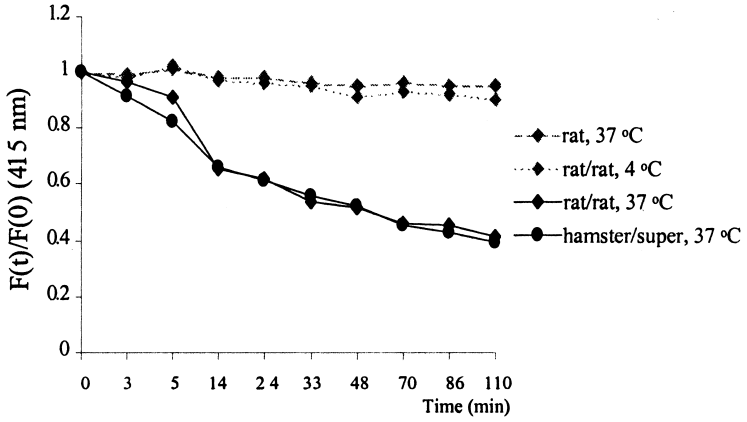

Fig. 6. Decrease in relative fluorescence due to subunit exchange as a function of time after mixing equal amounts of donor- and acceptor-labeled homomultimers at 37°C. Subunit exchange is monitored between recombinant AIAS-labeled hamster αA with recombinant LYI-labeled super αA (hamster/super 37°C) and between recombinant labeled rat homomultimers (rat/rat 37°C). Note that rat αA homomultimers do not show a change in emission spectra when kept at 4°C (rat/rat 4°C). No decrease in fluorescence intensity at 37°C could be observed when no energy acceptor was present in solution (rat 37°C).

exchange subunits with αA. The exchange rate is the same as between normal rat αA complexes labeled with energy donor and acceptor molecules at 37°C. For rat αA at 4°C, no exchange was detected (negative control). Rat αA labeled with the energy donor is used as a control for fluorescence at 37°C. No decrease in fluorescence is detected without an energy acceptor.

4. Discussion

Duplication of exon 3 due to exon shuffling has a major impact on the properties of super αA. Compared to the normal α-crystallin both structure and function are altered. From the results presented above we must conclude that the name 'super αA', as given in the previous paper because of its larger subunit and complex size [10], is less appropriate when we consider its greatly diminished chaperone-like capacity. At the time this name was introduced no data concerning its function were available yet, except that normal incorporation in the α-crystallin complexes and normal phosphorylation of super αA took place in C2C12 cells [10].

Protein sequence alignment of *Methanococcus jannaschii* Hsp16.5 with αA [20] and secondary structure prediction of αA-crystallin [21] led us to extrapolate the β-strands observed in the crystal structure of Hsp16.5 to super αA. From these data we conclude that part of the β2-strand and the β3-, β4- and β5-strands as found for Hsp16.5 [20] are duplicated in super αA. Obviously, this duplication does not seriously hamper the viability of super αA in vivo [10]. Similarly, our recombinant super αA behaves as a properly folded protein. This is most apparent from the fact that, as shown by the FRET-assay (Fig. 6), super αA readily exchanges subunits with normal αA, and thus must be structurally compatible. Also the limited increase of exposed hydrophobic patches seen in super αA suggests that the protein is correctly folded.

That such an insert of 41 residues can readily be accommodated in an otherwise normal αA subunit is not too surprising. Super αA is in that respect comparable to αA^{ins}-crystallin, a product of alternative splicing of the αA gene transcript in rodents and a few other mammals [22,23]. αA^{ins}-crystallin has a 23-residue insertion at the same position as in super αA, and is normally incorporated into the eye lens α-crystallin of these animals. αA^{ins}-crystallin forms, like super αA, larger complexes than normal αA and shows reduced chaperoning capacity [24].

Two parameters are considered to be most important for the chaperone-like capacity of sHSps: the exposure of hydrophobic areas, and the ability of sHSp complexes to dissociate and re-associate [12,13,18,21] In αA, hydrophobic patches involved in chaperoning have been pinpointed to residues 50–54 and 79–99 [25]. The first patch is encoded by exon 1, the second and larger one by exon 3. As to the second patch, a synthetic peptide corresponding to residues 70–88 shows chaperone-like activity by itself, confirming that this sequence in αA is a true chaperone site [26]. Despite the fact that this sequence is present twice in super αA, the protein has no chaperone-like activity at all. This suggests that in super αA these chaperone sites are not accessible because of conformational changes brought about by the duplication. Alternatively, or additionally, the greatly decreased thermostability of super αA – reflecting a lesser overall structural stability – likely contributes to the reduction of chaperone-like activity. We may conclude that for super αA no positive effect of exposure of hydrophobic patches on chaperone activity does exist, in accordance with the behavior of various other αA mutants [16]. Also Reddy et al. [27] recently confirmed that surface hydrophobicity is not the sole determinant of the chaperone activity of α-crystallin.

Chaperone assays reflect the differences between the kinetics of substrate aggregation and the binding of substrate to the chaperone. This dynamic model of chaperone function, as long as the exposure of binding sites is the rate limiting step in the assay, predicts a dependence of the chaperone efficiency on the stability of the oligomeric state. Although super αA is less heat-stable than normal αA, it exchanges subunits (a process that is supposed to be the rate limiting step in the chaperone-like activity [13,18,21]) as easily as normal αA. Therefore, the conclusion as stated by Koteiche and Mchaourab [21] 'when the dynamic threshold of function is lowered by an increase in temperature or a mutation-induced destabilization of the storage state one should expect an increase in the chaperone efficiency' is not tenable for super αA.

With regard to multimerization, heat stability and chaperone-like activity, super αA behaves like a mutant found in man (R116C) which also shows a larger complex size, reduced chaperone-like activity and lower heat stability [28]. The R116 is a buried amino acid in a stretch of residues 109–120 that have been shown to form contacts between equivalent strands from neighboring subunits [21]. Adding an extra exon that leads to a change in the direct environment of R116 may disrupt the two-fold symmetric interface. The 41-residue repeat, as well as the R116C mutation with a decrease in positive charge, may lead to an alternative dimerization motif in super αA. This leads to conclude that arginine 116 in human and its surrounding as seen in our mutant are very important in maintaining the structure and function of αA-crystallin.

Acknowledgements: This work was supported by EC-BIOMED (contract nr. BMH4-CT96-1593 and BMH4-CT98-3895) and in part by the Alcon Research Institute Award to one of us (H.B.).

References

[1] Boelens, W.C., van Boekel, M.A.M. and de Jong, W.W. (1998) Biochim. Biophys. Acta 1388, 513–516.
[2] Krief, S., Faivre, J.F., Robert, P., Le Douarin, B., Brument-Larignon, N., Lefrere, I., Bouzyk, M.M., Anderson, K.M., Greller, L.D., Tobin, F.L., Souchet, M. and Bril, A. (1999) J. Biol. Chem. 274, 36592–36600.
[3] de Jong, W.W., Caspers, G. and Leunissen, J.A. (1998) Int. J. Biol. Macromol. 22, 151–162.
[4] Bloemendal, H. (1977) Science 197, 127–138.
[5] Bhat, S.P. and Nagineni, C.N. (1989) Biophys. Res. Commun. 158, 319–325.
[6] Kato, K., Shinohara, H., Kurobe, N., Inaguma, Y., Shimizu, K. and Ohshima, K. (1991) Biochim. Biophys. Acta 1074, 201–208.
[7] Kato, K., Shinohara, H., Kurobe, N., Goto, S., Inaguma, Y. and Ohshima, K. (1991) Biochim. Biophys. Acta 1080, 173–180.
[8] Horwitz, J. (1992) Proc Natl. Acad. Sci. USA 89, 10449–10453.
[9] Klemenz, R., Fröhli, E., Steiger, R.H., Schäfer, R. and Aoyama, A. (1991) Proc. Natl. Acad. Sci. USA 88, 3652–3656.
[10] van Rijk, A.A.F., de Jong, W.W. and Bloemendal, H. (1999) Proc. Natl. Acad. Sci. USA 96, 8074–8079.
[11] Hendriks, W., Leunissen, J., Nevo, E., Bloemendal, H. and de Jong, W.W. (1987) Proc. Natl. Acad. Sci. USA 84, 5320–5324.
[12] Raman, B. and Rao, C.M. (1994) J. Biol. Chem. 269, 27264–27268.
[13] Haslbeck, M., Walke, S., Stromer, T., Ehrnsperger, M., White, H.E., Chen, S., Saibil, H.R. and Buchner, J. (1999) EMBO J. 18, 6744–6751.
[14] Merck, K.B., de Haard Hoekman, W.A., OudeEssink, B.B., Bloemendal, H. and de Jong, W.W. (1992) Biochim. Biophys. Acta 1130, 267–276.
[15] Farahbakhsh, T., Huang, Q.L., Ding, L.L., Altenbach, C., Steinhoff, H.J., Horwitz, J. and Hubbell, W.L. (1995) Biochemistry 34, 509–516.
[16] Smulders, R.H.P.H., Merck, K.B., Aendekerk, J., Horwitz, J., Takemoto, L., Slingsby, C., Bloemendal, H. and de Jong, W.W. (1995) Eur. J. Biochem. 232, 834–838.
[17] Birdsall, B., King, R.W., Wheeler, M.R., Lewis, C.A., Goode, S.R., Dunlap, R.B. and Roberts, G.K. (1983) Biochemistry 132, 353–361.
[18] Bova, M.P., Ding, L.L., Horwitz, J. and Fung, B.K.K. (1997) J. Biol. Chem. 272, 29511–29517.
[19] van Boekel, M.A.M., de Lange, F., de Grip, W.J. and de Jong, W.W. (1999) Biochim. Biophys. Acta 1434, 114–123.
[20] Kim, K.K., Kim, R. and Kim, S.H. (1998) Nature 394, 595–599.
[21] Koteiche, H.A. and Mchaourab, H.S. (1999) J. Mol. Biol. 294, 561–577.
[22] Cohen, L.H., Westerhuis, L.W., de Jong, W.W. and Bloemendal, H. (1978) Eur. J. Biochem. 89, 259–266.
[23] King, C.R. and Piatigorsky, J. (1983) Cell 32, 707–712.
[24] Smulders, R.H.P.H., van Geel, I.G., Gerards, W.L.H., Bloemendal, H. and de Jong, W.W. (1995) J. Biol. Chem. 270, 13916–13924.
[25] Sharma, K.K., Kumar, G.S., Murphy, A.S. and Kester, K. (1998) J. Biol. Chem. 273, 15474–15478.
[26] Sharma, K.K., Kumar, R.S., Kumar, G.S. and Quin, P.T. (2000) J. Biol. Chem. 275, 3767–3771.
[27] Reddy, G.B., Das, K.P., Petrash, J.M. and Surewitz, W.K. (2000) J. Biol. Chem. 275, 4565–4570.
[28] Schroff, N.P., Cherian-Shaw, M., Bera, S. and Abraham, E.C. (2000) Biochemistry 39, 1420–1426.