Differences in properties between human α A- and α B-crystallin proteins expressed in *Escherichia coli* cells in response to cold and extreme pH

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It has been reported that α A-crystallin has greater protective effects against apoptosis in lens epithelial cells than α B-crystallin [Andley, Song, Wawrousek, Fleming and Bassnett (2000) *J. Biol. Chem.* **275**, 36823–36831]. Because the α A-crystallin proteins are specifically expressed in the vertebrate lens, we examine the non-specific properties of both α A- and α B-crystallins in an *Escherichia coli* system. *E. coli* cells were transformed with the inducible protein expression vector pET-11a, harbouring the gene for either human α A- or α B-crystallin, and two other control plasmids, pET-11a vector alone or pGEX-2T vector encoding GST (glutathione S-transferase). These cells were exposed to various stress conditions, such as cold-shock at 4 °C or extremely low or high pH environments (pH 4.7 or pH 8.0) for 6 h, and survival of

the host cells and the solubility of the expressed target proteins in the cytosol were examined. Under these stress conditions, the cells expressing α B-crystallin protein demonstrated significantly improved survival when compared with the other cells, and the expressed protein in the cytosol was almost soluble, in contrast with the α A-crystallin protein. Differences in the amino acid sequence between the proteins in a phenylalanine-rich region next to the N-terminal consensus α -crystallin domain was considered to be responsible for chaperone activity and cell survival.

Key words: cold shock, extreme pH, human α A-crystallin, human α B-crystallin, solubility, cell survival.

INTRODUCTION

sHSPs (small heat-shock proteins) are ubiquitous in the cytosol of eukaryotes, and they are a diverse class of proteins that differ from other HSP families because they possess a certain short sequence motif, an α -crystallin domain. The domain is composed of approx. 90 residues which are highly conserved in the C-terminal region [1,2]. Their nuclear translocation in response to various stresses have been reported in several organisms [3,4]. In the higher organisms, seven kinds of sHSPs have been reported so far, such as α A-crystallin, α B-crystallin, Hsp27, Hsp20, MKBP, sHsp and HspL27. Amino-acid sequences of α A- and α B-crystallins are similar to those of sHSPs of diverse organisms, including plants, bacteria, fruitflies, and mammals.

α -Crystallin in the vertebrate eye has a function that shows chaperone-like activity to the aggregation of unfolded proteins *in vitro* when it is induced by various kinds of stresses, such as heat, osmotic, UV-radiation, arsenate or oxidative stress [5–8]. Crystallin protein in the human eye lens is a hetero-oligomer composed of the water-soluble α -, β - and γ -crystallin molecules, which has an average molecular mass of 800 kDa [9]. There are two α -crystallin isomers, α A- and α B-crystallin, each one characteristically found in specific organs [10,11]. Only in the eye lens do they form hetero-oligomers composed of α A- and α B-crystallins. Homology of amino acid sequences among α B-crystallin and Hsp25, two sHSPs from mouse, is approx. 40 % [12,13]. Expression of sHSP, including α -crystallin, confer cellular thermo-tolerance *in vivo* [14–21]. α B-crystallin is more vulnerable to heat-induced structural change than α A-crystallin [22] and shares approx. 60 % amino-acid-sequence homology

with that of α A-crystallin [23,26]. Despite the high homology, α A-crystallin is not stress-inducible and its presence in non-lenticular tissues is rather limited. Thermo-tolerance to heat-shock acquired by sHSPs is a very important reaction by which micro-organisms escape heat crisis and maintain their lives. However, experiments concerning cell survival in response to stresses such as cold-shock and extreme pH have not yet been performed in an *Escherichia coli* cell system.

α A-crystallin demonstrated more protective effects to stresses of staurosporine- or UVA-treatment than α B-crystallin in the lens epithelial cells [24]. However, the chaperone-like activity of α B-crystallin was greater than that of α A-crystallin when the substrate was alcohol dehydrogenase or insulin [25]. The apparent difference in the tissue specificity of α A- and α B-crystallins strongly suggests that these two proteins might have evolved to play distinct physiological roles. Therefore it is very important to understand how these differences correlate with the physico-chemical properties which are not dependent on tissue localization. Thus we used *E. coli* as a model system for humans, which is advantageous in experiments for extreme pH or cold-stress, since bacteria are generally more durable than mammalian cells.

MATERIALS AND METHODS

Culture and growth-rate measurement of *E. coli* cells

Human α A-crystallin and α B-crystallin expression vectors were kindly provided by Dr. Y. S. Sadakane (Biorecognition Chemistry Laboratory, Faculty of Pharmaceutical Sciences, Toyoma Medical

Abbreviations used: sHSPs, small heat-shock proteins; GST, glutathione S-transferase; IPTG, isopropyl 1-thio- β -D-galactoside; α A-crystallin cells, *E. coli* cells transformed with pET-11a harbouring human α A-crystallin gene; α B-crystallin cells, *E. coli* cells transformed with pET-11a harbouring human α B-crystallin gene; GST cells, *E. coli* cells transformed with pGEX-2T vector.

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and Pharmaceutical University, Toyama, Japan) [27] and Dr. A. I. Iwaki (Division of Disease Genes, Research Centre for Genetic Information, Medical Institute of Bioregulation, Kyushu University, Fukuoka, Japan) [28] respectively. These were reconstructed and inserted into pET-11a vector (Novagen) and transformed *E. coli*, BL21(DE3) competent cells (Novagen). *E. coli* cells transformed with either pET-11a harbouring human α A-crystallin gene (α A-crystallin cells) or human α B-crystallin gene (α B-crystallin cells) were grown on Luria-Bertani (LB) plates containing 100 μ g/ml ampicillin at 37 °C overnight. Each single colony containing the plasmid was inoculated and cultured overnight in liquid LB media with good aeration. To measure growth rates, 0.1 ml from 2 ml of overnight culture suspension was inoculated into 9.9 ml of fresh LB broth containing 100 μ g/ml ampicillin and was incubated at 37 °C. When the attenuation (*D*) of the cell suspension reached approx. 0.7 (at 600 nm), 10 μ l of 1 M IPTG (isopropyl 1-thio- β -D-galactoside; final concentration 1 mM) or the same volume of PBS was added and was cultured at 37 °C for 7 h. As controls, *E. coli* cells [BL21(DE3)] transformed with pET-11a vector alone (pET-11a cells) or pGEX-2T vector (Amersham Biociences) which expresses GST (glutathione S- transferase) protein (GST cells) were cultured in the same way as described above.

Solubility of expressed protein in *E. coli* cells

The α A- and α B-crystallin cells were cultured for 2 h at 37 °C after IPTG induction and the D_{600} was measured for each. Aliquots of the cell suspensions (1 ml) were centrifuged, washed with PBS, and then adjusted to $D_{600} = 0.5$ with PBS. Approximately equal numbers of α A- and α B-crystallin cells were centrifuged and resuspended in 100 μ l of Lysis buffer (20 mM Tris/HCl containing 1 mM 2-mercaptoethanol, 1 mM PMSF and 0.1 M NaCl, pH 8.0). The cells were disrupted by freeze–thawing twice and then passing through a 26-gauge syringe needle several times. To obtain the soluble fraction, the samples were centrifuged at 10^4 g for 5 min and the supernatants were boiled at 100 °C for 3 min for analysis by SDS/PAGE. Another analysis was performed for α A-crystallin cells to examine insoluble fractions. The cells were obtained in the same way and suspended in the Lysis buffer as described above, but were then sonicated at 4 °C (20 kHz, 5 min) in Laemmli Sample Buffer (containing 8 M urea) and then centrifuged at 10^4 g for 30 min at 4 °C. The supernatant was analysed by SDS/PAGE.

Cold-shock and pH stress

To analyse the survival of the transformed *E. coli* cells under cold-shock and extreme pHs, 10 ml of the transformed *E. coli* cells were incubated at 37 °C until D_{600} reached approx. 0.7. IPTG was then added to the cell suspension to a final concentration of 1 mM, or the same volume of PBS was added. The cell suspensions were chilled to 4 °C for 6 h, and then 1 ml of each suspension in the presence or absence of IPTG was treated as described above, and the soluble protein fractions were analysed by SDS/PAGE. For pH tolerance experiments, the transformed *E. coli* cells were grown in LB media, and 0.1 ml aliquots from 10 ml of cell suspension were inoculated into 9.9 ml of M9 minimal media which had been adjusted to pH 4.7 or 8.0 with sterile $\text{Na}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$ solutions respectively.

After inoculation, 10 ml of the M9 suspensions were incubated at 37 °C for 6 h in the presence or absence of IPTG respectively. Samples (1 ml) were taken at 2, 4 and 6 h and analysed by SDS/PAGE as described above.

Figure 1 Growth curves of transformed *E. coli* cells

Cells with (filled symbols) or without (open symbols) 1 mM IPTG are shown. *E. coli* cells were transformed as described in the Materials and methods section and are denoted as follows: α A-crystallin cells ($\circ$), α B-crystallin cells ($\square$), GST cells ($\triangle$), pET-11a cells ($\times$) in the absence of IPTG, and α A-crystallin ($\bullet$), α B-crystallin ($\blacksquare$), GST ($\blacktriangle$) in the presence of IPTG. Arrowheads indicate the time of IPTG induction.

Colony count of surviving *E. coli* cells under cold-shock and extreme pHs

The transformed *E. coli* cells were cultured for 72 h under stresses at each temperature or pH as described above, and 1 ml of cell suspension was plated on LB medium containing 1.2 % agar after dilution at 1×10^{-7} . The number of colonies was averaged among the three plates and the standard deviation was calculated.

Purification of over-expressed target proteins

α A- or α B-crystallin cells in 200 ml of LB medium were cultured and IPTG induction was performed as described above. Cells were harvested by centrifugation at 5400 g for 20 min using a Kubota 6930 centrifuge with an AG-2506 rotor. The harvested cells were resuspended in PBS, pH 7.4 (Nacalai Tesque, Kyoto, Japan), on ice. After two freeze–thaw cycles and sonication, the cell lysates were centrifuged at 23 700 g for 30 min. The extracted proteins were loaded on to a Superdex 200 HR 10/30 column (Amersham Biosciences). Elution was performed with PBS, and each 3 ml fraction was collected with monitoring at 280 nm.

RESULTS

Growth curves of *E. coli* cells in the presence or absence of IPTG

The cells were incubated at 37 °C and D_{600} was measured at the times indicated (Figure 1). In the absence of IPTG, the growth of α B-crystallin cells was as good as that with pET-11a cells and was apparently better than those with α A-crystallin and GST cells. Although the growth was inhibited by addition of IPTG, the degree of inhibition was not as severe as those with α A-crystallin and GST cells.

Solubilities of the expressed proteins

Small HSPs, including α -crystallin protein, are cytosolic and water-soluble and they are associated with cellular thermo-tolerance and viability under physiological stresses [14–21]. In the present study, we focused on the solubility of crystallin proteins in the cytosol of *E. coli* cells. In the presence of 1 mM IPTG, more α B-crystallin protein is expressed in a soluble form (Figure 2A, lane 2) when compared with α A-crystallin (Figure 2A, lane 1). In the absence of IPTG, a small amount of the two α -crystallins and

Figure 2 Expression and solubility of α A- and α B-crystallins and GST protein in the cytosol

Expression of two α -crystallins and GST in the presence (A) and absence (B) of 1 mM IPTG: α A-crystallin (lane 1), α B-crystallin (lane 2), GST (lane 3), pre-stained Bio-Rad SDS/PAGE standards markers (M). Arrows indicate α A-crystallin (20 kDa), α B-crystallin (20 kDa), and GST (26 kDa) in lanes 1, 2 and 3 respectively. (C) The insoluble fraction of α A-crystallin (right-hand side), purified as described in the Materials and methods section, is shown with a marker for α A-crystallin (20 kDa, left-hand side).

GST were expressed (Figure 2B). This 'leaky' gene expression without chemical inducer is frequently allowed even though the expression of the target protein is strongly regulated under the control of the T7 lac promoter in the pET expression system. When α A-crystallin expression was induced with 1 mM IPTG at 37 °C for 2 h, not much of the α A-crystallin was obtained by the physical disruption methods of freeze-thawing and passing the cells through a syringe. Therefore the cell sample was solubilized by sonication in the presence of 8 M urea, as described in the Materials and methods section, and most of the α A-crystallin protein was obtained in the insoluble fraction (Figure 2C, right).

Expression of the target proteins in the cytosol under cold-shock and extreme pH environments

α A- and α B-crystallin cells were cultured at 4 °C and 1 ml samples from 10 ml of cell suspension were examined by SDS/PAGE for soluble protein at 2, 4 and 6 h. As shown in Figures 3(A) and 3(B), very small quantities of α A-crystallin were expressed, whereas much more α B-crystallin was obtained at 4 °C. Small amounts of α A-crystallin were expressed at both pH 4.7 (Figure 3C) and pH 8.0 (Figure 3E), but large amounts of α B-crystallin were expressed at pH 8.0 (Figure 3F) and small amounts at pH 4.7 (D).

Survival of the transformed cells under cold-shock and extreme pHs

When cultured at 37 °C for 6 h, both the α A-crystallin and GST cells died, whereas the α B-crystallin cells survived (results not shown). When the cells were left at 4 °C for 6 h in the presence of 1 mM IPTG, α B-crystallin cells showed higher tolerance to the cold-shock than α A-crystallin and GST cells (Figure 4A). At extreme pHs (4.7 and 8.0), α B-crystallin cells showed sufficient survival, but no surviving cells were observed in the α A-crystallin and GST cells (Figures 4C and 4E). In the absence of IPTG, the α B-crystallin cells showed much higher tolerance than GST cells at pH 4.7 and pH 8.0 (Figures 4D and 4F), but this was not observed at 4 °C (Figure 4B), and they showed the same degree of tolerance as that of pET-11a cells at pH 8.0 (Figure 4F).

Figure 3 Expression of α A- and α B-crystallin protein in the soluble fractions from transformed *E. coli* cells under cold shock (4 °C) and extreme pHs (4.7 and 8.0)

The cells were treated as described in the Materials and methods section. Samples were taken at 2, 4 and 6 h (lanes 1, 2 and 3 of panels A–F respectively), and were analysed by SDS/PAGE. (A) α A-Crystallin samples at 4 °C. Lane 4 shows a purified α A-crystallin as a control. (B) α B-Crystallin samples at 4 °C. Lane 4 shows a purified α B-crystallin as a control. (C) α A-Crystallin sample at pH 4.7. (D) α B-Crystallin sample at pH 4.7. The arrowhead indicates an α B-crystallin. (E) α A-Crystallin sample at pH 8.0. (F) α B-Crystallin sample at pH 8.0.

Difference of amino acid sequences between α A- and α B-crystallin proteins

In order to investigate the chemical basis for the difference of solubility in the cytosol and enhanced survival of cells between α A- and α B-crystallins, we compared their amino acid sequences. Alignment of α A- and α B-crystallins is shown in Figure 5, and the number of hydrophobic residues in α B-crystallin is 75 out of a total number of 175 residues (43 %), whereas that of α A-crystallin is 67 out of 173 residues (39 %). A consensus α -crystallin domain that is shared in sHSPs [3,4] is underlined. Within the domain, the numbers of hydrophobic residues in α A- and α B-crystallins are almost same: 29 and 30 respectively.

DISCUSSION

Overproduction of foreign protein by induction in *E. coli* cells leads to an overloaded state in the cellular system, which results in deficiency of the essential proteins for the cells. By forming partially folded intermediates and misfolded proteins, which tend to aggregate themselves, the cells abolish their metabolic activities, and this leads to a lethal state. Furthermore, without induction, the cells express some target proteins as 'leak' and the expressed proteins burden the cells to some degree.

In our present study, as shown in Figure 2(B), target proteins were produced to some extent and growth of α A-crystallin cells was inhibited without IPTG, but that of α B-crystallin cells was not inhibited (Figure 1), possibly due to the effective chaperone activity of α B-crystallin protein. It can be considered that

Figure 4 Survival of transformed *E. coli* cells under cold shock and extreme pH environments

The transformed *E. coli* cells were incubated at 4 °C, or treated with extreme pHs as described in the Materials and methods section. (A) 4 °C, in the presence of IPTG; (B) 4 °C, in the absence of IPTG; (C) pH 4.7, in the presence of IPTG; (D) pH 4.7, in the absence of IPTG; (E) pH 8.0, in the presence of IPTG; (F) pH 8.0, in the absence of IPTG.

1	11	21	31	
MDVTIQHPWF	KRTLGPFY.P	SRLFDQFFGE	GLFEYDLLPF	-αA
MDIAIHHPWI	RRPFFPFHSP	SRLFDQFFGE	HLLESDLFPT	-αB
41	51	61	71	
LSSTISPYR	QSLFRTVLDS	GISEVSRDRD	KEVIFLDVKH	-αA
.STSLSPFYL	RPPSFRLAPS	WFDTLGLEMR	LEKDRESVNL	-αB
81	91	101	111	
ESPEDLTVKVO	DDEVEIHGK	HNERODDHGY	ISREFHRRYR	-αA
DVKHESPEELK	VKVLGDVIE	VHGKHEEROD	EHGEISREEH	-αB
121	131	141	151	
LPSNVDQSALS	CSLSDAGMLT	ECGPKIQTG	LDATHAERAI	-αA
RKYRIPADVDP	LTITSSLSDD	GVLTVNGPR	KQVSGPERTI	-αB
161	171			
PVSREEKPTS	..APSS	-αA		
PITREEKPAV	TAAPKK	-αB		

Figure 5 Sequence comparison of human αA- and αB-crystallins

In the sequence alignments of αA- and αB-crystallins, hydrophobic residues (in bold) and α-crystallin domains (underlined) are indicated. Alignment was performed using GENEWORKS software.

αB-crystallin cells were rescued by the chaperone activity under burdened state conditions by leaked foreign protein. GST protein is soluble, but has no chaperone activity and so could not rescue the host cells. The same degree of rescue in αB-crystallin cells to pET-11a cells must be due to the chaperone activity. These results are consistent with experiments for survival of cells under cold and extreme pH stresses. Unlike the case at 37 °C, GST cells at 4 °C were not so affected, because the metabolic level is lowered at this temperature (Figure 4B). When the target proteins were

induced with IPTG, αA-crystallin cells were rescued more efficiently than αB-crystallin cells at 4 °C, because expressed target proteins productions were limited at the temperature and chaperone activity of αA-crystallin was appropriately optimized in comparison with that of αB-crystallin. At extreme pHs, αB-crystallin cells showed fairly good survival with IPTG induction, in contrast with the αA-crystallin and GST cells. Thus the notable survival of the αB-crystallin cells may result from the excellent intracellular stability which occurs by the formation of a complex between unfolded protein and αB-crystallin, maintaining solubility of the complex in the cytosol under stresses. The representative roles of high-molecular-mass HSPs, such as Hsp100, Hsp90, Hsp70 and Hsp60, are to prevent protein aggregation and to assist protein folding *in vivo* [29], whereas sHSPs, including α-crystallin, have been shown to be associated with differentiation and multiplication of cells, and also with cellular thermo-tolerance [14–21]. sHSPs were not considered to be essential for the cell's activities, because cell growth was not affected by gene disruption experiments in *E. coli* and *Saccharomyces cerevisiae* cells [30]. However, the importance of sHSPs is now being recognized, because they show chaperone activity to unfolded proteins *in vitro*. In general, the critical molecular mass of foreign proteins in bacterial expression system is considered to be approx. 100 kDa. In spite of the high molecular mass of α-crystallin, which forms a large complex of 800 kDa [31], it is surprising that the solubility of IPTG-induced αB-crystallin is kept high in the cytosol (Figure 2A). It has been reported that a small heat shock protein stably binds heat-denatured model substrates and can maintain a substrate in a folding-competent state [32]. In spite of the high homology between αA- and αB-crystallins, solubility in the cytosol of *E. coli* cells is quite different, as shown in Figure 2. The difference is probably due to their different hydrophobicities. In general, the ratio of hydrophobic residues and the composition within proteins have significant effects on the tertiary structure. The hydrophobic residues are folded into the protein interior to avoid contact with water molecules, both *in vivo* and *in vitro*, and raise the solubility. Protein stability generally tends to decline by random hydrophobic bonds, especially when the number of hydrophobic residues is large. However, this general rule does not seem to fit to the chaperone proteins, because they preferentially bind to denatured and possibly exposed hydrophobic residues. This function is important, because digestion of α-crystallin by calpain is considered to be one of the causes of cataract in the human eye lens and loss of its chaperone activity induces aggregation of β- and γ-crystallins.

The elucidation of the crystal structure of MjHSP16.5, an sHSP from *Methanococcus jannaschii* which has an α-crystallin domain, revealed that the domain was important for oligomerization, and that the hydrophobic residues in the domain were involved in maintaining the tertiary structure [33]. Thermo-tolerance experiments performed in *E. coli* cells demonstrated that the α-crystallin domain alone did not confer thermo-tolerance [34].

Another related experiment showed that the α-crystallin domain alone is not sufficient for oligomerization and has no chaperone activity *in vitro* [35]. Mutational experiments of αB-crystallin reported that the phenylalanine-rich region located at the N-terminal of α-crystallin domain was necessary for chaperone activity for the process of self-oligomerization *in vitro* [36]. The number of hydrophobic residues in the phenylalanine-rich region of αB-crystallin is more than that of αA-crystallin, and they are considered to be responsible for its higher stability [30,34]. Another consideration on the difference in stabilities between αA- and αB-crystallin is the hydrophobicity at the

terminal part of α -crystallin [37]; the higher hydrophobicity of α B-crystallin may contribute to its ability as a molecular chaperone, which α B-crystallin can bind to denatured parts of proteins, such as exposed hydrophobic residues. Despite a lack of detailed mechanisms, our data are consistent with the results that the chaperone activity of the α B-crystallin is higher than that of the α A-crystallin *in vitro* [23,38].

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