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Chaperone activity of α-crystallins modulates intermediate filament assembly

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Intermediate filaments are generally regarded as one of the most insoluble and resilient cytoskeletal structures of eukaryotic cells. In extracts from the ocular lens, we noticed an unusually high level of vimentin in a soluble, non-filamentous form. Immunoprecipitation of this soluble vimentin resulted in the co-precipitation of α-crystallins. The α-crystallins are homologous to the small heat shock proteins (sHSPs) and have recently been identified as molecular chaperones, capable of preventing the heat-induced aggregation of proteins. We find that the α-crystallins dramatically inhibit the *in vitro* assembly of GFAP and vimentin in an ATP-independent manner. This inhibition is also independent of the phosphorylation state of the α-crystallin polypeptides and each one of the four polypeptides, either αA1-, αA2-, αB1- or αB2-crystallin, are equally effective in this inhibition. Furthermore, we show that α-crystallins can increase the soluble pool of GFAP when added to preformed filaments. Electron microscopy demonstrated that α-crystallin particles could bind to intermediate filaments in a regular fashion, the spacing coinciding with the molecular length of GFAP. This is the first report, as far as we are aware, of a chaperone being involved in intermediate filament assembly and implicates chaperones in the remodelling of intermediate filaments during development and cell differentiation.

Key words: α-crystallins/chaperone/GFAP/intermediate filaments/vimentin

Introduction

Intermediate filaments are ubiquitous cytoskeletal elements generally considered as one of the most insoluble and resilient structures of the eukaryotic cell. The discovery that several human inherited skin diseases are due to single point mutations in intermediate filaments, illustrates the consequence of a compromised intermediate filament network (for recent reviews see Coulombe, 1993; Liem, 1993). These studies also underline the importance of a correct cellular location of intermediate filaments and their integration into a functional array.

To date, over 45 different gene products have been found to constitute this multigene family. Currently, the intermediate filament protein family has been divided into six different protein types on the basis of sequence similarities and patterns of expression in tissues and cells. Intermediate filament protein expression is developmentally as well as differentially regulated and the characteristic patterns of expression provide a valuable catalogue of cell markers for use in medical diagnosis and in basic research (Osborn *et al.*, 1985; Quinlan *et al.*, 1985).

The major cell types of vertebrates, such as epithelia, mesenchyme, neurons, glia and muscle typically have one major intermediate filament protein type expressed in their fully differentiated state. For instance, neurons primarily express type IV intermediate filament proteins, namely the neurofilament proteins and internexin, whereas vimentin, a type III intermediate filament protein, is typically found in mesenchymal and mesenchymally derived tissues. Epithelia typically contain keratin intermediate filaments.

During the course of differentiation or development, the intermediate filament protein profile of a cell can change, sometimes involving the expression of completely different intermediate filament types. The mechanisms that enable the sorting of the respective filament systems have yet to be elucidated, but the existence of a soluble pool of intermediate filament subunits which is in dynamic equilibrium with the polymerized intermediate filaments in the cell (Söllner *et al.*, 1985) provides a framework for such investigations. Fluorescence energy transfer and fluorescence recovery after photobleaching (FRAP) studies confirm the dynamic nature of intermediate filaments, indicating that subunit exchange occurs along the whole filament length and is not restricted to the ends (reviewed in Stewart, 1993). Mechanisms that either alter the flux through the soluble pool or the equilibrium constant could elicit very rapid changes in the intermediate filament array of cells. An example of this is during mitosis where protein phosphorylation/dephosphorylation mechanisms operate to co-ordinate the disassembly and reassembly of the whole intermediate filament network (e.g. Eriksson *et al.*, 1992).

Dramatic rearrangements of intermediate filament arrays can also be induced experimentally either by heat shock, exposure to heavy metals or chemical poisons (Welch and Suhan, 1985; Collier and Schlesinger, 1986) and providing protein synthesis is not inhibited, this is a fully reversible event. These abuses are accompanied by the induction of the stress response proteins (Gething and Sambrook, 1992) of which there are several major classes, the stress-70, stress-90, chaperonin-60 and the small heat shock proteins (sHSPs). Of these, the role of the sHSPs is the most poorly defined. It is quite clear, however, that stress proteins are necessary for the successful recovery of the cells (e.g. Welch and Suhan, 1985)

The α-crystallins have been regarded as lens-specific proteins but the extra-lenticular expression of both αB- (Bhat and Nagineni, 1989; Dubin *et al.*, 1990; Iwaki *et al.*, 1990; Kato *et al.*, 1991b) and αA-crystallin (Kato *et al.*, 1991a; Srinivasan *et al.*, 1992) in a range of tissues suggests a general cellular function for these proteins. Sequence homology to the sHSPs (for a recent review see de Jong *et al.*, 1993), the recent discovery of the *in vitro* chaperone activity of α-crystallins (Horwitz, 1992) and the direct correlation between

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α-crystallin expression and thermotolerance (Klemenz *et al.*, 1991, 1993) in cells, confirms the fundamental importance of α-crystallins to the stress response of cells. Furthermore, the association of α-crystallins with various intermediate filament proteins in characteristic cellular inclusions in a range of human pathologies (e.g. Iwaki *et al.*, 1989; Kato *et al.*, 1992; Lowe *et al.*, 1992) has been well documented. These observations suggest that α-crystallins may function as chaperones of intermediate filament proteins.

Evidence for this arose during investigations in the ocular lens where α-crystallins are abundant and where several different intermediate filament protein types are expressed during development (Ramaekers *et al.*, 1982; Kasper and Viebahn, 1992), including two recently discovered lens fibre cell intermediate filament proteins called filensin (Gounari *et al.*, 1993; Remington, 1993) and CP49 (Hess *et al.*, 1993). Vimentin is selectively lost during lens fibre cell differentiation (Ellis *et al.*, 1984), whereas filensin and CP49 are maintained (FitzGerald and Gottlieb, 1989; Quinlan, 1991).

This report describes the association of αA- and αB-crystallins with intermediate filaments isolated from bovine lenses and identifies the specific association of αA- and αB-crystallin with soluble vimentin. The inhibition of the intermediate filament assembly *in vitro* by α-crystallins at sub-stoichiometric molar ratios is demonstrated as is their ability to increase the size of the soluble subunit pool when added to preformed intermediate filaments. Binding of α-crystallin complexes to preformed filaments is visualized by electron microscopy and can occur in a regular manner. The association of α-crystallins with intermediate filament aggregates in various human pathologies further suggests that these chaperone activities may be important steps in the response of cells to disease and other stresses. This is the first report of a chaperone being involved in the assembly of intermediate filaments and suggests another mechanism, other than direct post-translational modification of the filament proteins, which may be important in the observed remodelling of intermediate filament arrays during development and cell differentiation.

Results

Association of α-crystallins with a soluble pool of vimentin in extracts from the bovine lens

The ocular lens is an excellent source for the isolation of vimentin and the purification protocol (Geisler and Weber, 1981) takes advantage of the insoluble nature of vimentin intermediate filaments and their association with the lens fibre cell membranes. Figure 1a, however, shows that a sizable fraction of the vimentin remains in the 259 000 *g* supernatant of the lens extract (Figure 1a, track 2) and is thus normally discarded during the purification although this vimentin is assembly competent (R.A.Quinlan, unpublished observation). The identity of the vimentin in both the supernatant and the pellet fractions was confirmed by immunoblotting (Figure 1a, tracks 1′ and 2′). Qualitatively, the proportion of the vimentin in the soluble fraction in the lens is certainly more than the 0.05% estimated for vimentin in fibroblastic and muscle derived cell lines (Söllner *et al.*, 1985).

Vimentin was purified from the bovine lens membrane fraction essentially as described (Geisler and Weber, 1981) and assembled into filaments *in vitro*. The proportion of vimentin in the soluble and insoluble fractions after *in vitro*

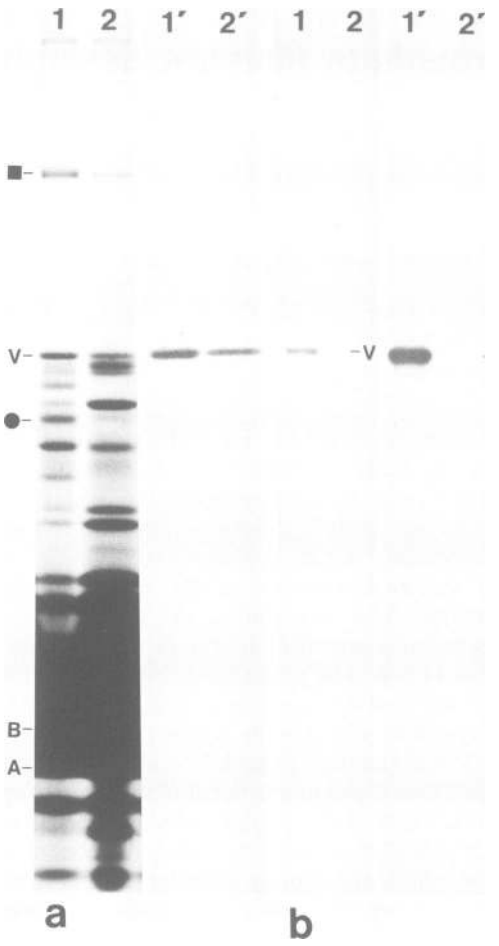


Fig. 1. The identification of the soluble pool of vimentin in the bovine lens. The pellets (tracks 1 and 1′) and supernatants (tracks 2 and 2′) from a 259 000 *g* supernatant of a bovine lens (**a**) extracted with an equal volume of 10 mM sodium phosphate pH 7.4, 100 mM KCl, 5 mM EDTA was compared with bovine vimentin purified from lens membranes reassembled *in vitro* (**b**) using SDS–PAGE (tracks 1 and 2) and subsequent immunoblotting with anti-vimentin antibodies (tracks 1′ and 2′). Note that the clone V9 antibodies to vimentin do not crossreact with lens crystallins including the αA- and αB-crystallins (track 1′). Vimentin (V), the 115 kDa lens fibre cell intermediate filament protein, filensin (closed square), the 49 kDa lens fibre cell intermediate filament protein, CP49 (closed circle), αB-crystallin (B) and αA-crystallin (A) are indicated (a, track 1). Note that the level of soluble vimentin seen in the total lens supernatant is significantly more than in the *in vitro* assembled lens vimentin preparation (compare panel a, tracks 2 and 2′ with panel b, tracks 2 and 2′).

assembly were determined (Figure 1b). The proportion of the vimentin remaining in the soluble fraction after the *in vitro* assay (Figure 1b, tracks 2 and 2′) was significantly less than that seen in the 259 000 *g* lens extract (Figure 1a, tracks 2 and 2′). A more efficient assembly process is therefore restored once the vimentin had been purified from the lens.

It was possible that vimentin could be associating with other lens proteins and that this was affecting the equilibrium of vimentin filament assembly and apparently elevating the soluble pool of vimentin in lens extracts. To investigate this possibility, we immunoprecipitated vimentin from the 30 000 *g* (Figure 2A, track 2) and the 259 000 *g* (Figure 2B, track 1) soluble fractions using a commercially available mouse monoclonal antibody (clone V9) to vimentin. Analysis of the immunoprecipiate by SDS–PAGE (Figure 2) revealed that

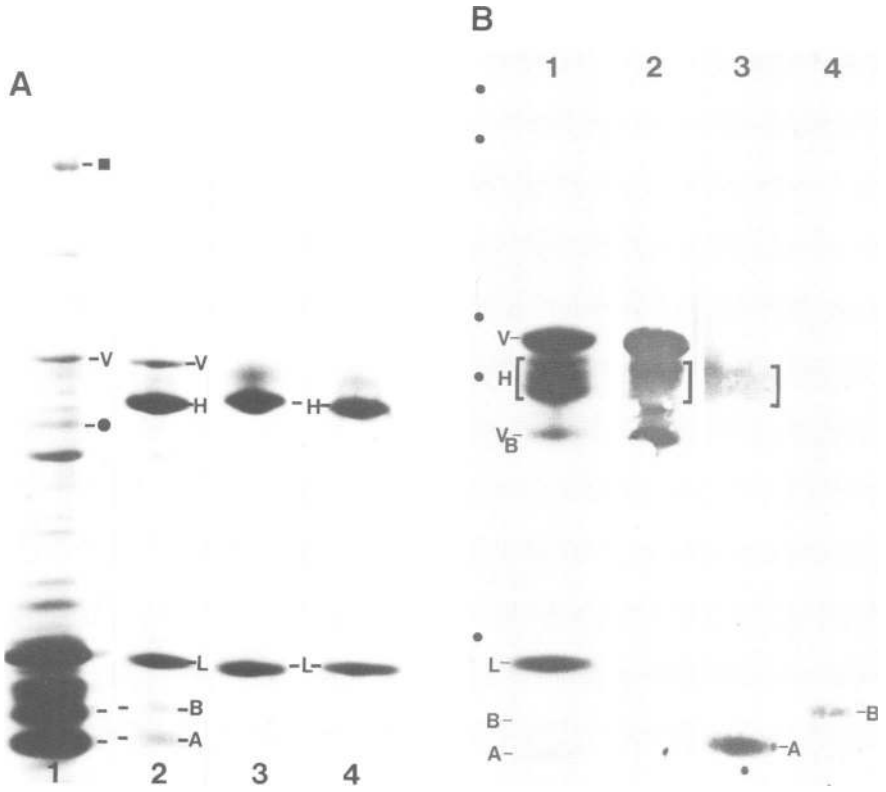


Fig. 2. Immunoprecipitation of vimentin from the soluble fractions of the lens. (**A**) 30 000 *g* supernatant after the extraction of lens membranes was analysed by SDS−PAGE (track 1) or used for immunoprecipitation with antibodies to vimentin (track 2), actin (track 3) or PCNA (track 4) prior to SDS−PAGE. These antibodies were all IgG1. Note only the vimentin antibodies immunoprecipitated proteins from the lens extract which had similar electrophoretic mobilities to vimentin (V) and the αA- (A) and αB-crystallins (B). The immunoglobulin heavy (H) and light (L) chains are indicated (tracks 2−4) In track 1, the horizontal bars indicate α-crystallins. Note that a band of similar mobility to filensin (track 2) is also just detectable by Coomassie blue staining in this immunoprecipitate. This is probably due to the entrapment of filamentous fragments in the immunoprecipitate which are not cleared from this fraction after centrifugation for 20 min at 30 000 *g*. Other symbols are as in Figure 1. (**B**) Western blot analysis of the proteins immunoprecipitated from the 259 000 *g* supernatant of a similar extract to that in panel a. The Coomassie blue stained immunoprecipitate from this high speed supernatant is shown in track 1 and here the only lens proteins to be detected are vimentin and its breakdown products along with αA- and αB-crystallin. Aliquots of the same were then analysed by Western blotting using anti-vimentin antibodies (track 2), monoclonal anti αA-crystallin (track 3) and rabbit polyclonal αB-crystallin (track 4) confirming the identity of αA- and αB-crystallin co-precipitating with vimentin. A major breakdown product of vimentin is indicated (V_B). Mouse immunoglobulin heavy chains of the vimentin monoclonal antibody are seen in tracks 2 and 3 (square brackets) because of the detection system used. Molecular weight markers are indicated (●) and are in order of increasing apparent mobility, β-galactosidase (116 kDa), phosphorylase b (97 kDa), pyruvate kinase (58 kDa), glutamic dehydrogenase (53 kDa) and bovine carbonic anhydrase (29 kDa).

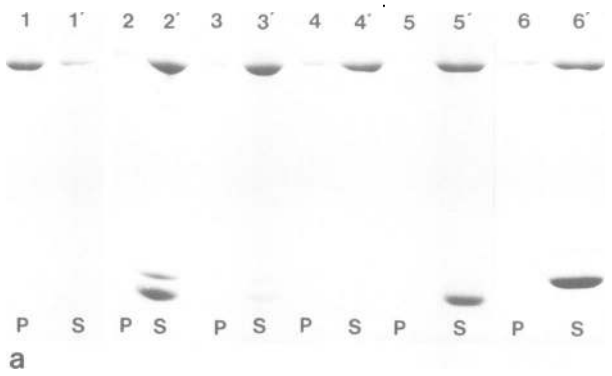
in addition to vimentin, two other low molecular weight proteins were co-precipitated (Figure 2A, track 2, 22 000 and 24 000 apparent molecular weights). These two proteins had a similar mobility on gradient SDS−polyacrylamide gels to lens αB- and αA-crystallins. This identity was confirmed using polyclonal and monospecific antibodies (Figure 2B, tracks 3 and 4). The lens is heavily enriched in α-crystallins (Figure 2A, track 1). Despite this abundance of αA- and αB-crystallin, immunoprecipitation experiments using other monoclonal antibodies of the same isotype as the vimentin monoclonal antibody, either to actin (Figure 2A, track 3) or to proliferating cell nuclear antigen (PCNA) (Figure 2A, track 4) produced no detectable immunoprecipitate and demonstrate that the association of vimentin with α-crystallins is specific.

The ratio of α-crystallins to vimentin in these immunoprecipitates is consistently sub-stoichiometric. There appears to be no preference for either αB- or αA-crystallin as the ratio of these proteins reflects the observed ratio of 1:3 moles respectively in the lens. Some variability in the ratio to vimentin is seen depending on the duration of the centrifugation conditions, the g-force used and the washing procedure after the immunoprecipitation step (compare Figure 2A, track 2 and Figure 2B, track 1). Size exclusion chromatography of the lens supernatant produced column fractions containing both vimentin and α-crystallins and no other lens proteins as detectable by Coomassie blue staining. αA- and αB-crystallins were co-precipitated with vimentin from these fractions (data not shown).

These results provide significant evidence for the association of vimentin with αA- and αB-crystallins in the lens. This association offers an explanation for the elevated soluble pool of vimentin, a suggestion which is amenable to experimentation *in vitro*.

The effect of αA- and αB-crystallin on in vitro assembly of GFAP

Previous studies on Alexander's disease, a neurodegenerative disease in humans, first revealed the association of αB-crystallin with filament aggregates of another type III intermediate filament protein called glial fibrillary acidic protein (GFAP; Iwaki *et al.*, 1989). This and the report that



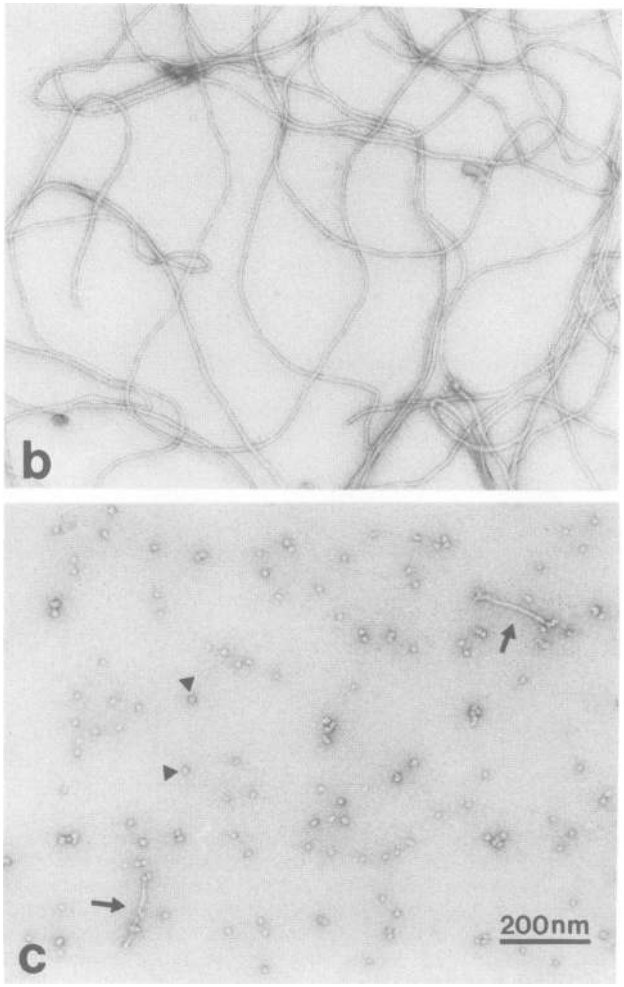


Fig. 3. Effect of αA-crystallin on the *in vitro* assembly of GFAP. (**a**) GFAP was assembled *in vitro* with additions of decreasing ratios of bovine α-crystallins (tracks 2−4′) or with αA_1- (tracks 5 and 5′) or αB_2-crystallin (tracks 6, 6′). The pellets (tracks 1−8, labelled P) and supernatants (tracks 1′−8′, labelled S) were analysed by SDS−PAGE. GFAP assembles efficiently into filaments under these *in vitro* conditions, driving most of the protein into the insoluble fraction (tracks 1, 1′). Addition of α-crystallins:GFAP on a mole ratio basis of 2:1 (tracks 2 and 2′), 0.4:1 (tracks 3 and 3′) or 0.1:1 (tracks 4 and 4′) inhibited the *in vitro* assembly. A similar result was obtained for αA_1- (tracks 5 and 5′) and αB_2-crystallin (tracks 6 and 6′) when added in a 2:1 molar ratio to GFAP. The inhibition is therefore independent of the α-crystallin polypeptide and the extent of α-crystallin post-translational modification. Negatively stained samples of a control GFAP assembly (**b**) and after assembly in the presence of α-crystallins (2:1 molar ratio of α-crystallins:GFAP) (**c**) confirm the sedimentation assay results that filament assembly is significantly inhibited. Note that some small filaments are seen (arrows) but the most abundant feature in panel c is the α-crystallin particles (arrowheads). Magnification in panels (b) and (c) was 60 000×.

desmin, a muscle-specific type III intermediate filament, assembled as filaments *in vitro* also binds αB-crystallin, albeit at acidic pH, (Bennardini *et al.*, 1992) suggests that the association of α-crystallins with vimentin is an example of the affinity of α-crystallins for the type III intermediate filament proteins in general. To test this suggestion, GFAP was selected for our detailed *in vitro* studies.

GFAP assembly *in vitro* was checked by sedimentation assay (Figure 3a) and electron microscopy (Figure 3b and c). Although a small proportion of the GFAP remained soluble (Figure 3a, tracks 1 and 1′), the GFAP assembled very efficiently (Figure 3b). Bovine α-crystallins were added to the GFAP assembly assay in 8 M urea buffer. The effect upon assembly was dramatic, almost completely inhibiting filament formation as determined by the sedimentation assay (Figure 3a, tracks 2−4′) and by electron microscopy (Figure 3c). Even over a 20-fold concentration range and at a level below detection by Coomassie staining of the SDS gel, α-crystallins are apparently a very effective inhibitor of the assembly process *in vitro*.

Immunoprecipitation experiments indicate that there is no preference for either αB- or αA-crystallin. Using either αA_1- or αB_2-crystallin, the non-phosphorylated and phosphorylated variants respectively of αA- and αB-crystallin, this inhibition was found to be independent of both the phosphorylation state and the particular α-crystallin component (Figure 3a, tracks 5−6′). Inclusion of ATP into the assembly assay did not appear to alter the effect. Similar results were obtained with purified vimentin, suggesting a general ability of α-crystallin to inhibit the assembly of type III intermediate filament proteins (data not shown).

In the *in vitro* assembly assay the conformation of the soluble GFAP apparently had little effect upon the inhibition as α-crystallins could be added to the assembly assay in 8 M urea, 4 M urea, 10 mM Tris−HCl pH 8.0 or 10 mM Tris−HCl pH 7.4, 1 mM $MgCl_2$, 25 mM 2-mercaptoethanol with similar effect (data not shown). This apparently contrasts other chaperone molecules which preferentially bind to refolding intermediates (Gething and Sambrook, 1992).

It is conceivable that the addition of any protein other than an intermediate filament protein to the assembly assay could inhibit GFAP assembly. In order to investigate this possibility, the effect of bovine carbonic anhydrase and bovine trypsin inhibitor upon GFAP assembly was analysed. Carbonic anhydrase is a normal component of the lens as well as most other tissues, including those expressing GFAP such as the Müller glia. Another reason for selecting these proteins was their ability to refold after denaturation (Creighton, 1977; Bergenhem and Carlsson, 1984) and because their difference in isoelectric points and tertiary structures would present a range of differentially charged interaction surfaces. Neither carbonic anhydrase nor trypsin inhibitor was able to inhibit the *in vitro* assembly of GFAP

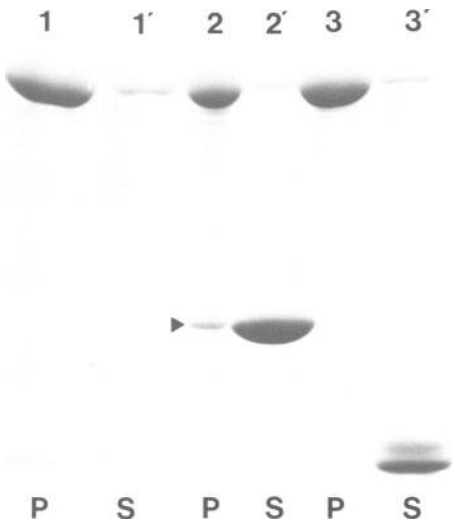


Fig. 4. Inhibition of the *in vitro* assembly of GFAP is specific to α-crystallins. GFAP was assembled *in vitro* (tracks 1 and 1') in the presence of carbonic anhydrase (tracks 2 and 2') and bovine trypsin inhibitor (tracks 3 and 3') in a mole ratio of 1:2 in both cases. The supernatants (S) and pellets (P) were then analysed by SDS−PAGE. Note the binding of carbonic anhydrase to the pelletable GFAP (track 2, arrowhead). Neither protein inhibited the assembly of GFAP.

as judged by the sedimentation assay (Figure 4) although carbonic anhydrase was able to bind to GFAP, albeit rather poorly and with no discernible effect (Figure 4, track 2).

Binding of α-crystallins to native lens intermediate filaments and to in vitro assembled filaments

Our *in vitro* studies confirmed the ability of isolated lens α-crystallins to inhibit type III intermediate filament protein assembly. We also investigated binding of α-crystallins to native and preformed intermediate filaments. The αA- and αB-crystallins are prominent components of bovine lens cytoskeleton preparations (Figure 5a, track 1). An intermediate filament rich fraction from the lens was also isolated (Quinlan, 1991; Quinlan *et al.*, 1992) and even though this was subsequently extracted with 0.5% v/v Triton X-100 and 1.5 M KCl, the α-crystallins remained associated with the intermediate filament fraction (Figure 5a, track 2). This fraction contains filensin and phakosin in addition to vimentin and the αA- or αB-crystallins. This result confirms previous immunoelectron microscopy (FitzGerald and Gottlieb, 1989; FitzGerald and Graham, 1991) and biochemical observations (Quinlan, 1991).

The addition of α-crystallins to preformed GFAP filaments assembled *in vitro* also resulted in a small fraction of the α-crystallins being recovered with the intermediate filaments in the sedimentation assay (Figure 5b, track 1). Significantly, the α-crystallins also increased the soluble pool of GFAP compared with the control assay where no α-crystallins were added (Figure 5b, compare tracks 2 and 4) indicative of an effect of α-crystallins on the assembly/disassembly equilibrium of *in vitro* filament assembly.

The binding of α-crystallins to preformed intermediate filaments was visualized by electron microscopy. Like the other stress proteins, α-crystallins also form large oligomeric particles with an estimated molecular weight of 800 kDa which were not pelleted under the conditions of the sedimentation assay. These are easily seen by electron microscopy (Figure 6b) as particles of 10−15 nm in

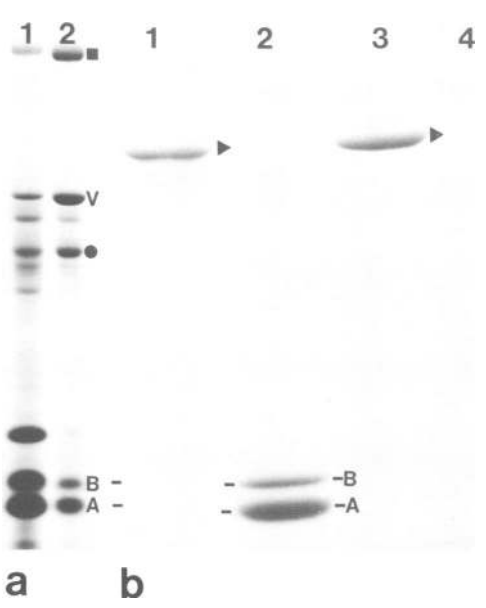


Fig. 5. Binding of α-crystallins to assembled intermediate filaments. (**a**) The bovine lens membrane fraction (track 1) and the intermediate filament-enriched fraction from the lens (track 2) were analysed by SDS−PAGE. Note that even after extraction of the intermediate filament-rich fraction with 1.5 M KCl and 0.5% v/v Triton X-100, αA- and αB-crystallins are still prominent components. (**b**) Purified α-crystallins were dialysed into 10 mM Tris−HCl pH 7.0, 50 mM NaCl, 25 mM 2-mercaptoethanol and then added in a mole:mole ratio of 2:1 to preassembled GFAP filaments at 37°C and left overnight. SDS−PAGE analysis of the pellet (track 1) and supernatant (track 2) showed an increase in the soluble GFAP in the supernatant when compared with the control (track 4). Some binding of α-crystallins to the pelletable material (track 1, bars) is seen when compared with the control (track 3).

diameter. Mixing α-crystallins with preformed GFAP filaments resulted in filaments that are decorated with such particles (Figure 6c). Higher magnification (Figure 6d) reveals that in some areas the association of the α-crystallin particles with the GFAP filaments appears to be regular, spaced every 50 nm. This value corresponds approximately to the length of one GFAP molecule in the filament (Quinlan *et al.*, 1989). The biochemical and electron microscopy results indicate that the *in vitro* binding only partially mimics the native complex of α-crystallins and lens intermediate filaments, strongly suggesting that other factors yet to be determined must contribute significantly to the binding.

Discussion

Role of α-crystallins in the assembly of vimentin and GFAP intermediate filaments

In this study we have demonstrated that there is an increased level of soluble vimentin in the lens, specifically associated with lens α-crystallins. The ability of α-crystallins to increase the soluble intermediate filament subunit pool is not restricted to vimentin but includes GFAP, another type III intermediate filament protein, as also shown in this study. Together, these results suggest that the assembly of vimentin and GFAP can be influenced by the chaperone activity of α-crystallins.

The *in vitro* assembly assays demonstrate the preferential association of α-crystallins for the intermediate filament subunits in the soluble fraction; however, *in vitro* some binding to assembled intermediate filaments was also detected (Figures 5b, 6c and d). This suggests that a docking

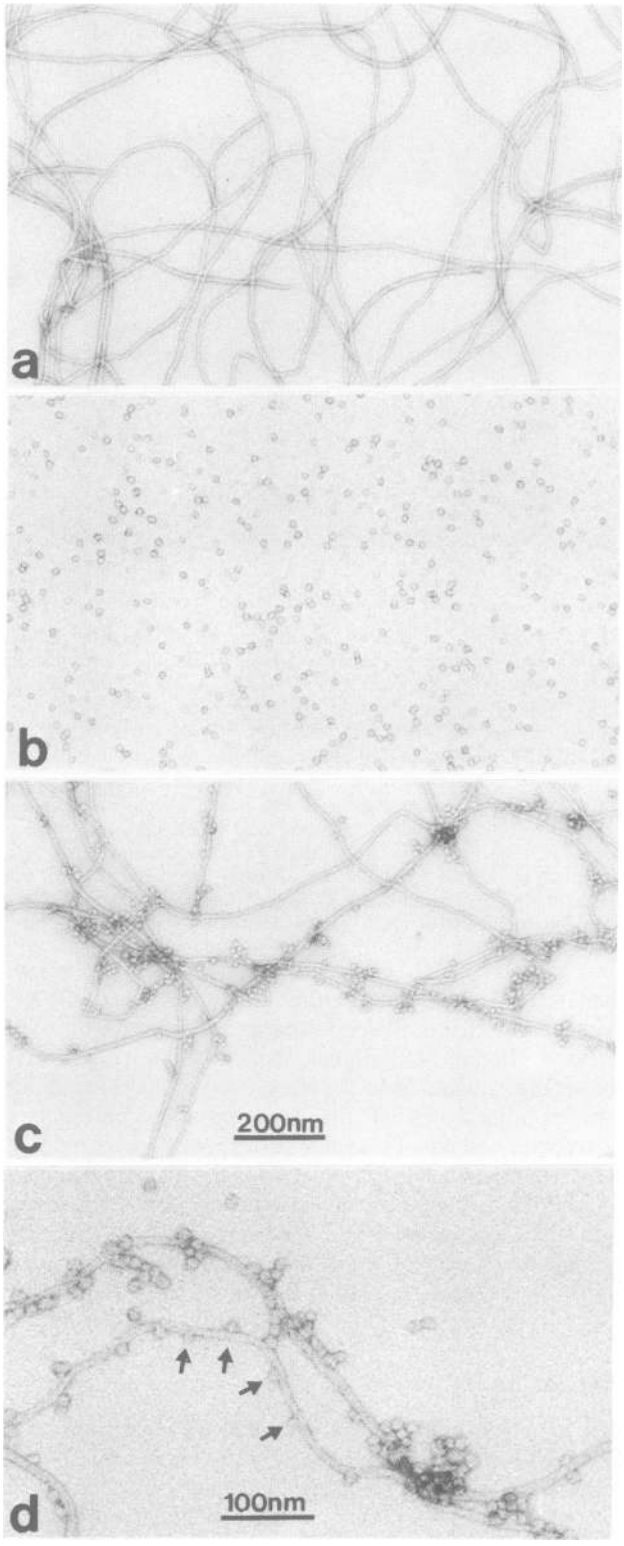


Fig. 6. Visualization of α-crystallin binding to GFAP filaments *in vitro* in negatively stained samples. *In vitro* assembled GFAP filaments (**a**), α-crystallin particles (**b**) and samples in which filaments and α-crystallin particles had been incubated together overnight (**c** and **d**) were viewed in the electron microscope by negative stain. These selected micrographs reveal that when mixed the α-crystallin particles associate with the GFAP filaments (c) which at higher magnification, is sometimes regular (d). Magnification in panels a–c is 60 000×. In panel (d) it is 120 000×.

motif for α-crystallins only becomes fully exposed when intermediate filament subunits dissociate from the filament. A low level of binding to assembled intermediate filaments would be consistent with this conclusion as not all of the protein monomers occupy the same geometrical position in the filament (e.g. Stewart, 1993) and some α-crystallin binding sites could still remain exposed. In some instances, the binding of α-crystallin particles to the *in vitro* assembled filaments appeared to be regular, occurring every 50 nm.

The high level of binding of α-crystallins to the insoluble intermediate filament fraction of the lens (Figure 5a) is in contrast to the low binding to GFAP filaments assembled *in vitro*. This appears to be specific to the lens as α-crystallins have so far not been recognized as major components of cytoskeletal preparations from other cells and tissues. In tissue culture cells prior to heat shock, αB-crystallin is to be found in the soluble fraction of the cell and is not apparently associated with the cytoskeleton. This would suggest that other factors, possibly the post-translational modification of lens intermediate filament proteins or the involvement of other proteins, contribute to the binding of α-crystallin. This awaits further investigation.

Our studies demonstrate that the interaction of α-crystallin with vimentin and GFAP is ATP-independent. This is not a unique feature for chaperones as HSP90 also does not require ATP for activity (Wiech *et al.*, 1992). It is interesting to note that HSP90 is one of the most abundant chaperones to be found constitutively expressed in cells. It is believed to be important in helping to maintain a subset of cellular proteins in an inactive form (Wiech *et al.*, 1992), similar to our observations on α-crystallins in the lens. HSP90 also recognizes only certain protein conformations and likewise α-crystallin activity is also sensitive to the intermediate filament protein conformation *in vitro* as it binds very efficiently to 'soluble' forms of intermediate filament proteins but not as well to preassembled filaments.

α-crystallins as intermediate filament protein chaperones in tissues other than the ocular lens

The observed level of αB-crystallin reported for soleus muscle, cardiac muscle, diaphragm and oesophagus in the rat (Kato *et al.*, 1991b) is well within the range of that which effectively inhibited GFAP assembly *in vitro*. Purification of αB-crystallin has been achieved from a variety of extra-lenticular tissues such as cardiac muscle (Longoni *et al.*, 1990; Bhat *et al.*, 1991), skeletal muscle (Atomi *et al.*, 1991; Kato *et al.*, 1992) and Rosenthal fibres (Iwaki *et al.*, 1989). As intermediate filaments are to be found in all vertebrate tissues, it is feasible that α-crystallins will also act as a chaperone for other intermediate filament protein types and not just the type III intermediate filament proteins. Indeed, the observed co-localization of αB-crystallin with keratin intermediate filaments (Lowe *et al.*, 1992) and with neurofilaments (Kato *et al.*, 1992) in specific human pathologies, strongly supports this suggestion.

Although the expression of αA- and αB-crystallin apparently do not overlap in tissues other than the lens (Kato *et al.*, 1991a; Srinivasan *et al.*, 1992), the association of these proteins is not a requirement of activity. The *in vitro* assembly of GFAP was equally affected by either one of the four protein variants of α-crystallin. The ability of αA- or αB-crystallin to form hybrid complexes with other sHSPs

such as HSP25 (Merck *et al.*, 1993) because of the high degree of conservation between these chaperone proteins (de Jong *et al.*, 1993), suggests that in those cells where these sHSPs are co-expressed, mixed complexes can exist (Kato *et al.*, 1992, 1993). The effect of this upon the activity of αA- or αB-crystallins is unknown.

A role for chaperone activities in intermediate filament protein exchange during development and cell differentiation

One of the proposed chaperone functions of α-crystallins in the lens is to elevate the pool of soluble vimentin. Overexpression of type III intermediate filament proteins, including vimentin, in the lens causes cataract in transgenic mice (reviewed in Bloemendal, 1991). One interpretation is that the overexpression and persistence of vimentin intermediate filaments in the more differentiated lens fibre cells is detrimental to cell function and contradicts the normal differentiation process where vimentin is apparently lost from the more mature lens fibre cells (Ellis *et al.*, 1984). The lens fibre cells express two differentiation-specific intermediate filament proteins filensin (Gounari *et al.*, 1993) and CP49 (Hess *et al.*, 1993) in addition to vimentin (Quinlan, 1991). In binding to vimentin and increasing the vimentin soluble pool, the proportion of vimentin in the lens intermediate filament population will be diminished providing a possible mechanism for the selective removal of the vimentin filament network.

The selective removal of specific intermediate filament proteins will be required at transition stages during differentiation and development. Recent observations on the network forming abilities of intermediate filament proteins in fully differentiated cell types indicate that a preformed filament array is necessary for the successful incorporation and assembly of the differentiation-specific complement of intermediate filament proteins. This concept is of considerable importance during neurogenesis (see discussion and references in Liem, 1993) and during terminal differentiation in the epidermis (Kartasova *et al.*, 1993). Protein chaperones provide a very attractive mechanism for the selective removal of one intermediate filament protein type because of the conformation and motif-specific requirements observed in general for chaperone activities (Gething and Sambrook, 1992).

The widespread expression of α-crystallins in tissues other than the lens suggests that the chaperone activities of α-crystallins are of more general importance. During development and at particular stages of cell differentiation the levels of α-crystallins increase, indicating that these stages are more vulnerable to non-specific protein−protein interactions due to the extensive remodelling of the cytoskeleton that accompanies these changes. For instance, in the chick embryo, αB-crystallin is elevated in expression in those regions undergoing the most dramatic morphological reorganization (Scotting *et al.*, 1991). These observations support major and important role(s) for α-crystallins during development.

Recovery from stress- and disease-induced intermediate filament collapse requires α-crystallin

Heat shock or other stresses upon cells causes the intermediate filament network to collapse around the nucleus (e.g. Welch and Suhan, 1985; Collier and Schlesinger, 1986). This aggregation is reversible provided that protein synthesis is not inhibited implying a role for HSPs in the redistribution of the intermediate filaments throughout the cytoplasm of the recovering cells (Collier and Schlesinger, 1986). In cell lines in which αB-crystallin is abundant, there is a transient relocation of αB-crystallin from the detergent soluble to the detergent insoluble phase of the cell upon heat shock (Klemenz *et al.*, 1991; Voorter *et al.*, 1992; Kato *et al.*, 1993). This would be consistent with an active role for αB-crystallin in the subsequent redistribution of intermediate filaments.

Intermediate filaments can be collapsed independently of the induction of stress proteins in cells (Welch and Feramisco, 1985) and so initially appears inconsistent with this hypothesis. All the major classes of heat shock proteins (HSPs), including the sHSPs, were not induced when the intermediate filament, microtubule and actin networks were disrupted using either colchicine or cytochalasin E. Some have interpreted such data as an indication that αB-crystallin is generally not needed for, or involved in, the recovery of intermediate filament networks after collapse (Voorter *et al.*, 1992). This assumes an equivalence of the collapsed filaments after both colchicine treatment and heat shock which may not necessarily be the case as, even morphologically, the collapsed intermediate filament networks appear quite different after the two treatments (Collier and Schlesinger, 1986; Voorter *et al.*, 1992). Heat shock produces a more severe phenotype with very compact filament aggregates. Drug treatment does not invoke the increased expression of HSPs and this has led to the suggestion that the recovery pathways could differ in the two cases (Collier and Schlesinger, 1986). The results from this study show that it is important to characterize the soluble pool of intermediate filament proteins before the involvement of the constitutively expressed HSPs can be excluded from the recovery of cells after drug treatment.

Extreme examples of the stress-induced redistribution of intermediate filament networks exist in human disease pathologies where aggregates of αB-crystallin and intermediate filament proteins are diagnostic features (Iwaki *et al.*, 1989; Lowe *et al.*, 1992). From these observations, it is clear that the reorganization of intermediate filament proteins into aggregates is sufficient to induce the association of chaperones and in particular αB-crystallin as part of the physiological response to enable the future unscrambling of these aggregates.

Elevated levels of α-crystallin are not always associated with pathologies as certain tissues such as cardiac muscle, skeletal muscle, Henle's loop and medullary collecting duct of the kidney and the lens clearly have high concentrations of αB-crystallin (Iwaki *et al.*, 1990; Kato *et al.*, 1991b). These tissues all experience their own particular stresses resulting from mechanical or osmotic extremes for muscle and kidney respectively, or the continuous exposure to environmental stresses such as UV radiation in the case of the lens. We suggest that in these instances αB-crystallin will chaperone any cytoskeletal repair or change in these tissues resulting from the specific tissue function.

Remodelling of the cytoskeleton uses several chaperone activities

A disadvantage of *in vitro* studies on protein assembly is that some of the factors required for protein assembly *in vivo*

can be overlooked. This has certainly been the case for the cytoskeleton, where assembly of filament subunits *in vitro* is expected *a priori* as proof of biological activity. The discovery that both tubulin and actin require chaperonins (TCP-1) during the synthesis of nascent proteins to ensure correct protein folding has only recently been discovered (Gao *et al.*, 1992; Yaffe *et al.*, 1992). No such activity has yet been described for intermediate filaments, but would have important consequences for the control of intermediate filament assembly because of the possibility of co-translational incorporation of nascent protein chains into intermediate filaments *in vivo* (Isaacs *et al.*, 1989).

Like actin, two different classes of HSPs directly associate with intermediate filaments. HSC70 was characterized as an intermediate filament associated protein (Green and Liem, 1989) although the function of this interaction has not yet been determined. We have demonstrated that α-crystallin, a closely related sHSP, binds to vimentin in the soluble pool as well as to intermediate filaments of the lens, leading us to propose that the chaperone activity of α-crystallin is functionally important in the differentiation-specific remodelling of the intermediate filament network in lens fibre cells. In the case of actin, the similarity in the three dimensional structure of the N-terminal 44 kDa portion of the HSP70s to actin (Flaherty *et al.*, 1991) is very suggestive of a direct role in controlling the distribution of actin. HSP25, a sHSP, was isolated as an inhibitor of actin assembly (Miron *et al.*, 1991). In conclusion, it would appear that HSPs are implicated in the control of the assembly and the remodelling of the cytoskeleton.

Materials and methods

Preparation of lens fractions

Bovine eyes were obtained from the local abattoir within 1 h of slaughter and from animals that were no more than 2 years old. Eyes were transported on ice. Lenses were isolated, decapsulated and lens extracts and cytoskeletal fractions were prepared as described (Quinlan, 1991; Quinlan *et al.*, 1992).

Purification of vimentin, GFAP and α-crystallins

A Merck–Hitachi Biochromatography system was used for the rapid purification of the proteins used in this study. Prepacked ion exchange columns, Fractogel EMD-DEAE and COO^- 650S (Merck Ltd, Poole, Dorset, UK) were substituted for the media described in the published methods. Vimentin was purified essentially as described (Geisler and Weber, 1981). GFAP was purified from either porcine or bovine spinal cord as described (Inagaki *et al.*, 1990). Bovine lens α-crystallins were purified by size exclusion chromatography using Fractogel HW-55S material (Merck Ltd, Poole, Dorset, UK) and subfractionated into αA1-, αA2-, αB1- and αB2-crystallins as described (de Jong *et al.*, 1984). Column fractions were checked for homogeneity by SDS–PAGE. Protein concentrations were determined by the Bradford protein assay.

Immunoprecipitation and immunoblotting

Lenses were extracted for immunoprecipitation with 7.5 mM sodium phosphate buffer pH 7.4, 2 mM EGTA, 1 mM $MgCl_2$, 0.5 mM DTT, 2 mM leupeptin and E-64 ('extraction buffer') at a ratio of 4:1 (buffer vol:lens wet weight). Sometimes 100 mM KCl and/or 0.5% Triton X-100 were included in the extraction buffer. This first extract was centrifuged at 30 000 *g* for 20 min at 4°C; the pellet was Dounce homogenized and treated again with extraction buffer plus 0.1 M KCl, 5 mM EDTA (instead of EGTA), ± 0.5% Triton X-100 (v/v) for 1 h and centrifuged at 30 000 *g* for 20 min at 4°C. The 30 000 *g* pellet was extracted once more to give the third 30 000 *g* supernatant and was used for the immunoprecipitation experiments. This third supernatant was also centrifuged at 259 000 *g* for 2 h at 4°C for some experiments. Monoclonal antibodies (ascites) to either vimentin (clone V9; Osborn *et al.*, 1984) actin (clone KJ43A; Skalli *et al.*, 1986) or PCNA (PC-1; Waseem and Lane, 1990) were added to supernatants and incubated for 2 h at 4°C. Gammabind G-agarose beads (Merck-BDH, Poole, UK) were added (200 μl of a 25% suspension to 1 ml supernatant) and incubated for 2 h at 4°C. Beads with their bound immune complexes were gently pelleted in a microfuge, washed at least four times with 1 ml buffer: 7.5 mM sodium phosphate pH 7.4, 0.1 M KCl, 5 mM EDTA, 0.5 mM DTT and 0.5% v/v Triton X-100. Additional washes with 'Ripa buffer' (50 mM Tris–HCl pH 7.4, 150 mM NaCl, 1% v/v NP40, 0.5% v/v deoxycholate, 0.1% v/v SDS) were sometimes included prior to the immunoprecipitate being dried and boiled in Laemmli's sample buffer. The solubilized immunoprecipitates were analysed by immunoblotting as described (Quinlan *et al.*, 1992). Primary antibodies used were: mouse monoclonal (tissue culture supernatants) anti-vimentin antibodies V9 (Osborn *et al.*, 1984) and anti-αA-crystallin antibodies (FitzGerald and Graham, 1991); rabbit polyclonal anti α-crystallin (Merck *et al.*, 1993) and anti αB-crystallin (Kato *et al.*, 1991a) antibodies.

Intermediate filament assembly assay

Purified GFAP or vimentin fractions were first dialysed into 8 M urea, 20 mM Tris–HCl pH 8.0, 5 mM EDTA, 25 mM 2-mercaptoethanol at 0.5–1.0 mg/ml. Bovine trypsin inhibitor (T-0256; Sigma, UK), carbonic anhydrase (C-7025; Sigma, UK) and α-crystallins were added to GFAP in the same buffer at the ratios indicated in the text. Protein mixtures were dialysed against 4 M urea, 20 mM Tris–HCl pH 8.0, 2 mM EDTA and 25 mM 2-mercaptoethanol, then 10 mM Tris–HCl pH 8.0, 25 mM 2-mercaptoethanol, then 10 mM Tris–HCl pH 7.0, 25 mM 2-mercaptoethanol, and finally against 10 mM Tris–HCl pH 7.0, 25 mM 2-mercaptoethanol plus 50 mM NaCl. Sometimes 1 mM $MgCl_2$ was included and in some cases, the concentration of NaCl in the final buffer was increased to 100 mM. Dialysis was performed at room temperature until the last step which was carried out at 37°C. Dialysates were layered onto a 0.85 M sucrose cushion and centrifuged at 80 000 *g* for 0.5 h at 20°C in a Beckman TLA-55 rotor using a TL100 benchtop ultracentrifuge. The pellet was dissolved directly in Laemmli sample buffer and the protein remaining in a portion of the supernatant was precipitated (Wessel and Flügge, 1984). The precipitate from the supernatant was dissolved in Laemmli's sample buffer, in a volume which was proportional to the initial sample size. In this way the relative levels of the protein in the pellet and the supernatant fractions are directly comparable.

SDS–PAGE

One-dimensional SDS–PAGE was performed according to Laemmli (1970) except that gradient slab gels from 7.5 to 17.5% w/v were used as resolving gels and the concentrating gel was 3% w/v acrylamide.

Electron microscopy

Protein samples were diluted to 100–200 μg/ml and negatively stained using 1% w/v uranyl acetate. Grids were viewed in a Jeol 1200EX TEM, using an accelerating voltage of 80 kV.

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