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Identification and electron microscopic analysis of a chaperonin oligomer from *Neurospora crassa* mitochondria

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A 7-fold symmetric particle has been identified in *Neurospora crassa* which is most probably the mitochondrial chaperonin. The particle, about 12 nm in diameter, appears in preparations of cytochrome reductase, and is shown to contain a 60 kd protein which cross-reacts with anti-GroEL antibodies. Results of STEM mass measurement suggest that the particle is composed of 14 subunits. A preliminary interpretation of the structure of the particle based on electron microscopy is given. Its quaternary structure and molecular weight are similar to those of the recently discovered family of particles called chaperonins, found in bacteria, chloroplasts and mitochondria.

Key words: chaperonin/structure/mitochondria/STEM mass-measurement

Introduction

Seven-fold rotational symmetry is rarely found in nature. We were therefore interested to observe a regular particle which exhibited this unusual symmetry, produced as a by-product of our crystallizations of the membrane protein cytochrome reductase from the mitochondria of *Neurospora crassa*. We initially assumed that these particles were breakdown products, composed of one or more subunits of cytochrome reductase. However,it is now clear that they are composed of another protein which co-isolates with it. The particles, on the evidence of symmetry, quaternary structure and molecular weight, are most probably a member of the family of proteins, termed 'chaperonins', which are thought to play an important role in the recovery of cells after heat shock, and may in addition be involved in the assembly of multi-subunit proteins in normal cells (Ellis, 1987).

The first chaperonin particle to be purified and characterized was bacterial groEL which is required for the assembly of bacteriophages λ and T4 (Hobin *et al.*, 1979; Hendrix, 1979). Analogues of bacterial groEL have now also been found in chloroplasts and mitochondria, Hemmingsen *et al.* (1988) showed that the amino acid sequence of groEL has 46% identity with a 60 kd chloroplast protein, 'RuBisCO large subunit binding protein', thought to be involved in the assembly of RuBisCO (ribulose bisphosphate carboxylase). The similarity extends beyond the sequence identity to a similarity in quaternary structure; RuBisCO large subunit binding protein is thought to occur as an $\alpha_7\beta_7$ oligomer. McMullin and Hallberg (1987, 1988) have recently published electron micrographs of an oligomeric mitochondrial protein with a molecular weight of ~60 kd which reacts with groEL antibodies and which has identical 7-fold symmetric and striated rectangular projections to groEL and to the protein we are studying. They show that similar proteins occur in the mitochondria of *Tetrahymena thermophila, Saccharomyces cerevisiae*, maize and human tumour cells, and propose that they are universally present in all mitochondria. These proteins are now thought to be members of a family which assists the folding of polypeptide chains and their correct assembly into multi-subunit structures and hence are termed 'chaperonins' (Hemmingsen *et al.*, 1988).

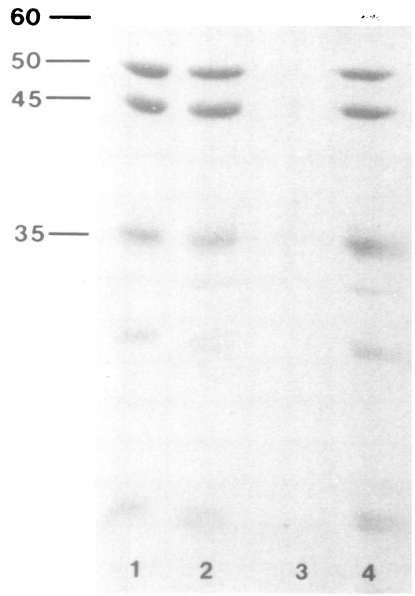

Fig. 1. Biochemical characterization of 7-fold particles. The results of SDS–PAGE on samples of (**a**) a complete cytochrome reductase preparation which does not yield 'stars'; (**2**) and (**4**) two different cytochrome reductase preparations which do yield stars under the conditions normally used for crystallization and (**3**) a preparation after crystallization which has been enriched in stars. The 60 kd band is present in the two preparations which give stars and absent in the preparation which does not. In the enriched sample the 60 kd band is present at approximately the same intensity as in the original preparation, while the other bands are considerably weaker.

Results

Biochemical characterization

Electron micrographs of cytochrome reductase membrane crystals prepared from Triton X-100 solubilized material frequently showed the presence of small regular particles ~12 nm in diameter with 7-fold symmetry in projection. In order to determine the subunit composition of these particles, we attempted to purify, or at least concentrate, the samples by removing the heavier crystalline cytochrome reductase vesicles by centrifugation. Separating them from lower molecular weight material proved to be more difficult but the final preparation as judged by electron microscopy was clearly enriched in 7-fold particles (see Figure 3).

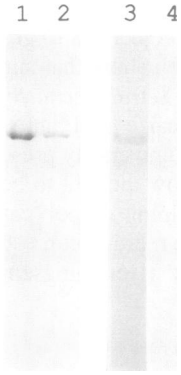

Fig. 2. Western blot anlaysis showing cross-reactivity with anti-GroEL antibody. (**1**) *Neurospora* 60 kd protein, (**2**) GroEL protein, both blots are stained with amido black; (**3**) *Neurospora* 60 kd protein incubated with anti-GroEL antibody, (**4**) control using non-specific antibody. In lanes 1, 3 and 4 the loading was 5 μg of Neurospora protein. In lane 2 the loading was 1 μg of GroEL.

The original and enriched samples were analysed by SDS−PAGE. We expected that gels would show that the particles were composed of one or more subunits of the cytochrome reductase molecule, most probably the largest (45 and 50 kd) subunits. As can be seen in Figure 1, the gel of the purified sample does indeed show bands corresponding to these subunits, but their intensities are weaker than that of a band corresponding to a protein of apparent molecular weight of 60 kd. This is higher than the molecular weights of any of the subunits of cytochrome reductase, and must therefore be due to a contaminant in the preparation. Integration of the gel peaks using a SEMPER routine written by Stanley and Pitt (1983) shows that, relative to the original sample, the staining intensities of the large subunit cytochrome reductase bands are reduced by a factor of ~18 in the enriched sample, while the intensity of the 60 kd is ~75% of its original value. It thus seemed that the protein component which was being enriched during the 'purification' was indeed this 60 kd contaminant.

The 7-fold particles were not present in every cytochrome reductase preparation—we were able to obtain further evidence for this conclusion by running gels of the different preparations. This showed that the observation of 7-fold particles in crystal preparations was directly correlated with the presence of the 60 kd band in the original protein. From these experiments we could conclude that the most likely constituent of the 7-fold particles was a 60 kd protein which could not be part of cytochrome reductase itself, but was nevertheless present as a contaminant in relatively large amounts.

The unusual 7-fold symmetry and a subunit molecular weight of ~60 kd is characteristic of so-called 'chaperonin'

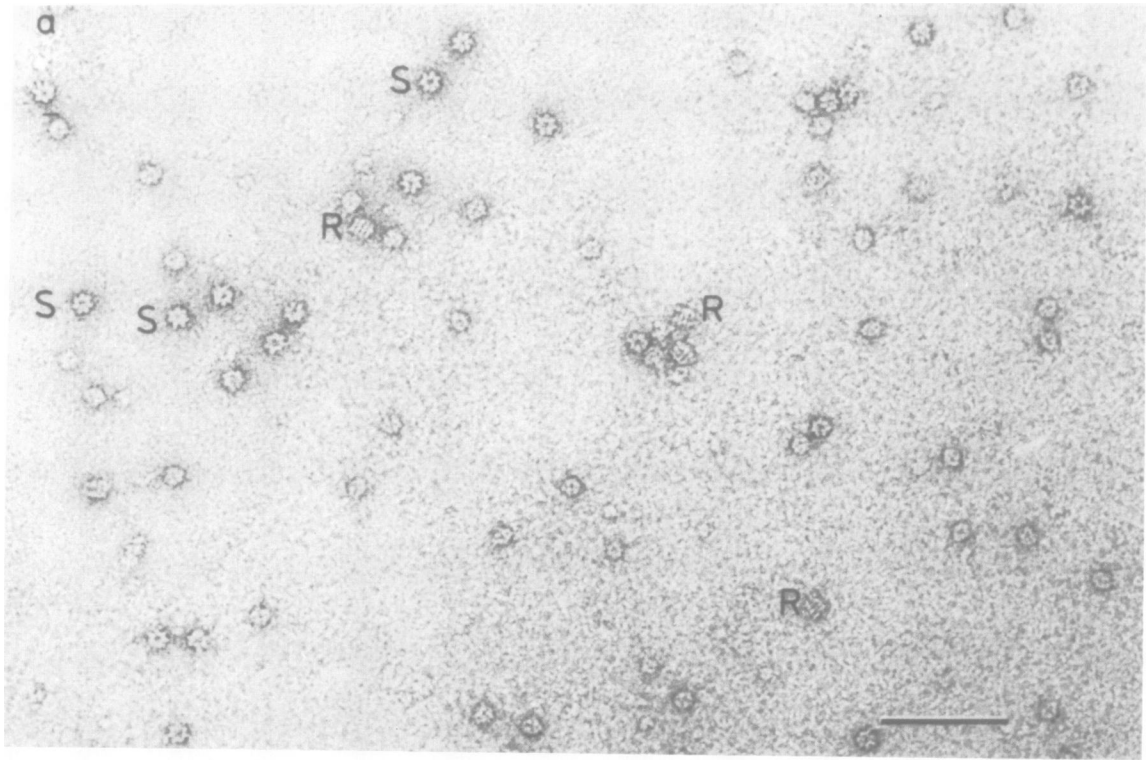

Fig. 3. Micrograph showing a field of the 7-fold particles negatively stained with 1% uranyl acetate. Particles with 7-fold rotational symmetry ('stars') and rectangular views are marked S and R respectively. The scale bar represents 100 nm.

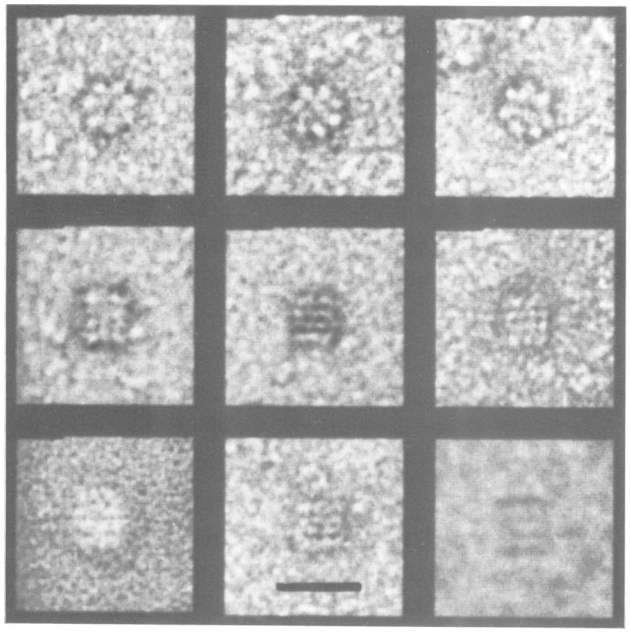

Fig. 4. Higher magnification 7-fold and rectangular particles. The top row shows three selected particles with well preserved structure. The middle and bottom rows show different rectangular views oriented with the striations horizontal. The left-hand image in the bottom row is a particle stained by the double carbon-sandwich technique which should lead to even staining around the particle. The right-hand image in the bottom row, which appears in reversed constrast, is a particle in vitreous water. This also shows the characteristic four-layered appearance. Scale bar is 20 nm.

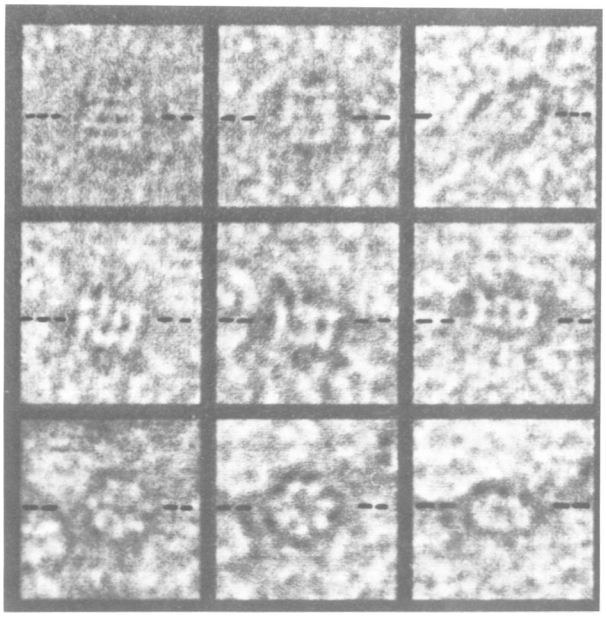

Fig. 5. Tilted view of particles negatively stained with uranyl acetate. Each row represents a single particle tilted through 0, 30 and 60° (left to right) about an axis which is approximately horizontal as shown. The image of a rectangular particle remains essentially unchanged (except for some distortion, probably caused by flattening) when tilted perpendicular to the striations (middle row). When a rectangular particle is tilted parallel to the striations, the striations disappear (top row) and the image has a similar appearance to that found on tilting the 'star' view (bottom row).

oligomers for which the best known example is the GroEL protein from *E.coli* (Ellis, 1987). Antigenicity to GroEL antibody was tested using affinity-purified polyclonal anti-groEL (Bochkareva *et al.*, 1988). The results of Western blot analysis (Figure 2) showed that the 60 kd mitochondrial protein cross-reacts with this antibody. A control using non-specific antibody was negative.

Electron microscopic analysis

A typical image of an enriched particle preparation, negatively stained with uranyl acetate, is shown in Figure 3. At least two distinct projections of the structure are visible. The most frequently observed view is a circular particle with a central hole and seven 'petals' protruding from it ('star view'). The image shown is typical, in that many of the particles look distorted, although at least ~7-fold rotational symmetry is evident in most of them. The second view, somewhat less frequently observed, is rectangular in appearance, with four equally spaced, approximately equally dense, transverse striations ('rectangular view'). Several other rounded views with less well defined structure may represent intermediate orientations. Selected higher magnification particles are shown in Figure 4. The rectangular views show considerable variation in detail from particle to particle, particularly in the density parallel to the striations. The interpretation of these two projections will be discussed in more detail later.

In order to investigate the possibility that the striated rectangular view is an artefact caused by asymmetric staining, we examined particles under different staining conditions. Striated views similar to those observed in specimens stained with uranyl acetate were also observed when the particles were stained with phosphotungstic acid (not shown), stained by the double carbon layer technique, and in frozen hydrated specimens (Figure 4).

We have also studied the effects of specimen tilting on the characteristic views of the chaperonin particles. Figure 5 shows examples of untilted particles and the same particles after tilting through 30 and 60°. The most clearly defined structure in the tilted images is that of the rectangular views which have been tilted about an axis perpendicular to the striations. These images retain their striated appearance on tilting to 60°, suggesting that the structure has approximate cylindrical symmetry along this tilt axis direction. When tilted about an axis parallel to the striations, they disappear completely, and the resulting projection is very similar to that observed on tilting the 7-fold views.

Rotational symmetry and dimensions

The apparent 7-fold symmetry of the 'star view' was confirmed by calculating the rotational symmetry. The results of this analysis for one particle are shown in Figure 6; all other particles analysed gave similar results. The correlation coefficient calculated for relative rotation angles $-\pi$ to π shows seven equally spaced peaks of approximately equal intensity. Thus, at least at the available resolution, the particles show 7-fold rotational symmetry. This was confirmed by imposing 6-, 7- and 8-fold symmetry on a selected particle. As can be seen from Figure 6b, only 7-fold averaging yields a particle with a clear structure which resembles the original particle.

The dimensions of the particles were estimated by calculation of projections across selected digitized images.

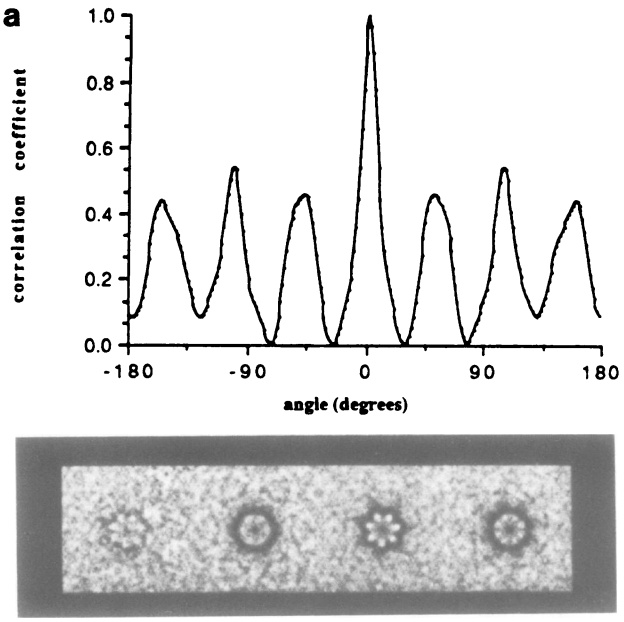

Fig. 6. Determination of the rotational symmetry of the particle. (**a**) Graph of the rotational correlation coefficient for the particle shown on the left in (b). (**b**) A selected particle and the same particle with 6-, 7- and 8-fold symmetry imposed (from left to right). The particle with 7-fold symmetry applied shows more clearly the structure of the original particle.

Table I. Dimensions of the 7-fold particles found in cytochrome reductase preparations and of a structurally similar protein from bacteria

	a(nm)	*b*(nm)	*c*(nm)
reductase particles	14.2 ± 1.1	11.3 ± 1.0	14.2 ± 0.9
bacterial groEL (i)	12.4 ± 0.8	10.2 ± 0.6	12.5 ± 0.5
bacterial groEL (ii)	13.4 ± 0.6	11.6 ± 0.6	12.9 ± 0.8

The measurements for bacterial groEL are the values reported in Hendrix (1979) (i) and Hohn *et al.* (1979) (ii).
a and *b* represent the dimensions of the rectangular view measured perpendicular and parallel to the striations. *c* represents the diameter of the 7-fold view.

The dimensions of the rectangular projections were measured to be 11.3 ± 1 by 14.2 ± 1.1 nm parallel and perpendicular to the striations (averaged data from 26 particles). The diameter of the circular projections was calculated to be 14.2 ± 0.9 nm (averaged data from 20 particles). The central hole of the stars is variable in diameter and, in some particles, totally absent. This may be due to variations in staining, or to slight tilting of the particles. A summary of the dimensions compared with published data for the similar bacterial groEL particles is given in Table I.

STEM mass measurement

We determined the mass of the particles by the well-established technique of STEM mass measurement using TMV as an internal standard (Freeman and Leonard, 1981). A typical dark-field image containing both unstained 7-fold particles and TMV is shown in Figure 7a. The distribution of mass initially found was bimodal, yet the mean mass for the principal, higher molecular weight peak was not a simple multiple of that corresponding to the lower peak, making it unlikely that the latter was due to breakdown of the

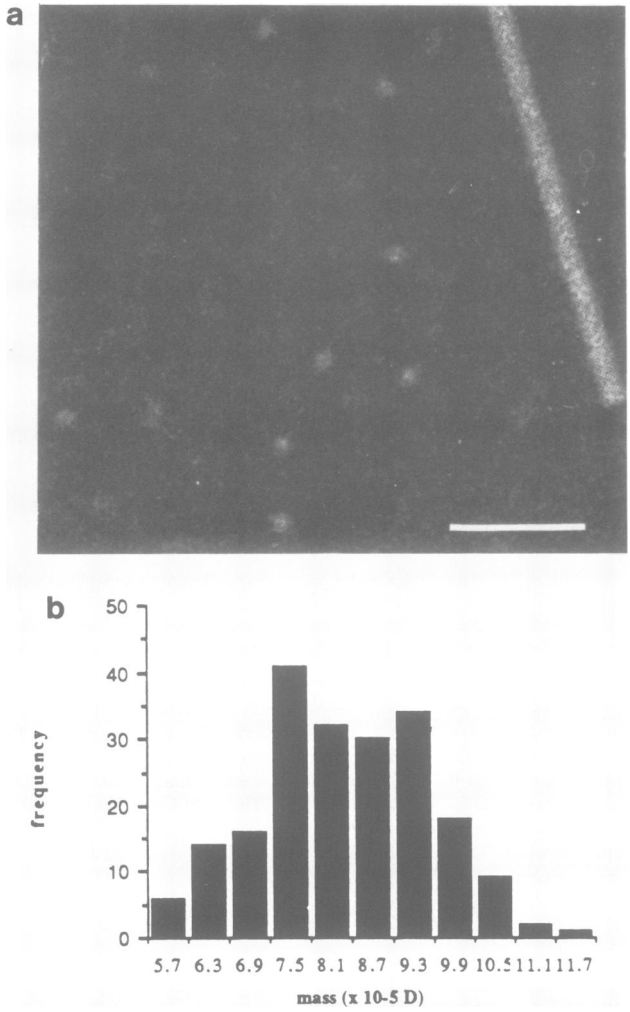

Fig. 7. STEM mass measurement. (**a**) A typical STEM dark-field image of an unstained specimen containing chaperonins and a TMV particle used as internal mass reference. The scale bar represents 100 nm. (**b**) Histogram showing mass distribution for 204 particles (see text). The mean value for the particle mass is 830 ± 130 kd.

particles. As was clear from negatively stained preparations, the sample used for STEM mass measurement was not completely pure and contained other small particles which were not chaperonins and which could have caused the wider distribution. If the lower molecular weight peak was excluded, the higher peak of the histogram, containing 204 out of the original 265 particles measured, had, by itself, a good standard deviation, giving a mean value of 830 ± 130 kd for the mass (Figure 7b).

Discussion

The biochemical and structural analyses of the particles we have isolated and their cross-reactivity with anti-GroEL antibodies makes it almost certain that the cytochrome reductase contaminant is a member of the 'chaperonin' family of heat inducible proteins of molecular weight of ~60 kd. This is the first time that a groEL-like protein has been found in *N.crassa*, although the recent work of McMullin and Hallberg (1987, 1988) makes it very likely that these proteins are to be found in almost every organism.

It is surprising that the chaperonin particles co-purify with

a detergent solubilized mitochondrial membrane fraction. GroEL and it analogues have been found as contaminants in a number of preparations of soluble proteins, including RNA polymerase (Hendrix, 1979), and glutamine synthetase (Burton and Eisenberg, 1980; Pushkin *et al.*, 1982). In these cases the contamination is most likely due to a combination of the similar molecular weights of the protein and contaminant and the fact that groEL is one of the most abundant bacterial proteins. The co-isolation of the *Neurospora* protein with cytochrome reductase may also result from its abundance in mitochondria, though other reasons cannot be excluded. If it were homologous to GroEL whose amino acid sequence contains a relatively high number of hydrophobic residues (7.5% leucine, 10.6% valine, 13.7% alanine), it may bind to the detergent. Another possibility is that there is a functional and stable interaction between the chaperonin and cytochrome reductase leading to their co-purification, but at the moment there is no experimental data to support this.

In order to understand the quaternary structure of these particles, we can make the assumption that the 'stars' and 'rectangles' are two mutually perpendicular projections of the same object. The simplest interpretation of the images would then be to assume that each particle is a cylinder with 7-fold rotational symmetry consisting of four layers, each layer corresponding to one of the four striations seen in the rectangular projection. If each layer is made up of seven subunits, there would be 28 subunits per particle which with the biochemically determined subunit molecular weight would give a total mass of 1680 kd. The STEM mass determination of 830 kd, however, supports the data of Hendrix (1979) and Hohn *et al.* (1979) who estimated the mass of the groEL particles by analytical ultracentrifugation to be ~700 kd, and concluded that they could consist only of 14 subunits. They proposed a model in which the 7-fold axis is parallel to the striations seen frequently in the rectangular view. They then explained the presence of four striations by assuming that the electron micrographs represent projections of 7-fold particles which have been preferentially stained on one side so that only one half (i.e. approximately four regions of density) can be seen.

Our results under several different conditions of staining and in vitreous water (Figure 4) and also the results of the tilting experiments (Figure 5) suggest, however, that one-sided staining is not a factor in producing the four striations seen in the rectangular view and that the 7-fold cylindrical axis is perpendicualr to them. If this were the case, then, even at this resolution, the particle as seen cannot be interpreted by any arrangement of 14 simple globular subunits. An alternative possibility would be that each monomeric unit is extended and made up of two roughly spherical domains which are separately resolved. The particle would then consist of two rings of seven subunits, each subunit consisting of two approximately equal sized domains of density.

In order to explore the possibility that the chaperonin subunit is made up of more than one domain, we studied the recently published amino acids sequence of the bacterial groEL protein (Hemmingsen *et al.*, 1988). The absence of any internal homologies excludes the possibility that the subunit protein is made up of two domains formed by gene duplication, although it could be made up of two unrelated domains. Support for the idea that there is a region within the sequence which constitutes a separate folding domain, is given by the strong sequence homology to a smaller protein required for the suppression of a temperature sensitive mutation in the *E.coli ams* gene (Chanda *et al.*, 1985). The mutation causes increased half-life of mRNA, which is lethal for the bacterium. The cloned gene codes for an amino acid sequence of ~17 kd of which a continuous stretch of 117 amino acids is, with the exception of two residues, identical to amino acids 307–424 of the groEL protein. The gene was originally assumed to be the *ams* gene itself; the recent paper by Hemmingsen *et al.* (1988) suggests that the cloned gene might actually be part of the groEL operon, though even this hypothesis does not completely explain all the data, in particular the 30 residues downstream of the homologous region which have little similarity to the groEL sequence. Although these points remain to be clarified, the ability of this fragment of the groEL protein to fold correctly and to function somehow as a destabilizer of mRNA might suggest that this region is a distinct structural domain.

We have recently obtained two-dimensional crystals of the chaperonin particles suitable for three-dimensional analysis by electron microscopy (Hofhaus *et al.*, in preparation). The 7-fold symmetry of the single particle also makes it a compelling object for analysis using methods such as those develped by Radermacher (1988) or Vogel and Provencher (1988). Clearly the most interesting aspect of this protein will be its function, which is, at present, only poorly understood. The relationship of function to the unusual quaternary structure may provide valuable insight into important cellular processes.

Materials and methods

Purification and gel analysis

The 7-fold particles appeared initially during crystallization of cytochrome reductase by the dialysis method at pH 5.5 (Hovmöller *et al.*, 1983). For biochemical analysis it was necessary to separate the particles both from larger and smaller material in the cytochrome reductase preparation. To remove the larger material, 100 μl of suspension, with an estimated protein concentration of 1 mg/ml were spun in a Beckman airfuge for 10 min at 100 000 *g*. This produced a pink precipitate which contained most of the crystalline and other large material. Grids of the supernatant were checked in the electron microscope, and contained little, if any large material. The supernatant was spun for a further 1 h at 100 000 *g* in the airfuge, and the pellet resuspended in 20 μl buffer (Tris–acetate, pH 5.5). Grids of the resuspended pellet contained substantial quantities of the 7-fold particles, though the lower molecular weight background material would not be completely eliminated by this method. We have now developed a method for purification in higher yield directly from *Neurospora* mitochondria (Hofhaus *et al.*, in preparation).

The biochemical compositions of the original and purified samples were determined by SDS–PAGE using the method of Laemmli (1970). Samples of purified and unpurified protein containing an estimated 2 μg of protein were run on microslab gels (Matsudaira and Burgess, 1978).

Electroblotting and immunostaining were performed by the method of Towbin *et al.* (1979) as modified by Burnette (1981). Samples were electrophoresed in microslab SDS–polyacrylamide gels and the proteins transferred to nitrocellulose sheets by electrophoresis in 10 mM Na-cyclohexylaminopropane sulphonate buffer (pH 11.0), 20% (v/v) methanol. Nitrocellulose strips were incubated for 1–2 h in the antibody. Blots were developed using horseradish peroxidase-conjugated goat anti-rabbit F(ab')2 (Jackson Immunoresearch Laboratories), and 4-chloro-1-naphthol (Sigma) and hydrogen peroxide as substrate (Hawkes *et al.*, 1982). Affinity purified anti-GroEL antibody was a gift of Dr A.S.Girshovich.

Electron microscopy

Negatively stained specimens were prepared by staining for 30 s using 1% uranyl acetate pH 5.5. or 1% sodium phosphotungstate pH 7.0 on glow discharged carbon-collodion grids. Specimens were also prepared by a double carbon sandwich technique using a modification of the Valentine technique

(see below) in which the floating carbon film was picked up on a carbon covered grid.

Frozen-hydrated specimens were prepared by the method of Dubochet *et al.* (1988). Specimens were observed in the microscope below the devitrification temperature (−130 to −140°C) under conditions of low electron dose.

Normal and tilted images were made in a Philips EM 400T electron microscope operating at 80 kv. Cryo-microscopy was carried out in a Philips EM 400 using a Gatan cold holder for transfer and observation of frozen hydrated specimens.

Image processing

Images selected for analysis were digitized on an Optronics rotating drum densitometer using either a 12.5 or 25 μm raster setting.

The rotational symmetry was determined using SEMPER (Saxton *et al.*, 1979). Well preserved particles were extracted from the densitometered images and centred. The symmetry was determined by calculating the correlation coefficient between a selected particle and the same particle rotated through an angle ψ such that $-\pi \leq \psi \leq \pi$. The correlation coefficient was calculated over a radius range which just included the complete particle. Having determined the symmetry in the above way, we imposed N-fold rotational symmetry by adding together images of a single particle rotated by $2\pi m/N$, $m = 1, N$ similar to the method used by Markham *et al.* (1963). The initial structure of the particle should be reinforced when the correct value of N is used, but, by itself, this method could potentially give misleading results and it was therefore only used to confirm the results obtained by the initial, more objective approach.

The dimensions of the particle were also calculated using SEMPER, both by calculation of the length of a line drawn from one edge of the particle to the other, and by projecting the particles, radially in the case of the stars and parallel to the axes of the rectangular views.

STEM mass measurement

The mass of the 7-fold particles was determined using STEM mass measurement with TMV as a standard. Unstained specimens were prepared using the Valentine technique (Valentine *et al.*, 1968). Thin carbon films were prepared by evaporation on to freshly cleaved mica. The films were floated off the mica on to a small volume of sample for 10 s, then on to a solution of the standard (TMV) for 10 s and finally washed briefly on water and picked up on a copper 600 mesh grid and left to dry. Dark-field images were obtained in a VG HB-5 STEM operating at 100 kV, magnification 83 000 ×, sample temperature −90°C and estimated electron dose 3 e/Å^2, conditions under which mass loss effects are negligible. Computer integration and background subtraction of stored images was carried out as described previously (Freeman and Leonard, 1981).

A total of 265 measurements of the relative mass were made and these were displayed as a histogram from which a value for the mass of the sample could be calculated.

Acknowledgements

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