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Transmission Electron Microscopy of GroEL, GroES, and the Symmetrical GroEL/ES Complex

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Two new 2-D crystal forms of the *Escherichia coli* chaperone GroEL (cpn60) 2 × 7-mer have been produced using the negative staining–carbon film (NS-CF) technique. These 2-D crystals, which contain the cylindrical GroEL in side-on and end-on orientations, both possess p21 symmetry, with two molecules in the respective unit cells. The crystallographically averaged images correlate well with those obtained by other authors from single particle analysis of GroEL and our own previous crystallographic analysis. 2-D crystallization of the smaller chaperone GroES (cpn10) 7-mer has also been achieved using the NS-CF technique. Crystallographically averaged images of GroES single particle images indicate considerable variation in molecular shape, which is most likely due to varying molecular orientation on the carbon support film. The quaternary structure of GroES does, nevertheless, approximate to a ring-like shape. The complex formed by GroEL and GroES in the presence of ATP at room temperature has been shown to possess a symmetrical hollow ellipsoidal conformation. This symmetrical complex forms in the presence of a 2:1 or greater molar ratio of GroES:GroEL. At lower molar ratios linear chains of GroEL form, apparently linked by GroES in a 1:1 manner, which provide supportive evidence for the ability of both ends of the GroEL cylinder to interact with GroES. The apparent discrepancy between our data and that of other groups who have described an asymmetrical "bullet-shaped" (holo-chaperone) GroEL/ES complex is discussed in detail.

INTRODUCTION

One of the most studied molecular chaperones is the *Escherichia coli* protein GroEL (Ellis, 1993; Hendricks and Hartl, 1993; Jaenicke, 1993), a cylinder-shaped protein containing a single 60-kDa subunit, which is arranged as two stacked rings each with sevenfold symmetry (Saibil and Wood, 1993; Zwickl *et al.*, 1990). GroEL has been shown to recognize the nonnative state of a multitude of proteins (Viitanen *et al.*, 1992), thereby preventing the irreversible aggregation of the substrate protein (Buchner *et al.*, 1991; Zahn and Plückthun, 1992). The complex between the chaperone and the substrate protein, in which complete tertiary and secondary structure is destabilized (Zahn *et al.*, 1994), dissociates in the presence of ATP (Jackson *et al.*, 1993) with the substrate protein now being able to fold into the native conformation (Laminet *et al.*, 1990; Martin *et al.*, 1991; Mendoza *et al.*, 1991; Zahn and Plückthun, 1992; Zeng *et al.*, 1993). The release reaction is accelerated by GroES, a smaller ring-shaped oligomer of a single 10-kDa subunit (Chandrasekhar *et al.*, 1986; Landry *et al.*, 1993). For some substrate proteins (Gray and Fersht, 1991, 1993; Schmidt and Buchner, 1992) a transient GroEL binding has been observed in the absence of ATP and GroES.

We have been concerned with the production of two-dimensional (2-D) crystals of GroEL for transmission electron microscopical (TEM) study and image processing (Zahn *et al.*, 1993). We have suggested that because of its diversion from the regular sevenfold rotational symmetry found in single particles, there may be considerable molecular plasticity, with subunit domain mobility. This is supported by the structural evidence of Saibil *et al.* (1993), from *R. spheroides* cpn60, that there is domain movement in the presence of ATP. An even more dramatic shape change occurs when GroEL interacts with GroES (Ishii *et al.*, 1992; Langer *et al.*, 1992; Saibil *et al.*, 1991, 1993), when in the presence of ADP or ATP. To date, there has been little electron microscopical work on GroES and although it is thought that this protein also possesses heptagonal symmetry, as suggested from biochemical titration studies, this is by no means directly apparent from the published electron micrographs (Chandrasekhar *et al.*, 1986). In view of the GroES subunit mass (10 kDa) and ring-like shape, the addition of one GroES to one GroEL oligomer (approximately the addition of one-twelfth of the GroES mass) might not be expected to generate such a pronounced shape change

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to the GroEL/ES complex. but, since this apparently occurs by the addition of a GroES ring to one end of the cylindrical GroEL, the resultant increase in mass was readily detectable electron microscopically as a curvature on one end of the cylinder, thereby generating an overall "bullet-like" shape (Ishii *et al.,* 1991, 1992; Langer *et al.,* 1992; Lissin *et al.,* 1992; Saibil *et al.,* 1991, 1993). Furthermore, Langer *et al.* (1992) showed that some conformational change also occurred at the opposite end of the GroEL to which the single GroES was apparently bound.

The concept that the GroEL oligomer is "cage-like" may not be particularly helpful structurally (Saibil *et al.,* 1993), in the knowledge that several other proteins possess a cylindrical quaternary structure (e.g., the 20S proteosome, the gastropod hemocyanin di-decamer, glutamine synthetase, invertebrate hemoglobins), except possibly for providing emphasis of the possibility that the actual functional role of GroEL in protein folding may occur within the central cavity (Braig *et al.,* 1993; Langer *et al.,* 1992), thereby preventing substate aggregation.

The production of 2-D protein crystals for electron microscopical study provides an alternative approach to single particle classification and image processing (e.g., Saibil *et al.,* 1993; Zwickl *et al.,* 1990). Often meaningful 2-D image averages can be obtained by crystallographic image processing, sometimes for a number of different specific protein orientations within such 2-D crystals (Cejka *et al.,* 1991; Harris, 1991; Harris and Holzenburgh, 1989; Harris *et al.,* 1992, 1993). This work has so far been performed using negatively stained 2-D protein crystals. Unfortunately, the associated problems of varying stain depth and protein–stain interaction can both greatly influence the images produced (Harris and Holzenburg, 1989; Harris and Horne, 1994; Harris *et al.,* 1993; Zahn *et al.,* 1993). Nevertheless, we have shown here that production of 2-D crystals by the negative staining–carbon film (NS-CF) procedure (Horne and Pasquali-Ronchetti, 1974; Harris, 1991) has enabled us to extend our preliminary structural investigations on GroEL (Zahn *et al.,* 1993) and indeed for the first time enabled 2-D crystals of GroES to be produced and analyzed, for comparison with images of randomly spread GroES molecules.

Although it might appear to be firmly established that the "bullet-shaped," asymmetrical GroEL/ES complex is the only complex which can be formed, there have been other reports of a more symmetrical or "football-shaped" form (G. Lorimer, personal communication; Todd *et al.,* 1993), although apparently these latter represent only a minor portion of the GroEL/ES complexes. In the present study we have demonstrated the formation of the *symmetrical* form of the GroEL/ES complex, when using a two or greater molar excess of GroES over GroEL in the presence of ATP (with or without the presence of KCl), under which conditions a similar conformational change is clearly demonstrated to occur at both ends of the GroEL molecule. The symmetrical GroEL/ES complex so formed can be structurally equated to a hollow ellipsoidal "football-shaped" body.

MATERIALS AND METHODS

Purification of GroEL and GroES

For the production of GroEL and GroES, *Escherichia coli* strain W3110 was freshly transformed with the plasmid pOF39 (Fayet *et al.,* 1989) and grown in a 50-liter fermenter (Bioengineering). The fermentation culture was inocculated with 1 liter of an overnight culture grown in the same LB medium (10 g/liter Bacto Trypton, 5 g/liter Bacto yeast extract, 5 g/liter NaCl) at 37°C containing 100 μg/ml ampicillin. After a growth period of 15 hr an OD_{550} of 14 was reached, the temperature was then reduced to 10°C and the cells (about 350 g) were harvested.

The following purification steps (Viitanen *et al.,* 1990) were carried out between 4 and 6°C. Cell paste (220 g) was thawed in 400 ml buffer A (100 m*M* Tris–HCL, 1 m*M* DTT, 0.1 m*M* EDTA, pH 8.1). DNase I and PMSF were added to a final concentration of 0.1 and 0.2 mg/ml, respectively. The cell suspension was then passed five times through a French Pressure Cell (Gaulin) and centrifuged at 13 700*g* for 1 hr. Ammonium sulfate (AS) precipitation of the supernatant was performed; the material precipitating between 40 and 70% saturation was collected. The pellet was dissolved in 500 ml buffer B (50 m*M* Tris–HCl, 1 m*M* DTT, 0.1 m*M* EDTA, pH 7.2) and dialyzed against the same buffer. The dialyzed protein solution was loaded onto a preequilibrated (buffer B) DEAE-Sephacel column (Pharmacia, 5 × 31 cm). After a washing step using 1.4 liters of buffer B, GroES and GroEL were eluted with a 0 to 500 m*M* NaCl gradient in buffer B (5 liters). GroES and GroEL eluted at 240 and 320 m*M* NaCl, respectively, and were then purified separately.

GroEL-containing fractions (420 ml) were precipitated with AS of 70% saturation and centrifuged (13 700*g,* 1 hr). The pellet was resuspended in buffer B (120 ml) and then loaded (10-ml aliquots) onto a preequilibrated (buffer B) Sephacryl S-300 column (Pharmacia 2.6 × 95 cm). GroEL was more than 98% homogeneous by SDS–PAGE at this stage and was stored after sterile filtration at 5 mg/ml at 4°C.

GroES-containing fractions (210 ml) were precipitated with AS at 70% saturation and centrifuged (13 700*g,* 1 hr). The pellet was resuspended in buffer C (5 m*M* potassium phosphate, 1 m*M* DTT, 0.1 m*M* EDTA, pH 7.6) and then loaded (75 ml) onto a preequilibrated (buffer C) DEAE-Sephacel column (Pharmacia, 3 × 21 cm). GroES was eluted with a 5–500 m*M* potassium phosphate gradient in buffer C (1 liter). GroES enriched fractions were collected between 225 and 265 m*M* potassium phosphate. After precipitation at 70% AS, resuspension, and dialysis against buffer B, GroES was loaded onto a Mono-Q column (Pharmacia, FPLC). GroES enriched fractions were eluted with a 0–500 m*M* NaCl gradient in buffer B, between 200 and 267 m*M* NaCl. After precipitation at 70% AS, resuspension, and dialysis against buffer B, GroES was loaded (2-ml aliquots) onto a preequilibrated (buffer B) Sephacryl S-300 column (Pharmacia, 2.6 × 95 cm). The GroES so obtained was more than 95% homogeneous by SDS–PAGE and was stored at 2 mg/ml at −20°C.

Preparation of GroEL/ES Complexes

Complexes of GroEL and GroES were prepared at room temperature using a 1.0 mg/ml solution of GroEL, diluted from the stock solution with 2 m*M* Tris–HCl buffer (pH 7.5), to which varying amounts of GroES were added. The molar ratios of GroEL to

GroES, based on 2 × 7-mer and 7-mer, respectively, and using the protein concentrations measured by quantitative amino acid analysis were determined. Experiments were performed using 1:1, 1:2, 1:4, and 1:10 molar ratios of GroEL:GroES. The GroEL–GroES solutions also contained 1 m*M* ATP, 2 m*M* $MgCl_2$, together with varying amounts of KCl (0, 1, 50, and 100 m*M*) in 5 m*M* Tris–HCl (pH 7.5). Aliquots were taken for EM specimen preparation at 15-min, 1-hr, 2-hr, 1-day, and 2-day intervals after mixing the GroEL and GroES.

Preparation of 2-D Crystals of GroEL and GroES

Ordered 2-D arrays and 2-D crystals of GroEL and GroES were prepared by the NS-CF procedure (Horne and Pasquali-Ronchetti, 1974), as applied to macromolecules (Ghiretti Magaldi *et al.,* 1985; Harris, 1982, 1991; Harris and Horne, 1991; Zahn *et al.,* 1993). GroEL concentrations were adjusted by dilution of the stock solution with 2 m*M* Tris–HCl (pH 8.0) to a protein concentration of 0.5 mg/ml; 10-μl quantities of GroEL were then mixed with 10 μl of 1% ammonium molybdate–0.2% polyethylene glycol (PEG) (M_r 1 500) at pH 8.0 and immediately spread on the surface of freshly cleaved mica. Protein crystallization occurred during slow air drying at room temperature. After coating *in vacuo* with a thin layer of carbon, the carbon film + adsorbed protein was floated onto the surface of 2% aqueous uranyl acetate. A number of specimen grids were then produced by bringing individual grids from beneath the floating film and removing the excess uranyl acetate with a filter paper. For GroES, an essentially identical procedure was adopted, but with a protein concentration of 0.1 and 0.2 mg/ml. In this case the ammonium molybdate–PEG solution had to be adjusted to pH 6.0 to promote crystallization.

Preparation of Negatively Stained Specimens

Negative staining was performed using the single droplet procedure (Harris and Horne, 1991). Carbon support films were briefly glow-discharged (20 sec) in a partial atmosphere to render them hydrophilic and adsorptive. GroEL, GroES, and GroEL/ES complexes were diluted to ca. 0.1 mg/ml with 2 m*M* Tris–HCl (pH 8) and applied to the carbon films. After 30 sec most of the protein solution was removed with a filter paper and replaced by a droplet of 2% uranyl acetate. After similarly removing the excess uranyl acetate, grids were dried at room temperature.

Electron Microscopy

Transmission electron microscopy was performed using the Philips EM400 and the Zeiss EM900 under conventional bright-field illumination conditions. Electron micrographs were recorded on Kodak type 4489 and AGFA Scientia 23D56 EM film, at instrumental magnifications between × 30 000 and × 105 000. Magnifications were calibrated using thin crystals of bovine liver catalase (Wrigley, 1968).

Image Processing

The crystallographic image processing system CRISP (Hovmöller, 1992) was used. 2-D crystal images were digitized using an ELMO CCD camera. Image areas of 516 × 516 pixels, containing more than 100 unit cells were processed for the fast Fourier transform. Manual and automated lattice indexing and refinement, symmetry determination based upon minimum phase residuals, and origin refinement were used, as provided by the CRISP PC-based image analysis system.

RESULTS

GroEL

It is well established that the cylindrical GroEL molecule possesses heptagonal rotational symmetry (Zwickl *et al.,* 1990). In common with all other cylindrical macromolecules it has a strong tendency to adopt two characteristic orientations on negatively stained specimens, whether these be produced by conventional negative staining or by the mica-spreading NS-CF procedure. Figure 1 shows a representative example of randomly spread GroEL molecules and small 2-D arrays of molecules, produced by the NS-CF procedure. The four-banded side-on images within the small 2-D arrays (small arrowheads, Fig. 1) immediately indicate that the nucleating molecules do not have a natural tendency to form absolutely straight lines end-to-end, although side-to-side the rows of molecules appear to be reasonably well in register. This is due to molecular misalignment, a feature already commented upon briefly by Zahn *et al.* (1993). The 2-D arrays of end-on molecules (large arrowheads, Fig. 1) indicate the possibility that larger and more highly ordered 2-D crystals might be produced with a similar molecular packing. It must be borne in mind that the side-to-side intermolecular interactions (Fig. 1) indicate this to be very specific and determined by the heptameric rotational symmetry of GroEL. Accordingly, this subtle interaction could also influence the arrangement of end-on molecules, for instance in the third dimension (c-direction) with respect to molecular tilt and/or height, with respect to the carbon support film.

The production of larger 2-D crystals of GroEL in the side-on orientation has proved to be technically possible (Zahn *et al.,* 1993), but most such crystals exhibit considerable disorder, due to lattice flexibility and slippage between rows of molecules (Fig. 2). Some such crystals do, however, possess sufficient structural order for image processing. Figure 3 shows such a 2-D crystal, which is lightly stained with uranyl acetate, together with the corresponding numerical transform and the crystallographically averaged image. The crystal symmetry is p21, with two slightly offset molecules comprising the unit cell. This feature is in immediate agreement with the 2-D crystal nucleation shown in Fig. 1 (small arrowheads). It is very likely that there is a variation in the axial rotation of the two molecules in the unit cell, together with negative stain depth variation and possibly height differences in the third (c) dimension. The unit cell parameters are a = 12.1 nm, b = 25.6 nm, and γ = 65.3°.

Several 2-D crystal forms containing the GroEL in the end-on orientation have been discussed in our previous communication (Zahn *et al.,* 1993). Data was then presented from three 2-D arrays, one of which was somewhat disordered and could not be considered a true crystal. The other two, which were true crystals, were of p2 and p4 symmetry, respectively. A significantly different 2-D crystal form, containing GroEL in the end-on orientation, was also frequently observed (Fig. 4). At first sight, it

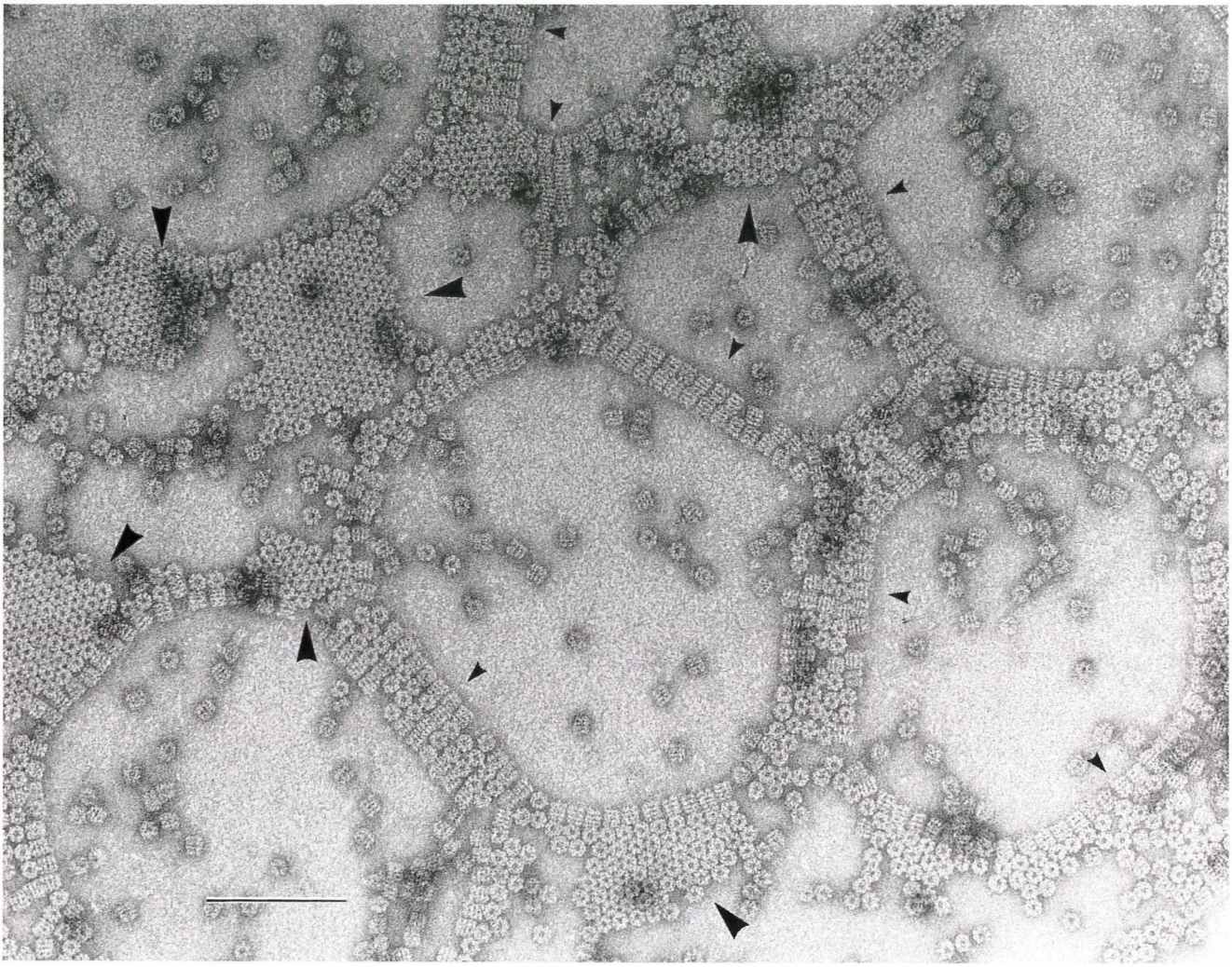

FIG. 1. A specimen of GroEL prepared by the NS-CF technique, exhibiting single molecules and small 2-D arrays. In both cases, the hollow cylindrical GroEL 2 × 7-mer is orientated side-on and end-on (small and large arrowheads, respectively). The side-on arrays show linear misalignment of molecules. Scale bar, 100 nm.

might appear that this crystal form possesses p1 symmetry, but on crystallographic analysis p21 symmetry is found, with unit cell parameters a = 12.8 nm, b = 22.6 nm, and γ = 89.2°. The small deviation from 90° is almost certainly due to crystal distortion produced during specimen preparation. The paired molecules comprising the unit cell are indicated by the alternating negative stain density in consecutive rows of molecules, a feature clearly obtained from the crystallographically averaged image (Fig. 4c, arrowheads). By careful inspection at an oblique angle, this crystal feature can be seen on the original image in Fig. 4a, where again the rows of paired molecules are indicated by arrowheads. This uneven negative staining of molecules closely adjacent to one another within a 2-D crystal lattice is likely to be an indication of slightly varying molecular tilt or height of alternate rows, in combination with the relatively shallow depth of the overall negative stain, as mentioned above. It appears that there is no deviation from the heptameric symmetry, i.e., molecular plasticity, present within this 2-D crystal form, in contrast to the 2-D crystals with p4 symmetry (Zahn *et al.,* 1993).

GroES

When conventionally negatively stained, the detailed structure of the relatively small 70-kDa GroES molecule is not readily defined. It has, however, routinely been found that the NS-CF technique does generate slightly superior specimens even with randomly dispersed molecules and regions containing 2-D crystals and disordered 2-D arrays. Figure 5a shows a region of randomly dispersed ring-like GroES molecules, and Fig. 5b shows dispersed molecules together with flexible rows of aggregated molecules. No clear "double-dot" images, characteristic of the side-on image generated from a

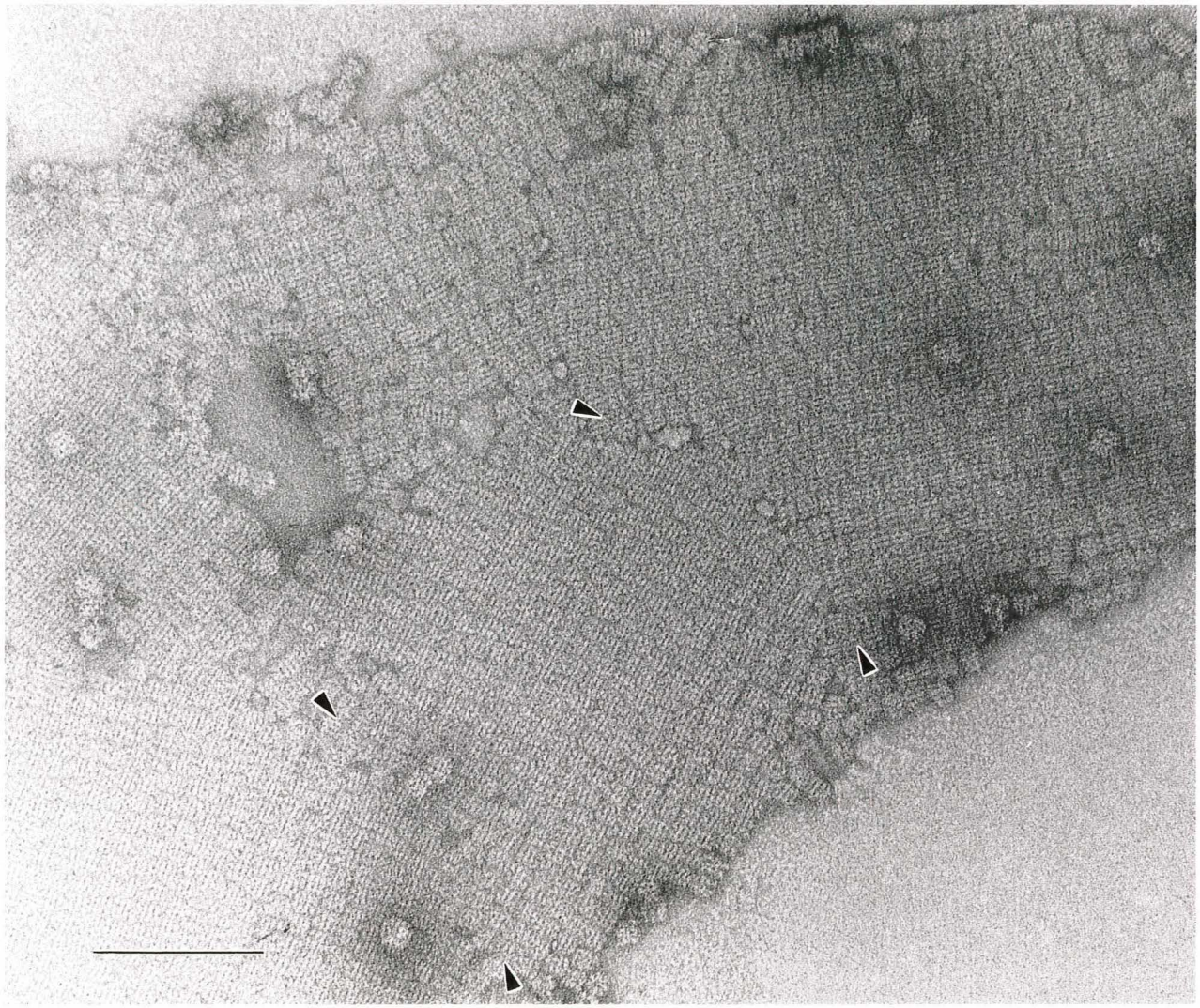

FIG. 2. 2-D crystals of GroEL in the side-on orientation exhibiting considerable lattice disorder, prepared by the NS-CF technique at pH 8.0. Lattice discontinuity is present in several places (between arrowheads), together with molecular slippage between the rows of molecules and a region of disorder. Scale bar, 100 nm.

ring-like protein (cf. the erythrocyte protein torin, Harris, 1969) have been detected. However, the penetration of negative stain into the centre of the flexible rows of molecules (arrowheads, Fig. 5b) is in agreement with this side-on image principle.

2-D crystals of GroES, produced by the NS-CF procedure, have been readily detected, if the protein was diluted to ca. 0.1 mg/ml to avoid overloading with protein and molecular superimposition. Representative examples of two GroES 2-D crystals are shown in Figs. 6a and 6b, each with the crystallographically averaged image included as an insert. The 2-D crystals of GroES formed to date contain considerable lattice disorder, and the power spectra contain only the second diffraction orders (resolution ca. 2.5 nm). The unit cell for the 2-D crystal in Fig. 6a appears to contain two molecules, whereas that in Fig. 6b contains one molecule. Neither of the averaged images (external dimension ca. 7.5 nm) shows a pronounced heptagonal rotational symmetry. Nevertheless, it is reasonably certain that the molecules are not hexagonal, as almost all published electron microscopical examples of hexagonal macromolecules create 2-D crystals with a very clear p6 symmetry (Cejka *et al.*, 1991; Peters *et al.*, 1992), but sevenfold symmetry remains a possibility for GroES. From our data it would appear that GroES does not possess a simple ring-like quaternary structure and that the seven 10-kDa subunits may be grouped in a more complex or flexible manner.

The GroEL/ES Complex

(i) GroEL/ES complexes prepared at room temperature with a 1:1 molar ratio of GroEL:GroES, in the

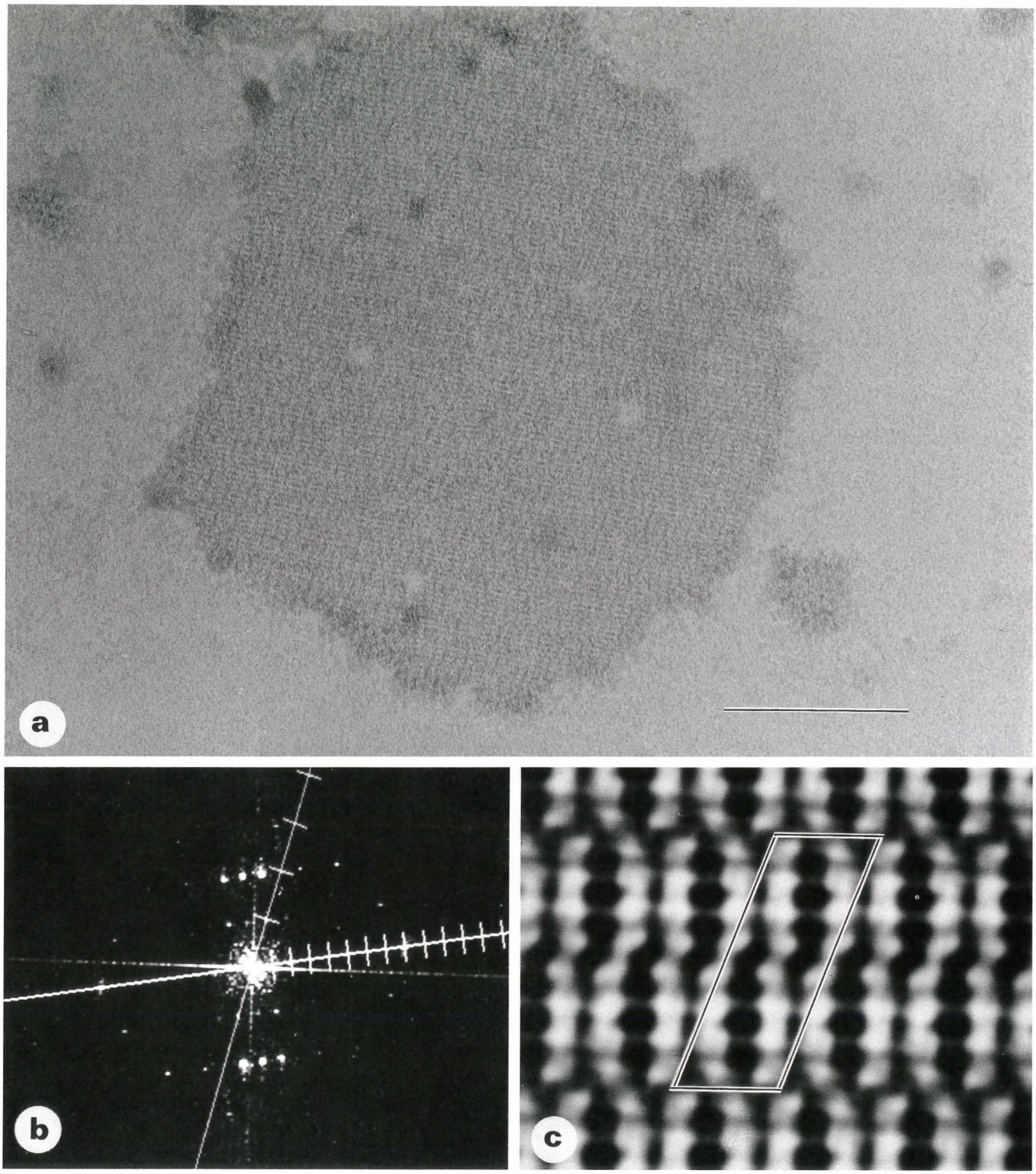

FIG. 3. A highly ordered 2-D crystal of GroEL orientated side-on, prepared by the NS-CF procedure at pH 8.0 (a). Insets show the indexed numerical transform/power spectrum (b) and crystallographic average (c) with the unit cell outlined. The unit cell parameters are a = 12.1 nm, b = 25.6 nm, and γ = 68.3°. Scale bar (a), 100 nm.

presence of 1 m*M* ATP, with or without the presence of KCl (see discussion), have been found to exhibit a symmetrical ellipsoidal shape, but some asymmetrical "bullet-shaped" complexes were present at short incubation times, together with short chains of GroEL molecules (Fig. 7a). Samples prepared after 15 min incubation did reveal the presence of some unreacted GroES and GroEL, but at longer incuba-

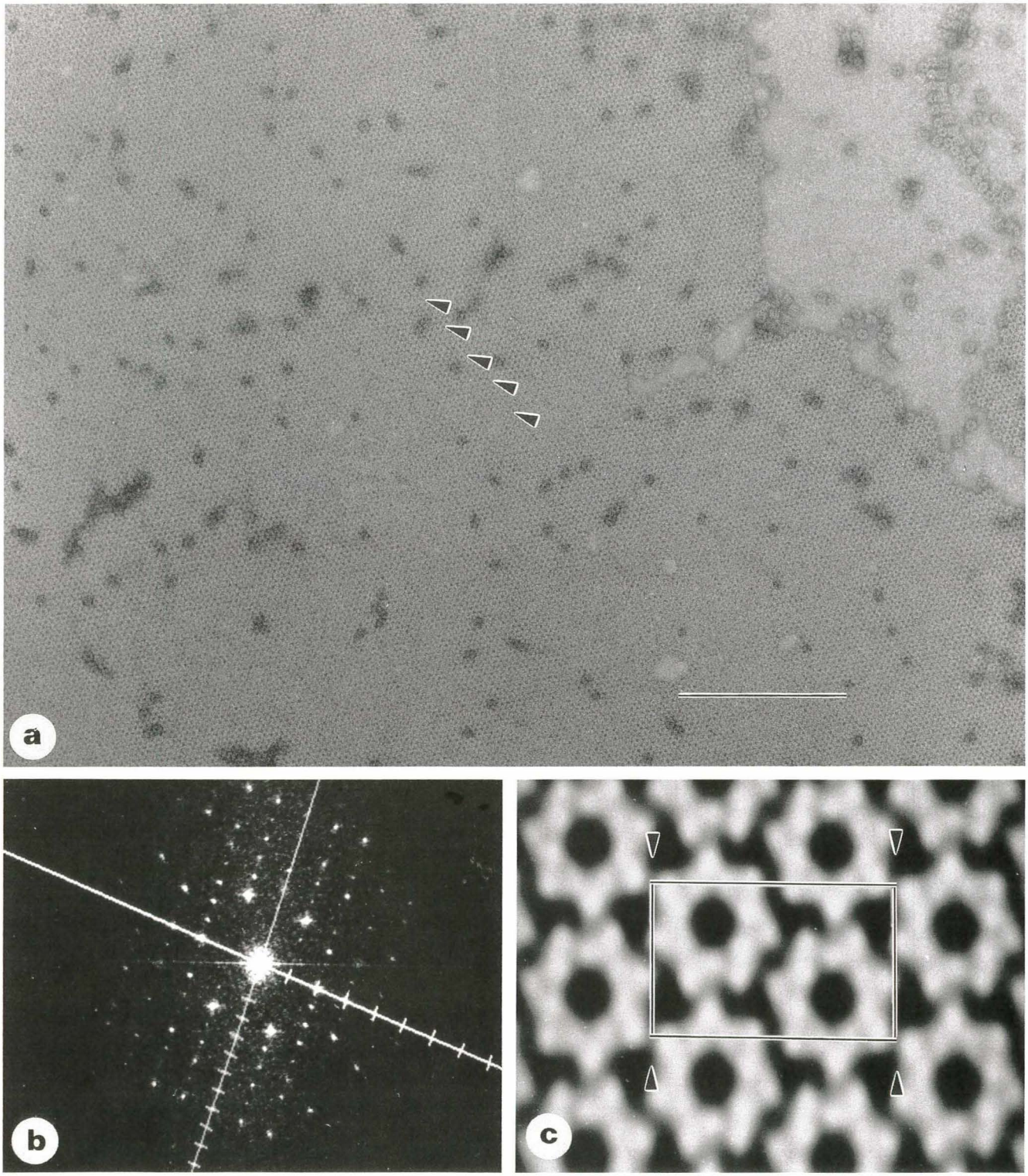

FIG. 4. A large and well-ordered 2-D crystal of GroEL containing molecules orientated end-on, produced by the NS-CF technique at pH 8.0 (a). Insets show the indexed numerical transform/power spectrum (b) and crystallographic average (c) with the unit cell outlined. Arrowheads (a and c) indicate the paired rows of molecules. The unit cell parameters are a = 12.8 nm, b = 22.6, and γ = 89.2°. Scale bar (a), 200 nm.

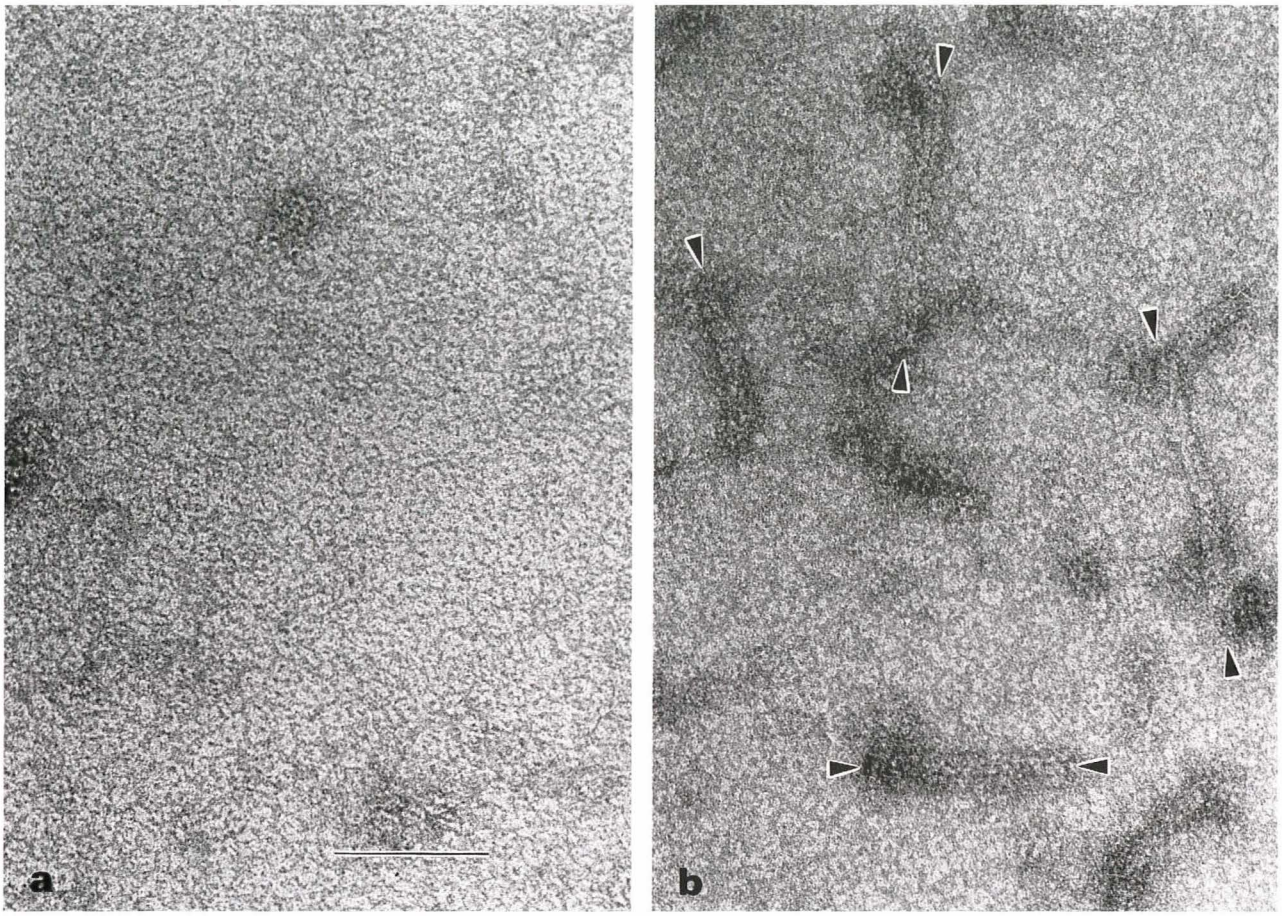

FIG. 5. (a) A high-magnification field of randomly dispersed GroES molecules, prepared by the NS-CF technique at pH 6, showing considerable shape variation. In (b) small flexible linear aggregates of GroES (ca. 7.5 nm width) are present with negative stain penetration centrally (arrowheads). Scale bar, 50 nm.

tion times free GroES was not detectable. With increasing time of incubation, it has been found that the asymmetrical GroEL/ES complexes were unstable and underwent further spontaneous aggregation into elongated chains, within which a single GroES molecule may be located between two GroEL molecules (Fig. 7b). After overnight incubation, extremely large filamentous/paracrystalline precipitates were formed (data not shown), leaving relatively few, yet apparently stable, ellipsoidal complexes remaining in free suspension. This behavior was not influenced by the presence of KCl, but may reflect the exhaustion of ATP by hydrolysis.

(ii) GroEL/ES complexes prepared with a molar ratio of GroEL:GroES of 1:2 have been found to generate a mixture of ellipsoidally symmetrical, rugby/American "football-shaped" complexes and linear aggregates, as shown in Fig. 8a. As with the complexes of 1:1 molar ratio, some protein paracrystalline aggregation occurred with prolonged incubation time (Fig. 8b), although somewhat less than with the lower molar ratio. In samples taken at the longer incubation times it was still possible to detect traces of free GroES in the negative stain surrounding the complexes.

(iii) GroEL/ES complexes prepared with a molar ratio of 1:4 and 1:10 GroEL:GroES have been found to exhibit predominantly the symmetrical ellipsoidal "football-shaped" conformation (Figs. 9 and 10). With these higher molar ratios, uncomplexed GroES readily became detectable within the background of negative stain surrounding the GroEL/ES complexes on all specimens. At both of these higher molar ratios of GroEL:GroES, no gross aggregation of the symmetrical GroEL/ES complexes was found to occur, even with prolonged incubation times (up to 2 days at room temperature). Nevertheless, in specimens of the 1:4 molar ratio, some short linear groups of GroEL/ES could readily be detected (arrowheads, Fig. 9a).

It is clearly seen in Figs. 9a and 10a that in shallow negative stain nearly all the symmetrical complexes adopt a side-on orientation within the negative stain, as would be expected from considerations of geometry. Only rarely would an ellipsoidal structure position itself stably upon its pointed end (viz. the rugby/American football), unless the structure

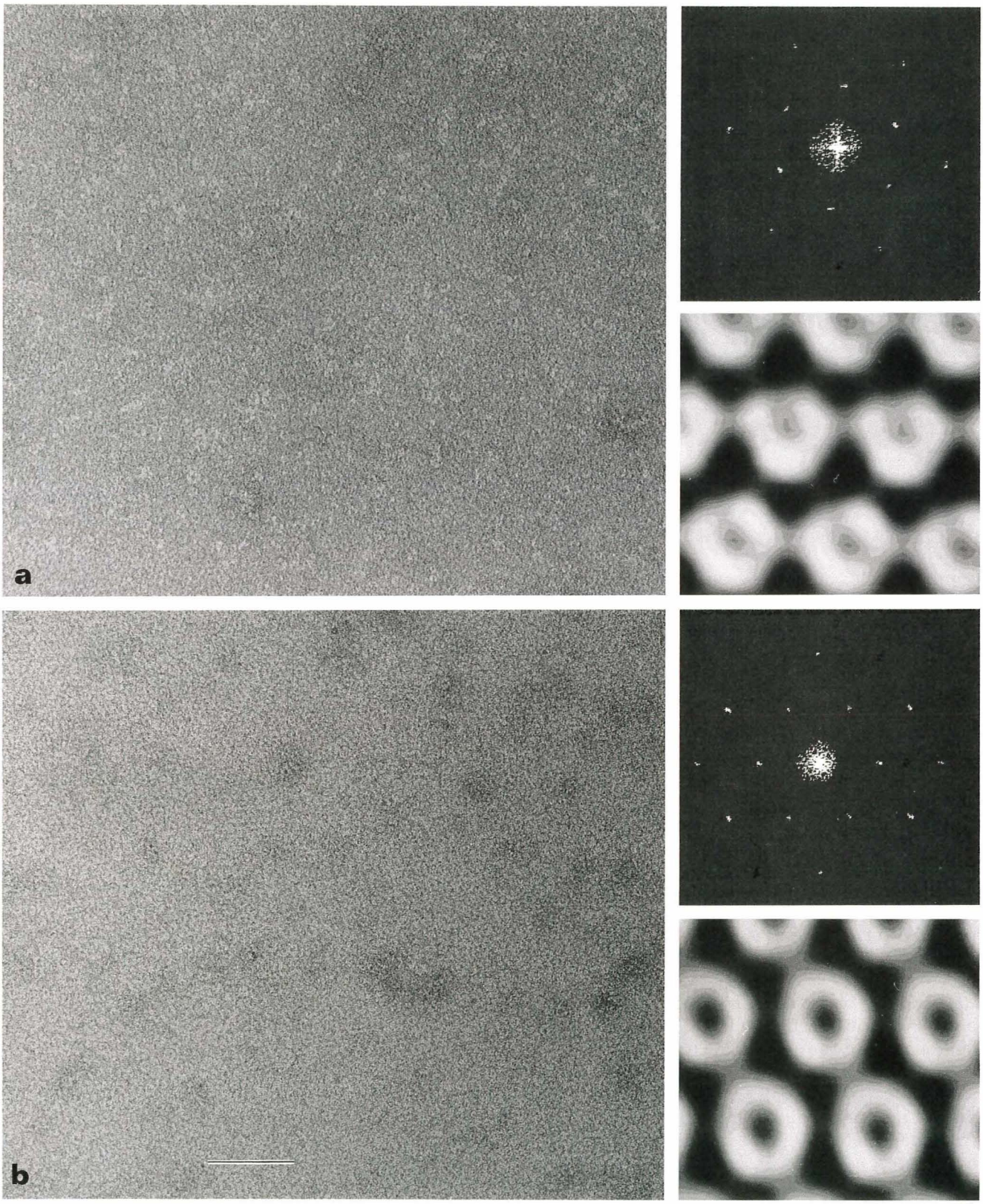

FIG. 6. (a and b) Examples of 2-D crystals of GroES, produced by the NS-CF technique at pH 6.0. Insets show the power spectra and crystallographically averaged images, one of which (a) contains two different image profiles, suggesting that two discrete molecular orientations are present within the crystal. The external dimension of the molecules is ca. 7.5 nm. Scale bar, 50 nm.

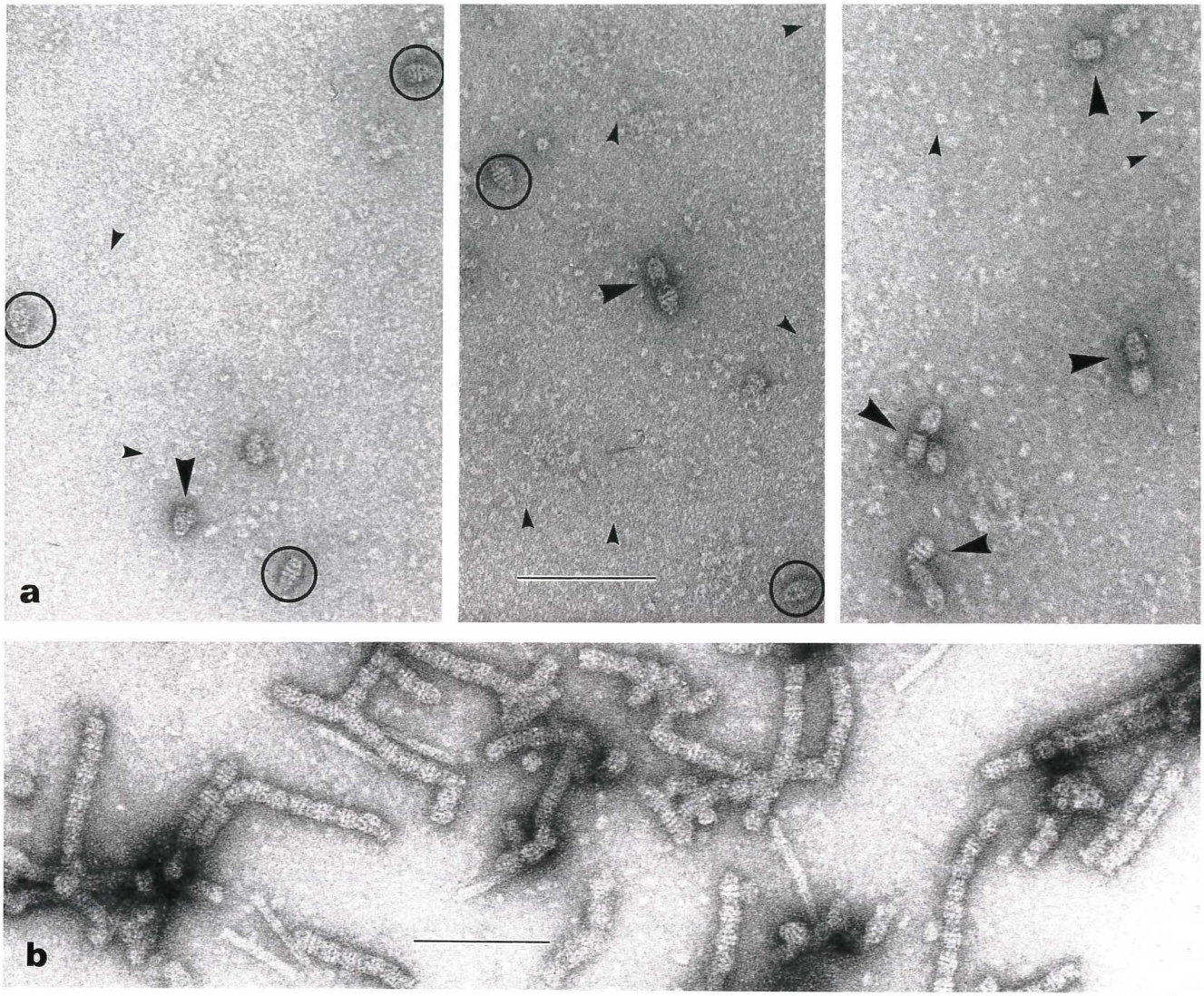

FIG. 7. The GroEL/ES complex prepared at room temperature from a mixture containing a 1:1 molar ratio, conventionally negatively stained with 2% uranyl acetate. In (a), after 15 min incubation some symmetrical hollow ellipsoidal GroEL/ES complexes are present (encircled), together with "bullet-shaped" asymmetrical complexes (large arrowheads), some free GroES (small arrowheads), and short linear chains of molecules. In (b), after longer incubation (1 hr), aggregation of the GroEL molecules into many elongated chains occurs, the interpretation being that single GroES molecules provide a cross-link between consecutive GroEL molecules. Scale bars, 100 nm.

could be supported, as can happen in regions containing many closely apposed complexes and in regions of deep negative stain (Figs. 9b and 10b). Within the deeper negative stain, there is an inherent variability between the side-on images of the symmetrical GroEL/ES complexes, as would be expected from the slightly varying tilt positions a three-dimensional ellipsoidal structure might adopt, when positioned on its side. In general, the symmetrical images of the GroEL/ES complex presented here (Figs. 8 to 10) show the complex to be ellipsoidal in overall conformation, particularly when the negative stain is rather deep. Within the complexes the double band of protein, from the paired cpn60 central domains of the hollow cylindrical GroEL 2 × 7-mer is always predominant, whereas the cpn60 end-domain protein bands of the GroEL cylinder appear to have become structurally modified following interaction with the GroES, to generate the two curved ends. In shallower negative stain (Fig. 9a), the linear outline of the ellipsoidal complexes is revealed more distinctly, again with a predominant central double band of protein (also, cf. the averaged image of uncomplexed GroEL, Fig. 3c). The occasional "bullet-shaped" complex has often been detected in both the higher molar ratio conditions. This may indicate that in our material there is a small population of molecules which are thermodynamically or structurally unable to react with GroES at both ends.

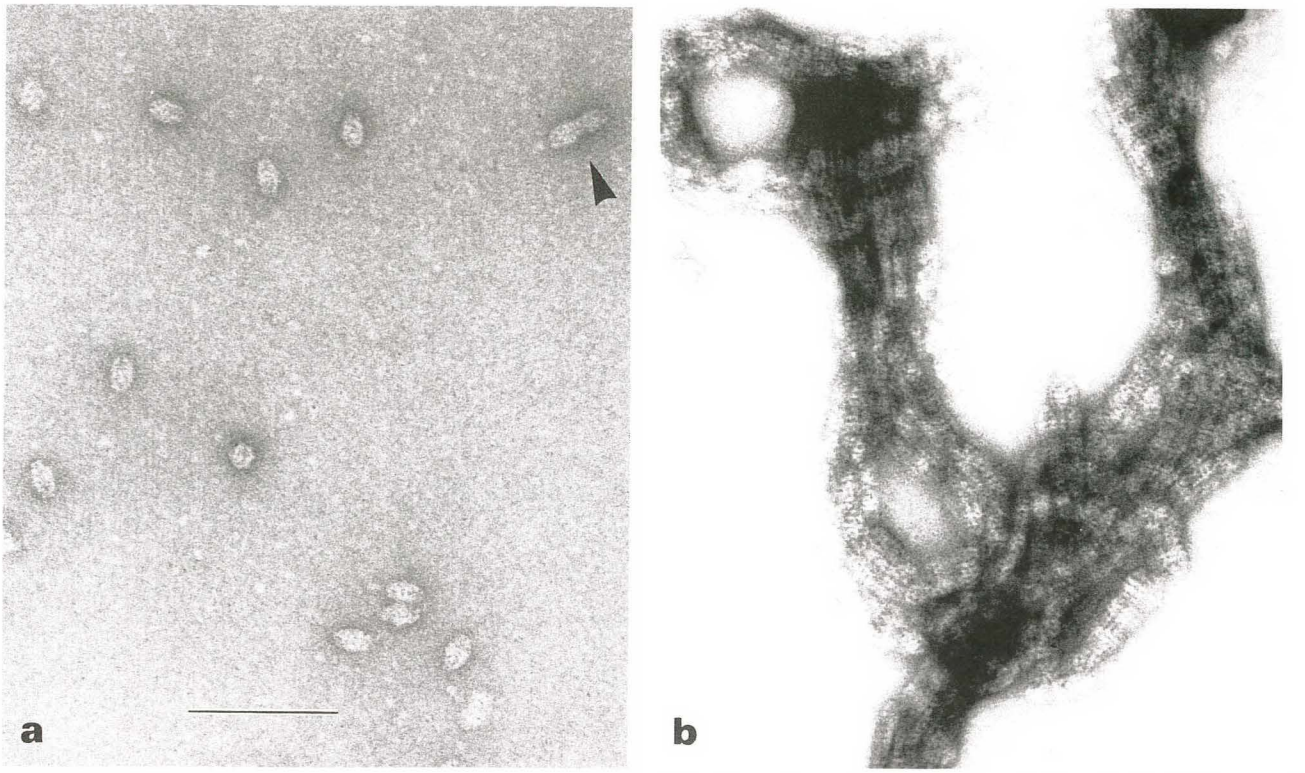

FIG. 8. GroEL/ES complexes prepared at room temperature from a 1:2 molar ratio, conventionally negatively stained with 2% uranyl acetate. Samples taken after 15 min incubation (a) contain numerous symmetrical hollow ellipsoidal complexes, together with short linear aggregates (arrowheads). Free GroES is just detectable at this short incubation time. At longer incubation times (i.e., 1 hr to 1 day) (b), larger linear aggregates are present, but in general these are somewhat smaller than those present in the 1:1 ratio of GroEL:GroES, at comparable times. Little free GroES is detectable at the longer incubation times. Scale bar, 100 nm.

DISCUSSION

The two crystallographically averaged 2-D images of GroEL we have presented agree very well with those obtained by single particle image processing (Saibil *et al.*, 1993; Zwickl *et al.*, 1990), clearly indicating that the NS-CF crystallization technique does not lead to molecular distortions. The side-on image average of GroEL (Fig. 3c) within the p21 crystal form appears to be most similar to the cpn60 "3-D section" average of Saibil *et al.* (1993), undoubtedly because the 2-D crystals used for our image processing were partly covered by relatively shallow negative stain. Within our side-on reconstructed image (Fig. 3c) there is no indication of additional protein within the central hollow channel of the molecule (cf. Saibil *et al.*, 1993; Saibil and Wood, 1993). Technically, it should be possible to produce imaged averages of protein molecules within crystals that are embedded in progressively increasing depths of negative stain. That such an approach may provide useful structural information has been suggested by Harris and Horne (1994).

The end-on image of GroEL (Fig. 4c) shows clearly the characteristic sevenfold symmetry of the molecule, despite the fact that there is again relatively shallow negative stain, only partly covering the molecules within the 2-D crystal. Very significantly, no indication of domain plasticity is apparent within this p21 crystal form (cf. Zahn *et al.*, 1993). In all probability the image obtained may be derived from only the lower half of the dimeric (2 × 7-mer) molecule, which is more completely surrounded by negative stain. Despite this limitation, the heptagonal reconstruction would appear to be a reasonably valid projection image of the complete molecule, particularly as it is in general agreement with other published data obtained from single particle analysis of negatively stained cpn60 molecules.

It is to be noted that the 2-D crystals of GroEL produced by the NS-CF technique in the presence of PEG were found to be greatly superior to the 2-D arrays produced from the *Bordetella pertussis* cpn60 homologue (the 22S antigen) in the absence of PEG (Harris, 1982). Nevertheless, in the presence of PEG both 2-D arrays of nucleating groups of molecules and somewhat disordered quasi 2-D crystals are found alongside genuine 2-D crystals (Harris, 1991; Harris *et al.*, 1993; Zahn *et al.*, 1993). Thus, the current NS-CF technique is also useful for defining the very early stages of molecular association leading to 2-D crystal nucleation.

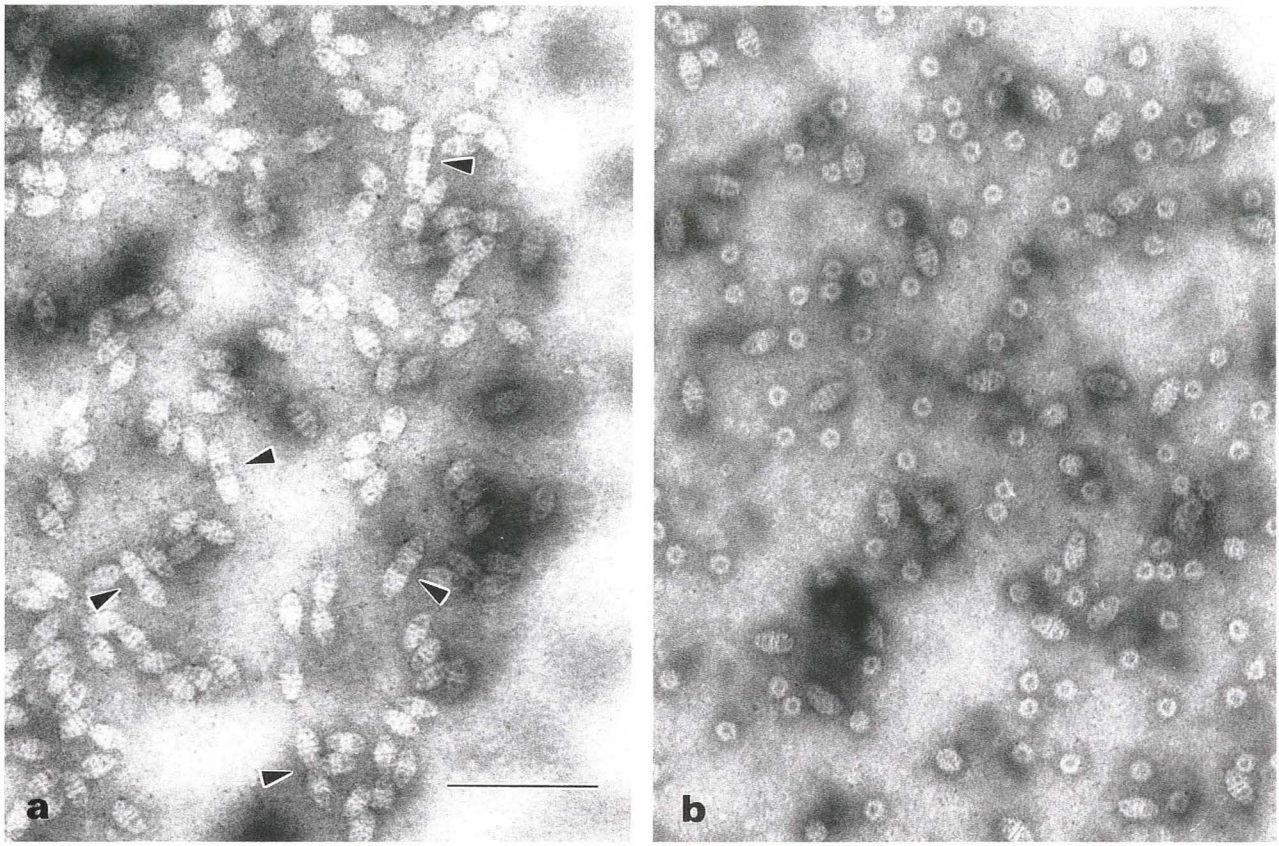

FIG. 9. GroEL/ES complexes prepared at room temperature from a 1:4 molar ratio, conventionally negatively stained with 2% uranyl acetate. The complexes formed under these conditions possess predominantly symmetrical hollow ellipsoidal shapes, with little difference between specimens prepared at increasing times of incubation. Free GroES is readily detectable, together with very short linear aggregates (arrowheads), that contain two or three GroEL molecules, with curved "bullet-shaped" ends. These small linear complexes are apparently stable over a period of days. In (a) the negative stain is relatively shallow and in (b) it is somewhat deeper. Scale bar, 100 nm.

It will be of interest to investigate further the 2-D crystallization of GroEL in the presence of bound ADP and ATP, again for comparison with the existing published data obtained by single particle analysis (Saibil *et al.,* 1993; Langer *et al.,* 1992), where a significant change in molecular shape was shown. It is clearly apparent that greater control of the depth of the negative stain surrounding macromolecules within 2-D crystals and indeed freely dispersed macromolecules is required. Harris and Horne (1994) have recently proposed that the inclusion of a low concentration of surfactant octyl-β-D-glucopyranoside may assist stain penetration and tend to combat any hydrophobicity associated with the carbon support film. In addition, it was proposed that trehalose-negative stain mixtures may be beneficial by providing increased protein stability, and at the same time a greater and controllable depth of negative stain, for both single protein particles and 2-D protein crystals (J.R.H., unpublished observations). It will therefore be important to be able to study the 2-D crystals of GroEL at low temperatures (eg. −175°C) using trehalose-negative stain mixtures and also when embedded in vitreous ice, for comparison with the ongoing X-ray crystallography of this protein (Spangfort *et al.,* 1993; Svenssen *et al.,* 1994).

However, both the side-on and end-on 2-D crystals with p21 symmetry are in principle suitable for future 3-D reconstruction, which will give more detailed information about the arrangement of subunits and domains within the GroEL molecule. In contrast to single particle averaging, such 3-D reconstruction from 2-D crystals of GroEL has the advantage that no molecular symmetry has to be imposed during the image processing. Thus, there is no loss of structural information during the image processing from a 2-D crystal, which may occur from single molecule averaging.

It will be of interest to investigate the variable negatively stained images obtained from the randomly dispersed GroES molecules, which are most likely to be due to a number of varying orientations of the molecule on the carbon support film (cf. human erythrocyte catalase, Harris and Holzenburg, 1989; Harris *et al.,* 1993). A ring-like molecule

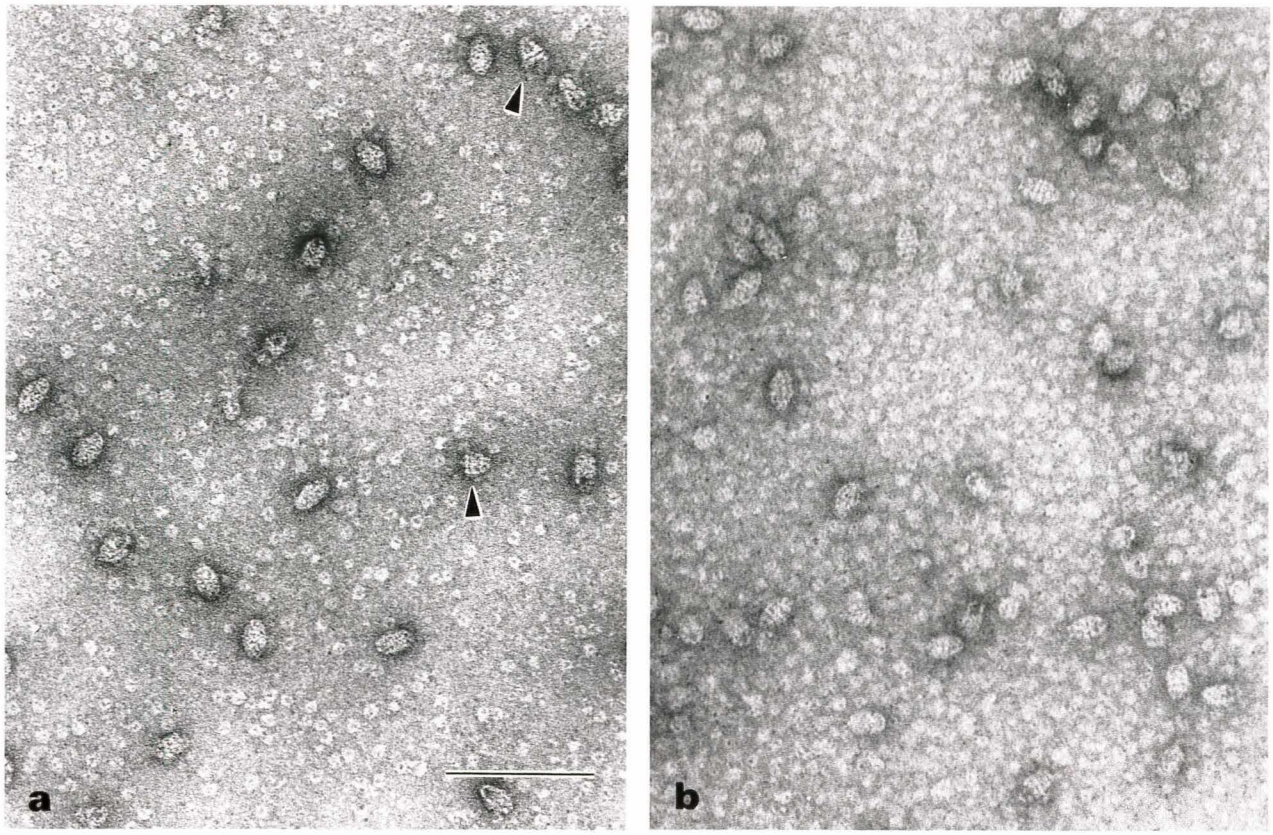

FIG. 10. GroEL/ES complexes prepared at room temperature from a 1:10 molar ratio, conventionally negatively stained with 2% uranyl acetate. The complexes exhibit almost totally the hollow ellipsoidal conformation, with no apparent difference between specimens prepared at increasing times of incubation. A large amount of free GroES is readily apparent and no short linear aggregates are present. In (a) the negative stain is relatively shallow and most of the ellipsoidal complexes are orientated side-on, whereas with slightly deeper negative stain (b) some complexes are oriented end-on. Even at this high molar ratio of GroEL:GroES, the occasional bullet-shaped complex can be detected (arrowheads, a). Scale bar, 100 nm.

would not be expected to generate a large variety of images (cf. Harris, 1969). However, deviation from a strictly ring-like quaternary structure could readily generate a number of different orientations for the molecule during the 2-D crystallization and negative staining procedures, resulting in a number of different projection images. There is no electron microscopical evidence to suggest that GroES has any tendency to dimerize or form rod-like multimers, as might be expected if it was a ring of seven 10-kDa subunits. We have, however, shown that GroES is able to create flexible linear aggregates. It is possible that the GroES molecule, although approximately circular, does possess an inherent rotational asymmetry, as has been described for the *E. coli* transcription terminating factor Rho (Gogol *et al.*, 1991) and is indicated in the images of the *Limulus polyphemus* sialic acid- and phospholipid-specific lectin (Fernández-Morán *et al.*, 1968; and J.R.H., recent unpublished observations).

This apparent lack of image uniformity expressed by GroES could also be due to other possibilities. The molecular quaternary structure may indeed be variable due to damage produced during purification and storage (Chandrasekhar *et al.*, 1986), or during the negative staining procedure, or, more likely, to inherent instability or structural plasticity. Recently, a mobile loop within the GroES 10-kDa subunit has been described (Landry *et al.*, 1993). Such flexibility may be required in the subunits or loops of the GroES 7-mer to enable them to bind, in the presence of ATP, simultaneously to sites on seven GroEL subunits, which also show molecular plasticity (Zahn *et al.*, 1993). The flexible arrangement of GroEL and GroES subunit or domains may promote the binding and/or release of substrate proteins and may be a prerequisite for the surprisingly large substrate promiscuity of the chaperone.

Future low dose–low temperature negative staining and cryoelectron microscopy of GroES single molecules and 2-D crystals is planned, together with single particle averaging, crystallographic image processing, and correlation averaging, in an attempt to obtain more accurate information concerning the quaternary structure of this small protein. Improved 2-D crystals of GroES are clearly required, in order

to obtain meaningful comparison of the EM data with that derived from X-ray diffraction analysis (Weaver *et al.,* 1993).

The formation of filamentous GroEL aggregates in the presence of a low molar ratio of GroES:GroEL (1:1 and 2:1) suggests that these aggregates may contain an alternating linear sequence of ES–EL–ES–EL–ES. It must be emphasised that filaments of this type have never been detected in the absence of GroES and that they can be clearly distinguished from the antibody-linked *B. pertussis* cpn60 linear chains shown by Cejka *et al.* (1993). Often, the filaments terminate with a curved "bullet-like" shape-transformed end, but by no means always. This observation indicates that GroEL has the ability to interact with GroES at both ends of the cylinder and furthermore that at the low molar ratios of GroES: GroEL, one GroES can be shared and thereby provide the cross-link between two GroEL molecules. Once an excess of GroES greater than 2-fold is present, our data suggest that the individual GroEL molecules favor the binding of GroES at both ends, thereby creating apparently stable (up to at least 3 days at room temperature) symmetrical complexes. Nevertheless, even in the presence of a molar ratio of 4:1 GroES:GroEL some very short linear GroEL–GroES chains have been reproducibly encountered, which terminate with two "bullet-shaped" ends, and which are apparently stable (i.e., they do not have any rapid tendency to dissociate, even though much free GroES is present). At 10-fold molar excess of GroES over GroEL, we observed predominantly the symmetrical complex, indicating that this state is preferred over the GroEL–GroES chains and the single GroEL–GroES bullets, under these conditions. Nevertheless, Langer *et al.* (1992) found an asymmetrical protease protection of GroEL even at a 10-fold molar excess of GroES over GroEL, when the excess GroES was not removed. In view of the fact that Martin *et al.* (1993) showed that both ends of the GroEL cylinder can bind GroES, and that GroES alternates dynamically between the two ends, the existence of a transient intermediate is reasonable. Under our experimental conditions, it is likely that this symmetrical intermediate has been stabilized.

In summary, with increasing concentrations of GroES relative to GroEL, there appears to be a shift in the equilibrium of association from the asymmetrical "bullet-shaped" complex and the long GroEL–GroES chains to shorter chains and the symmetrical "football-shaped" complex. This concentration-dependent binding of GroES by GroEL may explain the differences in our results compared to those of other groups. In contrast to others (i.e., Langer *et al.,* 1992), we used varying molar ratios, short, and progressively longer times of interaction, but did not include a gel filtration step to remove free/unbound GroES during our experimental procedure. The potassium concentration, which has been shown to regulate the inhibition of GroEL ATPase activity by GroES (Todd *et al.,* 1993), did not influence the complex formation in our experiments. We conclude that the symmetrical binding of two GroES molecules, i.e., to both ends of the GroEL cylinder under our conditions may give new insights into the mechanism of chaperone function and may reflect a more complete screening of bound substrate against other nonnative proteins or proteases.

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