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Electron microscopy and image analysis of the *GroEL*-like protein and its complexes with glutamine synthetase from pea leaves

Vladimir L. Tsuprun [a], Egbert J. Boekema [b], Alexander V. Pushkin [c] and Irina V. Tagunova [a]

[a] *A.V. Shubnikov Institute of Crystallography, USSR Academy of Sciences, Moscow (USSR),* [b] *BIOSON Research Institute, Groningen University, Groningen (Netherlands) and* [c] *A.N. Bach Institute of Biochemistry, USSR Academy of Sciences, Moscow (USSR)*

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The molecular structure of *GroEL*-like protein from pea leaves has been studied by electron microscopy and image analysis of negatively stained particles. Over 1500 molecular projections were selected and classified by multivariate statistical analysis. It was shown that the molecule consists of 14 subunits arranged in two layers with 72 point group symmetry. Side view projections of the molecule show a four-striation appearance, which subdivides both layers of seven subunits into two halves; this may be explained by a two-domain structure of the subunits. The presence in protein preparations of projections corresponding to one layer of subunits or half-molecules is consistent with the molecular structure suggested. Electron microscopic evidence for a specific association of *GroEL*-like protein and octameric glutamine synthetase, which was co-purified with this protein, was obtained.

Introduction

In 1982 during isolation of glutamine synthetase (EC 6.3.1.2) from pea leaves we found a high molecular weight protein, which was co-purified with the enzyme [1]. The structure and some properties of this protein were similar to the *GroEL*-protein of *E. coli*, which is required for correct assembly of some bacteriophages [2,3]. Lately very similar proteins were found in chloroplasts [4,5] and mitochondria [6,7]. The proteins of the *GroEL*-like family are thought to transiently bind to newly synthesized polypeptides. This association assists post-translational folding of polypeptides and assembly to the correct oligomeric form. Since these proteins are not a part of a final structure they have been termed chaperonins [4,8,9]. The quaternary structure of some chaperonins has been studied by electron microscopy [1–3,10]. It was shown that all of them were composed of 14 subunits arranged in two layers, but the models of the molecular structure were rather different. Therefore in this work we performed a more detailed electron-microscopic study of *GroEL*-like chaperonin from pea leaves using multivariate statistical image analysis (MSA) [11,12]. The MSA technique allows us to distinguish between slightly different projections arising from different positions of the molecule on the support film. The subsequent automatic classification groups the projections into classes. The images of each class are more similar to each other than to images of other classes which makes it possible to enhance the signal-to-noise ratio of the characteristic views of a particle.

During purification of glutamine synthetase from pea leaf cytosol we noticed an unusual co-migration of *GroEL*-like protein. In the present work we have thoroughly studied these preparations and revealed a stable association of glutamine synthetase and *GroEL*-like protein. The structure of these associates was analyzed.

Materials and Methods

Glutamine synthetase [13] and *GroEL*-like protein [1] were isolated and purified from leaves of 2–3-weeks old pea plants grown in open ground. The proteins were dissolved in 50 mm Tris-HCl buffer (pH 7.5).

Correspondence: V.L.Tsuprun, A.V.Shubnikov Institute of Crystallography, USSR Academy of Sciences, Leninsky pr., 59, 117333 Moscow, USSR.

containing 0.1 M $MgSO_4$, to a final concentration of 0.1–0.2 mg/ml. Negatively stained specimens were made on carbon-covered collodion films with the droplet method using 1% uranyl acetate. Electron microscopy was performed with a Philips EM 400 electron microscope at 80 kV. Images were recorded at 50,000 × magnification; the magnification was calibrated with orthorhombic catalase crystals. Selected micrographs were digitized with a Datacopy electronic digitizing camera using a step size of 25 μm corresponding to a pixel size of 0.5 nm on the object.

Image analysis was carried out on a Convex C1-XP mini-supercomputer using the IMAGIC software system [14]. The processing comprised several steps. Particles were selected interactively from the micrographs using a raster-scan image display system. A total of 1544 molecular images were extracted from 10 different micrographs using a window of 36 × 36 pixels. They were pretreated and normalized. A circular mask with a radius of 18 pixels was imposed upon the particles to remove unnecessary background. The average density inside the mask was set to zero and the variance of the densities was normalized. The next steps of the analysis were carried out essentially as described previously [15,16]. The pretreated images were aligned to each other by correlation methods. After alignment they were subjected to multivariate statistical image analysis (MSA) [11] and subsequent classification [12]. Finally the images belonging to the same class were averaged.

Results and Discussion

Two distinct types of molecular projections of *GroEL*-like protein could readily be observed in the micrographs (Fig. 1). The first type or top view has the form of a 'seven-pointed' star with a diameter of 13 ± 1 nm. The second type or side view looks like a rectangle of 12.5–15.0 nm length and 10.0–12.0 nm width with four stripes parallel to the short side. These two types are found more frequently than others and most probably correspond to the stable positions of the molecule on the support. Other projections arise more or less from slight deviations of these two orientations.

To study the structure of *GroEL*-like protein in more detail, the single particle projections were treated by MSA and classified. Since the two views (Fig. 1) differ substantially, the total data set of 1544 images was subdivided into two subsets (779 top views and 765 side views). These subsets were processed independently. The same scheme of subsequent steps was

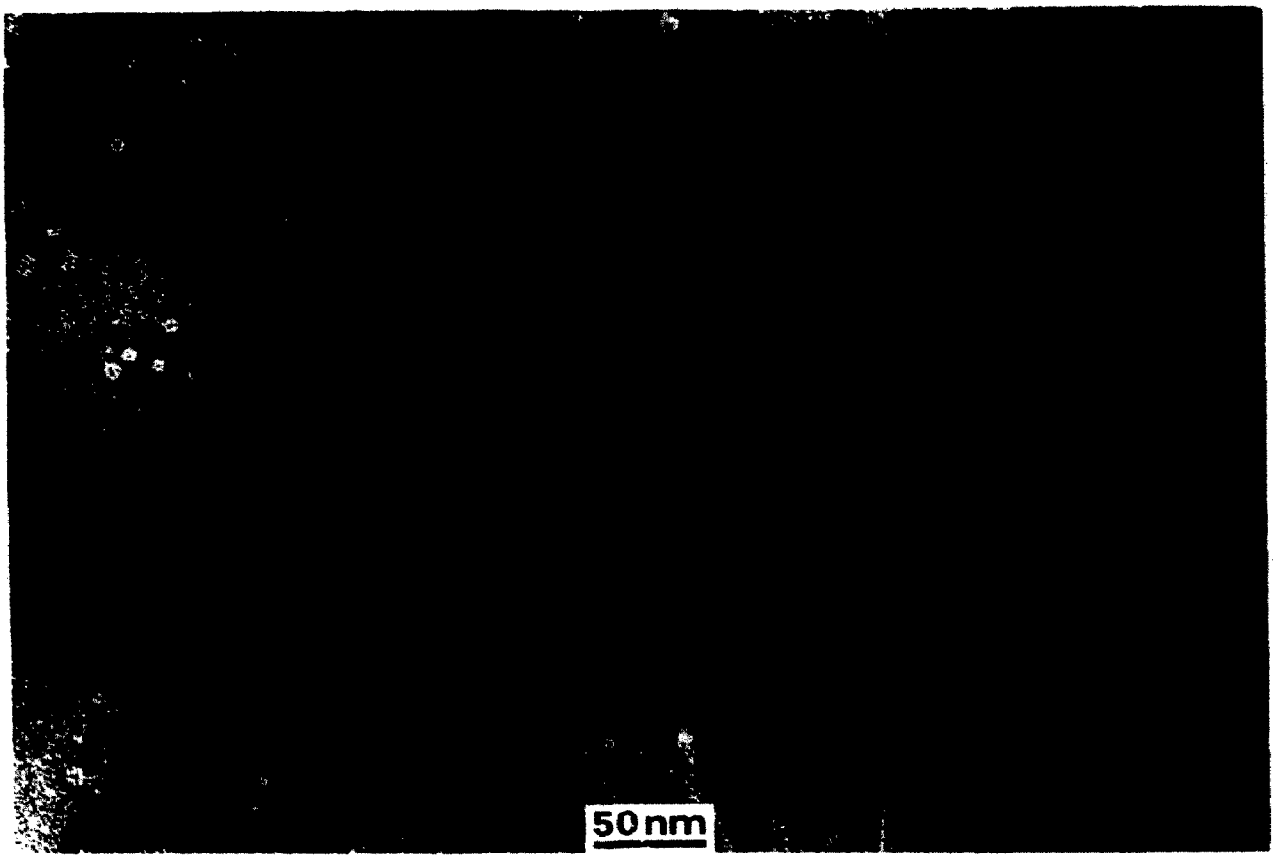

Fig. 1. Field of *GroEL*-like protein from pea leaf showing molecules used for image analysis of single particles. The particles indicated by arrows correspond to half-molecules.

followed in alignment of projections, MSA and classification. We started with an arbitrary particle as the first reference. All further alignments were carried out with improved intermediate references made from the previous alignments. Then it was followed by a first MSA and classification in which the two data sets were decomposed into 10–12 classes, automatically rejecting about 15% of the particles. The rejected images mainly seem to represent badly aligned particles and ones which were non-typically stained. The classes after the first MSA and classification were used as new references for the next cycle of multireference alignment and MSA procedure. The final classes of top and side view projections are shown in Fig. 2 and Fig. 3, respectively. They represent 75–80% of the particles used. Besides the rejection of 15% of the images during the first MSA procedure, 5–10% of the images were rejected after the second MSA step on the base of very low correlation between them and the reference. Visual examination of all these images showed that the latter ones were largely mis-aligned.

Analysis of top view projections

Fig. 2 shows 10 classes of averages of top view containing 40–98 members each. The sums are rather similar, but differ in size and shape. The classes A and J have the form of a seven-pointed star. The other classes are less symmetrical. The projections of classes B, E, I and J have a smaller size than the others. The projections of these classes are largely from the same subset of micrographs and their smaller size can be explained by variance in staining conditions of the particles on the support. The classes C, D, F, G and H are more similar and have a maximal diameter of 13 ± 0.5 nm, approximately equal to the diameter of class A (13–13.5 nm). They most likely correspond to

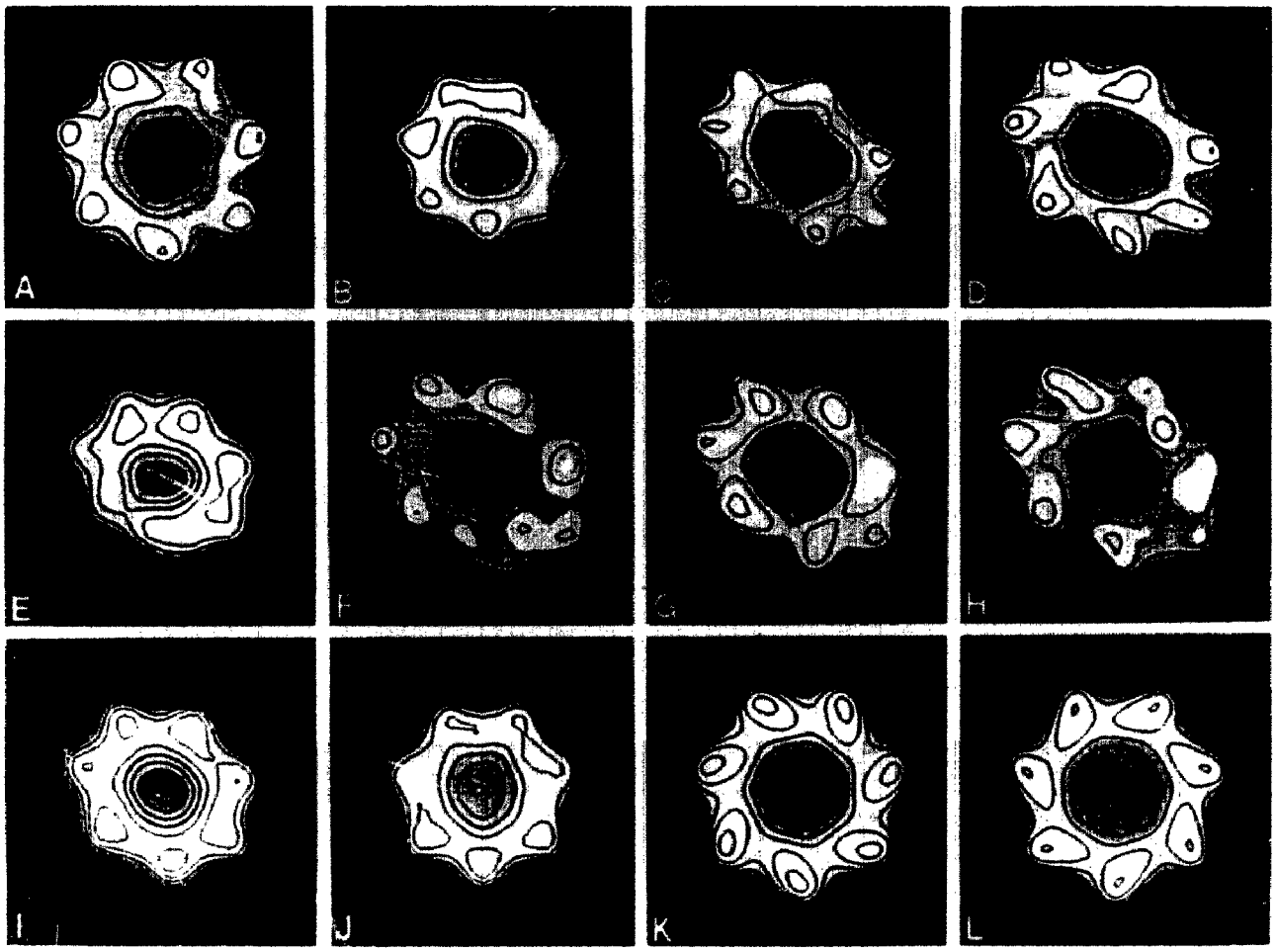

Fig. 2. Image analysis of the top views of *GroEL*-like particles. Averaged molecular projections were determined after multireference alignments, MSA and classification of 779 molecules: the final 10 classes (A-J) reflect the image variation present in the data set. Number of class members: A = 63, B = 98, C = 67, D = 67, E = 45, F = 56, G = 40, H = 80, I = 94 and J = 52. No symmetry was imposed upon the classes. 63 members of class A were further analyzed after seven-fold symmetrization; K and L are sums of two subclasses of 32 and 10 particles which showed a strong handedness. For representation the original 36 × 36 pixel images were interpolated to a 512 × 512 format and contoured with isodensity contour levels. The inner 420 × 420 pixels are shown.

projections from the class A position, but with the molecule rotated over an angle. Analysis of the individual particle images of the class A using auto-correlation functions shows that they are characterized by seven-fold rotational symmetry. Seven-fold averaging of each particle led to three groups of images: two of them have different handedness and the third one was close to 7 m symmetry. The particles of the first two groups were averaged separately. The averages (Fig. 2K and L) are mirror images and consist both of seven elongated stain-excluding (protein) regions. The appearance of these two groups of images can be explained by preferential staining of the back or front side of the molecule.

Analysis of side view projections

An examination of the micrographs showed that about one half of the particle projections were side views, clearly different from the top views. They were selected and analyzed. After repeated alignments on improved references and after the first MSA and classification, the classes obtained were used as intermediate references for a next multi-reference alignment. This was followed by final classification into 12 classes (Fig. 3), each class containing 27–81 individual molecular images. The projections A-L have a square or rectangular form and show a continuous variation of the morphology or variation of viewing angle. The projections J-L differ from other ones by their larger size in one direction which may be due to particle distortion on the support. Most of the classes show four stain-excluding striations.

Molecular structure of GroEL-like protein

The molecular mass of the protein determined by gel filtration and sedimentation equilibrium techniques, was found to be about 900–950 kDa [1]. With polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate, one type of subunit with a mass of about 60–70 kDa was found. The presence of

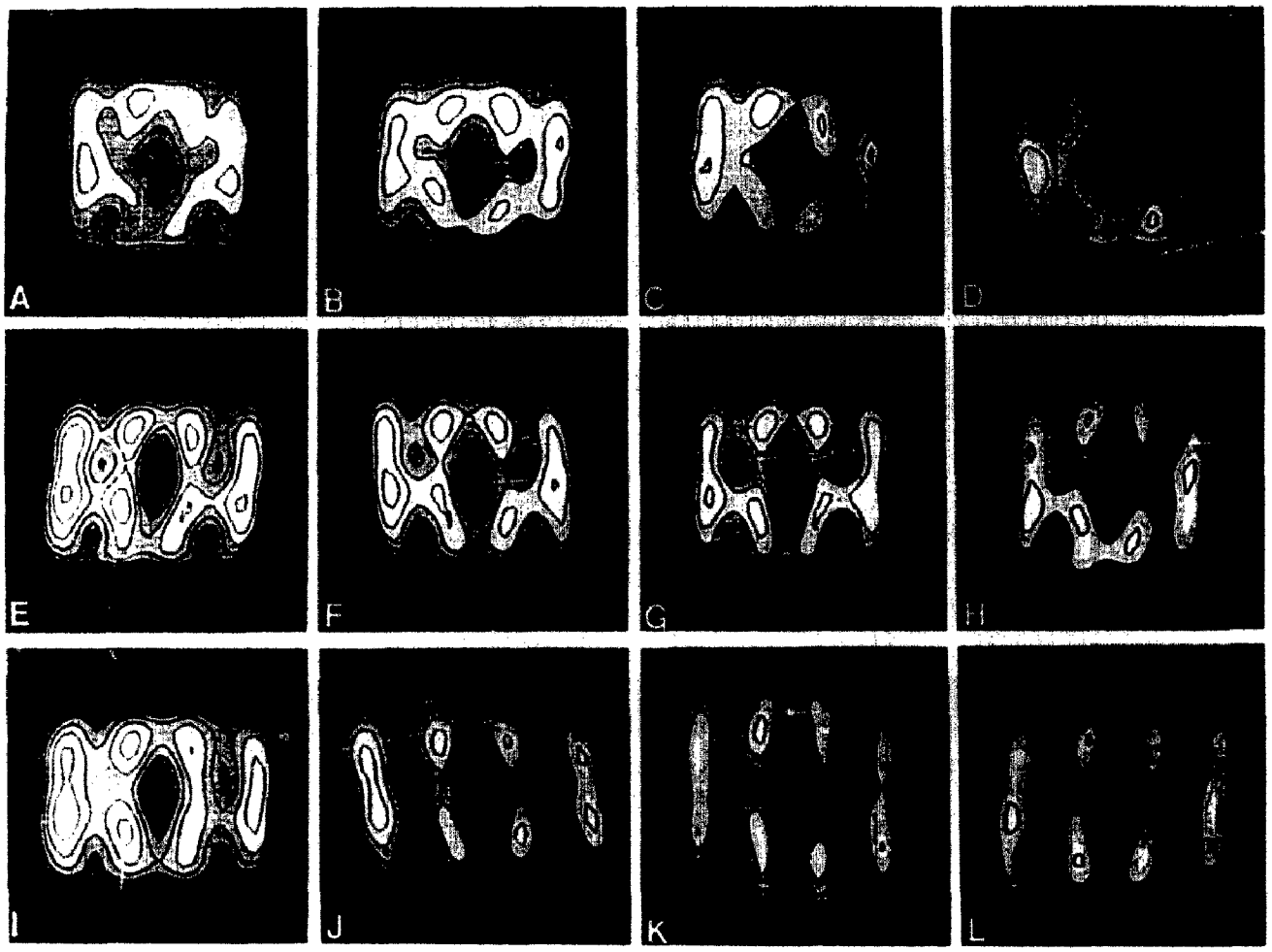

Fig. 3. Image analysis of side views of *GroEL-like* particles. The molecular projections were determined by the same procedure as for those of top views in Fig. 2, but the analysis was carried out independently. In the final classification step the data set of 765 projections was decomposed into 12 classes (A–L) with number of class members: A = 42, B = 37, C = 50, D = 36, E = 50, F = 48, G = 54, H = 45, I = 45, J = 66, K = 27 and L = 81.

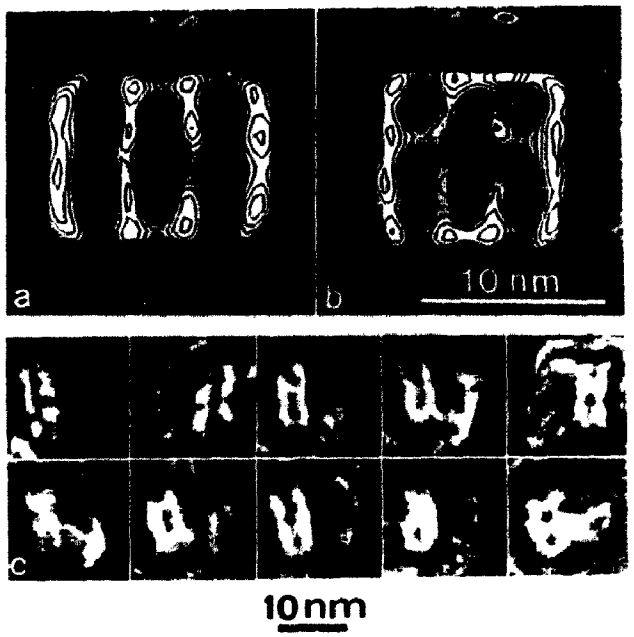

Fig. 4. (A,B) Two side view projections (classes I and G of Fig. 3) with imposed band-pass filter, which suppresses the very gross details. (C) Projections of half-molecules which align with their long axis parallel to the four striations as seen in the side view projection of the whole molecule.

seven-fold rotational symmetry in the top-view projection indicates that the *GroEL*-like protein should contain an integral multiple of seven subunits. Taking into account the molecular mass of the molecule and its subunits one can conclude that the protein consists of 14 subunits. The possibility of 28 copies (four layers of seven) can also be ruled out because of the dimensions. The *GroEL*-like protein particles (13 nm diameter and 12.5–15 nm in length) are too small to accommodate a mass of about 1800 kDa; for comparison: a F_1 ATP synthase molecule with a mass of 396 kDa has a diameter of 11 nm and a length of 8–9 nm [16]. The most important observation in side view projections with four striations is their division into two equal halves, approximately related by mirror symmetry. As a consequence of the seven-fold symmetry in the top views and the number of 14 subunits, each half must correspond to one layer with seven subunits. Such a two-layered structure may be characterized by 72 point group symmetry, in which the subunits in different layers are related by two-fold symmetry. The projections observed show that the molecule is cylindrically shaped with a diameter of about 13 nm and a height of 13 ± 1 nm measured from seven-fold (Fig. 2A) and rectangular (Fig. 4A) particle projections. The seven-fold axis is perpendicular to the striation on contrary to our previous work [1], where it was oriented parallel to the short side of the rectangle. The diameter of the top view is larger than the short side of the rectangular projection, which is about 11 nm (Fig. 4A). This can be explained by partial immersion of particles in 'side-on' projection in stain. The square projection (Fig. 4B) can be obtained by tilting of such particles (Fig. 4A) about an axis parallel to the striation. The four protein peaks which are seen in some strips after band-pass filtering (Fig. 4) may be also due to partial or one-sided staining. The presence of four protein strips in rectangular projections indicates that the protein is composed of four discs (two discs in each layer). The arrangement of seven subunits of each layer in two discs allowed us to suggest that each subunit consists of two different domains of equal size localized at different levels along the seven-fold axis. A similar subunit conformation was suggested for a *Neurospora crassa* chaperonin [10]. The division of each layer of seven subunits into two discs with the seven-fold axis normal to them is confirmed by the presence in the protein preparations of particles with two strips having a form and a size corresponding to a half of the molecule (indicated by arrows in Fig. 1). Probably the protein preparation can induce the dissociation of the molecules into halves of seven subunits. Fig. 4C shows such particles which turned out to be in one class after MSA procedure. A one-layer structure of seven subunits was suggested earlier for the mitochondrial chaperonin of a moth [17]. Our data on *GroEL*-like protein structure are compatible with electron microscopic study of heat shock protein from mitochondria using tilting experiments [10]. A very similar quaternary structure was found for *GroEL*-like protein from the eubacterium *Comamonas acidovorans* [18]. But we have not observed molecular forms with six-fold symmetry, observed for bacterial *GroEL*-chaperonins [19].

Association of GroEL-like protein with glutamine synthetase

GroEL-like proteins have been found as contaminants in preparations of some oligomeric enzymes, RNA-polymerase [2] and cytochrome reductase [10]. In our experiments the chaperonin molecules were co-purified with octameric glutamine synthetase of higher plants [1]. This allowed us to investigate the possibility of a stable interaction between this protein and glutamine synthetase. During the present work we have obtained experimental data which support this idea. Electron microscopy of a preparation of glutamine synthetase from pea leaf cytosol containing *GroEL*-like protein as a contaminant is shown in Fig. 5. The molecular projections of the two proteins differ sufficiently by their shape and size. The predominant view of glutamine synthetase particles was round, 12.5 ± 0.5 nm in size, with eight small protrusions grouped in pairs visible over their circumference. These images were interpreted in our work [13] as projections of a two-layered octameric molecule with two square tetramers only partially eclipsed down the four-fold axis.

Many *GroEL*-like protein molecules are oriented on their sides giving rectangular projections with four striations. The most interesting feature of these preparations is the visible association of *GroEL*-like protein to glutamine synthetase. The same complexes were also found after incubation of homogeneous glutamine synthetase with homogeneous *GroEL*-like protein. Complexes with different numbers of glutamine synthetase molecules bound to *GroEL*-like protein are present in our preparations (Fig. 5). We observed that only two sites of *GroEL*-like protein were frequently binding to glutamine synthetase: glutamine synthetase molecules are in contact with the protein in the middle of the short (Fig. 5A,C) or the long (Fig. 5B,C) side of the rectangle. Two groups of these images (Fig. 5A,B) were separately aligned by correlation methods and averaged. The results of averaging (Fig. 6A,B) demonstrate two 'sticky' sites of *GroEL*-like protein which are accessible to association with the enzyme. We have recently found very similar complexes between ribulose-1,5-bisphosphate carboxylase (Rubisco) subunit-binding protein (similar to *GroEL*-protein of *E. coli* and apparently identical to glutamine synthetase binding protein) and oligomeric Rubisco (L_8S_8) from pea leaf chloroplasts by electron microscopy [20,21]. There is some biochemical evidence on the involvement of Rubisco subunit-binding protein in the assembly of Rubisco in higher plants [22,23]. The *GroEL*-like protein may play a similar role in the case of glutamine synthetase. This suggestion is in agreement with data of Lubben et al. [5] and our previous data on the low yield of active octameric molecules of higher plant glutamine synthetase from subunits in vitro [24]. There were associations of *GroEL*-like chaperonins with unfolded

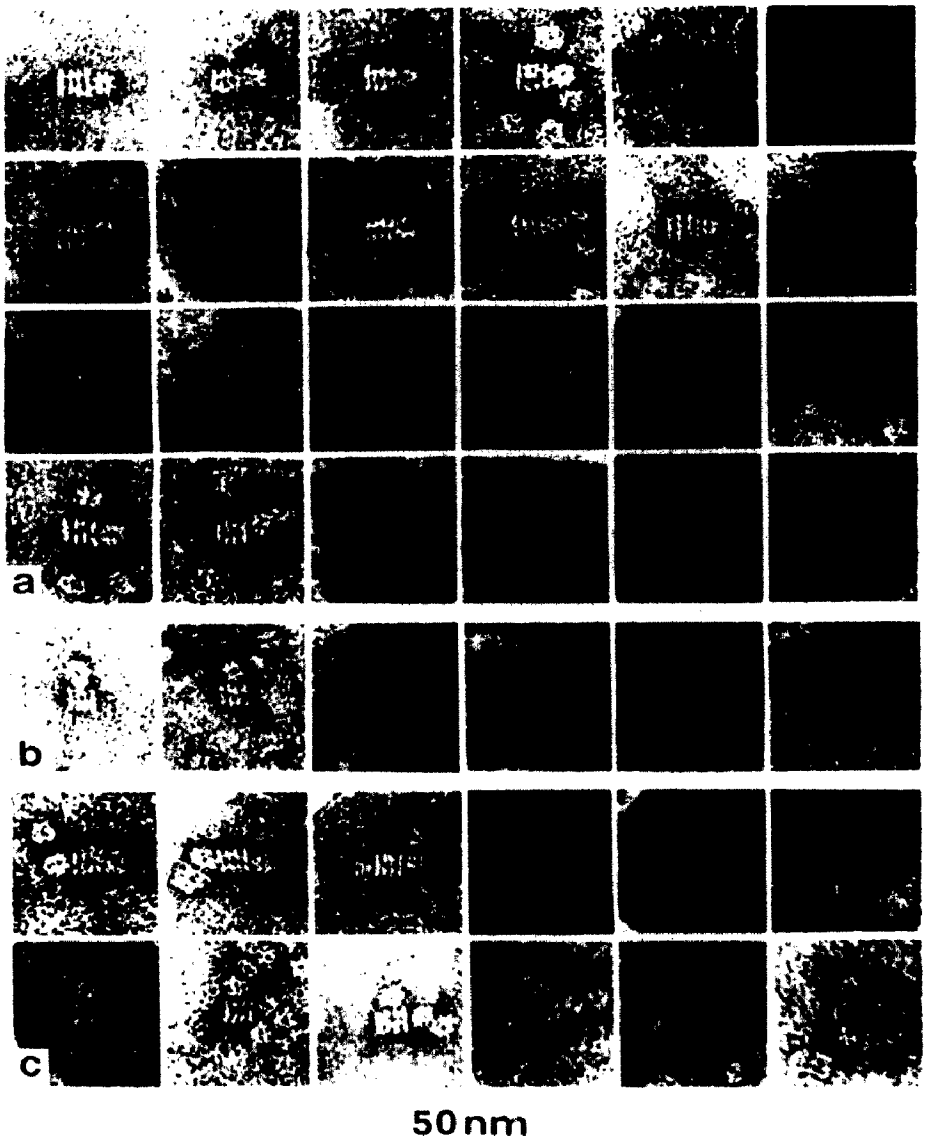

Fig. 5. Electron microscopy of a preparation of pea leaf cytosol glutamine synthetase containing co-purified *GroEL*-like protein. (A–C) Galeries of characteristic types of protein-enzyme complexes of glutamine synthetase and *GroEL*-like protein.

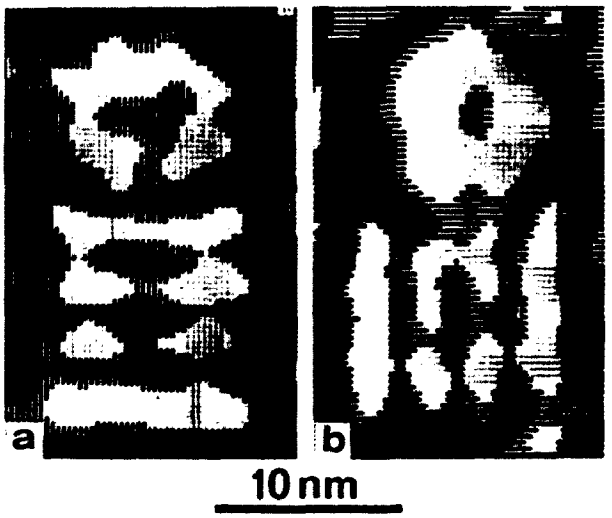

Fig. 6. (A,B) Computer averaged images of two variants of associations of *GroEL*-like protein and glutamine synthetase molecules (shown in Fig. 5A and B).

subunits of Rubisco [22,23,25] and pre-β-lactamase and chloramphenicol acetyltransferase [26]. One of the most interesting aspects of these problems now is to solve the functional part of the interaction between *GroEL*-like protein and other proteins. It is possible that such interaction may be necessary not only for protein folding and assembly but also for stabilization of their oligomeric structure.

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