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The three-dimensional structure of proteasomes from *Thermoplasma acidophilum* as determined by electron microscopy using random conical tilting

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The three-dimensional structure of proteasomes from the archaebacterium *Thermoplasma acidophilum* has been determined to a resolution of approximately 2 nm from electron micrographs of negatively stained preparations using the method of 'random conical tilting'. The particles turn out to be essentially cylinder-shaped barrels, 15 nm long and 11 nm wide, enclosing a tripartite inner compartiment. An account is given of some of the present limitations which prevent to attain a higher resolution and possible ways to overcome these limitations are indicated.

Proteasome; Archaebacterium; *Thermoplasma acidophilum*; Electron microscopy; Reconstruction 3-D; Conical tilting, random

1. INTRODUCTION

The proteasome or multicatalytic proteinase (MCP) is a high molecular weight protein complex of distinct size and shape; for recent reviews see [1–3]. By morphological, biochemical and immunological criteria proteasomes are identical with prosomes [4,5], 19–20 S ribonucleoproteins, supposed to be involved in the regulation of gene expression, and also with cylindrin described more than 20 years ago as a major component of human erythrocytes [6]. Proteasomes are ubiquitous in eukaryotic cells, while there is no evidence for their existence in eubacteria [7,8]. We have recently shown, however, that proteasomes occur in the archaebacterium *Thermoplasma acidophilum* [9]. Unlike eukaryotic proteasomes, which all appear to have a rather complex subunit composition with 8–16 different polypeptides, the archaebacterial proteasome is made of two polypeptides (α and β) only. In spite of the much simpler subunit composition of the archaebacterial proteasome, which facilitates a detailed structural analysis, proteasomes from eukaryotes and *Thermoplasma acidophilum* are almost identical in size and shape as shown by electron microscopy in conjunction with digital image processing [9,10].

The structural models suggested so far are based on the intuitive interpretation of electron micrographs, which usually show proteasomes in two basic orientations: ring-shaped and rectangular, the latter with a characteristic striation pattern. While some authors conclude from these two projections that the molecule is basically a hollow cylinder [6,11,12], others describe it as ring-shaped or as an ellipsoid [13]. Since two-dimensional (2-D) crystals suitable for three-dimensional (3-D) reconstruction by established electron crystallographic techniques are not available so far, we have performed a 3-D reconstruction from individual molecules using the method of random conical tilting [14]. This method takes advantage of the observation that proteasomes adsorb in a few preferred orientations onto specimen supports; moreover it allows the collection of a 3-D data set under low dose conditions.

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2. MATERIALS AND METHODS

Proteasomes from *Thermoplasma acidophilum* were purified to homogeneity as described previously [9]. The particles were negatively stained with 2% ammonium molybdate at a pH of 5.3. Micrographs were taken using a Philips EM 420 at a nominal magnification of 36000 and an electron dose of about 2000 e/nm^2. Two micrographs of a given specimen area were recorded, the first one with the specimen tilted to 60° and the second one with the specimen untilted.

Random conical tilt reconstruction was performed basically as outlined in [15]. This method is particularly suitable for the 3-D reconstruction of individual particles which occur on the specimen support in a single or in a few preferred orientations. On the micrograph of the untilted specimen each particle appears as a repeat of the same 'motif', differing from all the others only by translation, in-plane rotation, and noise. The micrograph of the tilted specimen provides a set of different particle projections which can be combined in order to obtain the 3-D reconstruction. When seen from the reconstructed body, the projection directions are randomly distributed on the surface of a virtual circular cone with its vertex centered in the reconstructed body. While the half angle of the cone equals the

specimen tilt angle, the azimuthal angles of the projection directions can be calculated from the in-plane rotation angles and from the azimuthal angle of the tilt axis. The method requires a 2-D alignment with respect to translation and rotation for all the individual particle images extracted from the micrograph of the untilted specimen.

Pairs of micrographs were selected for digital image processing according to the following criteria: both micrographs must cover the same or overlapping specimen areas and these must show a reasonable particle density and distribution. The focus setting and, in the case of the micrograph from the tilted specimen, the focus range must remain within tolerable limits. Such areas were digitized using an Eikonix 1412 camera system. The digitized pictures were represented by 2048 × 2048 pixel arrays with a pixel size of 15 μm which corresponds to 0.42 nm at the specimen level. From the micrograph with 0° tilt typically 200–300 particles were extracted interactively after suitable high and low pass filtering. As a result, a set of 64 × 64 pixel subframes was obtained, each one showing an individual proteasome. This set of data was subjected to a procedure for translational and rotational alignment using cross-correlation functions.

In order to define the tilt axis, the positions of about 10 particles were measured, both in the image of the tilted and of the untilted specimen. Assuming these coordinates to be related by a linear mapping, actual tilt angles and tilt axis azimuths were estimated by means of a least squares approximation. This procedure corrects for any offsets from nominal tilt angles, changes of the tilt axis azimuth and difference of magnification between the two images. With the knowledge of these parameters the position of each particle on the micrograph of the tilted specimen was calculated from its position in the corresponding 0° projection, making use of the positional refinement obtained in the course of the alignment. After extracting the subframes (64 × 64 pixel) from the nominal 60° tilt image, a set of projections was available for the 3-D reconstruction.

The 3-D reconstruction itself was effected by means of filtered back-projection. Because of the random distribution of projection directions, an exact filter function was calculated using the sinc-function [14]. Instead of handling each projection separately, the filtering was performed only once in three dimensions, thus reducing computing time. The software package 'EM' was used for the alignment and the 3-D reconstruction [16].

3. RESULTS AND DISCUSSION

On electron micrographs recorded with untilted specimens, the proteasomes from *Thermoplasma acidophilum* exhibit two basic aspects: ring-shaped, with a diameter of approximately 11 nm, and rectangular, with long and short axes of 15 and 11 nm, respectively. The preponderance of one or the other aspect apparently depends on a complex interplay of factors; amongst them are the pretreatment or 'conditioning' of the supporting carbon film, the particular negative stain used as well as the presence or absence of specific ions such as calcium. Tanaka et al. [13] conclude from electron micrographs of proteasomes negatively stained with uranyl acetate that the molecules are basically ring-shaped with a diameter of 16 nm and a central hole of 1–2 nm. They claim that phosphotungstate when used as negative stain induces the rings to assemble to cylinder-shaped structures of four rings [17]. We have previously shown that the low pH of uranyl acetate significantly alters the shape and the dimensions of the particles [10]. On the other hand, the use of several other negative stains as well as of embedding media such as aurothioglucose has invariably yielded images in which rectangular side-views and ring-shaped end-on views coexist, although at somewhat variable proportions. The same holds for freeze-dried and metal shadowed preparations [11]. Hence there is ample evidence that the two views are related to each other and represent one and the same particle in different orientations with respect to the supporting film. Therefore one can envisage the particle to a first approximation as cylinder-shaped, i.e. the ring-shaped aspect shows the cylinder end-on and the rectangular aspect shows it side-on with the cylinder axis parallel to the supporting film.

Previous 2-D analysis gave very distinct mass distributions within the particles upon averaging over large numbers of side-views of individual molecules. This indicates that particles in the side-on orientation do not freely rotate or wobble around the cylinder axis but rather prefer one or a few stable positions. This is a prerequisite for succesful use of the random conical tilting approach.

We have performed several independent reconstructions from particles in the side-on orientation; they all gave very similar results and therefore only one of them is fully documented in this communication. In Fig. 1a,b the same specimen area is shown as it appears in both, the image of the untilted object and the tilt image. These pictures represent the raw data of the 3-D reconstruction shown in Fig. 2. A total of 227 proteasome side-views was available after extracting particles from the whole 0° tilt image and subjecting them to the 2-D alignment procedure. The 2-D correlation average (Fig. 3a) of these 227 proteasome images is virtually identical with previously published averages [9]. Using the radial correlation function as a criterion the resolution is approximately 1.5 nm. Correction of tilt angle offsets as described in Materials and Methods gave an actual tilt angle of 57.2° for the micrograph of the tilted specimen, from which the corresponding 227 particle projections were extracted.

Although the reconstructions performed with several independent data sets turned out to be quite reproducible, the results are not yet entirely satisfactory. When 2-D averages of proteasome side views are compared with the corresponding projections through the 3-D reconstructions there is obviously some loss of resolution (Fig. 3a,b). As a result, several features of the 3-D reconstruction appear somewhat blurred, hitherto preventing a detailed interpretation of subunit shapes and positions. Some reasons for the loss of resolution encountered in proceeding from two dimensions to three dimensions are discussed below. Moreover, it is evident from the horizontal sections of the reconstruction displayed in Fig. 2 that in proceeding from top to bottom section the structure tends to fade out some distance below the plane of the cylinder axis, i.e. the bottom part of the molecule remains invisible. This is

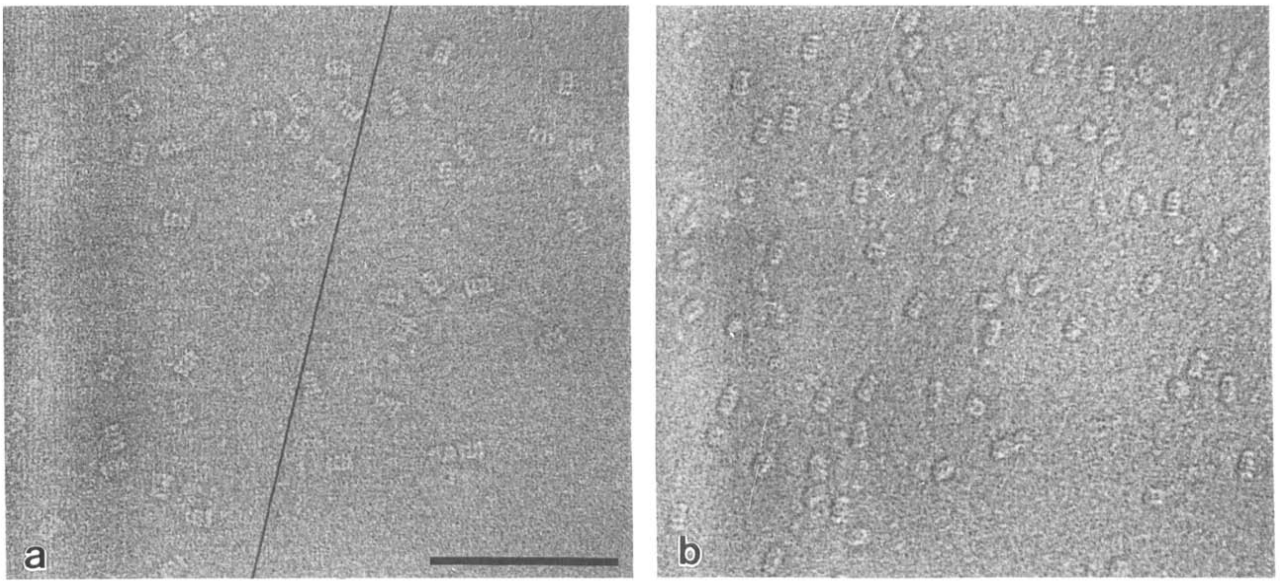

Fig. 1. Electron micrographs of negatively stained proteasomes from *Thermoplasma acidophilum*. Both images show the same specimen area, extracted (a) from the image of the untilted specimen, (b) from the image of the specimen tilted by 57.2°. The straight line in Fig. 1a indicates the direction of the tilt axis. Bar: 100 nm.

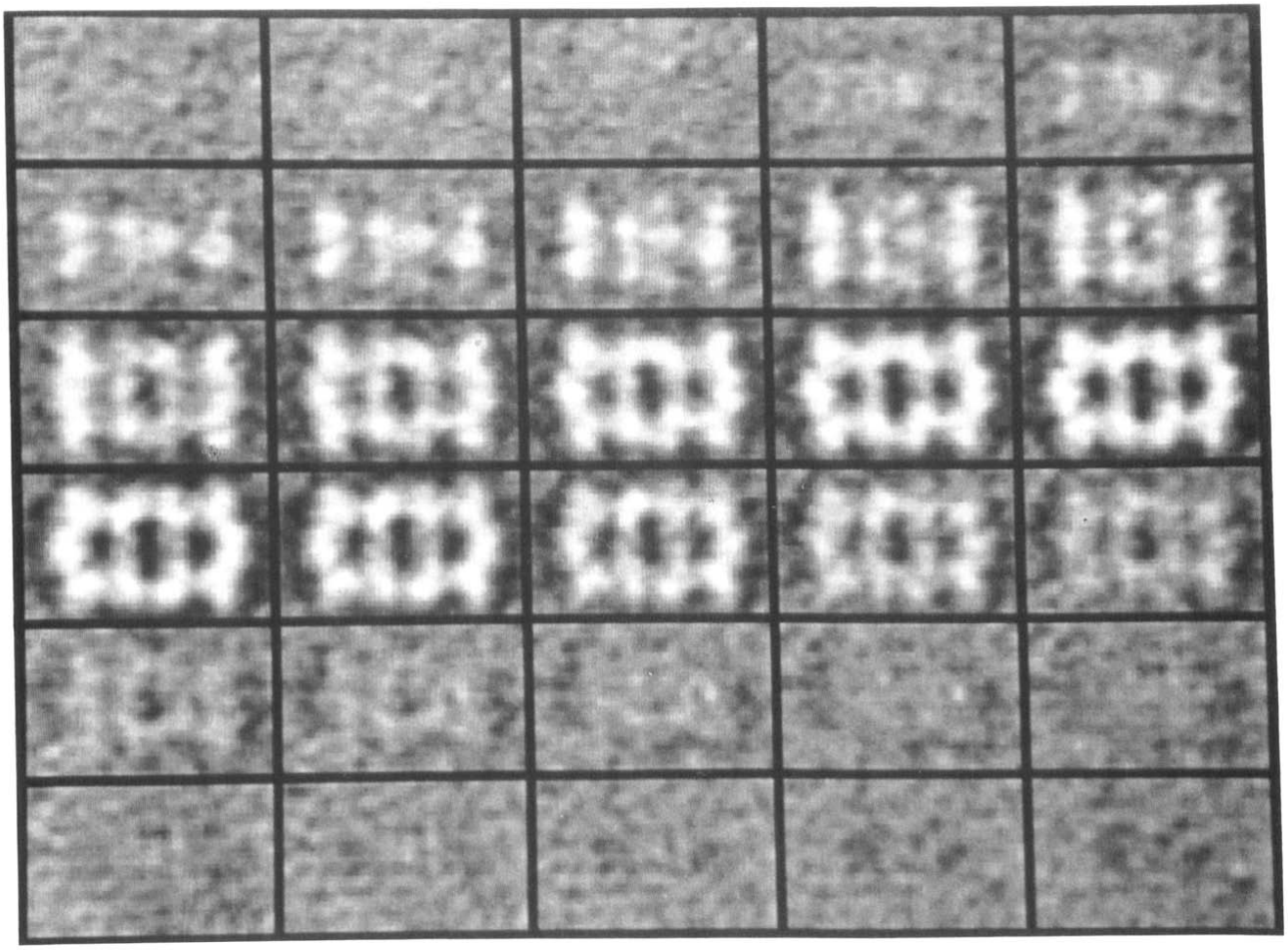

Fig. 2. 3-D reconstruction of the *Thermoplasma acidophilum* proteasome, represented as a gallery of sections 0.42 nm apart through the reconstructed object. Since the particle is in side-on orientation, the sections are parallel to the cylinder axis as well as to the specimen support. Bright regions indicate the presence of stain excluding material, i.e. protein.

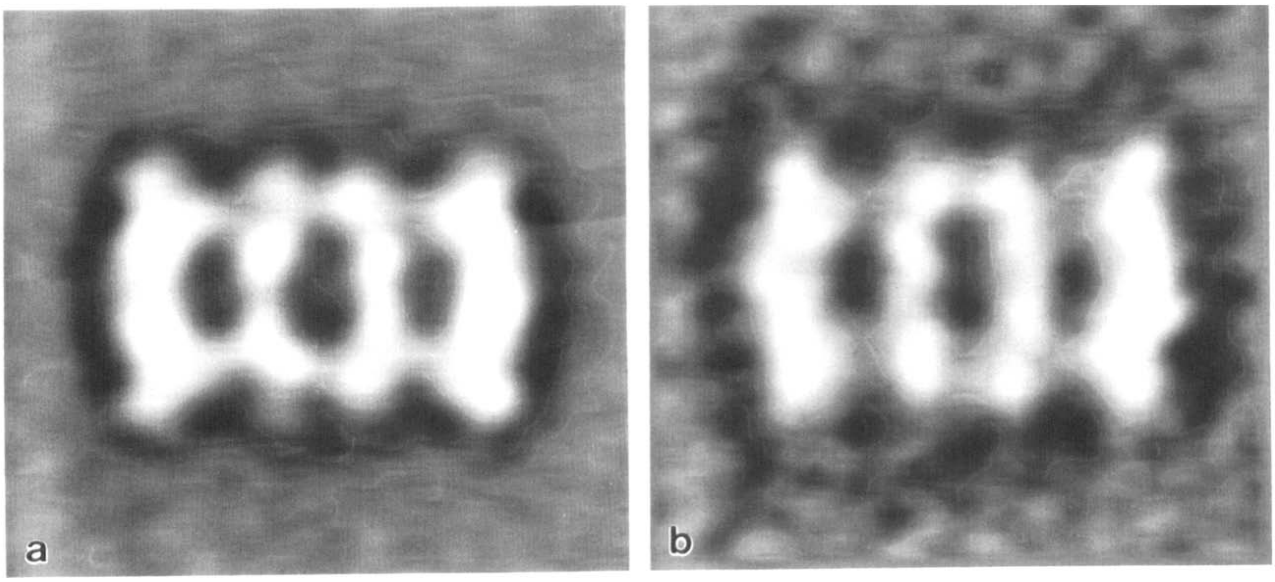

Fig. 3. Comparison of 0° projections: (a) 2-D correlation average of all particles extracted from the image of the untilted specimen. (b) Corresponding projection calculated from the 3-D reconstruction shown in Fig. 2.

probably due to an incomplete embedding of the particle in negative stain.

Nevertheless, the reconstruction reveals some new structural features of the proteasome which significantly change the prevailing structural concepts. The most important finding is that the proteasome cannot be pictured as a hollow cylinder with open ends made of four rings but rather as a cylinder-shaped barrel that is closed by two 'discs' containing most of the particle mass. This is in line with an earlier observation based on end-on views of molecules conically coated with platinum [11]. The inner 'discs' or 'rings', forming the central part of the particle, are less densely packed structures and apparently allow the negative stain to penetrate inside the particle. Although the 'rings' and 'discs' are too blurred to be interpreted in terms of subunit organization, a tripartite inner compartment can clearly be recognized: the central compartment is the largest and is bounded by the inner 'rings', and the two peripheral compartments are each delimited by one inner 'ring' and one outer 'disc'.

There are several possible reasons for the loss of resolution encountered in the 3-D reconstruction, each of which must be analyzed carefully in order to improve the situation. The three main factors are probably inaccuracies in the alignment, the influence of the focus gradient and inter-particle orientational changes. From image analysis including image classification by multivariate statistical analysis of the 0° tilt projection, it is unlikely with the present preparation that orientational changes, i.e. rotations around the cylinder axis, are a major cause for the loss of resolution since they would affect the 2-D averages equally. The accuracy of the alignment was examined in the course of an additional refinement of the 3-D alignment: calculating projections from the model with the use of all 227 actual projection directions permitted a comparison with the measured projections by cross-correlation functions (Fig. 4). The displacements indicated by the shifts of the cross-correlation peaks did not exceed one pixel in the average and, correspondingly there was essentially no improvement in the refined reconstruction. Apart from confirming a correct alignment, this result also proves that the specimen support is flat. Otherwise larger displacements should occur in some regions of the original image area.

Concerning the focus gradient, we have to expect a significant limitation of the resolution. The area of the digitized image corresponds to a square with a side length of 853 nm at the object level. Tilting the object by an angle of 60° creates a difference in height of 740 nm between two opposite sides of the square, assuming the tilt axis to be parallel to these sides (actually there is a 14° deviation). The effect of the corresponding focus difference depends on the absolute value of the focus. Calculating the contrast transfer function for extreme positions within the tilt image yields, in the worst case, a resolution limit of 2.5 nm due to intruding zeros. Although only a subset of the particles was imaged under these extreme conditions, the reconstruction probably suffers from degradation due to the focus gradient.

Due to these limitations, the result is in some aspects preliminary. We have attempted to overcome the incompleteness of the reconstructed particle originating from the imperfect stain embedding by a variation of the preparation technique, e.g. by mixing negative stain and particle suspension prior to the absorption onto the

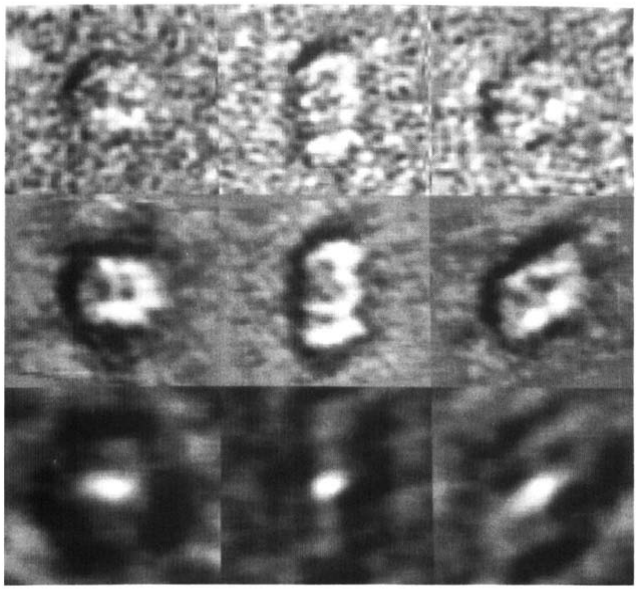

Fig. 4. Test of alignment. Upper row: three particles with different orientations extracted from the image of the tilted specimen. Middle row: projections calculated from the 3-D reconstruction using corresponding projection directions. Lower row: cross-correlation function between the measured projections of the upper row and the calculated projections in the middle row.

carbon film. The resulting 3-D reconstructions, not shown in this communication, appear to be more complete at the expense, however, of reduced resolution. This is probably caused by an increasing freedom of rotation around the cylinder axis and the resulting combination of misaligned particles in the 3-D reconstruction. Future work will involve a more sophisticated strategy including two essential improvements: firstly, negative staining must be replaced by preparation methods ensuring more complete embedding of the particles as well as an improved structure preservation, e.g. by using aurothioglucose. Secondly, the recently developed technique of dynamic focussing [18,19] will be used to correct for the focus gradient during image recording. With this method, a considerably larger number of molecular images can be extracted from the micrographs and used for image processing, due to the almost constant defocus everywhere in the tilt image. This is important because undesirable orientational changes are more likely to occur with such preparations. The availability, however, of a large number of particles, will allow to select particles sufficiently similar in orientation following classification by means of multivariate statistical analysis.

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REFERENCES

[1] Rivett, A.J. (1989) Arch. Biochem. Biophys. 268, 1-8.
[2] Orlowski, M. (1990) Biochemistry 29, 10289-10297.
[3] Scherrer, K. (1990) Mol. Biol. Rep. 14, 1-9.
[4] Arrigo, A.P., Tanaka, K., Goldberg, A.L. and Welch, W.J. (1988) Nature 331, 192-194.
[5] Falkenburg, P.-E., Haass, C., Kloetzel, P.-M., Niedel, B., Kopp, F., Kuehn, L. and Dahlmann, B. (1988) Nature 331, 190-192.
[6] Harris, J.R. (1968) Biochim. Biophys. Acta 150, 534-537.
[7] Arrigo, A.P., Simon, M., Darlix, J.L. and Spahr, P.F. (1987) J. Mol. Evol. 25, 141-150.
[8] Zwickl, P., Pfeifer, G., Lottspeich, F., Kopp, F., Dahlmann, B. and Baumeister, W. (1990) J. Struct. Biol. 103, 197-203.
[9] Dahlmann, B., Kopp, F., Kuehn, L., Niedel, B., Pfeifer, G., Hegerl, R. and Baumeister, W. (1989) FEBS Lett. 251, 125-131.
[10] Baumeister, W., Dahlmann, B., Hegerl, R., Kopp, F., Kuehn, L. and Pfeifer, G. (1988) FEBS Lett. 241, 234-245.
[11] Kopp, F., Steiner, R., Dahlmann, B., Kuehn, L. and Reinauer, H. (1986) Biochim. Biophys. Acta 872, 253-260.
[12] Kleinschmidt, J., Escher, C. and Wolf, D.H. (1988) FEBS Lett. 239, 35-40.
[13] Tanaka, K., Yoshimura, T., Ichihara, A., Ikai, A., Nishigai, M., Morimoto, Y., Sato, M., Tanaka, N., Katsube, Y., Kameyama, K. and Takagi, T. (1988) J. Mol. Biol. 203, 985-996.
[14] Radermacher, M., Wagenknecht, T., Verschoor, A. and Frank, J. (1986) J. Microsc. 141, RP1-RP2.
[15] Radermacher, M. (1988) J. Electr. Microsc. Tech. 9, 359-394.
[16] Hegerl, R. and Altbauer, A. (1982) Ultramicroscopy 9, 109-116.
[17] Tanaka, K. and Ichihara, A. (1990) Cell Struct. Funct. 15, 127-132.
[18] Zemlin, F. (1989) J. Electron Microsc. Techn. 11, 251-257.
[19] Typke, D., Pfeifer, G., Hegerl, R. and Baumeister, W. (1990) Proc. 11th Int. Congr. Electr. Micros., Seattle, pp. 244-245.