ELSEVIER

Ultramicroscopy 85 (2000) 123–129

ultramicroscopy

www.elsevier.nl/locate/ultramic

Parameters affecting specimen flatness of two-dimensional crystals for electron crystallography

Janet Vonck*

Max Planck Institut für Biophysik, Heinrich-Hoffmann-Strasse 7, D-60528 Frankfurt/Main, Germany

Received 1 February 2000; received in revised form 7 June 2000

Abstract

The flatness of two-dimensional (2D) crystals on the support film is a critical factor in protein electron crystallography. The influence of the carbon support film and of different grid makes and materials on flatness was investigated, using as a criterion the sharpness of diffraction spots perpendicular to the tilt axis of electron diffraction patterns of purple membrane tilted in the microscope at 45°. In a quantitative test, carbon film that had been evaporated without sparks forming gave a much larger proportion of flat crystals than "sparked" carbon. Titanium grids were superior to copper, probably because they introduce less cryo-crinkling of the carbon film when the sample is cooled to liquid nitrogen temperature, as their thermal expansion coefficient is closer to that of carbon. While the molybdenum grids from Plano were unsuitable for data collection because of their tendency of break the carbon, molybdenum grids from Pacific GridTech gave a much larger yield of flat crystals than the titanium grids. Scanning electron microscope images of the grids as supplied by the manufacturer showed that the Plano grids had very narrow and irregular grid bars, while the Pacific GridTech grids were very smooth with a large surface-to-hole ratio.

Keywords: Electron diffraction; Specimen preparation and handling; Cryomicroscopic methods

1. Introduction

One of the main problems in data collection of two-dimensional (2-D) protein crystals for electron crystallography is a lack of flatness of the specimens. The resulting overlap of data from crystal areas of slightly different tilt angles (visible as blurring of the diffraction spots in the direction perpendicular to the tilt axis) is especially severe at high tilt angles and effectively reduces the vertical resolution of a three-dimensional (3-D) map. It has been shown for bacteriorhodopsin that a tilt angle difference of 1° over a 20 μm crystal area is the maximum allowed [1]. For crystals with a larger unit cell and thus smaller spacing between the diffraction spots, the restrictions are more severe.

Lack of flatness can be due (i) to long-range bending of the support film, (ii) to short-range unevenness of the film, (iii) to small particles trapped between the support film and the crystal,

*Corresponding author. Tel.: +49-69-96769-357; fax: +49-69-96769-359.

E-mail address: vonck@mpibp-frankfurt.mpg.de (J. Vonck).

0304-3991/00/$ - see front matter
PII: S0304-3991(00)00052-8

or (iv) to improper attachment of the crystal to the support. These causes cannot be distinguished on a diffraction pattern, although some of them (e.g., broken support film, presence of foreign particles) can of course be recognized at the stage of a low-magnification search of the grid. For a successful data collection session all of these factors have to be controlled, and the specimen preparation has to be optimized in an empirical way for each specimen. However, many factors are common to all or many specimens and several approaches have been described which help to avoid one or another of the above mentioned sources of problems.

The properties of the carbon film play an important role in determining specimen flatness. Butt et al. [2] showed by AFM that the mica side of a carbon film is smoother than the air side, and that multiple evaporation produces smoother films than single-burst evaporation. Han et al. [3] found that flat purple membranes could be prepared only on films made from certain carbon stocks, although the reason for this (purity of the carbon?) was not evident from the information supplied by the manufacturers.

The problem of attachment of the crystals to the support film was initially tackled by varying the hydrophobicity of the carbon film either by ageing, glow discharge or baking [4–6] or by varying the amount of detergent-containing sample on the grid depending on the hydrophobicity [4]. An important improvement was the "back-injection method" [6], where the mica-facing side of the carbon film is used immediately after stripping the film from a small piece of mica onto a glucose solution. The carbon is picked up with a grid and the sample is applied through the grid bars. Blotting is done by pressing the grid against filter paper. This method is now routinely used in different laboratories [7–9]. Important factors that have to be optimized include the glucose (or other sugar) concentration, the amount of sugar remaining on the films, the amount of sample that is added, and the amount of sugar/sample mixture remaining before blotting.

Further, the attachment of the film to the grids plays a role. Wrinkling was found to be more pronounced on the shiny side of a copper grid than on the dull side [10].

The next problem is the so-called "cryo-crinkling", the effect that an initially flat film on a grid wrinkles when it is cooled to liquid nitrogen temperatures, due to the mismatch in thermal expansion coefficient between the carbon and the grid metal. Booy and Pawley [11] showed that this effect can be avoided by the choice of the right grid material. They found that molybdenum and tungsten were most effective. Molybdenum grids have been widely used since then [9,12–14]. The quality of molybdenum grids has been greatly variable, though, with some batches containing almost exclusive grids with a very rough surface, which not only is detrimental for specimen flatness, but also causes the carbon films to break.

Preparation of flat crystals is still a matter of trial and error. Many, often unknown, parameters influence the success rate. The factors influencing success can be roughly split up into three parts: the carbon film, the grid, and the preparation method. In this paper, a method is described that works for preparing flat purple membranes as well as LHCII crystals, and the relative contribution of several factors in the process is quantified. The suitability of different grids is compared, and the surface of the grids studied by scanning electron microscopy (SEM).

2. Experimental procedures

2.1. Materials

Carbon films of approximately 150 Å thick were prepared in an Edwards Auto 306 evaporator at a vacuum between 2×10^{-7} and 5×10^{-6} Torr onto freshly cleaved mica (Pelco Mica, Ted Pella). Evaporation was interrupted when the vacuum rose above this value and the voltage was adjusted so that no sparking was visible during evaporation. Films where sparking had occurred were also kept and used as a control. The carbon source were Hitachi Spectroscopic Graphite Electrodes (Hitachi Chemical Co. Ltd., Tokyo). Edwards rods E085-19-030 were also used.

Bacteriorhodopsin F219L mutant crystals were prepared and fused by standard methods [1,15].

LHCII from spinach was isolated and crystallised by standard methods [7].

Specimen support grids were 400 mesh copper grids (G2400C, Agar), 300 mesh titanium grids (1GT300, Plano), molybdenum grids 1GM200 and 1GM300 (Plano) and 300 mesh molybdenum grids from Pacific GridTech (Palo Alto, CA).

2.2. Specimen preparation

Grids were prepared essentially by the back-injection method [6] with some modifications. 3 mm × 3 mm pieces of carbon were floated off on a clean water surface, picked up from below with a clean grid, and transferred to the surface of a 4.5% glucose solution. Alternatively, the film was stripped off directly on the glucose solution and picked up from there. The grid plus carbon film were lifted away from the glucose, excess liquid removed until a thin layer remained, and 1 μl of purple membrane solution was added from the grid side and mixed with the glucose. The grid was blotted by pressing onto Whatman no. 4 filter paper, plunged into liquid nitrogen and transferred to a Gatan cryo specimen holder. LHCII grids were prepared by essentially the same method, using 0.5% tannin instead of glucose, and adding 2.5 μl of LHCII crystals. Preparations were done in a room with constant temperature and constant very low (10–20%) humidity.

2.3. Evaluation of flatness

Samples were investigated in a Philips CM12 or CM120 electron microscope at 120 kV. The grids were searched in defocused diffraction mode for suitable crystals that were not obviously wrinkled or bent. The flatness of those crystals was judged by visual inspection of their diffraction pattern at a tilt angle of 45°, either on a Gatan 626 TV system or on a TVIPS 1k × 1k slow-scan CCD camera, and comparing the presence and sharpness of spots along the tilt axis and perpendicular to the tilt axis. The illumination conditions were such that the patterns were visible for several minutes before fading. Flatness was graded on a four-step scale:

(a) Spots as sharp perpendicular as along the tilt axis.
(b) Spots perpendicular to the tilt axis slightly blurred.
(c) Spots perpendicular to the tilt axis badly blurred.
(d) Spots perpendicular to the tilt axis not visible or overlapping.

Examples are shown in Fig. 1.

The following combinations of conditions were tested:

(1) Sparkless carbon, floated off on water, picked up with a titanium grid.
(2) Sparkless carbon, floated off on glucose, picked up with a titanium grid.
(3) Sparked carbon, floated off on water, picked up with a titanium grid.
(4) sparkless carbon, floated off on water, picked up with a copper grid.

All conditions were used in a single day, and on different days the order of the samples was varied, to avoid the effect of atmospheric conditions varying between days or in the course of a day.

2.4. SEM

Grids as supplied by the manufacturer were examined in a Hitachi S5000 FEG scanning electron microscope at 5.0 kV at magnifications of 20–5000 × .

3. Results

By trial and error a method was found that yielded a reasonable proportion of flat crystals. Plano molybdenum grids (both 200 and 300 mesh) caused a very large proportion of broken carbon films and the crystals found on them were never flat. The most successful combination was Hitachi carbon evaporated without sparking, floated off on water, and picked up with a titanium grid and then transferred to a glucose or tannin solution. This procedure yielded a relatively large percentage of flat films for both bacteriorhodopsin and LHCII.

To check if all these factors were essential they were all varied independently and the number of

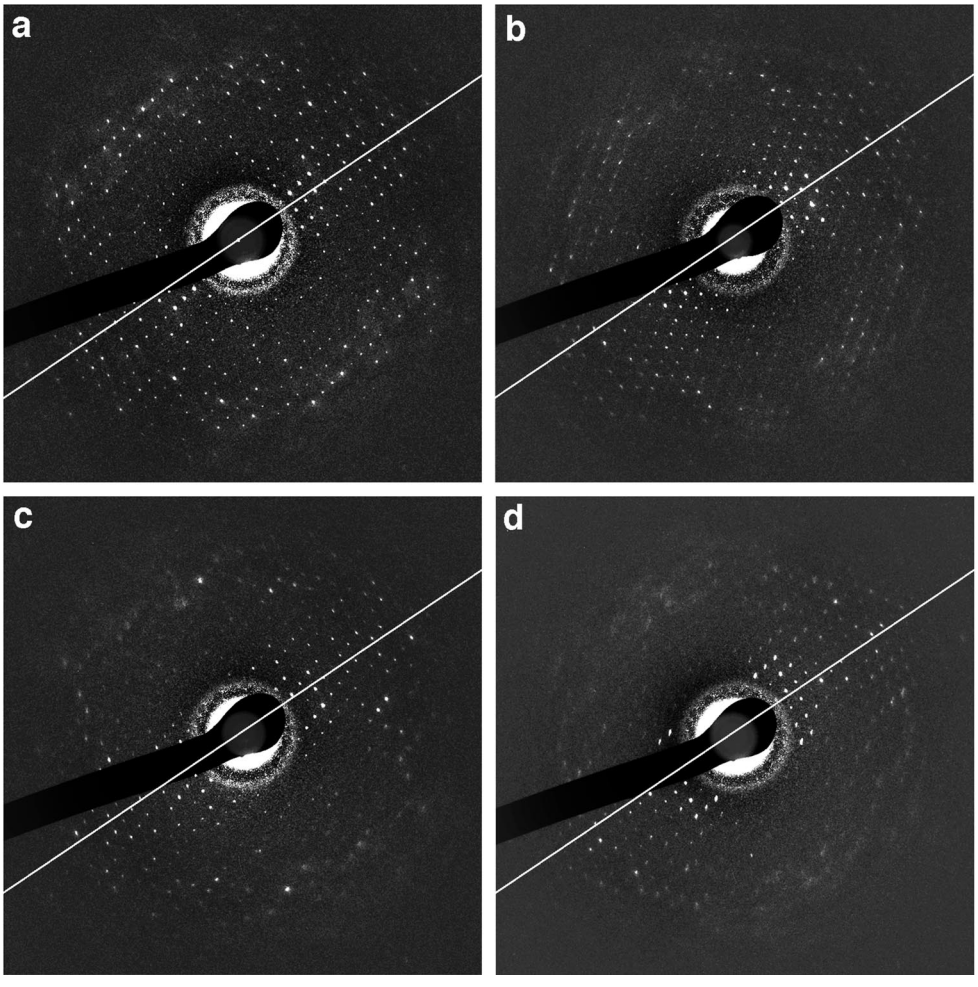

Fig. 1. Examples of diffraction patterns of bacteriorhodopsin crystals at 45° tilt. The patterns were collected on a slow-scan CCD camera, the radial background was subtracted. White lines indicate the tilt axis: (a) flat crystal (flatness grade "a"); (b) almost flat crystal (grade "b"); (c) wrinkled crystal (grade "c"); and (d) badly wrinkled crystal (grade "d").

flat crystals was counted, using bacteriorhodopsin as the test material. Examples of electron diffraction patterns are shown in Fig. 1. The results are shown in Fig. 2. It should be noted that the total number of crystals is not the same in all cases because on some grids much of the carbon was broken. Taking grade a and b together as "acceptable flatness" and c and d as "insufficient flatness" the success rate is 53% for the ideal conditions, 12% for the sparked carbon, 38% for the glucose-floating and 26% for the copper grids. These results were reproducible between days, as shown in Table 1.

Using the same preparation method with a batch of Pacific GridTech 300 mesh molybdenum grids, there was a much higher number of crystals that did not show any obvious signs of folds or wrinkles in a low-magnification search, probably because of the closer correspondence in expansion coefficients between molybdenum and carbon. Also, these grids gave a considerably higher proportion of very flat crystals than the titanium grids. This effect was difficult to quantify objectively, because in the test of the titanium and copper grids all the crystals that were obviously not flat in a low-magnification search, a very

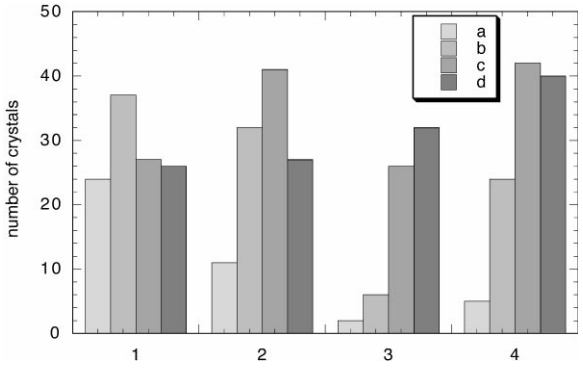

Fig. 2. Assessment of flatness of bacteriorhodopsin crystals prepared under different conditions: (1) sparkless carbon, floated on water, titanium grid; (2) sparkless carbon, floated on glucose, titanium grid; (3) sparked carbon, floated on water, titanium grid; and (4) sparkless carbon, floated on water, copper grid. Flatness categories a–d are as in Fig. 1.

substantial amount, were ignored. However, these grids were used for electron diffraction data collection of bacteriorhodopsin [16]; on average per grid 12 very flat (category a) single-layer crystals were found. The bacteriorhodopsin crystals have a large tendency to stack and single layers make up only about 20% of the sample, so on average overall ~ 60 flat crystals would have been present on molybdenum grids. As can be seen in Fig. 2, the titanium grids gave a total of 22 very flat (grade a) crystals over three grids, an average of ~ 7 per grid (both single layers and stacked crystals were counted in this experiment). From this, it can be concluded that the yield of flat crystals is $\sim 8\times$ higher on Pacific GridTech molybdenum grids than on titanium grids.

SEM inspection of the grid surfaces show that the Pacific GridTech grids are very smooth and have a relatively large metal area, with minimum width of the grid bars between round holes of approximately 10 μm (Fig. 3g and h). The Plano molybdenum grids, on the other extreme, are very rough and have a high number of extremely narrow grid bars of about 1 μm width (Fig. 3e and f). Copper grids are very smooth (Fig. 3a and b) whereas titanium grids are somewhat rough but have much flatter grid bars than the Plano molybdenum grids (Fig. 3c and d). Grid bars on both copper and titanium grids are approximately 10 μm.

Between Pacific GridTech molybdenum grids of the same batch, the hole size is somewhat variable (with all the holes on a single grid being very similar in size). It was found that carbon film breakage was much reduced on the grids with smaller holes and consequently larger surface-to-hole ratio.

4. Conclusions

Specimen flatness is one of the most important factors in determining the success of data collection from 2D crystals for electron crystallography. The combination of titanium grids, carbon evaporated without spark formation and floating off the carbon film on water prior to addition of glucose was shown here to give a reasonable success rate. The effect of each of these parameters separately was also investigated. It was found that all of these parameters had a positive effect, but to a reproducibly different degree.

The smallest effect was seen for floating off the carbon film on water versus glucose (the success rate went up from 38% to 53%). The reason for this is unclear. A possibility is a slight effect on the

Table 1
Percentage of crystals with acceptable flatness (grade a and b) under different preparation conditions for 3 independent experiments

	Water floating Sparkless carbon Titanium grid	Glucose floating Sparkless carbon Titanium grid	Water floating Sparked carbon Titanium grid	Water floating Sparkless carbon Copper grid
Day 1	50%	38%	0%	23%
Day 2	50%	21%	18%	33%
Day 3	62%	54%	8%	19%
Average	53%	38%	12%	26%

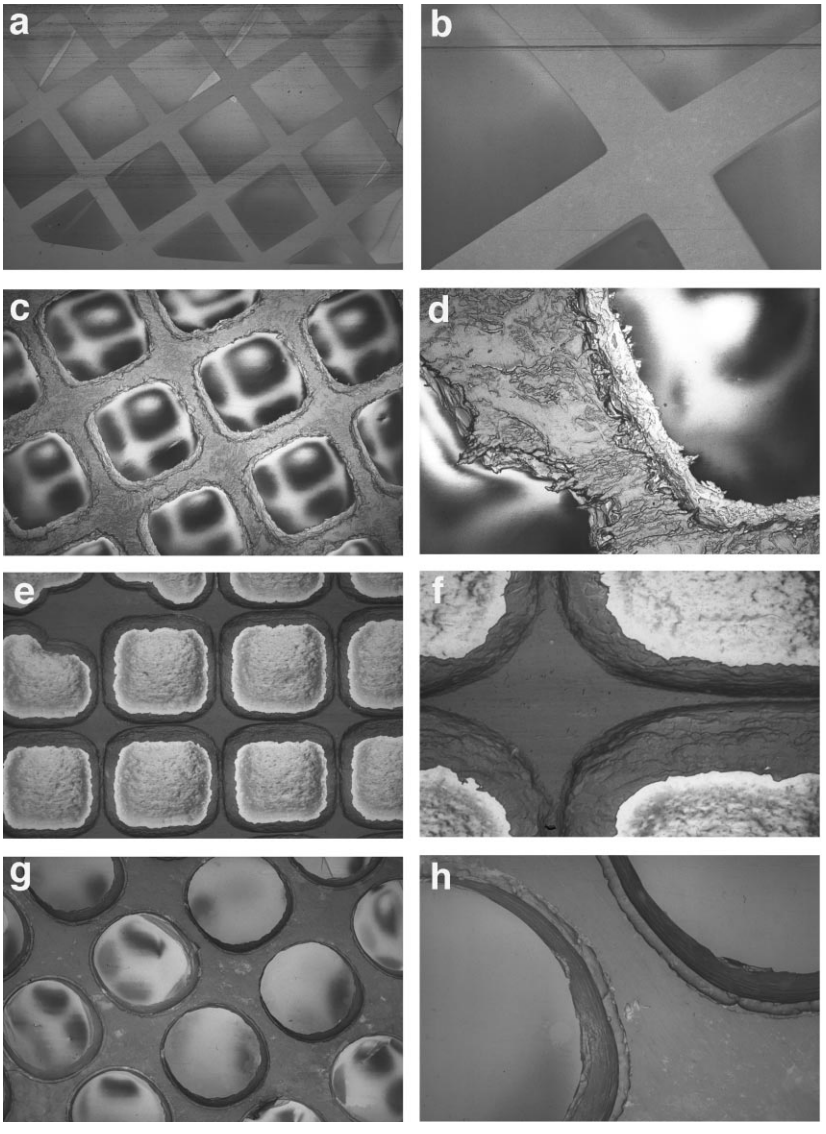

Fig. 3. SEM images of grids: (a, b) 400 mesh copper grid; (c, d) 300 mesh titanium grid 1GT300; (e, f) 300 mesh molybdenum grid 1GM300; (g, h) 300 mesh molybdenum grid from Pacific GridTech. Grid bars are 50 μm for the left panel and 10 μm for the right panel.

final glucose concentration, but as the glucose concentration is not controlled very accurately between different grids, this is difficult to test.

The most important contribution in obtaining flat crystals was the carbon film. On films obtained with sparking of the electrodes during evaporation, only 12% of the crystals were flat, compared to 53% on smooth carbon. In both cases, Hitachi carbon rods were used, because it proved very difficult to evaporate carbon without sparking from the Edwards rods. After an event of ''sparking'', macroscopic particles of carbon are found in the evaporation chamber. Probably smaller particles have also escaped from the rods and some must have reached the newly evaporated carbon film. In these experiments the sample is applied to the mica

side of the carbon, which was shown by AFM to be smoother than the air side [2]. The deposition of particles larger than the film thickness must also adversely affect the smoothness on the other side after floating off on a liquid surface.

A slightly smaller but highly significant effect was seen for copper versus titanium grids: 26% success with copper and 53% with titanium. This can be explained by the different expansion coefficients of carbon and the metals [11]. Titanium turns out to be a reasonable alternative for the previously used molybdenum grids, when no suitable molybdenum grids are available.

However, a further dramatic improvement is seen with Pacific GridTech molybdenum grids, compared to titanium grids, in the yield of flat crystals per grid. This confirms the finding that carbon showed less "cryo-crinkling" on molybdenum than on titanium grids [11].

SEM images reveal the difference between the Plano and the Pacific GridTech molybdenum grids. The latter have a very smooth surface and a large metal area between holes. The Plano grids, on the other hand, have a very irregular surface. Many grid bars are extremely narrow. It is easy to see why most of the carbon film breaks when deposited on these grids. The copper, titanium and Pacific GridTech molybdenum grids all have very similar surface areas with grids bars of about 10 μm wide which support the carbon film reasonably well, so that not much film breakage occurs. Some of the Pacific GridTech molybdenum grids have larger holes than the others at the same mesh size and these grids clearly contain less intact carbon. This confirms the importance of a large metal area for film integrity. These large-holed grids can be found and discarded before use by light microscope inspection. They comprise typically about 10% of a batch.

When the surface area of the grid is large enough, the yield of flat crystals is now no longer limited by the amount of material that can be found on a grid, but by the amount of cryo-crinkling, which is a function of the expansion coefficient. The low voltage SEM observation by Booy and Pawley [11] that molybdenum causes less crinkling of the carbon film than titanium and titanium less than copper is confirmed.

Acknowledgements

I thank Yoshinori Fujiyoshi for his advice on carbon evaporation and for the gift of Hitachi carbon, Kyong-Hi Rhee for the suggestion of using titanium grids, Werner Kühlbrandt for discussions, and Cécile Breyton for comments on the manuscript. I am grateful to Felicia Hendrickson for preparing the purple membrane stock, Ulrike Geldmacher-Kaufer for preparing LHCII, Deryck Mills for technical assistance, and Manfred Ruppel for performing the SEM microscopy. This work was supported by SFB Sonderforschungsbereich 472.

References

[1] J.M. Baldwin, R. Henderson, Ultramicroscopy 14 (1984) 319.
[2] H.J. Butt, D.N. Wang, P.K. Hansma, W. Kühlbrandt, Ultramicroscopy 36 (1991) 307.
[3] B.-G. Han, S.G. Wolf, J. Vonck, R.M. Glaeser, Ultramicroscopy 55 (1994) 1.
[4] R. Henderson, J.M. Baldwin, T.A. Ceska, F. Zemlin, E. Beckmann, K.H. Downing, J. Mol. Biol. 213 (1990) 899.
[5] R.M. Glaeser, A. Zilker, M. Radermacher, H.E. Gaub, T. Hartmann, W. Baumeister, J. Microscopy 161 (1990) 21.
[6] R.M. Glaeser, K.H. Downing, L.D. Peachy, D.B. Williams (Eds.), XIIth International Congress for Electron Microscopy, Seattle, San Francisco Press, Inc, 1990, p. 98.
[7] D.N. Wang, W. Kühlbrandt, J. Mol. Biol. 217 (1991) 691.
[8] E. Nogales, S.G. Wolf, I.A. Khan, R.F. Ludueña, K.H. Downing, Nature 375 (1995) 424.
[9] J. Vonck, Biochemistry 35 (1996) 5870.
[10] M. Schmutz, A. Brisson, Ultramicroscopy 63 (1996) 263.
[11] F.P. Booy, J.B. Pawley, Ultramicroscopy 48 (1993) 273.
[12] W. Kühlbrandt, D.N. Wang, Y. Fujiyoshi, Nature 367 (1994) 614.
[13] T. Walz, T. Hirai, K. Murata, J.B. Heymann, K. Mitsuoka, Y. Fujiyoshi, B.L. Smith, P. Agre, A. Engel, Nature 387 (1997) 624.
[14] A. Cheng, A.N.v. Hoek, M. Yeager, A.S. Verkman, A.K. Mitra, Nature 387 (1997) 627.
[15] D. Oesterhelt, W. Stoeckenius, Methods Enzymol. 31 (1974) 667.
[16] J. Vonck, EMBO J. 19 (2000) 2152.