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Two-dimensional crystals of the Kdp-ATPase of *Escherichia coli*

Atsuko Hikikoshi Iwane[1,a], Ikuko Ikeda[b,c], Yoshiaki Kimura[a,*], Yoshinori Fujiyoshi[c,d], Karlheinz Altendorf[e], Wolfgang Epstein[f]

[a]*Protein Engineering Research Institute (PERI) 6-2-3 Furuedai, Suita, Osaka 565, Japan*
[b]*Faculty of Engineering Science, Osaka University, Machikaneyama 1-3, Toyonaka, Osaka 560, Japan*
[c]*International Institute for Advanced Research, Matsushita Electronic Ind. Co. Ltd., 3-4 Hikaridai, Seika, Kyoto 619-02, Japan*
[d]*Department of Biophysics Faculty of Science, Kyouto University, Oiwake, Kitashirakawa, Sakyou-ku, Kyoto 606-01, Japan*
[e]*Faculty of Biology and Chemistry, Microbiology, University of Osnabrück, D-49069 Osnabrück, Germany*
[f]*Department of Biophysics and Theoretical biology/Department of Biochemistry, University of Chicago, Chicago, IL 60639, USA*

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Abstract A variant form of the Kdp-ATPase of *Escherichia coli* was overproduced to a level approaching 37% of the protein in the inner membrane of this organism. Membranes from overproducing cells were prepared with an inside-out orientation. Incubation of the membranes on ice for 1–2 weeks in the presence of sodium vanadate resulted in the formation of two-dimensional crystals of the Kdp-ATPase. The calculated projection map of the p1 crystal form showed three prominent density peaks at a resolution of 22 Å. This technique is a useful and simple method to obtain low-resolution structures of membrane proteins.

Key words: Kdp-ATPase; P-type ATPase; Two-dimensional crystal; Crystallization; Electron crystallography; Membrane protein; Structure; Projection map

1. Introduction

The Kdp-ATPase (Kdp) found in the inner membrane of *Escherichia coli* is a P-type ATPase such as Na^+,K^+-ATPase, H^+,K^+-ATPase and Ca^{2+}-ATPase [1]. Kdp transports K^+ into the cell to maintain internal K^+ ions at appropriate levels when other K^+ transport systems cannot do so. Kdp consists of three subunits, KdpA, 59 kDa; KdpB, 72 kDa; and KdpC, 21 kDa. The KdpB subunit resembles the large subunit of other P-type ATPase and KdpC is similar to the small subunit of the Na^+,K^+-ATPase. However, no similarity between KdpA and other P-type ATPase has been reported. KdpA is a highly hydrophobic subunit that spans the membrane 10 times [2]. Preliminary experiments have suggested that KdpA and KdpB are stably associated only in the presence of the KdpC subunit [1]. Determination of the structure of this ATPase would help in understanding its mechanism as a cation pump.

Only a few high-resolution structures of membrane proteins analyzed by X-ray crystallography have been reported, mainly because of the difficulty in crystallization. Recently, a two-dimensional (2-D) crystallization method has been developed [3]. Electron crystallography can provide atomic, or near atomic structure analysis of membrane proteins [4,5]. However, the determination of suitable conditions for forming 2-D crystals is tedious, requiring lengthy efforts by trial and error. Formation of 2-D crystals requires: (1) conditions favoring the assembly of protein molecules in a planar field; (2) a high concentration of the protein in the field; (3) lateral mobility of the proteins in the field; (4) non-denaturing conditions; and (5) interactions among the protein molecules and/or with lipids.

In addition to forming 2-D crystals in the membrane, as exemplified by well-ordered arrays of the bacteriorhodopsin of *Halobacterium halobium* [6], it is also possible to form 2-D crystals of membrane proteins purified after solubilization ([7] for the H^+-ATPase; [8] for the Ca^{2+}-ATPase). Intermediary methods have been used for *sn*-glycerol-3-phosphate acyltransferase [9] and for Na^+,K^+-ATPase [10].

Overproduction of a protein will favor its assembly into the regular array needed for crystallographic analysis, because it satisfies all 5 required crystallization conditions mentioned above. This report presents a convenient technique for 2-D crystallization of membrane proteins that are expressed at a high level by the use of recombinant DNA techniques.

2. Materials and methods

2.1. Bacterial strain and growth conditions

The strain used for this work, TK2242/pSR5, expresses the *kdpA42* form of Kdp, one with a normal rate of transport but markedly reduced affinity for potassium [2,11]. The *kdpA42* mutation is present both on the chromosome and on pSR5, the latter being a pBR322 derivative with a 5.7 kb insert that includes the 4.9 kb *Eco*RI fragment encoding the *kdpFABC* operon plus about 250 bp of chromosomal sequences upstream from the *Eco*RI site upstream of KdpA that include all the promoter sequences for this operon. Media used are the minimal, phosphate-buffered media described by their potassium concentration in mM [12] such as K1, K5, K115, with glucose, 2 (g/l) as carbon and energy source. Growth was at 37°C with vigorous agitation.

Cells of strain TK2242/pSR5 were maintained on K5 minimal plates. A single colony was inoculated into 2 ml of K1 medium and grown until the turbidity (600 nm) reached about 0.3. To express Kdp, 1 ml of this culture was added to 1 l of prewarmed K0.25 medium. Control cells not expressing Kdp were prepared by adding 1 ml of the culture to 1 l of K115 medium. The bacteria were chilled and collected by centrifugation ($9000 \times g$) when the turbidity reached 0.6–0.8, and immediately used to prepare vesicles. All subsequent steps were performed at 0–4°C.

2.2. Preparation of membrane vesicles

3 g wet weight of cells were washed once with 200 ml of buffer A (Tris-HCl (pH 7.5), 50 mM; sucrose, 250 mM; dithiothreitol, 1 mM; phenylmethylsulfonyl fluoride, 1 mM), collected by centrifugation and washed once with 200 ml of buffer B (buffer A plus sodium-EDTA, 1.5 mM). After centrifugation, the cells were suspended in 15 ml of buffer B and passed twice through an Aminco French Pressure cell

*Corresponding author. Biomolecular Engineering Research Institute (PERI has been reorganized as BERI), 6-2-3 Furuedai, Suita, Osaka 565, Japan. Fax: (81) (6) 872 8210. E-mail: kimuray@beri.co.jp

[1]*Present address:* Faculty of Engineering Science, Osaka Unviersity, Machikaneyama 1-3, Toyonaka, Osaka 560, Japan.

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(FA-030) at 7000 lb/inch^2. The resulting lysate was diluted with 55 ml of buffer B and centrifuged again (9000×g, 10 min) to eliminate unbroken cells and other debris. Membranes in the supernatant were collected by ultracentrifugation (190000×g, 2 h). The inner membrane fraction of the pellet was obtained by sucrose-gradient centrifugation according to [13]. The inner membrane fraction was diluted with 4 vols. of buffer B and pelleted by ultracentrifugation to eliminate sucrose. Protein concentration of the fraction was determined with the DC protein assay kit (Bio-Rad). Approx. 500 μg of inverted inner membranes were obtained from 3 g of wet cells. Membranes were stored in liquid nitrogen until used.

2.3. Electrophoresis and immunoblotting

Proteins were analyzed using sodium dodecyl sulfate polyacrylamide electrophoresis (SDS-PAGE) gels containing 10% (w/v) acrylamide and 0.27% bisacrylamide [14]. The gels were stained with Coomassie Brilliant Blue R-250 (Daiichi Pure Chemicals Co. Ltd.) or a silver staining kit (Daiichi Pure Chemicals, Co. Ltd.). The amounts of proteins in Coomassie-stained gels were estimated by a laser densitometer (UltroScan XL, Pharmacia-LKB). For immunoblotting, proteins were electrophoretically transferred to a nitrocellulose membrane with staining as described [15]. The membrane was incubated first with an anti-Kdp antibody, then with biotin-conjugated 2nd anti-rabbit antibody, avidin and finally with biotin-conjugated peroxidase (Vectastain Elute ABC system, Vector lab, Burlingame, CA). Diaminobenzidine was used as substrate of peroxidase. Antibodies to the Kdp complex as well as to its subunits have been described [16].

2.4. Two-dimensional crystallization and electron microscopy

Inverted vesicles were suspended at 3 mg protein/ml in Tris-HCl (pH 7.5), 50 mM, and Na_3VO_4, 150 mM, on ice for 3–14 days. This preparation was diluted 5-fold with the same buffer immediately before preparing grids for electron microscopy. The diluted mixture of 2 μl was placed on a 400 mesh copper grid coated with a continuous carbon film made hydrophilic by glow discharge, and left for 30 s. The specimen was stained with 2.5 μl of uranyl acetate (2%, w/v) for 20 s. Electron micrographs were taken at 100 kV acceleration voltage and 40000 or 50000 magnification.

2.5. Immuno-electron microscopy

To confirm the presence of the Kdp-ATPase, immuno-electron microscopy was performed as described in [17] using polyclonal rabbit anti-Kdp antibodies [16] and goat anti-rabbit antibodies conjugated to 20 nm gold spheres. Membranes were fixed with 1% glutaraldehyde in HEPES-Tris-buffer (10 mM HEPES-2 mM Tris-3 mM EDTA (pH 7.5)), on carbon-coated nickel grids (200 mesh) for 5 min at room temperature and then washed several times with Tris-buffered saline (TBS, Tris-HCl (pH 7.5), 100 mM NaCl). Grids were floated on gelatin 3% (w/v) in TBS for 60 min at room temperature. After further washings with TBS, the membranes were incubated for 90 min with rabbit anti-Kdp antibodies diluted 1:100 or with normal rabbit serum (2 ml/ml) similarly diluted. After several further washings, the grids were incubated for 90 min with the gold-conjugated antibodies, then washed repeatedly followed by stabilization with uranyl acetate, 2% (w/v), and air drying. The grids were examined by electron microscopy as described above.

2.6. Image processing

The electron micrographs selected for analysis were those that yielded the best optical diffraction patterns. 12 images were analyzed by digitization with Perkin Elmer 1010GM at 7 μm step size or with the Leafscan45 (Leaf Systems, Inc.) at 5 μm step size. Images were processed as described in [18]. The best images were then corrected for distortion by the cross-correlation method described in [20].

3. Results and discussion

3.1. Overproduction of the Kdp-ATPase

In accordance with earlier observations [11] and as shown in Fig. 1A, expression of Kdp in inner membrane preparations was observed when strain TK2242/pSR5 was grown in low potassium (0.25 mM) medium but not when grown in high potassium (115 mM). Three specific protein bands corresponding to 70, 42 and 23 kDa were seen only when grown in

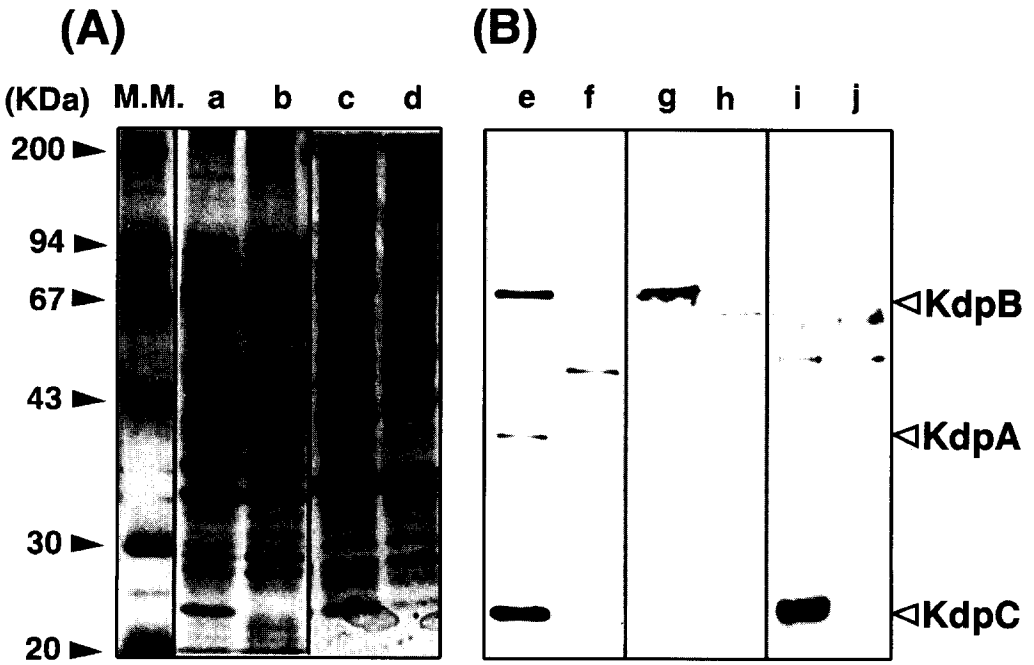

Fig. 1. SDS-PAGE of inner membranes of *E. coli* strain TK2242/pSR5 grown in media containing 0.25 mM KCl to express Kdp (a,c,e,g,i) or grown in media containing 115 mM KCl to prevents Kdp expression (b,d,f,h,j). Samples stained with Coomassie Brilliant Blue R-250 (a,b) or with silver (c,d). Immunoblotting with Kdp antibodies: anti-KdpABC (e,f); anti-KdpB (g,h); anti-KdpC (i,j). Approx. 40 μg protein was separated in lanes a,b; 8 μg in lanes c,d; 4 μg in lanes e,f.

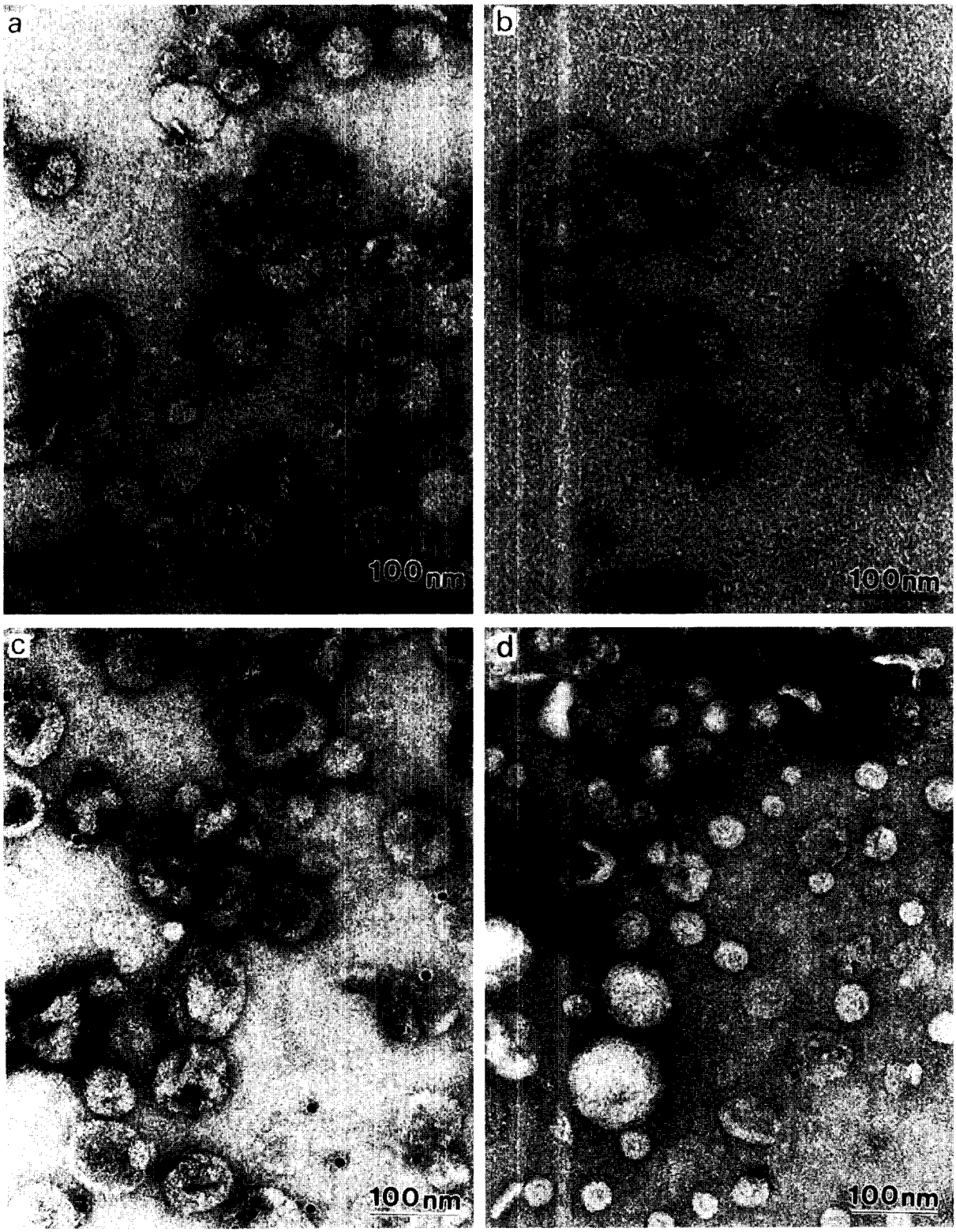

Fig. 2. Immunogold labeling of inner membranes grown in K0.25 medium to express Kdp (a,c) or in K115 medium to block expression of Kdp (b,d). Membranes were reacted with anti-KdpABC antibodies (a,b) or with anti-KdpC antibodies (c,d) and then with gold-conjugated goat anti-rabbit antibodies. Gold particles were observed only on the membranes grown in K0.25 medium (a,c); no significant gold particles could be observed on the membranes in K115 medium (b,d).

low potassium medium. These three represent the KdpB, KdpA and KdpC subunits, respectively, as demonstrated by the immunoblot of Fig. 1B. The low apparent molecular mass of KdpA, some 17 kDa smaller than its predicted size, is probably accounted for by its high hydrophobicity [16].

Coomassie-stained gels indicated that Kdp proteins repre-

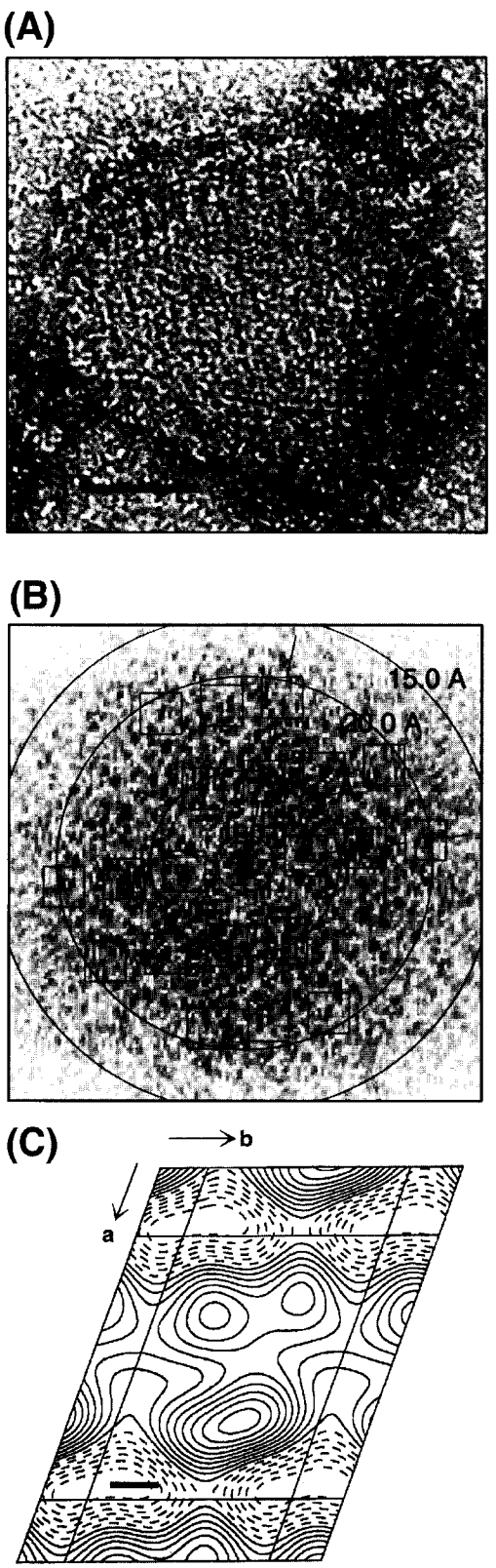

Fig. 3. (A) Electron micrograph of inner membranes expressing Kdp after incubation for 1 week in the presence of sodium vanadate. Membranes were stained with 2% uranyl acetate; the bar indicates 50 nm. (B) Fourier transform of the image in A. The squares indicate lattice positions. The observed lattice constants were $a = 65.8$ Å, $b = 46.8$ Å and $\gamma = 110°$. (C) Reconstituted projection map of a single unit cell of the crystals of A. The scale bar indicates 10 Å.

sent about 37% of the total protein of the inner membrane of the overproducing strain grown in low potassium medium but only a few associated with cells grown in high potassium medium (Fig. 2).

3.2. Crystallization of the Kdp-ATPase

Inside-out vesicles prepared from overproducing cells formed lattice structures after incubation in the cold with sodium vanadate for 1–2 weeks (Fig. 3A). No lattice structures were seen in right-side out vesicles of overproducing cells, or in inside-out vesicles from cells in which Kdp was not expressed. Vanadate favors the E2 conformation of P-type ATPases, and was chosen because it aided formation of 2-D crystals of the Na^+,K^+- and Ca^{2+}-ATPases [3].

3.3. Image analysis

Fourier transform analysis of the lattice structures revealed two types of crystals. One crystal type, of space group P1, had lattice constants of $a = 65.8$ Å, $b = 46.8$ Å, $\gamma = 110°$. The other type was of space group P2 with lattice constants of $a = 89.3$ Å, $b = 54.6$ Å, $\gamma = 90°$ (data not shown). The diffraction pattern of the P1 crystals showed strong reflections to a resolution of 20 Å (Fig. 3B) while the P2 crystal diffracted to a resolution of 35 Å (data not shown).

3.4. Structure of the Kdp-ATPase

The P1 type of crystal was analyzed by electron crystallography including crystal unbending and the final set of amplitudes and phases were used to calculate the projection map of Fig. 3C. The map shows one intense electron density peak and two of weaker but equivalent intensity. The lattice constants suggest that the unit cell consists of one Kdp-ATPase heterotrimer (153.5 kDa).

The crystals seen are undoubtedly related to Kdp, but their composition is not known since the protein was not purified. Specific antibodies were used to determine their location in the map of the acetylcholine receptor [19]. Better characterization of the composition in the crystals and identification in the final map is essential if this method is to be useful in determining the structure of membrane complexes consisting of more than one type of subunit.

References

[1] Altendorf, K., Siebers, A. and Epstein, W. (1992) Ann. N.Y. Acad. Sci. 671, 228–243.
[2] Buurman, E.D., Kim, K.-T. and Epstein, W. (1995) J. Biol. Chem. 270, 6678–6685.
[3] Kühlbrandt, W. (1992) Q. Rev. Biophys. 25, 1–49.
[4] Henderson, R., Baldwin, J.M., Ceska, T.A., Zemlin, F., Beckmann, E. and Downing, K.H. (1990) J. Mol. Biol. 213, 899–929.
[5] Kühlbrandt, W., Wang, D.N. and Fujiyoshi, Y. (1994) Nature 367, 614–621.
[6] Blaurock, A.E. and Stoeckenius, W. (1971) Nat. New Biol. 233, 152–155.
[7] Cyrklaff, M., Auer, M., Kühlbrandt, W. and Scarborough, G.A. (1995) EMBO J. 1416, 1854–1857.
[8] Toyoshima, C., Sasabe, H. and Stokes, D.L. (1993) Nature 362, 469–471.
[9] Wilkison, W.O., Walsh, J.P., Corless, J.M. and Bell, R.M. (1986) J. Biol. Chem. 261, 9951–9958.
[10] Tahara, Y., Ohnishi, S., Fujiyoshi, Y., Kimura, Y. and Hayashi, Y. (1993) FEBS Lett. 320, 17–22.
[11] Epstein, W., Whitelaw, V. and Hesse, J. (1978) J. Biol. Chem. 253, 6666–6668.
[12] Epstein, W. and Kim, B.S. (1971) J. Bacteriol. 108, 639–644.

[13] Osborn, M.J. and Munson, R. (1974) Methods Enzymol. 31, 642–653.
[14] Laemmli, U.K. (1970) Nature 227, 680–685.
[15] Harlow, E. and Lane, D. (1988) Antibodies, A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[16] Siebers, A. and Altendorf, K. (1988) Eur. J. Biochem. 178, 131–140.
[17] Kartner, N., Hanrahan, J.W., Jensen, T.J., Naismith, A.L., Sun, S., Ackerley, C., Reyes, E.F., Tsui, L.-C., Rommens, J.M., Bear, C.E. and Riordan, J.R. (1991) Cell 64, 681–691.
[18] Amos, L.A., Henderson, R. and Unwin, N. (1982) Prog. Biophys. Mol. Biol. 39, 183–231.
[19] Beroukhim, R. and Unwin, N. (1995) Neuron 15, 323–331.
[20] Henderson, R., Baldwin, J.M., Downing, K., Lepault, J. and Zemlin, F. (1986) Ultramicroscopy 19, 147–178.