G-protein-coupled receptors are integral membrane proteins that respond to environmental signals and initiate signal transduction pathways, which activate cellular processes. Rhodopsin, a well known member of the G-protein-coupled receptor family, is located in the disk membranes of the rod outer segment, where it is responsible for the visualization of dim light. Rhodopsin is the most extensively studied G-protein-coupled receptor, and knowledge about its structure serves as a template for other related receptors. We have gained detailed structural knowledge from the crystal structure (1), which was solved by X-ray crystallography in 2000 using three-dimensional crystals. Here we report a three-dimensional density map of bovine rhodopsin determined by electron cryomicroscopy of two-dimensional crystals. In our electron density map, all seven transmembrane helices were identified, and their arrangement is in agreement with the arrangement known from the crystal structure (1). In the retinal binding pocket, a density peak adjacent to helix 3 suggests the position of the $\beta$-ionone ring of the chromophore, and in its vicinity several of the bigger amino acids can be identified.

G-protein-coupled receptors (GPCRs) are a large group of integral membrane proteins that provide molecular links between extracellular signals and intracellular processes (2–7). Many neurotransmitters, hormones, and drugs produce their intracellular signaling through the mediation of G-protein-coupled receptors. The binding of a hormone or neurotransmitter causes a change in the structure of the receptor, which then activates a G-protein. Different members of the receptor family respond to different ligands, and the binding site for these ligands is in the membrane-embedded part of the protein (8, 9).

One of the most widely studied GPCRs is rhodopsin (1, 10–18). Rhodopsin, located in the retina disc membrane of the eye, is responsible for the visualization of dim light. It is composed of the protein opsin (~40 kDa) covalently linked to 11-cis-retinal through Lys296 of helix 7 (19). The chromophore 11-cis-retinal isomerizes upon light activation to its all-trans conformation, which changes the arrangement of the transmembrane helices (20). This triggers the signal transduction cascade via reactions of the G-protein transducin (21–24). Transducin (G$\text{t}$) transduces the visual stimuli by coupling the light-induced conformational change of rhodopsin to the activation of a phosphodiesterase that hydrolyzes cGMP to GMP. This leads to the closure of cGMP-dependent channels in the plasma membrane of the rod outer segment. The resulting hyperpolarization of the membrane lowers the rate of transmitter release in the synaptic part of the photoreceptor cell, triggering downstream responses in bipolar cells of the retina.

Knowledge of the structure of any of the receptors is crucial for the understanding of signal transduction. Rhodopsins have amino acid sequence and functional homology with other G-protein-coupled receptors and because the three-dimensional structures of membrane proteins are not readily available, computational modeling of GPCRs has been an active research area (25–28). A common structural feature of G-protein-coupled receptors is the presence of seven hydrophobic transmembrane helices. Conserved features in most G-protein-coupled receptors include sites of asparagine-linked glycosylation in the amino-terminal region (29), one or two palmitate-linked cysteines close to the carboxyl-terminal region (30), a disulfide bridge linking two extracellular loops (31, 32), and serine and threonine phosphorylation sites in the carboxyl-terminal region (12).

The seven transmembrane helices of rhodopsin were first observed by cryoelectron microscopy (cryo-EM) in a projection structure of two-dimensional crystals from bovine rhodopsin (33). Subsequently, a low resolution view of the helices was obtained from cryo-EM studies on two-dimensional crystals of frog rhodopsin (34). In addition, information was obtained by NMR (35–38), spin label EPR (39, 40), and disulfide formation rates (41, 42). Most detailed information we now gain from the crystal structure of rhodopsin, which was solved in 2000 (1),
indicates that rhodopsin is a prototypical member of subfamily A of GPCRs. They represent about 90% of all GPCRs. Here we report an electron density map of bovine rhodopsin obtained by cryoelectron microscopy of two-dimensional rhodopsin crystals with p222₁ symmetry. The usage of relatively small and not well ordered crystals made the process of structure determination challenging, although these crystals were better than the previous ones that were obtained from frog and bovine rhodopsin (43). Special attention was paid to the extraction of amplitudes and phases, since usable raw data were limited to a maximum tilt of 45°. In addition, in the refinement process an improved unbending procedure was applied. Data could be reconstructed to a final resolution of 5.5 Å in the membrane plane. Most important, the position of the molecule relative to the bilayer could be determined because of the symmetry of the two-dimensional crystal. All seven transmembrane helices were traced unambiguously, and in the retinal binding pocket we find additional density pointing out the position of the β-ionone ring. In order to be able to compare our electron density with the atomic data available today, we modeled the atomic x-ray structure (1) into our electron density map.

**EXPERIMENTAL PROCEDURES**

**Specimen Preparation and Electron Cryomicroscopy—**Isolation, crystallization, imaging, and preliminary crystal quality assessment of untilted specimen are described in more detail elsewhere (43). Briefly, rhodopsin was purified from bovine rod outer segments and solubilized in lauryldimethylamine oxide. The partially delipidated rhodopsin was dialyzed, and the rhodopsin electron diffraction data. A least squares fit was applied to calculate lattice lines, which were truncated manually at points where the scatter of the observed phases approached random or where the observed amplitudes were too small. A preliminary three-dimensional map was used to calculate references to unbend all images (e.g. distortions were removed according to the information given by these references) (program MAKETRAN (46)). Combination of all unbent images and subsequent refinement of all crystal parameters led to improved three-dimensional data sets, which themselves were used to again unbend all images. The quality of the data set was determined by analyzing the phase residuals of all images against the whole data set in resolution zones, calculating the overall average phase error, calculating the phase error between two independent halves of data, and calculating the R factor of the fitted lattice lines. Three cycles of unbending were applied, and in the final data set beam tilt was refined although this was only just significant. Phases to 4.6 Å were determined, but because of incompleteness of the data (42% below 5.3 Å) a resolution cut-off of 5.5 Å was chosen. The point spread function shows that the effective resolution perpendicular to the membrane plane is 12.4 Å (see inset in Fig. 2).

Electron density maps were calculated using the CCP4 program suite (45). Density maps were calculated at different resolution cut-off values to ensure that the refinement procedure and sharpening of the map did not introduce spurious density and to verify that features discussed here are reproducible. This was also checked on earlier maps calculated with fewer cycles of refinement. Solvent flattening applied for one cycle using a solvent content of 75% in the CCP4 program. DM had little effect on the appearance of the map. The electron density maps were displayed with the programs O (47) and VOLVIS (Research Foundation of the State University of New York). The fitting of ideal helices to the density map was performed in O (43). The schematic drawings were made using Rasmol (Rasmol Molecular Renderer, Roger Sayle), and Bobscreen (48).

The fitting of crystal data (1) into our map was done by eye with helices 4, 6, and 7 serving as anchor points. In that process, only the fit of the seven transmembrane helices was optimized. In general, in electron density maps from two-dimensional crystals density on both sides of the membrane is weak, and therefore information on both sides of the membrane was cut out from the crystal data for the fitting.

**RESULTS**

**Image Processing and Data Merging—**Dialysis of detergent-solubilized rhodopsin isolated from bovine rod outer segments resulted in the formation of rectangular two-dimensional crys-
Three-dimensional Structure of Rhodopsin by Cryo-EM

| Resolution range | Phase residuals for different refinement cycles | $\Delta$ degrees |
|------------------|--------------------------------------------------|------------------|
| 200–10 Å        | $1^*$                                             | 3.0              |
| 10–7.1 Å        | $2^*$                                             | 4.5              |
| 7.1–5.8 Å       | $3^*$                                             | 4.6              |
| 5.8–5.0 Å       | $4^*$                                             | 3.2              |

Each refinement cycle consists of re unbending all images using a reference created from the previous data set. Data from re unbending images were merged again, and original crystal tilt, and defocus refinement were performed until the phase residuals of the individual images did not improve any longer. Refinement cycle $1^*$ refers to a data set that consists only of images that were un bent in the conventional way; refinement cycle $4^*$ refers to the “final” data set.

Three-dimensional Density Map—The arrangement of the molecules in the bovine p22_2_1 symmetry. Lattice parameters were det ermined from untitled specimen to be 60.6 ± 0.8 and 86.3 ± 1.6 Å (43).

From a large number of images, 97 crystalline areas were chosen and analyzed, and an improved iterative re unbending of all images was applied (46). There references generated from a “pre liminary” merged structure were used to re unbend all images again. In Fig. 1, a comparison of phase residuals of the individual images is shown for images contained in the “pre liminary” data set (open circles) and for images contained in the “final” data set (black circles). The “pre liminary” data set con sists of images, which were un bent in the conventional way (program MASKTRAN (44)). The “final” data set was obtained after three additional rounds of refinement, each round of refinement consisting of (a) creating references from the previous data set, (b) unbending all images, (c) extracting amplitudes and phases, (d) merging, and (e) refinement of crystal origin, crystal tilt, and defocus values against this new data set. In each round of refinement, steps c and d were performed as long as phase residuals improved. This was checked statistically (see “Experimental Procedures”). In the next step, the three-dimensional data set was obtained as new reference for re unbending the images. The effects of this pro cedure on the phase error (calculated between two independent halves of data) are shown in Table I for the “pre liminary” data set and for the subsequent rounds of re unbending.

In Fig. 2, lattice lines from the final three-dimensional density map, calculated to 5.5-Å resolution, are shown. The density map was calculated using image-derived amplitudes and phase values, and the lattice lines were sampled at 0.005 Å$^{-3}$. Table II gives an overview of the crystallographic details.

Comparison with X-ray Data—In order to be able to interpret our data with the atomic structure data available, we fitted the crystal structure of rhodopsin (1) into our density data. The resulting fit is shown in Fig. 6a. The contouring of the map was chosen to enable a direct comparison of the EM map with the paths of the helices given by the crystal structure, which are colored. The position of the retinal in the x-ray structure is indicated in red. The almost perfect fit between the x-ray data of helices 6 (blue) and 7 (magenta) is shown in Fig. 6b in detail. On the extracellular side, connecting density between helices 6 and 7 is visible. Information about the retinal binding pocket is given in detail in Fig. 6, c and d. For clarity, a model of the retinal is shown in yellow; the position of the retinal is given by the x-ray coordinates (1), which were fitted into our map. Additional density on helix 7 of our map (Fig. 6c) indicates the position of the “starting point” of the retinal and additional density on helix 3 (Fig. 6d) indicates the position of the β-ionone ring. These additional density peaks indicate the position of the retinal in the cryo-EM map as is shown in Fig. 5.

Using the atomic x-ray data (1), electron density maps were simulated to the resolution of our three-dimensional data set (Fig. 7, b–d). For comparison, the electron density map obtained by cryo-EM is included as a projected view (Fig. 7a). The corresponding electron density map, calculated from the x-ray coordinates including information about the transmembrane helices only, is shown in Fig. 7, b and c, in a top view and side view with the cytoplasmic side on top. This side view should be compared with the side view of our cryo-EM map in Fig. 4a. In Fig. 7d, the whole crystal structure was simulated to 6 Å, pointing out the presence of helix 8 at the cytoplasmic side of the membrane.

Paths of Helices—In the following, we attempt to give a detailed description of our three-dimensional electron density map in relation to the known crystal structure (1). At a contour level of 1.0 σ, three highly tilted and curved neighboring density features span through the molecule. Helix 1 is visible from about −21 to +19 Å, and extending beyond weaker density is visible to −27 Å. Continuous density on the intracellular side links to helix 2, where they are −11 Å apart. This is in close agreement with the crystal structure data available today (1, 52), and in that case we are able to trace the loop region outside the membrane. The density of helix 1 is very broad and not

| Table I Overall improvement of phases during the refinement procedure |
|--------------------------|--------------------------|--------------------------|--------------------------|--------------------------|
| Resolution range         | Phase residuals for different refinement cycles | $\Delta$ degrees |
| 200–10 Å                | $1^*$                    | 3.0                      |
| 10–7.1 Å                | $2^*$                    | 4.5                      |
| 7.1–5.8 Å               | $3^*$                    | 4.6                      |
| 5.8–5.0 Å               | $4^*$                    | 3.2                      |
Three-dimensional Structure of Rhodopsin by Cryo-EM

Fig. 2. Fitted lattice lines of the final three-dimensional density map of bovine rhodopsin. Experimental phases (top panel) and amplitudes (bottom panel) and fitted curves for two reciprocal lattice lines are shown. Individual data points give amplitudes and phases from individual images. Error bars indicate the fitting error at the sampling points and are spaced 0.005 Å along the z* axis normal to the crystal plane. The figure includes all data with a signal/noise ratio of >1; the symbols refer to the strength of the spots (x, signal/noise ratio of >4; x, signal/noise ratio of >2; +, signal/noise ratio of >1.35; +, signal/noise ratio of >1). The inset shows the point spread function, indicating that the resolution perpendicular to the membrane plane is less accurate by a factor of 2.5 with respect to the resolution achieved within the membrane plane.

Table II

| Parameter                          | Value |
|-----------------------------------|-------|
| Symmetry                          | p22,2 |
| Lattice constants                 | a = 60.6 ± 0.8 Å, b = 86.3 ± 1.6 Å |
| Effective resolution cut-off       | 5.5 Å in plane, 12.4 Å perpendicular |
| Maximum tilt angle               | 50°   |
| Range of underfocus              | 1000–23,000 Å |
| No. of crystalline areas           | 97    |
| No. of independent structure factors | 4953 |
| Overall weighted phase error      | 22.1° |
| Overall weighted R factor         | 0.328 |
| Phase error in resolution zones   | 200–10.5 Å: 19.8%; 10.5–7.3 Å: 20.6%; 7.3–6.1 Å: 30.7%; 6.1–5.7 Å: 27.5%; 5.7–3.2 Å: 30.4%; 3.2–5.0 Å: 29.6% |
| Completeness of data              | 200–14 Å: 63%; 14–9.5 Å: 58%; 9.5–7.9 Å: 51%; 7.9–6.1 Å: 46%; 6.1–5.5: 45% |

* Distribution of tilt angles: 0–10° (29 images), 10–20° (23 images), 20–30° (17 images), 30–50° (28 images).

* Phase errors were calculated using all positive spots to a resolution of 5.5 Å, and weights were given according to the strength of the spots: signal to noise ratio (snr) > 7, wild type = 49; snr > 3.5, wild type = 27; snr > 2.3, wild type = 8.5; snr > 1.75, wild type = 4.2; snr > 1.4, wild type = 2.5; snr > 1.17, wild type = 1.7; snr > 1, wild type = 1.2; snr > 0.87, wild type = 0.25.

* A random value is expected at 45°.

straight, suggesting a kink at Pro215. This kink, however, is not very pronounced, and therefore helix 1 is simulated as a straight rod in Fig. 5. Density for helix 2 is visible from about +19 to −21 Å, and we also observe a slight kink near the center of the membrane. This observation seems to be related to the fact that helix 2 is kinked around Gly89 and Gly90 (1). Density assigned to helix 3 is visible from about −21 to 18 Å with the intracellular part outside the membrane being weak. According to the crystal data, however, this helix is rather long (1). We believe that because of the high tilt of helix 3 we are not able to pick a stronger signal outside the membrane in that region. Below the center of the membrane, a density feature (marked by an arrow head in Figs. 4a and 6a, marked by an asterisk in Fig. 4c) is visible. This additional density points toward the retinal binding pocket and will be discussed later. The extracellular part of helix 3 shows two density features; one is straight, and one is bent inwards. This is hard to interpret from our map alone. However, we know from the crystal structure that helix 3 is straight (1), and we therefore assign the weaker density in this region to the helix itself (Fig. 4d). The stronger density feature, visible in Fig. 4d (indicated with an asterisk) seems to correspond to parts of the loop between helices 4 and 5. This loop (EII) folds deeply into the center of rhodopsin, and a disulfide bridge is formed between Cys187 and Cys110 at the extracellular end of helix 3 (1).

Helix 4 is short, almost perpendicular to the membrane plane, and visible from about +18 to −17 Å, where it ends near helix 5 (Fig. 4d). At z = +2 to −4 Å density pointing toward helix 5 and a small kink at about −13 Å interrupt the straight character of this helix. These features seem to be related to Pro175, Pro171, and Trp161. Helix 5 seems to be slightly tilted according to our map. It is located close to helices 3 and 6 at the intracellular surface (Fig. 4b) and near helix 4 on the extracellular side (Fig. 4d), where it is visible to about −18 Å. No connection to another helix is visible on the intracellular side. We detect a bend at −4 Å that might suggest the position of Pro155, and therefore we allow a kink in our simulation at this position (Fig. 5), although in the crystal structure this helix seems to be rather straight (1). Interestingly, in the intracel-
lular half, this helix is weaker than all other helices. This observation was also made in the frog rhodopsin map (34), although there this helix was even weaker. In the frog rhodopsin map, the path of helix 5 could not be determined, but with the current data set a clear interpretation is possible. An explanation might be that in the crystal the helix is exposed to...
The transmembrane helices were approximated as straight rods, and bends toward helix 5 at about −20 Å, a connection between helices 6 and 7 corresponding to the third extracellular loop is visible on the extracellular surface (Fig. 6b). Helix 7 is visible from 23 to −22 Å ending with the carboxyl terminus at the intracellular end. The density assigned to this helix is very bulky, which is consistent with the presence of many amino acids with big side chains. A kink at −6 Å is observed, corresponding to Lys296, to which 11-cis-retinal is covalently linked (Fig. 6c). This observation may be compared with bacteriorhodopsin where a slight kink or bend has been observed near Lys216 on helix G of bacteriorhodopsin, where its chromophore is bound (53). Additional smaller kinks are observed at the two prolines (residues 291 and 303) at 10 and −20 Å.

We also detect density outside the membrane, which is not connected to any of the helices. In particular, we want to point out density observed on the extracellular surface between helices 3, 4, 5, 6, and 7. This density seems to close the retinal binding pocket at this side (Fig. 4d) and could be related to β₁ (described in Ref. 1), although detailed assignment is not possible at the present resolution of our map.

**DISCUSSION**

**Image Processing and Data Merging**—In this work, we have paid special attention to the problem of extracting as much information as possible from the images to try to extend the resolution of the data set. This is a very important aspect, since it is well known that two-dimensional crystals from membrane proteins are often not well ordered and small. These are clearly limiting factors in structure determination. One way around them seems to be the possibility of trying to extract more precise amplitudes and phases from the original raw data. In particular we tried to gain more information by applying an improved method of unbending, using a preliminary data set to create references to reunbend all images again. We also analyzed the progress of phases carefully during the whole refinement procedure in various different ways. In general, our rhodopsin crystals are less ordered than, for instance, bacteriorhodopsin crystals (53). In the case of the bacteriorhodopsin crystals, image data as well as diffraction data could be used to generate the three-dimensional map. The crystals of bovine rhodopsin, which we used in this study, were much better than previous crystals (see Fig. 6 in Ref. 43), but still they were not ordered high enough to give diffraction data on a regular basis, which we could have used for the generation of our three-dimensional map. Therefore, extracting as much information as possible from a set of images alone was of great importance in calculating a three-dimensional data set.

In our case, we could improve the phase errors of individual images over the whole range of data using the improved reunbending procedure. In general, successful application of reunbending led to stronger spots (better signal/noise ratio in all resolution zones) rather than more spots (Fig. 1). The achieved extension of phases leads to an overall increase of resolution. The phase improvement we observed was strongest in the lower resolution zone, less effect on the phases was observed near a covalently linked (Fig. 6c). This observation may be compared with bacteriorhodopsin where a slight kink or bend has been observed near Lys216 on helix G of bacteriorhodopsin, where its chromophore is bound (53). Additional smaller kinks are observed at the two prolines (residues 291 and 303) at 10 and −20 Å.

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Fig. 6. **Modeling of the crystal structure of bovine rhodopsin (1)** into our electron density map with helices 6 and 7 in the foreground. Shown are an overall view (a) and detailed fit (b) of helices 6 (blue) and 7 (magenta). The cytoplasmic side is at the top. Helices 1–7 of the crystal structure are colored in red, orange, yellow, green, cyan, purple, and magenta. The experimental density map of rhodopsin is shown in gray at a threshold to enable direct comparison with the x-ray data. Detailed views of the retinal binding pocket are shown in c and d. The retinal is shown in yellow at a position that is given by the fit of the crystal data into the EM map. Although the signal of the retinal itself is not picked in our map, additional density features (arrowheads) on helices 7 (c) and 3 (d) are clear indications of the presence of the retinal in our electron density map.

ing three additional rounds of re unbending, the resolution of our data set could be increased to better than 5 Å, although we decided to set the resolution limit to 5.5 Å. This was necessary because of weakness of the tilted images and consequent incompleteness of higher resolution data (see Table II).

**Crystal Packing**—In the p22121 crystal (Fig. 3), one unit cell has a volume of $1.04 \times 10^{-6}$ Å$^3$, and one molecule has approximate $x$, $y$, and $z$ dimensions of 28, 40, and 63 Å. The helices are assigned according to Ref. 26. Each molecule is closely packed, making contact with three neighbors. The closest contact occurs between helices 1 (red) of adjacent molecules, which are $-10$ Å apart across a 2-fold rotation axis, where both molecules face the same side (see Fig. 3d). Because of the helix tilt, they diverge, and at the intracellular side of the membrane they are about 35 Å apart. Another possible helix contact occurs between molecules, which are upside down. The intracellular part of helix 4 (green) is about 12 Å from the extracellular end of helix 6 (purple) and about 13 Å from the extracellular part of helix 7 (magenta) from the neighboring molecule. The extracellular part of helix 4 is slightly farther apart from the neighboring helices 6 and 7. In the middle of the membrane, close contacts are observed between helices 4 and 6 (11 Å). The distances are small enough to allow van der Waals contact between helices, but whether these contacts are side chain/side chain or side chain/main chain cannot be determined at this resolution.

**Paths of Helices**—In general, the paths of all transmembrane helices indicated by our density map agree with what is known from x-ray structures (1, 52). Starting and end points cannot be determined exactly, since we do not have atomic resolution in our map. However, their position relative to the membrane plane can be determined, which is difficult to ascertain from other sources. In our model, the helix paths show higher tilts than estimated from model calculations (27, 28). It is noteworthy that the current bovine rhodopsin map is in closer agreement with the model based on sequence analysis of about 500 G-protein binding receptors (26) than the previous frog rhodopsin map (34). This is due to the more complete and accurate phases, which lead to a lengthening of helices and an increase in helix tilts. Even the vertical helices now show a slight tilt, which could not be determined previously in the EM map of frog rhodopsin. Assuming that all helices are straight with endpoints at $-8$ and $+12$ Å, the tilt angles of the seven transmembrane helices (numbered 1–7) could be approximated as being 32, 27, 27, 4, 32, 9, and 16°, respectively.

**Retinal Binding Pocket**—It is known that in rhodopsin the chromophore 11-cis-retinal is attached through a protonated Schiff base bond to the ε-amino group of Lys$^{296}$ on helix 7 (19). The Schiff base linkage creates a formal positive charge on the chromophore, which is buried in the interior of the protein. This charge is balanced by the negatively charged carboxylate side chain of Glu$^{113}$ on helix 3 (1, 52).

According to this, the density we detect near helix 3 corresponds to the β-ionone ring of the chromophore (Figs. 4c and 6d). In the $z$ direction, it lies between sections 0 and $-6$ Å, since in these sections the density is separated from helix 3 at a contouring of the map of 1.7 σ. Thus, in our map the retinylide group is located closer to the extracellular side. The environment of the 11-cis-retinal in our EM map can be described as follows. The density map shows helix 6 bent with a density bulge facing one side of the helix (Phe$^{261}$, Trp$^{265}$, and Tyr$^{268}$). We also detect an indication of Trp$^{296}$; however, this we
would not be able to state with sufficient certainty from our map alone. Correlation with the crystal structure is necessary in that case. Since the kink in helix 6 indicates Pro267, we know that this proline lies extracellular to the β-ionone ring. The kink in helix 7 is at the position of Lys296 (Fig. 6c). This would place the lysine at −6 Å. Thus, from our EM map, it is possible to conclude that the transition dipole moment of the retinal chromophore is tilted relative to the membrane plane in the opposite direction to that in bacteriorhodopsin (53).

**Simulated Density Maps of Rhodopsin: Comparison with X-ray Data**—Generally, in electron microscopy maps, the resolution of data in the z direction is weaker because of the use of two-dimensional crystals. Therefore, no attempt is made to give a detailed description of these loops, and consequently we decided to leave out information of loop structures from our simulations. Comparisons of projection views of the cryo-EM map (Fig. 7a) with the x-ray data (Fig. 7b) and comparison of the corresponding side views (Fig. 4a, cryo-EM map; Fig. 7c, x-ray data) reveal that both structures are very similar regarding the arrangement of the seven transmembrane helices. However, the missing cone will make features elongated, which makes tilted features appear less tilted. Therefore, the helices seem slightly elongated in comparison with the crystal data available (1) (compare Fig. 4a, cryo-EM map, with Fig. 7c, simulated map from x-ray data).

In the crystal structure, an important additional helical feature was identified (1). Helix 8 is a short helical segment on the cytoplasmic side of the membrane surface (see Fig. 7d). Since this helix lies parallel to the membrane plane, we have difficulties in detecting this helix in our map of rhodopsin. This is due to the fact that we use two-dimensional crystals and therefore helices, which lie perpendicular to the membrane plane will be resolved best, whereas tilted helices will be resolved less perfect. The density of helices parallel to the membrane plane is extremely hard to detect. The fact that our crystals are weak and we could not include diffraction data limits our data, and as a consequence helix 8 could not be determined with sufficient certainty in the cryo-EM map.

**Implications from the Electron Density Map Obtained from Two-dimensional Crystals**—Most important is the information we gain about the position of the molecule in the membrane plane. In general, the position of a membrane protein within the membrane is not defined clearly, since the electron density map shows continuous density for the protein but no indication for the borders of the membrane bilayer. Therefore, the exact location (e.g. the center of the membrane) is hard to detect. In this study, we used two-dimensional crystals with p2221 symmetry. Because of the symmetry, neighboring molecules are upside down, but they must overlap in the center of the membrane plane (as outlined in Fig. 3, a–c). This clearly indicates the center of the membrane and the orientation of the molecule relative to the membrane plane. In the three-dimensional crystals, the lipid bilayer is not present, and therefore the information about the orientation of the molecule relative to the bilayer is lost. It is noteworthy, that, although there are “upside down” molecules present in the three-dimensional crystals, their relationship cannot be used to determine the position of the membrane plane. In the three-dimensional crystals, the two “upside down” molecules are related by a noncrystallographic axis, allowing the molecules to be different, and the presence of a NCS axis is not in any relation to the position of the molecules in the membrane.

Another very important piece of information we get is the information about neighboring dimers. The closest helix contact in our crystal occurs between helices 1 of adjacent molecules, which are ~10 Å apart across a 2-fold rotation axis where both molecules face the extracellular side (see Fig. 3d). Because of the helix tilt, they diverge, and at the opposite side of the membrane they are about 35 Å apart. This 1/1 contact is conserved in all two-dimensional crystal forms of bovine and frog rhodopsin (33, 34, 50) but differs from the dimer observed in the squid rhodopsin crystal (54). Although rhodopsin often crystallizes as a dimer, it is not clear whether the dimer has physiological relevance. However, in native mouse disc membranes, rhodopsin forms paracrystalline arrays of dimers (51) as well. Thus, we believe that the 1/1 dimer, as seen in this crystal form, might have some sort of physiological relevance. There might be a possibility that helices 8 are involved in this 1/1 contact, even if we cannot see them in our map. This would explain how the high angle between the helices is stabilized.

**Conclusions**—We have calculated a three-dimensional data set of bovine rhodopsin using two-dimensional crystals. Special attention was paid to the extraction of amplitudes and phases, since usable raw data were limited to a maximum goniometer tilt of 45° and the crystals used were disordered and small. In the refinement process, an improved unbending procedure was applied, which led to data to a final resolution of 5.5 Å in the membrane plane. This makes our map the most accurate data of a GPCR obtained by cryo-EM. Most important is the determination of the center of the membrane and the position of the
molecules relative to the bilayer, since this information cannot be retrieved from the crystal structure available (1). Contacts between neighboring rhodopsin molecules involve helices 1, and we believe that cytoplasmatic helix 8 might be involved in stabilizing the high angle between these helices. The map shows all seven transmembrane helices well resolved and in agreement with the crystallographic data (1, 52). We observe more density on the extracellular side than on the intracellular side, although interpretation of larger loop structures proved to be difficult. Density connecting helices 1 and 2 and helices 6 and 7 outside the hydrophobic zone of the bilayer could be identified. Extra density adjacent to helix 3 is consistent with the β-ionone ring of 11-cis-retinal.

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