from the conditions used in sample preparation for AFM.

Fast, transient, flash-induced photodichroism[1] first revealed the rapid rotational diffusion of rhodopsin *in situ*, and the kinetics of flash-bleaching recovery[2] indicated that rhodopsin undergoes rapid lateral diffusion in intact rods. Its random distribution in the membrane was evident from X-ray and neutron diffraction[3–5] and was confirmed by electron microscopy[6]. We therefore question whether the structures seen by Fotiadis *et al.*[7] in an osmotically burst disc membrane represent a native disc structure, or whether they can be explained by separation of the proteins and lipids during the 15-h incubation at 0 °C in 2 mM Tris–HCl in the preparation of samples for AFM[8].

A long exposure at low temperature will alter the native disc membrane structure in rods isolated from a warm-blooded vertebrate. In-plane X-ray diffraction revealed long ago that cooling cattle rods to 5 °C for only 15 min induces a partial phase transition of the lipids and a correlated segregation of the proteins[4]. By contrast, no phase separation in frog rods was ever observed by X-ray[4] or neutron[5] diffraction.

Low-temperature investigation of flash-induced transient photodichroism (R.C., unpublished results) has confirmed that frog rhodopsin can still undergo rapid rotational diffusion at temperatures close to 0 °C. Transient photodichroism cannot reliably distinguish between freely rotating dimers and monomers, but the packed rows of rhodopsin seen in the AFM images of Fotiadis *et al.*[7] are incompatible with rapid rotational diffusion. And if rhodopsin were oligomerized into rows *in vivo*, a stable or slowly decaying flash-induced dichroism would have been detected long ago in human vision and in isolated vertebrate retinae[1].

Electron-microscope images of snap-frozen, freeze-etched frog rods[6] were able to resolve rhodopsin monomers in a random array, with no evidence of dimers, although the rods had been incubated at 4 °C for 1 h. After digestion of these fragmented rods with phospholipase C, however, disc-membrane phospholipids were seen to segregate into droplets, whereas the packing of rhodopsin changed into concentrated, row-like arrays[6]. It is possible that these could be the equivalent of the double-row arrays that were observed by AFM[7].

All of these biophysical data are old, but remain valid. Until now, they have not been experimentally challenged or refuted. They exclude the occurrence of rhodopsin oligomers in native, unactivated, frog rods and provide evidence for the rapid phase separation of rhodopsin and lipids in mammalian disc membranes kept at low temperature.

Fotiadis *et al.* observe the same separation of rhodopsin and lipids when their samples are incubated at the higher temperature of 25 °C (see www.mih.unibas.ch/Nature/Fotiadis.html), but this is still below physiological temperature. Phase separation in a burst disc membrane may slow down under these conditions but will not be prevented. In osmotically disrupted cattle discs, for example, phase-separation artefacts were often visible in early X-ray patterns that required 15 h of exposure at room temperature[4].

We therefore maintain that native rhodopsin exists in monomeric form in the retinal rods of vertebrates. The double-row arrays seen in AFM[7,8] in isolated and osmotically disrupted mouse discs probably result from separation of the lipid phase from the proteins, which occurred rapidly at 0 °C and more slowly at 25 °C, during the long incubation and upon osmotic disruption of the discs.

Our contention excludes only constitutive oligomerization of non-activated rhodopsin in dark-adapted rods. After illumination, in an arrestin-mediated deactivation process, rhodopsin molecules may undergo pairing[9]. Oligomerization also occurs in other G-protein-coupled receptors[10] in the β-arrestin-mediated process of their inactivation and clustering in coated pits, but these processes are distinct from the oligomerization of non-activated receptors.

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Fotiadis et al. *reply* — Individual biological molecules can be imaged under physiological conditions by atomic force microscopy. Our results from AFM, supported by electron microscopy, revealed distinct rows of rhodopsin dimers and paracrystalline arrays in native murine disc membranes[1,2]. This supramolecular arrangement was also found for the light-activated form, opsin[1]. We counted 30,000–55,000 rhodopsin molecules per square micrometre, a packing density comparable to that measured by optical methods in amphibian discs[3]. From the lattice vectors describing the paracrystalline arrays, a maximum possible packing density of 62,900 rhodopsin molecules per square micrometre was calculated. The rhodopsin dimers seen in AFM topographs[2] have cytosolic protrusions separated by 3.8 nm, providing an ideal docking platform for arrestin, which has two binding grooves that are separated by 3.8 nm as well[1].

On the basis of data reported some 30 years ago, Chabre *et al.* are challenging our observations. Results from their and other[3] early experiments, which were all carried out on large ensembles of amphibian disc membranes, indicated that inactivated as well as activated rhodopsin diffuses freely as a monomer in the lipid bilayer. Chabre *et al.* now propose that the rhodopsin-packing arrangement that we observe by AFM[1,2] is induced by the segregation of proteins and lipids at low temperatures.

To respond to this criticism, we recorded electron micrographs as well as AFM topographs of rhodopsin paracrystals on native disc membranes that had been prepared and imaged at room temperature (see www.mih.unibas.ch/Nature/Fotiadis.html). Three different surface types could again be distinguished: rhodopsin paracrystals, rhodopsin rafts and the lipid bilayer; at increased magnification, rhodopsin dimers, the building block of the paracrystal, are clearly seen. This dimerization and higher-order organization of rhodopsin at room temperature argues against the claim by Chabre *et al.* that the packing arrangement we describe[1,2] is a result of low-temperature-induced protein–lipid segregation, particularly if we consider that the phase-transition temperature of the bovine-disc lipids is below 3 °C (ref. 4).

Freeze-fracture electron microscopy has also revealed paracrystalline rhodopsin arrays in *Drosophila* photoreceptive membranes[5] and in the plasma membrane of bovine-rod outer segments[6]. The concept of oligomerization in the presence or absence of ligands is generally accepted for many G-protein-coupled receptors[7], and rhodopsin is not an exception.

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