

OPTICAL PROBES OF INTRADISKAL PROCESSES IN ROD PHOTORECEPTORS I: LIGHT-SCATTERING STUDY OF ATP-DEPENDENT DARK REACTIONS

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(Received August 5, 1988; accepted November 16, 1988)

Keywords. Photoreceptor disk compartment, light-scattering changes in the dark, Mg-ATPase, proton translocation, $\Delta\mu(\text{H}^+)$.

Abbreviations

ATP	adenosine 5'-triphosphate
$\Delta\mu(\text{H}^+)$	electrochemical potential difference of protons
BICINE	<i>N,N</i> -bis(2-hydroxyethyl)glycine
CAPS	3-(cyclohexylamino)-1-propanesulphonic acid
cGMP	guanosine 3':5'-cyclic monophosphate
DCCD	<i>N,N'</i> -dicyclohexylcarbodiimide
DIDS	4,4-diisothiocyanatostilbene-2,2'-disulphonic acid
EGTA	ethylene glycol-bis(<i>b</i> -aminoethylether)- <i>N',N',N',N'</i> -tetraacetic acid
HEPES	(<i>N</i> -[2-hydroxyethyl]-piperazine- <i>N'</i> -[2-ethanesulphonic acid])
MES	2-(<i>N</i> -morpholino)-ethanesulphonic acid
NBD·Cl	7-chloro-4-nitrobenz-2-oxa-1,3-diazole
PEP	phosphoenolpyruvate
PIPES	1,4-piperazinediethanesulphonic acid
ROS	rod outer segment

Summary

ATP can cause dramatic structural changes in the outer segment of rod photoreceptors. These changes can be visualized by means of a concomitant light-scattering signal A_D , a decrease in scattered light intensity of over 20%. The large size of the signal suggests that major structural changes occur. The underlying molecular events may reflect an important, yet still unknown, part of the photoreceptor machinery.

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A_D signals reflect ATPase-driven transmembrane events which occur in and at the disk membrane. Their only structural prerequisite is the structural integrity of the disk compartment. The angular dependence of A_D , which can be mimicked by an osmotically-induced disk-swelling, suggests that the disk compartment swells during the production of the A_D signal.

A_D signals proceed with first-order kinetics (half-life = 1 min at 20 °C and ATP concentrations of greater than 100 μ M) and are accompanied by the hydrolysis of approximately 4 mol ATP (mol rhodopsin)⁻¹.

The A_D signal is inhibited by a number of transport ATPase inhibitors (quercetin, NBD·Cl, vanadate, DCCD), but not by oligomycin, azide and ouabain. The sensitivity to DCCD, together with the fact that except magnesium no other cation has to be present, points to a proton translocation. This proton transport appears to be electrogenic, since A_D signals require the presence of a permeant anion. In physiological saline this is chloride, and the chloride flux is facilitated by a DIDS-sensitive anion transport unit in the disk membrane.

1. Introduction

Our understanding of the mechanism of visual transduction has progressed considerably over the past few years. A sequence of biochemical events has been discovered, which starts in the disk membrane with the absorption of a photon by the pigment molecule rhodopsin, proceeds at the disk membrane surface with the transient activation of many G-proteins and, subsequently, phosphodiesterase molecules, and terminates with the blockage of cGMP-dependent sodium channels in the plasma membrane of the rod photoreceptor [1]. Within the framework of this model the disk membrane merely serves as a two-dimensional reaction vessel, facilitating the steric interaction between the various proteins. Its flatness affords a favourable orientation of the chromophore and the fact that rhodopsin is transmembrane provides for a solid anchoring. If this is so, why do disks form topologically closed membrane compartments (like thylakoids or mitochondria) and why does rhodopsin cause several light-induced intradiskal events? Moreover, what is the reason for the curious fact that the intradiskal loops of the rhodopsin molecule are among the best conserved sequences in vertebrate and drosophila rhodopsin [2]? These unanswered questions have suggested to us that the disk compartment may possibly play a role in visual transduction, a role which has yet to be determined.

A first step towards a substantiation of this idea has been undertaken in this and a following paper, where we have tried to identify further properties of the disk compartment and to measure dark and light reactions which take place inside the disk, rather than at its cytosolic surface. The physiological significance of our findings is, at present, unclear. However,

we believe that it is important to continue to gather information in order to try to produce a clearer picture of what is taking place.

Optical techniques can be used to study processes which occur in an unperturbed disk interior. Of these techniques, light scattering is probably the most sensitive. However, since it rarely leads to clear results a more logical approach has to be taken: assumptions must be made, further assumptions must be made which lead to further experiments (possibly justifying the previous assumptions) and a large amount of experimental evidence must be collected before a reasonably clear picture is obtained. The purpose of this and a following paper is to present large amounts of experimental data on the photoreceptor disk compartment and to demonstrate strategies for the extraction of information from these data.

Since our first systematic studies of time-resolved, light-induced, light-scattering transients from bovine ROS material [3 - 6], light scattering has been used to aid in the understanding of light-stimulated protein-protein and protein-membrane interactions, which all occur at the cytosolic disk surface. The basis for this progress has been the observation of Kühn *et al.* [7] that certain processes, involved in the light activation of rod photoreceptor enzymes, are accompanied by gross structural changes, and that these structural changes are reflected by the well-known light-scattering signals.

In the late 1970s a new class of light-scattering signals from ROS was discovered [8 - 13]. The signals are ATP dependent and, as is demonstrated in this and a following paper, they reflect trans-disk membrane processes rather than processes involving peripheral disk membrane proteins. These signals are a large decrease in light scattering (greater than 20%) in the dark (termed A_D) and a subsequent further decrease in light scattering (termed A_L) which is observed when ROS pre-incubated with ATP is flash illuminated.

In this paper we call the process which produces the A_D signal (and is a prerequisite for A_L) energization of the disk, simply because it builds up at the expense of metabolic energy, *e.g.* ATP. Evidence is provided later in this and the following paper that the ATP-driven energization of the disk actually reflects the development of an electrochemical proton potential difference $\Delta\mu(H^+)$ as in mitochondria and thylakoids.

2. Materials and methods

2.1. Chemicals

Ionophores and inhibitors were stored as concentrated ethanolic stock solutions in the dark at 4 °C. They were mixed with the test suspension using a microsyringe. The ethanol concentration in the reaction medium never exceeded 0.5%. X537A and nigericin were provided by Dr. W. E. Scott, Hoffmann LaRoche. A23187 was obtained from Calbiochem, tributyltin from Aldridge, Ficoll 400 from Pharmacia and all other reagents from Sigma.

2.2. Preparation of rod outer segment (ROS) material

2.2.1. Bovine ROS

Bovine ROS fragments with intact stacks of disks but perforated plasma membranes were prepared using a procedure described in ref. 14. Briefly, ROS fragments were released from fresh retinæ by vortexing for 5 s in argonated Ringer's solution. Crude ROS fragments were separated from retinal debris by filtering them through a Nylon mesh. They were purified further using discontinuous sucrose density gradient centrifugation. The usual yield was approximately 20 nmol rhodopsin per retina and the absorbance ratio A_{280}/A_{500} (indication of purity) was between 2.2 and 2.4. The ROS fragments were stored as small aliquots in liquid nitrogen and were thawed immediately before a particular experiment.

2.2.2. Disks

Isolated, freely floating disks were prepared from unfrozen ROS material using the Ficoll flotation procedure of Smith *et al.* [15]. They were harvested as swollen vesicles from the Ficoll-phosphate buffer interface (the overlaying with 100 mM sodium phosphate (pH 7.2) is a modification of the original Smith procedure), and were used immediately afterwards. It is important to note that A_D signals can only be obtained from disk preparations that have not been frozen. This seems to indicate that disks are stabilized by the stack and cannot withstand freezing when they are isolated.

Frequently in our experiments isolated disks were replaced by lysed ROS material. This was prepared by incubating the ROS for 20 min in 5 mM HEPES (pH 7.2) and 1 mM $MgSO_4$. Subsequently the material was pelleted in small Eppendorf vials, using an Eppendorf centrifuge, and resuspended in the particular measuring medium [14].

Most experiments reported in this paper were carried out using freeze-thaw-treated ROS. The experiments were reproduced using fresh material which was lysed immediately prior to the experiment.

2.2.3. Frog ROS

Bullfrogs (*Rana catesbiana*) were adapted to the dark over night and decapitated. The isolated retinæ were shaken vigorously in Ringer's solution identical with that used for bovine retinæ, except that the sodium concentration was only 90 mM. This procedure, which was performed within 5 min of the death of the animal, yielded a crude ROS suspension with mostly intact plasma membrane [16]. It was used without further purification in a number of experiments.

2.3. Light-scattering experiments

Light-scattering signals were recorded in a multiangle flash photolysis apparatus (MAFPA), the design of which has been described elsewhere [13]. It allows the simultaneous registration of light-scattering signals at eight scattering angles, has a time resolution of 32 μs and can resolve relative changes in light intensity as small as 0.004%.

The signals displayed in this paper usually reflect the decrease in scattered light intensity at an angle of 8° , where A_D signals have their highest amplitude. In Fig. 3 (see later) previous measurements are shown which were recorded at a fixed scattering angle of 10° . In Figs. 5, 6 and 11 (see later) the amplitudes of A_D were determined from the drop in transmission measured in a Zeiss PMQ-2 photometer. In all cases the wavelength of the monitoring light was 840 nm.

To avoid multiple scattering, a rhodopsin concentration of $1 \mu\text{M}$ was chosen. In a cuvette with a path length of 5 mm this corresponds to a transmission of approximately 80%.

Unless otherwise indicated in the figure captions, the measuring buffer contained 100 mM KCl, 5 mM PIPES (pH 7.2) and 1 mM MgCl_2 . The usual Mg-ATP concentration was $100 \mu\text{M}$ and the temperature was 22°C .

2.4. ATPase activity measurement

The ATPase activity was determined using an ATP-regenerating system (phosphoenolpyruvate–pyruvate kinase) coupled to the NADH-dependent reduction of pyruvate to lactate [17]. The rate of the absorbance decrease at 340 nm, corrected for light-scattering effects, was used as a measure of ATPase activity [11]. It was either determined in an Aminco DW-2 dual wavelength spectrophotometer (wavelength pair: 340 and 400 nm) or, in previous experiments (Fig. 3, see later), in a Zeiss PMQ-2 with manual light-scattering correction, using the same set of wavelengths.

3. Results

When ROS fragments with a perforated plasma membrane are incubated in the presence of Mg-ATP, their light-scattering behaviour changes dramatically with 5 min [10]. Figure 1 shows such an A_D signal, monitored as a decrease in turbidity at a scattering angle of 0° and as a decrease in scattered light intensity at a scattering angle of 8° . Since non-hydrolysable ATP analogues cannot substitute for ATP, and even suppress A_D when added together with ATP, we conclude that the underlying structural phenomenon requires the hydrolysis of ATP [9]. Neither GTP nor creatine phosphate produce a similar effect.

3.1. A_D signals are physiological

A_D -type signals can also be observed in freshly prepared (frog) ROS with completely intact plasma membrane (data not shown). Here, because of the presence of endogenous ATP (we verified this for our preparation using a luciferase–luciferin assay; data to be published), the addition of exogenous ATP is no longer required. Instead, the ROS must be kept on ice for a while and then warmed up in order to initiate A_D . The ATP-dependent energization of the disk is apparently unstable at low temperature (because the ATPase activity is sufficiently slowed down) and so the energization can take place again on warming. After perforation of the plasma membrane

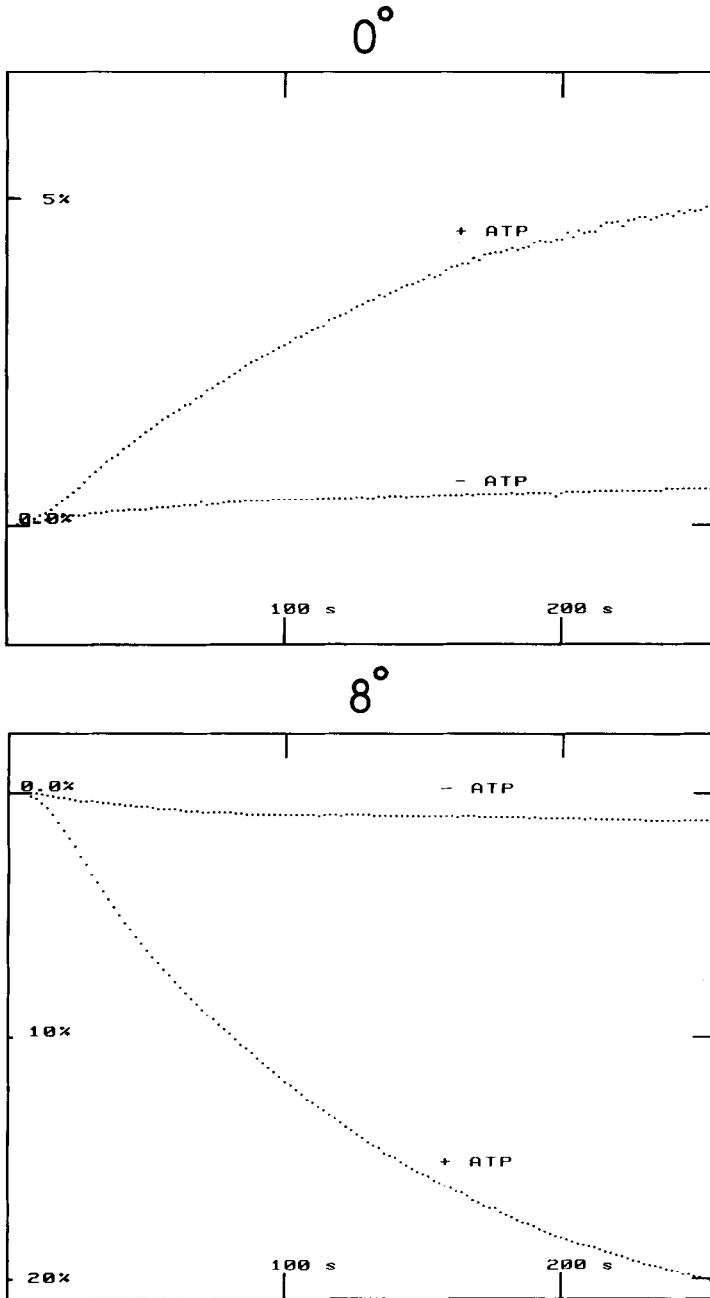


Fig. 1. A_D signals at 0° (increase in transmission) and at a scattering angle of 8° (decrease in scattered light intensity). The rhodopsin concentration was $1 \mu\text{M}$ Mg-ATP ($100 \mu\text{M}$) was added immediately prior to measurement. The signal obtained at 0° is small because the turbidity of the suspension was very low to avoid multiple scattering in the cuvette. Charges of up to 40% can be obtained when the rhodopsin concentration is increased tenfold.

by a freeze-thaw cycle, the frog ROS material still produces an A_D -type signal on resuspension in warm measuring buffer. This signal, like A_D , can be inhibited by vanadate. It is also blocked by a large excess of glucose and hexokinase. It follows that even broken ROS material retains sufficient amounts of ATP to drive A_D signals to completion [16].

3.2. ATPase activity during A_D

Figure 2 shows that ROS material exhibits ATPase activity which runs in parallel with the A_D signal. It can be monitored as an initial burst of ATPase activity on top of a constant time-independent activity. The initial activity corresponds to the hydrolysis of about 4 mol ATP (mol rhodopsin)⁻¹. Its inhibitor specificity is identical with that of A_D (Fig. 3), suggesting that there is a link between the two processes.

The second persistent phase of ATPase activity, however, which is completely blocked by quercetin, is only slightly sensitive to vanadate and DCCD and is therefore distinct. We assume that its major, quercetin-sensitive, vanadate-insensitive part reflects the Mg-ATPase activity described by Thacher [18], whereas its small vanadate-sensitive fraction reflects the ATP consumption required for the maintenance of the energized state. The amplitude of the latter was determined in a number of experiments (data not shown) to be 0.2 - 0.3 mol ATP hydrolysed min⁻¹ (mol rhodopsin)⁻¹. This corresponds to a decay rate of the previous energization of 7% min⁻¹ or a half-life of de-energization of 10 min (at 22 °C).

The fact that A_D is inhibited by the transport ATPase inhibitors DCCD and quercetin (Fig. 3) and also by NBD·Cl (data not shown) points to a transport ATPase; the DCCD sensitivity suggests that proton translocation is involved and the insensitivity to azide precludes the possible participation of an F_1/F_0 -type enzyme. This, together with the huge size of the light-scattering signal, clearly establishes that the underlying events originate in the ROS and not in contaminating (mitochondrial) material.

3.3. Kinetic characterization of A_D

Micromolar amounts of ATP are sufficient for A_D signals to occur [10]. Their rate increases with increasing ATP concentration in a manner predicted by the Michaelis Menten equation when a 1:1 stoichiometry between ATPase and ATP is assumed. The K_M value was determined to be approximately 60 μ M, and the activation energy is 45 kJ mol⁻¹ [16].

3.4. The structural origin of A_D

Thacher [19] has characterized A_D signals from frog ROS. He detected no signs of a disk swelling during A_D , using both light microscopy and radioactive tracer experiments. He therefore concluded that the ATPase in question is not a transport ATPase. He suggested that it may be a dynein-type ATPase similar to those connected with the microtubular structures of eucaryotic flagellae. These ATPases are also sensitive to vanadate, and ciliary structures have been reported to exhibit similarly pronounced light-

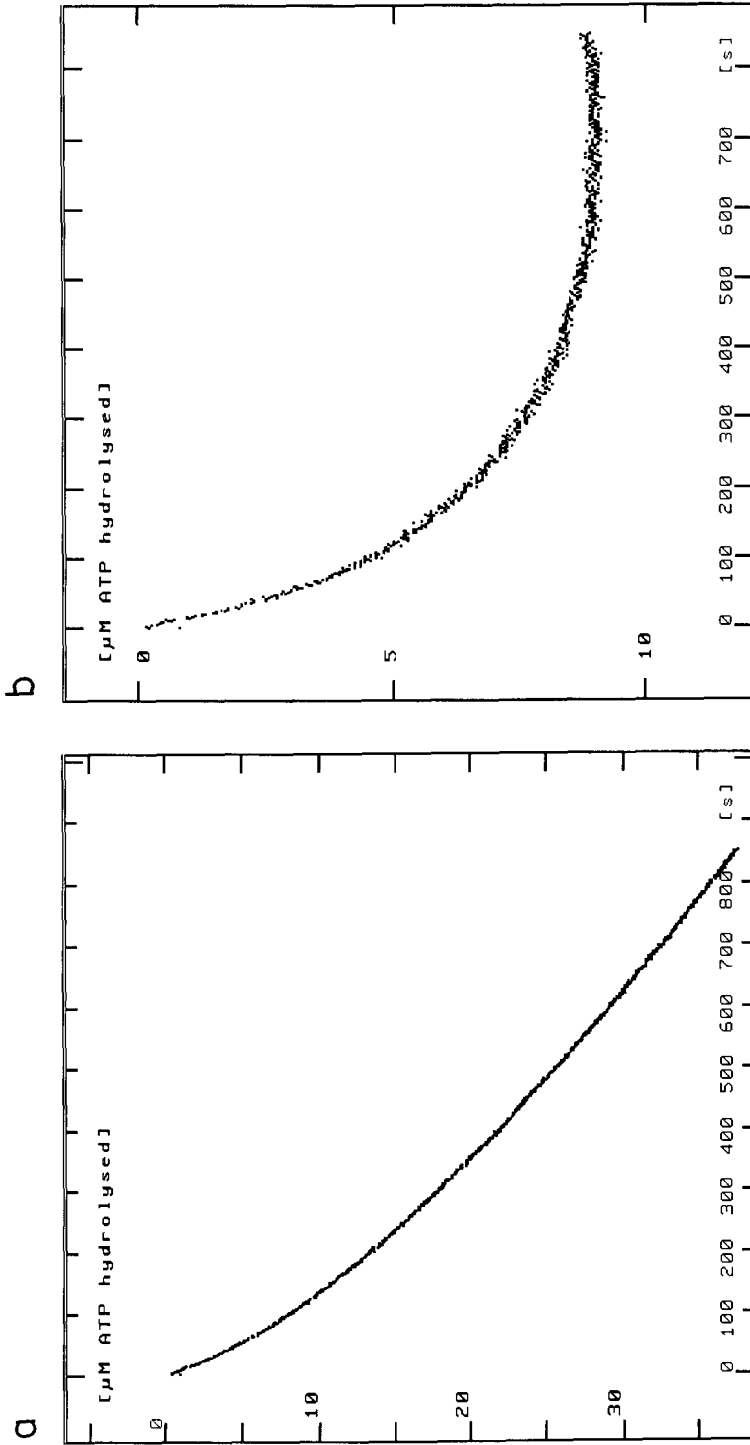


Fig. 2. ATPase activity during A_D . Experimental conditions as in Fig. 1 (except a rhodopsin concentration of $3 \mu\text{M}$). In addition, the medium contained $100 \mu\text{M}$ PEP, $100 \mu\text{M}$ NADH, 10 U pyruvate kinase and 10 units lactate dehydrogenase. The total ATPase activity (a) was determined from the consumption of NADH, measured as the decrease in absorbance at 340 nm . To correct for light scattering the absorbance at 400 nm was subtracted using an Aminco DW-2 dual wavelength spectrophotometer. The initial phase of activity (b), which is attributed to the A_D signal, was obtained by digitally correcting for the residual constant slope after 8 min . The levelling off of the slope is not due to a lack of substrate: when, after completion of a measurement, ADP is added, it is converted into ATP with the same rate as if it had been added prior to the start of ATPase activity.

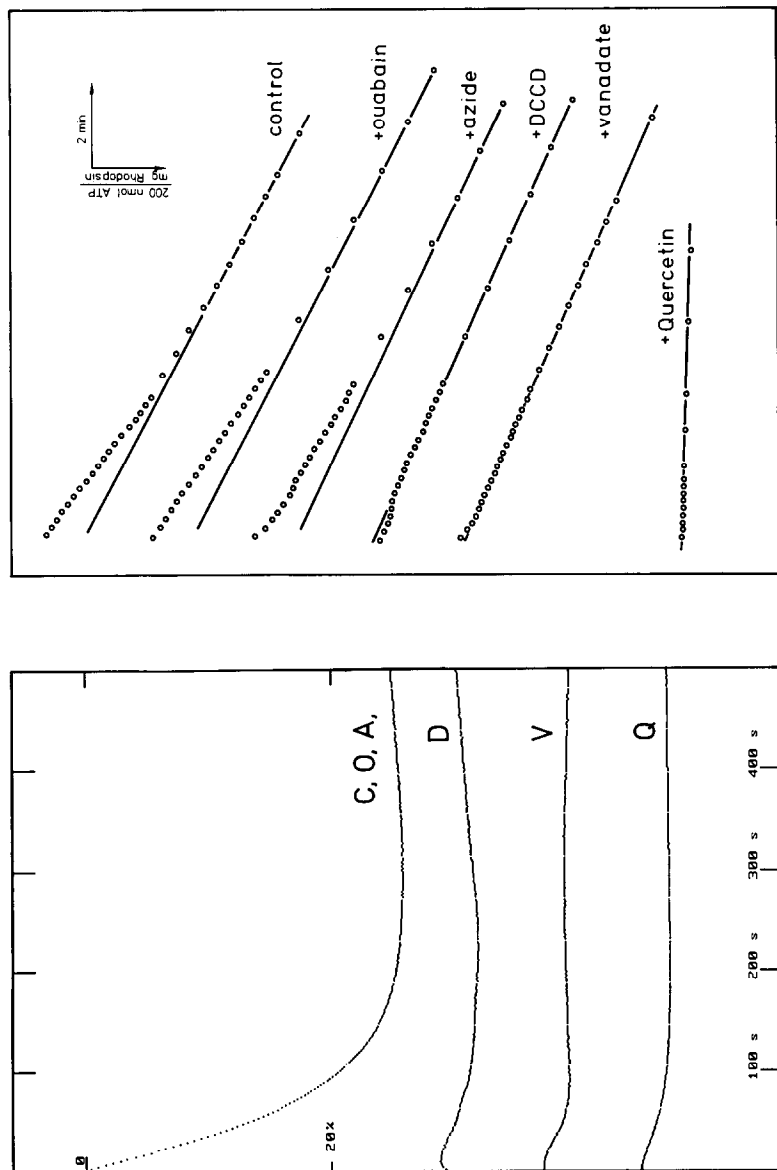


Fig. 3. A_D (left) and concomitant ATPase activity (right) in the presence of various inhibitors: C, control, no additions, medium as in Figs. 1 and 2, rhodopsin concentrations $3 \mu\text{M}$, ATP concentration $100 \mu\text{M}$; O, $+10 \mu\text{M}$ ouabain; A, $+100 \mu\text{M}$ azide; D, $+20 \mu\text{M}$ DCCD; V, $+20 \mu\text{M}$ vanadate; Q, $+100 \mu\text{M}$ quercetin.

scattering signals in the presence of ATP (see ref. 19). The recently discovered filaments connecting the rim of adjacent disks [20] would be candidates for such structures and could hence be responsible for A_D . However, the results of this and a following paper do not support this interpretation.

We have also attempted to clarify the structural origin of A_D and we have therefore determined its angular dependence (Fig. 4). It is basically the same for stacked disks, lysed ROS (data not shown) and freely floating, isolated disks, thus ruling out the participation of vesicle aggregation effects and demonstrating that neither a disk stack nor peripheral ROS proteins are essential. The only structure which all three preparations have in common (see also the electron micrographs in refs. 14 and 15) is the disk itself, indicating that it must be the disk structure that undergoes the ATP-dependent changes.

Interpreting light-scattering data from ROS material by theoretical means is exceedingly difficult [21] and has hardly ever led to meaningful conclusions. Therefore, we tried to mimick the ATP-dependent light-scattering changes during A_D by osmotically-induced volume changes. An identical angular dependence is observed when disk swelling is produced by the addition of the potassium ionophore valinomycin ($2 \mu\text{M}$) to disks suspended in a medium containing KSCN (Fig. 4). Thiocyanate is a lipid-soluble anion which promotes net salt uptake, provided that there is a conductance for the particular cation. The similarity of the angular profiles leads us to the conclusion (which will be verified repeatedly later on) that the structural origin of A_D is also a swelling of the disk compartment due to the uptake of salt and water.

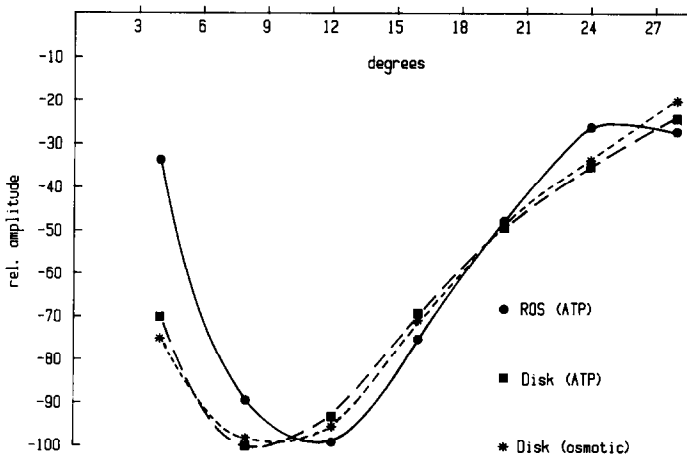


Fig. 4. Angular dependence of A_D signals obtained from ROS and from freely floating, isolated disks. For comparison the angular dependence of an osmotically-induced disk swelling is also shown.

3.5. A_D signal and disk volume

The disk compartments of ROS fragments, suspended in media of different osmotic strength, behave as (not ideal) osmometers [22]. Figure 5 shows that the amplitude of A_D depends strongly on the osmolarity and therefore on the disk volume. The peculiar behaviour in sucrose medium, *i.e.* the increase in amplitude at very high osmolarities, is consistent with the observation of Berman *et al.* [23] that ROS disks are swollen in media of high sucrose concentration. There is, so far, no explanation for this effect, which seems to indicate that, under certain conditions, sucrose can enter the disk. It would also provide a plausible explanation for the observation of Thacher [19] that the internal volume of the disk, as determined by tracer experiments (using labelled sugars), shows unrealistically high values and no increase on addition of ATP.

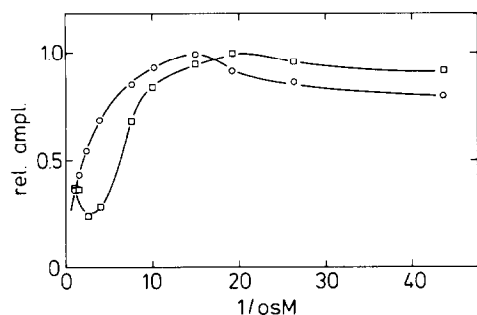


Fig. 5. Amplitude of A_D as a function of osmolarity and hence disk volume; \circ , KCl medium; \square , sucrose medium. Experimental conditions as in Fig. 1, except that the respective osmolarities were adjusted using KCl or sucrose.

Figure 6 shows the dependence of the disk volume, as determined by light scattering, on the osmolarity of the medium. When the ROS fragments are incubated in the presence of ATP prior to the osmotic manipulation, the

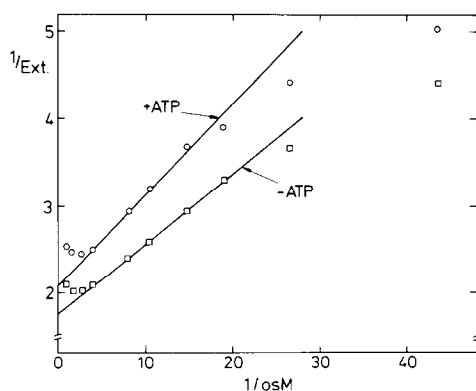


Fig. 6. Turbidity of a ROS suspension, before and after the completion of A_D , as a function of the osmolarity of the medium. Osmolarities were adjusted with KCl. The reciprocal extinction value is a measure of the disk volume [21].

disk volume assumes enhanced values at all tested osmolarities, suggesting that the number of osmotically active ions in the disk interior has increased. However, the extrapolation of the turbidity to infinite osmotic pressure (this should be a measure of the maximally compressed disk volume) also yields an increased value after completion of A_D . This implies that osmotic effects alone are not sufficient to explain A_D .

A possible reason for the observed difference in the "compressible volume" of the disks could be an increase in surface potential within the disk, which would impede a closer approach of the two adjacent disk membranes. However, proton transport into the disk interior, as inferred from other experiments, would tend to decrease the surface potential rather than increase it. Moreover, since A_D signals are identical in the presence and absence of 50 mM ammonium acetate (data not shown) (a membrane-permeable salt that has been reported to destroy any surface potential inside the disk [24]), it must be assumed that surface potentials do not play a major role in the underlying processes of A_D .

3.6. Ion specificity of A_D

3.6.1. Divalent and trivalent cations

The substrate of the ATPase responsible for A_D is Mg-ATP. Calcium and barium cannot substitute for magnesium and manganese can only substitute to a small extent (data not shown). Moreover, in the presence of equal amounts of magnesium and calcium A_D is inhibited. However, calcium inhibition can be overcome by simply increasing the magnesium concentration. EGTA does not have an inhibitory effect on A_D ; it sometimes stimulates it slightly. Trivalent cations, such as lanthanum, block A_D completely, even at very low (50 μ M) concentration.

3.6.2. Monovalent cations

Figure 7 shows that A_D signals, recorded in the presence of potassium and choline chloride, are almost identical. The same is true (data not shown) for sodium chloride. However, when the disk membrane becomes permeable to potassium due to the presence of 5 μ M valinomycin, the signal is markedly enhanced in KCl and is reduced in choline chloride. This clearly demonstrates that A_D is accompanied by transmembrane processes and points to the participation of diffusion potentials. Further support for this comes from the observation that A_D can be destroyed by minor amounts of detergents or lysolecithins (data not shown), substances known to cause leaks in membranes.

A_D can also be observed in the presence of membrane-permeant cations (Fig. 8). The permeable cations used in this study (TRIS, ammonium and imidazolium) are all weak bases. In the presence of a pH gradient Δ pH they redistribute between two aqueous phases according to the proton gradient. A proton uptake by the disk during the course of the A_D signal should lead to a concomitant enhanced swelling and hence increase the signal amplitude. The amplitude of the A_D signal increases with decreasing

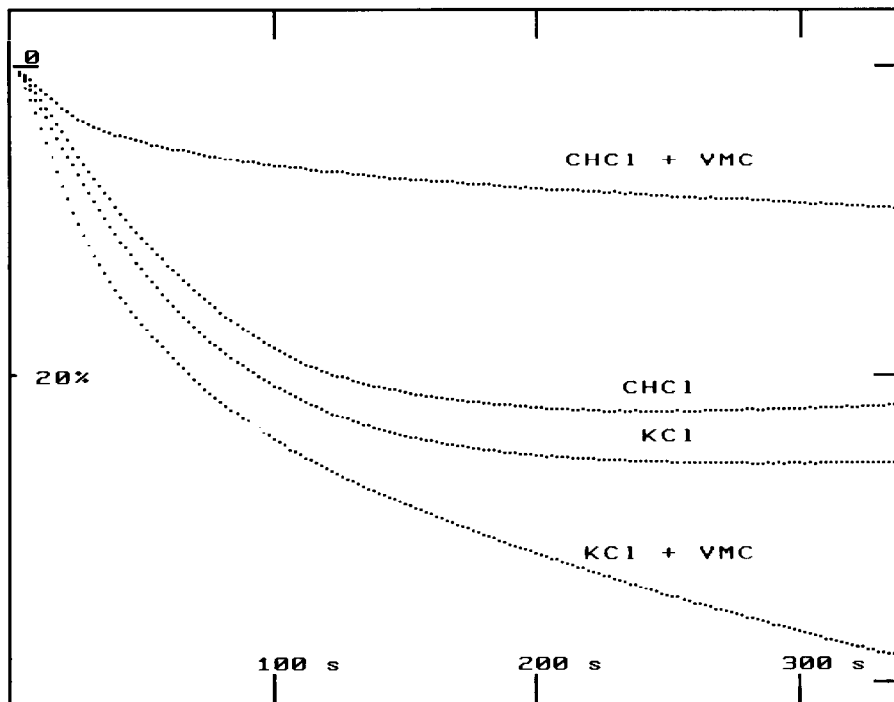


Fig. 7. Effect of $5 \mu\text{M}$ valinomycin on A_D signals: ROS fragments were suspended in 100 mM choline chloride (ChCl) or 100 mM potassium chloride (KCl).

pK value of the permeable cation. Since the fraction of the lipid-soluble, unchanged form also increases with decreasing pK (at a pH of 7.2, 0.1% of the ammonia, 10% of the TRIS and 50% of the imidazolium molecules are unchanged) this is further support for the assumption of proton uptake by the disk.

Following the same reasoning, A_D signals should decrease in size in the presence of lipid-permeable, weak acids. They should leave the disk interior as a consequence of the proposed proton uptake. This has been observed using maleic acid (data not shown).

3.6.3. Anion specificity

The anion specificity was tested using the potassium salts of a number of different anions (Fig. 9). The greatest amplitudes are observed in the presence of lipid-soluble anions (thiocyanate, iodide), whereas in the presence of the impermeable, zwitterionic anion PIPES, A_D is completely suppressed. The same result is obtained when PIPES is replaced by glutamate (data not shown). From this we can conclude that the light-scattering signal is accompanied by anion uptake. It is interesting to note that A_D signals are enhanced, but not accelerated, in the presence of lipophilic anions. This points to the ATPase activity as the rate-limiting factor in the production of the A_D signal.

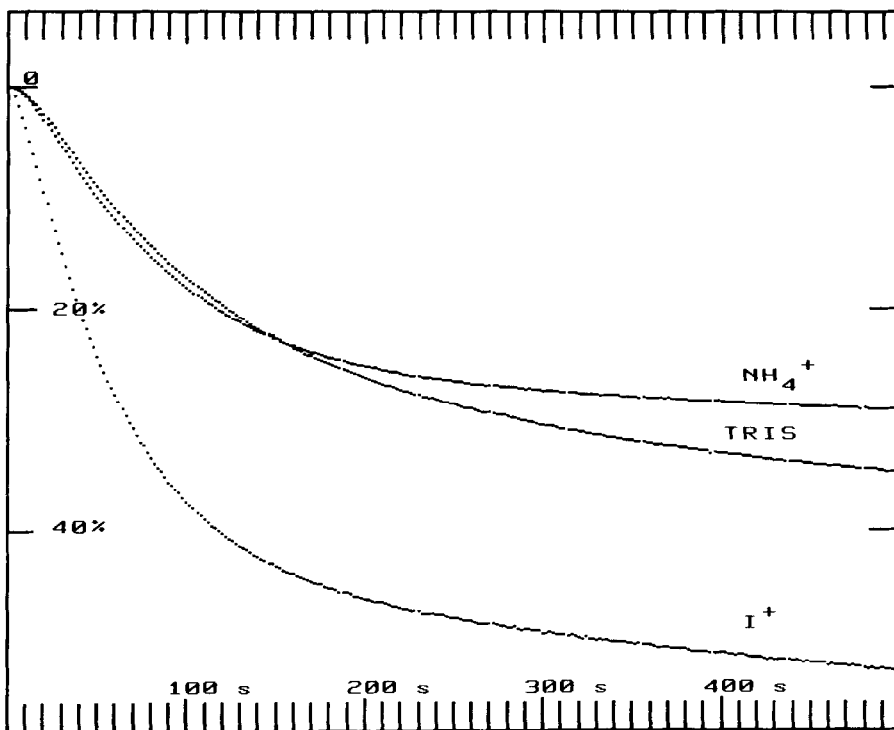


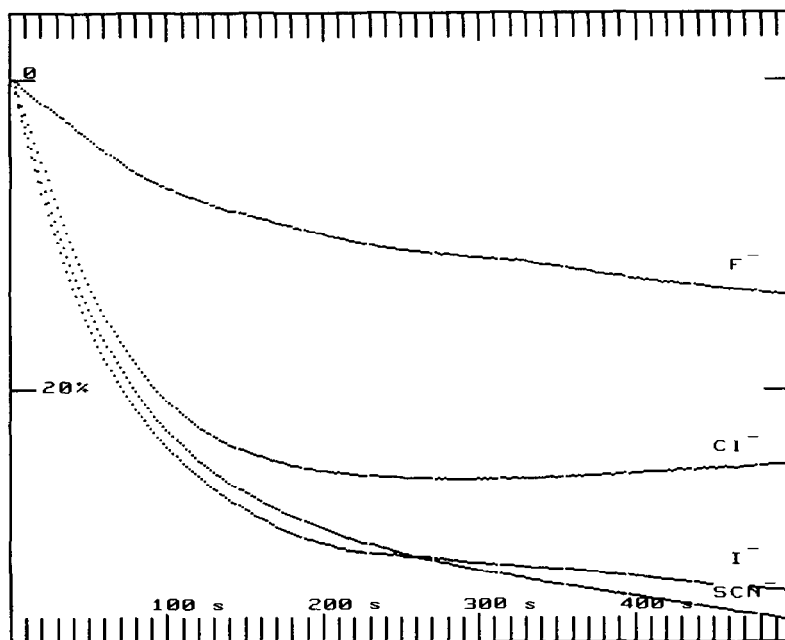
Fig. 8. A_D signals obtained in the presence of various permeant cations at pH 7.0: I^+ , 100 mM imidazolium chloride; NH_4^+ , 90 mM ammonium chloride; TRIS, 100 mM tris-HCl.

3.6.4. Action of the anion channel blocker DIDS

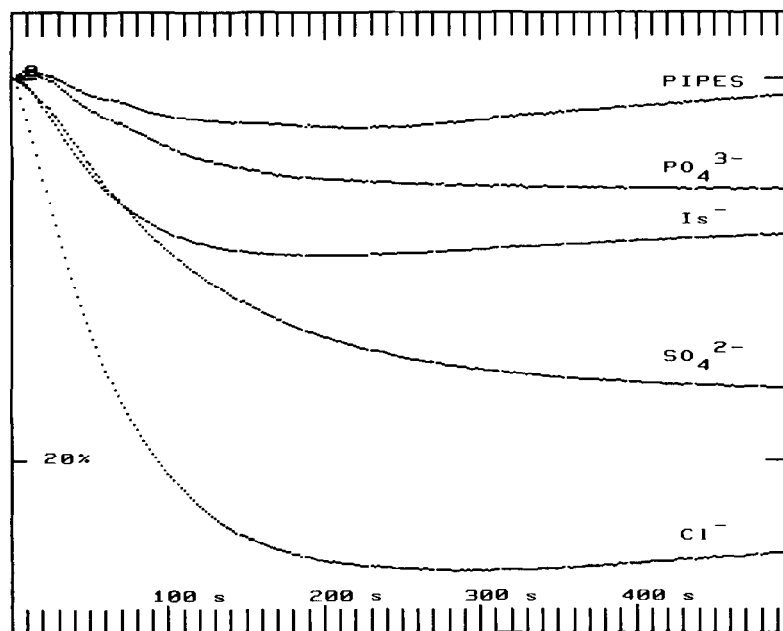
Further evidence for the participation of anion transport during A_D comes from the finding that the anion transport blocker DIDS inhibits A_D in the presence of chloride, but not in the presence of the lipophilic thiocyanate anion (Fig. 10).

3.6.5. Action of ionophores

In Fig. 10 A_D signals are shown in the presence and absence of the ionophores tributyltin (a chloride- OH^- exchanger) and nigericin (a potassium-proton exchanging antibiotic [22]). The former destroys the signal completely (presumably by reversing the ATP-driven uptake of HCl), whereas the latter increases it (it allows the transformation of a proton gradient into an osmotically more effective potassium gradient, hence causing increased disk swelling). Consequently, if the ATPase itself is rate limiting, the rate of swelling should increase twofold. Figure 10 demonstrates that this is so.



(a)



(b)

Fig. 9. A_D signals in the presence of various anions: (a) 90 mM of the various potassium salts; (b) the potassium salt concentration of the various anions was chosen such that the total osmolarity was 200 mosM. The abbreviation Is^- denotes isethionate.

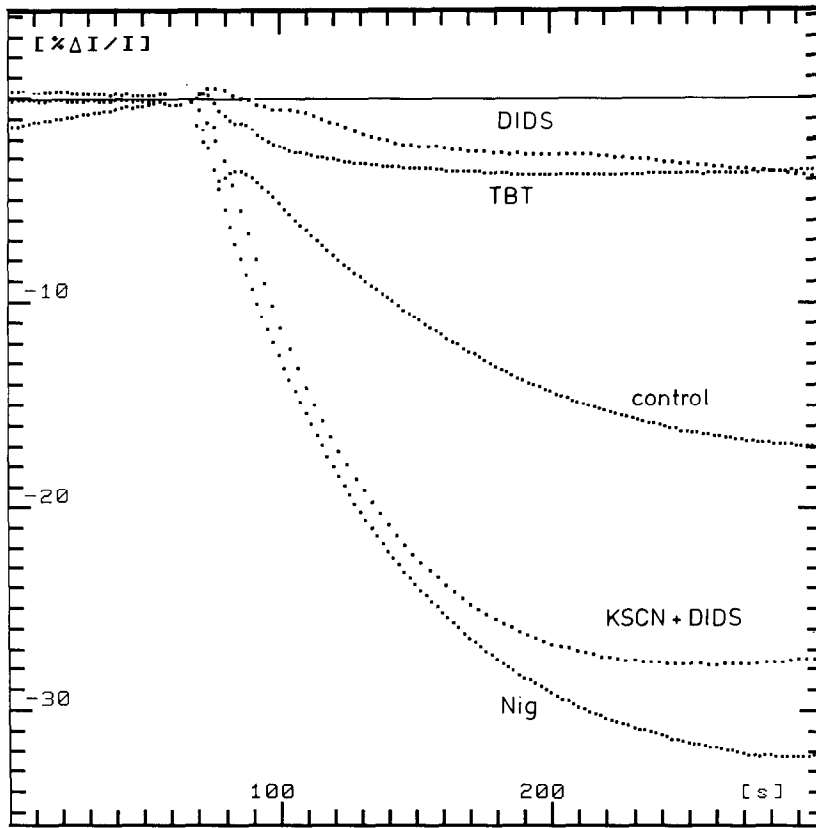


Fig. 10. A_D signals in 100 mM KCl, in the absence (control) and presence of 2 μ M nigericin (Nig), 1 μ M tributyltin (TBT), 50 μ M DIDS or 100 mM KSCN + 50 μ M DIDS. ROS fragments were incubated for 5 min in the presence of the ionophores or the inhibitor before Mg-ATP was added.

3.7 Dependence on pH

Between pH 5.5 and 8.0, A_D is independent of the pH of the medium. Both above pH 8 and below pH 5.5 its amplitude decreases; below pH 4.0 its sign is reversed (Fig. 11).

3.8. Dependence on light

Qualitatively identical A_D signals were obtained from unbleached and bleached ROS. The amplitude was usually slightly enhanced with bleached ROS (data not shown).

3.9. Dependence on taurine

There have been reports in the literature on an ATP-dependent, taurine-stimulated calcium uptake by rod disks [25]. Therefore we tested the effect of taurine (50 mM) on A_D . No effect was found (data not shown).

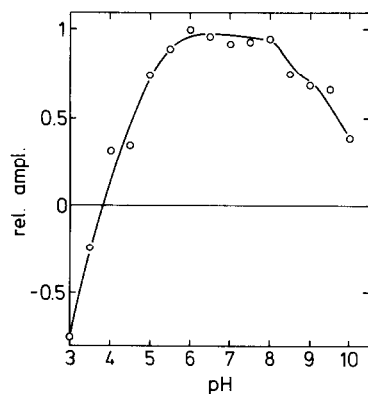


Fig. 11. Amplitude of as a function of pH A_D signal. The following buffers were used: pH 3.0 - 4.5; 5 mM citric acid; pH 5.0 - 6.0; 5 mM MES; pH 6.5 - 7.5; 5 mM PIPES; pH 8.0 - 9.0; 5 mM BICINE; pH 9.5 - 10.0; 5 mM CAPS.

4. Discussion

A scattering signal as large as A_D (20% - 40%), which can be detected with the naked eye, clearly reflects a major structural reorganization. The experiments with freshly detached frog ROS indicate that in the living retina the energized state of the disk, *i.e.* its state after completion of the underlying processes of A_D , prevails and must therefore be considered to be physiological. This would seem to indicate that part of the huge metabolic energy supply in the photoreceptor cell is used for the setting up and maintenance of the proposed disk energization. Rod outer segments contain a nucleotide buffering system, and their usual nucleotide complement is 20 mM creatine phosphate, 3 mM ATP and 3 mM GTP [26].

There are two classes of transport ATPases in biological systems. The F_1/F_0 -type ATPases of energy-conserving membranes, and the less complex transport ATPases found in other membranes. The latter are usually sensitive to vanadate, have no soluble protein subunits (like the F_1 complex) and form stable, phosphorylated intermediates [27]. Our ATPase still functions after hypotonic washing procedures and is inhibited by vanadate. It is probably identical with the vanadate-sensitive, phosphorylated, 160 kDa protein described by Thacher [28]. It therefore belongs to the second category.

Most ATPases are transport ATPases, *i.e.* they promote transmembrane ion transport. The observation that A_D signals can be obtained from freely floating, isolated disks indicates that the responsible ATPase is an integral part of the disk membrane and rules out a dynein-type ATPase located in connecting filaments [19]. This and the fact that typical transport ATPase inhibitors can block A_D and the concomitant ATPase activity, points to a

transport ATPase. Moreover, A_D is affected by ionophores in an ion- and ionophore-specific way and is completely destroyed by reagents that break through the structural integrity of the disk compartment.

The question as to which ions are transported during the production of the A_D signal can be answered, although not absolutely conclusively, on the basis of the light-scattering experiments. A vast amount of experimental evidence points to the proton as the primary transported ion: neither specific cations (apart from magnesium) nor specific anions are necessary for A_D signals to occur. The DCCD sensitivity of the effect also confirms this, and the dramatic increase in signal amplitude in the presence of weak permeant bases such as imidazole suggests the same.

Proton transport across membranes can be electrogenic or electro-neutral. The requirement for a permeant anion (either a lipid-soluble anion, such as thiocyanate, or chloride which obviously possesses specific carrier-mediated pathways in the disk) points to an electrogenic mechanism in the case of A_D .

When all the evidence is combined the following picture emerges. There is a vanadate- and DCCD-sensitive Mg-ATPase in the disk, which pumps protons into the disc interior. The proton uptake is electrically compensated by an anion uptake. Under physiological conditions this anion is chloride, which can cross the disk membrane through a specific DIDS-inhibited transport unit. As a result of the proton translocation a proton-motive force $\Delta\mu(H^+)$ develops across the disk membrane and also a chloride gradient. The HCl uptake leads to an osmotic swelling of the disks and it is this swelling which gives rise to the light-scattering signal A_D . Consequently, conditions which increase the (osmotically visible) ΔpH component of $\Delta\mu(H^+)$ (thiocyanate or iodide instead of chloride) enhance the amplitude of A_D . A similar enhancement is observed when valinomycin is administered in the presence of potassium: the swelling due to the uptake of HCl will dilute the internal potassium concentration and this will induce further swelling due to the uptake of KCl. However, when valinomycin is administered in the presence of an impermeable cation, internal monovalent cations, which can be transported by valinomycin, are exchanged for the pumped protons and this, in turn, decreases the swelling and hence A_D .

When conditions are chosen such that KCl (in the presence of nigericin) or imidazolium chloride can be taken up instead of HCl, the swelling is also enhanced due to the fact that only a small fraction of the internal protons are not bound and therefore osmotically active. In addition, under these conditions the ATPase can act longer and transport more ions before a $\Delta\mu(H^+)$ value has developed which is large enough to slow down the ATPase.

The rate-limiting step in the production of A_D could either be the ATPase activity or the chloride transport. The fact that A_D is not accelerated when chloride is replaced by thiocyanate points to the first possibility. Moreover, both A_D signals and the concomitant ATPase activity start with a maximal rate. Therefore the electric field component of $\Delta\mu(H^+)$ should

develop slowly during the production of A_D . This has been verified experimentally using field-sensitive dyes (data to be published).

Assuming a 1:1 or 2:1 stoichiometry between the number of pumped protons and the number of hydrolysed ATP molecules, there are approximately 4 - 8 protons taken up per rhodopsin molecule during the production of A_D . This will be sufficient to protonate most or all of the available proton binding sites inside the disk [24].

The persistence of a small vanadate-sensitive (and DCCD-sensitive) ATPase activity after the completion of A_D suggests the existence of a small leak in the disk membrane, most probably for protons. It corresponds to a flux of 280 - 560 ions $\text{disk}^{-1} \text{s}^{-1}$. This low value is not in conflict with our previous observation of a strikingly low proton permeability of the disk [22]: even if the proposed $\Delta\mu(\text{H}^+)$ value amounts to only 10 mV, the leakage conductance of an entire disk will be in the range 4 - 8 fS!

It is interesting to note that Bennett [29] has reached similar conclusions about A_D , namely that ATP causes a chloride-dependent swelling of the disks. She has shown that this swelling is a prerequisite for a specific, light-induced, G-protein-related light-scattering signal. This signal, however, is distinct from the ATP-dependent light-scattering signal A_L , which probes the energized disk state with flashes of light, and which will be dealt with in detail in a following paper.

Acknowledgment

This work was supported by a research grant from the Deutsche Forschungs-Gemeinschaft (DFG, UH30/6,7) and a Heisenberg fellowship to R.U.

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