Evidence for aerobic metabolism in retinal rod outer segment disks

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The disks of the vertebrate retinal rod Outer Segment (OS), devoid of mitochondria, are the site of visual transduction, a very energy demanding process. In a previous proteomic study we reported the expression of the respiratory chain complexes I–IV and the oxidative phosphorylation Complex V (F1,F0-ATP synthase) in disks. In the present study, the functional localization of these proteins in disks was investigated by biochemical analyses, oxymetry, membrane potential measurements, and confocal laser scanning microscopy. Disk preparations, isolated by Ficoll flotation, were characterized for purity. An oxygen consumption, stimulated by NADH and Succinate and reverted by rotenone, antimycin A and KCN was measured in disks, either in coupled or uncoupled conditions. Rhodamine-123 fluorescence quenching kinetics showed the existence of a proton potential difference across the disk membranes. Citrate synthase activity was assayed and found enriched in disks with respect to ROS. ATP synthesis by disks (0.7 μmol ATP/min/mg), sensitive to the common mitochondrial ATP synthase inhibitors, would largely account for the rod ATP need in the light.

Overall, data indicate that an oxidative phosphorylation occurs in rod OS, which do not contain mitochondria, thank to the presence of ectopically located mitochondrial proteins. These findings may provide important new insight into energy production in outer segments via aerobic metabolism and additional information about protein components in OS disk membranes.

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1. Introduction

Light captured by visual pigment Rhodopsin (Rh) in rod Outer Segments (OS) of the vertebrate retina triggers phototransduction (Stryer, 1996; Pepe, 2001; Ridge et al., 2003; Lamb and Pugh, 2006). Rods, associated with scotopic vision, have reached the utmost sensitivity to light (Fain et al., 2001), being triggered by a single photon (Lamb and Pugh, 2006). A unique property of OS is the presence of a stack of floating membranous disks surrounded by plasma membrane, housing the integral and peripheral membrane proteins that perform photon capture and visual transduction (Ridge et al., 2003). Disks are synthesized disks at the base of the rod and move toward the tip, where the upper disks are eventually phagocytosed by the pigment epithelium (Bok, 1985; Finnemann et al., 1997). This localization is corre-
lated to a decrease in membrane cholesterol content in parallel with the acquisition of functionality (Albert and Boesze-Battaglia, 2005).

The exciton phase of the vertebrate rod photoreceptor leads to a light-stimulated enzymatic cascade culminating in the hydrolysis of guanosine 3′-5′-cyclic monophosphate (cGMP), generating the neuronal signal. The recovery of photoreceptor is due to a guanyllyl cyclase stimulated by the lowered Ca2+ levels following photoexcitation (Koch and Stryer, 1988; Ridge et al., 2003; Lamb and Pugh, 2006). Current hypotheses for continual GTP supply in OS include its production via glycolysis or its diffusion from the Inner Segment (IS) (Carretta and Cavaggioni, 1976; Ames et al., 1986; Hsu and Molday, 1994; Pepe, 2001). Although anaerobic glycolysis may provide enough ATP, i.e. GTP thanks to the conversion to ATP to GTP by the activity of a nucleoside diphosphate kinase (NDP) (Orlov et al., 1996), for cGMP turnover in dark adapted rods, illumination increases cGMP turnover by 5-folds, exceeding the glycolytic capacity of rods to produce ATP (Biernbaum and Bownds, 1985; Ames et al., 1986; Pepe, 2001). There is a mismatch between the energy that can be produced by glycolysis to supply the OS (Hsu and Molday, 1994; Pepe, 2001) which sets up the requirement for another energy source.

Our recent proteomic studies have identified several subunits of the four redox complexes and of F0-F1-ATPase in enriched outer segment disk membrane preparations (Panfoli et al., 2008). A proteomic analysis of rod OS was recently reported (Kwok et al., 2008) focused on proteins implicated in vesicle trafficking and membrane fusion.

In the present paper, we show that purified disks aerobically synthesize ATP at a rate which largely accounts for the need of energy during phototransduction, by employing a transmembrane proton (H+) potential (ΔΨH+).
2.4. ATP synthesis assays

2.4.1. Spectrophotometric determination

ATP formation from ADP and inorganic phosphate ($P_i$) in disks was studied by an upgrading of the experimental protocol previously reported (Panfoli et al., 2008), according to Mangiullo et al. (2008). Disks (0.03 mg protein/ml) were incubated for 5 min at 37 °C in 50 mM Tris/HCl (pH 7.4), 100 mM KCl, 1 mM EGTA, 5 mM MgCl$_2$, 0.6 mM ouabain, 0.4 mM of the adenylyl kinase inhibitor di(adenosine-5') penta-phosphate (Ap$_5$A), and ampicillin (25 μg/ml). ATP synthesis was then induced by adding 5 mM KH$_2$PO$_4$, 20 mM succinate, 0.35 mM NADH, and 0.1 mM ADP (or 0.3 mM ADP for time course analyses) at the same pH of the mixture. After stopping the reaction with 7% perchloric acid, the ATP concentration in each sample was measured in a luminometer (Lumi-Scint, Bioscan) by the luciferin/luciferase chemiluminescent method with ATP standard solutions between 10$^{-3}$ and 10$^{-7}$ M for calibration (Roche Diagnostics Corp., Indianapolis, IN). NADP reduction was followed in a dual-beam spectrophotometer (UNICAM UV2, Analytical S.n.c., Italy).

2.4.2. Bioluminescent luciferase ATP assay

ATP synthesis in bovine retinal mitochondria was conducted first incubating samples (0.03 mg protein/ml) for 5 min at 37 °C in 10 mM Tris/HCl (pH 7.4), 100 mM KCl, 1 mM EGTA, 2.5 mM EDTA, and 5 mM MgCl$_2$ with 0.2 μM di(adenosine-5') penta-phosphate, 0.6 mM ouabain, 50 mM Cyclosporin A and ampicillin (25 μg/ml). ATP synthesis was then induced by adding 5 mM KH$_2$PO$_4$, 20 mM succinate, 5 mM pyruvate/2.5 mM malate and 0.1 mM ADP at the same pH of the mixture. After stopping the reaction with 7% perchloric acid, neutralized and clarified supernatant was added to a mixture containing 2 mM MgCl$_2$, 0.5 mM NADP, 5 mM Glucose, 100 mM Tris/HCl pH 7.4 and 7 U/ml of a mix of hexokinase and glucose-6-phosphate dehydrogenase (Roche Diagnostics Corp., Indianapolis, IN). NADP reduction was followed in a dual-beam spectrophotometer (UNICAM UV2, Analytical S.n.c., Italy).

2.5. Spectrofluorimetric measurements of disk membrane potential

$\Delta \mu_{\text{H}^+}$ of isolated disk was assay using Rhodamine 123 (RH-123) [Emaus, 1989] (Excitation 503 nm-Emission 527 nm). Fluorescence was detected at 25 °C with a thermocostatically controlled PerkinElmer (MA, USA) LS 50B spectrofluorometer, under continuous stirring. 0.020 mg bovine rod disks were added to buffer (2 ml final volume) containing: 10 mM HEPES pH 7.2, 5 mM MgCl$_2$, 0.1 mM ouabain, 50 mM KCl, 0.2 mM Ap$_5$A, and an ADP-regenerating system (10 mM glucose and 2.5 U hexokinase), and incubated for 15 min. Then 50 mM RH-123 was added, dissolved in ethanol (kept below 0.4%).

2.6. Oxymetric measurements

Respiration rates (0.04 mg protein/1.7 ml) were measured at 22 °C in a closed chamber according to [Aicardi and Solaini, 1982], using a thermostatically controlled oxygraph apparatus equipped with an amperometric electrode (Unisense--Microrespiration, Unisense A/S, Denmark) that determines the μM of O$_2$ of any solution. Oxymeter was connected to a PC running dedicated proprietary datalogger software (MicOx, Unisense) converting data in Excel files. An electromagnetic stirrer bar was used to mix the contents of the chamber. Additions were conducted by Hamilton syringes, through a rubber cup in a volume of no more than 0.05 ml. Electrode was equilibrated with the appropriate medium before each experiment, until the oxygen consumption remained constant. For disks the incubation medium was: 50 mM HEPES, 120 mM KCl, 2 mM MgCl$_2$, 5 mM KH$_2$PO$_4$, 2.5 μg/ml ampicillin, and 0.25 mM Ap$_5$A, pH 7.3 (Sgobbo et al., 2007). For retinal mitochondria-enriched-fractions standard medium was: 75 mM sucrose, 30 mM Tris--HCl, 40 mM KCl, 2 mM MgCl$_2$, 5 mM KH$_2$PO$_4$, 0.5 mM EDTA, 25 μg/ml ampicillin, and 0.2 mM Ap$_5$A, pH 7.4 (Sgobbo et al., 2007). In mitochondria to stimulate the Complex I+III+IV pathway, 5 mM pyruvate and 2.5 mM malate were employed as oxidative substrates. The respiratory control ratios (RCR) of mitochondria-enriched fractions, calculated as the ratio between state 3 respiration and state 4 respiration, were above the value of 5. To observe the ADP-stimulated respiration rats 0.19 mM ADP was added. Respiratory rates are expressed in μM/mg/min. For uncoupled measurements, Nigericin (5μM) and Valinomycin (10μM) were added to the suspension after the membrane additions.

2.7. Spectrophotometric assay of disk redox complexes

Complex I (NADH-ubiquinone oxidoreductase) activity was measured on 50 μg of protein following the reduction of ferricyanide at 420 nm (Sottocasa et al., 1967). Complex II (Succinic dehydrogenase) was assayed at 600 nm, in 2 mM EDTA, 0.2 mM ATP, 20 mM succinate 0.5 mM cyanide, 80 μM dicloroindophenol (DCIP), 50 μM demicyubiquinone, 40 μM antimycin A and 10 μM rotenone and 10 mM Phosphate buffer, pH 7.2, with 50 μg of protein (Janssen et al., 2007). Complex III (Cytochrome c reductase) activity was measured following the reduction of oxidized Cytochrome c at 550 nm with 50 μg of protein (Sottocasa et al., 1967). Complex IV (Cytochrome c oxidase) activity was measured following the oxidation of Ascorbate-reduced Cytochrome c at 550 nm with 5 μg of protein (Baracca et al., 2003).

2.8. Confocal Laser Scanning Microscopy of disks

Treatments on disks (60 μg protein/50 μl) were conducted in solution, as reported (Ravera et al., 2007a). 2 μg/ml of anti-Rh, -Na+/K-ATPase, -TIM, or -ANT were used as primary Ab, Secondary Ab were: Cy3-labeled anti-mouse, or -rabbit IgG, Alexa Fluor® 488-labeled anti-goat IgG (Molecular Probes, Invitrogen Corporation). CLSM imaging was performed as reported (Panfoli et al., 2008) on an inverted LEICA TCS SP5 AOBS confocal laser scanning microscope (Leica Microsystems CMS, Mannheim, Germany). In controls, disks were treated with secondary Ab only, and yielded negligible immunoreactivity (data not shown).

2.9. TEM microscopy

Disks were fixed in 3% paraformaldehyde and glutaraldehyde (GA) 0.2% included in gelatine and freezed in liquid nitrogen. Ultrathin sections (20 nm thick) obtained with a microtome were put onto classical copper grids (1 mm x 1 mm). Disk sections were labeled with anti-Rh (1:200) as primary Ab. Primary Ab was recognized by rabbit anti-mouse secondary Ab and protein-A bound to colloidal gold (15 nm) (Amersham Biosciences, Piscataway, NJ).

2.10. DNA extraction from retinas and disk fractions

DNA from small aliquots of fresh whole bovine retinas and purified disks (16 μg of total protein) was obtained according to standard procedure (Maniatis et al., 1982). The final pellet was
resuspended in PCR-grade water. A NanoDrop® (NanoDrop® ND-1000 Spectro-photometer, NanoDrop Technologies) was used to measure UV absorbance spectra, 260/280 absorbance ratios and concentrations of DNA extracted from disks bovine and retinas. Stock DNAs were stored at −20 °C.

2.11. Primers and PCR amplification

A primer pair, to specifically detect bovine mitochondrial DNA, was designed by the on-line Primer3 software program (Rozen and Skaletsky, 2000). The sequences for primers were forward 5′-GCTAGACCCCAACTGGGATT-3′, reverse 5′-AGCCCATTTCTTCCATTTC-3′. Amplification reactions were carried out in a total volume of 20 μl, and reaction mixtures containing 5X PCR-Buffer, 2 mM MgCl₂, 1 pmol of each primer, 0.2 mM deoxynucleoside triphosphates, and 1 U of AmpliTaq DNA polymerase (PerkinElmer, Emeryville, Calif.). Starting from an equal amount of DNA (0.3 μg), the two samples were subjected to a PCR program consisted of 1 cycle at 95 °C for 2 min, then 12–27 cycles at 95 °C for 30 s for denaturing, 56 °C for 45 s for annealing, and 72 °C for 30 s for extension, and finally 1 cycle at 72 °C for 7 min. PCR products were separated on 1% agarose gels in Tris-acetate-EDTA buffer and stained with ethidium bromide (0.4 mg/ml).

2.12. Citrate synthase assay

Citrate synthase (CS, EC 4.1.3.7) activity was assayed in disks, rod OS, mitochondria and mitochondria prepared as disks by the reduction of acetyl-CoA to CoA with 5-5′-dithio-bis-2-nitrobenzoic acid (DTNB); thionitrobenzoic acid (TNB) formation was monitored at 412 nm. The reaction mixture was composed by 100 mM Tris–HCl pH 8.0, 0.1 mM DTNB, 0.4 mm acetyl-CoA and 10 mM of oxaloacetate. In order to allow the accessibility of the substrate to the enzyme, for mitochondria and mitochondria prepared as disks 0.1% Triton X-100 was added to the reaction mixture while disks and ROS were subjected to 15 passages through a needle (25 G). Reaction was started by addition of samples (20 μg of protein).

2.13. Standard procedures

Protein concentration was determined by the BCA protein assay from Pierce Biotechnology, Inc.

3. Results

3.1. Characterization of the disk fractions

The osmotically intact disk fractions were collected at the interface between a 5% Ficoll solution and water, after ultracentrifugation (Smith and Litman, 1982). The sediment contained the cellular material that bursts upon 5 h permanence in hyposmotic solution. Disks swell but do not burst by hypotonic shock. Their osmotic resistance is due to the phospholipid disk environment (Lamba et al., 1994). Purity of the disk preparations was evaluated by Transmission Electron Microscopy (TEM) imaging. Results are shown in Fig. 1. Panel A, is a TEM image of the disk fraction, representative of three experiments on different disk preparations. Flattened disks and sealed vesicles were observed. Careful, examination of the 12 × 3 shots taken in all the experiments showed absence of RIS organelles, such as mitochondria, endoplasmic reticulum, or melanosomes. Vesicles of other nature or resealed pieces of plasma membranes may be present, however would not float up (Smith and Litman, 1982). Fig. 1 (panel B) is a immunogold TEM image of disks labeled with anti bovine Rh Ab. Panel C is an overview of a single labeled disk. Panels B and C show that the visual pigment is distributed on disks.

3.2. ATP synthesis by rod disks

Fig. 2 shows the extra vesicular ATP formation determined after incubation of disks (0.03 mg of protein/sample) in the presence of ADP and inorganic phosphate (P₁). A maximal activity of 0.7 ± 0.1 μmol/min/mg of protein was detected in the presence of 0.35 mM NADH, 20 mM succinate and 0.1 mM ADP. Inset shows that the time course of the reaction was linear within 2 min. ATP synthesis was inhibited by the mitochondrial F₁Fₒ-ATPase/H⁺-pump inhibitor oligomycin (75%). The oligomycin-sensitive extra-vesicular ATP production was depressed by DCCD, inhibitor of passive H⁺ conduction (66%), the oxidative phosphorylation uncoupler FCCP (95%), and by the H⁺-K⁺/ionophores Nigericin/Valinomycin (97%). Additions of medium in which some
3.3. Assay of proton gradient on disks by Rhodamine 123 fluorescence quenching

Data suggests that the putative disk surface ATP synthase employs a transmembrane electrochemical $\Delta \mu_{H^+}$, whose existence has been previously envisaged (Kaupp et al., 1981; McConnell et al., 1968; Uhl et al., 1979; Uhl and Desel, 1989). The actual presence of a $\Delta \mu_{H^+}$ across the disk membrane was assessed monitoring fluorescence quenching of RH-123 (Baracca et al., 2003). In isolated organelles, RH-123 electrophoretically accumulates where a steep $\Delta \mu_{H^+}$ is present (Uckermann et al., 2004; Poot et al., 1996; Kahler et al., 2008) with a fluorescence quenching, due to aggregation of the dye (Emaus, 1986), proportional to $\Delta \mu_{H^+}$.

Fig. 3 shows the effect of a series of additions on the dye fluorescence. 50 nM RH-123 was added to the medium containing 0.02 mg/ml disks and an ADP-regenerating system. No fluorescence due to disks alone was detected. Addition of ADP induced an enhancement of steady-state fluorescence, indicating a decrease of $\Delta \mu_{H^+}$. Rotenone, a specific inhibitor of NADH dehydrogenase, caused a further fluorescence increase, likely due to the membrane potential dissipation. Disk membrane potential could be recovered by addition of succinate, which induced the quenching of the dye. Inhibition of ATP synthase by oligomycin induced a further increase of the membrane potential, causing decrease of fluorescence quenching. Addition of antimycin A, an inhibitor of Complex III of the respiratory chain, allowed a recovery of fluorescence, likely as a consequence of $\Delta \mu_{H^+}$ disappearance. Dynamic RH-123 redistribution across the disk membrane is likely a consequence of its potential changes.

3.4. Intact disks consume oxygen

The rate of oxygen ($O_2$) consumption by disks was measured at 23 °C with an amperometric electrode (Unisense A/S, Denmark). Fig. 4(panel A) reports a typical amperometric tracing of Nigericin/Valinomycin-uncoupled respiration rates in disks. Additions were: NADH (0.35 mM); ADP (0.19 mM); rotenone (0.1 mM); succinate (20 mM); antimycin A (0.05 mM); ascorbate (10 mM); KCN (0.5 mM); (B) as (A) but with coupled disk in the presence of oxidative substrates [NADH and succinate] and ADP. Insets report the mean activity of the classical states of respiration, expressed as mean $\mu$M O$_2$/s ± S.E. from five independent experiments. In both cases 0.04 mg of total disk proteins were used, in 1.7 ml volume. Rotenone, antimycin A inhibited oxygen consumption, while the addition of medium (ethanol) alone had no effect (not shown).

III of the respiratory chain, allowed a recovery of fluorescence, likely as a consequence of $\Delta \mu_{H^+}$ disappearance. Dynamic RH-123 redistribution across the disk membrane is likely a consequence of its potential changes.

A series of analogous experiments performed separately allowed to couple disk respiration to ATP production oxidative
phosphorylation efficiency (P/O ratio). The P/O ratio was calculated as the ratio between the nanomoles of ADP consumed and the nanomoles of O consumed during the ADP-stimulated respiration (conditions analogue to mitochondrial state 3). P/O ratio with NADH was 2.8 ± 0.15; and with succinate was 1.7 ± 0.11. Addition of ethanol alone elicited no effects (data not shown).

3.5. Respiratory complex assays

Fig. 5 shows the time course of the activities of the redox complexes in disks, in the absence or presence of specific inhibitors (rotenone for NADH-ubiquinone oxidoreductase, 72% inhibition, panel A; antimycin A for Cytochrome c reductase, 75% inhibition, panel C; cyanide for Cytochrome c oxidase, 99% inhibition, panel D). Succinic dehydrogenase activity is in panel B. Activity of each complex in disks was comparable to retinal mitochondria-enriched fractions in the same experimental conditions (data not shown).

3.6. Evaluation of the results in terms of the entity of mitochondrial contamination

The extent of contamination of the disk sample by IS organella was evaluated by immunofluorescence CLSM imaging of disks, using a new technique that we have developed (Ravera et al., 2007a). By this technique, we showed that ATP synthase is expressed as IU/mg (μmol of reduced DCPIP/min/mg of total protein). The presence of total DNA, and of mitochondrial DNA was assessed with Semiquantitative PCR. The chemiluminescent signal was calculated as the ratio of den- sitometric value of the band to that of total protein in each lane of the SDS-PAGE gel (as stained with Colloidal Blue Coomassie, shown in panel F). The presence of total DNA, and of mitochondrial DNA was assessed in disk samples, in comparison to bovine retinal aliquots, being 1645.5 and 51.4 ng/μl in retinas and disks, respectively. The ratio between the absorption at 260 vs 280 nm was 1.8 ± 0.02 for retinal DNA and 1.67 ± 0.02 for disks. The smooth absorbance spectrum from retina (green) showing an absorbance maximum at 260 nm demonstrates the high quality of the extracted DNA (Fig. 1S). The flat absorbance spectrum of disks (red in Fig. 1S), without any evident absorbance maximum, likely depends on the sensibly lower starting material and suggests the presence of nucleic acid degradation.

The presence of mitochondrial DNA in disks, was assessed with a primer pair specific for bovine mitochondrial DNA (see Section 2). Semiquantitative PCR assays conducted on an equal amount of DNA of the samples showed that while the amplification product of retinal mitochondrial DNA was visualized after 15 cycles and augmented with the increase in cycle number, disk mitochondrial DNA was detectable only after 18 cycles. Overall, the amplification of disk DNA confirmed the poor quality of DNA extracted from disks (Fig. 25).
3.7. Comparison among mitochondria and "mitochondria prepared as disks"

Fig. 7, shows that coupled mitochondria prepared and assayed in the presence of 50 nM Cyclosporin A conduct ATP synthesis (1.2 ± 0.2 μmol/min/mg of protein) sensitive to inhibition by oligomycin (95%), DCCD (93%), and FCCP (97%) (Fig. 7). Conversely, "mitochondria prepared as disks" (i.e.: diluted in Milli-Q water in the absence of nutrients and of Cyclosporin A, for 1 h (see Section 2)), synthesize a negligible amount of ATP (bar 5). Table 1 reports the mean values of ATP production expressed as μmol/min/mg of protein.

Table 1

<table>
<thead>
<tr>
<th></th>
<th>μmol ATP synthesized/min/mg protein</th>
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<tr>
<td>Mitochondria-enriched fraction</td>
<td>1.2 ± 0.2</td>
</tr>
<tr>
<td>&quot;Mitochondria prepared as disks&quot;</td>
<td>0.009 ± 0.002</td>
</tr>
</tbody>
</table>

Table shows ATP production from ADP and P_i at 37° C by coupled mitochondria, or "mitochondria prepared as disks". Data are the mean of four separate experiments ± S.D.
Fig. 8. Respiration rates in retinal mitochondria-enriched fraction and in “mitochondria prepared as disks”. (A) Respiration rates in retinal coupled mitochondria (0.04 mg/1.7 ml) prepared and assayed in the presence of 50 nM Cyclosporin A. (B) Respiration rates in coupled “mitochondria prepared as disks” (0.04 mg/1.7 ml) (see Section 2). Additions: pyruvate (2.5 mM); malate (5 mM); ADP (0.19 mM); rotenone (0.1 mM); succinate (5 mM); antimycin A (0.05 mM); ascorbate (10 mM); KCN, potassium-cyanide (0.5 mM).

Fig. 9. Semiquantitative Western Blot analysis of the four respiratory complexes. WB analysis was conducted on disks (D), rod OS (R) mitochondria (M) and mitochondria prepared as disks (Md) using a primary Ab against NADH-ubiquinone oxidoreductase (A), Succinate Dehydrogenase (B), Cytochrome c reductase (C) and Cytochrome c oxidase (D). (E) Reports the semiquantitative densitometric analysis of these signals. The protein pattern of boiled samples is in (F). Panels are representative of three experiments.

Fig. 8 (panel A) is a representative amperometric recording trace of respiration rates of coupled mitochondria prepared and assayed in the presence of 50 nM Cyclosporin A. “Mitochondria prepared as disks” (panel B) are unable to consume O$_2$ likely due to the opening of the MTP (Berman et al., 2000). Table 2 shows the mean O$_2$ consumption of mitochondria-enriched fractions and “mitochondria prepared as disks”, in coupled conditions. Rates were calculated from the first derivative of the oxygraph traces. Substrates and inhibitors were the same used for disks.

3.8. Semiquantitative Western Blot analysis of the four respiratory complexes

Fig. 9 shows the chemiluminescent WB signal of the presence on disks of NADH-ubiquinone oxidoreductase (panel A); Succinic dehydrogenase (panel B); Cytochrome c reductase, (panel D); Cytochrome c oxidase (panel D), monitored with specific Abs in disks, OS, retinal mitochondria-enriched fractions, and mitochondria prepared as disks, the latter two used as a control. Relative quantification (panel E) of chemiluminescent band signal was calculated as the ratio of the densitometric value of each band to that of total protein in each lane of the SDS-PAGE gel (as stained with Colloidal Blue Coomassie, shown in panel F, where OS and disks proteins were boiled before the run, in order to eliminate Rh).

Table 2

<table>
<thead>
<tr>
<th>Samples</th>
<th>Oxygen consumption (μM O$_2$/min/mg protein)</th>
<th>Complex I + III + IV</th>
<th>Complex II + III + IV</th>
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<tr>
<td>Disks</td>
<td>151 ± 12</td>
<td>253 ± 21</td>
<td></td>
</tr>
<tr>
<td>Mitochondria-enriched fraction</td>
<td>243 ± 23</td>
<td>160 ± 10</td>
<td></td>
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<tr>
<td>“Mitochondria prepared as disks”</td>
<td>0.23 ± 0.03</td>
<td>0.56 ± 0.07</td>
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</table>

Respiration rates in coupled disks, mitochondria and “mitochondria prepared as disks”. To stimulate the Complex I + III + IV pathway, NADH was substituted by 5 mM pyruvate and 2.5 mM malate. Data are the mean ± SD of at least eight experiments.
3.9. Citrate synthase assay

Citrate synthase (CS) was assayed in ROS, disks, mitochondria and mitochondria prepared as disks, as an example of a mitochondrial matrix protein. Its expression in disks, together with that of other Krebs cycle enzymes had been reported in our previous proteomic paper (Panfoli et al., 2008). The presence of a number of cytosolic proteins identified by Mass spectrometry of the 2-DE gels, among is justified by the fact that disks purified by Ficoll flotation are aggregated, entrapping some ROS cytosol (Ravera et al., 2007a). Fig. 10 shows that CS activity (panel A) is consistent, and enriched in disks with respect to OS, that is a less purified fraction, indicating that it may not be a contaminant. Mitochondria and mitochondria prepared as disks (Md) were used as a control. WB analysis (panel B) with a specific antibody confirmed the presence of the enzyme in all of the fractions. Relative quantification (panel G) of chemiluminescent band signal, calculated as the ratio of densitometric concentration in OS 3 mM, about 85% of the disk protein), one order of magnitude in excess with respect to the photoreceptor need of 30 μM/μm²/h (where disks and OS were boiled before the run.

4. Discussion

The present biochemical metabolic studies suggest that rod OS disks possess a respiratory capacity (Figs. 4 and 5) which can build up a $\Delta\mu_{H^+}$ (Fig. 3) to drive ATP synthesis (Fig. 2) from ADP and F$_1$, accordingly to our recent proteomic studies that identified several subunits of the mitochondrial Electron Transfer Chain (ETC) complexes and of ATP synthase in enriched outer segment disk membrane preparations (Panfoli et al., 2008).

ATP synthesis and O$_2$ consumption by disks were sensitive to the classical inhibitors (Figs. 2 and 4). Biochemical assays (Fig. 5) and semiquantitative Western Blotting analyses with specific antibodies (Fig. 9) show that the ETC complexes are functionally expressed in disks. Respiration rates of disks were similar to those observed in mitochondria (compare Figs. 4 and 8), but not sensitive to the potential loss that mitochondria suffer. In fact, disks were prepared and assayed in the absence of inhibitors of the opening of the MTP (Crompton et al., 1988) (Beutner et al., 1996). The ability of disks to use NADH as a respiring substrate indicates a free access to Complex I (Figs. 2–4), suggestive of a sidedness of the ETC and of ATP synthase similar to that of submicrochondrial particles, therefore devoid of substrate permeability barriers.

The hypothesis that putative disk ATP synthase employs a transmembrane $\Delta\mu_{H^+}$ to synthesise ATP like in mitochondria (Boyer, 1997), is supported by the redistribution of RH-123 across the disk membrane (Fig. 3) and by the inhibition of ATP synthesis by uncouplers (Fig. 2). RH-123 fluorescence quenching kinetics gives reliable and sensitive evaluation of $\Delta\mu_{H^+}$ (Emaus et al., 1986, Uckermann et al., 2004). We had found that the OS selectively stains with mitochondrial vital dyes ex vivo, in a manner sensitive to pharmacological disruption of the disk $\Delta\mu_{H^+}$ (Bianchini et al., 2008).

Observations dating since 1968 are consistent with the notion of disks as organelles able to store and release H$^+$ and unusually impermeant to H$^+$ (McConnell et al., 1968, Kaupp et al., 1981). A H$^+$ uptake by disks (2.8 mol H$^+$/mol Rh) stimulated by ADP at acidic pH, and a H$^+$ release inhibited by KCN at neutral pH were observed (McConnell et al., 1968, Kaupp et al., 1981). The $\Delta\mu_{H^+}$ across disk membranes was studied by light scattering experiments (Uhl and Desel, 1989; Uhl et al., 1989). Signals (half life, 1 min) were accompanied by ATP hydrolysis and inhibited by oligomycin and DCCD. Caretta and Cavaggioni performed ATP turnover experiments with isolated frog OS, and reported that addition of the uncoupler 2,4-dinitrophenol caused a decrease in ATP content (Caretta and Cavaggioni, 1976).

Mammalian retina consumes an elevated O$_2$ amount: OS have a 3-fold greater O$_2$ consumption than the inner retina (Braun et al., 1995). Anaerobic glycolysis may provide enough ATP for cGMP turnover in dark adapted rods, but illumination increases cGMP turnover by 5-folds, exceeding the glycolytic capacity of rods to produce ATP (Biernbaum and Bownds, 1985, Ames et al., 1986, Pepe, 2001). ATP need was calculated to be 127 μM/s in the light (Ames et al., 1986). The average diffusion time for cGMP (smaller than ATP) through the entire outer segment was estimated to be about six minutes by fluorescent probes (Olson and Pugh, 1993) while the time involved in phototransduction reactions is of the order of milliseconds.

It is tempting to presume that ATP supply for phototransduction in OS be carried out mainly by oxidative phosphorylation. The aerobic ATP synthesis of 0.7 ± 0.1 μmol/min/mg here reported corresponds to about 1330 μM/s (assuming M.W. of Rh 39 kDa, and its concentration in OS 3 mM, about 85% of the disk protein), one order of magnitude in excess with respect to the photoreceptor need of 127 μM ATP/s in the light (Ames et al., 1986). The great membrane surface area of the OS disks would be consonant to both light and O$_2$ capture.

The presence of the ATP synthase in ectopic locations has been reviewed (Chi and Pizzo, 2006), however, most Authors ascribe to ectopic ATP synthase functions different from ATP synthesis (Arakaki et al., 2003) (Martinez et al., 2003). Besides, the membrane of a cell bears an extracellular positive charge, not favourable for the functioning of an ATP synthase with an outward facing F$_1$ moiety (ecto-F$_1$-ATPase). In fact, only ATP hydrolysis by ecto-F$_1$-ATPase was reported in hepatocyte cultures (Fabre et al., 2006). Besides, if ATP synthase is not coupled to ETC, that carries out proton pumping and electron transfer (Wallace, 1999), its activity produces a pH unbalance.

An extracelluar ATP synthesis on the surface of hepatomes (Mangiullo et al., 2008) and of the human umbilical endothelial cells (HUVECs) (Arakaki et al., 2003) was reported. However, Quillen and colleagues of the ectopic ATP synthesis ranged from picomoles to nanomoles. By contrast, disks synthesise micromoles of ATP. To the best of our knowledge the present is the first study reporting such a consistent ectopic ATP synthesis. It must also be noted that activities are
referred to total protein, including Rh which accounts for about 85% (Stryer, 1996).

The disk ecto-F$_i$ would face a vesicle side bearing a negative charge, as disks form as invaginations of the plasma membrane, favouring the outward H$^+$ flow (Capaldi et al., 1994) to the OS cytosol. Interestingly, mitochondrial disorders, a group of human diseases characterized by defects of the OXPHOS, primarily affect visual system and the CNS and PNS (Zeviani and Di Donato, 2004).

The possibility that our results are due an artefact causing a fusion of mitochondrial vesicles with disks may not be ruled out. However, the quality of the disk preparations seems good (Figs. 1 and 6). Moreover, a contamination would not account for the absence of ANT, TIM, TOM and Na$^+$/K$^+$-ATPase (Fig. 6). Even if disks preparations were composed mostly of intact mitochondria, not visible by TEM analysis (Fig. 1), these would be uncoupled due to the procedure of disk isolation (Smith and Litman, 1987), as demonstrated in Figs. 7 and 8. Contaminating mitochondria, if any, would not respire after 3 h incubation in water in the absence of cyclosporin A (Mitchell, 1961) (Boyed, 1997).

Finding of ETC and ATP synthase in the purified retinal rod OS disks may mean that these be regarded as some particular mitochondrial nevertheless, the absence of assembling factors in the 2-DE gel of disks (Panfoli et al., 2008) and the observation that mitochondrial DNA is a contaminant in disks (Figs. 15 and 2S) may rule out the possibility that the assembly of complexes would proceed in the OS. Rather it may be speculated that the ETC and ATP synthase are transferred from the preformed inner mitochondrial membranes, fused to thenascent disks. In fact, the antibodies against the redox chains I to V recognized the proteins in both the redox complexes I, II and IV are thought to form a supercomplex in vivo (Genova et al., 2008), the assembly of the ETC may take place in mitochondria. It seems that the putative fusion would bring also some matrix proteins, considering the consistent activity of CS that we report (Fig. 10). This may mean that some proteins of the mitochondrial matrix be functionally expressed in the OS cytosol. Indeed, it is conceivable that the ETC be continuously fed by Krebs cycle directly within the interdiskal space. Matrix proteins may be transferred into OS cytosol during an hypothetic fusion of mitochondria with disks in formation. On the other hand, an artifactual fusion during experiments would be ruled out by the consideration that even if a membrane embeds the respiratory complexes it may not be able to synthesize ATP, unless it is impermeant to protons, an essential prerequisite to build the proton gradient (whose presence across disks is shown in Fig. 3), but a very unusual characteristic restricted to respiring organella.

A physiological recruitment of mitochondrial proteins into disk membranes may be hypothesized. Mitochondria may then fuse with disk membranes during their formation, as recently discussed for myelin sheath in our report of ectopic ATP synthesis and O$_2$ consumption by intact myelin vesicles (Ravera et al., 2009). In our proteomic study by ESI-MS/MS of the monodimensional gel one protein related to fusion: dynamin 1 (gi12320374) was found. Further studies may lead to a deeper understanding of the mechanism of exportation of mitochondrial proteins on extramitochondrial membranes.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.biocel.2009.08.013.

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