Photoactive mitochondria: *In vivo* transfer of a light-driven proton pump into the inner mitochondrial membrane of *Schizosaccharomyces pombe*

[bacteriorhodopsin/mitochondrial signal sequence/photocycle/2',7'-bis(2-carboxyethyl)-5 (and -6)-carboxyfluorescein/glucose uptake]

Astrid Hoffmann, Volker Hildebrandt*, Joachim Heberle, and Georg Büldt

Forschungszentrum Jülich, Institut für Biologische Informationsverarbeitung, IBI-2: Biologische Strukturforschung, D-52425 Jülich, Germany

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ABSTRACT   The light-driven proton pump bacteriorhodopsin (bR) from *Halobacterium salinarium* has been genetically transferred into the inner mitochondrial membrane (IM) of the eukaryotic cell *Schizosaccharomyces pombe*, where the archaebacterial proton pump replaces or increases the proton gradient usually formed by the respiratory chain. For targeting and integration, as well as for the correct orientation of bR in the IM, the bacterioopsin gene (*bop*) was fused to signal sequences of IM proteins. Northern and Western blot analysis proved that all hybrid gene constructs containing the *bop* gene and a mitochondrial signal sequence were expressed and processed to mature bR. Fast transient absorption spectroscopy showed photocycle activity of bR integrated in the IM by formation of the M intermediate. Experiments with the pH-sensitive fluorescence dye 2',7'-bis(2-carboxyethyl)-5 (and -6)-carboxyfluorescein revealed bR-mediated proton pumping from the mitochondrial matrix into the intermembrane space. Glucose uptake measurements under anaerobic conditions showed that yeast cells containing photoactive mitochondria need less sugar under illumination. In summary, our experiments demonstrate the functional genetic transfer of a light energy converter to a naturally nonphotoactive eukaryotic organism.

In the past, several efforts were made to obtain functional expression of the bacterioopsin gene (*bop*) in a heterologous (1–3) or in a homologous system (4–7). In previous papers, it was shown that the fission yeast *Schizosaccharomyces pombe* is the only heterologous organism suitable for functional expression of bacteriorhodopsin (bR) in high yield (8, 9), without isolation and reconstitution of the protein in artificial membranes as is necessary for the *Escherichia coli* expression system (3). The retinal protein bR expressed in *S. pombe* with its original leader sequence is located in the cell membrane as in the natural host *Halobacterium salinarium*. To obtain functional bR in the fission yeast, bacterioopsin (bO) is regenerated *in vivo* by the addition of retinal to the culture medium (8, 9).

Until now the combination of bR with the mitochondrial energy-converting system has only been carried out *in vitro*. bR was reconstituted in liposomes together with the F_1F_0-ATPase (10), or it was fused with submitochondrial particles (11) or integrated into mitochondria by a freeze–thaw technique (12). This paper, however, describes the *in vivo* transfer of bR into the inner mitochondrial membrane (IM) of *S. pombe*.

Mitochondrial protein targeting has been investigated in a variety of systems (13, 14). More than 90% of the mitochondrial proteins are encoded by nuclear genes and are synthesized in the cytoplasm as precursors with N-terminal signal sequences that mediate protein transport from the cytosol to the mitochondria. During import, signal sequences are removed to generate the mature proteins (13, 14). To target the heterologously expressed bR into the IM, the *bop* gene was fused to signal sequences employed by mitochondrial proteins. For targeting and integration as well as for the correct orientation of bR in the IM (C-terminal end in the matrix and N terminus in the intermembrane space of the mitochondria), the following three distinct signal sequences from proteins with different positions in the IM and the intermembrane space were selected: (*i*) the signal sequence of the β subunit of the F_1F_0-ATPase (15) from *S. pombe* (ATP2), (*ii*) the signal peptide of the Rieske Fe/S protein (RIP1) (16), and (*iii*) the signal sequence of the cytochrome *c* oxidase subunit IV (17) from *Saccharomyces cerevisiae* (COX IV). Transcription and translation products of the hybrid gene constructs were analyzed, and the proper function of the light-driven proton pump in mitochondria was demonstrated. The present report proves that bR molecules targeted to mitochondria form a proton gradient that is used for energy production in the same way as the proton gradient produced by the respiratory chain.

MATERIALS AND METHODS

Microbial Strains, Growth Media, and Yeast Expression Vector. For transformation, *S. pombe* cells (*leu1-32 h⁻*) were grown in yeast extract medium (9). Transformed yeast cells were cultured in minimal medium with 1% (wt/vol) glucose as described (9). For isolation of mitochondria, the glucose concentration was reduced to 0.3%. *H. salinarium* strain ET1001 was grown in complex medium for the isolation of purple membrane (PM) (18). The hybrid gene constructs were ligated into the fission yeast expression vector pEVP11 (19), resulting in the following plasmids: pEVp-ATP2, pEVm-ATP2, pEVp-RIP1, pEVm-RIP1, pEVp-COX IV, and pEVm-COX IV.

Isolation of Membranes and Mitochondria. Isolation of PM from *H. salinarium* was performed according to the protocol in ref. 18. Membranes from *S. pombe* were prepared as described elsewhere (9). *S. pombe* mitochondria were isolated as in ref. 20. *In vivo* regeneration of bR in *S. pombe* cells, by adding retinal to the culture medium, was performed in 6-h intervals to a final concentration of 2 μM before the isolation of mitochondria and during the glucose uptake measurements.

Northern and Western Blot Analysis. Total cellular yeast RNA was extracted as described (9). RNA (for each sample,

Abbreviations: bR, bacteriorhodopsin; bO, bacterioopsin; IM, inner mitochondrial membrane; PM, purple membrane; BCECF, 2',7'-bis(2-carboxyethyl)-5 (and -6)-carboxyfluorescein (free acid); BCECF/AM, acetoxymethyl ester of BCECF.
*Present address: Howard Hughes Medical Institute, University of California, 5-748 MacDonald Building, 10833 Le Conte Avenue, Los Angeles, CA 90024-1662.

5 μg of total RNA) was fractionated on a 1.5% formaldehyde/agarose gel and subsequently transferred to a nylon membrane. To obtain a probe for the Northern blot, radioactive labeling was done by nick-translation of the *bop* gene. Hybridization and washing were performed as specified by Nitsch *et al.* (21). SDS/PAGE and Western blot analysis were carried out as reported (9).

Fast Transient Absorption Spectroscopy. Laser flash-induced changes in absorbance were recorded as described (22, 23). Measurements on isolated mitochondria of clone pEVp-COX IV and pEVP11 were performed at 10°C because the organelles are more stable at lower temperatures. In addition, photocycle kinetics are slowed down, which results in a higher signal-to-noise ratio at longer time scales. Due to the high turbidity and the low concentration of bR in the pEVp-COX IV sample compared to a PM sample, an electronic low-pass filter (rise time, 1 μs) had to be used, which limited the time resolution. After Percoll density gradient centrifugation, the samples were suspended in buffer S (600 mM mannitol/2 mM EGTA/10 mM Tris maleate) at pH 6.8. Data of 200 successive recordings were averaged.

Measurements with the Acetoxymethyl Ester (AM) of 2′,7′-Bis(2-carboxyethyl)-5 (and -6)-carboxyfluorescein (BCECF), a pH-Sensitive Fluorescence Dye. BCECF (free acid, final concentration of 5 μM) was added to buffer M (650 mM mannitol/0.36 mM EGTA/1 mM Mops/1 mM MgCl$_2$/1 mM K$_2$HPO$_4$) at pH 7.4, 7.1, 6.8, and 6.4 to illustrate the pH sensitivity of the dye. Isolated mitochondria (0.5 mg/ml) were incubated with BCECF/AM (final concentration of 5 μM) for 30 min at 25°C in buffer M. The metabolic substrates and inhibitors were present in the corresponding sample at the following final concentrations: 10 mM succinate, 100 μM ADP, 2 μM carbonylcyanide *p*-trifluoromethoxyphenylhydrazone, or 0.1% (vol/vol) Triton X-100. Excitation spectra were detected at 504 nm, and emission spectra were detected at 600 nm. To monitor the pumping ability of bR in mitochondria by BCECF, respective probes were illuminated for 1 min before recording the spectra.

Glucose Uptake Measurements. The glucose concentration in the media of anaerobically growing cultures with and without illumination was measured by a blood glucose test (Haemo-Glukotest 20-800 R, Boehringer Mannheim). The test was performed as described by the company. Anaerobic culture conditions were achieved by cultivating the cells under nitrogen.

RESULTS AND DISCUSSION

Hybrid Gene Constructs. Previous investigations revealed that the purple color of bR in the plasma membrane of *S. pombe* was more intense when bO was expressed with its own presequence (9). Therefore two types of constructs were made: one type of construct contained a mitochondrial signal sequence (ATP2, RIP1, or COX IV, signal peptide) ligated in front of the *bop* gene *with* the original 13 amino acid presequence of bO (*bop* constructs: pEVp-ATP2, -RIP1, and -COX IV); another type contained a mitochondrial signal sequence ligated to the *bop* gene *without* the presequence of bO (*bom* constructs: pEVm-ATP2, -RIP1, and -COX IV). To achieve in-frame fusions, oligonucleotides were placed between the DNA fragment coding for the signal peptide and the *bop* or *bom* gene. With respect to the ATP2, and RIP1 constructs, a part of the corresponding structural gene was also added. All hybrid gene constructs (Fig. 1) were constitutively expressed by the *S. pombe adh* promoter in the shuttle vector pEVP11 (19).

Expression of the Hybrid Gene Constructs and Processing of the Translation Products. Northern blot analysis of all hybrid genes shows a single band for each construct (Fig. 2A), indicating that a single messenger RNA species is transcribed
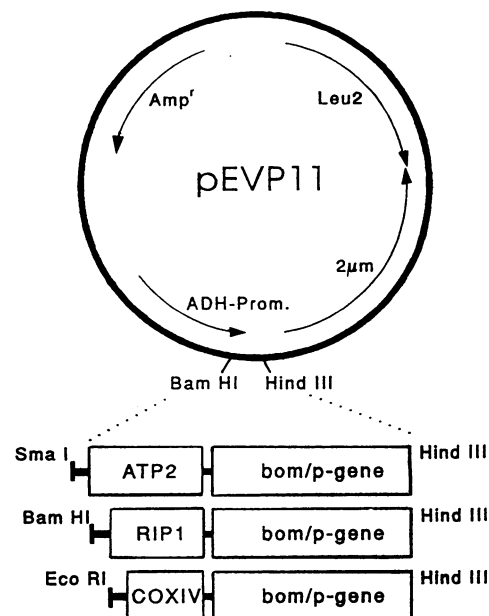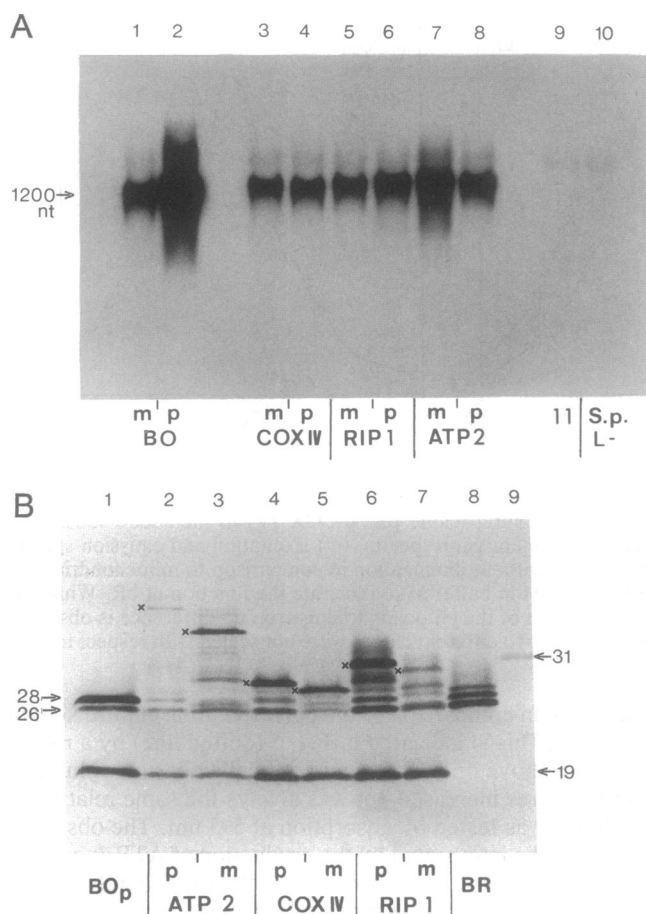

FIG. 1.   Hybrid gene constructs. The three mitochondrial signal sequences (ATP2, RIP1, and COX IV signal peptides) were fused to *bom* or *bop* gene (encoding the mature bO or the precursor of this protein). The hybrid gene cassettes were cloned into the fission yeast vector pEVP11 behind the constitutive *adh* promoter of *S. pombe*, resulting in (*i*) pEVp-ATP2, -RIP1, and -COX IV and (*ii*) pEVm-ATP2, -RIP1, and -COX IV. Amp^r, ampicillin resistance.

from each hybrid gene. The translation products of all hybrid gene constructs were determined by Western blot analysis of total membrane protein (Fig. 2B). The size of the upper band, marked by an "x" (Fig. 2B, lanes 2–7), is consistent with the calculated molecular mass of the corresponding hybrid bR or hybrid pre-bR (Table 1). In addition, a 26-kDa band is observed, suggesting that all hybrid proteins are processed to mature bR (26.5 kDa). The band pattern shown in Fig. 2B, lanes 2–7 does not represent unspecific cross-reactions of the bR antibody with yeast protein, because this pattern is not observed in lane 1 of Fig. 2B. A band of about 19 kDa (Fig. 2B, lanes 1–7) represents a cleavage product of bR in *S. pombe*, which appears if there is not sufficient retinal in the culture medium. The same band was observed in the retinal-deficient halobacterial strain *H. salinarium* JW5 (22) and is probably identical with the C-1 fragment after digestion of bR with α-chymotrypsin (24). The processing pattern of hybrid bR and/or hybrid pre-bR indicates that the hybrid gene products were targeted and inserted into mitochondrial membranes. Observations obtained from experiments with *Saccharomyces cerevisiae* and *Neurospora crassa* indicate that mitochondrial protein processing proceeds in multiple steps (13) involving several processing enzymes (25).

The presence of bR in the mitochondrial membrane was indicated by electron micrographs of immuno-gold-labeled thin sections from *S. pombe* protoplasts transformed with the hybrid gene constructs. Proteolytic digestion studies with papain and α-chymotrypsin on isolated mitochondria and mitoplasts revealed the orientation of bR in the IM with the C-terminal end protruding into the matrix and the N terminus directed toward the intermembrane space (data not shown).

Kinetics of the Photocycle of bR Integrated into the IM. Fig. 3 depicts the time course of changes in absorbance of bR in the IM of clone pEVp-COX IV. The recovery kinetics after bleaching with a nanosecond laser pulse of ground-state bR are well resolved (Fig. 3, lower trace). The appearance of the M intermediate of the photocycle of bR is clearly detected (Fig. 3, upper trace). Samples of mitochondria without bR

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A

B

Table 1. Molecular masses of unprocessed hybrid bR and/or hybrid pre-bR as calculated from the sequence of the hybrid genes and determined from the SDS gel

Hybrid gene construct	Molecular mass, kDa	
	Calculated	Determined from SDS gel
pEVp-ATP 2	38.39	37
pEVm-ATP 2	36.57	35
pEVp-COX IV	33.67	31
pEVm-COX IV	31.85	30
pEVp-RIP1	31.32	29
pEVm-RIP1	29.51	28

ever, based on the amount of bR undergoing the photocycle, a concentration of 0.02% (wt/wt) bR per total mitochondrial protein was estimated.

Monitoring Light-Driven Proton Translocation Across the IM by BCECF. The change in pH in the matrix of isolated *S. pombe* mitochondria was followed by the pH-sensitive fluorescence dye BCECF. This dye is taken up into the matrix of mitochondria as a BCECF/AM and is processed by esterases inside the organelle to the free acid BCECF. The free acid form is trapped in the matrix and shows fluorescence depending on the local pH (27). Fig. 4A illustrates that the fluorescence intensity of BCECF (free acid) increases with pH (28). Fig. 4B demonstrates that BCECF can faithfully monitor the mitochondrial matrix pH under various physiological conditions (27). Four different samples, each containing mitochondria from clone pEVp-COX IV, were measured. When succinate is added to isolated mitochondria (Fig. 4B, long dashes), respiration is turned on, and the proton gradient across the IM increases. In contrast, the addition of ADP (Fig. 4B, dotted line) leads to an acidification of the matrix due to the influx of protons and results in ATP synthesis. This acidification is reflected by a decreased fluorescence of BCECF, as compared to succinate-treated mitochondria. Incubation of mitochondria and proton translocators (uncouplers) like carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (Fig. 4B, solid line) reduces the proton electrochemical potential, resulting in a decrease in BCECF fluorescence. When Triton X-100 is added to isolated *S. pombe* mitochondria (Fig. 4B, dashed-dotted line), the fluorescence intensity

FIG. 2. (*A*) Northern blot analysis of total RNA isolated from *S. pombe*. The following clones were investigated: lanes 1 and 2, *S. pombe* containing the plasmid with *bom* or *bop* gene, respectively (clone mBO or pBO); lanes 3–8, clones transformed with the hybrid gene constructs (clone m- and p-COX IV; m- and p-RIP1; m- and p-ATP2); lane 9, yeast cells transformed with the plasmid pEVP11 (clone 11, containing neither the *bom* nor the *bop* gene); lane 10, untransformed leucin-negative *S. pombe* cells (clone S.p. L-). In each lane about 5 μg of total cellular yeast RNA were fractionated. The nick-translated *bop* gene was used as the hybridization probe. The size of the represented RNA from clones mBO and pBO is about 1200 nt as estimated elsewhere (9). (*B*) Western blot analysis of total membrane proteins from *S. pombe* transformed with the hybrid gene constructs grown in the absence of retinal, purified PM from *H. salinarium*, and cytoplasmic membrane from *S. pombe* containing bR. Lane 1, 10 μg of membrane protein of clone pBO (precursor bR molecular mass of 28 kDa, mature bR molecular mass of 26 kDa). Lanes 2–7, 10 μg of total membrane protein from *S. pombe* transformed with the hybrid gene constructs; the band of the corresponding unprocessed hybrid bR or hybrid pre-bR is marked by an "x." Lane 8, 0.1 μg of bR from *H. salinarium*. Lane 9, 6 μl of molecular size marker (Rainbow marker; Amersham). The antiserum was directed against the C-terminal end of bR. Molecular sizes (in kDa) are indicated.

(clone pEVP11) did not exhibit any changes in absorbance at 412 nm (Fig. 3, middle trace). The photocycle kinetics of bR in the IM (clone pEVp-COX IV) are similar to those observed for the pigment inserted into the plasma membrane of *S. pombe* (data not shown; see ref. 22 for the kinetics at 20°C). Compared with bR in its native membrane (26), the decay of the M intermediate as well as the ground-state recovery time are slower in the IM of *S. pombe*. This effect has also been observed for bR reconstituted in liposomes, which differ in the lipid composition and the state of aggregation of bR.
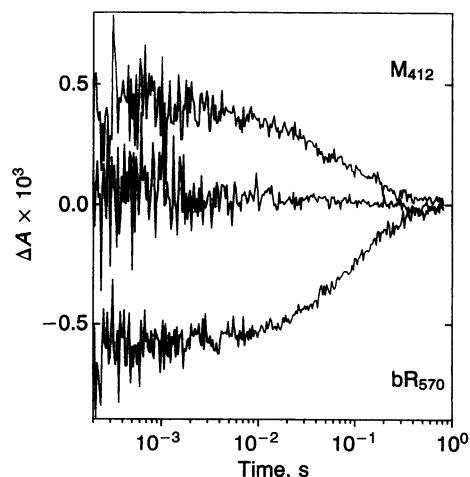
Due to the high turbidity of the samples, it was not feasible to record a steady-state absorption spectrum of bR. How-

FIG. 3. Light-induced changes in absorbance (ΔA) detected at 412 nm (absorbance maximum of the M intermediate of bR; ΔA positive, upper trace) and at 570 nm (absorbance maximum of the ground-state of bR; ΔA negative, lower trace) for mitochondria of clone pEVp-COX IV. The middle trace depicts a measurement (at 412 nm) of a sample where no bR is incorporated into the IM (mitochondria of clone pEVP11).
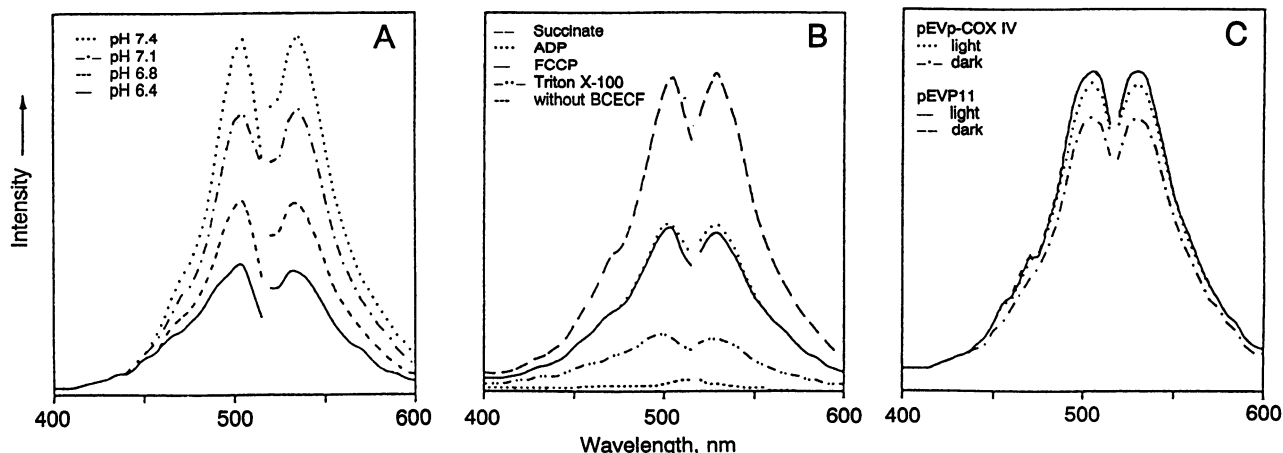
FIG. 4. (*A*) Sensitivity test of the dye BCECF (free acid) with respect to the pH of buffer M, showing changes in the fluorescence of excitation and emission spectra. (*B*) Response of the matrix pH of isolated *S. pombe* mitochondria (clone pEVp-COX IV) to metabolic substrates, uncoupler, and detergent in buffer M detected by BCECF fluorescence excitation and emission spectra. (*C*) Excitation and emission spectra monitored on isolated, bR-containing mitochondria (clone pEVp-COX IV) with and without illumination in comparison to mitochondria with no bR (clone pEVP11). Mitochondria of both clones were incubated with BCECF/AM in buffer M to illustrate the function of bR. While the difference of the two spectra of clone pEVp-COX IV show the pumping of protons out of the mitochondrial matrix, no difference is observed in the spectra of clone pEVP11 (the two spectra are superimposed). The spectra of the two different clones were not scaled with respect to each other. FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone.

drops to a minimum. As can be seen in Fig. 4*B* (short dashes), the background fluorescence is low.

Fig. 4*C* demonstrates the function of the light-driven proton pump bR in the IM. As a representative of the hybrid gene constructs, again mitochondria from clone pEVp-COX IV were investigated. Using the same sample, the matrix pH was determined with and without illumination. With light (dotted line), the concentration of protons in the matrix of mitochondria containing bR decreased, as indicated by the increase of BCECF fluorescence in comparison to the same sample kept in the dark (dash-dotted line). Mitochondria of clone pEVP11, which contain no bR, were used in control experiments (Fig. 4*C*; solid line, illuminated mitochondria; dashed line, mitochondria kept in the dark; the two lines are superimposed). Mitochondria from this control clone do not respond to light. There is no difference in the intensity of fluorescence before and after illumination of this sample (Fig. 4*C*, solid line and dashed line). The spectra recorded from these two clones (pEVP11 and pEVp-COX IV) are not scaled with respect to each other. Therefore, the fluorescence spectra obtained from the samples without illumination are not superimposable.

The orientation of bR in the IM can be inferred from the direction of the light-induced proton flux across the IM of bR-containing mitochondria. Accordingly, the C-terminal end of bR is located in the matrix, and the N terminus is directed toward the intermembrane space. This result was confirmed by experiments on suspensions of isolated bR-containing mitochondria, which showed an acidification of the suspension upon illumination measured by a pH electrode.

How Does Functional Integration of bR into the IM Influence the Physiology of *S. pombe* Cells? Transformed yeast cells (clone pEVp-COX IV) were grown under anaerobic conditions with and without light. Glucose was given to the culture medium as an energy source. The glucose concentration in the medium was measured during growth by the blood glucose test. The ratio of glucose concentrations in the culture media of cells grown with and without illumination is plotted in Fig. 5 for clone pEVp-COX IV (dotted line). The glucose concentrations from which these ratios were calculated are listed in Table 2. About 20 h after inoculation, when fermentation of this clone starts, the concentration of glucose in the culture medium of these cells, grown with light,

increased in comparison to the corresponding culture kept in the dark. This is indicated in Fig. 5 (dotted line) by a rise in the ratio above one. The number of cells per unit volume in both cultures increased but was always the same relative to each other as tested by absorption at 595 nm. The observed effect can be interpreted by the production of ATP due to the light-induced proton gradient across the IM. Therefore, less ATP resulting from anaerobical glucose consumption is needed. Hence, the glucose uptake by the cell is down-regulated under illumination, and the concentration of glucose in the medium increases relative to transformed yeast growing in the dark. It is important to note that the opposite effect is obtained for *S. pombe* cells containing bR in the plasma membrane (clone pEV-BOp). In this case, protons are transported out of the cell under illumination, creating an elevated proton gradient across the plasma membrane (22). Due to a glucose–proton 1:1 symporter in the plasma membrane (29), more glucose is taken up by cells growing in light than by cells kept in the dark. This results in a change in the concentration ratio below one (Fig. 5, solid line). As a control, pEVP11-transformed cells, which contain no bR, do
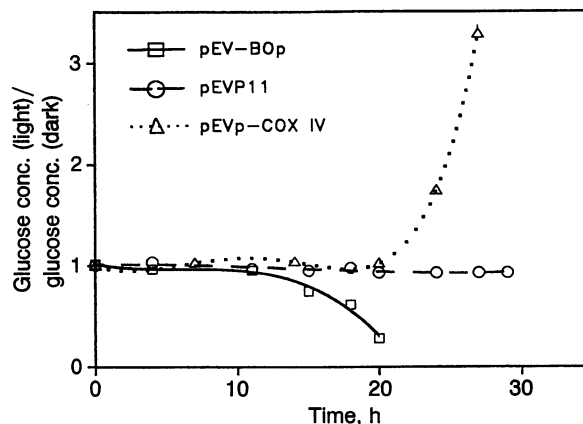

FIG. 5. Ratio of glucose concentrations in the growth medium for anaerobically growing *S. pombe* cultures with and without illumination [glucose conc. (light)/glucose conc. (dark)]. This ratio is plotted for three clones (pEV-BOp, pEVP11, and pEVp-COX IV). The absolute values of glucose concentrations are listed in Table 2.

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Table 2. Absolute glucose concentrations that went into the calculation of the ratios plotted in Fig. 5

Time, h	Glucose, mg/dl		Ratio of light:dark
	Light	Dark	
Clone pEV-BOp			
0	1220.0	1200.0	1.017
4	1050.0	1085.0	0.968
11	565.0	590.0	0.958
15	231.0	309.0	0.748
18	54.5	88.5	0.616
20	11.0	39.0	0.282
Clone pEVP11			
0	1210.0	1205.0	1.004
4	1125.0	1085.0	1.037
11	755.0	775.0	0.974
15	525.0	550.0	0.955
18	303.5	310.5	0.977
20	192.5	206.5	0.932
Clone pEVp-COX IV			
0	1065.0	1070.0	0.995
7	925.0	900.0	1.028
14	685.0	660.0	1.038
20	495.0	485.0	1.021
24	273.5	157.0	1.752
27	100.0	30.5	3.279
29	45.0	10.0	4.500

The data are representative examples of several measurements.

not respond to illumination; the ratio remains constant (Fig. 5, dashed line).

Conclusion. This paper describes the successful genetic transfer of a photoactive proton gradient generator into the IM and demonstrates its proper function in connection to the energy-converting machinery of the mitochondria. Further experiments (to be published elsewhere) gave additional evidence that the signal sequences of mitochondrial proteins directed bR to this organelle, where it is inserted into the IM as a functional proton pump: (*i*) Electron micrographs of immuno-gold-labeled thin sections from *S. pombe* protoplasts transformed with hybrid-gene constructs demonstrate the presence of bR in the mitochondrial membrane. (*ii*) Proteolytic digestion studies with papain and α-chymotrypsin on isolated mitochondria and on mitoplasts revealed the orientation of bR in the IM with the N-terminal end protruding into the intermembrane space and the C-terminal end into the matrix. (*iii*) When illuminating a suspension of isolated mitochondria from transformed *S. pombe* cells, an acidification of the suspension was measured by a pH electrode. (*iv*) An increased malate import into mitochondria caused by illuminating bR containing mitochondria was observed.

bR integrated into the IM may become an optimal model system for the study of membrane protein targeting and folding since its spectroscopic properties and proton-pumping abilities are very sensitive parameters to test for a functional molecule. In addition, *in vivo* studies of bioenergetic processes in mitochondria become more accessible. It will be possible to carry out time-resolved experiments on the import of various metabolites into mitochondria due to the generation of an additional proton gradient by bR.

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