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The light-driven proton pump, cruxrhodopsin-2 in *Haloarcula* sp. arg-2 (bR^+, hR^-), and its coupled ATP formation

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Abstract

Haloarcula sp. arg-2, a natural bacterial isolate from Andes heights, has a light-driven proton pump but not a light-driven anion pump. We have cloned and sequenced the gene encoding for the proton pump which has been named cruxrhodopsin-2. The gene consists of 768 bp encoding 255 amino acids with a molecular mass of 27544 Da. The deduced amino acid sequence of cruxrhodopsin-2 is 77%, 50%, 48% and 48% identical to those of cruxrhodopsin-1, bacteriorhodopsin, archaerhodopsin-1 and archaerhodopsin-2, respectively. The charged amino acids important for the proton pump function were conserved among all these molecules. Cruxrhodopsin-2 accounted for 0.05 nmol/mg protein in arg-2, which was 20–30-fold less than the proportion of bacteriorhodopsin in *Halobacterium salinarium* R_1M_1. In contrast to R_1M_1, under anaerobic conditions, arg-2 showed light-induced proton extrusion concomitant with an increase in ATP level without transient proton uptake. Dicyclohexylcarbodiimide enhanced the rate and extent of proton extrusion and inhibited ATP formation in the light. The apparent stoichiometry of H^+/ATP was estimated to be more than three in this natural bR^+hR^- strain.

Keywords: Halobacterium; Light-driven proton pump; Cruxrhodopsin-2; ATP synthesis; (*Haloarcula*)

1. Introduction

Bacteriorhodopsin (bR) of *Halobacterium salinarium* (*halobium*) is a light-driven proton pump and an extensively studied and well-characterized membrane protein [1]. Archaerhodopsin-1 and -2 (aR-1 and aR-2) are also proton pumps first isolated from two new halobacterial strains collected in Western Australia [2], both of which have been proposed to be classified into a new genus *Halorubra* (Mukohata, Y. et al., unpublished results). Alignment of amino acid sequences revealed that amino acid residues essential for the proton pump function were identical among these proteins [3,4]. However, a few amino acid residue substitutions in the retinal pocket seemed to affect their respective physicochemical properties [5]. Comparative studies on the structure/function relationships of light-driven proton pumps have begun with these natural bR analogues [6,7].

Recently, we collected several strains of halobacteria from salt pans located at Andes heights and the Peninsula Valdez in Argentina, and found novel retinal proteins distinguishable from bR and aRs. One of these has been named cruxrhodopsin-1 (cR-1) and has been reported previously [8]. In the present study, we have characterized another of these molecules, cruxrhodopsin-2 (cR-2), from an Andes isolate, strain arg-2. The amino acid sequence of cR-2 deduced from the DNA sequence of its gene was homologous to that of cR-1 but distinct from those of bR and aRs. Both strains, arg-1 and arg-2, have been classified into the genus *Haloarcula* (Mukohata, Y. et al., unpublished results). The finding of cR-2 supports our proposal that the proton pumps in halobacteria should be classified into at least three groups which diverged concomitantly with the differentiation into each genus [9].

Abbreviations: aR, archaerhodopsin; bR, bacteriorhodopsin; CCCP, carbonylcyanide-*m*-chlorophenylhydrazone; DCCD, dicyclohexylcarbodiimide; (k)bp, (kilo)base pair; MEGA-9, nonanoyl-*N*-methylglucamide; octyl-glucoside, n-octyl-β-D-glucopyranoside; SDS-PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate.

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Along with halobacterial rhodopsins, our interest has also concentrated on the photophosphorylation elements in halobacteria [10]. Threshold membrane potentials for ATP synthesis were demonstrated to be −100 mV in *Hb. salinarium* R_1mR (bR^-hR^+) vesicles under steady-state conditions [11]. In contrast, bR-mediated ATP synthesis could not be investigated separately from that mediated by hR because most halobacteria express both bR and hR [12], and a bR^+hR^- strain is required for this purpose. Arg-2 cells contained the light-driven proton pump cR-2, at levels as low as hR in *Hb. salinarium*, and, furthermore, appeared to have no hR-like anion pump. We present here the first observation of the initial proton translocation coupled to photophosphorylation in these naturally occurring bR^+hR^- cells.

2. Materials and methods

2.1. Bacterial strains

Haloarcula sp. arg-2 is one of the isolates collected at Salinas Grandes, Province of Jujuy, Argentina and was grown as described previously [2]. *Escherichia coli* JM 83 was used for the gene manipulation.

2.2. Preparation of cR-2

Cell envelope vesicles were prepared by the sonication method [13] and suspended in 4 M NaCl. KCl-loaded vesicles and Na_2SO_4-loaded vesicles were prepared by the previously described method [14]. CR-2 was prepared according to the purification method for hR [15] with slight modifications. Buffer solutions used for a purification of cR-2 were as follows: Buffer A, 2 M NaCl, 0.4% (w/v) Na-cholate and 25 mM Tris-HCl (pH 7.2); Buffer B, 2 M NaCl, 0.5% (w/v) MEGA-9 and 25 mM Tris-HCl (pH 7.2); Buffer C, 1 M NaCl, 1 M $(NH_4)_2SO_4$, 1.5% (w/v) Na-Cholate and 25 mM Tris-HCl (pH 7.2). The cell envelope vesicles were solubilized by adding Na-cholate at a final concentration of 1.5% (w/v) and loaded on a phenyl-Sepharose column equilibrated with Buffer A. After washing with Buffer A, reddish-purple pigments were fractionated by eluting with Buffer B. Pooled fractions were concentrated by ultrafiltration, diluted with ten volumes of Buffer C and loaded on an octyl-Sepharose column previously equilibrated with Buffer C. After washing with Buffer C, cR-2 was eluted with Buffer B.

2.3. Determination of the partial amino acid sequence

cR-2 (20 μg) in Buffer B was dialyzed against 200 volumes of 0.2% SDS and 20 mM Tris-HCl (pH 8.5) and incubated with *Achromobacter lyticus* proteinase I (0.2 μg) at 37°C for 20 h. Peptide fragments were separated by SDS-PAGE and electroblotted onto polyvinylidenedifluoride (Immobilon-P, Millipore) membranes. Protein bands stained with Coomassie Brilliant blue were cut out and analyzed with an automated protein sequencer (Applied Biosystems 476A).

2.4. Cloning of the cop-2 gene

Genomic DNA was isolated from *Haloarcula* sp. arg-2 by the method described previously [16]. *Pst* I-digested DNA was ligated into pUC18 and transformed into *E. coli* JM 83. Positive clones were selected by colony hybridization [17] using a 5′-^{32}P-labeled oligonucleotide mixture as a probe. The DNA sequence was determined for both strands by the dideoxy termination method [18]. Oligonucleotides were synthesized with a DNA synthesizer (Applied Biosystems 392) and used as probes for hybridization and as a primer for DNA sequencing.

2.5. Measurements of the proton pumping activity and cellular ATP contents

The light-induced proton pumping activity of intact cells was measured with a combination pH electrode at 30°C. ATP content of intact cells was determined by the luciferine-luciferase method as described previously [19].

2.6. Reagents

Restriction endonucleases were obtained from Nippon Gene and Toyobo. *Achromobacter lyticus* proteinase I and MEGA-9 were from Wako Pure Chemicals. Phenyl-Sepharose and octyl-Sepharose were from Pharmacia/LKB. DNA ligation kit and T4 polynucleotide kinase were from Takara Shuzo. [γ-^{32}P]ATP and [α-^{32}P]d-CTP were from ICN Biochemicals. T7 DNA polymerase (Sequenase) was from United States Biochemicals.

3. Results and discussion

3.1. Arg-2 has a light-driven proton pump but no anion pump

Cell envelope vesicles of *Haloarcula* sp. arg-2 showed CCCP-sensitive light-driven proton pumping activity as reported previously [8]. Although spontaneous pH increases of the acidified pH signal in the light were observed [8], the absence of light-induced proton uptake in the presence of CCCP suggested that arg-2 has no, or very little hR-like anion pump activity. This was supported by the observation that Na_2SO_4-

loaded vesicles showed the light-induced proton extrusion which diminished in the presence of CCCP, whereas no proton uptake occurred even after addition of 100 mM NaCl in the presence of CCCP (data not shown).

The presence of a gene for an hR-like protein in the arg-2 genome was tested using 400 bp DNA fragments of two types of the halo-opsin (*hop*) genes as probes; the *hop* gene from *Halobacterium salinalium* and the cruxhalo-opsin-3 (*chop-3*) gene from *Haloarcula vallismortis* (Kitajima, T. et al., unpublished results), 60% homologous to the *hop* gene. Southern hybridization revealed no positive bands in restriction digests of the arg-2 genomic DNA even under weaker stringency than the control strains. Furthermore, no DNA fragment was amplified by polymerase chain reaction (PCR) under the same conditions used for amplification of the *hop* and *chop*-3 genes. These observations strongly suggest that arg-2 cells have no light-driven anion pump and no copy of a gene for an hR-like protein.

3.2. Purification of the proton pump and isolation of the gene encoding for it

Membrane fragments corresponding to purple membrane or claret membrane [2] could not be prepared from arg-2. Unlike bR or aRs, the pigment of arg-2 should be solubilized by sodium cholate and adsorbed onto phenyl- or octyl-Sepharose columns in a high ionic strength medium as well as hR [15,21]. The adsorbed pigment was released by exchanging sodium cholate with MEGA-9 or octyl-glucoside. By repeating octyl-Sepharose column chromatography, 1.6 mg of the pigment was prepared from 10 l of culture, and its absorption spectrum (Fig. 1) indicated that the pigment contained bacterioruberin in proportions as high as in the claret membrane of aRs [2]. SDS-PAGE indicated that the protein part of the pigment was a single polypeptide of 26 kDa. The amino-terminal amino acid could not be determined by automated Edman degradation. An *Achromobacter* proteinase I-digested fragment of 18 kDa was thus isolated and analyzed for its amino-terminal amino acid sequence, which was determined as FYIATIMIAAIAFVNYLSMAL. This sequence was homologous to those of helix B of other proton pumps, especially of cR-1, but not identical to any previously reported pump. Therefore, this arg-2 molecule represents a novel proton pump protein, and it was designated cruxrhodopsin-2 (cR-2) as a member of the cR family proton pumps (in *Haloarcula*) [10].

A genomic library, constructed from the complete *Pst* I digest of arg-2 DNA, was screened using a mixture of the 24-mer degenerate oligonucleotide 5′-AAGTTCTACAT(A/C/T)GCNACNAT(A/C/T)ATG-3′ corresponding to the amino acid sequence KFYIATIM. A positive clone, pCR-2, had a 12 kbp insert which contained the entire gene for cR-2. The coding region of the crux-opsin-2 (*cop*-2) gene consists of 768 bp and encodes a protein of 255 amino acids (Fig. 2). Since the amino terminus of cR-2 could not been determined by Edman degradation, Gln-3 would be a possible candidate for the amino terminus and presumably present as a pyro-glutamate in the mature protein as in bR. The carboxy terminus has not been determined at present. Hydropathy analysis suggested seven transmembrane segments in cR-2 as in other retinal proteins.

3.3. Comparison of the amino acid sequence of cR-2 with those of other proton pumps

Alignment of amino acid sequences revealed that cR-2 was 77%, 50%, 48% and 48% identical to cR-1, bR, aR-1 and aR-2, respectively. cR-2 is thus much more closely related to cR-1 than to bR or aRs. This supported our previous proposal that the halobacterial proton pumps could be classified into three groups [9]. The homology of 77% between cR-2 and cR-1 is rather low compared with that (89%) between aR-1 and aR-2. This would be mostly due to the evolutionary distance between the strains carrying these pumps. The relatedness of the 16S rRNA gene (Ihara, K. et al., unpublished results) showed that *Haloarcula* sp. arg-2 is relatively close to the *Haloarcula* strains (homology 88%) than any other genus tested (80%), whereas *Halorubra* sp. aus-1 for aR-1 and *Hr.* sp. aus-2 for aR-2 are very close (homology 97%) (Mukohata, Y. et al., unpublished results).

The amino acid residues of the proton channel and the retinal binding pocket [22] not conserved in five natural proton pumps are listed in Table 1. Important amino acid residues for proton pumping such as Arg-82, Asp-85, Asp-96, Tyr-185, Asp-212 and Lys-216 are conserved in cR-2 as well as in the other proton pumps. Effects of substitutions in cR-1 (V49I, M145L),

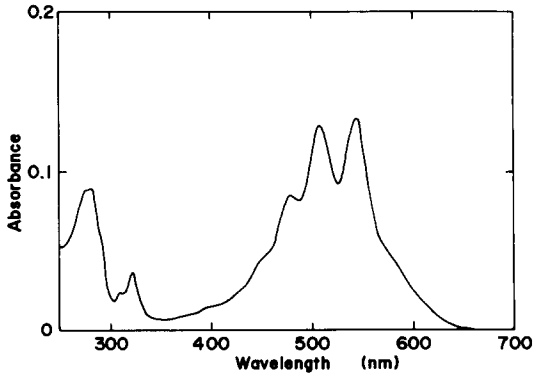

Fig. 1. Absorption spectra of the purified cR-2 in Buffer B. Three peaks in the visible region were characteristic of carotenoids, and the shoulder at 560 nm was due to a retinal pigment. An unidentified peak was observed at 322 nm.

Table 1
Amino acid residues of the proton channel and the retinal binding pocket not conserved among five natural proton pumps

Proton pump	Proton channel			Retinal pocket	
bR	Glu-166	Thr-171	Arg-227	Val-49	Met-145
aR-1	Glu	Thr	Arg	Val	Met
aR-2	Glu	Thr	Arg	Val	Phe
cR-1	Asp	Thr	His	Ile	Leu
cR-2	Asp	Lys	His	Ile	Leu

cR-2 (V49I, M145L) and aR-2 (M145F), all of which have comparable proton pumping activity, have been tested for photochemical processes (to be published) as well as the light-dark adaptation process in aRs [7].

It should be noted that the loop region connecting helices D and E in cR-1 and cR-2 had six extra amino acids compared to bR, aR-1 and aR-2. The amino acid sequence of cR-2 was 35 and 24% identical to those of hR and sR, respectively.

3.4. Light-induced pH changes and ATP formation in strain arg-2

ATP formation in anaerobic R_1 and R_1M_1 cells in the light have been reported to follow the initial transient proton uptake [23–25], leading to the finding of hR [13]. Later proton uptake could be resolved into three components, one of which seemed to be coupled with ATP formation [26]. However, both bR and hR in these strains have been shown to function as primary pumps and establish membrane potentials immediately after onset of illumination which drives the H^+-ATPase [12,13,29]. Therefore, interpretation of the precise energetic features of proton inflow in *Hb. salinarium* was difficult. More recently, with *Hb. salinarium* Pho81BR,

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                                                                  CCGACG
      GACGAACCGCTCTCCCGGTGTCTGTGTTTGATTCGCGTCTCCTCGCCGGGCTCGGGAAGG
      CGTACCAATCCCTGTTGGCATGGGCTGTTTAAGTGTCGGGCCGTTATTACAACATGAACC

    1 ATGTTACAGTCTGGCATGAGCACGTACGTACCGGGCGGCGAAAGCATATTCCTGTGGGTC
      M  L  Q  S  G  M  S  T  Y  V  P  G  G  E  S  I  F  L  W  V

   61 GGGACGGCAGGAATGTTCCTGGGGATGTTGTACTTCATCGCCCGGGGCTGGAGTGTCTCC
      G  T  A  G  M  F  L  G  M  L  Y  F  I  A  R  G  W  S  V  S

  121 GACCAGCGTCGCCAGAAGTTCTACATCGCGACGATCATGATCGCCGCGATCGCCTTCGTG
      D  Q  R  R  Q  K  F  Y  I  A  T  I  M  I  A  A  I  A  F  V

  181 AACTACCTCTCGATGGCACTGGGTTTCGGTGTCACGACCATCGAACTCGGCGGCGAGGAA
      N  Y  L  S  M  A  L  G  F  G  V  T  T  I  E  L  G  G  E  E

  241 CGGGCGATCTACTGGGCGAGATACACCGACTGGCTGTTCACGACGCCGTTGCTGCTGTAC
      R  A  I  Y  W  A  R  Y  T  D  W  L  F  T  T  P  L  L  L  Y

  301 GACCTCGCCCTGCTGGCGGGGGCCGACCGGAACACGATCTACTCGCTGGTCGGTCTGGAC
      D  L  A  L  L  A  G  A  D  R  N  T  I  Y  S  L  V  G  L  D

  361 GTGCTGATGATCGGTACCGGCGCGCTCGCGACGCTGTCGGCGGGTTCGGGCGTCCTCCCG
      V  L  M  I  G  T  G  A  L  A  T  L  S  A  G  S  G  V  L  P
                                                          •  •

  421 GCGGGTGCCGAACGCCTCGTCTGGTGGGGTATCAGCACCGGCTTCCTGCTGGTCCTGCTG
      A  G  A  E  R  L  V  W  W  G  I  S  T  G  F  L  L  V  L  L
      •  •  •  •

  481 TACTTCCTGTTCAGCAACCTCACCGACCGAGCGAGCGAGCTCTCGGGTGACTTGCAGTCG
      Y  F  L  F  S  N  L  T  D  R  A  S  E  L  S  G  D  L  Q  S

  541 AAGTTCTCGACGCTGCGGAACCTGGTGCTGGTCCTGTGGCTCGTGTACCCCGTCCTGTGG
      K  F  S  T  L  R  N  L  V  L  V  L  W  L  V  Y  P  V  L  W

  601 TTGGTCGGGACCGAGGGCCTCGGACTCGTCGGCCTCCCGATCGAGACGGCCGCGTTCATG
      L  V  G  T  E  G  L  G  L  V  G  L  P  I  E  T  A  A  F  M

  661 GTGCTCGACCTGACCGCCAAGATCGGCTTCGGTATCATCCTGCTCCAGAGTCACGCGGTC
      V  L  D  L  T  A  K  I  G  F  G  I  I  L  L  Q  S  H  A  V

  721 CTCGACGAGGGCCAGACCGCGTCGGAAGGCGCAGCCGTCGCCGACTGATCGCGGCTCCCC
      L  D  E  G  Q  T  A  S  E  G  A  A  V  A  D  *

      TCTCGCCACTCCAGTCGAAACACTCAGGGCGATCTGCCCGATAGTGTGACCGT
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Fig. 2. Nucleotide sequence of the *cop*-2 gene. The underlined amino acids correspond to the sequence obtained by amino acid analysis of the proteinase-digested fragment. An open reading frame starts at a nucleotide position 1 with an ATG codon and ends at nucleotide 768 with a TGA codon. The amino acids in the extra loop region between helix D and E are dotted.

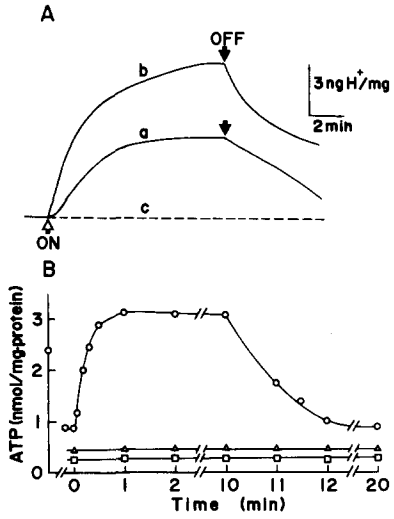

Fig. 3. Light-induced pH changes of the cell suspension (A) and ATP level of arg-2 cells (B). *Haloarcula* sp. arg-2 cells (5 ml basal salt, 2 mg protein/ml) were incubated in a vessel under argon at 30°C. The suspension was first illuminated with a 750 W slide projector for 20 min and then kept in the dark for 30 min. (A) ON and OFF indicate the points at which light was turned on and off, respectively. The pH of the suspension was continuously monitored for the intact cells in the presence (trace c) or absence (trace a) of CCCP and for the DCCD-treated cells (trace b). After each experiment, 5 μl of 0.01 M HCl was added for calibration. The initial pH was 6.5. (B) At zero time, the light was turned on, and was turned off after 10 min. Fifty μl of cell suspensions were sampled for ATP determination at the indicated times. (○) Intact cells. (△) Intact cells in the presence of CCCP. (□) DCCD-treated cells. DCCD (10 mM in ethanol) was added 30 min before the start of the experiment to a final concentration of 50 μM. CCCP (10 mM in ethanol) was added to a final concentration of 10 μM.

a recombinant $bR^+hR^-sR\text{-}I^-sR\text{-}II^-$ strain, a DCCD-sensitive gated-secondary proton uptake was demonstrated to be mostly attributable to H^+-ATPase [28]. In contrast to R_1, R_1M_1 and Pho81BR, under anaerobic conditions the pH of the suspension of the freshly isolated arg-2 cells decreased and reached a steady-state within 4 min in the light. Typical results are shown in Fig. 3A. The proton extrusion rate was 0.03 ng H^+/mg protein per s for the first 30 s, after which it increased to 0.1 ng H^+/mg protein per s. Fifty μM DCCD, a H^+-ATPase inhibitor, enhanced both the rate and extent of light-induced proton extrusion. The initial proton extrusion rate of DCCD-treated cells was 0.3 ng H^+/mg protein per s, which value showed slight differences between different cell preparations. The decreased pH returned to the original pH in the dark (Fig. 3A). Proton inflow rate after turning off the light was faster in DCCD-treated than in untreated cells. This can be easily explained because the proton extrusion due to the hydrolysis of ATP by H^+-ATPase occurred concomitantly with the proton inflow after turning off the light in untreated but not in DCCD-treated cells (see below). 10 μM CCCP completely abolished the light-induced pH changes. It was reported previously that light-induced transient proton uptake occurs in Pho81BR irrespective of conditions such as freshness of the cell preparation, light intensity and preillumination [28]. In contrast, arg-2 cells showed light-induced proton extrusion even after a storage for more than one week at 4°C in the dark. Instead, when grown under vigorous aeration, arg-2 showed a small light-induced transient proton uptake (data not shown). Therefore, the light-induced transient proton uptake may vary depending on cell growth conditions and the relative abundance of the proton pump and Na^+/H^+ antiporter in each cell.

Under anaerobic conditions in the dark, cellular ATP level decreased to 30% of that under aerobic conditions within 20 min. Upon actinic illumination, the ATP level increased and reached a steady-state level within 30 s, which was maintained in the light (Fig. 3B). The initial rate of ATP synthesis was 0.09 nmol ATP/mg protein per s in this arg-2 preparation, which also varied slightly between individual cell preparations. R_1M_1 and aus-1 showed comparable ATP synthesis activity under our experimental conditions. Turning off the light, the ATP level decreased to the dark-anaerobic level within 2 min. The initial rate of ATP hydrolysis in the dark was 0.03 nmol/mg protein per s. Fifty μM DCCD and 10 μM CCCP decreased anaerobic ATP level and completely inhibited light-driven ATP synthesis (Fig. 3B). The light-induced proton extrusion and ATP synthesis of the intact and the DCCD-treated cells were measured under various light intensities (Fig. 4). The initial rates of ATP synthesis of the intact cells increased linearly with increases in the light intensity. Although the initial proton extrusion rates of the intact cells were constant, those of the

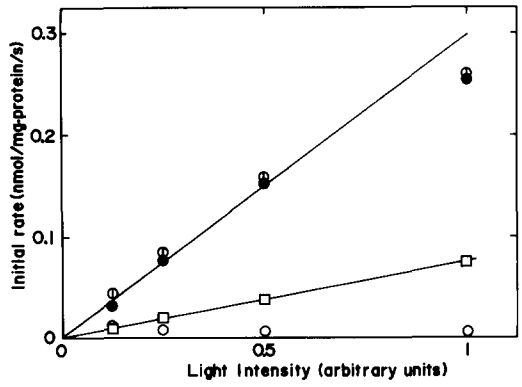

Fig. 4. Effects of light intensity on the initial rates of proton extrusion and ATP synthesis of arg-2 under anaerobic conditions. Light-induced proton extrusion and ATP synthesis were measured as in Fig. 3. Light intensity was changed using the neutral density filter and shown relative to that with no filter. The initial rates of light-induced proton extrusion of intact cells (○) and 50 μM DCCD-treated cells (⊙) are plotted against light intensity. The difference between the latter and the former is shown as (●). The same results were obtained for 100 μM DCCD-treated cells. The initial rates of ATP synthesis (□) are shown for the intact cells.

DCCD-treated cells increased with increases in light intensity, and deviated from linearity at high light intensities. The DCCD-sensitive proton uptake rate (ΔH^+), that is, the difference in proton extrusion rates between the DCCD-treated and intact cells, was 4-times larger than the rate of ATP synthesis at the low light intensities. This is consistent with the previous observation of steady-state ATP synthesis [11], in which the H^+/ATP stoichiometry was estimated to be more than three. The present results also suggest that ΔH^+ includes another component of proton uptake which was blocked by DCCD.

The amounts of cR-2 in arg-2 cells were estimated to be 0.05 nmol/mg of cell protein by measuring the differences in spectra before and after NH_2OH bleaching, which was 20–30-fold smaller than bR in R_1M_1 cells [29]. The present results thus indicated that the relatively small amounts of this novel proton pump in arg-2 cells could generate a protonmotive force sufficient to synthesize ATP in the light. These observations were inconsistent with the previous report that the rate of ATP formation under non-saturating illumination was proportional to the bR content in R_1M_1 (bR^+hR^+) cells [25], and we are unable to offer a conclusive explanation for this discrepancy at present. However, the following discussion may be adequate. Assuming the membrane capacitance (C_m) of $4.45 \cdot 10^{-14}$ farad/cell and $2 \cdot 10^9$ cells/mg protein [29], the time required to increase the negative membrane potential by ΔE (V) could be calculated by Eq. (1).

$$\Delta t = C_m \times \Delta E \times 1/\Phi F \quad (1)$$

where Φ is the proton pumping rate (mol/cell per s) and F is the Faraday constant. When the proton extrusion rate was 0.3 ng H^+/mg protein per s, Δt was 0.37 s at $\Delta E = 0.12$. Since the photocycling rate of cR-2 was comparable to that of bR (approx. 20 ms), it is expected that the membrane potential necessary for ATP synthesis could be established when 12% of cR-2 molecules actively pumped out protons for 0.37 s. Thus, arg-2 has sufficient cR-2 for photophosphorylation, and provides a suitable experimental system for studying the relationship between the rate of generation of proton motive force and onset of ATP formation (Sugiyama, Y. et al., unpublished results).

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