Journal of Bioenergetics and Biomembranes, Vol. 24, No. 6, 1992

Minireview

Photophosphorylation Elements in Halobacteria: An A-type ATP Synthase and Bacterial Rhodopsins

Yasuo Mukohata,[1] Yasuo Sugiyama,[1] and Kunio Ihara[1]

Received May 30, 1992; accepted June 15, 1992

Photophosphorylation in halobacteria is carried out by two rather simple elements: an A-type ATP synthase and light-driven ion-pumping bacterial rhodopsins. The unique features of halobacterial ATP synthase, mostly common to archaebacteria (A-type), and of new members of the bacteriorhodopsin family are introduced along with studies performed in the authors' laboratory. This is the story of how we found that the A-type ATP synthase is close to V-type ATPase but far from F-type ATPase, although all three ATPases are believed to have the same ancestor. Archaerhodopsins, the new members of the proton-pumping retinal proteins, were found in Australian halobacteria and have been used in a comparative study of bacterial rhodopsins.

KEY WORDS: Halobacteria; halophilic archaebacteria; photophosphorylation; ATP synthesis; A-type ATPase; bacterial rhodopsins; archaerhodopsin; bacteriorhodopsin.

INTRODUCTION

Photophosphorylation in *Halobacterium halobium* [*salinarium* (Larsen, 1984)] was first reported in 1974 (Danon and Stoeckenius, 1974), and was said to be energized by a then newly found protein, bacteriorhodopsin, which pumps out protons in the light (Oesterhelt and Stoeckenius, 1971). We then found photophosphorylation in a halobacterium which cannot express bacteriorhodopsin but contains another light-energy transducer (Matsuno-Yagi and Mukohata, 1977, 1980). We named this second retinal protein halorhodopsin (Mukohata *et al.*, 1980). Bacteriorhodopsin and halorhodopsin build up the proton-motive force (ΔpH and/or $\Delta\psi$) in the light and drive ATP synthesis.

Of the actual machinery of ATP synthesis in halobacteria, little was known in the late 1970's. The well-known light-driven ATP formation by the reconstituted vesicles with bacteriorhodopsin and F_0F_1 (F-type) ATP synthase of rat liver mitochondria (Racker and Stoeckenius, 1974) not only gave strong support to the chemiosmotic theory (Mitchell, 1961) but also provided the preconception that the same elements play the same role in halobacteria, which we show here to be untrue. The ATPases of archaebacteria can be categorized as A-type ATPase (Mukohata and Yoshida, 1987b; Mukohata and Ihara, 1990), which is closely related to V-type ATPase and much more remotely to F-type ATPase.

Of the driving machinery of photophosphorylation in halobacteria, the structure and the proton pumping mechanism of bacteriorhodopsin have been extensively investigated. Another line of investigation began after our finding of new proton pumps [archaerhodopsins (Mukohata *et al.*, 1988)] which share less than 60% of the amino acid sequence identities with bacteriorhodopsin. Presently, four sequences are known for proton pumps which can be used to assign the conserved (probably essential) amino acid residues. Comparative studies on the physicochemical properties of the pumps have pointed out the possible amino acid residues which cause the differences in their properties and can be the target of site-directed mutagenesis, as suggested in this paper.

[1] Department of Biology, Faculty of Science, Nagoya University, Nagoya 464-01, Japan.

0145-479X/92/1200-0547$06.50/0

ATP SYNTHESIS IN CELLS AND CELL ENVELOPE VESICLES

Halobacterium halobium (*salinarium*) is an aerobic archaebacterium which metabolizes amino acids (Larsen, 1984). The cellular ATP level under aerobic conditions is depressed when air is replaced with nitrogen (Danon and Stoeckenius, 1974; Mukohata and Kaji, 1981a,b). The rate of oxygen consumption of intact cells is reversibly lowered by light illumination (Hartmann *et al.*, 1980), which suggests that the light-activated bacteriorhodopsin plays the same functional role as respiration, i.e., formation of the proton-motive force.

Light activation of the substrate-stuffed envelope vesicles containing bacteriorhodopsin and/or halorhodopsin results in the production of ATP from Mg-ADP and Pi (Mukohata *et al.*, 1986; Mukohata and Yoshida, 1987a,b). The base–acid transition (pH jump) applied on these envelope vesicles in the dark also resulted in ATP production (Mukohata *et al.*, 1986). These results imply that an ATP synthase driven by the proton-motive force synthesizes ATP in halobacteria.

The ATP synthesis in halobacterial vesicles (Mukohata *et al.*, 1986), as much as in intact cells (Danon and Stoeckenius, 1974; Mukohata and Kaji, 1981a; Mukohata *et al.*, 1987a), was inhibited by DCCD just as is F_0F_1-mediated ATP synthesis. However, to our surprise, halobacterial ATP synthesis was not inhibited by azide (Mukohata and Yoshida, 1987a). Azide is an inhibitor known to be specific to F_0F_1 ATP synthase (F-type ATPase) which had been believed to be distributed ubiquitously in all aerobic organisms: in mitochondria, chloroplasts, and eubacteria, and also in archaebacteria. The ATP synthesis was also insensitive to vanadate (specific to P-type ATPase), nitrate (specific to V-type ATPase), AP_5A (specific to adenylate kinase), and quercetin at the concentrations fully effective on the corresponding enzymes (Mukohata and Yoshida, 1987a).

In addition to the azide insensitivity, the active pH range for the ATP synthesis lies between pH 6.3 and 7.3 (Mukohata *et al.*, 1986), which is much narrower than the ATP synthesis by F_0F_1 ATP synthase. The threshold proton-motive force for the ATP synthesis was estimated to be -90 to ~ -100 mV (Mukohata *et al.*, 1986), which is much lower than that for F_0F_1 ATP synthase [-180 mV (Hirata *et al.*, 1986)]. The K_m value for ADP was around 300 μM.

ATPASE FROM *H. halobium (salinarium)*

Fractions of the homogenates of halobacterial cells were traced for ATP hydrolyzing activity, and one fraction of 320-kDa protein (estimated by gel filtration) was isolated and purified (Nanba and Mukohata, 1987) and then shown to be the (catalytic) head piece of the ATP synthase (Mukohata and Yoshida, 1987b). The enzyme contained two kinds of polypeptides, 64 (α) and 56 (β) kDa from the amino acid sequences (Ihara and Mukohata, 1991) [86 and 64 kDa by SDS-PAGE estimation (Nanba and Mukohata, 1987)], suggesting a composition of $\alpha_3\beta_3$. The enzyme has an optimum between pH 5.3 and 6.3, and is 10 times more active in 1.5 M Na_2SO_4 than in 4 M NaCl or KCl at pH 5.8. The isolated ATPase was inactivated in low salt media or by storage at 4°C for one night, although this low-temperature inactivation does not take place on ATPase *in situ*, i.e. ATP synthase. The ATPase was inhibited by nitrate (K_i = 3 mM), which is known to be specific to V-type ATPase, but not by azide specific to F-type ATPase (Nanba and Mukohata, 1987; Mukohata and Ihara, 1990).

It is noteworthy that nitrate inhibits only ATP hydrolysis (but not synthesis) noncompetitively to ATP, and the inhibition is protected by chloride (Ihara *et al.*, 1992a). Nitrate seems to affect the enzyme only when the head piece is released (as ATPase) from the ATP synthase. On the other hand, the inhibitory effects of other chaotropic anions, such as iodide, trichloroacetate, periodate, and thiocyanate, are not appreciable on ATPase, but they are degradative on ATP synthesis (Ihara *et al.*, 1992a). This inhibitory effect on the ATP synthesis may include deterioration of the vesicle membrane. The effect of nitrate on halobacterial ATP synthase is thus different from that on the membrane-bound V-type ATPase which seems to be much more sensitive to the chaotropic action of nitrate (Bowman and Bowman, 1986; Moriyama and Nelson, 1989).

The antibodies raised against the isolated ATPase were applied on the ATPases of various origins (Western blotting). The antibodies cross-reacted, in a simultaneous assay, with the ATPase of *Sulfolobus acidocaldarius* as much as they did with the antigen ATPase of halobacteria. To our surprise, they also cross-reacted with the V-type ATPase of red beet tonoplasts, though not with the F-type ATPase of spinach chloroplasts (Mukohata *et al.*, 1987b). The above enzymological differences from F-type ATPase

and some similarities to V-type ATPase were thus confirmed immunochemically as proteins. In order to make discussion easier, we categorized all archaebacterial ATPases as A-type ATPase (Mukohata and Yoshida, 1987b; Mukohata and Ihara, 1990). Later, the cross-reactivities were examined in more detail for various kinds of ATPases and fundamentally the same relatedness was found among A-type, F-type, and V-type ATPases (Konishi *et al.*, 1991; Mukohata *et al.*, 1991a).

The ATPase from *Halobacterium saccharovorum* seems to differ from that reported here, although it is in the same A-type ATPase family. This ATPase and those of *Haloarcula vallismortis*, *Haloferax mediterranei*, and *volcanii* are active in 3 M NaCl but not in 1.5 M Na_2SO_4 (Ihara *et al.*, 1992a). Western blotting with the antibodies raised against each one of the α and β subunits of *H. salinarium* ATPase shows that the sizes of the α subunits of the ATPase of *H.saccharovorum* and also of the above-mentioned *Haloarcula* and *Haloferax* are similar (M_r = 86 kDa by SDS-PAGE) to the corresponding subunit of *H. salinarium* ATPase; those of the β subunits are different, however (60 to 70 kDa but not 64 kDa) (Ihara *et al.*, 1992a). Although such an apparent difference in SDS-PAGE may come from protein nature such as acidic amino acid content, some differences in enzyme properties have also been reported (Hochstein *et al.*, 1987; Schobert and Lanyi, 1989).

THE A-TYPE ATPASE FAMILY

The genomic DNAs coding the major subunits of the A-type ATPases of the three major groups [thermoacidophiles (Denda *et al.*, 1988a,b), methanogens (Inatomi *et al.*, 1989), and extreme halophiles (Ihara and Mukohata, 1991)] were isolated and sequenced. It was noted that the GC contents of the ATPase genes differ among these three archaebacteria. They range from 65% (*Halobacterium salinarium*) to 34% (*Sulfolobus acidocaldarius*) with 52% (*Methanosarcina barkeri*) in between, suggesting that archaebacteria have diverged in different extreme habitats and come to use different codons to code the same enzyme. This implies that the conserved amino acid residues (e.g., 258 out of about 580 residues of the α subunit of the above three ATPases) should have essential roles as protein and/or enzyme.

The primary structures of the ATPase subunits were deduced from those DNA sequences. The percentage identity of the amino acid sequences of ATPases (Table I) numerically confirm again the relatedness observed enzymologically and immunochemically. The percentages are large among the largest (α) subunits of A-type and V-type ATPases, and among the β subunits as well. The values between these two types and F-type are much smaller and almost similar to those between the α and β subunits.

The major two subunits, α and β, of H^+-translocating (A-, F-, and V-type) ATPases can be postulated as they were formed by gene duplication. Therefore, the diversity of these two subunits from this time point (the root) of the gene duplication can be analyzed by the maximum-likelihood method and/or the neighbor-joining method with reference to *Sulfolobus* ATPase (Gogarten *et al.*, 1989), *Sulfolobus* and *Metanosarcina* ATPases (Iwabe *et al.*, 1989), and the three major A-type ATPases (Mukohata *et al.*, 1990). The results show that the F-type ATPases in the endosymbionts (mitochondria and chloroplasts) and eubacteria make one large cluster and that the V- and A-type ATPases make the other (Fig. 1). In most analyses, V- and A-type ATPases are likely to be separated as independent clusters, i.e., they are monophyletic (Iwabe *et al.*, 1989; Hasegawa *et al.*, 1990; Miyata *et al.*, 1991). This "rooted" phylogenetic tree suggests that the distance between archaebacteria and

Table 1. Numerical Comparison of the Three A-type ATPases

ATPase origin		*H. halobium*[1]		*S. acido.*[2]		*M. barkeri*[3]	
Subunit size		α	β	α	β	α	β
(kDa)		64	52	66	51	64	50
Identity (%)							
H. halobium[1]	α	—	23	49	27	63	26
	β	23	—	27	55	23	65
S. acido.[2]	α	49	27	—	25	50	25
	β	27	55	25	—	25	57
M. barkeri[3]	α	63	23	50	25	—	28
	β	26	65	25	57	28	—
N. cras. V[4]	α	50	24	48	23	53	26
	β	28	54	27	53	27	56
E. coli F[5]	α	27	23	23	23	27	26
	β	25	24	26	28	25	25

[1] *Halobacterium halobium* (extreme halophile); [2] *Sulfolobus acidocaldarius* (extreme thermophile); [3] *Methanosarcina barkeri* (obligatory anaerobe); [4] *Neurospora crassa* (mold, tonoplast) V-type ATPase; [5] *Escherichia coli* (eubacteria) F-type ATPase. The subunit sizes are obtained by amino acid compositions. The N- and/or C-termini have not been identified for some ATPases. The identity values were obtained from the amino acid sequences of the major two subunits, α and β, of these ATPases.

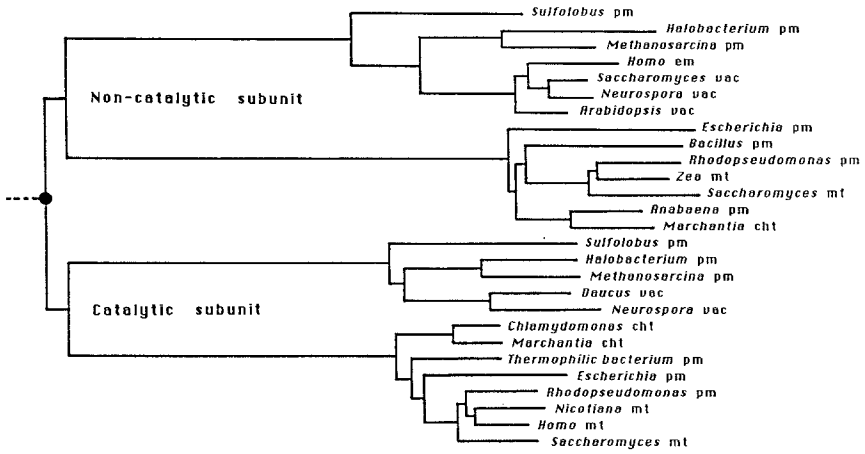

Fig. 1. The "rooted" composite phylogenetic tree of the H^+-translocating ATPase/synthase. The two major subunits (α and β) of A-, V-, and F-type H^+-translocating ATPases/synthases were analyzed as the two daughter subunits made by the gene duplication by the neighbor-joining method. Location of the enzyme is: pm, plasma membrane; em, endosomal membrane; vac, vacuolar membrane; mt, mitochondrial inner membrane; cht, chloroplast thylakoid membrane. For details, see Mukohata *et al.* (1990).

eukaryotes is much closer than that between either one of the two and eubacteria. Such close relatedness between archaebacteria and eukaryotes was first argued on the basis of the study of "unrooted" trees of 5S rRNA (Hori and Osawa, 1979), in response to archaebacteria urkingdom (Woese and Fox, 1977).

Archaebacterial progenitors would thus be possible candidates for the host of endosymbiosis (Margulis, 1968; Cavalier-Smith, 1987) when the relatedness between A- and V-type ATPases and the orientation of the ATPase on the membrane (i.e., all the ATPases extrudes into the "original" cytoplasm) are considered (Nelson and Taiz, 1989; Mukohata *et al.*, 1990).

However, since an exceptional V-type ATPase was found in an extremely thermophilic eubacterium, *Thermus thermophiles* (Yokoyama *et al.*, 1990), in order to argue the evolution of living things, we need more data on the "rooted" trees of other markers to find the most probable tree.

BACTERIAL RHODOPSINS AS LIGHT-ENERGY TRANSDUCERS

Bacteriorhodopsin (Oesterhelt and Stoeckenius, 1971) is a membrane protein of purple color, the color being due to the chromophore retinal interacting with the protein part. This is a chromoprotein which pumps protons in the light from its C-terminal side (cytoplasmic side *in situ*) to the N-terminal (periplasmic) side. Its amino acid sequence is known (Ovchinnikov *et al.*, 1979; Dunn *et al.*, 1981).

Bacteriorhodopsin in the light builds up a proton-motive force which is used to synthesize ATP, to rotate a flagellar motor, to exclude sodium ions through Na^+/H^+ antiporter, and so on. The light-driven mechanism of proton pumping has been extensively studied by such means as electron micrograph imaging (Henderson *et al.*, 1990), time-resolved FTIR (Engelhard *et al.*, 1985; Rothschild, 1988), site-directed mutagenesis (Khorana, 1988), and so forth. The mechanism contains sequential association and dissociation of protons to and from Asp96, Asp85 (or Asp212), and the Schiff's base formed between the retinal and the ε-amino residue of Lys216. In the detailed structure of the seven transmembrane helix segments (Henderson *et al.*, 1990), 30 amino acid residues relevant to the path of protons (the proton channel) and 21 relevant to the envelop of retinal (the retinal pocket) are assigned.

Bacteriorhodopsin and then halorhodopsin (Matsuno-Yagi and Mukohata, 1977) were found, as light-energy transducers, in one strain of *Halobacterium halobium* (*salinarium*) which also contains sensory rhodopsin (Tsuda *et al.*, 1982; Bogomolni and Spudich, 1982) and phoborhodopsin (Takahashi *et al.*, 1985) as light sensors. The idea that new bacterial rhodopsins would exist in new strains of halobacteria

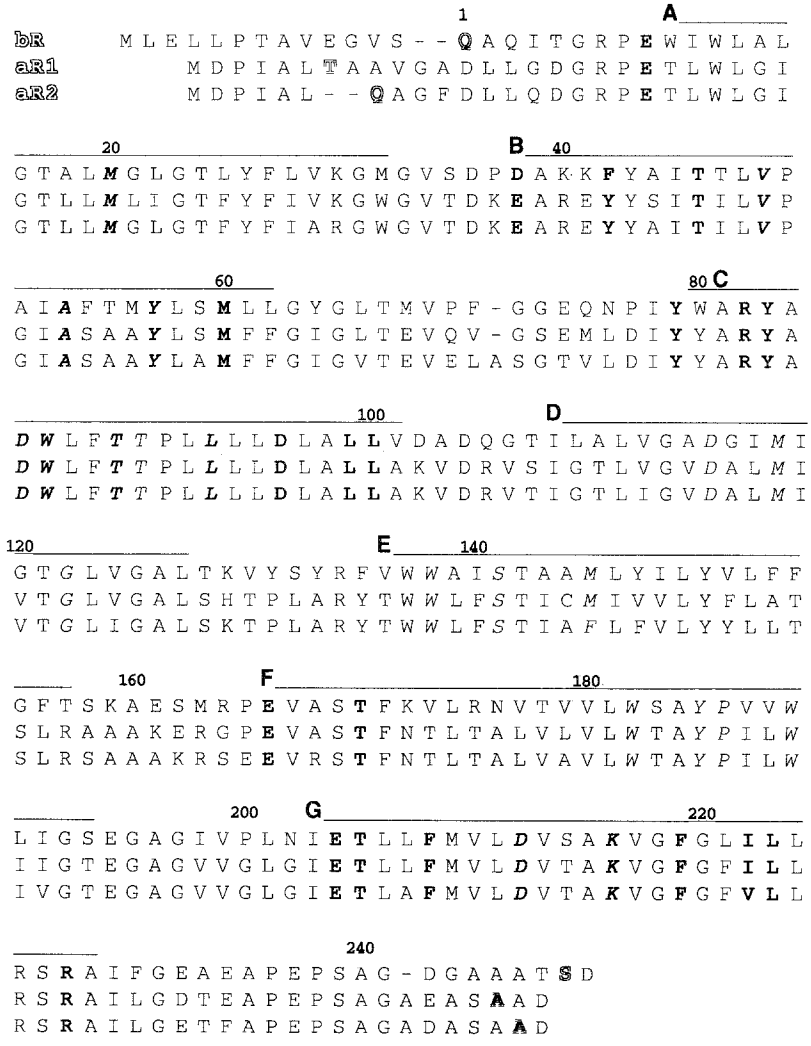

Fig. 2. The amino acid sequences of three proton pumps. The amino acid sequence of bacteriorhodopsin (bR) (Dunn *et al.*, 1981), archaerhodopsin-1 (aR1) (Sugiyama *et al.*, 1989), and archaerhodopsin-2 (aR2) (Uegaki *et al.*, 1991) are aligned in the single letter expression so as to give conceivable identities. The numbers are based on the sequence of the bacteriorhodopsin protein. The amino acid residues assigned for the proton channel are in bold letters and those for the retinal pocket (Henderson *et al.*, 1990) are in italics. The N- and C-termini are in outline letters. The regions assumed for the helix A to G are indicated.

was proved when we found new light-driven ion pumps of retinal protein in the extremely halophilic archaebacteria collected in playas of Western Australia (Mukohata *et al.*, 1988, 1991b).

Upon actinic illumination, the new isolates showed reversible change of the pH of the suspension concomitant with an increase of cellular ATP level. The claret-colored membranous fractions obtained from the homogenates of the isolates contained retinal proteins of around 27 kDa. Their genomic DNAs were cloned and sequenced, and the amino acid sequences were deduced (Sugiyama *et al.*, 1989; Uegaki *et al.*, 1991). Presently, we have two proton pumps to discuss, named archaerhodopsin-1 and -2, whose amino acid sequences are nearly 90% identical to one another. The sequences of both pumps, however, are less than 60% identical to bacteriorhodopsin (Fig. 2). Archaerhodopsin-3 differs only 3 nucleotides from bacteriorhodopsin (unpublished results).

The arrangement of the sequences of three proton

pumps (Fig. 2) points out the conserved amino acid residues, which would be essential for the structure and function of the pump. Almost all the residues assigned by various means to be important on bacteriorhodopsin are found in these conserved residues. The number of the conserved residues will become smaller as the sequences of more new pumps become available.

Another line of investigation with natural members of the bacteriorhodopsin family is to determine differences in their physicochemical properties and to reduce these to the differences in their structures. For example, we found a difference in the light-dark adaptation process (the thermal isomerization process of the chromophore retinal of 100% all-*trans* configuration in the light-adapted bacterial rhodopsins to the 13-*cis* form, in the dark-adapted rhodopsins these two forms of isomers are in equilibrium) of bacteriorhodopsin and archaerhodopsins. The dark-adapted bacteriorhodopsin contained 65% of 13-*cis* retinal, while the dark-adapted archaerhodopsin-1 and archaerhodopsin-2 contained only 50 and 25%, respectively, of 13-*cis* isomer. This difference was explained kinetically by the faster rate of back-reaction (13-*cis* to all-*trans*) in the isomerization equilibrium especially in the case of archaerhodopsin-2 (Ihara *et al.*, 1992b). Among the amino acid residues proposed for the retinal pocket (Fig. 2), only Met 145 in bacteriorhodopsin and archaerhodopsin-1 is replaced with Phe in archaerhodopsin-2. This replacement would cause such a difference in the rate of isomerization. This amino acid is thus the definite candidate for the site-directed mutagenesis which is now in progress.

Halorhodopsin pumps in halide ions in the light (Schobert and Lanyi, 1982) and builds up a membrane potential (MacDonald *et al.*, 1979), negative inside, which can drive the A-type ATP synthase mentioned above. Recently, a purple protein in a haloalkaliphilic strain, *Natronobacterium pharaonis*, was found to carry another halorhodopsin-like protein (Lanyi *et al.*, 1990). All those halide-ion pumps still remain to be investigated. They could be investigated in ways similar to those described here.

ACKNOWLEDGMENTS

The authors are indebted to Dr. Akemi Matsuno-Yagi, Yoshio Kaji, Masaharu Isoyama, Ayumi Fuke, Takuya Watanabe, Yasushi Enokido, and Manabu Yoshida of the Department of Biology, Faculty of Science, Osaka University, as well as Ken-ichiro Sugimura, Tomoyasu Taguchi, Tomohiro Amemiya, Yukiya Miyashita, Eiji Asahara, and Tadahiro Abe in our present institution, all of whom have worked together with us at various times over the past two decades. The collaboration of Dr. Masasuke Yoshida (Tokyo Institute of Technology) in the immunochemical examination of A-type ATPases and of Drs. Takashi Miyata and Naoyuki Iwabe (Kyoto University) and Dr. Masami Hasegawa (Institute of Statistical Mathematics) in the analyses of phylogenetic assay are greatly appreciated. We also thank Dr. Masamitsu Futai (Osaka University) and Dr. Kimitoshi Denda (Tokyo Institute of Technology) for their initial guidance in gene technology.

This work was supported in part by the grants-in-aid for special project research (Mechanisms of biological energy transduction) and the grants-in-aid for scientific research on priority areas (Bioenergetics) from the Ministry of Education, Science and Culture of Japan. The field research in Australia for new halobacteria (1983) was fully supported by The Japanese Society for the Promotion of Science (JSPS).

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