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Homology Modeling of an Algal Membrane Protein, *Heterosigma Akashiwo* Na^+–ATPase

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The three–dimensional structure of *Heterosigma akashiwo* Na^+–ATPase (HANA) was predicted by means of homology modeling based on the crystal structure of the K^+–bound form of shark Na^+/K^+–ATPase (PDB ID: 2ZXE). The overall structure of HANA appears to be similar to that of shark Na^+/K^+–ATPase. Both contain three characteristic cytoplasmic domains, A, N and P, which are unique to P–type ATPases. HANA has a long TM7–8 junction as a large extracellular domain, in place of the β–subunit of shark Na^+/K^+–ATPase. Two putative K^+–binding sites in the transmembrane domain of HANA were identified by means of valence mapping based on the constructed structure. The presence of K^+–binding sites and the reported ion requirements for ATPase activity and EP formation indicate that HANA may transport K^+ ions in the same manner as animal Na^+/K^+–ATPases.

Key words : *Heterosigma akashiwo* ／ marine alga ／ Na^+/K^+–ATPase ／ homology modeling ／ K^+–binding sites

1. Introduction

Most living cells maintain a low intracellular concentration of Na^+ ions, despite the generally higher Na^+ ion concentration in the extracellular milieu. Plant cells have been believed to extrude intracellular Na^+ ions primarily through the combined action of a Na^+/H^+-antiporter and H^+–ATPase, rather than via a sodium pump. However, *Heterosigma akashiwo*, a wall–less unicellular marine alga, was found to contain Na^+–ATPase on the plasma membrane[1, 2)]. The ATPase activity is greatly stimulated in the presence of 100 mM NaCl, 10 mM KCl and 5 mM $MgCl_2$, and is inhibited by orthovanadate, a specific inhibitor of P–type ATPases. ATP–dependent Na^+ transport was also demonstrated using reconstituted vesicles containing *H. akashiwo* plasma membrane[3)]. The ATPase formed phosphorylated enzyme intermediates of approximately 140–kDa in the presence of Na^+ and Mg^{2+} ions[1, 2, 4)]. An antibody raised against pig kidney Na^+/K^+–ATPase reacted with the 140–kDa polypeptides[4)]. The full-length cDNA of *H. akashiwo* Na^+–ATPase (HANA) has been cloned[5)], and the putative product shows about 40 % identity with animal Na^+/K^+–ATPase α–subunits and contains several conserved P–type ATPase sequences, including ATP binding site and phosphorylation site. Moreover, considerably higher identity (65 % on average) was found between the 10 putative transmembrane domains of HANA and those of animal Na^+/K^+–ATPase α–subunits. These data strongly suggest that HANA is a Na^+ and K^+ transporting P–type ATPase, and its reaction mechanism might be similar to that of Na^+/K^+–ATPases, though K^+ transport by HANA has not yet been demonstrated.

Recently, the X–ray crystal structure of K^+–bound

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form of shark rectal gland Na^+/K^+–ATPase has been elucidated at 2.4–Å resolution[6]. We utilized this structure to predict the location of K^+–binding sites in HANA by means of homology modeling. In this report, we discuss the putative K^+–binding sites of HANA, and propose that HANA transports K^+ in the same manner as animal Na^+/K^+–ATPases.

2. Materials and Methods

2.1 Homology modeling of HANA

The amino acid sequence of HANA (UniProt ID: Q9SXK5) was aligned with that of shark rectal gland Na^+/K^+–ATPase α 1 (PDB ID: 2ZXE) by using a maximum matching program (Genetyx Co. Ltd., Tokyo). With the crystal structure of shark rectal gland Na^+/K^+–ATPase as a template, the 3D structure of HANA was generated by the modeling software MODELLER 9v7[7]. Energy minimization was done with Swiss Protein Database Viewer[8]. The calculated structure was evaluated by PROCHECK[9]. PyMol (http://www.pymol.org) was used for visualizing chemical bonds and calculating the distances between atoms. The valence values for K^+ were calculated based on the equation $s = (R/R_1)^{-N}$, where s is the bond valence, R the cation-oxygen bond length, and R_1 and N are empirical parameters according to Brown and Wu[10]. The valence mapping was carried out at 0.1–Å intervals throughout the constructed model using VALE software, kindly provided by Di Cera[11]. Threshold valence for the calculation was set at 0.90. To take water molecules into consideration, Fold–X ver.2.52 software (http://foldx.crg.es/) was used for predicting the positions of structural water molecules in protein structures.

3. Results

3.1 Modeling of HANA

Protein sequence alignment of HANA and shark Na^+, K^+–ATPase α–subunit showed that both sequences contain 10 TMs at similar positions, and the lengths of the TMs and loops TM2–3, TM3–4, TM5–6, TM6–7, TM8–9, and TM9–10 are essentially the same in the two sequences. But, HANA has a six–fold longer TM7–8 loop (290 aa) than shark Na^+, K^+–ATPase α–subunit (41 aa). Overall, HANA showed remarkable similarity to shark Na^+/K^+–ATPase α1–subunit, with

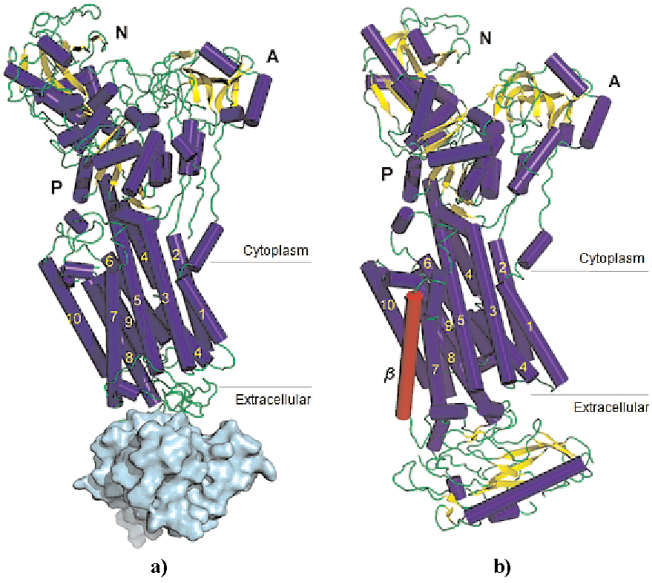

Fig. 1 The 3D structures of HANA and Na^+/K^+–ATPase. Cylinders and ribbons represent α–helices and β–strands, respectively. The numbers of transmembrane regions, and the A–, N– and P–domains are indicated. a) Model of HANA generated by means of homology modeling. An amorphous mass on the extracellular side indicates a long loop of the TM7–8 region of HANA. b) Atomic structure of shark Na^+/K^+–ATPase (PDB ID: 2ZXE). β indicates the β–subunit.

an identity of 55.0 % and a homology of 79.7 % at the amino acid level (Supplementary Figure). Taking advantage of this high homology, the 3D structure of HANA was constructed based on the crystal structure of the K^+–bound form of shark Na^+/K^+–ATPase. A long loop of the TM7–8 region of HANA was shown as an amorphous mass on the extracellular side. Fig. 1 shows the predicted overall architecture of HANA, and the crystal structure of shark Na^+/K^+–ATPase. Both structures appear quite similar, having 10 transmembrane segments TM1–10, and three intracellular characteristic domains, *i.e.* A, N, and P. At the extracellular side, HANA has a longer TM7–8 loop region in place of the Na^+/K^+–ATPase β–subunit. The predicted model of HANA was evaluated with the PROCHECK program, which showed that 86.8 % of amino acid residues in the model reside in the most favored region and 10.7 % in the additionally allowed region of the Ramachandran plot. The percentage of amino acid residues in the disallowed region was less than 1.2 %. The overall goodness factor, which represents the plausibility of covalent bond-angles and bond–distances of

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TM4
HANA      :  299  NLVFLISIIVANVPEGLLATVTVCLTLTA  327
Na+K+_Sa  :  320  AVIFLIGIIVANVPEGLLATVTVCLTLTA  348
Na+K+_Ss  :  318  AVIFLIGIIVANVPEGLLATVTVCLTLTA  346
Na+K+_Hs  :  320  AVIFLIGIIVANVPEGLLATVTVCLTLTA  348
Na+K+_Gg  :  318  AVIFLIGIIVANVPEGLLATVTVCLTLTA  346
Na+K+_Aa  :  319  AVIFLIGIIVANVPEGLLATVTVCLTLTA  347
Na+K+_Dm  :  338  AVIFLIGIIVANVPEGLLATVTVCLTLTA  366

TM5
HANA      :  819  SICYTLTSNIPEISPFLCFIVIGT  842
Na+K+_Sa  :  775  SIAYTLTSNIPEITPFLVFIIGNV  798
Na+K+_Ss  :  773  SIAYTLTSNIPEITPFLIFIIANI  796
Na+K+_Hs  :  775  SIAYTLTSNIPEITPFLIFIIANI  798
Na+K+_Gg  :  773  SIAYTLTSNIPEITPFLIFIIANI  796
Na+K+_Aa  :  774  SIAYTLTSNIPEITPFLLFIIANI  797
Na+K+_Dm  :  793  SIAYTLTSNIPEISPFLAFILCDI  816

TM6
HANA      : 846  LSTVLILGIDLGTDMVPAISMAY  868
Na+K+_Sa  : 802  LGTVTILCIDLGTDMVPAISLAY  824
Na+K+_Ss  : 800  LGTVTILCIDLGTDMVPAISLAY  822
Na+K+_Hs  : 802  LGTVTILCIDLGTDMVPAISLAY  824
Na+K+_Gg  : 800  LGTCTILCIDLGTDMVPAISLAY  822
Na+K+_Aa  : 801  LGTVTILCIDLGTDMVPAISLAY  823
Na+K+_Dm  : 820  LGTVTILCIDLGTDMVPAISLAY  842
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Fig. 2 Comparison of amino acid sequences in TM4, TM5 and TM6 among HANA and animal Na^+/K^+–ATPases (Sa; *Squalus acanthias*, Ss; *Sus scrofa*, Hs; *Homo sapiens*, Gg; *Gallus gallus*, Aa; *Anguilla anguilla*, Dm; *Drosophila melanogaster*).
The residues directly contributing to K^+–binding are shown on a gray background. Numbers flanking each sequence indicate the residue numbers at the start and end of the sequence.

the estimated structure, and which should exceed – 0.5 for a reliable model[7], was found to be – 0.21 in the case of HANA.

3.2 Identification of K^+–binding sites

Fig. 2 shows the alignments of amino acid sequences of TM4, 5 and 6, which contribute to K^+ binding[6, 12], among shark, human, chicken, eel and *Drosophila* Na^+/K^+–ATPase α1–subunits and HANA. A comparison of the transmembrane sequences of HANA with the corresponding shark sequences revealed identities of 86.2, 70.8 and 82.6 %, in M4, M5 and M6, respectively, as shown in Table 1. The amino acid residues directly contributing to K^+ binding in shark Na^+/K^+–ATPase[6] are completely conserved in HANA, as shown in Fig. 2. We then attempted to predict the coordination geometry of the amino acid residues in the putative K^+ binding sites of HANA by using homology modeling. As a water molecule is involved in K^+ coordination in the case of shark Na^+/K^+–ATPase, locations of structural water molecules on the structure of HANA were predicted, the locations of bound K^+ were determined by using valence mapping. Fig. 3 a, and b show the putative K^+–binding sites of HANA from different angles. The predicted K^+ binding sites are similar to those of shark Na^+/K^+–ATPase (Fig. 3a and b[6]). In HANA, the amino acid residues whose oxygen atoms lie within 4Å–distance from the K^+ ions were Thr823, Asn827, Asp855 and Asp859 for site I , and Val308, Ala309, Val311, Glu313, Asn827, Glu830 and Asp855 for site II . There mostly correspond well with the residues involved in K^+ binding in HANA and shark Na^+/K^+–ATPase, except that Ser826, corresponding to Ser782 in shark Na^+/K^+–ATPase, is placed quite far from K^+–binding site I in the constructed model.

Table 1 Similarity and Identity of HANA versus Shark Na^+/K^+–ATPase

region	residues	HANA vs. Shark Na^+/K^+-ATPase			
		Similarity		Identity	
		residues	%	residues	%
TM1	22	18	81.8	13	59.1
TM2	20	19	95.0	13	65.0
TM3	23	21	91.3	14	60.9
TM4	29	28	96.6	26	86.2
TM5	24	21	87.5	17	70.8
TM6	23	21	91.3	19	82.6
TM7	26	22	84.6	15	57.7
TM8	22	18	81.8	13	59.1
TM9	26	19	73.1	12	46.2
TM10	16	13	81.3	8	50.0
Total	231	200	86.6	149	64.5

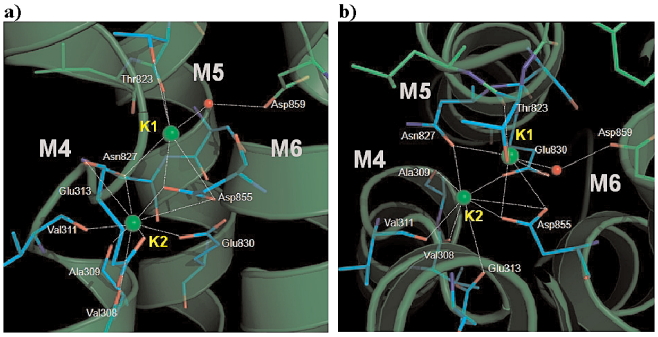

Fig. 3 Coordination geometry of K^+–binding sites in HANA and Na^+/K^+–ATPase. Green sphere represents K^+ ion and red sphere oxygen atom. a) K^+–binding sites of HANA viewed in parallel to the membrane. b) K^+–binding sites of HANA viewed from the cytoplasmic side.

Table 2 Coordinating oxygen atoms around bound K^+ ions within 4.0 Å–distance and valences.

	HANA		
site	residue	distance (Å)	valence for K^+
I	Thr823	3.59 (OG)	0.016
	Thr823	2.46	0.493
	Asn827	3.31	0.033
	Asp855	3.65 (OD1)	0.014
	Asp855	3.00 (OD2)	0.081
	Asp859	2.48 (water)	0.458
	sum		1.094
□	Val308	3.11	0.058
	Ala309	2.71	0.204
	Val311	2.63	0.268
	Glu313	3.56	0.017
	Asn827	2.76	0.173
	Glu830	2.74	0.185
	Asp855	3.31 (OD1)	0.033
	Asp855	2.75 (OD2)	0.179
	sum		1.118

Table 2 summarizes the distances between bound K^+ ions and coordinating oxygen atoms and the valence values.

4. Discussion

In the previous paper, Na^+ transport was measured using proteoliposomes reconstituted with purified HANA. ATP was added extravesicularly and uptake of $^{22}Na^+$ into the vesicles were measured. We examined a similar approach to detect ATP–dependent K^+–transport in the same system, by measuring decrease in intravesicular K^+ concentration or increase in extravesicular K^+ concentration. However, this proved not to be feasible, because of the low K^+ transport activity of HANA. Therefore, we decided to search for putative K^+ binding sites by means of homology modeling.

The 3D structure of HANA was predicted by means of homology modeling. The results of an evaluation program and the obtained value of goodness factor (– 0.21) indicate that the constructed model of HANA is reliable.

The overall structure of HANA has considerable similarities with that of Na^+/K^+–ATPase. Both have 10 transmembrane segments, TM1–10, and three characteristic cytoplasmic domains, A, N and P. Because the residues whose oxygen atoms contribute to K^+–binding in Na^+/K^+–ATPase were completely conserved in HANA, we were able to estimate the positions of bound K^+ ions in HANA. In the predicted coordination geometry of K^+ in HANA, the sums of valences were 1.094 and 1.118 for K^+–binding sites Ⅰ and Ⅱ, respectively, as shown in Table 2. As the sums of valence values were close to 1.0, the ideal value, K^+ ions should be bound with high affinity.

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HANA       :923  PPHILPGLGRGGF  935
Na+K+_Sa   : 71  DRVAPPGLSHAPY  83
Na+K+_Ss   : 70  DRVAPPGLTQIPQ  82
Na+K+_Hs   : 75  DRLATPGLMIRPK  87
Na+K+_Gg   : 74  DRISSPGLMISPK  86
Na+K+_Aa   : 70  DRVAPPGLSHTPR  82
Na+K+_Dm   : 86  LIGTNPGLGFRPL  97
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Fig. 4 Comparison of amino acid sequences in TM–8 loop of HANA with animal Na^+/K^+–ATPases β–subunits (Sa; *Squalus acanthias*, Ss; *Sus scrofa*, Hs; *Homo sapiens*, Gg; *Gallus gallus*, Aa; *Anguilla anguilla*, Dm; *Drosophila melanogaster*). Numbers flanking each sequence indicate the residue numbers at the start and end of the sequence.

Homology modeling is a useful means to analyze molecular structure of membrane proteins because of the difficulty of crystallizing them. The structures of the K^+ binding sites of human Na^+/K^+–ATPase were predicted by means of homology modeling using the Ca^{2+} unbound form of Ca^{2+}–ATPase as template by Ogawa[13]. Our model was constructed based on the K^+ bound form of shark Na^+/K^+–ATPase. As there is a very high homology between HANA and shark Na^+/K^+–ATPase, the obtained structure of K^+ binding sites should be reliable. The 3D model of Na^+ binding sites in HANA was not constructed, because of low homology in amino acid sequence between HANA and Ca^{2+}–ATPase (PDB ID: 1SU4).

Na^+/K^+–ATPase β–subunit was reported to play a critical role in K^+ binding[14, 15]. HANA lacks a β–subunit, but has a longer TM7–8 junction consisting of 290 amino acid residues, with a length similar to that of the Na^+/K^+–ATPase β–subunit. This loop contains Pro-Gly–Leu (PGL), which is present in most β–subunits (Fig. 4). The PGL sequence in the β–subunit is located in the region involved in interaction with the α–subunit[6]. Taking account of the extracellular localization of the TM7–8 junction, this junction might play a role equivalent to that of the Na^+/K^+–ATPase β–subunit.

HANA is faced with 450 mM Na^+ of seawater as well

as shark rectal gland Na^+/K^+–ATPase. The Na^+/K^+–ATPase in marine environment was reported to have more positive amino acids in extracellular Na^+ exit pathway, to reduce its sensitivity to external Na^+ ions[16]. Gly319 on TM3–4 loop in Loligo Na^+/K^+–ATPase, conserved among the Na pumps of marine animals and one of the important residues for the high Na^+ adaptation. The Gly319 was also conserved as Gly294 in HANA and as Gly314 in shark Na^+/K^+–ATPase.

It was reported that HANA was highly activated in the presence of both Na^+ and K^+, and the steady-state level of phosphoenzyme was high in the presence of Na^+, but very low in the presence of K^+ [2]. These characteristics resemble those of Na^+/K^+–ATPase, so HANA might transport K^+ ions in the same way as animal Na^+/K^+–ATPases, though K^+ transport has not yet be demonstrated.

These kinetic and structural features of putative K^+ binding in HANA strongly indicate that HANA transports K^+ as well as Na^+, and that its mechanism of action is similar to that of Na^+/K^+–ATPase of animal cells.

Na^+/K^+–ATPases had long been believed to be present exclusively in animal cells. In 1995, however, cells of marine alga *H. akashiwo* were found by us to exhibit Na^+/K^+–ATPase–like activity[2], and lately aquatic fungus *Blastocladiella emersonii* was found to contain Na^+/K^+–ATPase–like activity by Flavio et al.[17] Moreover, the genes encoding animal Na^+/K^+–ATPase α–subunit like molecules have been cloned from many organisms, containing alga, fungus and even archaebacteria[18]. Then, an ancestral molecule of Na^+/K^+– ATPase may have emerged an early stage of evolution, and the possibility should be considered that molecules with Na^+/K^+–ATPase–like activity might also be present in plants as well as animal kingdom.

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Supplementary Figure Alignment of amino acid sequences of HANA (Q9SXK5) and the shark Na^+/K^+–ATPase α1–subunit (2ZXE). Boxes indicate transmembrane regions. Asterisks indicate identical amino acids, and colons and periods indicate highly and weakly similar amino acids, respectively.