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ORIGINAL ARTICLE

Interhelical Hydrogen Bonds in Transmembrane Region Are Important for Function and Stability of Ca^{2+}-Transporting ATPase

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Abstract

Ca^{2+}-transporting adenosine triphosphatase (ATPase) of sarcoplasmic reticulum couples ATP hydrolysis with ion transport. Phosphorylation of the cytosolic region of the calcium-bound conformation (E1) of the protein leads to drastic conformational rearrangements of the transmembrane helices and the release of Ca^{2+}. The resulting calcium-free conformation (E2) is less stable than the E1 form. The changes in van der Waals interactions and interhelical hydrogen bonding in the E1 and E2 conformations were compared. Conformational changes in the transmembrane region concomitant with the release of Ca^{2+} mainly affect the number of interhelical hydrogen bonds, which is reduced to half of that in E1 form, whereas the number of interhelical atomic pairwise contacts reflecting van der Waals interactions experience little change. The interhelical hydrogen bonds in Ca^{2+}-transporting ATPase can be divided into two groups according to their roles: those that play a structural stabilizing role and those that are important for the correct geometry of the Ca^{2+} binding site. Interhelical hydrogen bonds in the transmembrane regions play important roles for the stability and specificity of helix–helix interactions in proteins where change of conformation is required for transport of ions or small molecules.

Index Entries: Membrane protein; Ca^{2+}-transporting ATPase; helix packing; hydrogen bond.

INTRODUCTION

Transmembrane (TM) proteins play important roles in signal transduction, transport of ions and small molecules, volume regulation, light harvesting, and many other physiologic processes. Important issues in membrane protein studies are how TM helices assemble and examination of the relative roles of van der Waals interactions and interhelical hydrogen bonds. In this study, we examine the details of packing interactions of the Ca^{2+}-bound and Ca^{2+}-free conformations of Ca^{2+}-transporting adenosine triphosphatase (ATPase). We find that interhelical hydrogen bonds may be important for the stability and proper function of membrane proteins that experience conformational change in the process of transport of ions and small molecules.

Integral membrane protein is composed of a transmembrane region that is buried in the

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phospholipid bilayer and "soluble" regions that are exposed to polar environment. The environment shapes these two different regions of the protein and influences their amino acid composition. The transmembrane region is formed either as bundles of α-helices or as β-barrels composed of mostly hydrophobic amino acid residues, with a significantly smaller fraction of polar and ionizable residues than expected in soluble proteins. The amino acid composition of the solvent-exposed region resembles that of soluble proteins.

The solvent-exposed region and the transmembrane region of integral membrane proteins are both essential for biological function. Binding of ligands to receptors often causes conformational changes in the solvent exposed regions, which are then relayed through the membrane domain. For Ca^{2+}-transporting ATPase, binding of ATP followed by phosphorylation of the cytosolic region results first in significant conformational changes of the cytosolic part of the molecule, which then propels conformational changes in the transmembrane region. These conformational rearrangements are necessary to transfer Ca^{2+} ions across the membrane against a gradient of calcium concentration.

Compare to soluble proteins, the number of known membrane protein structures is small. Structures that capture the TM proteins in different conformational states are even more scarce. Recently, Toyoshima et al. solved the structure of Ca^{2+}-transporting ATPase in two conformations, E1 *(1)* and E2 *(2)*, where E1 is the calcium bound form and E2 is the calcium-free form of the protein. These structures provide important source of information for understanding membrane protein function and stability.

Ca^{2+}-transporting ATPase (SERCA1a) belongs to the large family of P-type ion-translocating ATPases, which couple ATP hydrolysis to ion transport. Among them are Na^+, K^+-ATPase and H^+, K^+-ATPase that are important therapeutic targets *(3)*. Ca^{2+}-ATPase pumps two calcium ions across the sarcoplasmic reticulum (SR) membrane for each ATP molecule hydrolyzed. At the same time, two or three H^+ ions are countertransported. The mechanism of transport is well studied and reviewed in *(4,5)*. The transport of Ca^{2+} ions is thought to be a multistep process in which two main conformations are recognized: a stable E1 conformation with high affinity for Ca^{2+} ions and a less stable E2 conformation with low affinity for Ca^{2+} ions. Significant conformational changes accompany calcium release and binding: six of ten TM helices rearrange *(2)* with extensive repacking of helices. The X-ray structure of the protein in E2 conformation (1IWO) is maintained by the potent inhibitor thapsigargin, which keeps the enzyme from denaturing in the absence of Ca^{2+}. Toyoshima et al. *(2)* suggested that the thermal movements of transmembrane helices become deleteriously large without the support of lipids or the cohesion provided by bound Ca^{2+} ions.

Transmembrane helical domains of integral membrane proteins are generally packed tighter than α-helical soluble proteins *(6,7)*. Well-packed amino acid side chains that maximize van der Waals interactions are important for the folding, stability, and specificity of TM proteins *(8)*. Additionally, experimental studies showed that interhelical hydrogen bonds play critical roles for helix oligomerization *(9,10)*. Recent statistical survey found that almost every TM helix in a set of 13 membrane proteins with available X-ray structures forms at least one interhelical hydrogen bond with its neighbors and that the helical pairs with interhelical hydrogen bonds have a tendency to pack tighter than their counterparts without interhelical H-bonds *(11)*. The roles of hydrogen bonds and higher order interactions in membrane protein assembly are discussed elsewhere *(12,13)*.

We further examine structures of Ca^{2+}-transporting ATPase in E1 and E2 conformations and compare the contribution of van der Waals interactions and interhelical hydrogen bonds on stability in both states. We also compare mutational data of residues involved in hydrogen bonding and find that interhelical hydrogen bonds can be roughly divided into those that are critical for function and those that are

mostly important for stability of transmembrane helices. We suggest that the decrease in interhelical hydrogen bonding may be an important factor that affects the stability of Ca^{2+}-ATPase in E2 form.

METHODS

Transmembrane Helices and Calculation of Interhelical Hydrogen Bonds

Two protein structures are used in this study: 1EUL *(1)* and 1IWO *(2),* representing E1 and E2 states of Ca^{2+}-transporting ATPase, respectively. All loops in the soluble regions are manually removed, leaving only the alpha helices in the TM regions. As a result, each protein is represented by a bundle of TM helices. Transmembrane helices were designated as follows: M1: residues 50–76, M2: 87–112, M3: 253–277, M4: 291–315, M5: 756–781, M6: 789–809, M7: 831–856, M8: 894–916, M9: 931–951, M10: 963–987. The lengths of helices vary from 20 (M9) to 26 (M1) residues.

H-bonds are identified by HBPLUS program *(14)* using default parameters and allowing exchange of the nearly symmetrical side chains of residues His, Gln, and Asn, because nitrogen, oxygen and carbon atoms are indistinguishable in electron density maps. Potential H-bonds that would be formed if histidine CD2 was actually ND1, CE1 was NE2 and the oxygens and nitrogens in asparagines and glutamines were the other way around, were counted.

Computation of Pairwise Interhelical Contacts

Using the alpha shape application program interface kindly provided by Prof. Edelsbrunner and colleagues, a program INTERFACE-2 has been implemented to compute interhelical pairwise atomic interactions as described previously *(7).* INTERFACE-2 uses precomputed Delaunay triangulation and alpha shape. The Delaunay triangulation of membrane proteins is computed using the DELCX program *(15,16),* and the alpha shape is computed using the MKALF program *(15,17).* Both can be downloaded from the website of NCSA (http://www.ncsa.uiuc.edu). The van der Waals radii of protein atoms are taken from Tsai et al. *(18).* The advantage of using INTERFACE-2 compared to distance cut-off methods is that only the nearest neighbor atoms in physical contacts are counted *(7).*

RESULTS

Packing and van der Waals Interactions

The atomic interhelical pairwise contacts provide a measure of the extent of packing interactions *(19).* Stronger van der Waals interactions are expected for helices with extensive interhelical contacts between amino acid residues. A common heuristic approach to studying van der Waals interactions is to define a cut-off distance and search for all atoms and residues within this distance and count them as contact partners *(20,21).* The problem with this approach is that in order to identify all contacting nearest neighbor atoms, a large enough distance will have to be chosen, which may lead to the inclusion of many noncontacting neighbors. Here, we use a method based on computational geometry that accurately identifies the nearest atomic neighbors in physical contact *(7,22,23).* Because there is some degree of uncertainty in the atomic coordinates obtained by X-ray crystallography, which affects the alpha shape calculations, it is necessary to account for this uncertainty to ensure the reliability of results of geometry calculations.

To assess how the size of the probe radius affects the number of pairwise contacts in the different structures of the same protein, we chose bacteriorhodopsin (bR) (residues 8–152 and 167–226), because this transmembrane protein has the largest number of X-ray structures solved at different resolution. Figure 1 shows the dependence of the total number of pairwise atomic (Fig. 1A) and residue (Fig. 1B) interhelical contacts in bR structures with different resolution vs the probe radius. The number of pairwise contacts increases with the probe radius used in alpha shape calculations. Overall, the high-resolution structures 1M0L

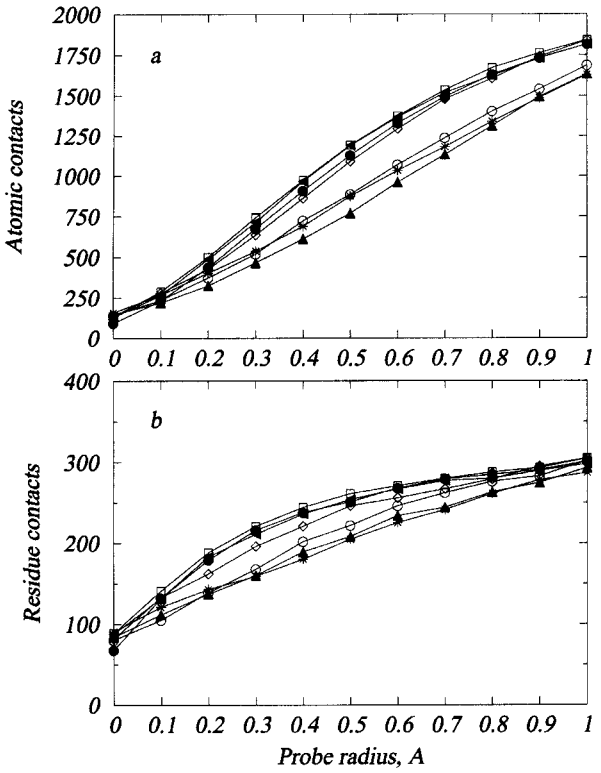

Fig. 1. Interhelical pairwise atomic **(A)** and residue **(B)** contacts for bacteriorhodopsin structures calculated with different probe radii.

(1.47 Å), 1C3W (1.55 Å), 1QHJ (1.9 Å), and 1BRX (2.3 Å) have larger number of interhelical pairwise atomic and residue contacts for every given probe radius when compared with the low-resolution structures 2AT9 (3.0 Å), 1FBB (3.2 Å), and 2BRD (3.5 Å). No two structures give identical curves. The number of contacts is not 100% identical for any pair of structures using any probe radius, including the structures with the highest resolution (1M0L, 1.47 Å and 1C3W, 1.55 Å), except for a single point at the probe radius of 0.7 Å, where the number of residue but not atomic contacts is identical. There is always a number of pairwise contacts for any two structures of the same protein that are different. This is independent of the probe radius used.

To assess van der Waals interactions in low-resolution structures, it is common to use a probe with a radius that enables sufficient sampling of the surrounding atoms to account for the uncertainty in the atomic coordinates and to minimize the influence of background variation of contact interactions as seen in the structures of bR. Figure 2A compares residue-residue contacts in three structures of bacteriorhodopsin with resolution 3.0 Å (2AT9), 3.2 Å (1FBB), and 3.5 Å (2BRD), which are similar to the resolution of Ca^{2+}-transporting ATPase structures in E1 and E2 forms. The upper curves show that the total number of unique interhelical residue pairs in these structures all increases with the probe radius. The curves with empty and filled circles (2AT9 and 2BRD) are almost identical at probe radii 0.3 Å and up to 0.8 Å, whereas the curve for 1FBB structure marked with "X" differs from them. At this level of structure resolution, we estimate that the difference in pairwise contacts of up to 13% for different structures of the same protein in the same functional state reflects the average variation and error in the atomic coordinates determined experimentally. We further examine contacting residue pairs that are present in only one of the structures. Curves with empty and filled diamonds show the number of pairwise interactions that are found only in 2AT9 and 2BRD structures, respectively. This number is rather constant and fluctuates around 50 for calculations with different probe radii. The number of different pairwise contacts is smaller in the higher resolution structures, e.g., 1M0L and 1C3W (Fig. 2B, empty and filled diamonds). The fraction of differing contacts falls as the probe radius increases (Fig. 2A, filled and empty triangles), whereas the total number of pairwise contacts increases. The small probe radii (0.0 Å–0.3 Å) seem to be a good choice according to the curves on Fig. 1A,B, because the number of atomic and residue pairwise contacts has a small variation. On the other hand, the fraction of different pairwise contacts is high at this range of probe radii, which reaches up to 65% for 2AT9 and 2BRD structures (*see* Fig. 2A) and up to 25% for 1M0L and 1C3W structures (*see* Fig. 2C). Analysis of curves on Fig. 2A and B indicates that probe radii 0.5Å-0.7Å are most

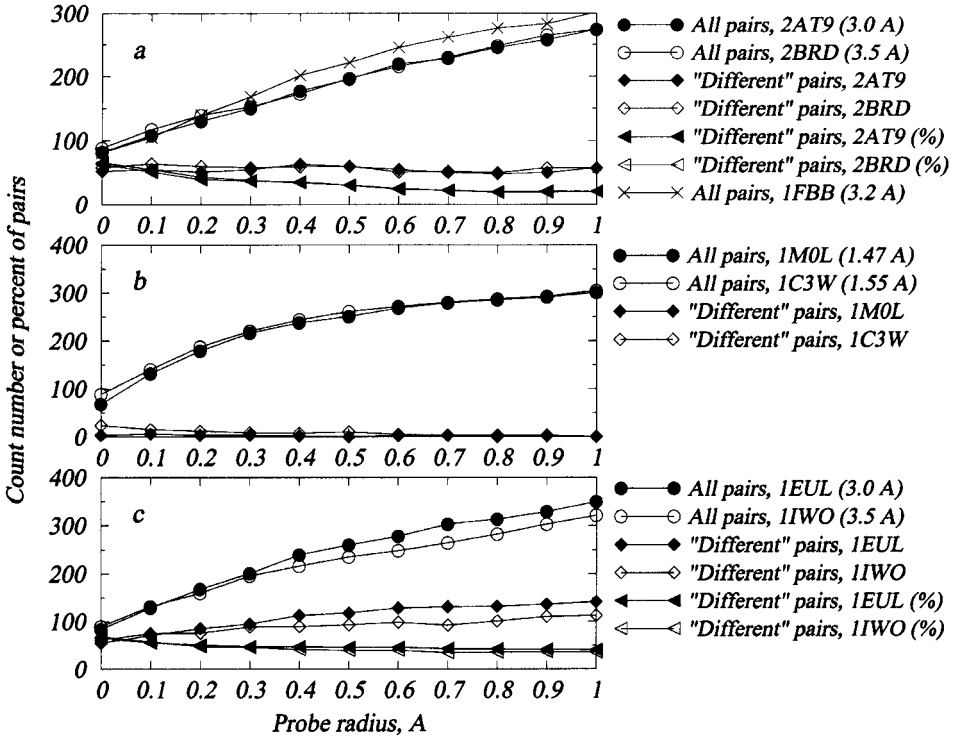

Fig. 2. Interhelical pairwise residue contacts in high- and low-resolution protein structures vs probe radius: **(A)** 2AT9 (3.0 Å), 1FBB (3.2 Å) and 2BRD (3.5 Å); **(B)** 1M0L (1.47 Å) and 1C3W (1.55 Å); **(C)** 1EUL (3.0 Å) and 1IWO (3.5 Å). Empty and filled circles and "X" signs represent a total number of interhelical pairwise contacts, empty and filled diamonds represent pairs that are different when two structures are compared with each other, empty and filled triangles represent the percentage of pairs present only in one structure ("different pairs").

appropriate for the purpose of assessing van der Waals interactions, because the fraction of different contacts decreases in this interval of probe radii, while their small sizes still reflect the physical picture of atomic interactions.

Figure 2C shows the total number of all pairwise residue contacts (filled and empty circles) and "different" pairwise contacts (filled and empty diamonds) for probe radii 0.0 Å–1.0 Å for Ca^{2+}-transporting ATPase structures in E1 and E2 forms. Both curves are similar to those for low-resolution structures of bacteriorhodopsin 2AT9 and 2BRD, with the only difference that the number of "different" contacts increases with the increase of the probe radius and the lower resolution structure 1IWO has a fewer number of contacts for probe radii 0.4 Å and up. Although we have not calculated the exact magnitude of van der Waals interactions in these structures, comparison of plots on Fig. 2A and C suggests that the total number of residue contacts in the TM region does not change much upon release of Ca^{2+} ions: The difference of 25 in residue contacts between 1EUL (E1) and 1IWO (E2), which includes the change of 12 H-bond interactions discussed below, is comparable to the difference of 17 residue contacts observed between two structures of bR in the same ground state (1FBB, 3.2 Å and 2BRD, 3.5 Å).

Based on the previous analysis, we chose the probe radius of 0.5 Å, as reported in *(24)* for assessing van der Waals interactions by examining atomic contact interactions. The number of interhelical atomic contacts obtained from

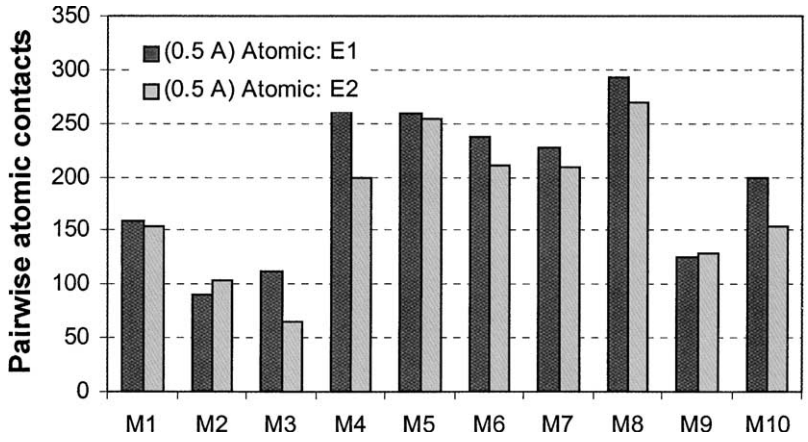

Fig. 3. Number of interhelical pairwise atomic contacts that were calculated by INTERFACE-2 program for every TM helix of Ca^{2+}-transporting ATPase using probe radius 0.5 Å.

alpha shape calculations with probe radius 0.5 Å for each of the 10 TM helices of Ca^{2+}-transporting ATPase in E1 and E2 conformations are shown on Fig. 3. Helices M4-M8 have the highest number of interhelical pairwise contacts and, consequently, the tightest packing. All of these helices, excluding M7, contain residues that participate in Ca^{2+} binding. There are several known mutations of hydrophobic residues from helices M4-M8 that affect the calcium transporting activity when the size of their side chain is changed. In most cases, either increase or decrease in the size of side chain results in a loss of Ca^{2+}-transporting activity, although the mutations affect different steps in the calcium pumping pathway. For example, mutations L777A (helix M5), A900F (helix M8) block transition from E1P to E2P state, whereas mutation F760G as well as Y763L affect Ca^{2+} affinity and mutation G770V blocks the formation of phosphoenzyme from ATP to P*i (25).*

The helices M1-M3 and M9-M10 have a smaller number of interhelical pairwise contacts and are less tightly packed. Helices M1-M3 experience dramatic structural rearrangement *(2).* Despite of the movements of helices relative to one another and relative to the membrane plane, the number of interhelical pairwise atomic contacts did not significantly change either for the more mobile helices M1-M6, or for the more static helices M7-M10 (*see* Fig. 3). There are more polar-polar interactions in E1 (number count: 60, or 6.1%) than in E2 state (39, or 4.5%), but in both conformations they constitute a small fraction of total interhelical contacts.

Interhelical Hydrogen Bonds That Are Preserved in Both E1 and E2 Forms of Ca^{2+}-Transporting ATPase

Table 1 lists interhelical hydrogen bonds that are identified in the TM region of the structure of Ca^{2+}-transporting ATPase in E1 (Table 1, A) and in E2 (Table 1, B) conformations. There are 21 interhelical H-bonds in E1 state and only 11 or 12 in E2 state. (Structure 1IWO [E2 conformation] does not contain H-bond between side chains of His 944 and Trp 794, although we think that this interaction is preserved in both E1 and E2 states. We present our arguments in support of this later.) The extent of the rearrangement of TM helices is so significant that there are only three interhelical H-bonds preserved in both E1 and E2 conformations. These are marked with the "*" in Table 1 and are found in helical pairs that contain at least one helix that did not significantly move during the calcium transport. The residues involved in preserved hydrogen bonds are Ser 766, Ser 767, Asn 911, Trp 794, Ser 902 and His 944.

Table 1
Interhelical Hydrogen Bonds in Ca^{2+} ATPase in E1 and E2 Forms

A. E1 H-bonds:								
1.	M1	Glu	58	(OE1)	M4	Glu	309	(OE2)
2.	M4	Lys	297	(NZ)	M1	Ser	72	(OG)
3.	M4	Lys	297	(NZ)	M2	Glu	90	(OE1)
4.	M2	Gln	108	(NE2)	M6	Thr	805	(OG1
5.	M3	Cys	268	(SG)	M4	Ala	299	(O)
6.	M3	Trp	272	(NE1)	M4	Ala	292	(O)
7.	M5	Asn	768	(ND2)	M4	Ala	306	(O)
8.	M5	Asn	756	(ND2)	M6	Gly	808	(O)
9.	M5	Arg	762	(NH1)	M8	Asn	914	(O)
10.	M5	Tyr	763	(OH)	M6	Thr	799	(O)
11.	M5	Glu	771	(OE2)	M8	Glu	908	(OE1)
*12.	M8	Asn	911	(ND2)	M5	Ser	766	(OG)
13.	M8	Asn	911	(ND2)	M5	Ser	767	(OG)
14.	M5	Asn	768	(ND2)	M6	Asp	800	(OD2)
*15.	M6	Trp	794	(NE1)	M9	His	944	(ND1)
16.	M9	Ser	940	(OG)	M6	Val	798	(O)
17.	M6	Thr	799	(OG1)	M8	Glu	908	(OE2)
18.	M7	Tyr	843	(OH)	M10	Ile	973	(O)
*19.	M8	Ser	902	(OG)	M9	His	944	(NE2)
20.	M8	Asn	914	(ND2)	M10	Val	977	(O)
21.	M10	Trp	967	(NE1)	M9	Tyr	949	(OH)
B. E2 H-bonds:								
1.	M4	Tyr	295	(OH)	M3	Asn	275	(O)
2.	M5	Asn	768	(ND2)	M4	Ala	305	(O)
*3.	M8	Asn	911	(ND2)	M5	Ser	766	(OG)
4.	M6	Asp	800	(OD2)	M5	Ser	767	(OG)
5.	M6	Asn	796	(OD1)	M5	Glu	771	(OE2)
6.	M5	Cys	774	(SG)	M7	Thr	848	(OG1)
7.	M9	Ser	940	(OG)	M6	Thr	799	(O)
8.	M7	Trp	832	(NE1)	M10	Leu	984	(O)
9.	M7	Arg	836	(NH1)	M10	Asp	981	(OD1)
*10.	M8	Ser	902	(OG)	M9	His	944	(NE2)
11.	M10	Ser	974	(OG)	M8	Val	903	(O)
*12.	M6	Trp	794	(NE1)	M9	His	944	(ND1)

The side chain of residue Asn 911 (M8) forms two interhelical H-bonds with the side chains of Ser 766 (M5) and Ser 767 (M5) in E1 conformation. This is a "polar clamp" spatial motif *(11)*, where polar groups from three amino acid side chains form two interhelical hydrogen bonds. The calcium release is accompanied by the rotation of the part of M6 helix containing Ca^{2+}-binding residues Asn 796, Thr 799, and Asp 800 by nearly 90 degrees *(2)*. As a result of this rotation, residue Ser 767 forms interhelical H-bond with Asp 800 in the E2 form. Consequently, only one of the two (S766-N911) interhelical H-bonds is preserved in E2 form.

Substitutions of Ser 766 or Ser 767 with Ala decreased Ca^{2+} transport activity by 20% and 70% of wild type, respectively. The effect of mutation of Ser 767 was more pronounced because it stabilizes the low affinity E2 state by forming an H-bond with Asp 800. On the other hand, the mutation of residue Asn 911 had no effect on Ca^{2+} transport and binding *(26)*, which implies that hydrogen bonds formed by Asn 911 with Ser 766 and Ser 767 in E1 conformation are not critical for Ca^{2+}-transport.

The remaining two preserved H-bonds involve helices M6, M8, and M9. Both of these hydrogen bonds involve a common and highly conserved residue His 944 (M9). There is little change in conformation between helices M8 and M9 (RMSD to mean on all Cα = 0.8 Å for 44 residues) and a slight change between helices M6 and M9 (RMSD to mean on all Cα = 1.3 Å for 42 residues). In the first preserved hydrogen bond, NE2 atom of the imidazole ring of His 944 donates hydrogen to hydroxyl oxygen of highly conserved and mutation-sensitive Ser 902 *(25)*, while in the second hydrogen bond the ND2 atom accepts the hydrogen from Trp 794 (M6). The latter hydrogen bond is very clear from the structure in E1 conformation (1EUL). The Trp residue is located in the middle of the TM region with the indole ring situated parallel to the membrane plane and partially exposed to phospholipids by its hydrophobic side. However, the Trp side chain is flipped around 180 degrees in 1IWO structure (E2 conformation) with NE1 atom facing the phospholipids. As a result, it seems that a hydrogen bond is not preserved. There is a small chance for the flip of the bulky indole ring in the context of tightly packed TM helix M6. We suspect that NE1 group of Trp 794 still faces the TM helices and probably forms a hydrogen bond with His 944 side chain in the E2 conformation as well.

Hydrogen Bonds Formed by Mutation-Sensitive Polar Residues

There are several mutation-sensitive polar residues that are involved in interhelical hydrogen bonds in the TM region of Ca^{2+}-transporting ATPase. Both Ca^{2+} binding sites are stabilized by hydrogen-bond network between the coordinating residues *(1)* and residues residing nearby the binding sites. For example, Asn 768 (M5) forms interhelical H-bonds with Ala 306 (M4) and Asp 800 (M6), connecting together three different helices. Interhelical hydrogen bonds formed by main chain oxygens of Thr 799 also connect three different helices: Thr 799 (M6), Tyr 763 (M5) and Glu 908 (M8). Residue Glu 309 (M4) forms H-bonds with Glu 58 (M1) (*see* Table 1).

The calcium binding site II is mainly formed by residues from helix M4 *(1)*, which moves significantly in the process of Ca^{2+} transport *(2)*. The drastic conformational changes of helix M4 require a mechanism that controls and stabilizes the correct helical positioning in the process of transition from one state to another. There are two mutation-sensitive residues with polar groups at the lumenal side of helix M4 that are not involved in Ca^{2+} binding: Tyr 295 and Lys 297. Figure 4 illustrates hydrogen bond interactions on the transmembrane luminal side of Ca^{2+}-transporting ATPase. In E1 conformation, Lys 297 forms two hydrogen bonds with the side chains of residues Ser 72 and Glu 90, whereas residues Tyr 295 and Asn 275 do not have any polar interactions at all. In E2 conformation, helices M3 and M4 move and Tyr 295 forms hydrogen bond with main chain oxygen of Asn 275. At the same time, residues Lys 297, Ser 72, and Glu 90 are separated far from each other and do not interact. In the repetitive process of ion pumping, each set of hydrogen bonds specifically stabilizes helix M4 in a correct position that is either optimized for Ca^{2+} binding in E1 conformation or for Ca^{2+} release in E2 conformation.

Mutational data confirm the importance of these hydrogen bonds. Substitution of Lys 297 with glycine resulted in a complete loss of Ca^{2+}-transporting activity. Substitutions of Lys 297 with amino acids of large hydrophobic side chains (mutants K297M and K297F) resulted in 80–90% loss of maximal Ca^{2+} transport activity. However, the mutants K297R and K297E, both of which have hydrogen-bonding capabilities,

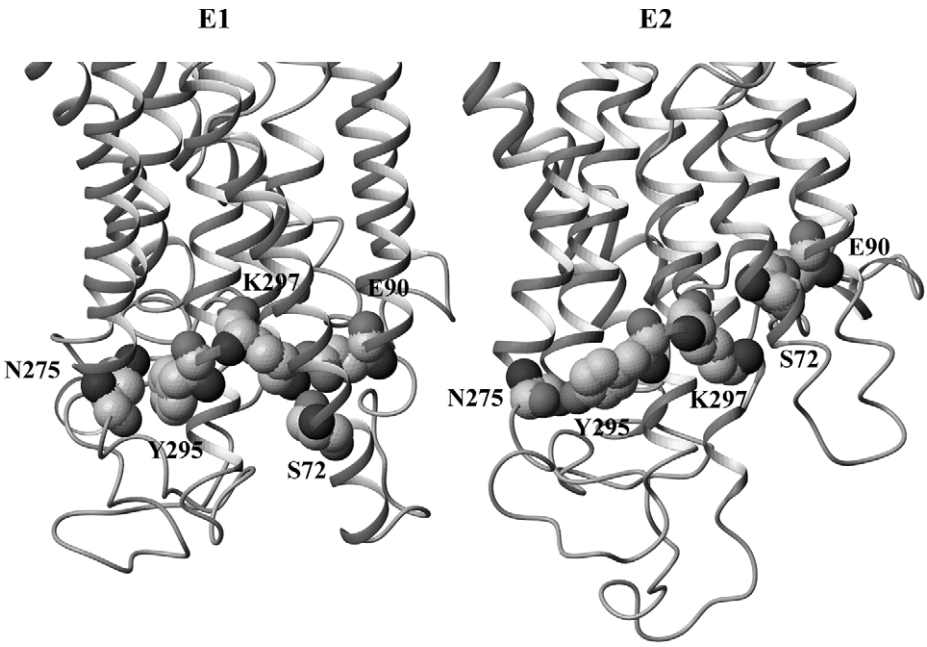

Fig. 4. Interhelical hydrogen bonds involving two residues from TM helix M4 in E1 and E2 conformations. E1 conformation: side chain of Lys 297 (M4) forms two interhelical hydrogen bonds with side chains of residues Ser 72 (M1) and Glu 90 (M2). E2 conformation: interhelical hydrogen bond between side chains of Tyr 295 (M4) and carbonyl oxygen of Asn 275 (M3).

retained 60–70% of activity *(27).* Mutational studies of Tyr 295 are not as extensive as that of Lys 297, but available data suggest the important role of this residue in stabilizing E2 conformation: mutant Y295A loses 70% of the wild-type Ca^{2+}-transporting activity *(28).*

Hydrogen Bonds Formed by Mutation-Insensitive Polar Residues

In addition to the hydrogen bonds formed between Asn 911 and Ser 766, Ser 767 in E1 conformation, there are several other examples when the loss of hydrogen bonding properties in single-point mutants of residues forming H-bonds in either E1 or E2 conformation do not significantly affect Ca^{2+}-transporting activity. For example, mutants Y843F (forms H-bond in E1 conformation, highly conserved) and S940A (forms H-bond in E1 and E2 conformations, highly conserved, only Thr substitution is found in sequence alignments) both retain 100% activity *(26).* The high degree of the conservation of these residues suggests that they are selected under purifying pressure during evolution. Their major role is probably in stabilizing the TM region. We propose that the effect of a single mutation may not be detected, but the loss of several interhelical H-bonds may be deleterious.

DISCUSSION

The release of calcium ions and changes in conformation affect the stability of Ca^{2+} ATPase. In this study, we investigate what factors besides the loss of Ca^{2+} ion binding account for the decreased stability of this protein in low affinity state. It is postulated that there are two possible driving forces for transmembrane helix association: van der Waals interactions and interhelical polar interactions *(29).* The packing or van der Waals interactions are known to mediate the oligomerization of TM helices. For example, mutations that disrupt the optimal packing significantly affect

the stability of glycophorin A dimer *(30,31).* Tightly packed regions of glycophorin A form "hot spots" of interactions. The introduction of clashes or the removal of favorable packing interactions disrupts or eliminates weakly attractive van der Waals interactions, which results in the loss of stability *(32).* The Ca^{2+}-transporting ATPase is less stable in E2 conformation than in E1 conformation. Our calculations indicate that during the transition from E1 to E2 conformation that accompanies the pumping of Ca^{2+} ions, the total number of pairwise interhelical contacts experience little change, hence, there is no significant changes in van der Waals interactions. We conclude that change in van der Waals packing interactions is not among the factors that affect stability of the Ca^{2+}-transporting ATPase in E2 conformation.

There is a growing experimental evidence suggesting that interhelical hydrogen bonds are important for stability and specificity of helical association in the TM regions of membrane proteins. One series of experiments demonstrated strong association of the engineered transmembrane helices derived from GCN4 leucine zipper containing Asn residue. Importantly, the dimers of the TM helices with polar residue that form interhelical hydrogen bond were found to be more stable than those where only van der Waals interactions contribute to oligomerization *(9,10,33).* Another set of experiments using randomized library of transmembrane interfaces showed that specific motifs of serine and threonine residues can drive association of transmembrane helices *(34).* Computational modeling of the structures of the most stable sequences produced structures containing multiple interhelical hydrogen bonds *(34).*

In Ca^{2+}-ATPase, the total number of interhelical hydrogen bonds decreases almost by a factor of two on the transition from a calcium bound state E1 to a calcium free state E2. We speculate that in addition to the loss of Ca^{2+} ions, decrease in interhelical hydrogen bonds is the main factor that affects the stability of Ca^{2+}-transporting ATPase in E2 state. We find that interhelical H-bonds can be classified into two types according to their roles: those that play mostly stabilizing role and those that are important for the correct geometry of the Ca^{2+} binding site. The polar residues involved in both types of interactions are usually highly conserved. However, residues that are important for the correct geometry of the calcium binding site are mutation-sensitive, while polar residues that play mostly stabilizing role are often mutation-insensitive. We also suggest that hydrogen bonding stabilization of transmembrane helices may be cooperative in nature: the mutation of one residue may not significantly affect the stability of the protein, but multiple mutations of residues involved in hydrogen bonding network may be deleterious. The cooperative nature of interhelical hydrogen bonds was also discussed in the oligomerization of TM helices containing serine and threonine residues *(34),* where it was showed that single serine or threonine side chains cannot promote a strong helix-helix association, but several residues that presumably form multiple interhelical H-bonds can.

CONCLUSIONS

We show that conformational changes in the transmembrane region accompanying calcium pumping by Ca^{2+}-transporting ATPase of sarcoplasmic reticulum mainly affect the number of interhelical hydrogen bonds. This contributes to a decreased stability of the protein in calcium-free E2 form. We propose that interhelical hydrogen bonds in TM regions may play important roles for the stability and specificity of helix-helix interactions in proteins where change of conformation is required for transport of ions or small molecules. Our results may help to design further experimental studies to elucidate the role of interhelical hydrogen bonds in membrane protein stability and function.

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REFERENCES

1. Toyoshima, C., Nakasako, M., and Nomura, O. H. (2000) Crystal structure of the calcium pump of sarcoplasmic reticulum at 2.6 A resolution. *Nature* **405,** 647–655.
2. Toyoshima, C., and Nomura, O. H. (2002) Structural changes in the calcium pump accompanying the dissociation of calcium. *Nature* **418,** 605–611.
3. Perlin, D. S. (1998) Ion pumps as targets for therapeutic intervention: old and new paradigms. *Electronic J. Biotech.* **1,** 55–64.
4. MacLennan, D. H., Rice, W. J., and Green, N. M. (1997) The mechanism of Ca^{2+} transport by sarco(endo)plasmic reticulum Ca^{2+} ions-ATPases. *J. Biol. Chem.* **272,** 28815–28818.
5. Lee, A. G., and East, J. M. (2001) What the structure of a calcium pump tells us about its mechanism. *Biochem. J.* **356,** 665–683.
6. Eilers, M., Patel, A. B., Liu, W., and Smith, S. O. (2002) Comparison of helix interaction in membrane and soluble α-bundle proteins. *Biophysical J.* **82,** 2720–2736.
7. Adamian, L., and Liang, J. (2001) Helix-helix packing and interfacial pairwise interactions of residues in membrane proteins. *J. Mol. Biol.* **311,** 891–907.
8. MacKenzie, K. R., Prestegard, J. H., and Engelman, D. M. (1997) A transmembrane helix dimer: structure and implications. *Science* **276,** 131–133.
9. Choma, C., Gratkowski, H., Lear, J. D., and DeGrado, W. F. (2000) Asparagine-mediated self-association of a model transmembrane helix. *Nat. Structural Biol.* **7,** 161–166.
10. Zhou, F. X., Cocco, M. J., Russ, W. P., Brunger, A. T., and Engelman, D. M. (2000) Interhelical hydrogen bonding drives strong interactions in membrane proteins. *Nat. Structural Biol.* **7,** 154–160.
11. Adamian, L., and Liang, J. (2002) Interhelical hydrogen bonds and spatial motifs in membrane proteins: polar clamps and serine zippers. *Proteins* **47,** 209–218.
12. Liang, J. (2002) Experimental and computational studies of determinants of membrane protein folding. *Curr. Opin. Chem. Biol.* **6,** 878–884.
13. Adamian, L., Jackups, R., Binkowski, T. A., and Liang, J. (2003) Higher order interhelical spatial interactions in membrane proteins. *J. Mol. Biol.* in press.
14. McDonald, I. K., and Thornton, J. M. (1994) Satisfying hydrogen bonding potential in proteins. *J. Mol. Biol.* **238,** 777–793.
15. Edelsbrunner, H., and Mucke, E. P. (1994) 3-dimensional alpha-shapes. *ACM Transactions Graphics* **13,** 43–72.
16. Edelsbrunner, H., and Shah, N. R. (1996) Incremental topological flipping works for regular triangulations. *Algorithmica* **15,** 223–241.
17. Facello, M. A. (1995) Implementation of a randomized algorithm for Delaunay and regular triangulation in 3 dimensions. *Comput. Aided Geom. D.* **12,** 349–370.
18. Tsai, J., Taylor, R., Chothia, C., and Gerstein, M. (1999) The packing density in proteins: standard radii and volumes. *J. Mol. Biol.* **290,** 253–266.
19. Berezovsky, I. N., Esipova, N., Tumanyan, V. G., and Namiot, V. A. (2000) A new approach for the calculation of the energy of van der Waals interaction in macromolecules of globular proteins. *J. Biomolecular Structure Dynamics* **17,** 799–809.
20. Langosch, D., and Heringa, J. (1998) Interaction of transmembrane helices by a knobs-into-holes packing characteristic of soluble coiled coils. *Proteins* **31,** 150–159.
21. Berezovsky, I. N., and Trifonov, E. N. (2001) Van der Waals locks: loop-n-lock structure of globular proteins. *J. Mol. Biol.* **307,** 1419–1426.
22. Liang, J., Edelsbrunner, H., Fu, P., Sudhakar, P. V., and Subramaniam, S. (1998) Analytical shape computation of macromolecules: I. Molecular area and volume through alpha shape. *Proteins* **33,** 1–17.
23. Liang, J., Edelsbrunner, H., Fu, P., Sudhakar, P. V., and Subramaniam, S. (1998) Analytical shape computation of macromolecules: II. Inaccessible cavities in proteins. *Proteins* **33,** 18–29.
24. Singh, J., and Thornton, J. M. (1992) *Atlas of Protein Side-Chain Interactions.* IRL Press, Oxford.
25. Rice, W. J., and MacLennan, D. H. (1996) Scanning mutagenesis reveals a similar pattern of mutation sensitivity in transmembrane sequences M4, M5, and M6, but not in M8, of the Ca^{2+}-ATPase of sarcoplasmic reticulum (SERCA1a). *J. Biol. Chem.* **271,** 31412–31419.
26. Clarke, D. M., Loo, T. W., and MacLennan, D. H. (1990) Functional consequences of alteration to polar amino acids located in the transmembrane domain of the Ca^{2+} ATPase of sarcoplasmic reticulum. *J. Biol. Chem.* **265,** 6262–6267.
27. Chen, L., Sumbilla, C., Lewis, D., et al. (1996) Short and long range functions of amino acids in

the transmembrane region of the sarcoplasmic reticulum ATPase. *J. Biol. Chem.* **271,** 10745–10752.
28. Adams, P., East, J. M., Lee, A. G., and O'Connor, C. D. (1998) Mutational analysis of transmembrane helices M3, M4, M5 and M7 of the fast-twitch Ca^{2+}-ATPase. *Biochem. J.* **335,** 131–138.
29. Engelman, D. M., and Steitz, T. A. In: *The Protein Folding Problem* (Wetlaufer, D. B., ed.). westview, Boulder, Colorado, 1984, pp. 87–113.
30. Lemmon, M. A., Flanagan, J. M., Treutlein, H. R., Zhang, J., and Engelman, D. M. (1992) Sequence specificity in the dimerization of transmembrane α-helices. *Biochemistry* **31,** 12719–12725.
31. Lemmon, M. A., Treutlein, H. R., Adams, P. D., Brunger, A. T., and Engelman, D. M. (1994) A dimerization motif for transmembrane α-helices. *Nature Structural Biol.* **1,** 157–163.
32. Fleming, K. G., and Engelman, D. M. (2001) Specificity in transmembrane helix-helix interactions can define a hierarchy of stability for sequence variants. *Proc. Natl. Acad. Sci. USA.* **98,** 14340–14344.
33. Gratkowski, J., Lear, J. D., and DeGrado, W. F. (2001) Polar side chains drive the association of model transmembrane peptides. *Proc. Natl. Acad. Sci. USA.* **98,** 880–885.
34. Dawson, J. P., Weinger, J. S., and Engelman, D. M. (2001) Motifs of serine and threonine can drive association of transmembrane helices. *J. Mol. Biol.* **316,** 799–805.