Second transmembrane domain of human uncoupling protein 2 is essential for its anion channel formation

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Abstract **Uncoupling proteins (UCP) are known to transport anions, such as Cl^-, in addition to H^+ transport. Although H^+ transport by UCP is clearly involved in thermogenesis, the mechanism of its anion transport is not clearly understood. In this study, we examined the anion channel characteristics of the six individual helical transmembrane (TM) domains of the human UCP2. The second TM domain peptide (TM2) forms multi-state channels by assemblies of conductive oligomers. Furthermore, the TM2 exhibited voltage-dependent anion channels with properties comparable to those of UCP1 chloride channel. However, the other five TM peptides did not form UCP1-like channels. Moreover, an analog of TM2 in which two Arg residues were substituted by Ala residues did not form stable channels, implying the significance of Arg residues for anion transport. These results suggest that the anion channel structure of UCP2 protein is oligomeric and the second TM domain is essential for the voltage-dependence of this anion channel.**

Keywords: Uncoupling protein; Ion channel; Transmembrane domain; Pipette-dipping patch-clamp method; Synthetic peptide

1. Introduction

Uncoupling protein (UCP) family from brown adipose tissue is a membrane protein and a member of mitochondrial anion carrier family. UCP1 is an isoform of UCP family and plays an important role in thermogenesis by allowing dissipation of energy from the proton electrochemical gradient across the inner mitochondrial membrane generated by respiratory activity [1–4]. UCP1 is activated by a free fatty acid (FA). In addition to H^+ transport activity, it has been also known that UCP1 can transport anions [2–6], in particular Cl^-, in a FA-independent manner [3,5,6]. Although H^+ transport by UCP1 is clearly involved in thermogenesis, detailed mechanism of anion transport is not well understood in this protein.

On the basis of its primary structure, UCP1 has six putative helical transmembrane (TM) domains [3]. This structure is supported by recent X-ray structure of the ADP/ATP carrier, also a member of the mitochondrial anion carrier family [7]. Site-directed mutagenesis studies have shown that Asp27 in the first TM domain of hamster UCP1 affect H^+ transport, but not Cl^- transport [8], while Arg83 and Arg91 in the second TM domain are important for Cl^- transport, but not H^+ transport [6]. Moreover, UCP1 reconstituted into giant liposomes formed voltage-dependent anion channels in a FA-independent manner [5]. These studies indicate that the mechanisms between H^+ transport and Cl^- transport are different. It is known that in many ion channel proteins, TM domains participate in the pore structure through TM–TM interaction. The overall pore structure is considered to be a fundamental determinant for characteristics of ion channels, such as ion selectivity [9]. Although the roles of Asp27, Arg83 and Arg91 of UCP1 in the formation and function of the pore structure are not clearly known, the pore structure of UCP1 may be lined, at least in part, by residues from TM domains.

The synthetic peptide segments of TM domains have been used to investigate the structure-function relationships of several integral membrane proteins. Studies of the TM peptides of bacteriorhodopsin [10] and glycophorin A [11], membrane proteins of known three-dimensional structure, indicate that the folding of these TM peptides is largely identical with the overall conformation of proteins in lipid membranes. Moreover, the peptides representing the TM domains of ion channel proteins, e.g., nicotinic acetylcholine receptor [12] or CFTR [13], have shown the ion channel properties comparable to the native proteins. These studies have demonstrated that the TM peptides can assume membrane-integrated conformations and form native-like TM–TM interactions leading to ion channel formation [14].

New UCPs have been discovered recently. Interestingly, TM domains are highly conserved among mammalian UCPs. UCP2, 59% identical with UCP1, is more widespread than UCP1, and maps to regions of human chromosome 11 that have been linked to hyperinsulinemia, obesity [15] and diabetes [16], suggesting that UCP2 influences the body-mass index [17]. A recent study has shown that UCP2 regulates cellular oncosis, a form of cell death induced by energy depletion and initially characterized by cell swelling [18]. There is a possibility that UCP2 can function as a Cl^- channel in this swelling

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Abbreviations: CD, circular dichroism; DPhPC, diphytanoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; DPPG, dipalmitoylphosphatidylglycerol; FA, fatty acid; SDS, sodium dodecyl sulfate; SUVs, small unilamellar vesicles; TFE, trifluoroethanol; TM, transmembrane; UCP, uncoupling protein

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phenomenon. In this study, to elucidate the anion channel properties and conformation of individual TM domains of human UCP2 (hUCP2), six peptides corresponding to each TM domain were synthesized. Consequently, it is found that all six TM peptides form helical conformations in lipid model membranes, but only the second TM peptide (TM2) exhibits stable voltage-dependent anion channel activities.

2. Materials and methods

2.1. Materials

Peptides were synthesized by solid-phase peptide synthesis using 9-fluorenylmethoxycarbonyl chemistry and purified by reversed-phase high-performance liquid chromatography. Peptides were characterized by mass spectrometry and amino acid analysis. Diphytanoylphosphatidylcholine (DPhPC) was obtained from Avanti Polar Lipids (Alabaster, AL). Dipalmitoylphosphatidylcholine (DPPC) and dipalmitoylphosphatidylglycerol (DPPG) were from Sigma (St. Louis, MO). All other chemicals from Wako Pure Chemicals (Osaka, Japan) were of special grade and used without further purification.

2.2. Sample preparations

Peptide stock solutions were prepared by dissolving the peptides in 30% (v/v) trifluoroethanol (TFE)/water solution. Sodium dodecyl sulfate (SDS) micellar peptide solutions were prepared by dissolving the appropriate amount of peptides and SDS in 5 mM Tris–HCl (pH 7.4). Small unilamellar vesicles (SUVs) were prepared by probe sonication of DPPC/DPPG (3:1 molar ratio) dispersions in 5 mM Tris/Mes 100 mM KCl (pH 7.4). The final concentrations of TFE in all aqueous peptide solutions used in circular dichroism (CD) and single-channel measurements were less than 1.5% and 0.15%, respectively. TFE at these concentrations did not affect the secondary structures and channel formation abilities of peptides.

2.3. Circular dichroism spectra and single-channel measurements

Circular dichroism spectra were measured on a Jasco J-720 spectropolarimeter (Tokyo, Japan), as described previously [19–21]. The helicity of peptides was calculated using the SELCON program [22]. Patch-clamp experiments were performed using a pipette-dipping technique, as described previously [19–21]. DPhPC zwitterionic lipids were used in all patch-clamp experiments. The electrolyte solutions for patch-clamp experiments were comprised of 100 mM KCl solutions buffered with 5 mM Tris/Mes (pH 7.4). Blank solutions with TFE concentrations comparable to those of peptide solutions were used as controls. Formation of ion channel like conductance patterns was not observed in these controls. The reversal potential was measured in the presence of asymmetric KCl concentrations across the bilayer (100 mM KCl in the *cis*-side and 20 mM KCl in *trans*-side).

3. Results

3.1. TM peptide design

TM peptides of integral membrane proteins have very low solubility in aqueous solvents due to their hydrophobic nature and, therefore, it is difficult to apply conventional characterization and purification procedures to them. Many of the reported TM peptides have charged and/or polar residues at both N- and C-terminals [10,23]. Synthetic TM peptides of hUCP2 of this study include all the intramembrane residues plus a few residues (some charged or polar) of the flanking sequences at both terminals (Table 1). To avoid the problem of disulfide bond formation between Cys residues, these residues in TM1, TM4 and TM5 were replaced with Ser residues. It has been reported that the activity of UCP1 is not affected by Cys residues [24].

Table 1
Amino acid sequences of TM peptides of hUCP2

Peptide	Residue	Sequence[a]
TM1	5–38	KATDVPPTATVKFLGAGTAASIADLITFPLD-TAK
TM2	72–106	TEGPRSLYNGLVAGLQRQMSFASVRIGLYDS-VKQF
TM3	115–141	SIGSRLLAGSTTGALAVAVAQPTDWK
TM4	173–204	RGLWKGTSPNVARNAIVNSAELVTYDLIKDAL
TM5	211–239	TDDLPSHFTSAFGAGFSTTVIASPVDWK
TM6	265–295	GPRAFYKGFMPSFLRLGSWNWMFVTYEQLK

[a] Underlined sequence indicate the putative TM domains from the SWISS-PROT database (accession number: P55851). Cys25 in TMl, Cys191 in TM4 and Cys216 and Cys227 in TM5 were replaced with Ser residues [24].

3.2. Conformations of TM peptides

Fig. 1 shows CD spectra of six TM peptides in buffer, the inner mitochondrial model membranes [1 mM DPPC/DPPG (3/1) SUVs] and membrane-mimetic micelles (20 mM SDS). Although all peptides are largely unstructured in buffer, they adopt helical conformations with red shifts and increased negative ellipticities at 220 nm in both model membrane environments. The CD spectrum of TM5 in DPPC/DPPG is quite similar to that of the peptide in buffer, suggesting weak peptide-lipid affinity. This weak affinity can be caused by unfavorable interactions between the overall negative charge on TM5 and negatively charged DPPC/DPPG vesicles. Interestingly, TM2, TM4 and TM6 conformations in both lipid model membranes showed higher helicities compared to those of TM1, TM3 and TM5. The increase in helicity does not seem to correlate with the chain-length, overall charge and hydrophobicity of TM peptides.

3.3. Ion channel formation of TM peptides

To investigate the ion channel formation of individual TM domains, single-channel current recordings were carried out by the pipette-dipping patch-clamp method. The electrolyte solution is composed of symmetrical 100 mM KCl in 5 mM Tris/Mes (pH 7.4) and does not contain FAs. This buffer solution is similar to the solution previously used to study the anion channel properties of hamster UCP1 protein [5]. Fig. 2 shows conductance patterns of all six TM peptides at +100 mV membrane potential. It seems that TM1, TM3, TM4 and TM6 partition into the lipid bilayers, however, only erratic and fluctuating drifts in membrane currents, and not channel like conductance, were observed with these peptides. These currents did not show discrete events ($n \geqslant 10$) and formation of channel like conductance patterns was not observed in these peptides even at membrane potentials as high as ± 200 mV. In contrast, TM2 exhibited conductance patterns with clear transition between the open and closed states. The relative frequency of channel formation (number of discrete ion channels/number of experiments) was 0.36 ($n = 11$). The main and most frequent conducting state of TM2 (283 ± 6 pS) was larger than the value reported for the chloride channel in UCP1 (75 pS). TM5 also formed ion channels, however, the conductance (47 pS) and the relative frequency for detecting the conducting states were lower compared to those of TM2 ($\leqslant 0.09$, $n = 11$). These results indicate that of all TM peptides of hUCP2, TM2 forms stable ion channels.

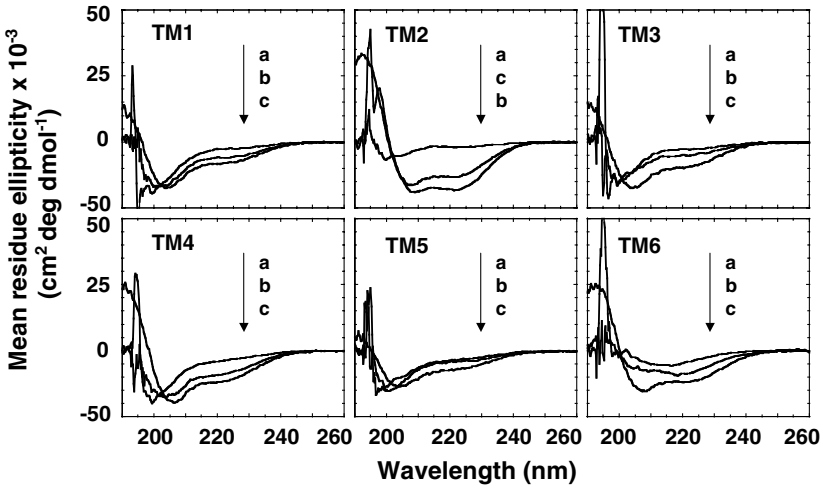

Fig. 1. CD spectra of TM peptides (10 μM) in (a) 5 mM Tris/Mes 100 mM KCl (pH 7.4), (b) 1 mM DPPC/DPPG (3/1) in 5 mM Tris/Mes 100 mM KCl (pH 7.4) and (c) 20 mM SDS in 5 mM Tris–HCl (pH 7.4) at 25 ± 1 °C. The spectra are the average for two independent preparations, and errors were within 500 cm^2 deg $dmol^{-1}$ at 220 nm.

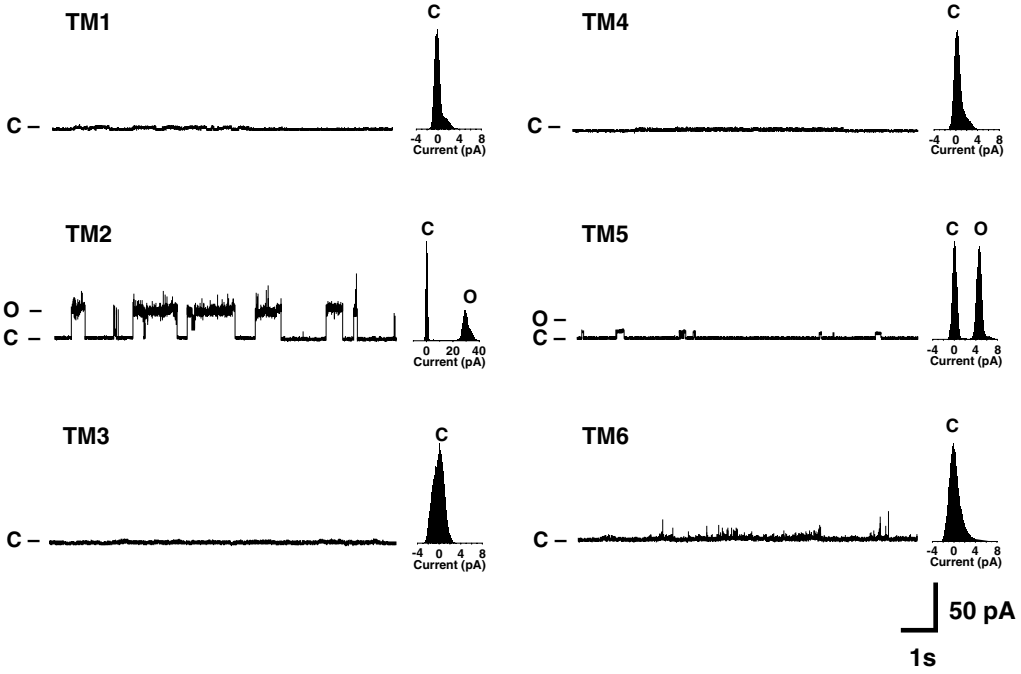

Fig. 2. Conductance patterns of TM peptides. Peptides (1 μM) were added to the *cis*-side of the DPhPC bilayer, separating symmetrical 100 mM KCl in 5 mM Tris/Mes at pH 7.4 on both sides. The membrane potential was +100 mV. Measurements were carried out at 25 ± 2 °C. The states of open and closed, determined from the current amplitude histogram, are represented as O and C, respectively.

3.4. Properties of the TM2 ion channel

Fig. 3A shows conductance patterns of TM2 at various membrane potentials. Multi-state channels were observed at different membrane potentials. The current amplitude histograms at different membrane potentials showed the occurrence of two discrete current states (O_1 and O_2, in Fig. 3) with the lower current state (O_1) occurring more frequently (Fig. 3A). As shown in Fig. 3B, the two discrete current states were non-linearly increased with the increase in membrane potential, indicating that TM2 forms voltage-dependent ion channels. Moreover, Fig. 3B shows that the higher current states (O_2) are not merely the sum of two simultaneously opened lower current states, (O_1). Different current levels of TM2 channels imply the existence of non-covalent conductive oligomeric units (composed of TM2 monomers) with different pore sizes [12,13,19].

Anion selectivity of TM2 pore structure was measured by using NaCl and K^+Asp^- as electrolytes at −50 mV in symmetric distribution on both sides of the lipid membrane (Fig. 4). In NaCl solution, TM2 showed a comparable conductance pattern with that in KCl solution (Fig. 4A and B). Similarity of conductance in NaCl and KCl suggests no

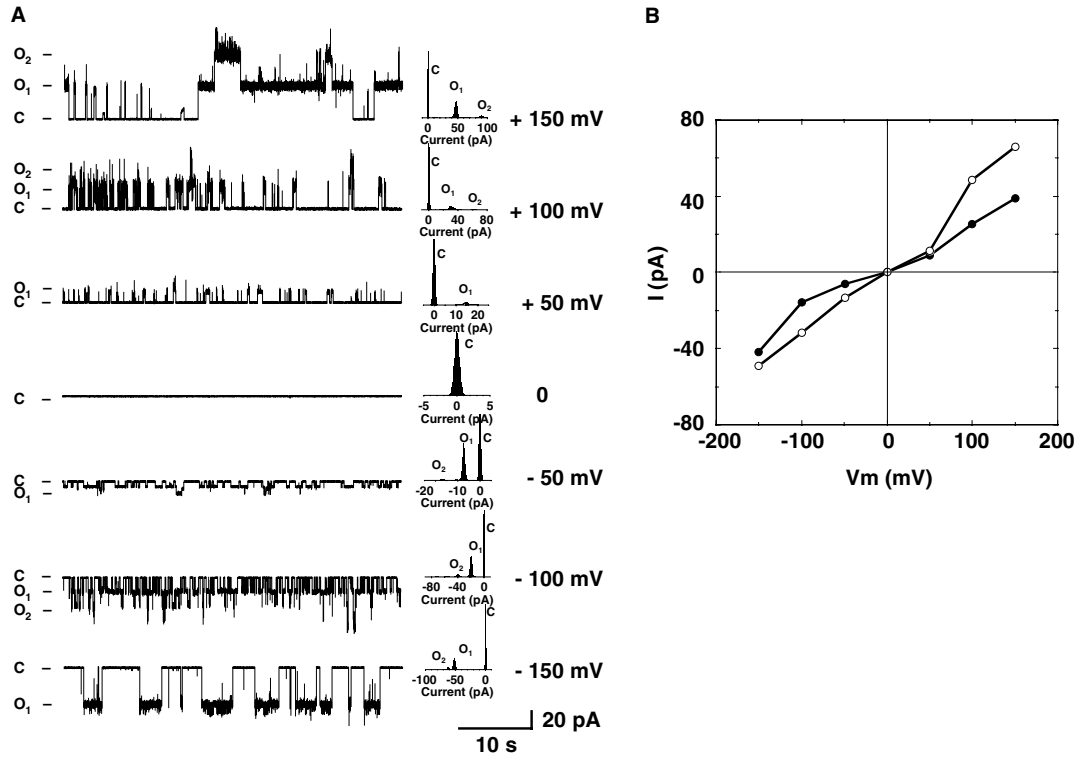

Fig. 3. Current–voltage relationships of channels formed by the TM2 peptide. The holding membrane potential was changed from −150 to +150 mV. Experimental conditions were the same as those of Fig. 2. (A) Conductance patterns at various holding membrane potentials. Current histograms generated from continuous records lasting 60 s are shown to the right of the respective patterns. (B) Current–voltage (I–V) relationship. The two current states O_1 (closed circle) and O_2 (open circle) were plotted against the membrane potential. The graph shows the mean values of at least four experiments.

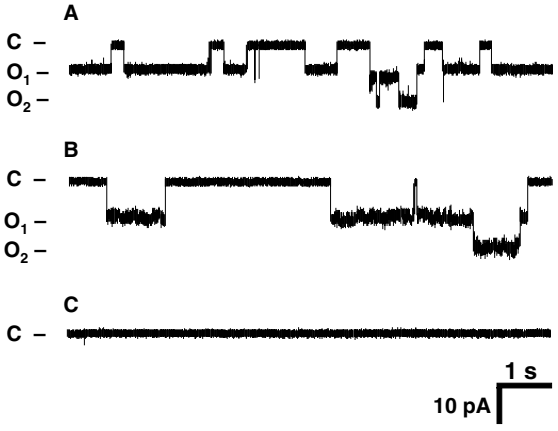

Fig. 4. Conductance patterns of the TM2 peptide in 100 mM (A) KCI, (B) NaCl and (C) L-aspartic acid potassium salt buffered with 5 mM Tris/Mes (pH 7.4). The membrane potential was at −50 mV. Experimental conditions were the same as those of Fig. 2.

intra-cation selectivity. However, in K^+Asp^- solution (Fig. 4C), TM2 did not exhibit channel-like conductance patterns ($n = 3$). This can imply that TM2 pores are anion-selective with a limited size that are permeable to the smaller Cl^-, but not to the larger Asp^-. The anion selectivity was also confirmed by measuring the reversal potential in the presence of asymmetric KCI concentrations across the bilayer. The Goldman–Hodgkin–Katz equation [25] leads to the permeability ratio: $P_{Cl}/P_K = 3.6$. These results suggest that TM2 forms voltage-dependent anion channels with properties comparable to those of UCP1 chloride channels.

3.5. Role of intrahelical Arg residues in TM2

Intrahelical Arg residues (Arg83 and Arg91) in TM2 domain of hamster UCP1 are important for Cl^- transport [6]. To assess the effect of Arg residues of TM2 peptide on the channel formation, TM2 analog with substituted Ala for two Arg residues (R88A/R96A TM2) was synthesized. CD spectra in SDS micelles revealed that TM2 and R88A/R96A TM2 have comparable helical conformations (spectrum not shown). R88A/R96A TM2 formed channels with similar conductance (154 pS) to TM2 (151 pS) at −50 mV in 100 mM KCI (conductance pattern not shown). The relative probability for detecting the conducting states was also comparable with that of TM2 (0.38, $n = 13$). However, almost all conductance patterns were irregular fluctuations with erratic patterns that did not depend on membrane potentials.

4. Discussion

Recently, X-ray structure of ADP/ATP carrier, one of the most studied and characterized members of the mitochondrial anion carriers, has been determined in its carboxyatractyoside-inhibited form [7]. Although the structure explains the inhibitor-binding site and typical threefold sequence repeats, including TM domains, the molecular complex was initially crystallized as a monomer in detergent. It is well known that

the functional carrier is a homodimer or a coupled dimer (tetramer) [26]. Consequently, the complete structure and detailed mechanism of ion transport in the mitochondrial carrier family, including UCPs, remain unclear. Moreover, the mitochondrial anion carriers are known to switch from specific carriers to channels/pores [27]. However, this alterable function and mechanism are not also understood. As a step towards studying the structure-function relationships in UCPs, we have synthesized the TM domains of hUCP2, and examined the conformation and channel formation of these domains in lipid membranes.

On the basis of CD results in lipid membranes, all six TM peptides formed helical conformations to different degrees. Among the TM peptides, TM2, TM4 and TM6 exhibited distinct highly helical conformations (Fig. 1). TM2 formed stable channels with clear transition between the open and closed states (Fig. 2). In contrast, the peptides with sequences of TM1, TM3, TM4 and TM6 did not form channels under our experimental conditions. These results suggest that the abilities of ion channel formation cannot be directly correlated with the helical conformation of the peptides or their affinity for lipid membranes. In addition to TM2, TM5 also showed channel-like conductance patterns in zwitterionic DPhPC lipid bilayers (Fig. 2). However, compared to TM2, TM5 is less helical in lipid membranes (Fig. 1), which implies lower affinity for membrane insertion. Consequently, TM5 peptides seem to be located on the membrane interface and their formation of ion channels is, therefore, less spontaneous and could be induced by other factors such as differences in TM potential. It has been reported that the Asp209 and Asp210 pair, near the fifth TM domain in the hamster UCP1, affects H^+ transport [8]. Both Asp residues are conserved in hUCP2 and are present in the synthetic TM5 peptide (Table 1). It is therefore, possible that the negatively charged Asp residues in TM5 participate in the formation of cation channel pore structures. Due to low stability of TM5 channels, we could not characterize their ion channel properties in detail at this stage.

The self-association of TM helices is a key step for their channel formation in lipid membranes. The amphipathic nature of the helix is one of the important factors for self-association of TM helices in the channel proteins or channel forming peptides [9,12,14,19]. Of all transmembane helices, TM2 helix has a more pronounced amphipathic nature with Arg side chains aligned along one side and hydrophobic residues along the opposite side. High amphipathicity of TM2 helices influences the intramembrane formation of the ion conducting aggregates and could account for their higher stability. This assumption is further supported by the ion conductance measurements of the mutant R88A/R96A TM2 peptide. Replacement of the two Arg residues with Ala residues in TM2 decreases the stability of the ion channels. The amphipathic structure of TM2 suggests that the Arg residues contribute, at least in part, to the formation, stable conductance and selectivity of the pore structure.

Hamster UCP1 exhibited the voltage-dependent anion channels [5]. The side chains of the positively charged amino acid residues, such as Lys and Arg, in the TM domains of ion channel proteins can function as anion-selective regions. In ClC chloride channel family, a highly conserved sequence, G(K/R)EGP, appears to line the pore [28]. Recently, X-ray structures of two prokaryotic ClC chloride channels were determined [29]. Arg residues in the conserved sequence of these proteins are located on the entire channel surface. It is indicated that this distribution of positive charge creates an electrostatic potential that probably funnels chloride ions into the pore entry ways [29]. In CFTR chloride channel, the second and sixth TM peptides form the anion-selective channels after being reconstituted in lipid membranes [13]. Arg residues in these domains are apparently important for the anion-selectivity process [13]. UCPs have four Arg residues in their TM domains (two Arg for TM2, one Arg in each of TM4 and TM6 domains). Site-directed mutagenesis studies suggested the contribution of only two Arg residues (Arg83 and Arg91) in TM2 domain of UCP1 for Cl^- transport activity of the protein [6]. In contrast, three Arg residues, Arg182 (TM4), Arg276 (TM6) and also Arg83 (TM2), are essential for the nucleotide binding, which inhibits its ion transport activity [6,30]. These results are in agreement with our study of TM peptides of hUCP2. In addition, further observation proved that TM2 peptide could be the anion-selective pore structure. The observed voltage-dependence behavior of TM2 peptide channel (Fig. 3) is also comparable to that of the UCP1 protein. It is, therefore, plausible that TM2 in UCPs are critical for the pore formation of the proteins and can transport anions.

Single-channel measurements showed that TM2 has a conductance of 283 pS at +100 mV, which is larger than the value for the UCP1 protein (75 pS). As described above, the functional mitochondria anion carriers are homodimers or coupled dimers. Cross-linking experiments suggested that UCPs form a homodimer structure [31]. If UCPs generally form homodimers to form anion channels, then the TM2 domain has to form heterooligomers with other TM domain(s). This suggestion is supported by the CD data. The helicity of TM2 in lipid model membranes (~50%) is smaller than the percentage of helicity necessary to span the membranes, i.e., 66% (23 of 32). Interaction of TM2 with other TM domain(s) or other region(s) of the UCP molecule can increase its helicity and somehow stabilize the channel structure. In the case of the isolated synthetic TM2 peptide, the same stabilizing role is played by mutual interaction of the peptide monomers, which also leads to increase in the TM2 peptide helicity. Mutation of Glu167 near the fourth TM domain in hamster UCP1 also inhibited Cl^- transport [8], suggesting that this region may be a component of the pore structure. These structural features suggest that pore structure of UCPs can be an oligomer of TM domains, including TM2 domain. Other TM domains stabilize TM2 helix, which is necessary for the formation of the ion-conducting units.

In conclusion, our results indicate that TM2 domain of hUCP2, highly conserved with other UCPs, is an essential TM domain involved in the formation of voltage-dependent anion channels in lipid membranes. TM2 domain is a plausible candidate to line the ion-conducting path of the UCPs channel. Further investigations into the relationships of TM–TM interactions and transport functions will certainly contribute to the understanding of structure and transport mechanism of the UCP family.

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