Molecular mechanism of anion permeation through CFTR channel pore

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TECHNOLOGICAL
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**Molecular Mechanism of Anion Permeation
through CFTR Channel Pore**

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SCHOOL OF BIOLOGICAL SCIENCES

2009

Molecular Mechanism of Anion Permeation through CFTR Channel Pore

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School of Biological Sciences

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Abbreviations

ABC	ATP-binding cassette
AMFE	anomalous mole fraction effect
AMP-PNP	adenylyl imidodiphosphate
ASF	airway surface fluid
BHK	baby hamster kidney
CAL	CFTR-associated ligand
CF	cystic fibrosis
CFTR	cystic fibrosis transmembrane conductance regulator
CHO	chinese hamster ovary
DMEM	Dulbecco's modified Eagle's medium
DPC	diphenylamine-2-carboxylic acid
E_{Rev}	reversal potential
<i>E. coli</i>	Escherichia coli
ECL	extracellular loop
ENaC	epithelial sodium channel
FRAP	fluorescent recovery after photobleaching
GlyH	glycine hydrazide
GSH	γ -glutamyl-cysteinyl-glycine
ICL	intracellular loop
I/I_0	control current remains
IRK	inwardly rectifying potassium channel
K_d	dissociation constant

LB	Luria-Bertani
MEM	Minimum Essential Medium
MRP	multidrug resistance protein
MSD	membrane-spanning domain
MTS	methane thiosulfonate
MTSEA ⁻	methane thiosulfonate ethylammonium
MTSES ⁻	methane thiosulfonate ethylsulfonate
MTSET ⁻	methane thiosulfonate thyltrimethylammonium
NBD	nucleotide binding domain
ORCC	outwardly rectifying chloride channel
P _o	open probability
PKA	protein kinase A
PKC	protein kinase C
PPi	pyrophosphate
RD	regulatory domain
ROMK	renal outer medullary potassium channel
TES	N-tris[hydroxymethyl] methyl-2-aminoethanesulfonate
TM	transmembrane segment
t-SNARE	target soluble N-ethylmaleimide-sensitive factor attachment protein receptor
WT	wild type

Abstract

Cystic fibrosis is an inherited disease caused by mutations in a single gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR), a member of ATP-binding cassette (ABC) proteins superfamily. Several approaches have been applied to determine the pore-lining domains or residues including mutagenesis study of residues in transmembrane (TM) segments by identifying their contribution to CFTR channel functional features. However, we do not know which and how many TMs contribute to the channel pore of CFTR. For the purpose of revealing the architecture of CFTR channel pore, we used site-directed mutagenesis to construct single and double mutated CFTR channel and then investigated their functional variation by using patch clamp recording. Our results suggested that TM1 and TM6 played very important roles in CFTR channel pore forming and the amino acid residue T338 in TM6 could interact with the residues K95 and Q98 in TM1. Therefore, we proposed that these residues form a selectivity filter of the CFTR channels. Subsequently, we found that the residue T338 in TM6 could also interact with the residues T1142A and W1145A in TM12. Since the mutations of T1142A and W1145A did not impact the channel current of CFTR, we suggested that the residues T1142 and W1145 in TM12 might support the wall structure of CFTR channel pore and affect the channel function indirectly by interacting with T338 in TM6.

Besides the TMs, we questioned if the glycosylation on extracellular loop 4 of CFTR may also contribute to the functional property of the channel because of its pivotal roles in protein trafficking, localization and protein-protein interaction. To provide further insight into the effects of glycosylation on the functions of CFTR channel pore, we

mutated either one or both extracellular glycosylation sites and then analyzed the functional properties of the mutated CFTR chloride channel. Single channel analysis revealed that eliminating one of the glycosylation sites (N894D and N900D) could induce a subconductance level of CFTR channel open state. Abolishment of both the glycosylation sites (N894D/N900D) increased the occurrence of the subconductance open. Macroscopic current analysis showed that both partially glycosylated and fully nonglycosylated CFTR displayed functional differences compared with the wild type. We concluded that glycosylation affected CFTR gating properties and CFTR conformation transition of channel pore.

Finally, in the sidedness study of CFTR channel pore, we found that the large anions can pass through the pore easier from cytoplasmic side to external side than from the opposite direction. To explain the asymmetric large anion permeation through CFTR channel pore, we proposed a novel “fishing basket” model. This model was based on the hypothesis that the pore is flexible enough to perform a conformational change under appropriate pressure after the channel is open. To test it, we used the extreme negative membrane potential to analyze the voltage dependence of blockade by the intracellular open CFTR channel blocker $\text{Au}(\text{CN})_2^-$. The data demonstrated that a proportion of $\text{Au}(\text{CN})_2^-$ ions were pushed out from the cytoplasmic side of the pore to the external side under the extreme negative membrane potential. Therefore, we concluded that the CFTR channel pore is really flexible and the fishing basket model is suitable to explain the mechanism for asymmetric large anion permeation in CFTR pore.

1. Literature Review

1.1 The story of ion channels

The history of the ion channel should be traced back to 1791 when Galvani observed that electricity could induce a biological response in a dead frog's leg [1]. In Galvani's time, the existence of "ions" was still largely in doubt, as was the concept of cell membrane. However, albeit indirectly, it could be said that he was the first one in studying ion channels. His work encouraged many to devote their whole life to exploring the phenomenon underlying nerve and muscle cell messaging.

Technological improvements in instrumentation made sensitive measurement possible, and helped those investigators to unveil the chemical mechanism in nature underlying nerve and muscle cell excitation. Using a modified galvanometer, Helmholtz determined the velocity of the electric signal on a nerve cell in 1850 [1]. He found that the electrical current conducted slower on nerves than on metal wires which suggested that the nerves did not conduct electrical signal in the same way as a metal wire did. His findings made a real contribution to the emerging of the ionic theory and the concept of cell membrane. The next improvement in instrumentation took place in the late 1940s. The glass microelectrode was invented to make an aperture in the cell membrane and then detect variation of ionic composition between the intracellular and extracellular solutions [2]. Not long after this, Cole placed a second glass electrode inside the cell to "voltage clamp" the interior of the cell [3]. Their efforts brought up the explanation by Hodgkin, Huxley and Katz of resting potential and action potential in terms of movement of specific ions

(Na⁺, K⁺, Cl⁻) through ion channels in neuron cell membranes [4]. In 1976, Neher and Sakmann invented the “patch clamp” method based on the improvement in amplifiers and cell culturing techniques [5, 6]. Having the patch clamp, the investigators were able to make direct observations about ion channels individually and then distinguish different types of ion channels from one another. After the crystal structure of the potassium channel KscA was published by Doyle [7], we got the chance to take a rough “look” at the ion channel. Since then, there have been several other ion channel structures solved in atomic resolution in parts, such as the mechanosensitive channel of small conductance (MscS) [8], several members of the chloride channel (CLC) family [9, 10], the cystic fibrosis transmembrane conductance regulator (CFTR) [11], the voltage-dependent K⁺ channel (KvAP) [12]. However, there are still many difficulties and pitfalls in resolving the crystal structure of large membrane proteins [13]. The next step, we should establish a protocol to produce a series of crystal structures of an ion channel and compose a “video” of a channel in action. Before that, patch clamp is still the main method in studying the functional and structural properties of ion channels.

1.2. Nomenclature of ion channels

Based on how they are regulated, ion channels have been classified into three main categories: voltage-gated channels, extracellular ligand-gated channels and intracellular ligand-gated channels. The mechanosensitive and cell volume-regulated ion channels have their own group, which we also called “miscellaneous 1”, since they are still in the process of being classified. A fifth group, miscellaneous 2, has been made to include all ion channels that are not included in the above groups [14].

Voltage-gated ion channels are mostly distributed in neuron and muscle cells, such as the sodium and potassium channels in the nerve axons and calcium channels in the muscle cells. They are diverse in the gating kinetics. Potassium ion channels are the most diverse of all ion channels, even more so than chloride and calcium channels. Extracellular ligand-gated ion channels are often named after the ligand they bind to, such as nicotinic acetylcholine receptors (nAChRs), glutamate receptors, and gamma-aminobutyric acid (GABA) and glycine receptors. nAChR was the first sequenced ion channel isolated from the electric organ of fish *Torpedo* [15]. The nAChRs together with the GABA and glycine chloride channels form a superfamily of neurotransmitter ligand-gated ion channels based on strong sequence similarity. And they all have 4 distinct transmembrane segments [4]. Intracellular ligand-gated ion channels are regulated by changing the intracellular concentrations of various second messengers as well as alteration of various coupling proteins and cofactors. Cells use them as a strategy to modulate the incoming signals individually. The common intracellular ligands which activate these kinds of channels include calcium, ATP, cAMP, GMP and phosphatidylinositol (PI). This group includes ATP-sensitive potassium channels, CFTR, epithelial sodium channel (ENaC), transient receptor potential (TRP) family members [16], and aquaporin channels [17].

1.3. Anion channels

Quite different from highly specialized cation channels, anion channels are relatively nonselective and allow the passive diffusion of various negatively charged ions along their electrochemical gradient [18-20]. Since chloride is the most abundant anion in cells

and hence is the predominant permeating anion under most circumstance, anion channels are often called chloride channels, even though they may conduct other anions better than chloride. So far, the most popular nomenclature of chloride channels is based on their molecular structures. However, the genes of many biophysically identified chloride channels have not yet been identified. There are three well-established gene families of chloride channels: the CLC chloride channel family which has at least nine members [21]; the CFTR which is the only member of the ATP-binding cassette (ABC) transporters superfamily that is known to function as an ion channel [22, 23]; and ligand-gated GABA- and glycine-receptor chloride channels which is the largest chloride channel family [24]. In addition, an intracellular chloride channel (CLIC) family has been identified [25-28]. Interestingly, the members in this family only have one transmembrane domain. Another gene family that encodes proteins with four or five transmembrane domains was suggested to encode Ca^{2+} activated Cl^- channels (CLCA) [29, 30].

1.4. CF and CFTR

CFTR was the first chloride channel that has been cloned [22]. It is located primarily in the apical membrane, where it provides a crucial pathway for the salt and water secretory response of many epithelial tissues [31-33]. Dysfunction of CFTR chloride channels in the monogenetic disease cystic fibrosis (CF) disrupts transepithelial anion secretion and subsequently leads to the wide-spread symptoms which include salty sweat, airway inflammation, meconium ileus, and pancreatic failure [34]. Clogging and infection of bronchial passages impede breathing [35]. The infections progressively destroy the lungs.

Plugging of small bile ducts impedes digestion and disrupts liver function in about 5% patients [36]. Occlusion of ducts prevents the pancreas from delivering critical digestive enzymes to the bowel in 65% of patients and diabetes can result as well [37, 38]. Obstruction of the gut by thick stool necessitates surgery in about 10% of newborn patients [38]. Absence of fine ducts in reproductive tract renders 95% male patients infertile [39]. Malfunctioning of sweat glands causes perspiration to contain excessive salt [40]. Measurement of chloride in sweat is a mainstay of diagnosis [40]. Among them, the airway inflammation is the dominant syndrome that can result in death. It appears that the CFTR related dysregulation in the inflammatory responses to pathogens and the coordinated cytokines network is more or less involved in CF [41-43]. Researchers observed decreased production of the cytokine IL-10 in CF [44]. Moreover, the CFTR channels are proposed to assist the transportation of glutathione [45, 46], which functions as an important antioxidant in the mucus [47-49]. In an absorptive model, the ionic strength of airway surface liquid is increased in CF patients due to the diminished salt absorption, which ultimately reduces the killing activity of antibacterial substances on the airway surface [50]. In another model, it has been proposed that pathology in the airway results from Na^+ and fluid hyperabsorption caused by dysregulated Na^+ absorption [51]. According to this, CF symptoms are caused not only by the dysfunction of CFTR protein but also by the consequent dysfunction of other related proteins, particularly the epithelial Na^+ channel (ENaC).

1.5. Domain structure of CFTR

The proposed domain structure of CFTR is summarized in Figure 1.1. As a member of the ATP-binding cassette (ABC) transporters super-family [23], CFTR contains two

membrane-spanning domains (MSDs), two nucleotide binding domains (NBDs), and a regulatory domain (R domain) [22]. Each MSD contains six transmembrane segments (TMs) that are predominantly α -helix, but their arrangement is still unknown. As a glycoprotein, CFTR contains two Asn-linked N-glycosylation sites at positions N894 and N900 in the fourth extracellular loop between TM 7 and TM 8. Knowledge of the contribution that these domains make to the overall function of CFTR has been summarized from the functional studies of wild type CFTR and site-directed CFTR mutations: the transmembrane segments contribute to the formation of the channel pore [52-55]; the NBDs bind ATP and hydrolyze ATP to regulate channel gating [56-60]; and the phosphorylation of R domain controls channel activity [61-64].

Figure 1.1. The proposed domain structure of CFTR. Channel gating is modulated by R domain phosphorylation and the ATP hydrolysis at NBDs. MSD, membrane-spanning domain; NBD, nucleotide binding domain; R domain, regulatory domain. This picture is modified from Sheppard and Welsh [34].

1.6. Structure of the CFTR channel pore: Methodology and achievements

Although a rough crystal structure of CFTR has been reported [11], the pore architecture of the channel is still unclear. As stated above, each of the two membrane-spanning domains has six transmembrane segments (TMs). All the TMs have the possibility to participate in the formation of channel pore. In addition, there are six extracellular loops (ECLs) which connect the TMs at the predicted external side [22] and may form the pore similar with the loops of potassium channel [7]. Since it is not easy to resolve the structure of CFTR at a high resolution, multiple functional assays comparing the electrophysiological properties of wild type and mutated CFTR channel has been used to determine the amino acid residues that are important for maintaining the functional properties of CFTR channel, and subsequently identify the pore-lining residues.

1.6.1. Site-directed mutagenesis

Site-directed mutagenesis has been widely employed as a means to gain functional and structural information of ion channels. Using this method, the amino acid sequence of protein can be mutated at the selected site. To obtain information about the functional significance of the mutated amino acid residues in a protein, mutants need to be characterized by functional assays by using patch clamp. This so-called “structure-function” strategy is based on the interesting fact that mutation of just one residue of the protein may have profound effects on the protein function. Summarized from previous studies, we know that mutations of amino acids that affect single channel conductance or the binding of blockers which bind to the open state of the channel are generally involved

in the channel pore forming [54, 65], while mutations that mainly affect channel gating are most likely to locate in regulatory regions [56, 57, 66]. Although this strategy has the potential to obtain penetrating insights into the inner workings of ion channels, a detailed interpretation of the functional consequences may require detailed information about the relation of mutated residue to nearby residues in the peptide backbone which can only come from a three-dimensional structure. However, it has been possible to make useful postulation about specific structural features of channel proteins using this approach in the absence of crystal structures. For example, MacKinnon hypothesized the location and functional significance of the reentrant loops for the pore properties of cation selective channels using the “structure-function” strategy before he confirmed this assumption by obtaining the three-dimensional structure of a potassium channel [7, 67, 68].

1.6.2. Pore blockers of CFTR channel: probes for pharmacologic studies

In recent years, to find out a new therapeutic strategy for cystic fibrosis, researchers have paid much attention on the pharmacology of the CFTR chloride channel. In addition to therapeutic applications, the CFTR inhibitors are widely used as probes to identify the pharmacologic properties of wild-type and site-directed mutated CFTR channel, and subsequently obtain the functional and structural information of the channel. This strategy is proved useful in deciphering the three-dimensional structure of the selectivity filter of the bacterial potassium channel [7, 69]. Several classes of compounds have been identified as useful probes of CFTR to date. These include the arylamino benzoates such as diphenylamine-2-carboxylic acid (DPC) [70-72], sulfonylureas such as glibenclamide [65, 73-76], the disulfonic stilbenes such as DIDS and DNDS [77, 78], certain

polyatomic anions such as $\text{Au}(\text{CN})_2^-$ and $\text{Pt}(\text{NO}_2)_4^{2-}$ [79-83], and glycine hydrazides [84]. In addition, the thiol-reactive reagents such as the derivatives of methane thiosulfonate (MTS) [85-89] and Cd^{2+} [85] have been used as probes in cysteine scanning studies to suspect pore-lining residues.

The CFTR pore blockers have several common characteristics: most of them are lipophilic anions and have a large size. Generally, they inhibit CFTR by two different mechanisms: open channel block and allosteric block [90, 91]. Some of the large anions such as glibenclamide and $\text{Au}(\text{CN})_2^-$ are open channel blockers of the CFTR channel pore. These compounds block CFTR by binding in the larger vestibule at the intracellular side of the CFTR channel pore [52, 92] and subsequently prevent Cl^- flow by occluding the permeation pathway [81]. In contrast, some blockers such as genistein, which may potentiate Cl^- currents through CFTR under certain concentrations and has been investigated as clinical drug to correct the primary defect in cystic fibrosis [93-97], may inhibit CFTR channel function under higher concentrations under an allosteric mechanism [90, 98, 99]. These blockers inhibit CFTR by interacting with NBDs and subsequently slowing the rate of channel opening [98]. Since the NBDs seem to function in a head-to-tail dimer [56, 100, 101], these allosteric blockers may inhibit channel opening by preventing dimer formation [102]. In addition, another newly identified open channel blocker, glycine hydrazide (GlyH), inhibits CFTR channel by occupying the shallow extracellular vestibule [84].

These open channel and allosteric blockers can be distinguished by the characteristics of CFTR channel inhibition. Blockade of CFTR by open channel blockers is voltage dependent and enhanced by reducing the external Cl^- concentration, but not affected by nucleotide analogue pyrophosphate (PPi) [80, 81]. In contrast, blockade of CFTR by allosteric blockers is independent from voltage and not affected by the external Cl^- concentration, but relieved by PPi [90]. GlyH has a characteristic macroscopic current-voltage relationship, which makes it different from other open channel blockers. Most open channel blockers of CFTR cause an outward rectification of CFTR Cl^- currents, indicating that Cl^- flow from the intracellular to the extracellular side of the cell membrane is more strongly attenuated than that in the opposite direction [81]. In contrast, a trace dosage of GlyH ($< 30 \mu\text{M}$) caused an inward rectification of CFTR Cl^- currents, indicating that Cl^- flow from the extracellular to the intracellular side of the cell membrane is more strongly attenuated than that in the opposite direction [84].

1.6.3. Cysteine accessibility

Cysteine scanning is a widely used method and offers opportunities for engineering new binding sites at the locations we suspected to be within the channel pore, and then determining the accessibility of the molecules that are predicted to enter the pore. This approach relies on the introduction of cysteine residues into the selected locations by site-directed mutagenesis and the use of thiol-reactive reagents as probes. Akabas and his colleagues synthesized three charged MTS derivatives by adding to MTS the negatively charged ethylsulfonate (MTSES^-) or the positively charged ethylammonium (MTSEA^+) or thyltrimethylammonium (MTSET^+) [89]. These reagents can react with the SH group

of cysteines to form disulfide bonds only on a water accessible surface. Therefore, the probe is most likely to react with cysteines that face the water-filled channel pore. By using this strategy, Akabas and his colleagues scanned nine consecutive residues in TM1 of CFTR and found that in three TM1 mutants, G91C, K95C, Q98C, all of which are predicted to locate on the same face of the TM1 α -helix, the conductance was irreversibly altered by either MTSES⁻ or MTSEA⁻ [89]. In subsequent studies, they reported that 9 of 24 cysteine-substituted mutants in TM6 exhibited inhibition of CFTR conductance when exposed to either MTSES⁻ or MTSEA⁻ and concluded that TM6 might take an important role in CFTR channel pore forming [88, 103]. Using the same strategy, TM6 has been verified as a pore-lining helix and its conformational change was coupled to CFTR channel gating mechanism in a recent study [85].

1.6.4. Current achievements in framing the pore

Since it is still technically difficult to obtain an accurate structure of CFTR channel pore, the pore-lining amino acid residues has been determined by comparing the electrophysiological properties of wild type and mutated CFTR channels. Several lines of evidence have demonstrated that the sixth transmembrane segment (TM6) in the first membrane spanning domain (MSD1) plays an important role in CFTR channel pore formation. Mutagenesis studies indicate that mutations in TM6 affect CFTR functional properties such as single channel conductance [54, 104, 105], the shape of macroscopic I-V relationship [54, 106, 107], anion selectivity [55, 79, 108], blockers sensitivities [65, 80, 82] and multi-ion pore behavior [83, 109, 110]. As mentioned above, cysteine scanning assays, in which the selected residues are systematically substituted by single

cysteines and the effects of hydrophilic thiol-reactive reagents on anion permeation are assayed, also suggested that TM6 is most likely to be a pore-lining α -helix [88, 103].

Most effective TM6 mutants have lower single channel conductances than that of wild type CFTR, such as F337A, R334K [54], R334W, R347H [104], K335E [109], R352A, and R352Q [86]. However, single mutated T338A significantly increased CFTR channel conductance and this effect was enhanced in T338A/T339A double mutant [79, 105]. Also, the permeabilities to several large anions were increased [79]. Given these data, it was proposed that T338 locates at the narrowest part of the channel pore and the conductance should be elevated due to the wider dimension of this part [79]. By employing $\text{Au}(\text{CN})_2^-$ as a probe, Gong and his colleagues reported significant changes of $\text{Au}(\text{CN})_2^-$ binding affinity in the CFTR mutants K335A, F337S, T338A, S341A, I344A, and R352Q, which were expected to locate at the same surface of TM6 α -helix [80]. And subsequently, they conformed their findings by using another probe $\text{Pt}(\text{NO}_2)_4^{2-}$ [82].

A model in which the amino acids located at the distal side of TM6 α -helix fold back into the membrane was proposed because of the accessibility of cysteines engineered in this part to extracellularly applied methane sulfonate (MTS) reagents [103]. Consistent with this model, mutations that altered the charge at R352 in the proposed re-entrant loop dramatically altered anion selectivity over cation [111]. However, further study suggested that loss of positive charge in R352Q mutant caused an endogenous cysteine to become available to MTSEA⁺ and implicated that the channel pore structure might be damaged due to mutation [86]. It was previously suggested that a hypothetical salt bridge between

R347 in TM6 and D924 in TM8 should be essential for normal channel conductance and loss of positive charge in R347 might alter pore geometry [112]. Taken together, it seems that R347 and R352 may serve a similar role in preserving the structure of CFTR channel pore.

Several TMs other than TM6 have been investigated as well and were also suggested contributing to the CFTR channel pore formation, such as TM1 [54, 55, 89, 113], TM2 [114], TM3 [115], TM5 [113, 116], TM11 [117] and TM12 [53, 65, 118]. TM5 was supported to be important in pore formation since the mutations at G314 and V317 altered conduction properties of the pore [113, 116]. By contrast, several residues in TM1 were suggested to make contributions to defining the anion permeability properties of the open channels [54]. Mutating S1118 in TM11 affects both anion permeation and channel gating, suggesting it also contributes to the pore [117]. In addition, TM12 probably lines the pore, but makes less contribution than TM6, since alanine substitutions at T1134, M1137, N1138, S1141 and T1142 have little or no effect on anion permeation [53, 65]. Among these candidates, TM1 has the strongest evidence to form the pore together with TM6. The lysine at the position 95 in TM1 has been suggested to be a pore-lining residue by using several different methods [54, 55, 89, 113]. The alanine substitutions at Q98 and P99 significantly reduce single channel conductance of the channel pore [54]. Cysteine scanning research shows that G91, K95 and Q98 are pore-lining residues [89]. A research focused on the contribution of proline residues in the CFTR molecule implies that P99 may contribute to CFTR pore [119].

Based on a series of electrophysiological studies, a model of the CFTR pore has been proposed, in which chloride ions are attracted into the channel pore from the extracellular side by R334 in TM6 and from the intracellular side by K95 in TM1 [120]. However, there has no direct biochemical evidence yet for any pore-lining residue and also the functional data obtained from some mutants are controversial and difficult to reconcile. For example, deleting the N-terminus and TMs 1–4 apparently has little effect on CFTR channel function [121], but there are several mutations in this region that can dramatically alter channel properties [54, 89, 113]. Mutations in TM12 affect sensitivity to the open channel blocker chlorodiphenylphosphine (DPC), suggesting that it takes a role in forming the channel pore [118], but the N-terminal half of CFTR molecule, which only contains MSD1, NBD1, and the R domain, can form a normal functional channel without the C-terminal half [122]. However, these functional investigations are still important in revealing the structure and permeation mechanism of the CFTR Cl⁻ channel pore.

1.7. Control of CFTR channel gating

CFTR is activated by both phosphorylation of its R domain by protein kinase A (PKA) and ATP binding at its NBDs. The NBDs are hallmarks of the ATP-binding cassette transporter (ABC) superfamily, indicating that CFTR is a member of it. On the other hand, CFTR is the only ABC molecule that contains an R domain and forms an ion channel.

1.7.1. Functions of the two NBDs

The NBDs of CFTR contain highly conserved Walker A, Walker B and LSGGQ motifs that were predicted to bind and hydrolyze intracellular ATP [123, 124]. Structural and functional studies of ATPases and ABC transporters suggested that the lysine in Walker A (K464 in NBD1 and K1250 in NBD2) can interact with either the α - or γ -phosphate of ATP and is essential for ATP hydrolysis [124, 125], while the aspartate in Walker B (D572 in NBD1 and D1370 in NBD2) can coordinate Mg^{2+} in MgATP and is required for ATP binding [126, 127]. Similarly, studies of GTP-binding proteins suggest that the conserved glutamine in the LSGGQ motif plays a dominant role in GTP hydrolysis [128]. Although it is now clear that the ATPase activity of CFTR is not indispensable for its channel activity, CFTR channel gating is actually controlled by the interactions of ATP with the NBDs [60]. The structures of NBDs in CFTR are based on those in the bacterial ABC transporters such as LivG, HisP, LolD and MalK [100, 129]. It has been proposed that the NBDs probably form a dimer with ATP molecules bound to catalytic sites which situate at the interface between NBD1 and NBD2 [58]. Isolated NBDs of prokaryotic ABC proteins dimerize upon binding ATP and ATP hydrolysis causes dissociation of the dimer [58, 59, 129]. Using single channel recording on intact CFTR molecules, Vergani and his colleagues monitored the opening and closing of the channel gates and related these occurrences to ATP mediated events in the NBDs [56]. These results directly linked ATP driven dimerization of NBDs to ion channel opening in MSDs (Figure 1.2).

Figure 1.2. The hypothesis of CFTR channel opening by ATP-driven dimerization of its nucleotide binding domains. This series of cartoon illustrates the proposed mechanism coupling the opening of CFTR channel pore in the membrane spanning domains to the hydrolysis cycle through the dimerization of NBD1 and NBD2.

Several kinetic models have been proposed to explain the ATP-dependent gating mechanism of CFTR channel in early reports and reviews [57, 130-132]. Although the detailed mechanism of CFTR gating by its two NBDs remains unclear, some agreements have been achieved regarding how ATP binding at the NBDs leads to the opening and closing of the channel. Since mutations of the Walker A lysine at NBD2 (e.g., K1250A) abolish ATP hydrolysis and subsequently result in channels with prolonged opening burst durations, it is proposed that ATP hydrolysis at NBD2 leads to channel closing [66, 130, 133, 134]. However, it is still unclear what role NBD1 plays in CFTR gating. Some researchs suggested that ATP hydrolysis at NBD1 was involved in the opening of CFTR channel since mutations of the Walker A lysine at NBD1 (e.g., K464A) decrease the channel opening rate [66, 130, 133]. However, a later study reported no significant difference in the channel opening rate between K464A mutated and wild-type CFTR [135]. In addition, biochemical studies suggest that association and dissociation rate of ATP from NBD1 is too slow ($\leq 1 \text{ s}^{-1}$) to play a role in the CFTR channel opening transitions that are recorded by electrophysiological instrument [136, 137].

To find out more details about the effect of nucleotide binding on CFTR gating, researchers focused on CFTR mutants with impaired ATP hydrolysis by using both macroscopic voltage dependent current recording and single channel trace analysis. A rightward shift of the ATP dose response was reported when one of the Walker A lysines (K464 or K1250) was substituted to alanine [57]. Moreover, a similar result was obtained for the mutation at the Walker B aspartate at NBD2 (D1370). Substitution this aspartate to asparagine (D1370N) results in channels with prolonged opening and closing burst

durations [57, 133]. Taken together, it could be proposed that ATP binding at both NBDs is required for CFTR channel opening, and ATP hydrolysis at NBD2 leads to channel closing. However, this model is still not suitable to explain a decrease of channel opening time by ADP [138].

1.7.2. Regulation by phosphorylation: the R domain

Using excised inside-out membrane patches from cells expressing recombinant wild type CFTR, Anderson and his colleagues indicated that MgATP could regulate CFTR channel activity only when the molecule has first been phosphorylated with PKA [139, 140]. PKA favors the consensus phosphorylation sequence $R-R/K-X-S^*/T^* > R-X-X-S^*/T^* = R-X-S^*/T^*$, where X is any amino acid, and the phosphorylation site is indicated by an asterisk [34]. Nine of the ten predicted dibasic ($R-R/K-X-S^*/T^*$) PKA consensus sequences within CFTR are in the R domain including eight serines (S660, S686, S700, S712, S737, S768, S795, S813) and one threonine (T788). Site-directed mutagenesis studies demonstrated that disabling the dibasic and most monobasic PKA sites in various combinations strongly inhibited channel activity, but did not abolish it [62, 64, 141]. It seems that PKA regulation is redundant since no one PKA site was essential for channel activation [142]. Interestingly, replacing S737 or S768 with alanines increased channel activity, which suggests that phosphorylation of these two sites could inhibit CFTR channel activation [143, 144].

Several lines of evidence have demonstrated that the responses of CFTR to PKA stimulation could be modulated by PKC with unknown mechanism [145-150]. In these

studies, exposing to PKC or PKC activators enhanced the rate and magnitude of subsequent activation of CFTR channel by PKA. However, even though the main PKC phosphorylation sites S686 and S790 were disabled, the modulation of CFTR activity by PKC was not changed [148]. Early studies of a mutant lacking all nine PKC sites on the R domain demonstrate that PKC modulation of channel gating requires direct phosphorylation of CFTR itself rather than an ancillary protein, and at least one of the nine PKC sites also mediates the partial activation induced by PKC alone [151]. Removing the PKA and PKC sites may inhibit ATPase activity since phosphorylation and dephosphorylation influences the ATPase activity of CFTR [140]. However, the nucleotide-binding and ATPase activities of CFTR with mutated phosphorylation have not yet been assayed biochemically.

How does phosphorylation by PKA modify CFTR function? The earliest suggestion was that the unphosphorylated R domain is an inhibitor which functions to keep the channel in a closed state [64, 152, 153]. This scheme is similar to that of the inhibitory “ball” of Shaker K⁺ channels [154, 155]. It is in an agreement with the finding that the addition of an unphosphorylated recombinant R domain to CFTR molecule blocked its channel function [153]. However, this simplest scheme is controversial, since CFTR Δ R channels, from which most of the R domain has been removed (residues 708-835), remain closed until they are provided with ATP [139, 156]. Moreover, with the presence of ATP, the channel activity of phosphorylated CFTR significantly exceeded that of CFTR mutants which could open without phosphorylation [156-158]. Mutation of the phosphorylation sites altered the relationship between ATP concentration and open state probability (P_o) at

low ATP concentrations (between 0.1 mM to 1 mM), while all mutants had the same P_o as the wild type CFTR at high ATP concentrations above 1 mM [158]. Another report confirmed that phosphorylation might enhance the ATPase activity of CFTR [140]. Taken together, it is reasonable to say that the R domain has a novel regulatory role in CFTR channel gating. Once phosphorylated, the R domain is released from the inhibition site and simultaneously facilitates interactions of the NBDs with ATP. However, a recent study indicates that phosphorylation regulates CFTR by promoting the association of the R domain with other domains rather than by causing its dissociation from an inhibitory site [159]. In one word, the regulation of CFTR channel gating is more complicated than previously believed.

1.8. Anion conduction and selectivity of CFTR

This section focuses particularly on the anion selective channel function of CFTR and the mechanism of ion conduction through the channel pore. For this purpose, Ohm's law is used to describe the behavior of the CFTR anion channel. In which the flow of anions, expressed as an electric current (I), is written as the product of the conductance of the channel (γ) and the total electrochemical driving force, as showed in the following equation,

$$I = \gamma (V_m - E_{rev}) \quad (1)$$

where γ is the conductance of a single channel (in pS), V_m is the membrane potential referenced to the outside of the cell membrane (in mV), and E_{rev} is the reversal potential defined as the value of V_m at which $I = 0$ (in mV). In the presence of a net driving force, the magnitude of flow is determined by the conductance γ , a concise description of the

anion translocation from one side of the cell membrane to the other. In the investigation of CFTR channel conduction properties, both of the channel conductance and reversal potential for single channel current are fundamental parameters for determining anion conduction mechanisms and subsequently providing principal information about the architecture of the channel pore.

Reversal potential measurements with different NaCl concentration gradients indicate that CFTR is highly selective for monovalent anions over cations. Previous studies have shown that the permeability for Na^+ over Cl^- ($P_{\text{Na}}/P_{\text{Cl}}$) is in the range of 0.03 – 0.1 [20, 55, 160]. Substituting Na^+ by other cations including K^+ made no change on the permeability ratio. Thus it is likely that the CFTR channel has little cation permeability. To investigate the anion conduction by CFTR, researchers compared the permeability ratios ($P_{\text{X}}/P_{\text{Cl}}$) and conductance ratios for permeant anions and found that anion channels, compared with cation channels, tend to be relatively nonselective and allow the passive diffusion of a large number of inorganic and organic anions that differ widely in their size and shape [20, 46, 79, 161]. Moreover, certain anions may bind within the pore acting as the blockers of the channel [83, 110, 113, 162]. To determine the permeability sequence of CFTR, under strictly bi-ionic conditions and assuming zero cation permeability, with anion X^- in the intracellular solution and Cl^- in the extracellular solution, the permeability ratios of various anions X relative to that of Cl^- are calculated by using a modified form of the Goldman-Hodgkin-Katz voltage equation,

$$P_{\text{X}}/P_{\text{Cl}} = \exp (E_{\text{Rev}} F/RT) \quad (2)$$

where E_{Rev} is the estimated current reversal potential, F is the Faraday constant ($9.648 \times 10^4 \text{ C/mol}$), R is the Gas constant ($8.314 \text{ J/K}\cdot\text{mol}$), and T is the temperature (in Kelvin) [163].

The anion permeability sequence of CFTR was first determined by using the whole cell patch technique and the sequence is $\text{Br}^- \geq \text{Cl}^- > \text{I}^- > \text{F}^-$ [55]. This anion permeability sequence of CFTR is obviously different from other epithelial Cl^- channels having a higher permeability to I^- than Cl^- , such as the ORCC channel [19]. However, subsequent study of CFTR Cl^- channels using excised inside-out membrane patches suggested that the apparent I^- permeability relative to that of Cl^- is affected by the interactions between I^- and Cl^- within the pore [20]. The single channel data of Tabcharani and his colleagues suggest that I^- is more permeable than Cl^- . Since I^- blocks the pore under some conditions, it seems that I^- is less permeable than Cl^- . Depending on whether the block effect of I^- is considered and the various experimental conditions, different anion selectivity sequences have been suggested [164]. Therefore, it was proposed that the CFTR pore has a “weak field strength” selectivity filter [34, 165] as other Cl^- channels such as ligand-gated Cl^- channels in neurons [18] and ORCC channels in epithelia [19].

When being analyzed by using bi-ionic reversal potential measurements, the permeability sequence for polyatomic anions with known dimensions is $\text{NO}_3^- > \text{Cl}^- > \text{HCO}_3^- > \text{formate} > \text{acetate}$ [79]. In contrast, pyruvate, propanoate, methane sulfonate, ethane sulfonate, and gluconate are not measurably permeant ($P_X/P_{\text{Cl}} < 0.05$). The relationship between permeability ratio and ion diameter suggests that the minimum diameter of the

CFTR pore is about 5.3 Å [166], similar to that of other Cl⁻ channels [18, 19]. Among all the permeable anions, HCO₃⁻ is a major anion transported in the pancreatic duct and may also be important in airway surface fluid secretion [167]. Although HCO₃⁻ permeates through the CFTR channel worse than Cl⁻, it accounts for about half of the anion secretion by airway submucosal glands [79, 168]. Interestingly, extracellular HCO₃⁻ inhibits CFTR-mediated Cl⁻ current dramatically [169]. Such inhibition may be important during pancreatic bicarbonate secretion since it would reduce dissipation of the HCO₃⁻ gradient when luminal HCO₃⁻ concentration is increased to about 150 mM, which is sixfold higher than plasma and tenfold higher than cytoplasm [169, 170]. In addition, water, urea, and ATP have also been reported to permeate CFTR Cl⁻ channels, though the evidence for ATP permeation is controversial [171-176]. The halide anion permeability sequence for CFTR proposed by Tabcharani and his colleagues indicates that the permeability sequence of polyatomic anions follows a lyotropic sequence, which suggests that anion permeation is determined by the hydration energy of anions in CFTR [20]. This idea was further confirmed by the study of CFTR permeation using polyatomic pseudohalide ions [177].

Several lines of evidence suggest that CFTR Cl⁻ channel is a multi-ion pore that can hold more than one anion simultaneously. Firstly, wild type CFTR shows anomalous mole fraction effect (AMFE) when bathed with symmetrical Cl⁻/SCN⁻ mixtures [109]. The conductance decreases from 7 to 2 pS as the SCN⁻ mole fraction is increased from 0% to 7%, and then increases again as the SCN⁻ mole fraction is increased further to 97%. Secondly, the inhibition of the CFTR pore by intracellular gluconate is relieved by raising

the extracellular Cl^- concentration, suggesting that Cl^- can expel gluconate from the pore [178, 179]. Thirdly, the inhibition of CFTR by I^- only occurs when I^- is present on one side of the membrane and Cl^- is present on the other side, suggesting that interactions between Cl^- and I^- within the pore are responsible for the low $P_{\text{I}}/P_{\text{Cl}}$ values reported for CFTR [20]. Finally, the repulsive interactions between Cl^- and the channel blockers $\text{Au}(\text{CN})_2^-$ or $\text{Pt}(\text{NO}_2)_4^{2-}$ in the CFTR channel pore show that multiple anions can bind simultaneously within the pore and repel each other to exit from the pore [80, 82, 110]. The same repulsive interactions are also found between impermeant blocking anions such as between $\text{Pt}(\text{NO}_2)_4^{2-}$ and $\text{Fe}(\text{CN})_6^{3-}$ [83]. Collectively, these data provide strong evidence for interactions between anions within the CFTR pore. It is also suggested that the CFTR pore may contain at least two anions simultaneously. The relative permeability of different anions is principally controlled by some aspect of the interaction between permeant anions and the walls of the channel pore. Although many controversies remain unsettled, most laboratories agree on an overall anion selectivity sequence for human wild type CFTR of $\text{SCN}^- > \text{NO}_3^- > \text{Br}^- > \text{Cl}^- > \text{I}^- > \text{HCOO}^- \geq \text{ClO}_4^- > \text{F}^- > \text{Gluconate}$.

Anion selectivity mostly depend on the heights of energetic barriers and the dehydration energy of anions, whereas anion conductance mainly depend on anion concentration and anion binding affinity [108]. Therefore, permeability and conductance ratios measured for SCN^- and some other anions are quite different [79]. Several laboratories have emphasized the importance of anion binding sites in CFTR channel pore [52, 164, 180]. Previous studies demonstrate that channel conductance is more strongly affected by mutations than relative permeability ratios, which suggest that the anion conductance

may be more sensitive to the structural properties of the pore [54, 108, 113]. Mutations at K95, Q98 and P99 in TM1 dramatically reduce the single channel conductance as well as the mutations at R334, K335 and F337 [54, 55, 109]. However, substitutions at T338 by alanine and serine enhances the conductance significantly by about 30% [105]. Cysteines substituted at R334 and K335 also cause a decrease in covalent modification with MTSES⁻ [181]. Thus, the positively charged side chains of R334 and K335 may influence anion conductance by increasing Cl⁻ concentration near the external mouth of the CFTR channel pore. At the intracellular side of the cell membrane, K95 may have the same function. Since anion permeation is usually interpreted by assuming that anions are translocated between sites that are separated by energetically unfavorable barriers within the pore, a three binding sites model is proposed based on the experimental results of current-voltage relationships under various conditions, single channel recording, anion selectivity, block by intracellular Au(CN)₂⁻, and AMFEs in mixtures of SCN⁻ and Cl⁻ [52, 54, 120, 178]. Although such models are highly speculative and require many assumptions, it is heartening that most assumptions concerning the functional properties of K⁺ and CLC Cl⁻ channels that obtained from electrophysiological experiments are strongly supported by the corresponding crystal structures [7, 9].

1.9. CFTR: a multifunctional molecule

In addition to play a role as a chloride channel, CFTR is predicted to help regulate the following channels: epithelial sodium channel (ENaC) [182, 183], outwardly rectifying chloride channel (ORCC) [176, 184, 185] and renal outer medullary potassium channel (ROMK) or other inwardly rectifying potassium channels (IRK) [186, 187].

Figure 1.3. Epithelial cell model summarizing multiple functions of cystic fibrosis transmembrane conductance regulator (CFTR). CFTR is a multifunctional protein which can 1) function as a Cl^- channel, 2) facilitate ATP release from intracellular side to extracellular side, 3) positively regulate outwardly rectifying chloride channel (ORCC), 4) negatively regulate epithelial sodium channel (ENaC), 5) modulate renal outer medullary potassium channel (ROMK) sensitivity to sulfonylureas, 6) regulate the intracellular compartment acidification and protein processing, and 7) regulate the vesicle trafficking. This picture is modified from Schwiebert et al [188].

1.9.1. CFTR-ENaC interaction

Previous studies implicated that a consistent Na^+ absorption acted as the dominant ion flux in airway epithelia [189-191]. Two additional characteristics of CF airway epithelial ion transport could not be directly explained by dysfunction of the CFTR cAMP-dependent Cl^- channel. First, the magnitude of basal Na^+ absorption was two to three times greater than in normal airway epithelia [192, 193]. Second, increased intracellular cAMP stimulated further Na^+ absorption in freshly excised CF airway epithelia, while there was no effect on the magnitude of Na^+ absorption in normal airway epithelia [183]. These abnormalities in Na^+ transport in CF airways seem to indicate a negative modulatory effect of CFTR on the rate-limiting step in Na^+ absorption by ENaC, the apical membrane amiloride-sensitive sodium channel.

Although the molecular basis for negative regulation of ENaC by CFTR has not been identified, several possibilities are proposed. First, it has been suggested that CFTR might play a role in the export of intracellular ATP [176]. After released into the extracellular domain, ATP could inhibit apical membrane Na^+ channels [194]. In addition, some related ABC transporters, such as *mdr* (multidrug resistance protein), move phospholipids across membranes [195-197]. Subsequently, phospholipid messengers may play a role in modulation of ENaC by CFTR. Second, CFTR may interact directly with one or more ENaC subunits [198-201]. Although the functional regulation of ENaC by CFTR seen in the airways was not seen in the sweat duct epithelium where CFTR and ENaC should be expressed simultaneously, the direct protein-protein interaction is supposed to be involved [202]. The third possibility is that CFTR and ENaC may interact through membrane-associated cytoskeleton. Although the details of this mechanism have not been

established fully, both CFTR and ENaC have been shown to be affected by interactions with actin, one cytoskeletal component [203, 204]. Thus, the aforementioned tissue-specific regulation of ENaC by CFTR could be explained by the variable expression of a key regulatory protein or proteins linking CFTR or ENaC to the cytoskeleton or to each other in different tissues.

1.9.2. CFTR interactions with potassium channels

Studies have reported that the expression of CFTR alters K^+ currents in several cell types [186, 187, 205, 206]. For example, the expression of CFTR is associated with cAMP-dependent regulation of inwardly rectifying K^+ currents in an immortalized CF-affected pancreatic epithelial cell line [206]. Inwardly rectifying K^+ (IRK) channels are believed to play a role in K^+ recycling on the basolateral cell membrane in airway epithelia, but no abnormalities in their function have been reported in CF patients. To provide a driving force for apical chloride channel activation and maintain normal Na^+ and Cl^- balance in the airway, it has been suggested that similar K^+ channels may exist on the apical membrane of airway epithelial cells [206]. One of these apical K^+ channels ROMK1 (Kir1.1) has been identified in the lung [207].

The ROMK family is made up of ATP-sensitive K^+ channels derived from kidney and has several isoforms (ROMK 1, 2, and 3) [207, 208]. In renal tissue, ROMK channels are presented in the apical membrane of distal nephron segments of the mammalian kidney, where they play a major role in K^+ homeostasis [208, 209]. Immunohistochemical studies have shown that ROMK2 is presented in the apical membrane of the cortical collecting

duct [210]. Moreover, it has been demonstrated that CFTR is also presented in the apical membrane of the cortical collecting duct [211]. Thus, it is reasonable to suppose that CFTR may play a regulatory role in ROMK2 channel activity. Previous patch clamp studies of the coexpressed CFTR and ROMK2 have demonstrated that CFTR altered only the glibenclamide sensitivity of ROMK2 channel [187, 212]. After rephosphorylation by addition of PKA and ATP, glibenclamide had no inhibitory effect on ROMK2 K⁺ channel activity [187]. In contrast, when expressed with CFTR, the single channel conductance of ROMK1 was significantly decreased and ATP sensitivity of ROMK1 was also altered [205]. Given this data, it was suggested that CFTR may provide a necessary domain for the ROMK1 channel's ATP sensor. As stated above, CFTR may regulate other ion channels directly or indirectly via several protein-protein or signaling interactions. Moreover, CFTR may also be capable of conferring effects of agonists or antagonists on other proteins.

1.9.3. CFTR-ORCC interaction

Previous studies demonstrated that ORCC were presented in CF epithelial cells but these channels could not be activated by PKA or PKC without the normal regulatory function of CFTR [213-215]. Complementation studies showed that reintroduction of wild-type CFTR into a CF cell corrected defective protein kinase regulation of ORCC [185]. Moreover, patch-clamp studies demonstrated that ORCC were presented but insensitive to PKA or PKC in airway epithelial cells devoid of CFTR [184]. Taken together, these results suggested that kinase regulation of ORCC required the multifunctional CFTR molecule. But why CFTR expression is essential for kinase stimulation of ORCC?

To answer this question, a convenient assay was conducted to record the activity of CFTR and ORCC chloride channels simultaneously [216]. In this study, only CFTR chloride channels were stimulated with 1 mM intracellular ATP, while both CFTR and ORCC chloride channel currents could be measured when the intracellular concentration of ATP increased to 5 mM. However, other studies utilizing planar lipid bilayer system revealed that the intracellular ATP had to pass through the cell membrane to the extracellular side to exert its effects on ORCC [176, 217]. Without the cAMP stimulation or CFTR expression, extracellular ATP at nanomolar concentration activated ORCC in both normal and CF airway epithelial cells [176]. The simplest interpretation was that CFTR could function as an ATP channel and conduct the ATP itself [218]. However, other unclear mechanisms should exist for how CFTR facilitated ATP release or efflux from the cells.

1.10. The studies about interval loops and terminuses in CFTR molecule

The CFTR molecule is predicted to be 77% present at the intracellular side of the cell membrane including intracellular loops (ICLs), NBDs, R domain, N- and C-terminuses, 4% of its total reside in the outside environment of the cell as extracellular loops (ECLs), and 19% in membrane-spanning domains [22]. The interval loops of CFTR channel usually consist of hydrophilic amino acids. It has been shown by engineering N-glycosylation consensus sequences (NXS/T) into all predicted intracellular and extracellular loops, that the originally predicted CFTR structure based on hydropathy algorithms is so far correct for the location of the loops [219].

1.10.1 Function of extra- and intracellular loops

When compared to the intracellular loops, the six extracellular loops of CFTR are predicted to be extremely short and less than 5 residues except ECL1 and ECL4, which contains about 15 and 31 residues respectively [220]. ECL1 and ECL4 have been studied in details to view whether they have any function other than simply joining the TMs. Some studies suggest that the external vestibule of the CFTR channel pore may be formed in part by ECL1 [104, 221]. Mutations R117C/H/L/P are widely known mild disease causing mutations, suggesting that the R117 in ECL1 is important for the function of CFTR channel. Mutating this arginine to histidine reduces single channel conductance by 70% and results in a channel open probability approximately equaling to 1/3 that of wild type CFTR [104]. It has also been suggested that the mutant R117H is involved in sensing extracellular pH and subsequently influencing conduction [104]. A recent study shows that mutations of two positively charged arginines in ECL1, R104 and R117, result in an inwardly rectified current-voltage relationship and significantly decreased single channel conductance [221]. In contrast, the mutations of the positively charged residues in other ECLs have no significant effect on the permeation properties of the CFTR channel pore [221]. Collectively, it is suggested that ECL1 contributes to the formation of CFTR channel pore's external vestibule and the positively charged residues in this loop function to attract anions into the pore.

ECL4 is the only loop in CFTR where could be glycosylated. Glycosylation is one of the common protein post-translational modifications and plays an important role in protein

folding, protein trafficking and localization, ligand recognition, and protein-protein interactions [222, 223]. CFTR has been previously described in three different forms with apparent weights of approximately 127, 131, and 160 kDa on an SDS-PAGE gel, representing nonglycosylated, core glycosylated, and complex glycosylated, fully mature CFTR, respectively [224]. The most common mutation in CF, $\Delta F508$, causes mislocation and glycosylation failure of CFTR [224]. It sounds that only the mature, fully glycosylated CFTR can be transported to the cell membrane, however, functional studies of mutants lacking the N-glycosylation sites (e.g., N894/900D) reveal that glycosylation is not necessary for normal channel activity or proper folding of CFTR, though distinct reduction of CFTR expressing level in cell membrane was verified [219]. Interestingly, previous study has demonstrated that a disease related mutant T908N created an extra consensus sequence for N-glycosylation and subsequently resulted in a reduced rate of Cl^- efflux and a noisy single channel opening [225]. In addition, mutations at six different positions in ECL1 and single position in ECL2 and ECL4 all destabilized the channel open state in the unitary current traces, some of them severely, indicating that the ECLs of CFTR not only contribute to its trafficking and maturation but also contribute to the stability of the CFTR Cl^- channel pore [220].

The intracellular loops (ICLs) of CFTR are generally more highly conserved than the ECLs or TMs when compared among different species and speculated to be critical for CFTR protein processing [219, 224]. Moreover, the ICLs are predicted to be involved in channel gating and stabilization of conductance states by interacting with R domain [226]. Disease associated mutation E193K in ICL1 significantly decreases the channel opening

probability of CFTR [226]. Although none of the ICL1 and ICL2 mutants appears to contribute to the CFTR pore formation, interestingly, the ability of the non-hydrolysable ATP analogue adenylyl imidodiphosphate (AMP-PNP) to lock the CFTR channel in the open states was abolished by two mutations in ICL1, I148T and G178R [226]. Some mutations associated with pancreatic sufficiency, such as E193K in ICL1, H949Y in ICL3, and R1070Q in ICL4, have no effect on chloride current but reduce bicarbonate transport by 50-60% [227]. Deletion of exon 5 which codes for 30 amino acids (residues 163-193) in ICL1 of CFTR affects both processing and functions of the CFTR Cl⁻ channel [228]. This splice mutation of CFTR results in mis-targeting to cell membrane when expressed in epithelial cells [228, 229]. When recording the single channel trace in intracellular vesicles, it shows more subconductance state behavior (6 and 3pS) along with a reduced open probability to the wild type single channel conductance of about 8 pS [228]. Similar to the exon 5 deletion mutant, purified CFTR with 19 residues deletion in ICL2 also shows a subconductance state of 6 pS and this subconductance is exhibited more frequently than in wild type [230]. T351, R352, and Q353 in the ICL between TM6 and NBD1 are highly conserved among CFTR of different species, and have been proposed to form a re-entrant loop back into the pore [103, 111].

The high resolution structure of an ABC superfamily member MsbA clearly shows that the ICLs have distinct α -helices, and the loop between TM6 and the NBD domain act as a bridge between the NBDs and the TMs [231]. In another words, it could be inferred that the ICLs of CFTR function to transmit energy from ATP hydrolysis at the NBDs into a conformational change of the pore. Since it is widely believed that CFTR exists as a

duplication of a protein similar to MsbA which functions as a homodimer, it is believed that ICL3 and ICL4 is the mirror image of ICL1 and ICL2 in CFTR [231]. Mutants S945L and G970R in ICL3 show slight outward rectification of current-voltage relationships over the range ± 80 mV which indicates that ICL3 may be physically close to the inner mouth of the pore [232]. Moreover, the inhibition of forskolin activated CFTR Cl^- current by glibenclamide is abolished by charge neutralizing mutations K978A/Q/S in a recent study [76]. Although mutations in these four loops all reduce the channel open probability of CFTR, generally, mutations in ICL1 and ICL2 tend to affect the function of CFTR by increasing the channel close time [226, 228, 230], whereas mutations in ICL3 and ICL4 tend to affect the channel by decreasing the channel open time [232-234]. It is suspected that ICL1 and ICL2 help to open the pore by coupling NBD1 to the pore, while ICL3 and ICL4 help to hold it in the open state by interacting with NBD2.

1.10.2 The function of CFTR N- and C-terminuses

CFTR has no apparent N-terminal signal peptide. However, deleting the 80 amino acids of the cytosolic amino-terminus disrupts normal trafficking of CFTR from ER to the cell membrane, demonstrating that this domain is critical for CFTR biosynthesis [235]. Site-directed mutations in this region such as P5L, S50P, E60K, and R75Q severely affect the channel function [236]. The N-terminus of CFTR has been found to interact with the target soluble N-ethylmaleimide-sensitive factor attachment protein receptors (t-SNAREs) such as syntaxin 1A [237], SNAP23 [238], and syntaxin 8 [239], which effectively couple CFTR regulation to membrane traffic machinery. The interaction of the t-

SNAREs and the CFTR N-terminus inhibits exocytosis of CFTR to the cell membrane and negatively regulates CFTR channel gating [237, 238, 240-242]. Moreover, a highly conserved and highly charged motif from D44 thru E60 in the N-terminus of CFTR is found to be involved in regulation of channel gating by interacting with the R domain [159, 243, 244]. Inhibition of CFTR channel activity by syntaxins may due to the disruption of the interaction between N-terminus and R domain [243-245]. It is suggested that the N-terminus directly controls the access of the phosphorylated R domain to inhibitory or stimulatory sites within the pore region [246]. Zhang and his colleagues demonstrated that mutations in N-terminus could promote abnormal CFTR folding by disrupting the interaction of N-terminus to the molecular chaperone cysteine string protein [247, 248]. Finally, mutant S13F is reported to disrupt the interaction between CFTR N-terminus and the cytoskeletal filamin proteins and subsequently affect the stability and trafficking of CFTR [249].

Many members in the ABC transporters superfamily have PDZ-protein binding motifs at their C-terminuses, such as CFTR, ABCA1, ABCA7, MRP2 and MRP4 [250]. The PDZ domains help to mediate protein-protein interactions, cluster membrane proteins in specific regions, determine the polarized localization and modulate cytosolic trafficking of some proteins [251]. The C-terminus of CFTR protein has come under intense interest in the last ten years since the discovery of the PDZ-binding motif with the sequence of DTRL. This motif mediates the interacting of CFTR to several PDZ-domain protein, including Na⁺/H⁺ exchanger regulatory factor isoform-1 (NHERF1, also named EBP50, ezrin-binding protein, 50 kDa), NHERF2, NHERF3 (Also named CAP70, CFTR-

associated protein, 70 kDa), NHERF4 and CFTR-associated ligand (CAL) [252-255]. NHERF1 and NHERF2 have an ERM-binding domain, which binds ezrin, radixin or moesin to localize the NHERF-binding proteins such as CFTR to the apical cytoskeleton in epithelial cells [256, 257]. Fluorescent recovery after photobleaching (FRAP) studies demonstrate that PDZ-domain proteins interact with CFTR in the cell membrane and the interaction is highly dynamic on a time scale of milliseconds [258]. By interacting with the C-terminus of CFTR molecule, NHERF1 and CAP70 enhance the single channel current and potentiate Cl^- permeability through CFTR channel pore [259-261]. It is suggested that NHERF1 and CAP70 mediate CFTR dimerization and then alter channel conformation and function [259, 260]. Dimerization may facilitate the interaction between the NBDs and R domains [262]. However, neither NHERF1 nor NHERF2 were found to induce the dimerization of CFTR in a recent study. In contrast, it is reported that PKA induces the formation of CFTR dimers [263]. In addition, the PDZ-domain proteins are the platforms or scaffolds CFTR uses to interact with other proteins and regulate other ion channels [264-266].

1.11. Objectives

As we have mentioned above, CFTR molecule is a multi-functional protein localized in the apical membrane of epithelial cells. Although fruitful results have been obtained concerning the Cl^- channel function, interaction with other proteins, pharmacological properties, biosynthesis, disease-associated mutants and even the rough structure of CFTR, the molecular mechanism that determines anion permeation through CFTR channel pore is still largely unclear.

There are many controversies concerning the components which form CFTR channel pore. In this project, we used patch clamp recording and employed a strategy of co-mutagenesis to gain more powerful evidence to determine which TMs are involved in CFTR channel pore forming. We constructed double mutations from two potential pore-forming transmembrane regions to investigate the involvement of these two TMs by comparing the effects of single and double mutations on the functional properties of CFTR Cl⁻ channel. Since the role of residue T338 in TM6 in CFTR channel pore forming is strongly suggested by enormous evidence, we used the mutant T338A, in which the threonine at site 338 in TM6 was substituted by alanine, as a control mutation and constructed double mutations on this background to scanning other potential pore lining residues that can interact with T338. This could be an important step in refining models of the three dimensional architecture of the CFTR channel pore.

Moreover, other potential components which could be involved in CFTR channel pore forming besides those transmembrane segments were also tested in present study. CFTR contains two evolutionary conserved N-glycosylation sites (N894 and N900) in the fourth extracellular loop between the seventh and eighth transmembrane segments. We know that glycosylation may play an important role in protein folding, trafficking, and protein-protein interactions. So we wonder if these oligosaccharides play a role in CFTR channel function. To test the channel functional role of glycosylation and identify potential molecular correlates accounting for the functional difference, we constructed three mutant CFTRs in which the glycosylation sites were abolished by site-directed

mutagenesis to delete either one or both extracellular glycosylation sites and examined the effects of deletion on CFTR channel function.

Finally, the physical properties of the wild-type CFTR channel pore were revisited in present study to collect more information in terms of sidedness and flexibility of the pore. Since current ion channel models are unsuitable to explain many pharmacological properties of CFTR, a new model should be established to understand the asymmetric anion permeation and large organic anion permeation through CFTR channel pore.

We hope this project could raise a new strategy to study the pore architecture of ion channels by constructing double mutations. Our findings should contribute to demonstrate a three dimensional model of the CFTR channel pore. In addition, we hope that the flexibility and conformational changes of the pore will be considered in future studies concerning channel gating and the mechanisms of anion permeation.

2. Materials and Methods

2.1. Site-directed mutagenesis

2.1.1. Bacterial strain and culture media

Escherichia coli DH5 α was purchased from Invitrogen (Carlsbad, CA, USA). Kanamycin sulphate was purchased from US Biological (MA, USA). Dissolve 100 mg of kanamycin in 10 ml of Milli-Q water. Filter-sterilize and store at -20 °C. Add 3 ml per 1 L medium. Luria-Bertani (LB) medium was prepared by dissolving 10 g bacto-tryptone, 5 g bacto-yeast extract and 5 g NaCl in 1000 ml ddH₂O. Adjust to pH 7.0 with 1N NaOH and autoclave at 121 °C for 20 mins. For preparing LB agar plates, 2% agar was added in the LB medium and autoclaved. Plates were poured after the LB agar cooled to about 50-60 °C. After sterilization, antibiotics can be added aseptically to the medium if necessary. SOC medium was prepared by dissolving 20 g bacto-tryptone, 5 g bacto-yeast extract, 0.584 g NaCl, 0.186 g KCl, 2.033g MgCl₂, 2.468 g MgSO₄ and 3.604 g glucose in 1000 ml ddH₂O. 10% glycerol was prepared by dissolving 100 ml of glycerol in 1000 ml ddH₂O. All the media and solutions were sterilized by autoclave at 121 °C for 20 mins.

2.1.2. Plasmids and subcloning

CFTR in the pNUT vector was kindly provided by Dr Lap-Chee Tsui, Hospital for Sick Children, Toronto, Canada. To visualize cells transiently transfected with CFTR, the CFTR cDNA was subcloned from the pNUT vector into the bicistronic pIRES2-EGFP

vector (Figure 2.1, Clontech, USA). pIRES2-EGFP was first digested with *EcoR* I and *BamH* I at 37 °C for 2 hrs. The 5300 bp fragment was purified through gel extraction and used as backbone. The targeting CFTR cDNA fragment (4700 bp) from pNUT-CFTR was also digested with *EcoR* I and *BamH* I. The stoichiometry of targeting fragment to backbone was 3:1 in 20 µl ligation reactions with 1 µl T₄ DNA ligase (Roche). The reactions were kept at 16 °C overnight and then mixed with 100 µl DH5α competent cells. The mixture was kept at 0 °C for 30 min, followed by heat shock at 42 °C for 2 min, then incubated at 0 °C for 5 min; cells were recovered by adding 880 µl LB media and shaking at 37 °C, 230 rpm for 40 min. After incubation, bacteria cells were centrifugated at 2000xg for 10 min. Supernatant was discarded and cell pellet was resuspended with 100 µl fresh LB media and plated onto LB agar plates with 30 µg/ml kanamycin. LB agar plate was incubated at 37 °C overnight. Isolated colonies were inoculated into LB media with 30 µg/ml kanamycin. Cultures were incubated at 37 °C, shaking 250-300 rpm overnight. The plasmid was purified by mini-prep kit (Qiagen) and analyzed by digestion and sequencing. The product plasmid was named as pIRES2-EGFP-wt-CFTR.

2.1.3. Primer designing and site-directed mutagenesis

To construct different site-specific CFTR mutants, we designed the mutagenesis primers based on the transcription codes of amino acids. All the primers we have used in this project were summarized in Table 2.1.

Figure 2.1. Restriction Map and Multiple Cloning Site (MCS) of pIRES2-EGFP

Vector. Unique restriction sites are in bold.

Table 2.1. The primers used for construction of CFTR mutants (Research Biolabs)

Mutant	Primer (5'-3')	
K95Q	Forward	GGCGAAGTCACCCAAGCAGTACAGC
	Reverse	GCTGTACTGCTTGGGTGACTTCCCC
A96V	Forward	GGGGAAGTCACCAAAGTAGGTACAGCCTCTCTTAC
	Reverse	GTAAGAGAGGCTGTACTACTTTGGTGACTTCCCC
Q98A	Forward	ACCAAAGCAGTAGCGCCTCTCTTACTGGGAAG
	Reverse	CTTCCCAGTAAGAGAGGCGCTACTGCTTTGGT
T338A	Forward	CCTCCGGAATAATATTCGCCACCATCTCATCTGC
	Reverse	GCAGAATGAGATGGTGGCGAATATTTTCCGGAGG
N1138A	Forward	CTTTAGCCATGGCTATCATGAGTAC
	Reverse	GTACTCATGATAGCCATGGCTAAAG
S1141A	Forward	CATGAATATCATGGCTACATTGCAGTGGG
	Reverse	CCCACTGCAATGTAGCCATGATATTCATG
T1142A	Forward	GAATATCATGAGTGCATTGCAGTGGG
	Reverse	CCCACTGCAATGCACTCATGATATTC
W1145A	Forward	GTACATTGCAGGCGGCTGTAAACTC
	Reverse	GAGTTTACAGCCGCCTGCAATGTAC
N894D	Forward	CTCTTCAAGACAAAGGGGATAGTACTCATAGTAG
	Reverse	CTACTATGAGTACTATCCCCTTTGTCTTGAAGAG
N900D	Forward	GTACTCATAGTAGAGATAACAGCTATGCAG
	Reverse	CTGCATAGCTGTTATCTCTACTATGAGTAC
Mutants based on T338A-Using T338A mutated plasmid as template		
K95Q/T338A	Forward	GGCGAAGTCACCCAAGCAGTACAGC
	Reverse	GCTGTACTGCTTGGGTGACTTCCCC
A96V/T338A	Forward	GGGGAAGTCACCAAAGTAGGTACAGCCTCTCTTAC
	Reverse	GTAAGAGAGGCTGTACTACTTTGGTGACTTCCCC
Q98A/T338A	Forward	ACCAAAGCAGTAGCGCCTCTCTTACTGGGAAG
	Reverse	CTTCCCAGTAAGAGAGGCGCTACTGCTTTGGT
N1138A/T338A	Forward	CTTTAGCCATGGCTATCATGAGTAC
	Reverse	GTACTCATGATAGCCATGGCTAAAG
S1141A/T338A	Forward	CATGAATATCATGGCTACATTGCAGTGGG
	Reverse	CCCACTGCAATGTAGCCATGATATTCATG
T1142A/T338A	Forward	GAATATCATGAGTGCATTGCAGTGGG
	Reverse	CCCACTGCAATGCACTCATGATATTC
W1145A/T338A	Forward	GTACATTGCAGGCGGCTGTAAACTC
	Reverse	GAGTTTACAGCCGCCTGCAATGTAC
Mutants based on N894D-Using N894D mutated plasmid as template		
N894D/N900D	Forward	GTACTCATAGTAGAGATAACAGCTATGCAG
	Reverse	CTGCATAGCTGTTATCTCTACTATGAGTAC

The mutation sites were shaded in this table.

Site-directed Mutagenesis of CFTR was then carried out within the pIRES2-EGFP vector using the QuickChange XL Site-Directed Mutagenesis Kit (Stratagene, USA) as previously described [81, 82, 107, 110, 267]. Briefly, the thermal cycling mixture contained a final concentration of 100-200 ng pIRES2-EGFP-CFTR plasmid DNA, 1.5 x *Pfu* reaction buffer, 250 ng of each of two synthesized complementary oligonucleotide primers which contain the designed mutation, 500 μ M each of dNTPs and 5 U *Pfu* Turbo DNA polymerase. Temperature cycling was performed using an Eppendorf Master Cycler (Eppendorf, Singapore), with a short (30 s) denaturing step at 95 °C followed by 20 cycles of denaturation (95 °C for 30 s), annealing (55 -60 °C for 60 s) and extension (68 °C for 20 min). Following cycling, DNA was treated with *DpnI* for 2 h at 37 °C to digest template DNA, transformed into competent *Escherichia coli* DH5 α cells and grown overnight on LB agar plates containing 30 μ g ml⁻¹ kanamycin. Five to ten separate colonies were selected and expanded, and plasmid DNA was isolated for confirmation of the desired mutation by DNA sequencing (Research Biolab Pte. Ltd, Singapore).

2.1.4. Preparation of DH5 α competent cells

To prepare the competent *Escherichia coli* DH5 α cells for electro-transformation, 1-10 μ l bacteria stock stored at -80°C was picked out and incubated in 5 ml LB (no antibiotics) medium at 37°C with shaking overnight. And then, transferred all 5 ml overnight culture medium into 200 ml LB medium in a 500 ml flask and incubate at 37°C until OD₆₀₀ = 0.5 ($\pm$ 0.05). Centrifuged at 6000 rpm for 10 min at 4°C to obtain the bacterial pellet. Resuspended the pellet in 100 ml 0.1 M MgCl₂ with vigorous vortex and put on ice for 5 min. Centrifuged at 6000 rpm for 10 min at 4°C and resuspended the pellet in 20 ml 0.1

M CaCl₂. Incubated at 4°C overnight. Spinned at 4000 rpm for 10 min at 4°C. The cells were carefully resuspended using a serological pipette in 10ml of 86% CaCl₂ (w/v, 0.1 M), 14% glycerol (w/v) until the pellet was completely homogenized. And then, aliquoted 100 µl cells into 0.6 ml eppendorf tubes and stored at -80°C.

2.1.5. Transformation by electroporation

The electroporation voltage was set at 2500 V. 1 µl (5 pg to 0.5 µg) plasmid DNA was added into the microcentrifuge tube containing 100 µl prechilled DH5α competent cells. Mixed and transferred the mixture into an prechilled electroporation cuvette. Gently Shaked the cuvette to settle the cells to the bottom and placed the cuvette into the sample chamber immediately. To apply the 2500 V pulse, the “Pulse” button was pressed twice. After the beep signal, removed the cuvette from the chamber and immediately transferred the cells into 1 ml SOC medium for cell recovery. 100 µl original, 30×, 900× and 2700× diluted cells were spreaded onto labelled LB agar plates containing 30 µg ml⁻¹ kanamycin and incubated overnight at 37°C. 3 single colonies of transformed cells were aseptically inoculated and amplified in 5 ml of LB medium containing kanamycin. Two 100 µl aliquots of transformed cells were aseptically transferred in microcentrifuge tubes. 100 µl 100% glycerol was added into each tube and mixed well. Then, the transformed cells were kept at -80 °C for future use. The rest of the culture was used for DNA extraction by Mini-prep (Invitrogen) or Maxi-prep kits (Qiagen).

2.2. Cell culture and transfection

Experiments were carried out on Chinese hamster ovary (CHO) and baby hamster kidney (BHK) cells from American Tissue Culture Collection (ATCC), transiently transfected with wild type or mutated human CFTR, prepared as described previously[80, 82, 106, 107, 110, 267, 268]. BHK cells were grown at 37°C in 5% CO₂ in phenol red Dulbecco's Modified Eagle Medium supplemented with 5% fetal bovine serum, 100 U ml⁻¹ penicillin-streptomycin, and 0.25 µg ml⁻¹ fungizone (all from Life Technologies, USA). CHO cells were routinely maintained in phenol red Minimum Essential Medium Alpha Modification (Life Technologies) supplemented with 8% FCS, 100 U ml⁻¹ penicillin-streptomycin and 0.25 µg ml⁻¹ fungizone. For patch clamp recording, cells were seeded onto 22 mm glass coverslips and transfected with the pIRES2-EGFP vector containing wild-type or mutated CFTR cDNA. Briefly, plasmid DNA was pre-incubated with Lipofectamine 2000 reagent (Invitrogen) or Superfect reagents (Qiagen) for 15 min. Complexed DNA was then diluted in supplement-free medium to a final concentration of 0.5 µg ml⁻¹ and added to the cell culture dishes. After 3-6 hours at 37 °C and 5% CO₂, the medium was completely replaced with growth medium. Transiently transfected CHO or BHK cells could be identified by fluorescence microscopy within 24 hours and used for patch clamp recording 1 to 3 days after transfection.

2.3. Patch clamp recording

Electrophysiological data were recorded from excised, inside-out patches of membrane from the transfected CHO or BHK cells using the patch clamp technique, as described in

detail previously[80, 82, 106, 107, 110, 267, 268]. Briefly, CFTR channels were activated after patch excision by exposure to 50 nM protein kinase A catalytic subunit (PKA) plus 1 mM MgATP. For single channel recording, the extracellular (pipette) solution contained: 150 mM Na gluconate, 10 mM N-tris[hydroxymethyl] methyl-2-aminoethanesulfonate (TES), 2 mM MgCl₂. Intracellular solution contained: 150 mM NaCl, 10 mM N-tris[hydroxymethyl] methyl-2-aminoethanesulfonate (TES), 2 mM MgCl₂. For macroscopic current-voltage relationship recording, symmetric concentration of chloride was added at both sides of the excised cell membrane. To estimate anion permeability, NaCl in the extracellular solution (pipette) was replaced by 150 mM NaX, where X⁻ represents one of the monovalent anions we used other than Cl⁻ such as 150 mM NaBr or 150 mM NaSCN. For the iodide permeability experiment, the intracellular (bath) solution containing 150 mM NaCl was replaced by 150 mM NaI to avoid electrode oxidation in pipette. Dicyanoaurate (Stock solution = 200 mM, used final concentrations were indicated in the results) was used as probe to characterize the physical properties of the CFTR channel pore [81]. All of the solutions were adjusted to pH 7.4 with NaOH or HCl and filtered with 0.22 μ m syringe filter (Life Technologies).

Given voltages have been corrected for liquid junction potentials calculated using Clampex 9.2 software (Axon Instruments, USA). Pipette resistances were 6-10 M Ω for single channel recordings and around 2 M Ω for macroscopic excised patches. Currents were filtered at 50 Hz for single channel recording or 1 kHz for the macroscopic inside-out recording using an 8-pole Bessel filter (Frequency Devices, USA), amplified using an Axopatch 200B amplifier (Axon Instruments), digitized at 250 Hz for single channel

recording or 5 kHz for the macroscopic inside-out recording using a DigiData 1322A interface and analyzed using Clampfit 9.2 (Axon Instruments). Macroscopic current-voltage (I - V) relationships were constructed using depolarizing voltage ramp protocols as described previously[80, 110, 268]. Macroscopic current were elicited with voltage ramp protocol from -100 mV to 60 mV within 500 ms. Current amplitude was at the beginning (20 ms) and end (480 ms). The 20 ms-time point was selected to ensure that the cell membrane capacitive current did not contribute to the current measured and was used for calculating the inhibition ratio of $\text{Au}(\text{CN})_2^-$ blockade and the rectification ratio. Currents in the absence of PKA have been subtracted so that the data shown are only of currents through PKA-activated CFTR channels. The macroscopic IV curve can be directly used to measure the rectification ratio and the reversal potential to study the bidirectional ion permeation and selectivity in the channel pore. And also, the macroscopic membrane patch provides an ideal way to study the pharmacological properties in a large channel population.

2.4. Data analysis

Rectification of the I - V relationship was quantified as the rectification ratio, the slope conductance at -50 mV as a fraction of that at +50 mV. The macroscopic current reversal potential (E_{Rev}) was estimated by reading the zero current potential of the leak-subtracted I - V relationship. Under strictly biionic conditions and assuming zero cation permeability, with monovalent anions A in the intracellular solution and B in the extracellular solution, the relative permeability of these two anions was given by Goldman-Hodgkin-Katz voltage equation as described in literature review:

$$P_A/P_B = \exp (E_{\text{Rev}} F/RT) \quad (2)$$

Where E_{Rev} is the estimated current reversal potential, F is the Faraday constant (9.648×10^4 C/mol), R is the gas constant (8.314 J/K·mol), and T is the absolute temperature in Kelvin [163].

The dissociation constant of the blocker (K_d) was calculated by the Woodhull model of voltage-dependent block [106] using SigmaPlot 9.0:

$$I/I_0 = K_d(V) / \{K_d(V) + [B]\} \quad (3)$$

$$K_d(V) = K_d(0) \exp (-z \delta VF/RT) \quad (4)$$

Where I/I_0 is the fraction of the control current remaining following addition of blocker, $[B]$ is the blocker concentration (μM), $K_d(V)$ is the voltage dependent dissociation constant, $K_d(0)$ is the dissociation constant of the blocker at zero membrane potential, z is the valence of the blocker (a value of -1 is assumed for dicyanoaurate), V is the membrane potential, δ is the fraction of the transmembrane electric field transversed by blockers in reaching their binding site within the pore from the intracellular solution, and F , R and T have their normal thermodynamic meanings [269].

Experiments were performed at room temperature, 20-25 °C. Mean values were given as mean $\pm$ S.D. Statistical comparisons between mutants were carried out using Student's two-tailed t -test, with $P < 0.05$ being considered statistically significant. Preliminary structural studies of the transmembrane α -helical segments and structural hypotheses of the CFTR channel pore were displayed by using the software Swiss-PDB viewer[270,

271] and 2 D helical wheels. Unless stated otherwise, all chemicals were from Sigma-Aldrich, USA.

3. Co-mutagenesis study in TM1 and TM6

3.1. Introduction

Site-directed mutagenesis has been widely used to obtain information about the functional significance of amino acid within ion channel, as this structure-function strategy would offer mechanistic insights into the inner working of ion channel. In CFTR, mutations of amino acid residues that are conserved in the CFTR showed significant alternations in channel activity and anion permeation as compared to wild type CFTR, thus these mutations are associated with clinical cystic fibrosis [180]. As such, this site-directed mutagenesis strategy has also been widely employed as a means of creating clinical important mutants and obtaining information about the functional significance of amino acid residues in CFTR chloride channel as well. Unlike cation channels, there are re-entrant loops in forming the cation-selective pore [9, 272-276], the structure-functional studies concerning anion channel pore have focused on the transmembrane regions which are presumed to form the physical pores of the anion channels.

A number of laboratories including our own have used mutagenesis to identify amino acid residues that are critical for CFTR chloride channel conductance and anion permeation [80, 110, 267]. It is apparent that there have been a lot of hits on TM6, so that this TM emerges as the most studied, and therefore at this stage the TM with the most clearly demonstrable importance to the properties of CFTR channel pore [180, 277, 278]. Several studies have demonstrated that some residues in TM1 also played a role in pore formation [55, 89, 113, 121]. Evidence for involvement of the remaining ten TMs is

minimal [180], although limited data has been put forward supporting a pore-forming role for TM3 [115], TM5 [113], TM11 [117], and TM12 [53, 118, 279, 280]. However, these mutation-induced CFTR functional changes often provide little direct insights into the structural features, such as the CFTR pore architecture.

Guided by our previous findings that TM1 and TM6 are major contributors to the CFTR channel pore and have strong implications for emerging structural models of CFTR proteins [54, 80, 82, 267], in this work, we investigated the architecture of the CFTR channel pore by using a strategy of co-mutagenesis combined with patch clamp recording techniques. We then characterized these double mutated CFTR variants using a range of functional assays. Consistent with previous reports [54], our results reinforce the notion that TM1 and TM6 play important roles in forming the channel pore and determining its functional properties. Furthermore, we provide evidence that the amino acid residue threonine (T338) in the TM6 may locate closely with the amino acids lysine (K95) and glutamine (Q98) in the TM1. We show that the CFTR channel anion selectivity, conductance and anion current rectification are controlled by the functional cooperation of the residue K95 in TM1 with the residue T338 from TM6. We propose that these residues form a selectivity filter of the CFTR channels. Based on our findings, we propose a partial structural model of CFTR channel pore that encompasses the position of amino acid residues from TM1 and TM6.

3.2. Construction of the double mutants from different TMs

To investigate the pore architecture of the CFTR chloride channel, we designed three double mutations (K95Q/T338A, A96V/T338A, and Q98A/T338A). We constructed the three double mutants from TM1 and TM6 to test upon our hypothesis that if these residues are closely located and may interact with each other, thus influence functional properties of the double mutated CFTR channels. A prerequisite for employing a double mutation for revealing the pore architecture of CFTR channel is to identify at least one residue that clearly contributes to the CFTR pore formation and if the mutation of this residue shows distinguishable response from that of wild type CFTR channels. To identify this residue, we examined several residues of TM6. We selected TM6, as this TM play a dominant role in CFTR channel pore formation suggested by the effects of site-directed mutations in TM6 on anion binding and permeation [109, 118], current rectification [78, 80, 106, 107, 109, 110, 118, 268], and blockade by CFTR channel blockers [78, 80, 106, 109, 118, 268]. Among these residues (R334, K335, F337, T338, S341, R347, and R352) from TM6, we found that the polar but uncharged threonine residue (T338) outstands other residues since it played a very important role in anion binding [107], determining the single channel conductance [105] and anion selectivity [105, 277] of the pore, perhaps by contributing to the narrowest region of the pore [105, 120, 277]. Therefore, we used T338A as the CFTR control channel variant and constructed all double mutations on this background.

Some previous studies [281, 282] suggest that CFTR channel pore is function as a monomer even though the CFTR dimer is identified by natural PFO-PAGE and electron

microscope [283-285]. Our results in another research on stoichiometry of CFTR channel (Yang Jie's PhD thesis) also demonstrated that CFTR channel pore is formed by a single protein. In Yang Jie's project, we cotransfected CHO cells with equal amounts of R334K (small conductance) and T338A (large conductance) mutated CFTR proteins. The observed single channel trace showed two distinct channel currents for R334K and T338A respectively. No hybrid channel (intermediate or any other than expected single channel conductances of the transfected mutants) was detected from at least 10 patches. So CFTR channel pore could only be formed by parallel or antiparallel array of its own transmembrane segments, just as the pore of the GABAR channels lined by portions of five helices packed so as to provide a channel lumen of ~ 5.8 Å in diameter [18, 286]. Therefore, we proposed at least another TM together with TM6 must also play a role in the CFTR channel pore formation. Among these transmembrane regions, TM1 is most likely to cooperate with TM6 in channel pore formation [54, 89, 120] and substitutions of several amino acid residues such as G91 [89, 113], K95 [54, 55], A96 [54], Q98 [54, 89], and P99 [54, 119] affected the functional properties of CFTR channel. Here, we have chosen three residues K95, A96 and Q98 in TM1 for mutagenesis, as they were proposed to be the pore-lining residues [54, 89].

3.3. Single channel properties of single and double mutants

To investigate the possible interactions of TM1 and TM6, we compared the functional properties of these three double mutants (K95Q/T338A, A96V/T338A, and Q98A/T338A) with that of the wild type and four single mutants (T338A, K95Q, A96V, and Q98A). Expression of wild type or mutant CFTR in Chinese hamster ovary (CHO)

cells led to the appearance of PKA- and ATP-dependent chloride channel currents in excised inside-out membrane patches (Figure 3.1). For the single mutants, A96V and Q98A showed similar unitary current to that of wild type as indicated from the all-points histograms (right panel in Figure 3.1). However, the unitary conductance was drastically reduced in the mutant K95Q (Figure 3.1). In contrast, T338A led to a significant increase in channel conductance as described previously [105]. For the double mutants, we found the enhancing effect of T338A single mutation on unitary current was mitigated by simultaneously mutated A96V (Figure 3.1 and Table 3.1). However the unitary current of double mutant Q98A/T338A was similar to that of T338A single mutant. Very interestingly, the unitary current of K95Q/T338A double mutant exhibited a smaller single channel current amplitude (0.09 ± 0.04 pA) than that of its two single mutants T338A (0.75 ± 0.02 pA) and K95Q (0.16 ± 0.05 pA), a synergic effect of two single mutants (Figure 3.1 and Table 3.1. Data are presented as mean $\pm$ standard of deviation of 3-7 patches and 20 opening amplitudes were measured from each patch. $p < 0.01$, the significance were analysed by using student's t test). This result suggests these two residues are very closely located within CFTR protein and suggests a functional cooperation between the amino acid residues K95 and T338.

Figure 3.1. Single channel current amplitudes of the wild type CFTR, single mutants from TM 6 (T338A), TM1 (K95Q, A96V and Q98A) and double mutants from TM1 and TM6 (K95Q/T338A, A96V/T338A, Q98A/T338A). Unitary currents were recorded in inside-out configuration at a membrane potential of -50 mV in each case. For each trace the short dashed line represents the current level when CFTR channels are closed. The unitary current amplitudes are demonstrated from all point histograms (right panel) prepared from these current traces, and as indicated. PKA (5 nM) and ATP (1 mM) were added at the bath solution to activate the CFTR channels.

Table 3.1. Functional properties of the CFTR wild type and mutants

Mutants	Unitary current (pA)	Rectification ratio	Relative permeability of Br⁻	Relative permeability of SCN⁻	Relative permeability of I⁻
Wild type	0.48 ± 0.05	1.05 ± 0.05	1.57 ± 0.06	2.08 ± 0.09	0.92 ± 0.04
T338A	0.75 ± 0.02	1.22 ± 0.08	2.00 ± 0.04	7.50 ± 0.60	3.16 ± 0.34
K95Q	0.16 ± 0.05	0.58 ± 0.11	1.54 ± 0.03	4.65 ± 0.45	1.62 ± 0.09
A96V	0.52 ± 0.05	1.56 ± 0.07	1.22 ± 0.09	3.20 ± 0.28	0.69 ± 0.07
Q98A	0.51 ± 0.02	1.76 ± 0.08	1.44 ± 0.13	3.72 ± 0.55	1.23 ± 0.02
T338A/K95Q	0.09 ± 0.04	0.23 ± 0.06	2.01 ± 0.03	6.73 ± 0.23	3.02 ± 0.15
T338A/A96V	0.62 ± 0.09	1.47 ± 0.16	1.84 ± 0.06	7.32 ± 0.14	3.26 ± 0.41
T338A/Q98A	0.72 ± 0.03	1.65 ± 0.19	1.61 ± 0.16	5.09 ± 0.14	2.91 ± 0.21

Data are presented as mean ± standard of deviation of 3-7 patches. The unitary currents were recorded at the membrane potential of -50 mV. To calculate the unitary currents of WT and mutated CFTR channel, 20 opening amplitudes were measured from each patch. Rectification ratio was quantified by the slope conductance at -50 mV as a fraction of that at +50 mV. Relative permeabilities (P_X / P_{Cl}) of Br⁻, SCN⁻ and I⁻ in wild type CFTR and mutants were calculated by Goldman-Hodgkin-Katz voltage equation: $P_A/P_B = \exp (E_{Rev} F/RT)$, where E_{Rev} is the estimated current reversal potential; F is the Faraday constant (9.648×10^4 C/mol); R is the gas constant (8.314 J/K·mol); and T is the absolute temperature in Kelvin (K).

3.4. Macroscopic current properties

The macroscopic current properties were studied in inside-out patches following maximal channel activation in presence of 1 mM ATP, 50 nM PKA and 2 mM pyrophosphate (Figure 3.2). Examples of leak-subtracted macroscopic CFTR I-V relationships are shown in Figure 3.2 (control currents). Here, we used rectification ratio [107, 110] to compare the asymmetric permeation of Cl^- caused by mutations with wild type CFTR channel (Table 3.1, Figure 3.2 and Figure 3.3). In most cases (T338A, A96V, Q98A, A96V/T338A and Q98A/T338A) macroscopic I-V relationships were linear or weakly inwardly rectifying in the presence of symmetrical chloride concentrations as shown in Figure 3.2 and Figure 3.3. T338A single mutation showed a slight inward rectification, but no significant difference with that of the wild type CFTR channel (Figure 3.3, $p > 0.01$). The mutants A96V, Q98A and their double mutants, A96V/T338A and Q98A/T338A, showed statistically significant inward rectification effects compared with that of the wild type CFTR (Figure 3.3, $p < 0.01$). Consistent with the previous report [54], K95Q showed strong outward rectification (Figure 3.3). The double mutant K95Q/T338A demonstrated the characteristics of the single mutant K95Q. Very significantly, the double mutant K95Q/T338A exhibited stronger outward rectification (a ratio of 0.23 ± 0.06) than that of K95Q (0.58 ± 0.11), even that T338A showed slightly inwardly rectifying (1.22 ± 0.08) (Table 3.1 and Figure 3.3, $p < 0.01$).

$\text{Au}(\text{CN})_2^-$ has been used as a high affinity probe of anion binding sites in the CFTR pore and to identify TM6 residues contributing to the CFTR pore formation [80, 107, 110]. As published previously [81], in wild type CFTR chloride channel, the fractional unblocked

current (I/I_0) following addition of 100 μM and 1 mM $\text{Au}(\text{CN})_2^-$ increased in a voltage dependent manner during voltage changed from -100 mV to +60 mV (Figure 3.4). The fractional unblocked current of T338A single mutant was also voltage dependent, but showed a U-shaped plot with highest blockade near to zero membrane potential (Figure 3.4). It was proposed that $\text{Au}(\text{CN})_2^-$ could pass through the T338A single mutated channel under negative voltages which suggests the mutation reduced the binding affinity of $\text{Au}(\text{CN})_2^-$ to the channel pore. $\text{Au}(\text{CN})_2^-$ caused a voltage-dependent block of wild type and mutant forms of CFTR under both concentrations of 100 μM and 1 mM as demonstrated in the mean fractional I-V relationships (Figure 3.2 and Figure 3.4). The weak block and less voltage dependence were observed in the single mutants K95Q, A96V and Q98A and their corresponding double mutants K95Q/T338A, A96V/T338A and Q98A/T338A (Fig. 4). However, the fractional unblocked current (I/I_0) of K95Q by $\text{Au}(\text{CN})_2^-$ was flat under the voltage range tested, and no voltage dependence has been shown. We also found that the double mutant K95Q/T338A showed very weak blocking effect at the membrane potential near 0 mV, weaker than that of the single mutants K95Q and T338A, while Q98A/T338A double mutant exhibited a weaker block at more negative membrane potential around -100 mV, than its two single mutants Q98A and T338A (see the third row panel in Figure 3.4).

Figure 3.2. $\text{Au}(\text{CN})_2^-$ block of macroscopic CFTR currents. Examples of leak-subtracted macroscopic currents recorded in inside-out membrane patches following maximal activation with 50 nM PKA, 1 mM MgATP and 2 mM sodium pyrophosphate under symmetrical 154 mM Cl^- concentrations. In each case, currents were recorded before (Control) and after additions of 100 μM and 1 mM $\text{Au}(\text{CN})_2^-$ to the intracellular solution.

Figure 3.3. Effect of mutations on rectification of the macroscopic I - V relationship.

Rectification was quantified as the rectification ratio as described under “Materials and Methods”, the slope conductance at -50 mV as a fraction of that at +50 mV. A ratio greater than one reflects inward rectification and a ratio less than one indicates outward rectification of the I - V curve. Asterisks indicate a significant difference from wild type ($P < 0.01$). Mean of data from five to seven patches.

Figure 3.4. Effects of mutations on the voltage-dependent block by 100 μM and 1 mM intracellular $\text{Au}(\text{CN})_2^-$. For the first two rows, each panel shows the mean fractions of control current remaining (I/I_0) following additions of 100 μM (dark circle) and 1 mM (hollow circle) $\text{Au}(\text{CN})_2^-$ to the intracellular solution as a function of membrane potential. For the panels in the third row, each panel shows the superimposed comparisons of an average remaining fraction of two single mutants (dark circle and square) with the control current remaining from their corresponding double mutants (hollow triangle and diamond). Note that the double mutants (K95Q/T338A and Q98A/T338A) show synergistic effects (weaker than that of their individual mutants) of $\text{Au}(\text{CN})_2^-$ block under negative membrane potentials. Mean of data from five to seven patches.

3.5. Anion selectivity

CFTR is associated with a lyotropic anion selectivity pattern [180] that has been shown to be disrupted by mutagenesis within TM6 [80]. Mutagenesis studies of CFTR have also suggested that the selectivity of anion permeation is relatively resistant to the effects of mutagenesis unless that the residues mutated are within the “selectivity filter” [80]. In present study, we also estimated three anions Br^- , SCN^- , and I^- permeability from measurements of the macroscopic current reversal potential in inside-out excised patches. According to the reversal potentials under biionic condition with anion X^- containing solution on one side and Cl^- containing solution on the other side, the relative permeabilities of X^- ($P_{\text{X}}/P_{\text{Cl}}$) in wild type and mutated CFTR channels are showed in Figure 3.5 and are compared in Table 3.1. From Figure 3.5 and Table 3.1, we found that all of single mutants (K95Q, A96V, Q98A and T338A) played relative important roles in the relative permeability of the anions tested. However, T338A mutant played a dominant role on CFTR anion selectivity in all of its three double mutations. Bromide permeability was slightly increased in T338A and its double mutants (A96V/T338A and Q98A/T338A), while slightly reduced in Q98A (Figure 3.5.A). Thiocyanate permeability was increased with different intensity in all the mutants (Figure 3.5.B). The pattern of iodide permeability was similar to that of bromide permeability, but these mutants showed more sensitive to iodide than to bromide (Figure 3.5.C).

Figure 3.5. Relative permeabilities (P_X / P_{Cl}) of Br^- , SCN^- and I^- in wild type and CFTR mutants. Br^- and SCN^- were contained in pipette solution (extracellular); however I^- was added in bath solution (intracellular) to avoid electrode oxidation in pipette which could cause baseline drift. Asterisks indicate a significant difference from wild type ($P < 0.01$). Mean of data from four to six patches. Data were calculated by equation 2 (see Section 2.4)

4. Co-mutagenesis study in TM12 and TM6

4.1. Introduction

Mutagenesis studies demonstrate that mutations of some residues in TM6 affect CFTR channel functions in single channel conductance [54, 104, 105], the voltage dependence of Cl^- current [54, 106, 107], monovalent anion selectivity [55, 79, 108], blockers sensitivities [65, 80, 82] and multi-ion interaction inside the pore [83, 109, 110]. Moreover, cysteine scanning assays also suggested that TM6 is most likely to be a pore-lining α -helix [88, 103]. Many TMs other than TM6 were also investigated and suggested to make some contributions to the CFTR channel pore forming, such as TM1 [54, 55, 89, 113], TM2 [114], TM3 [115], TM5 [113, 116], TM11 [117] and TM12 [53, 65, 118]. Recently, Linsdell proposed a model of the CFTR pore formed by TM1 and TM6, in which chloride ions are attracted into the channel pore from the extracellular side by R334 in TM6 and from the intracellular side by K95 in TM1 [120]. Our results that we have described above also supported this model by creating T338A/K95Q co-mutated CFTR mutants (see Section 3). However, other TMs may also take a part in channel pore forming, though no convincing evidence has been provided. For example, mutating G314 and V317 in TM5 [113, 116] and S1118 in TM11 altered conduction properties of the pore [117], suggesting they also contribute to the pore. In addition, alanine substitution at N1138 and T1142 in TM12 significantly enhanced glibenclamide block which implicates that TM12 probably lines the pore, but makes less contribution than TM6, since those mutations including T1134A, M1137A, N1138A, S1141A and T1142A have little or no effect on anion permeation [53, 65].

To find out which TM other than TM1 and TM6 are most likely to be channel pore lining domains, we used Swiss-PDB viewer [270, 271] to analyze the distribution of hydrophilic residues on the surface of all 12 transmembrane α -helices. Based on the preliminary result that indicates a high possibility of TM12 to act as a pore lining domain (Figure 4.2 and Table 4.2), we designed four alanine substituted mutants at the sites predicted to locate at the same hydrophilic surface, including N1138A, S1141A, T1142A and W1145A. And then, we used the co-mutagenesis strategy to construct double mutated mutants of CFTR from TM6 and TM12 and observe whether these co-mutations induced synergic effects. Since the alanine substitution at T338 in TM6 caused a outstanding change in functional properties [54, 287], we chose this single mutant T338A which has distinguishing single channel properties for comparison and created co-mutated double mutants based on T338A single mutant to find out whether a direct interaction exists between residues from TM6 and TM12. Since the single channel current is a very sensitive parameter to detect a structural modification inside the channel pore [161], we scanned all the mutants using patch clamp single channel recording to determine the role of TM12 and its probably functional cooperation with TM6.

4.2. Hydrophilic residues distribution in all 12 TMs of CFTR

In present study, we first used the 2 D helical wheels to generate the 12 transmembrane α -helices based on the primary structure that published by Riordan [22] and test the hydrophobicity of these 12 TM domains of CFTR Cl^- channel. The sequences we have input for the TMs are stated in the following table.

Table 4.1. The inputting sequences of TM1 to TM12 for hydrophobicity analysis

	Region	Flank	Transmembrane segments	Flank
TM1	79-103	WR	FMFYGIFLYLGEVTKAVQPLL	LG
TM2	116-140	ER	SIAIYLGIGLCLLFIVRTLLL	HP
TM3	193-217	EG	LALAHFVWIAPLQVALLMGLI	WE
TM4	219-243	LQ	ASAF CGLGFLIVLALFQAGLG	RM
TM5	306-329	NS	SAFFFSGFFVVFLSVLPYALI	K
TM6	330-353	G	IILRKIFTTISFCIVLRMAVT	RQ
TM7	836-860	SL	IFVLIWCLVIFLAEVAASLVV	LW
TM8	910-934	TS	SYVVFYIYVG VADTLLAMGFF	RG
TM9	989-1013	LT	IFDFIQLLLIVIGAIAVVAVL	QP
TM10	1013-1036	Y	YIFVATVPVIVAFIML RAYFL	QT
TM11	1079-1103	MR	IEMIFVIFFI AVTFISILTTG	EG
TM12	1128-1152	RV	GIILTLAMNIMSTLQWAVNSS	ID

As have been shown in Figure 4.1, the predicted combos of hydrophilic residues aligned in the same phase of the α -helices were indicated. The summary of all the predicted combos of hydrophilic residues and evaluated possibilities of the TMs to form water filled chamber were given in Table 4.2. From the preliminary analysis results, we found that TM12 is highly qualified to be a channel pore forming domain besides TM1 and TM6, as more than six hydrophilic residues are aligned in the same phase in TM12 (Figure 4.1). These residues are most likely to form the channel pore, especially the continuously located residues T1134, N1138 and S1141 which we have indicated by shading in table 4.2. Interestingly, the mutation N1138A and T1142A significantly strengthened glibenclamide block and abolished the dependence of block on the

extracellular Cl⁻ concentration, which suggested these two residues may facilitate the interaction between glibenclamide and Cl⁻ ions within the pore directly or indirectly [65]. By substituted the residues to cysteine, cross-links could be formed between M348-T1142, T351-T1142, and W356-W1145, which implicated that TM6 and TM12 are nearly located and may form the channel pore together [288]. In fact, a cooperation between TM6 and TM12 has already been suggested by creating a triple mutant S341A/M1140I/T1142F [118]. Since no significant variations have been reported for mutant T1134A concerning macroscopic current-voltage relationship, single channel conductance and voltage dependence of block by SCN⁻ and glibenclamide [53, 65], we made a little rotation to obtain a more sensitive combo N1138, S1141, T1142 and W1145, which are also continuously located at the same side of the TM12 α -helix. Based on these preliminary results, we designed four alanine substituted mutants including N1138A, S1141A, T1142A and W1145A. And then, we created co-mutated double mutants based on the single channel current enhancing mutation T338A in TM6 including N1138A/T338A, S1141A/T338A, T1142A/T338A and W1145A/T338A to investigate the coordination between TM6 and TM12 in the possible CFTR pore formation.

Table 4.2. The summary of the hydrophilic residues combos in the 12 TMs of CFTR

TMs	Hydrophilic residues		Predicted combo aligned in the same face of the α -helix	Pore-forming Possibility
	Total	Flanking region		
TM1	5	R80	R80, T94, K95, Q98	High
TM2	5	R117, H139	R117, T135, H139	Low
TM3	4	E193, E217	E193, H199, Q207, E217	Moderate
TM4	4	Q220, R242	NO	Low
TM5	6	N306, S307, K329	NO	Low
TM6	9	R352, Q353	R334, T338, S341, R352, Q353	High
TM7	3	S836	S836, E851	Low
TM8	6	T910, S911, R933	S911, T925, R933	Low
TM9	4	T990, Q1012	NO	Low
TM10	4	Q1035, T1036	T1019, R1030	Low
TM11	6	R1080, E1102	E1082, T1093, T1100	Moderate
TM12	10	R1128, D1152	T1134, N1138, S1141, N1148, S1149, D1152	High

The shaded areas indicate the continuously aligned hydrophilic residues combos in the same phase of the α -helix.

Figure 4.1. The 2 D helical wheels of twelve transmembrane segments in CFTR chloride channel.

4.3. Single channel properties of wild type and mutated CFTR molecules

After the construction of all single and double mutants, we used single channel recording protocol of patch clamp software Clampex 9.2 to observe the characteristics of CFTR channel opening after fully activated by PKA and ATP. In order to obtain enough current amplitude for comparison, we used isosmotic sodium gluconate solution as extracellular solution and held the membrane potential at -50 mV. As shown in Figure 4.3 we found that all the single mutants from TM12 exhibited similar unitary current to that of wild type CFTR channel as indicated from the all-points histograms (right panel in Figure 4.2). However, T338A led to a significant increase in unitary channel current from 0.65 ± 0.02 pA to 0.81 ± 0.02 pA ($p < 0.05$) as described previously [105]. Since no effect on unitary current of CFTR Cl⁻ channel has been detected for all the TM12 single mutants, we proposed that all the co-mutated CFTR channels would function as T338A single mutated one if they have no synergic interaction. As we expected, the unitary currents of double mutants N1138A/T338A and S1141A/T338A showed no significant variation from that of T338A single mutant though N1138A/T338A slightly mitigated the unitary current to 0.76 ± 0.04 pA (Figure 4.3, $p > 0.05$). However, very interestingly, we found that the enhancing effect of T338A on single channel current was reinforced by simultaneously mutated T1142A and W1145A to 1.03 ± 0.03 pA and 0.98 ± 0.02 pA respectively (Figure 4.3, $p < 0.05$). That is a synergic effect of two single mutants (see Section 3), which suggests these two residues are very closely located within CFTR protein and implies an interaction between these two amino acids.

Figure 4.2. The unitary current traces of the wild type CFTR, single mutants T338A from TM 6, N1138A, S1141, T1142A and W1145A from TM12. Unitary currents were recorded in inside-out configuration at a membrane potential of -50 mV in each case. For each trace the short dashed line represents the current level when CFTR channels are closed. The unitary current amplitudes are demonstrated from all point histograms (right panel) prepared from these current traces, and as indicated. PKA (5 nM) and ATP (1 mM) were added at the intracellular solution to activate the CFTR channels.

Figure 4.3. The unitary current traces of the wild type CFTR, single mutant T338A from TM 6, and double mutants N1138A/T338A, S1141/T338A, T1142A/T338A and W1145A/T338A from TM12 and TM6. Unitary currents were recorded in inside-out configuration at a membrane potential of -50 mV in each case. For each trace the short dashed line represents the current level when CFTR channels are closed. The unitary current amplitudes are demonstrated from all point histograms (right panel) prepared from these current traces, and as indicated. PKA (5 nM) and ATP (1 mM) were added at the bath solution to activate the CFTR channels.

4.4. Macroscopic and pharmacological properties of T1142A and W1145A single mutated CFTR Cl⁻ channels

Since we obtained synergic effects between the mutant pairs T338A-T1142A and T338A-W1145A, whereas the single mutations T1142A and W1145A made no apparent effect on single channel current, we wonder if these two single mutated CFTR channels have some functional variations from wild type CFTR except the single channel current.

The macroscopic current properties were studied in inside-out membrane patches following maximal channel activation in presence of 1 mM ATP, 50 nM PKA and 2 mM pyrophosphate (Figure 4.4). Examples of leak-subtracted macroscopic CFTR I-V relationships are shown in Figure 4.4 (control currents). All the control current lines were almost linear and no apparent rectification was caused by T1142A and W1145A mutations (Upper row of Figure 4.4). Applying $\text{Au}(\text{CN})_2^-$ as a blocker [81], the fractional unblocked current of T1142A single mutant at the dosage of 100 μM was significantly lower than that of wild type CFTR (Figure 4.5, $K_d(0)$ of T1142A = $329 \pm 83 \mu\text{M}$, $K_d(0)$ of wild type = $582 \pm 56 \mu\text{M}$, $p < 0.05$), which means the T1142A mutation strengthened $\text{Au}(\text{CN})_2^-$ binding in the channel pore. As was reported previously, T1142A mutation also strengthened glibenclamide block [65]. In mutant W1145A, the voltage dependence of the blocker $\text{Au}(\text{CN})_2^-$ was dramatically weakened (Figure 4.4). So we conclude that the alanine substitutions of residues T1142 and W1145 in TM12 do affect the anion binding affinity in CFTR channel pore in spite of the normal single channel Cl^- currents.

Figure 4.4. Comparison of the macroscopic and pharmacological properties of T1142A, W1145A single mutated and wild type CFTR Cl^- channels. The upper plots are the schematic representations of the macroscopic I-V relationships of wild type and mutant CFTR Cl^- currents in the absence (control) and presence (with the dosage of 100 μM and 1 mM respectively) of $\text{Au}(\text{CN})_2^-$ under the condition of symmetric Cl^- concentration. Control currents are activated by intracellular 50 nM PKA and 1 mM ATP and locked open by 2 mM PPI. The lower plots illustrate the voltage dependent control current remains (I/I_0) by applying 100 μM (triangular dots) and 1 mM (circular dots) $\text{Au}(\text{CN})_2^-$ respectively. $K_d(0)$ is the dissociation constant of the blocker at zero membrane potential. The values were calculated using the equation 3 and 4 (see Section 2.4). Means are averaged from 4-6 patches.

5. The mutagenesis study on the CFTR glycosylation sites

5.1. Introduction

The CFTR protein contains two Asn-linked glycosylation consensus sites at positions N894 and N900 in the fourth extracellular loop. This membrane glycoprotein does function as a regulated chloride channel in epithelial cells which are involved in the pathology of CF [289, 290]. The most common mutation in CF, $\Delta F508$, causes mislocation and glycosylation failure of CFTR [224].

Glycosylation is one of the common protein post-translational modifications and plays an important role in protein folding, protein trafficking and localization, ligand recognition, and protein-protein interactions [222, 223]. CFTR have been described in three different forms with apparent weights of approximately 127, 131, and 160 kDa on an SDS-PAGE gel, representing nonglycosylated, core glycosylated, and complex glycosylated, fully mature CFTR, respectively [224]. It sounds that only the mature, fully glycosylated CFTR can be transported to the cell membrane. However, functional studies of mutants lacking the N-glycosylation sites (e.g., N894/900D) reveal that glycosylation is not necessary for normal channel activity or proper folding of CFTR, though distinct reduction of CFTR expressing level in cell membrane was verified [219].

Since the alterations in the glycosylation of CFTR are commonly considered to be involved in the pathogenesis of the CF disease [291, 292], we doubt that glycosylation may also inflect the channel function in molecular level besides reducing surface

expression of CFTR protein. Interestingly, previous study has demonstrated that a disease related mutant T908N created an extra consensus sequence (N-X-S/T) for N-glycosylation and subsequently resulted in a reduced rate of Cl^- efflux and a noisy single channel opening [225]. In addition, it has been proposed that extracellular loops of CFTR not only contribute to its trafficking and maturation but also to the stability of the CFTR channel pore [220]. To provide further insight into the effects of glycosylation on the properties of CFTR channel pore, we designed three mutations (N894D, N900D and N894D/N900D) based on the two N-glycosylation sites in extracellular loop 4, and then analyzed the functional properties of the mutated CFTR chloride channel by using excised inside-out patch clamp recording.

5.2. Single channel results

In this study, we used single channel recording of inside-out patches excised from transiently transfected CHO cells to observe the characteristics of CFTR channel opening. The membrane potential has been held at 0 mV or -50 mV to test the characteristic of the pore's performance depending on the voltage. Under the same experimental conditions, we found diverse channel opening with quite different conductance showed up randomly in the single channel traces of all three mutated CFTR channels (Figure 5.1-5.3). Among them, we distinguished two channel open states: the most abundant one has the same conductance with wild-type CFTR channel, which we named full open state; the smaller one was named half open state since its conductance is approximately half of the full open state. Interestingly, some opening events can hold at the half open state for a moment before fully opened or totally closed (Figure 5.1-5.3). The similar event has been

described previously in R334C mutated CFTR channel, which has been used as an evidence to support the monomer hypothesis of the channel pore [87]. Quite earlier before that, some researchers proposed a CFTR gating model depending on ATP hydrolysis to explain the two open conductance states that they observed in single channel traces of wild-type CFTR [133].

Figure 5.1. Single channel subconductance levels induced by the glycosylation site mutation (N894D). **A.** Single channel currents recorded from the wild type CFTR at 0 mV and -50 mV. Current amplitude histograms are shown at the right panels from the corresponding patches at the membrane potentials 0 mV and -50 mV. **B** and **C.** Example single channel currents recorded from the mutation N894D-CFTR at 0 mV and -50 mV, respectively. The current amplitudes are demonstrated in the all point histograms (right panel) prepared from these single channel current traces from the corresponding patches. For each trace the short dashed line represents the current level when CFTR channels are closed. The dotted lines represent the open states as indicated; C for the closed state, H for a half opened state, F for a full opened state. The unitary currents were recorded in inside-out configuration in each case. PKA (50 nM) and ATP (1 mM) were added at the bath solution to activate the CFTR channels.

Figure 5.2. Mutation at the glycosylation site (N900D) locks CFTR into an open conductance state of prolonged duration. A and B, Example single channel currents recorded from the mutation N900D-CFTR at 0 mV and -50 mV, respectively. Note that N900D-CFTR single channel was locked at the half open state shown at the first trace in panel A. Current amplitudes are demonstrated in the all point histograms (right panel) prepared from these single channel current traces from the corresponding patches. For each trace the short dashed line represents the current level when CFTR channels are closed. The dotted lines represent the open states as indicated; C for the closed state, H for a half opened state, F for a full opened state. The unitary currents were recorded in inside-out configuration in each case. PKA (50 nM) and ATP (1 mM) were added at the bath solution to activate the CFTR channels.

Figure 5.3. Modification of both glycosylation sites (N894D/N900D) increases the half opened conductance state. A and B, Example single channel currents recorded from the mutation N894D/N900D-CFTR at 0 mV and -50 mV, respectively. Note that the locked half opened conductance states could be recorded at both membrane potentials, 0 mV (first trace in A) and -50 mV (first trace from B). Current amplitudes are demonstrated in the all point histograms (right panel) prepared from these single channel current traces from the corresponding patches. For each trace the short dashed line represents the current level when CFTR channels are closed. The dotted lines represent the open states as indicated; C for the closed state, H for a half opened state, F for a full opened state. The unitary currents were recorded in inside-out configuration in each case. PKA (50 nM) and ATP (1 mM) were added at the bath solution to activate the CFTR channels.

However, under the same condition, in present study, the percentage of half opening events in wild-type CFTR, which is around 0.67% (Table 5.1), is obviously lower than previously reported [133]. It seems that the conformational change during wild-type CFTR channel open happened too fast to be recorded. After we abolished one of the two glycosylation sites of CFTR molecule in mutants N894D and N900D, the percentage of half opening events dramatically increased to 10.13% and 7.76% respectively (Table 5.1). Moreover, this percentage increased upwardly to about 37.58% when we mutated both glycosylation sites. Given these data, we could directly couple the glycosylation level of CFTR to the gating conformational change rate of the channel pore.

Table 5.1. Transition topology of channel opening in wild type and mutated CFTR at the glycosylation sites

Transition Topology	Wild Type	N894D	N900D	N894D/N900D
	(Counts in 21 traces)	(Counts in 25 traces)	(Counts in 16 traces)	(Counts in 28 traces)
C↔H	7	123	67	473
C↔F	1034	1331	856	824
C↔H↔F	0	27	5	23
Total Events	1041	1481	928	1320
Percentage of half openings	0.67%	10.13%	7.76%	37.58%

5.3. Macroscopic patch clamp results

To explore the effect of glycosylation on channel pore structure, we studied the macroscopic voltage dependent currents of wild-type and mutated CFTR following activated by ATP and PKA and subsequently locked open by PPI [81]. Two working concentrations of $\text{Au}(\text{CN})_2^-$ (100 μM and 1 mM), which has been used as a probe to characterize the physical property of the opened channel pore [110], were applied to plot the fractional unblocked currents (I/I_0) in present study (Figure 5.4). The results show that the binding affinity of $\text{Au}(\text{CN})_2^-$ in the N894D and N900D single mutated CFTR channel pore is significantly reduced under zero membrane potential (Figure 5.5.A). However, compared to wild-type CFTR, the voltage dependent response was mitigated in the mutated N900D and N894D/N900D CFTR channel (Figure 5.5.B). Since the conformational changes in CLC-0 channel pore caused quite different blocker binding affinity in the open and close state [293], we collectively suggest that the glycosylation of ECL4 may facilitate the conformational change in the CFTR channel pore and help to stabilize pore structure, as has reported previously [220].

Figure 5.4. Glycosylation affects $\text{Au}(\text{CN})_2^-$ blockade of the macroscopic CFTR currents. Examples of leak-subtracted macroscopic currents recorded in inside-out membrane patches following maximal activation with 50 nM PKA, 1 mM MgATP under symmetrical 154 mM Cl^- concentrations. In each case, currents were recorded before (*Control*) and after additions of 100 μM and 1 mM $\text{Au}(\text{CN})_2^-$ to the intracellular solution. For the second row, each panel shows the mean fractions of control current remaining (I/I_0) following additions of 100 μM and 1 mM $\text{Au}(\text{CN})_2^-$ to the intracellular solution as a function of membrane potential. Mean of data from five to seven patches.

Figure 5.5. Effect of different mutations at the glycosylation sites on the apparent affinity and voltage dependence of block by intracellular $\text{Au}(\text{CN})_2^-$. (A) $K_d(0)$ is the dissociation constant of the blocker at zero membrane potential. (B) δ is the fraction of the transmembrane electric field transversed by blockers in reaching their binding site within the pore from the intracellular solution. This coefficient can be used to evaluate the voltage dependence of blockade. All the values were calculated using the equation 3 and 4 (see Section 2.4) and the data from individual patches on the blocking effects of 100 μM intracellular $\text{Au}(\text{CN})_2^-$ as in Figure 5.4. In both cases, * indicates significantly different from wild type CFTR ($p < 0.05$). Mean of data from five to seven patches.

6. The mechanism for large anion permeation in CFTR channel pore

6.1. Introduction

The published paper has demonstrated that the current-voltage relationship of the CFTR channel with symmetrical Cl^- concentration is a straight line [294]. This linear and zero point crossing trace suggests that the chloride ions conduct through the CFTR channel pore in a bidirectional symmetric behavior. However, the asymmetric conduction of large organic anions through CFTR reveals that the mechanism of anion permeation in CFTR channel is still largely in doubt [166].

Previously, it has been proposed that the structure of CFTR channel pore is asymmetric with a relatively large internal vestibule [90, 120, 178]. In addition, several anion binding sites were determined and distributed within the pore asymmetrically [52, 120]. It was widely accepted that the residue T338 in the sixth transmembrane (TM6) α -helix of CFTR is located near the narrowest part of the pore and take a role in forming the selectivity filter [79, 120]. Moreover, the mutation of the positively charged K95 in TM1 caused strong outward rectification which suggests this residue should form an anion binding site locating closely at the cytoplasmic side of the selectivity filter [52, 54]. As was indicated by the predicted α -helical structure of transmembrane segments, the locations of T338 in TM6 and K95 in TM1 are obviously closer to the external mouth of the pore [22, 54]. Through the investigation of the multi-ion behavior and the voltage dependent block in CFTR channel pore, it was further verified that the chemicals entered the pore from the external mouth and internal mouth in a quite different manner [83].

Therefore, it is reasonable to assume that the anion permeation properties of CFTR channel is asymmetric from two sides. But how could this structural and functional asymmetry contribute to the large anion permeation in CFTR channel pore?

We believed that the CFTR channel allows the dissipative diffusion of many inorganic and organic anions that differ widely in size and shape [20, 161]. Especially, CFTR can help to translocate the biomedical important anionic tripeptide glutathione (γ -glutamyl-cysteinyl-glycine, GSH) from the cytosol into the airway surface fluid (ASF), where it functions as the most important extracellular antioxidant, and maintain it in a 50 times higher concentration than that in the cytosol [46, 49]. In this study, we hope to uncover the mechanism for CFTR to translocate large anionic chemicals such as GSH. For this purpose, we should collect more information in terms of sidedness and flexibility of the pore to establish a new anion permeation model to reason the asymmetric large anion permeation through CFTR channel pore. Therefore, we compared the permeation properties of several representative anions in CFTR when they presented intracellularly and extracellularly by employing the relative permeability ratios (P_X/P_{Cl}) as described previously [79] and then tested the flexibility of CFTR channel pore.

6.2. Bidirectional anion permeability ratios

As we have known, the activation trace of wild type CFTR illustrated by I-V relationship is almost linear and the reversal potential is close to zero under symmetric Cl^- concentration (Figure 6.2.A). This has been used as an evidence to support that CFTR is a functionally symmetric channel. However, like most anion channels, CFTR channels

allow the permeation not only of Cl^- , but also of many other monovalent anions. In order to gain more insight into the mechanism of anion permeation in wild type CFTR channel pore, we compared the permeability ratios of ten anions (I^- , Br^- , F^- , SCN^- , gluconate, formate, ClO_4^- , NO_3^- , HCO_3^- and glutathione) relative to that of Cl^- when contained intracellularly or extracellularly (Table 6.1 and Figure 6.1). The data were summarized in table 6.1.

From the macroscopic I-V relationships recorded under biionic condition with I^- in bath or pipette solution (Figure 6.1), we estimated the reversal potential and permeability ratio of I^- relative to that of Cl^- ($P_{\text{I}}/P_{\text{Cl}}$). The mean $P_{\text{I}}/P_{\text{Cl}}$ ratios were 0.30 ± 0.01 and 0.92 ± 0.05 with external and internal I^- respectively (Table 6.1), suggesting that I^- could pass through the pore easier from cytoplasmic side to extracellular side than from opposite direction. The mean $P_{\text{SCN}}/P_{\text{Cl}}$ ratios calculated from the activation traces with SCN^- contained in bath or pipette solution (Figure 6.1) displayed a similar profile as that of I^- . Moreover, the reversal potentials of extracellular reduced glutathione (GSH) can not be measured under current experimental conditions as that of gluconate (Figure 6.1). However, the intracellular glutathione shows 0.15 ± 0.03 relative permeability ratios to that of Cl^- , which means that the glutathione can pass from cytosol to airway surface fluid through the CFTR channel pore (Table 6.1 and Figure 6.1). Relative permeability ratios of intracellular and extracellular anions are compared in table 1, from which we found there were no significant differences between the relative permeabilities of internal and external F^- . However, the asymmetric anion permeation in CFTR could be verified by significant variances between the intracellular and extracellular permeabilities of Br^- , I^- ,

SCN^- , ClO_4^- , NO_3^- , HCO_3^- , HCOO^- and GSH^- (Table 6.1, $p < 0.01$). The data demonstrate that most anions can pass through the CFTR channel pore easier from cytoplasmic side to extracellular side than from opposite direction. Nevertheless, the overall permeability sequence of anions, $\text{SCN}^- > \text{NO}_3^- > \text{Br}^- > \text{Cl}^- > \text{I}^- > \text{HCOO}^- > \text{ClO}_4^- > \text{GSH}^- > \text{HCO}_3^- > \text{F}^- > \text{Gluconate}$, have not been significantly changed regardless of the direction of the anion gradients (Table 6.1). Based on these findings, we suggest that the asymmetric pore structure of CFTR results in a functional asymmetry of anion conduction. However, the biionic experimental conditions may be unequalized from opposite directions. Therefore, we used the symmetric concentration of the same anion at both sides to view whether these anions permeate through the CFTR channel pore in a way as Cl^- .

Table 6.1. The asymmetric relative permeabilities of various anions through wild type CFTR channel pore when contained in extracellular and intracellular solutions

Anion	Intracellular			Extracellular		
	E_{rev} (mV)	P_X/P_{Cl}	n	E_{rev} (mV)	P_X/P_{Cl}	n
Chloride	0.1 ± 0.65	1.01 ± 0.04	6	0.1 ± 0.65	1.01 ± 0.04	6
Iodide	-2.4 ± 1.2	0.92 ± 0.05	5	31.3 ± 0.5	0.30 ± 0.01	3
Bromide	13.2 ± 0.7	1.68 ± 0.05	5	-10.3 ± 0.7	1.50 ± 0.03	5
Fluoride	-60.6 ± 1.2	0.09 ± 0.01	4	59 ± 4.3	0.10 ± 0.020	4
Thiocyanate	38.0 ± 3.6	4.35 ± 0.12	6	-18.7 ± 0.7	2.08 ± 0.10	3
Nitrate	15.8 ± 0.1	1.86 ± 0.03	5	-7.8 ± 2.6	1.37 ± 0.12	5
Perchlorate	-29.7 ± 0.1	0.31 ± 0.01	4	57.2 ± 3.4	0.11 ± 0.01	4
Formiate	-27.6 ± 2.6	0.35 ± 0.05	4	43.6 ± 2.9	0.18 ± 0.02	5
Gluconate	-62.8 ± 2.5	0.09 ± 0.01	4	NA	< 0.1	4
Bicarbonate	-52.3 ± 1.2	0.13 ± 0.01	6	60.0 ± 1.9	0.10 ± 0.006	5
Glutathione	-45.6 ± 5.7	0.15 ± 0.03	7	NA	< 0.1	6

Reversal potentials (E_{rev}) measured from the wild type CFTR channel current-voltage relationships running with various anions contained intracellularly and extracellularly. The relative permeabilities (P_X/P_{Cl}) were calculated from the reversal potentials by using the equation 2 that we have described in Materials and Methods (see Section 2.4). n represents the number of patches we measured for each anion. Mean values were given as mean $\pm$ S.E.

Figure 6.1. Macroscopic activation curves of wild type CFTR voltage dependent currents under biionic condition with Cl^- on one side of the membrane and the indicated anion on the opposite site in the same concentration. Curves were leak-subtracted macroscopic currents recorded in inside-out membrane patches following fully activated by intracellular PKA (50 nM) and ATP (1 mM) and locked open by PPI (2 mM). The traces were recorded as the membrane potential was ramped from -100 mV to +60 mV in 500 milliseconds. The upper panel shows the reversal potentials (the membrane potential where the net current through the channel pore is zero) when iodide, thiocyanate and glutathione presented intracellularly. The lower panel displays the reversal potentials when these anions presented at the extracellular side.

6.3. Asymmetric anion conduction

The macroscopic I-V relationships of three anions (SCN^- , NO_3^- and HCOO^-) presented on both sides of the membrane patches were also recorded (Figure 6.2) so as to determine whether or not the permeation properties of these anions are the same as Cl^- . Compared to the activation trace of CFTR under symmetric Cl^- concentration that was almost linear as showed in Figure 6.2, the traces under symmetric concentration of the other three anions illustrated quite different characters. The trace for HCOO^- on both sides was inwardly rectified (Figure 6.3), which suggests that HCOO^- could pass through the channel easier from inside to outside of the membrane patches than from outside to inside when presented at both sides under symmetric concentration. In contrast, the trace for NO_3^- on both sides was outwardly rectified (Figure 6.3). It was not consistent with the former results which suggested that nitrate could pass through the channel easier when contained internally (Table 6.1). However, this controversy could be explained by multi-ion interaction that the permeation of one ion may be facilitated or obstructed by the other ion when these two ions located in the channel pore simultaneously [287]. As shown in Figure 6.2, the reversal potentials for NO_3^- and HCOO^- were about zero, which means the flows under symmetric NO_3^- and HCOO^- concentration were balanced at zero membrane potential. However, the reversal potential for SCN^- was about -7 mV and the net flow at zero membrane potential under symmetric SCN^- was positive (anion flowed from extracellular side to intracellular side). That means SCN^- can flow from extracellular side to intracellular side through CFTR channel pore under symmetric concentration on both sides without additional pressures. Despite it is hardly to explain these unexpected

phenomena with current data, we have demonstrated that the anion permeation through CFTR channel pore is asymmetric even under symmetric anion concentration.

Figure 6.2. The schematic representations of the macroscopic I-V relationships of wild type CFTR currents under symmetric chloride, nitrate, formate and thiocyanate concentrations respectively. Traces were recorded following addition of PKA (50 nM), ATP (1 mM) and PPi (2 mM). The reversal potentials of CFTR channel currents under symmetric chloride, nitrate and formate are around zero membrane potentials. However, under symmetric thiocyanate, the reversal potential is about -6.26 ± 1.18 mV (average from 5 patches).

Figure 6.3. Rectification ratios of the macroscopic wild type CFTR channel I-V relationships under symmetric chloride, nitrate, formiate and thiocyanate concentrations respectively. The rectification ratios were quantified as the slope conductance at -50 mV as a fraction of that at +50 mV as described in materials and methods. Means of data were averaged from 3 to 5 patches. Asterisks indicate a significant difference from 1 ($p < 0.05$).

6.4. Extremely high voltage pushes $\text{Au}(\text{CN})_2^-$ through CFTR channel pore

The lyotropic pseudohalide anion dicyanoaurate ($\text{Au}(\text{CN})_2^-$) has been used as specific high affinity probes of CFTR channel pore [81]. Applying $\text{Au}(\text{CN})_2^-$ at the cytoplasmic side of the cell membrane strongly inhibited Cl^- current in CFTR channel [81]. However, when applied externally, the inhibition ratio of $\text{Au}(\text{CN})_2^-$ with the same concentration was obviously weakened [81, 177]. It was suggested that $\text{Au}(\text{CN})_2^-$ has a relatively higher binding affinity with the intracellular ion binding site in CFTR channel pore [80, 107]. Otherwise, the relatively large internal vestibule of the pore may facilitate the entrance of $\text{Au}(\text{CN})_2^-$ into the channel [120]. Mutation of several proposed pore-lining residues in the sixth transmembrane segment dramatically altered the $\text{Au}(\text{CN})_2^-$ intrapore anion binding affinity [80]. Especially, the relative $\text{Au}(\text{CN})_2^-$ permeability was significantly enhanced in T338A mutated CFTR channel [54, 80]. Since the mutation T338A was suggested to change the functional properties of the channel pore dominantly by widening the narrowest part in the pore [79, 105, 108], we suggest that the alteration of $\text{Au}(\text{CN})_2^-$ binding affinity and permeation in CFTR channel pore is also due to the conformational change of the pore that resulted from mutation.

Currently, we have no idea about the mechanism for CFTR to conduct the large organic anions such as the reduced glutathione (GSH). Here, we used $\text{Au}(\text{CN})_2^-$ as a probe to test the voltage dependence of the large anion permeation. In wild type CFTR channel, the inhibition ratio of $\text{Au}(\text{CN})_2^-$ reached the highest value and saturated at about -70 mV membrane potential (Figure 6.4.A). In T338A mutated CFTR channel, the voltage dependent fractional unblocked current trace showed a U-shaped plot with lowest value

near to zero membrane potential (Figure 6.4.B), which implicates that Au(CN)_2^- could pass through the T338A mutated channel under the driving force of minus membrane potential. Interestingly, when we extended the minus voltage scale to the limited value of patch clamp protocol at -200 mV, we found that the control current remains increased again under extreme negative membrane potential lower than -170 mV (Figure 6.4.C). The macroscopic I-V relationships ramping from -200 mV to +60 mV under symmetric Cl^- concentration in wild type CFTR demonstrated that the fully activated Cl^- current have not steeply strengthened at extreme negative voltage, whereas the remaining current after applying 1 mM Au(CN)_2^- showed a suddenly enhancement when membrane potential was lower than -170 mV (Figure 6.4.D). These results suggested that the intrapore Au(CN)_2^- began to dissociate from the binding site under the pressure of the extremely high membrane potential. In other words, Au(CN)_2^- , the open channel blocker of CFTR Cl^- channel, could be pushed through the channel from cytoplasmic side to extracellular side by applying appropriate driving force.

Figure 6.4. The voltage-dependent block by 100 μM and 1 mM intracellular Au(CN)_2^- in CFTR channel Cl^- current under symmetric Cl^- concentration. For the upper two plots, the membrane potential was ramped from -100 mV to +60 mV in 500 milliseconds. For the lower plots, the membrane potential was ramped from -200 mV to +60 mV in the same duration. The control currents were activated by intracellular PKA (50 nM) and ATP (1 mM) and locked open by PPI (2 mM). The triangular and circular dots show the mean fractions of control current remains (I/I_0) as a function of membrane potential following additions of 100 μM and 1 mM Au(CN)_2^- to the intracellular solution respectively. Plot A shows the control current remains reach the lowest values at about -70 mV membrane potential in wild type CFTR Cl^- channel. However, when we extended the scale of membrane potential to -200 mV, as shown in C and D, the control current remains increase again under higher minus membrane potential. In T338A mutated CFTR channel, the fractional unblocked current showed a U-shaped plot with lowest value near to zero membrane potential as shown in plot B. The data used in plots A to C were averaged from five to seven recordings of macroscopic I-V relationships as plot D.

7. Discussion

7.1. Revealing the functional cooperation between TM1 and TM6 of the CFTR Chloride Channel through Co-Mutagenesis study

In this study, we found that TM1 and TM6, interacting together, play important roles in forming the channel pore and determining its functional properties by comparing the single channel conductance, anion binding, and anion selectivity properties of different co-mutated channel variants. The results of these investigations are summarized in Table 1. Our finding reinforces previous notion that TM1 and TM6 are involved in the CFTR channel pore formation. Furthermore, we demonstrated that the amino acid threonine (T338) in the TM6 located closely with the amino acids-lysine (K95) and glutamine (Q98) in the TM1. Although the nature of CFTR channel pore remains unclear and many mechanistic details have yet to be clarified, base on our findings, we propose that these two residues, K95 from TM1 and T338 from TM6, form a selectivity filter of the CFTR channels. The emerging picture of CFTR channel pore architecture let us present a partial structural model that supports this view (Figure 7.1).

Several approaches including site-directed mutagenesis [82, 107], using potential probes of the pore [80, 106], cysteine accessibility scanning [89, 115], have been applied to define the pore-lining domains from the existing 12 transmembrane (TM) domains, and the residues that play important roles in establishing the functional profile of CFTR Cl⁻ channel. Although significant progress has been made concerning the involvement of the 12 transmembrane regions in the formation of CFTR channel pore, it is not very sure

about the identity of the pore-forming TMs. We do not clearly understand how these pore-forming TMs or the residues from these TMs work together to construct the architecture and therefore determine functional properties of the CFTR channel pore. Numerous prior studies have illustrated the role of TM6 in controlling CFTR pore properties such as unitary conductance [104, 108, 118, 181, 267], binding of permeant anions [107, 110], and anion selectivity [105, 164, 277]. Previously, we have proposed that anion selectivity is determined primarily by anion binding around F337 and T338 in TM6 [80] and that this central “selectivity filter” is flanked by other binding sites involving R334 and S341. Substitution of these four amino acid residues dramatically changes the CFTR channel pore properties [80], indicating the critical roles in CFTR pore formation played by these residues. Indeed, we found that these four amino acid residues are located in the same hydrophilic facet of the TM6 α -helix (Figure 7.1). Together with other reports [55, 89, 113, 119], we have examined other TMs 1-5 to obtain an overview of the functional roles of these TMs [54] and concluded that TM1 make an important contribution to the pore when it is included in an intact CFTR molecule [54].

In this study, we further investigated the architecture and arrangement of the TM1 and TM6 of CFTR channels. To test our hypothesis that TM1 and TM6 are physically closely located within CFTR protein and confirm our conclusion that TM1 and TM6 are involved in CFTR pore formation, we applied the co-mutagenesis strategy based on the following intuitive principle. If the two mutated residues are independent of one another (that is, if they do not interact), then the effect of the double mutant (two residues from TM1 and TM6, respectively) should demonstrate an additive effects of its two single mutants.

Otherwise, a synergic outcome is expected if two residues form a strong and specific interaction with each other. For example, through co-mutagenesis, previously we found that simultaneous mutations of two close residues (T338A/T339A or T338/S341A) near the middle of TM6 significantly changed single channel conductance, which was even stronger than that of their individual single mutants (T338A and T339A) [79], or was weaker than that of their individual single mutants (T338A and S341A, unpublished data). Another example shows the energetic coupling (interaction) between two CFTR residues (R555 and T1246), expected to lie on opposite sides of the predicted NBD1-NBD2 dimer interface [56]. The unexpected result caused by simultaneous mutations of two neighbour residues show a good example of residue interaction when they are closely located. Taken similar assumption, in present study, we planned to find out whether a direct interaction exists between residues from TM1 and TM6. To achieve our objectives, we chose T338A as an appropriate mutant to study its interactions with other pore lining residues in TM1. Given its outstanding functional properties of T338A-CFTR channel [54, 105, 287], as substitution of the threonine to alanine causes very characteristic functional properties of the T338A-CFTR channels in single channel conductance [287], anion permeability [287] and channel blocker's binding affinity [80, 287], we first selected the mutant (T338A) to characterize the effects of the double mutants (another one from TM1). Next, we chosen several candidate residues from TM1 using T338A as the CFTR control channel variant and constructed all double mutations on this background. The most expected site in TM1 which may have an interaction with T338 is lysine at position 95 (K95), since K95 is located at the same layer as T338 does when these two TMs are arranged together in antiparallel [54]. Previous mutagenesis studies

have implicated that G91, K95 and Q98 were pore-lining residues [89], and the positively charged side chain of K95 may be involved in attracting Cl^- ions and thus contributing to Cl^- ion binding site inside the CFTR channel pore [52, 55]. Therefore, we chose the most extensively studied mutants of these two sites, T338A and K95Q, to construct a double mutated mutant of CFTR protein, in which the threonine at position 338 was substituted by alanine and the lysine at position 95 was substituted by glutamine simultaneously. A direct interaction should be expected if these two residues are located closely enough, as that have been showed in T338A/T339A [79, 105] T338A/S341A and R555K/T1246N [56] double mutations. To find out whether the residues in TM1 interacts with the T338 residue in TM6, we compared the effects of the representative single mutations of CFTR, such as K95Q, A96V and Q98A, with the effects of double mutants on CFTR channel properties. Finally, we interpreted our double mutant results in terms of their single mutant characteristics.

We found that the structural change caused by T338A mutation amplified the outward rectification effect of K95Q single mutation (Figure 3.3). There can be two possible explanations for this phenomenon: first, anion influx increased under positive membrane potential; second, anion efflux decreased under negative membrane potential. Since the single channel current of K95Q/T338A double mutant was smaller than that of K95Q single mutant at -50 mV (Table 3.1), the conclusion could be made that T338A mutation can further reduce the anion efflux caused by K95Q mutation. Unlike the additive interactions between A96V and T338A or Q98A and T338A which we could consider as functional overlapped, the effects of T338A mutation on K95Q single mutant were quite

different from those on wild type CFTR channel. As expected, we could propose that the site K95 in TM1 has a stronger interaction with T338 in TM6 than A96 and Q98, which might due to a direct cooperation within the same anion binding site through hydrogen bond or polar contact. But how does the mutation T338A reduce the anion efflux in K95Q mutant, while increase the single channel conductance in wild type CFTR Cl⁻ channel? Our hypothesis is that the lost of positively charged side chain in K95Q mutant restrained the recruit of anion around the cytoplasmic side of the channel pore [52], and then reduced the efflux of anion. As an important residue in the anion binding site, the hydroxyl group of T338 may assist K95 to recruit anions. In T338A single mutated CFTR channel, the major recruit residue K95 remained and the size of the anion binding site was widened by the substitution, so we got an increase of channel conductance. However, when the major anion recruiter residue K95 was dysfunctional, T338 should play a more important role in anion recruiting than in determining the size of the pore. Therefore, a subdued unitary conductance caused by T338A mutation in K95Q mutant should be expected, if we assumed that K95 and T338 form an anion binding site together near the cytoplasmic side of the CFTR Cl⁻ channel pore.

As a control mutant, we expected no effects of A96V on functional properties of CFTR channel would appear in present study. However the additional side chain added by the substitution may contribute to a structural change of the near anion binding site, and subsequently affected the properties of anion selectivity (Figure 3.5). We also found that the effect of T338A single mutation on unitary current was compromised by simultaneously mutated A96V, but the unitary current of double mutant Q98A/T338A

was the same as that of T338A single mutant. Since this phenomenon could be explained by the structural complement of two near residues, we could propose that the residue A96 in TM1 might locate closer with T338 in TM6 than the site Q98. This could be another evidence to support the hypothesis that K95 might locate closely with T338.

The unusual functional properties on the single channel conductance, anion permeability, macroscopic current rectification and binding affinity of CFTR channel blocker $\text{Au}(\text{CN})_2^-$ of the double mutant K95Q/T338A are caused by the consequences of the strong interaction between K95 in the TM1 and T338A in the TM6. These consequences suggest the possibility that TM1 and TM6 are physically closely arranged within the CFTR protein. Our approach on co-mutation will lead rapidly to a more complete picture of the real architecture of the CFTR channel pore. These findings lead us to propose the following model for the pore of CFTR channel (Figure 7.1). In this model, two α -helices of TM1 and TM6 (may include other TMs) participate in the CFTR channel pore. The hydrophilic facets of TM1 and TM6 face each other (Figure 7.1.C). TM1 and TM6 α -helices are arranged in antiparallel manner, similar to the arrangement of the ClC Cl⁻ channels [9, 275]. TM1 and TM6 helices may be tilted in a small angle so that residues K95, Q98 in TM1 and F337 and T338 can form a helical selectivity filter (Figure 7.1.B). The narrowest part of the pore near T338 may locate in the mid-to-outer regions of the TM1 and TM6. Two experimental findings indicate that the selectivity filter is lined by these four amino acid side chains. Firstly, substituting alanine for threonine at 338 resulted in a significant broadening of the channel diameter, indicating that the amino acid side chain at this point sticks out into the pore and determines its narrowest passage

[287]. Secondly, cysteine introduced at position K95 reacted rapidly with MTS reagents, indicating that the thiol group of cysteine protrudes into the water-filled pore [89]. R334 and S341 residues together with the residues from TM1, most likely P99 and G91 constitute two anion binding sites, one in the outer mouth and another in the intracellular side of the CFTR channel. The results from our study provide the first structural view of the CFTR channel pore, and may form the basis of future studies aimed at the unravelling of the exact mechanisms underlying CFTR anion conduction. Nevertheless, it should be noted that our proposed model refers only to TM1 and TM6, which according to our model constitute the channel and selectivity filter of the pore, and gives no information about the involvement of other TMs. Thus, it will be of great interest to investigate whether the pore of CFTR channel contains other TMs together with TM1 and TM6.

Figure 7.1. A proposed model of the interaction between TM1 and TM6 in forming CFTR channel pore. In this model, we propose that the positively charged residue lysine (K95) and the polar but uncharged residue glutamine (Q98A) in TM1 can form an anion binding site together with the polar residue threonine (T338) in TM6. They are also implied in the formation of the “selectivity filter” of the CFTR channel pore.

7.2. Co-mutations involving residues in TM6 and TM12 reveal a supporting role of TM12 in CFTR channel pore forming

Ion channels allow ions conduction through cell membrane by forming an aqueous pathway. The functional properties of these hydrophilic channel pores are determined by the structure of the pore lining domains and the interaction between permeating ions and these domains. Normally, the channel pore is formed by several homologous domains from different subunits as in ligand gated channels [295] or by repeated motifs in a single peptide as in voltage gated sodium and calcium channels [296]. In CFTR, which contains two roughly repeated halves, it is clear that TM1 and TM6 play important roles in forming the channel pore [120]. However, there is currently lacking strong evidence that supporting a similar role for any other TM. For example, the TM12, which locates at the same place in the second membrane-spanning domain (MSD2) as TM6 in MSD1, was reported to have relative minor contribution to CFTR channel pore forming [53]. It means the contributions of TM6 and TM12 to determining pore properties are highly unequal in despite of their symmetrical positions within the CFTR molecule. However, in present study, we found that the TM12 is a potential candidate to be a pore lining domain since it contains a perfect combo of hydrophilic amino acids, the side chains of which continuously aligned in the same interface of the TM12 α -helix (Table 4.2 and Figure 4.1).

The unitary conductance of CFTR Cl^- channel could be dramatically altered by mutation of several TM6 residues, such as R334 [104], K335 [109], F337 [108], T338 [105], S341 [118], R347 [109] and R352 [111]. But only one of these single mutants T338A has

previously been reported to significantly increase the unitary Cl^- conductance of CFTR [105]. It was suggested that this residue T338 contributes to the narrowest region of the pore. This hypothesis has also been supported by the unitary conductance enhancing effect of the T338A/T339A co-mutation [79]. Interestingly, in present study, we obtained the same enhancing effects as T338A/T339A double mutant by co-mutating two residues from TM6 and TM12. Despite no significant alteration of unitary conductance has been achieved by alanine substitution at the sites T1142 and W1145 (Figure 4.2), we found the enhancing effect of T338A on unitary conductance was further enhanced by simultaneously mutated T1142A and W1145A (Figure 4.3). As we have discussed in Chapter One, this kind of synergic effect of two residues might implicate these two residues are very closely located and have a direct interaction between each other (see Section 3).

Originally, based on the preliminary evaluation, we planned to evaluate the role of TM12 in CFTR channel pore forming in this study. Although we have chosen several different residues in TM12 to design the single mutants, we achieved the same conclusion that mutations in TM12 made no significant effect on single channel conductance as was reported previously [53]. However, further experiments demonstrated that T1142A and W1145A altered the pharmacological properties of CFTR channel in anion binding affinity and voltage dependence of block (Figure 4.4). We have known that the mutation of some residues in many TMs could more or less affect CFTR channel function such as the G314 in TM5 [116] and S1118 in TM11 [117]. But it is still uncertain whether the substitutions of these residues altered the channel function directly or indirectly.

Nevertheless, it seems that TM12 is less important than its “mirror-image” TM6 in forming the pore. Some researchers have suggested that the CFTR channel pore may be formed as homodimer by its two roughly repeated halves [11, 231, 282]. Whatever, these two halves, especially the MSD1 and MSD2, should have some interactions as was suggested recently [288]. The 3-D crystal structure of CFTR implicates a same homodimerization model of CFTR molecule as CLC Cl⁻ channel [9, 11]. This homology model of the membrane-spanning domains was supported by the results that cross-links can be formed between the residue pairs M348-T1142, T351-T1142 and W356-W1145 from TM6 and TM12 respectively [288]. Taken together, it is reasonable to suppose that TM12 may locate near TM6 and affect the functional properties of CFTR channel indirectly by interacting with TM6 (Figure 7.2). By using the co-mutagenesis strategy in current study, we found the synergic effects between the residue pairs T338-T1142 and T338-W1145. The results indicated that T1142 and W1145 in TM12 locate closely to T338 in TM6 and have direct interactions with it. But how could these two proposed non-pore-lining residues cooperate with the crucial pore-lining residue T338 and subsequently affect the single channel conductance? That might be due to the structural variation between wild type and T338A mutated CFTR channels.

Unlike the T1142 and W1145 in TM12, mutating K95 in TM1, which also interplayed with T338, caused a significant reduction in single channel conductance and an outward rectification of current-voltage relationship (see Section 3). And also, K95 was suggested to be a pore-lining residue by several lines of evidence [54, 55, 89, 120]. Therefore, the interaction between K95 and T338 should occur within the pore and have synergic effects

on the channel pore properties (see Section 3). However, for those non-pore-lining TM12 residues T1142 and W1145, they might only interplay with T338 elsewhere beside the conduction way in the pore and affect the pore structure by reducing the supporting force on the wall of the channel pore. Previous studies have demonstrated that $\text{Au}(\text{CN})_2^-$ can binding within the CFTR channel pore firmly from the intracellular vestibule [81]. In contrast, the $\text{Au}(\text{CN})_2^-$ binding affinity was dramatically reduced in T338A mutated CFTR channel [80, 287]. Therefore, we assume that the wild type CFTR channel pore was rarely affected by the alanine substitutions at T1142 and W1145 since it has a relatively firm structure. After the alanine substitution at T338 in TM6 weakened the wall to enhance the permeating anion conduction, the side chains of neighboring residues T1142 and W1145 may help to maintain the wall structure at their contact interface (Figure 7.2). Based on this assumption, co-mutating one of these two supporting residues together with T338A may cause a further modification of the pore conformation and then widen the dimension of the pore. Subsequently, we obtained enhancing Cl^- current in single channel recording due to the wider pore dimension. Here, we suggest that the residues T1142 and W1145 in TM12 functions as a scaffold to support the wall structure of CFTR channel pore by interacting with T338 in TM6.

Figure 7.2. The proposed model for TM12 and TM6 interaction in CFTR channel.

Red indicates continuously aligned hydrophilic amino acids in TM12 and TM6. T338 in TM6 is believed to be a very important pore-lining residue. T1142 and W1145 in TM12 may may help to maintain the wall structure of the pore after alanine substitution of T338.

This study implicates that the co-mutagenesis strategy is very useful and sensitive in scanning cooperative residue pairs. By determining the cooperation residue pairs from various domains, we can image the spatial arrangement of these domains in one molecule and infer the functional roles of these domains. For example, the single mutations R555K in NBD1 and T1246N in NBD2 significantly altered the open probability (P_o) of CFTR channel, whereas co-mutating these two residues restored the energetic coupling or interaction between these two closely located sites [56]. This finding helped Vergani to establish the dimer interface between NBD1 and NBD2 and explain the mechanism of CFTR channel gating by ATP-driven dimerization of its NBDs [56]. In previous study, we found the synergic effect of K95 in TM1 and T338 in TM6 and inferred that TM1 and TM6 may form the CFTR channel pore together (see Section 3.4). In present study, the cooperation between the residue pairs T338-T1142 and T338-W1145 suggested the supporting role of TM12 in maintaining the CFTR pore structure. Therefore, we think it should be a good idea to use the co-mutagenesis strategy together with patch clamp functional study to investigate the interaction between amino acid residues, if we have any doubt concerning the structural or functional cooperation among various domains of an ion channel.

7.3. Glycosylation affects CFTR conformational states associated with channel gating

We have demonstrated that incomplete glycosylated, especially nonglycosylated, CFTR channel significantly increased the occurrence of subconductance behavior in its single channel traces (Table 5.1) and altered the binding affinity of $\text{Au}(\text{CN})_2^-$ in the channel

pore (Figure 5.4). These subconductance states were also reported with exon 5 deleting CFTR channel, which lacks 30 amino acids in the first intracellular loop [228]. Moreover, in an earlier study, deleting 19 amino acids from the second intracellular loop showed more subconductance behavior of the CFTR channel [230]. Some suggested that the ICLs are predicted to be involved in channel gating and stabilization of conductance states by interacting with R domain [226, 262]; the others predicted that the intracellular loops act as a bridge between the NBDs and the TMs to transmit energy from ATP hydrolysis at the NBDs into a conformational change of the pore [228, 231] since applying nonhydrolyzable nucleoside triphosphate prolonged the subconductance state in wild-type CFTR Cl⁻ channel [133, 294]. Based on the data described above, it seems that the CFTR molecules also need the oligosaccharides on ECL4 to facilitate the energy transmission and conformational changes that cause channel open and close. To reveal the mechanism for this kind of modulation, the following facts should be considered.

Opening and closing of CFTR channel pore have been linked to ATP binding and hydrolysis at two nucleotide-binding domains (NBDs) of CFTR [66, 133]. Lots of evidences have demonstrated that NBDs can dimerize upon binding ATP [58, 59] and this ATP-driven dimerization of NBDs was supposed to be involved in CFTR channel opening [56, 100]. Whether the dimerization occurred between the two NBDs in the same CFTR protein or between NBDs from different molecules remains unclear. Besides NBDs, a large regulatory domain (R domain) also locates intracellularly, which connects the two halves of CFTR molecule [22]. As a widely accepted hypothesis, CFTR chloride channel is activated by phosphorylation of the R domain via cAMP activated protein

kinase A (PKA) and ATP binding and hydrolysis at NBDs [132, 297]. This large and irregularly formed R domain may need a complicated intermolecular cooperation to perform its function in delicate regulation of channel gating because of the scattered distributed phosphorylation sites in R domain of CFTR protein [141, 149]. Such an intermolecular cooperation can also provide more chances for NBDs dimerization which was supposed to be important in channel gating [56].

Previous studies have defined that glycosylation play a dominant role in apical clustering of most glycoproteins expressed in epithelial cells [298, 299]. The clustered distribution of glycoprotein may affect channel gating under unclear mechanism, such as the modulation of AMPAR synaptic clustering affected AMPAR channel gating by desensitization and deactivation [300-302]. Therefore, a social interplay among this kind of clustering glycoprotein could be expected to play a role in channel gating modulation. In addition, the oligosaccharides on the CFTR glycoprotein can be recognized by some ligands [223, 303] and make it possible to communicate with other molecules at the extracellular side. In another word, the oligosaccharides on the ECL4 of CFTR may function like a bridgehead to interplay with others. Here, we have drawn a graph to illustrate the interaction among CFTR proteins (Figure 7.3).

After we disabled the two N-glycosylation sites at positions N894 and N900, such kind of intermolecular communication became difficult. Low surface expression level of CFTR protein caused by incomplete glycosylation [219, 224] dramatically reduced the chance of intermolecular cooperation between CFTR proteins. The clustering CFTR glycoprotein

scattered after losing the cooperation platform. Since the CFTR have to do the channel function as a monomer or cooperate with others just by chance, we obtained more possibilities to record down the transition incompletely activated channel opening.

Collectively, we conclude that disabling the CFTR glycosylation sites N984 and N900 significantly elongate the transition half open state during channel opening, which indicates intermolecular cooperation might be involved in channel gating of CFTR chloride channel. This work has implicated a network model for CFTR molecule conducting its channel function.

Figure 7.3. The clustering distribution and protein-protein interaction among CFTR proteins mediated or facilitated by the oligosaccharides on the ECL4. The “bridge protein” mediates the clustering distribution of CFTR proteins by adhering to the oligosaccharides on them, and facilitates protein-protein interaction via the PDZ domain binding motif in the C-terminal of CFTR protein. The clustering of CFTR proteins may facilitate the homo- or hetero- dimerization of nucleotide binding domains (NBDs) and the multiple sites phosphorylation of regulatory (R) domain.

7.4. Asymmetric anion permeability through wild type CFTR chloride channel reveals a flexible conformation of the pore

This study demonstrates that the CFTR Cl⁻ channel pore is asymmetric in terms of anion permeation. Comparison of the relative permeability ratios of intracellular and extracellular anions to that of Cl⁻ shows that most anions, especially the large organic anions, can pass through the CFTR channel pore easier from cytoplasmic side to extracellular side than from opposite direction. Furthermore, the anion permeation properties are also asymmetric from opposite directions under symmetric SCN⁻, NO₃⁻ and HCOO⁻ concentrations. Particularly, we studied the permeation of the large organic open channel blocker dicyanoaurate (Au(CN)₂⁻) to reveal the mechanism for large anion permeation in CFTR channel pore.

We found that the reduced glutathione (GSH) can pass through the CFTR channel pore easier from cytosol to airway surface fluid (ASF) than from opposite direction as was reported previously [45, 46]. GSH is the most important extracellular antioxidant that have found in ASF and presents in the concentration of approximate 400 μM which is about 50 times the concentration in plasma and the external fluids of many other tissues [48, 50]. In the ASF of cystic fibrosis patients the concentration of GSH is dramatically reduced [47, 49], which suggests that the function of CFTR for GSH permeation is crucial to release the oxidative stress that accumulated from chronic inflammation of the airway [49]. However, the mechanism for GSH permeation in CFTR is still uncertain.

Functional studies of CFTR channel suggests that permeating anions can bind to several discrete binding sites in the pore [110, 177]. Mutagenesis studies shows that the positively charged side chains of K95 in TM1 [52, 54] and R334 in TM6 [107, 181] may help to attract anions into the pore and contribute to form different anion binding sites respectively located closely at the cytoplasmic side and external side of the selectivity filter in CFTR channel pore [52, 54]. Since it is unreasonable to have absolutely same binding affinities of anions on different binding sites that formed asymmetrically within the pore, we may assume that the GSH enters the pore easier from cytoplasmic side and binds to the internal binding site tighter. Then, the local anion concentration near the internal mouse of the pore should be maintained at a higher level due to the more powerful attracting force provided by the binding site in the cytoplasmic vestibule. Previous studies have suggested that CFTR channel pore has a relatively larger intracellular vestibule [90, 120, 178] and several positively charged residues that located in this big chamber such as R347 and R352 may help to maintain the local concentration of anions near the internal mouse of the pore. Therefore, GSH can pass through the CFTR channel pore easier from cytosol to external ASF through the concentration gradient. However, this hypothesis cannot explain the symmetric permeation of Cl^- and many other anions in CFTR pore when presented at both sides with symmetric concentration. Since the reversal potential of the voltage dependant currents is minus under symmetric SCN^- concentration, it was suggested that SCN^- should flow from extracellular side into intracellular side at zero membrane potential. Moreover, we have demonstrated that the asymmetric permeation of other anions such as SCN^- , NO_3^- and HCOO^- acts quite differently from each other. This kind of variation among monovalent

anions cannot be explained only by the electrogradient and the dynamic properties of the binding sites. For GSH itself, the local electrogradient induced by CFTR channel pore is hardly to maintain about 50 times higher concentration of GSH in external ASF. Taken together, we think other structural properties of CFTR channel pore may also contribute to translocate GSH⁻ from cytosol to ASF and prevent it flow back.

Several lines of evidence have implicated that CFTR conducts a conformational change in the pore during channel gating [85, 133, 294]. Unlike the inhibitory “ball” channel gating scheme of Shaker K⁺ channels [154, 155], CFTR channel pore has been supposed to open by the conformational change of the membrane spanning domains (MSDs) driven by the dimerization of nucleotide binding domains (NBDs) [56]. Our results also demonstrated two opening states of CFTR channel by eliminating the glycosylation sites (see Section 5). The subconductance states we have recorded in unitary current traces might be caused by incomplete conformational change of the pore during CFTR channel opening (see Section 5). Therefore, the wall of the pore should have a flexible structure that can conduct a conformational change during channel gating. Does this flexible structure of CFTR channel pore take a role in large anion permeation? To answer this question, we should choose an appropriate probe to test the stability of the wild type CFTR channel pore.

The pharmacological properties of some CFTR channel blockers indicated that these blockers inhibit CFTR Cl⁻ current by occluding the internal vestibule at the open state [91]. So we called these blockers open channel blockers which includes various large

organic anions such as glibenclamide [74] and dicyanoaurate ($\text{Au}(\text{CN})_2^-$) [81]. Since applying $\text{Au}(\text{CN})_2^-$ intracellularly caused an outward rectification of the voltage dependent Cl^- currents in CFTR channel [80, 81], we believe that $\text{Au}(\text{CN})_2^-$ cannot pass through the channel pore into the external solution due to the structural restriction of the pore [90, 91]. However, these large organic blockers also have the opportunity to permeate through the pore by damaging the structure of the pore. For example, mutating T338 in TM6 caused an enhanced permeation of $\text{Au}(\text{CN})_2^-$ under negative membrane potential by widening the dimension of CFTR channel pore [80]. We have proposed that CFTR channel pore has a flexible structure that can undergo a conformational change during channel opening. If the opened CFTR channel also has a flexible wall that can transform under certain pressure, we may push the large anions through it by applying enough driving force. To access this hypothesis, in present study, we used the well established open channel blocker $\text{Au}(\text{CN})_2^-$ to test the stability of the opened CFTR channel pore.

Interestingly, we found that the intrapore $\text{Au}(\text{CN})_2^-$ could be pushed through CFTR channel by using extreme negative membrane potential. Since we have discussed the contribution of the electrogradient and the binding sites above, we suggest that the applied driving force can push the large anion against the flexible wall and subsequently press the pore wider to squeeze the anion through it. Here, we proposed a scheme that CFTR channel applies to translocate large organic anions, especially the GSH^- . As we have mentioned, the CFTR channel pore contains a deep wide intracellular vestibule and a shallow external vestibule separated by a narrow selectivity filter [90, 120]. The results

indicate that large organic anions can pass through CFTR pore easier from the cytoplasmic side to extracellular side than from opposite direction. In addition, the intrapore open channel blocker $\text{Au}(\text{CN})_2^-$ can be squeezed out to external fluid by extremely minus membrane potential. Taken together, we suggest that the CFTR channel pore contains a flexible wall as the entrance bars of a fishing basket (Figure 7.4). As we have shown in figure 6.5, the fish can easily pass the flexible sloping bars by applying a little force. After they enter the trap, they have no way to press the bars since there is no slope provided inside. The deep wide intracellular vestibule in CFTR channel pore may function as the sloping bars of fishing basket to facilitate the permeation of large organic anions from cytoplasmic side to extracellular side (Figure 7.5). However, from opposite direction, the shallow external mouth of the pore occludes these large anions outside. By using this fishing basket model, in Figure 7.5, we demonstrated the mechanism for asymmetric GSH permeating in CFTR channel pore and explains why they can maintain 50 times higher concentration in airway surface fluid (ASF). Under normal conditions, CFTR functions as a chloride channel which is regulated open by PKA and ATP and can be blocked by $\text{Au}(\text{CN})_2^-$ (Figure 7.5.A-C). However, if the membrane potential provides enough pressure on some large anionic chemicals, the CFTR channel pore could be squeezed wider to act as a large anion transporter (Figure 7.5.D-E).

A. The bamboo fishing basket contains a circle of flexible bars that can bend under appropriate force.

B. Allured by the bait, the fish struggles against the flexible bars and bends them to enter the snare.

C. The flexible bars spring back to the former positions after the fish passed through them and occlude the fish inside the snare.

Figure 7.4. The illustration of the fishing basket model. This graph series demonstrates the working mechanism of a fishing basket.

Figure 7.5. The mechanism for large anion permeation in CFTR channel explained by the fishing basket model. (A) The closed CFTR channel pore. The channel contains a relatively large intracellular vestibule and a shallow external vestibule separated by the selectivity filter. Chloride ions cannot pass the closed CFTR pore. (B) Driven by the hydrolyzation of ATP and the PKA catalyzed phosphorylation, CFTR channel pore performs a conformational change to the open state and begins to conduct Cl^- . (C) When we applied the open channel blocker $\text{Au}(\text{CN})_2^-$ intracellularly, this large anion inhibits the Cl^- current in CFTR by occluding the intracellular vestibule. (D) Under extremely minus membrane potential, the intrapore $\text{Au}(\text{CN})_2^-$ is squeezed out from the intracellular side to external side, which implicates that CFTR channel pore contains a flexible wall. (E) Other large organic anions such as GSH may also pass through the CFTR pore by using the same strategy as $\text{Au}(\text{CN})_2^-$. (F) In the fishing basket model, we assume that the flexible wall of the CFTR channel pore may act as the entrance bars of the fishing basket and control the direction of large anion permeation by using the same strategy as we have demonstrated in Figure 7.4.

CFTR has been suggested to evolve from transporters since it belongs to the ABC transporter superfamily [304]. The multidrug resistance protein (MRP), a member of ABC transporters [305], mediates the active transport of phospholipids and chemotherapeutic drug across cell membranes [195-197]. Similar to CFTR which can translocate GSH, MRP can also transport GSH conjugates *in vitro* [306]. Here, we suppose that CFTR may be in the intermediate stage evolving from a transporter to an ion channel. The function of CFTR for large organic anion conduction might be inherited from its transporter ancestors. As we have demonstrated in Figure 6.6, the fishing basket model for large anion permeation is only effective for the opened CFTR channel pore. Therefore, the large anions can be translocated through the pore only if the CFTR channel has been activated depending on the ATP-driven dimerization of NBDs [56, 57]. Albeit that is very less, CFTR still need to consume energy for large anion translocation. So we can assume that CFTR may both function as a chloride channel and a large anionic chemical transporter. Of course, we need more evidence to support it. However, the fishing basket model provides us more options in proofing this assumption and also in considering the working mechanism of the large anion transporters, voltage dependent ion channels and mechanosensitive ion channels.

This work has implications for the mechanism of anion permeation in CFTR and other anion channels. Currently, we are used to analyze the pharmacological properties of anion channels by the Woodhull model [307]. This two-barrier model was supposed originally to analyze and interpret the voltage dependence of blockade on sodium channels. Other researchers also suggested that the ion selectivity of cation channel may

originate from simple physical mechanisms [308]. Actually, all the currently used models are based on one assumption: the structure of the opened channel pore is fixed when it conducts ions. For cation channels, Woodhull model has been successfully used to analyze the relationship between the fractional unblocked current and membrane potential. However, we could not explain the large anion permeation and the multi-ionic behavior in CFTR channel by using currently available models and rate theories. By suggesting the fishing basket model, we separate the mechanism of ion conduction of anion channels apart from that of cation channels. The pore of cation channel is very firm and is regulated to open in an all or nil model. We only need to consider the electrostatic interactions in simulating the ion permeation in cation channels. In contrast, the anion channel has a soft pore and is modulated to open step by step. Under some conditions, we have to consider the physical driving force to explain the anion permeation in anion channels. The fishing basket model we proposed here may help to explain the relative nonselective anion permeation and large organic anion conduction in anion channels.

7.5. Summary

The project presented in this thesis aimed to reveal the molecular determinant of anion permeating mechanism in the channel pore of cystic fibrosis transmembrane conductance regulator (CFTR). For this purpose, we have determined several pore-forming domains by using the co-mutagenesis strategy and confirmed several conformational stages of the pore during channel opening.

To obtain insight of the architecture of CFTR channel pore, we used a strategy of co-mutagenesis from two potential pore-forming domains to investigate the involvement of these two TMs. Our results on a range of specific functional assays which compared the single channel conductance, anion binding, and anion selectivity properties of different co-mutated channel variants suggest that TM1 and TM6 do play vital roles in forming the channel pore and thus in determining the channel functional properties. Furthermore, we provide functional evidence that the residue T338 in TM6 interacts with the amino acids K95 and Q98 in TM1. By using the same strategy of co-mutagenesis from TM6 and TM12, we found that mutations of T1142A and W1145A did affect the functional properties of CFTR channel in terms of the apparent binding affinity and voltage dependence of blockade by intracellular $\text{Au}(\text{CN})_2^-$. On the other hand, the T1142A and W1145A single mutated CFTR channel had the same single channel current as wild type CFTR. Interestingly, the single channel currents of T1142A/T338A and W1145A/T338A double mutated CFTR channels were dramatically strengthened and were even bigger than that of T338A single mutated CFTR channel which suggest there are synergic effects between the residue pairs T1142-T338 and W1145-T338. Collectively, we suppose that the amino acids K95 and Q98 from TM1 and T338 from TM6 form a selectivity filter of the CFTR channels and the residues T1142 and W1145 from TM12 may function as a scaffold to support the wall structure of CFTR channel pore by interacting with T338 from TM6.

Besides the transmembrane segments (TMs), extracellular loops (ECLs) were also considered as the candidates for CFTR channel pore formation. The glycosylation on

extracellular loop 4 of CFTR was reported to contribute to the stability of the CFTR channel pore. To evaluate the effects of glycosylation on the functional properties of CFTR, we constructed three mutations that abolished either one (N894D and N900D) or both (N894D/N900D) extracellular glycosylation sites. Our results showed that both partially glycosylated or fully nonglycosylated CFTR displayed functional variations from wild type concerning the anion binding affinity and voltage dependence of blockade by intracellular $\text{Au}(\text{CN})_2^-$. Single channel traces revealed that elimination of either one or both of the glycosylation sites could induce a subconductance level of CFTR channel opened state. Abolishment of both the glycosylation sites (N894D/N900D) increases the event frequency of subconductance level. We concluded that the glycosylation on ECL 4 plays a vital role in CFTR channel gating and CFTR conformational transition of the pore.

CFTR allows the permeation of various anions that differ widely in size and shape. To reveal how CFTR help to asymmetrically translocate large anionic chemicals, we compared the relative permeation ratios of various anions to that of Cl^- and showed that most anions could pass through the CFTR channel pore easier from cytoplasmic side to external fluid than from opposite direction. To reveal the mechanism of the asymmetric large anion permeation (for example, GSH) in CFTR channel pore, we proposed a new “fishing basket” model which is based on the hypothesis that the CFTR channel pore is flexible and can perform a further conformational change under appropriate pressure after channel opened. To test it, we used the extreme negative membrane potential to analyze the voltage dependence of blockade by the intracellular open CFTR channel blocker $\text{Au}(\text{CN})_2^-$. As we expected, the control current remains (I/I_0) reached the lowest value at

about -170 mV and increased again when the membrane potential reduced lower than -170 mV. The data demonstrated that a proportion of $\text{Au}(\text{CN})_2^-$ ions were pushed out from the cytoplasmic side of the pore to the external side under the extreme negative membrane potential. Therefore, we concluded that the CFTR channel pore is flexible and the fishing basket model is suitable to explain the mechanism for asymmetric large anion permeation in CFTR pore. Taken together, we found that CFTR pore has several conformational stages including the close stage, subconductance stage, open stage for small anion permeation and squeezed wider open stage for large anion permeation. Therefore, we suggested that the CFTR channel gating is modulated by the conformational change of membrane spanning domains (MSDs) coupled with the phosphorylation and ATP hydrolysis at NBDs.

This study implicates that the co-mutagenesis strategy is very useful and sensitive in scanning cooperative residue pairs. It should be a good idea to construct a double mutant if we want to test the functional or structural cooperation between amino acid residues from various domains of an ion channel. For example, we propose there should have some synergic effects between the residues T338 in TM6 and G314 in TM5 of CFTR. We can construct a double mutant T338A/G314A to evaluate our proposal by the functional study of patch clamp. Step by step, after we screen out all the cooperative residue pairs from various domains of the CFTR channel, we can infer the functional roles of these domains and image the spatial arrangement of them in a whole molecule.

This work also has implications for the mechanism of anion permeation in CFTR and other anion channels. Our fishing basket model for the large organic anion conduction in CFTR channels could be tested for the working mechanism of the large anion transporters (such as MRPs) and mechanosensitive ion channels (such as ClC channels). Moreover, this model may help us to determine the relationship between ion channels and transmembrane transporters.

8. Reference

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