^{1}H-NMR and Circular Dichroism Spectroscopic Studies on Changes in Secondary Structures of the Sodium Channel Inactivation Gate Peptides as Caused by the Pentapeptide KIFMK

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ABSTRACT The pentapeptide KIFMK, which contains three clustered hydrophobic amino acid residues of isoleucine, phenylalanine, and methionine (IFM) in the sodium channel inactivation gate on the cytoplasmic linker between domains III and IV (III-IV linker), is known to restore fast inactivation to the mutant sodium channels having a defective inactivation gate or to accelerate the inactivation of the wild-type sodium channels. To investigate the docking site of KIFMK and to clarify the mechanisms for restoring the fast inactivation, we have studied the interactions between KIFMK and the fragment peptide in the III-IV linker GGQDIFMTEEQK (MP-1A; G1484-K1495 in rat brain IIA) by one- and two-dimensional ^{1}H-NMR and circular dichroism (CD) spectroscopies. KIFMK was found to increase the helical content of MP-1A in 80% trifluoroethanol (TFE) solution by ~11%. A pentapeptide, KIFMT, which can restore inactivation but less effectively than KIFMK, also increased the helical content of MP-1A, but to a lesser extent (~6%) than did KIFMK. In contrast, KDIFMTK, which is ineffective in restoring inactivation, decreased the helical content (~−4%). Furthermore, we studied the interactions between KIFMK and modified peptides from MP-1A, that is, MP-1NA (D1487N), MP-1QEA (E1492Q), or MP-1EQA (E1493Q). The KIFMK was found to increase the helical content of MP-1EQA to an extent nearly identical to that of MP-1A, whereas it was found to decrease those of MP-1NA and MP-1QEA. These findings mean that KIFMK, by allowing each of the Lys residues to interact with D1487 and E1492, respectively, stabilized the helical structure of the III-IV linker around the IFM residues. This helix-stabilizing effect of KIFMK on the III-IV linker may restore and/or accelerate fast inactivation to the sodium channels having a defective inactivation gate or to wild-type sodium channels.

INTRODUCTION

Voltage-gated sodium channels are membrane-spanning proteins that pass sodium ions through the membrane from extracellular to cytoplasmic sides in response to action potentials (Hille, 1992). The inward sodium current is terminated by the closing of an inactivation gate; this process is called a fast inactivation. Subsequently, the channels return to an initial resting state. The inactivation gate is considered to be formed from three clustered hydrophobic amino acid residues of isoleucine, phenylalanine, and methionine (I1488, F1489, and M1490 in rat brain type IIA) on the cytoplasmic linker between domains IIIS6 and IVS1 (III-IV linker) as an inactivation particle that occludes the intracellular mouth of the pore (West et al., 1992; Patton et al., 1992), and from their receptors, which are composed of short S4-S5 loops of both domains III (Smith and Goldin, 1997) and IV (Tang et al., 1996; Lerche et al., 1997; McPhee et al., 1998). In the inactivation particle of IFM residues, F1489 plays the most important role in occluding the pore; if F1489Q-mutated sodium channels are employed, the fast inactivation is removed (West et al., 1992).

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Recently, Eaholtz et al. have reported interesting findings in which a synthetic pentapeptide containing the IFM sequence and two lysine residues on both sides of it (KIFMK) restores fast inactivation to the mutant sodium channels (F1489Q) having a defective inactivation gate and to wild-type sodium channels having inactivation slowed by α-scorpion toxin (Eaholtz et al., 1994, 1998). The pentapeptide KIFMK also competes with the intrinsic inactivation particle and binds to and blocks open sodium channels in a voltage- and frequency-dependent manner, accelerating the inactivation of the wild-type sodium current. They also reported that KDIFMTK is ineffective in restoring inactivation, whereas the pentapeptide KIFMT, having a single positive charge, restores inactivation but is less effective than KIFMK (Eaholtz et al., 1994). The aspartic acid (D) and threonine (T) residues before and after the IFM motif in KDIFMTK are those following the sequence of wild-type sodium channels. Summarizing their results, it is suggested that both the IFM motif and two Lys residues are indispensable for a synthetic peptide to be effective in restoring fast inactivation. The amino acid sequence of the III-IV linker reported (rat brain type IIA) is as follows: DNFNQQKKKF-GGQDIFMTEEQKKYYNAMKKLGSKKPQKPIPRPAN-KFQGMVFD(1474–1526) (Noda et al., 1986). For this sequence, we notice that except for the N- and C-terminal Asp residues, there are negatively charged amino acids only on both sides of the IFM residues, i.e., D1487, E1492, and E1493. Importantly, this finding is also true for human brain

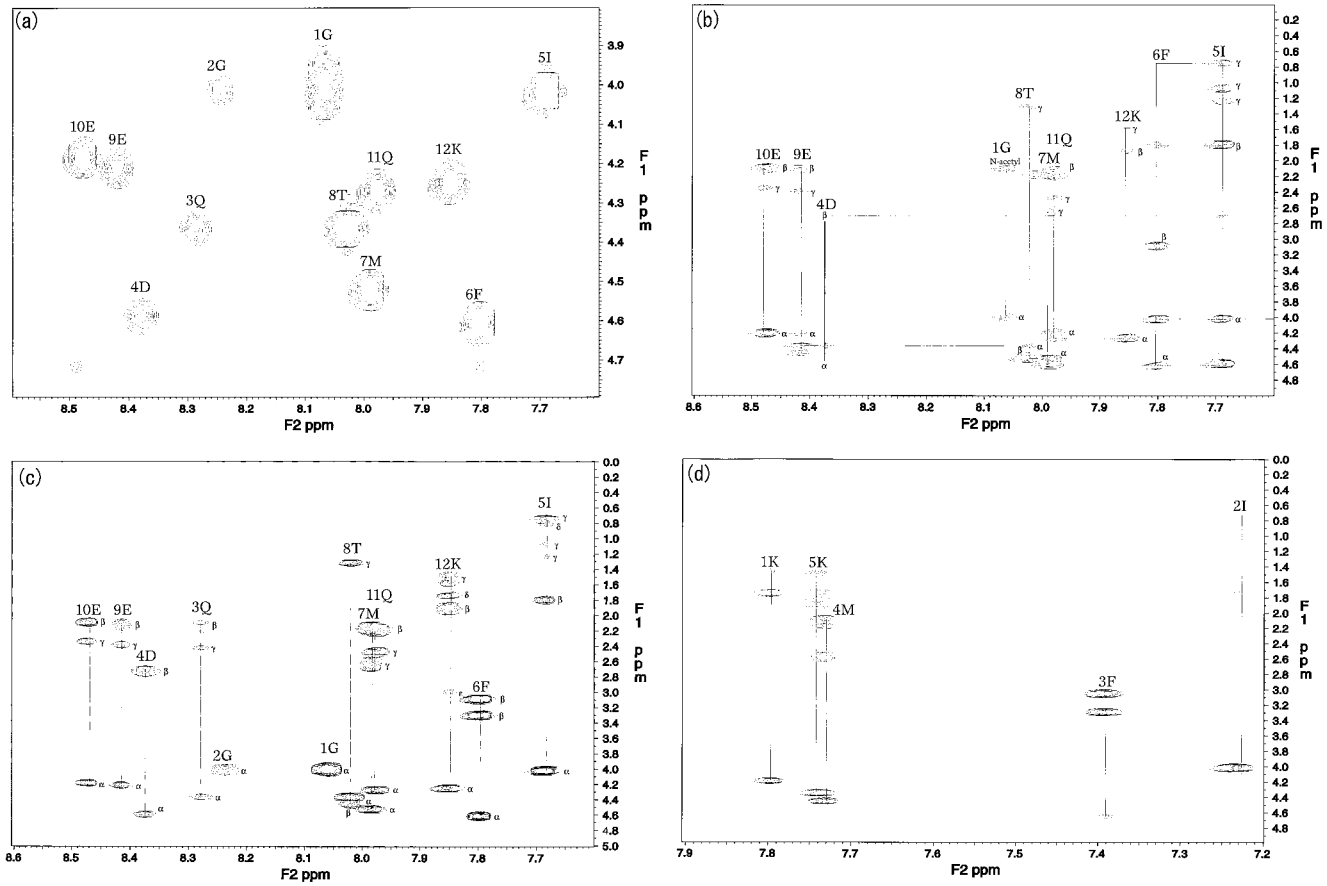

FIGURE 1 Two-dimensional (*a*) COSY, (*b*) ROESY and (*c*) TOCSY spectra of MP-1A in 80% TFE-d_2-20% H_2O solution, and (*d*) TOCSY spectrum of KIFMK in 80% TFE-d_2-20% H_2O solution. In *a–c*, 1G-12K correspond to Gly-1484–Lys-1495.

(Ahmed et al., 1992), human heart (Gellens et al., 1992), and human skeletal muscle (George et al., 1992) sodium channels. Based on these findings, we speculate that the pentapeptide KIFMK, especially its two Lys residues, may be interacting with those negatively charged amino acids located before and after the IFM motif. In the present study, we synthesized a fragment peptide that includes the IFM motif and the negatively charged amino acid residues, i.e., acetyl-GGQDIFMTEEQK-amide (MP-1A), as a model peptide for the III-IV linker. We studied the interactions of MP-1A with KIFMK, KIFMT, or KDIFMTK by ^{1}H-NMR and CD spectroscopies; these three peptides were chosen, respectively, as examples having activities that are fully effective, partly effective, and ineffective in restoring and/or accelerating fast inactivation. Interactions between MP-1A and the pentapeptide KIFMK, which was synthesized using D-Lys instead of L-Lys, have also been studied to elucidate the importance of the two lysine residues in the electrostatic interactions with the negatively charged amino acid residues. Moreover, to reveal which negatively charged amino acids are important to the interaction with the two lysine residues, we also studied the interactions between KIFMK and some modified peptides from MP-1A, that is, MP-1NA (D1487N), MP-1QEA (E1492Q), and MP-1EQA (E1493Q). The ^{1}H-NMR chemical shift changes revealed that KIFMK stabilizes the helical conformation of MP-1A, whereas KDIFMTK destabilizes it. The CD spectra gave supporting evidence for these findings.

MATERIALS AND METHODS

Materials

All of the peptides were synthesized automatically by the solid-phase method, using Fmoc chemistry on an Applied Biosystems 433A peptide synthesizer; their N-termini were acetylated (denoted by Ac-), and their C-termini were amidated (denoted by -NH_2). They were purified on a reverse-phase C_{18} high-performance liquid chromatography column using a gradient of 90% A, 10% B to 70% A, 30% B, where A is 0.1% trifluoroacetic acid (TFA) in water and B is 0.1% TFA in acetonitrile; the rate of decrease in A was 20%/40 min. The peptides were characterized by ion spray mass spectrometry. The amino acid sequences of the peptides were MP-1A, Ac-GGQDIFMTEEQK-NH_2; MP-1NA, Ac-GGQNIFMTEEQK-NH_2; MP-1EQA, Ac-GGQDIFMTEQQK-NH_2; MP-1QEA, Ac-GGQDIFMTQEQK-NH_2; KIFMK, Ac-KIFMK-NH_2; KIFMT, Ac-KIFMT-NH_2; KDIFMTK, Ac-KDIFMTK-NH_2; KIFMK, Ac-KIFMK-NH_2 (K means D-Lys).

Trifluoroethanol-d_2 (TFE-d_2; 2,2,2-trifluoroethyl-1,1-d_2 alcohol) was obtained from ISOTEC (Miamisburg, OH).

TABLE 1 ^{1}H chemical shifts (in ppm) of MP-1A and KIFMK in 80% TFE-d_2 solutions

Residue	NH	α H	β H	γ H	Other
			MP-1A		
1G	8.07	4.01			
2G	8.25	4.02			
3Q	8.28	4.36	2.21	2.42	6.57 (δ NH_2)
4D	8.38	4.59	2.74		
5I	7.69	4.03	1.80	0.75, 1.07, 1.23	0.81 (δ CH_3)
6F	7.80	4.61	3.09, 3.30		7.34 (phenyl)
7M	7.99	4.52	2.18	2.46, 2.59	
8T	8.03	4.38	4.46	1.31	
9E	8.42	4.19	2.10	2.39	
10E	8.48	4.18	2.08	2.34	
11Q	7.98	4.27	2.21	2.47	6.62 (δ NH_2)
12K	7.85	4.24	1.90	1.48, 1.58	1.73 (δ CH_2), 2.99 (ε CH_2)
			KIFMK		
1K	7.79	4.17	1.82	1.46	1.72 (δ CH_2), 2,98 (ε CH_2)
2I	7.23	4.02	1.73	0.72, 0.98, 1.13	0.80 (δ CH_3)
3F	7.39	4.65	3.06, 3.30		7.35 (phenyl)
4M	7.73	4.43	2.18	2.56, 2.61	
5K	7.74	4.35	1.93	1.50	1.82 (δ CH_2), 3.00 (ε CH_2)

^{1}H-NMR measurements

The peptide (3 mM) was dissolved into an isotonic (310 mOsm, 150 mM) phosphate buffer, 50% TFE-d_2-50% isotonic phosphate buffer, or 80% TFE-d_2-20% H_2O solutions. All of the solutions were adjusted to pH 7.0 (meter reading). One- and two-dimensional ^{1}H-NMR spectra were observed on a Bruker AM-600 (600.13 MHz) spectrometer equipped with an Aspect X32 computer. The digital resolution for observing changes in the chemical shifts was 0.25 Hz/point; all of the changes in the chemical shifts are expressed in units of Hz at 600.13 MHz. The ambient probe temperature was 27°C. Two-dimensional absolute value COSY (Nagayama et al., 1980), phase-sensitive TOCSY (Bax and Davis, 1985a), and phase-sensitive ROESY (Bax and Davis, 1985b) spectra were observed for the purpose of assigning the ^{1}H-NMR spectra of the peptides; observed free induction decays were transferred from the Aspect X32 computer to a Silicon Graphics Indigo workstation and processed using the NMRPipe program provided by the National Institutes of Health (Delaglio et al., 1995). The chemical shifts were referenced to TSP (3-trimethylsilyl-propionic acid-d_4 sodium salt).

CD spectra

Stock peptide solutions (1–2 mM) were prepared by dissolving the samples in 80% trifluoroethanol-20% H_2O (pH 7.0). CD spectra (190–250 nm) were measured on a Jasco J-720 spectrometer interfaced to an NEC PC-9801 microcomputer at room temperature after the instrument was calibrated with d-camphor-10-sulfonate. A 0.5-mm-path-length quartz cell was used for a 50 μM sample solution. Eight scans were averaged for each sample; the averaged blank spectra were subtracted. The helicity (α) was determined from the mean residue ellipticity [Θ] at 222 nm according to the relation $\alpha = ([\Theta]_{222} + 2000) \times 100/-30{,}000$ (%) (Wu et al., 1981).

RESULTS

^{1}H-NMR spectroscopy

Assignments of ^{1}H-NMR spectra

Assignments of ^{1}H-NMR spectra were made based on sequential assignment procedures (Wüthrich, 1986). Typical examples of a set of COSY, ROESY, and TOCSY spectra of MP-1A and a TOCSY spectrum of KIFMK in 80% TFE-d_2-20% H_2O solutions are shown in Fig. 1, *a, b, c,* and *d*, respectively. The cross-peak assignments are shown by the one-letter code for amino acids; the numbering denotes amino acid residue number from the N-terminus to the C-terminus, and a Greek letter specifies side-chain protons. Observed chemical shifts of MP-1A and KIFMK are summarized in Table 1.

Interactions between MP-1A and KIFMK in a phosphate buffer and 50% TFE-d_2-50% phosphate buffer solutions

Based on ^{1}H-NMR spectral assignments, we have observed changes in the ^{1}H-NMR chemical shifts of MP-1A caused by KIFMK, expecting that the resonances due to the Asp and Glu residues or nearby residues may shift more than do the other amino acid residues. In Fig. 2, we summarize graphically (in units of Hz at 600.13 MHz) the results for side-chain protons (Fig. 2 *a*), α-protons (Fig. 2 *b*), and amide protons (Fig. 2 *c*) of MP-1A in 50% TFE-d_2 solution, together with the results, which will be described later; the positive sign means a high-frequency (downfield) shift, and the negative one a low-frequency (upfield) shift. The side-chain protons chosen were those located at the terminal positions. On the whole, the magnitudes of changes in the chemical shifts were small. In a phosphate buffer, the chemical shift changes were much smaller than those shown in Fig. 2.

Interactions between MP-1A and KIFMK, KIFMT, or KDIFMTK in 80% TFE-d_2-20% H_2O solutions

We observed changes in the ^{1}H-NMR chemical shifts of MP-1A in 80% TFE-d_2 solution (Fig. 2, *a–c*). Evidently, in this medium, MP-1A and KIFMK interacted with each other much more strongly than in a phosphate buffer or in a 50% TFE-d_2 solution, and as a result, MP-1A underwent large chemical shift changes. This result suggests that interactions

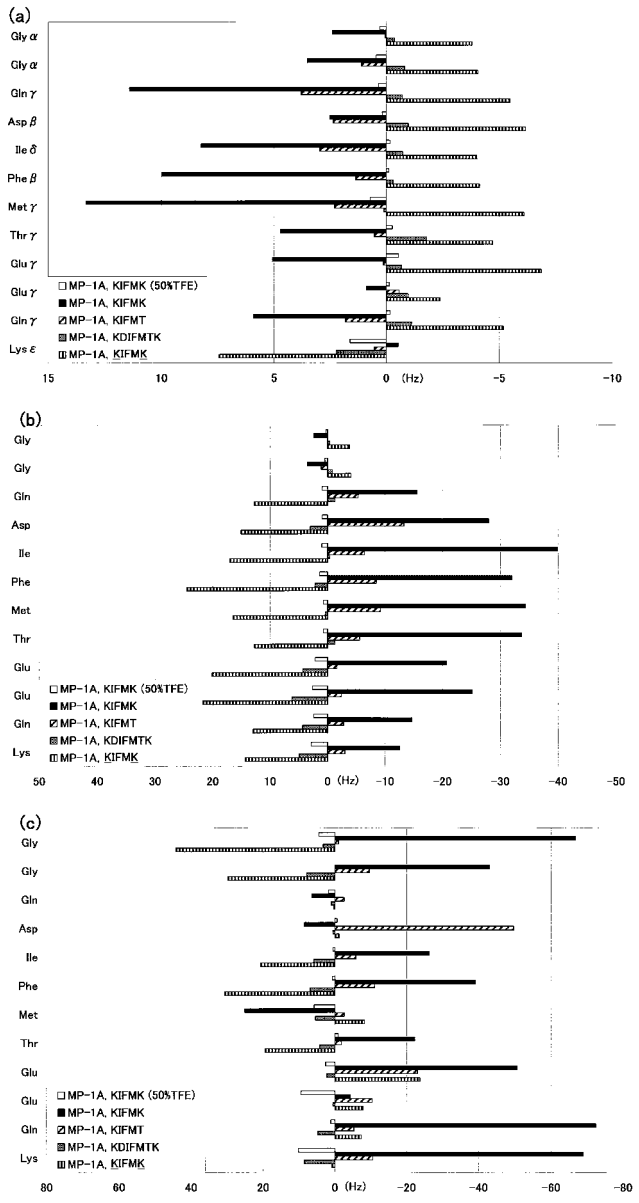

FIGURE 2 Changes in chemical shifts (in Hz at 600.13 MHz) of MP-1A as a result of the interactions with KIFMK in 50% TFE-d_2 solution, and with KIFMK, KIFMT, KDIFMTK, and KIFMK (K means D-Lys) in 80% TFE-d_2 solutions: (*a*) side-chain protons, (*b*) α-protons, and (*c*) amide protons. The concentrations of all of the peptides were 3 mM.

between MP-1A and KIFMK are electrostatic in nature, as expected, because the electrostatic interactions are reinforced by the increasing hydrophobicity of the medium (Israelachvili, 1992). Interestingly, the observed trends of high/low frequency shifts for side-chain protons (Fig. 2 *a*), α-protons (Fig. 2 *b*), and amide protons (Fig. 2 *c*) are unusual in a sense that almost all resonances among the amino acid residues shifted in the same direction; side-chain protons shifted to higher frequencies, α-protons and amide protons to lower frequencies. The changes in ^{1}H-NMR chemical shifts caused by mutual interactions of peptides may be divided into two groups of changes due to the following causes. One is the intermolecular interactions that cause changes in chemical shifts with each other. For example, hydrogen bonds (Pople et al., 1959) between them may largely affect the chemical shift values for both MP-1A and KIFMK; this is especially so for resonances due to amide protons and side-chain protons located at terminal positions in Gln, Asp, Thr, Glu, and Lys. Ring-current effects, magnetic anisotropy (Pople et al., 1959), and electric field effects (Buckingham, 1960) due to the peptide and polar groups in KIFMK may also affect the chemical shift values of MP-1A. Although all of these effects may be more or less reflected in the results shown in Fig. 2, we cannot imagine the mode of interactions that can cause changes in chemical shifts on the average in the same direction among amino acid residues. Evidently, we must invoke another (the second) reason, that is, changes in the secondary structure of MP-1A caused by KIFMK. The conformation-dependent chemical shifts of proteins and peptides have been extensively surveyed by Wishart et al. (Wishart et al., 1991, 1992; Wishart and Sykes, 1994). They found that on the average among various amino acid residues, α-protons resonate at a higher field by ~0.38 ppm in a helix structure than in a random coil structure, whereas the α-protons resonate at a lower field by ~0.38 ppm in a β-strand structure; a theoretical consideration was given by Williamson et al. for these secondary structure-dependent shifts in α-proton resonances (Williamson et al., 1992). Amide protons show the same trend as that for α-protons, with an upfield shift (−0.19 ppm) for helices and a downfield shift for β-strands (0.29 ppm) (Wishart and Sykes, 1994). They also found that side-chain protons have opposite trends; helices cause a downfield shift (0.10 ppm), whereas β-strands cause an upfield shift of roughly equal magnitude (Wishart et al., 1991). Keeping these relationships between chemical shifts and secondary structures in mind, we found from inspection of the data in Fig. 2 that KIFMK induced a conformational change in MP-1A in a way that increases the helical content in solution.

We also observed changes in ^{1}H-NMR chemical shifts of MP-1A caused by KIFMT and KDIFMTK in 80% TFE-d_2 solutions. Because KIFMT has a threonine residue instead of lysine at its C-terminus but is still weakly effective as an open channel blocker, using this pentapeptide can be a good test for confirming the importance of lysine residues in the interaction with MP-1A. Indeed, Fig. 2 shows that the interaction of KIFMT with MP-1A was weaker than that of KIFMK, as expected. Interestingly, however, KIFMT induced the same sort of general trends as in KIFMK for the chemical shift changes of side-chain, α-, and amide protons, indicating that KIFMT also increased the helical content of MP-1A in solution. In contrast, the heptapeptide KDIFMTK has lysine residues on both its N- and C-termini, but it is ineffective as an open channel blocker. If we consider the amino acid sequence of MP-1A (GGQDIFMTEEQK), it might be expected a priori that the heptapeptide may interact more strongly with MP-1A than does KIFMK, because the two lysine residues in KDIFMTK match exactly in its

amino acid sequence with the aspartic acid (D) and C-terminus side glutamic acid (E) of MP-1A, respectively, whereas those of KIFMK are mismatched (for K-D, but K-T). Fig. 2 clearly shows that unfortunately this was indeed not true; the heptapeptide interacted with MP-1A much more weakly than did KIFMK. The interaction appears to be even weaker than in the case of KIFMT. The general trends found from chemical shift changes are interesting, however. The heptapeptide shifted resonances due to MP-1A in opposite directions, i.e., the side-chain protons to lower frequencies, the α- and amide protons to higher frequencies. This result means that the heptapeptide, which is ineffective as an open channel blocker, decreased the helical content of MP-1A in solution. It thus can be concluded that a peptide that is effective as an open channel blocker works for the inactivation gate peptide in the III-IV linker as an agent that stabilizes its helical structure, whereas one that is ineffective as an open channel blocker works as an agent that destabilizes the helical structure.

Interactions between MP-1A and KIFMK

The importance of lysine residues on both sides of the IFM motif in the interaction with MP-1A has also been verified using the pentapeptide KIFMK instead of KIFMK. This pentapeptide was synthesized using D-Lys (denoted as K) instead of L-Lys. In this case, we can expect the following two situations: one is that the interaction between KIFMK and MP-1A would be very weak and would provide no useful information on the changes in the secondary structure of MP-1A and/or on the mode of interaction between them. The other is that they would interact with each other in a way similar to that in the case of KIFMK and would increase the helical content of MP-1A in solution. The experimental results are also shown in Fig. 2. Evidently, contrary to our first expectation, KIFMK interacted with moderate strength compared with that in the case of KIFMK. More interestingly, however, the observed general trends in the chemical shift changes indicate that the helical content of MP-1A was decreased. These results clearly show that for KIFMK to interact in a way that stabilizes the helical structure of MP-1A, first, both of the two lysine residues and the IFM motif are absolutely necessary, and second, these must have an appropriate stereochemical configuration relative to each other.

Interactions between KIFMK and MP-1NA, MP-1QEA, or MP-1EQA in 80% TFE-d_2-20% H_2O solutions

MP-1A involves three negatively charged amino acids. To clarify which negatively charged amino acids are effective in the interaction with the two lysine residues of KIFMK, we studied the interactions between KIFMK and some modified peptides from MP-1A, that is, MP-1NA (D1487N), MP-1QEA (E1492Q), and MP-1EQA (E1493Q). Observed changes in chemical shifts for MP-1NA, MP-1QEA, and MP-1EQA are shown in Fig. 3, *a* (side-chain protons), *b* (α-protons), and *c* (amide protons). Evidently, the observed general trends in the chemical shift changes show that KIFMK decreased the helical contents of MP-1NA and MP-1QEA, whereas it increased that of MP-1EQA. These results mean that aspartic acid and glutamic acid, which is adjacent to threonine, are indispensable for stabilizing the helical structure of MP-1A.

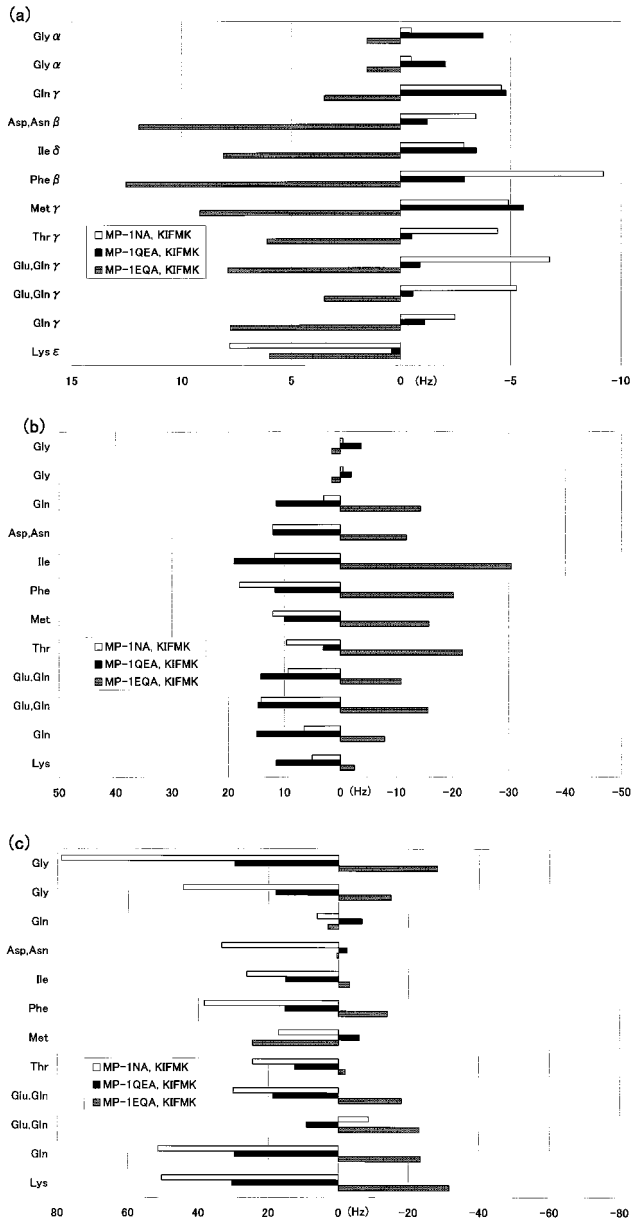

FIGURE 3 Changes in chemical shifts (in Hz at 600.13 MHz) of MP-1NA, MP-1QEA, and MP-1EQA as a result of the interactions with KIFMK in 80% TFE-d_2 solutions: (*a*) side-chain protons, (*b*) α-protons, and (*c*) amide protons. The concentrations of all of the peptides were 3 mM.

Changes in ^{1}H-NMR chemical shifts of KIFMK, KIFMT, KDIFMTK, and KIFMK

Because ^{1}H-NMR resonances due to the open channel blocking peptides in 80% TFE-d_2 solutions with various

inactivation gate peptides were in all cases somewhat broadened and sometimes buried in those due to the inactivation gate peptides, it was difficult to descern the changes in the chemical shifts of α- and amide proton resonances. Thus we summarized the changes in the chemical shifts only for side-chain proton resonances. Fig. 4 shows a summary of changes in the chemical shifts of the side- chain proton resonances of KIFMK and other peptides (i.e., KIFMT, KDIFMTK, and KIFMK) studied so far. Interestingly, all of the side-chain proton resonances due to both the N- and C-termini lysine residues of KIFMK, KDIFMTK, and KIFMK and the N-terminus lysine residue of KIFMT shifted to higher frequencies. These results clearly indicate that in all of these cases, the positively charged ϵ-amino groups of the lysine residues are interacting with the inactivation gate peptides, probably with the negatively charged side chains of D1487, E1492, and/or E1493. Another interesting finding from these graphs is that KDIFMTK (Fig. 4 *d*) and KIFMT (Fig. 4 *c*), which did not interact appreciably with MP-1A, showed relatively large chemical shift changes, whereas KIFMK, which interacted considerably with MP-1A (Fig. 4 *b*) and MP-1EQA (Fig. 4 *h*), showed only small chemical shift changes. Because KIFMK in 50% TFE-d_2 solution, in which interactions with MP-1A were intrinsically weak, showed very small chemical shift changes (Fig. 4 *a*), there may be some reasons for this finding, although they are at present unexplained.

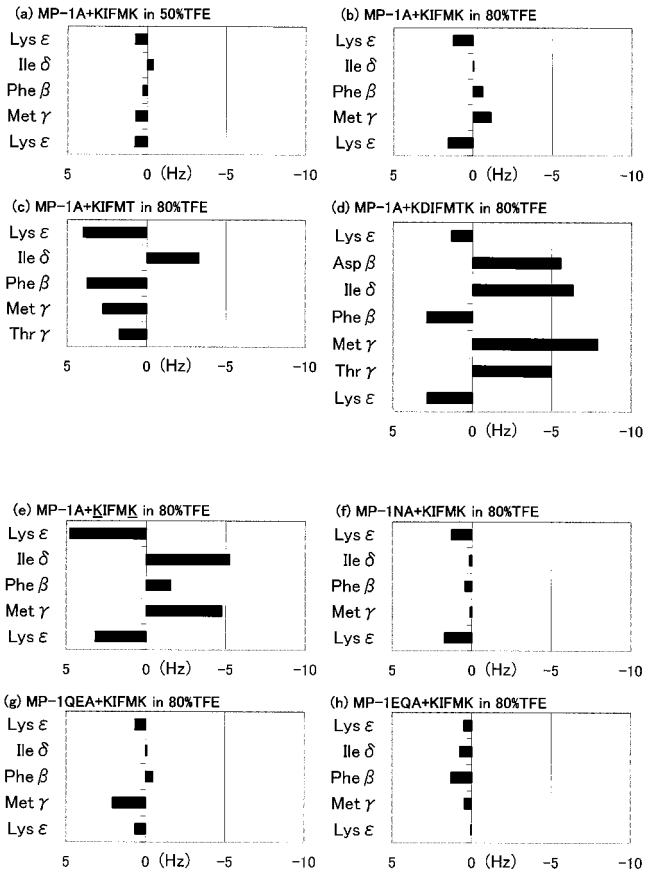

FIGURE 4 Changes in chemical shifts (in Hz at 600.13 MHz) for side-chain proton resonances of (*a*) KIFMK in 50% TFE-d_2 solution with MP-1A, (*b*) KIFMK in 80% TFE-d_2 solution with MP-1A, (*c*) KIFMT in 80% TFE-d_2 solution with MP-1A, (*d*) KDIFMTK in 80% TFE-d_2 solution with MP-1A, (*e*) KIFM*K* (K means D-Lys) in 80% TFE-d_2 solution with MP-1A, (*f*) KIFMK in 80% TFE-d_2 solution with MP-1NA, (*g*) KIFMK in 80% TFE-d_2 solution with MP-1QEA, and (*h*) KIFMK in 80% TFE-d_2 solution with MP-1EQA. The concentrations of all of the peptides were 3 mM.

Chemical shift index

In accordance with Wishart et al. (1992), we summarize in Fig. 5 all of the chemical shift index data for the α-protons of the peptides studied so far. The downward arrow specifies that the index is -1, i.e., the chemical shift of an α-proton is smaller (i.e., resonates at a higher field) than the shift range given by Wishart et al. for the amino acid in question, and the upward arrow specifies that the index is $+1$, i.e., it is larger than the corresponding shift range. The chemical shift ranges of Wishart et al. presented for the α-proton resonances of various amino acids are for the peptides assuming random coil conformations in aqueous solutions, and thus a caution is given by Wishart et al. in applying their values to peptides and proteins in TFE or

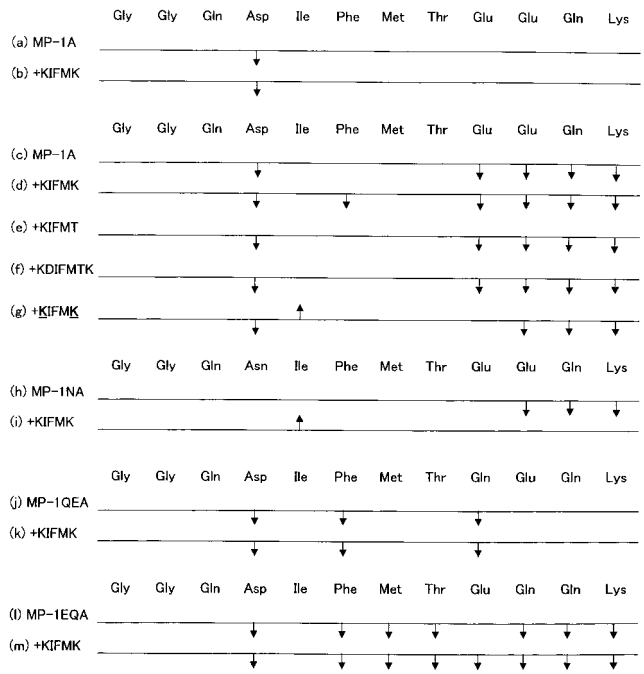

FIGURE 5 Chemical shift index data for (*a*) MP-1A in 50% TFE-d_2 solution, (*b*) MP-1A in 50% TFE-d_2 solution with KIFMK, (*c*) MP-1A in 80% TFE-d_2 solution, (*d*) MP-1A in 80% TFE-d_2 solution with KIFMK, (*e*) MP-1A in 80% TFE-d_2 solution with KIFMT, (*f*) MP-1A in 80% TFE-d_2 solution with KDIFMTK, (*g*) MP-1A in 80% TFE-d_2 solution with KIFMK (K means D-Lys), (*h*) MP-1NA in 80% TFE-d_2 solution, (*i*) MP-1NA in 80% TFE-d_2 solution with KIFMK, (*j*) MP-1QEA in 80% TFE-d_2 solution, (*k*) MP-1QEA in 80% TFE-d_2 solution with KIFMK, (*l*) MP-1EQA in 80% TFE-d_2 solution, and (*m*) MP-1EQA in 80% TFE-d_2 solution with KIFMK. The concentrations of all of the peptides were 3 mM.

other organic solvents (Wishart and Sykes, l994). However, shortly afterward, Merutka et al. found that the α-proton chemical shift generally shows negligible change on addition of TFE (Merutka et al., 1995). Inspection of Fig. 5 shows that MP-1A and MP-1EQA in 80% TFE-d_2 solutions and those including KIFMK have a tendency to assume a helical conformation at their C-terminal half-sides, whereas MP-1NA and MP-1QEA and those including KIFMK solutions assume random coil conformations. However, CD data described later showed that the conformations of all of the inactivation gate peptides and KIFMK are essentially α-helix.

Estimation of changes in helical contents of inactivation gate peptides in 80% TFE-d_2 solutions based on ^{1}H-NMR chemical shifts

According to Wishart et al. (1991), the secondary shift ($\Delta\delta_{helix}$) for an α-proton resonance relative to the corresponding random coil value differs from another, depending on the kinds of amino acid residue. For example, the $\Delta\delta_{helix}$ value of threonine is -0.48 ppm, whereas that of aspartic acid is -0.17 ppm (Wishart et al., 1991). Thus, to discuss the relative magnitudes of changes in ^{1}H chemical shifts among different amino acids, we have calculated the amounts of increase or decrease in helical contents from the $\Delta\delta_{helix}$ value for each amino acid residue and from the observed changes in ^{1}H chemical shifts, assuming that all of the observed changes in ^{1}H-NMR chemical shifts are due solely to changes in their secondary structures. Table 2 shows a summary of the changes in the helical contents (Δf_H(%)) of MP-1A caused by KIFMK, KIFMT, KDIFMTK, and KIFMK. Inspection of Table 2 shows that KIFMK stabilized the helical structure of MP-1A for a wide range of amino acids in its sequence (Q1486-K1495). On average, the helical content of MP-1A was increased by 11.9%. On the other hand, KIFMT stabilized only at around D1487 of MP-1A, and on the average it increased the helical content of MP-1A by 2.9%. These results suggest that the lysine residue in KIFMT is interacting with D1487 but not with E1492 or E1493. Thus it is conceivable that the N-terminal lysine residue in KIFMK is also interacting with D1487 and that, consequently, the C-terminal lysine residue is interacting with E1492, although helix-helix dipole interactions, if any, may favor the reverse situation. On the other hand, KIFMK destabilized the helical structure of MP-1A for a wide range of amino acids (Q1486-K1495).

Table 3 shows a summary of changes in helical contents (Δf_H(%)) of MP-1NA, MP-1QEA, and MP-1EQA caused by KIFMK. In MP-1EQA, KIFMK again stabilized the helical structure of MP-1EQA for a wide range of amino acids in the sequence Q1486-K1495.

CD spectroscopy

CD spectra of MP-1A and KIFMK in 80% TFE solutions

To obtain supporting evidence for the interpretation of changes in ^{1}H-NMR chemical shifts described above, we have observed CD spectra. It would be expected that if KIFMK really stabilized the helical structure of MP-1A in 80% TFE solution, the increase in the helical content should also be reflected in their CD spectra. Fig. 6 clearly shows that this is, in fact, observed. Fig. 6 shows the CD spectra of MP-1A and KIFMK in 80% TFE solutions, respectively (spectra a and b). The CD patterns show that both MP-1A and KIFMK are assuming helix conformations. Fig. 6 (spectrum c) shows the sum of the CD spectra of MP-1A and KIFMK observed separately in 80% TFE solutions, and spectrum d is the CD spectrum of MP-1A and KIFMK in an 80% TFE solution; the vertical scale of spectrum c was adjusted to the mean residue ellipticity. Interestingly, the negative ellipticity at the minimum absorption peak at 204 nm (π-π*-transition) increased in spectrum d more than that

TABLE 2 Changes in helical contents (Δf_H(%)) of MP-1A caused by interactions with KIFMK, KIFMT, KDIFMTK, or KIFMK in 80% TFE-d_2 solutions estimated from the observed changes in ^{1}H chemical shifts and CD spectra

MP-1A	$\Delta\delta_{helix}$ (ppm)*	KIFMK Δf_H(%)	KIFMT Δf_H(%)	KDIFMTK Δf_H(%)	KIFMK Δf_H(%)
G1484	−0.17	−2.4	−0.1	+0.3	+3.7
G1485	−0.17	−3.5	−1.1	+0.8	+4.0
Q1486	−0.29	+8.9	+3.1	+0.7	−7.3
D1487	−0.17	+27.5	+13.0	−3.0	−14.8
I1488	−0.47	+14.1	+2.3	+0.1	−6.0
F1489	−0.42	+12.7	+3.4	−0.9	−9.7
M1490	−0.25	+22.9	+6.1	−0.3	−11.0
T1491	−0.48	+11.7	+1.9	+0.4	−4.4
E1492	−0.22	+15.7	+1.2	−3.3	−15.2
E1493	−0.22	+19.0	+1.8	−4.7	−16.5
Q1494	−0.29	+8.4	+1.6	−2.5	−7.5
K1495	−0.28	+7.5	+1.8	−2.9	−8.6
Average#		+11.9	+2.9	−1.3	−7.8
CD spectra		+11.1	+6.2	−4.4	−2.8

*Taken from Wishart et al.'s table (Wishart et al., 1991).
#Averaged value among G1484–K1495.

TABLE 3 Changes in helical contents (Δf_H(%)) of MP-1NA, MP-1QEA, and MP-1EQA caused by interactions with KIFMK in 80% TFE-d_2 solutions estimated from the observed changes in ^{1}H chemical shifts and CD spectra

MP-1NA, MP-1QEA, MP-1EQA	$\Delta\delta_{helix}$ (ppm)*	MP-1NA Δf_H(%)	MP-1QEA Δf_H(%)	MP-1EQA Δf_H(%)
G1484	−0.17	+0.5	+3.7	−1.5
G1485	−0.17	+0.5	+2.0	−1.5
Q1486	−0.29	−1.7	−6.6	+8.3
D(N)1487	−0.17 (−0.27)	−7.4	−11.8	+11.6
I1488	−0.47	−4.2	−6.7	+10.8
F1489	−0.42	−7.1	−4.6	+8.0
M1490	−0.25	−8.0	−6.6	+10.5
T1491	−0.48	−3.3	−1.1	+7.6
E(Q)1492	−0.22 (−0.29)	−7.0	−8.2	+8.3
E(Q)1493	−0.22 (−0.29)	−10.7	−11.1	+9.0
Q1494	−0.29	−3.7	−8.6	+4.6
K1495	−0.28	−3.0	−6.8	+1.5
Average#		−4.6	−5.5	+6.4
CD spectra		−8.6	−7.4	+5.5

*Taken from Wishart et al.'s table (Wishart et al., 1991).
#Averaged value among G1484–K1495.

in spectrum c. The negative ellipticity at 222 nm (n-π*-transition) showed that the helical content of MP-1A and KIFMK in the 80% TFE solution was 41.3%, whereas that calculated for spectrum c was 30.2%. Thus the helical content was increased by ~11% by mixing MP-1A and KIFMK in the 80% TFE solution. This value closely agrees with that estimated from changes in the ^{1}H chemical shift (Table 2) for MP-1A. The changes in the helical contents of MP-1A by KIFMT, KDIFMTK, and KIFMK were calculated in the same manner for KIFMK and are shown in Table 2. Furthermore, because the CD spectra of the other inactivation gate peptides were also characteristic for helix conformations (spectra not shown), we have estimated the increase/decrease in the helical contents of MP-1NA, MP-1QEA, and MP-1EQA in the same manner. The results are shown in Table 3. Inspection of Tables 2 and 3 shows that the magnitudes of changes in helical contents estimated from ^{1}H-NMR chemical shifts (average values) and those from CD spectra agree satisfactorily with each other.

The concentration dependence of the helix-stabilizing effects of KIFMK on MP-1A

To document the specificity of the interaction between MP-1A and KIFMK, we have investigated the concentration dependence of the helix-stabilizing effects of KIFMK on MP-1A; the results are shown in Fig. 7. Evidently the graph shows that the interaction is specific, because the helix-stabilizing effects of KIFMK showed a saturation in the curve. Moreover, MP-1A and KIFMK are interacting with each other, with a 1:1 stoichiometry, as expected.

DISCUSSION

The inactivation gate of sodium channels is assumed to close by a mechanism called a "hinged-lid" model (West et al., 1992). In this model, three clustered hydrophobic amino acids, I1488-F1489-M1490 (West et al., 1992), and T1491 (Kellenberger et al., 1997a) in the intracellular linker con-

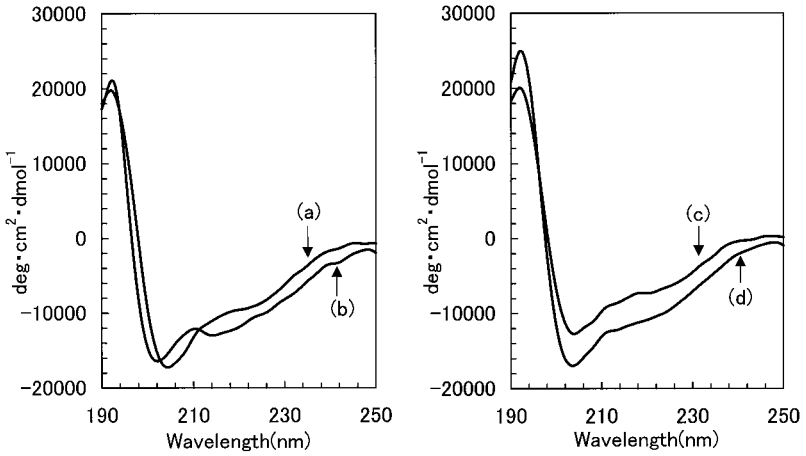

FIGURE 6 CD spectra of (*a*) MP-1A in 80% TFE solution, (*b*) KIFMK in 80% TFE solution, (*c*) sum of those of MP-1A and KIFMK observed separately in 80% TFE solutions, and (*d*) MP-1A and KIFMK in 80% TFE solution. The concentrations of MP-1A and KIFMK were 50 μM.

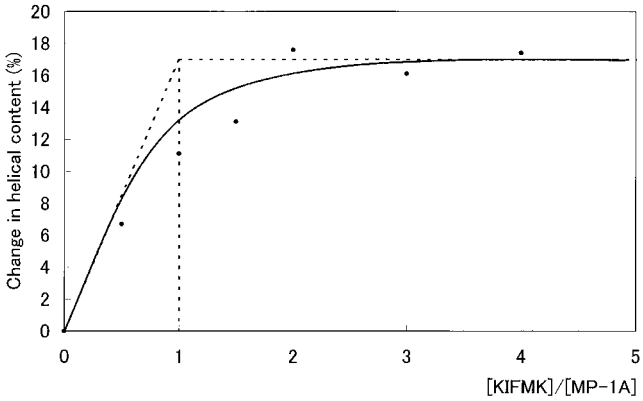

FIGURE 7 The concentration dependence of the helix-stabilizing effects of KIFMK on MP-1A: MP-1A, 50 μM; KIFMK, 25, 50, 75, 100, 200, 300, and 400 μM.

necting domains III and IV work as a lid and occlude the pore, making use of G1484, G1485, P1512, P1514, and P1516, which are located on either side of the IFM motif, as hinges that allow the inactivation gate to move in and out of the channel pore (Kellenberger et al., 1997b). The receptor for the lid is considered to be composed of short S4-S5 loops of both domains III (Smith and Goldin, 1997) and IV (Tang et al., 1996; Lerche et al., 1997; McPhee et al., 1998) and of three hydrophobic amino acids, V1774-I1775-L1776, which are located near the C-terminal side of segment 6 of domain IV (IVS6) (McPhee et al., 1994). Because both the lid and its receptor are made up of hydrophobic amino aicd residues, the driving force that moves the lid toward its receptor is assumed to be hydrophobic interaction. Fig. 8 shows a schematic drawing of the interactions among the S4-S5 loops of domains III and IV and the III-IV linker (G1484-K1495) in a sodium channel α-subunit. Eaholtz et al. have shown that a synthetic pentapeptide, KIFMK, restores fast inactivation to the mutant sodium channels (F1489Q) having a defective inactivation gate and to wild-type sodium channels having inactivation slowed by α-scorpion toxin (Eaholtz et al., 1994). The pentapeptide KIFMK competes with the intrinsic inactivation, accelerating the inactivation of the wild-type sodium current (Eaholtz et al., 1994). The KIFMK also restores fast inactivation to the sodium channels having a defective inactivation gate caused by mutations for the receptor of the IFM motif. Tang et al. have shown that KIFMK restores fast inactivation to the mutants substituted at M1651M1652/QQ or MM/AA in the S4-S5 loop of domain IV (Fig. 8) of human heart (hH1) sodium channels (Tang et al., 1996). McPhee et al. have shown that KIFMK restores fast inactivation of open channels to the F1651A/L1660A mutant in the S4-S5 loop of domain IV (Fig. 8) of rat brain IIA sodium channels (McPhee et al., 1998). Furthermore, McPhee et al. have shown that KIFMK restores fast inactivation to the mutant substituted at F1764A/V1774A (IVS6) of rat brain type IIA sodium channels (McPhee et al., 1995).

We have shown in the present study that the relative potency as an open channel blocker among KIFMK,

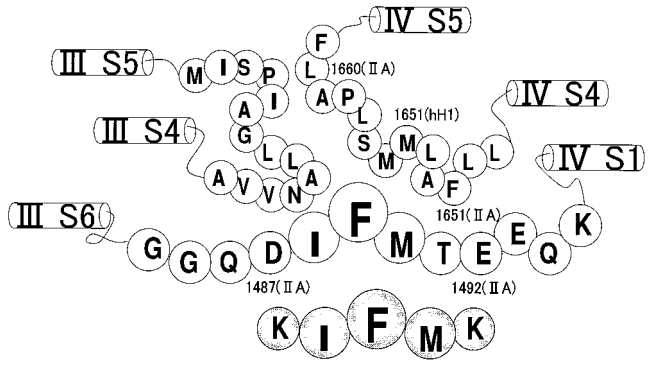

FIGURE 8 A schematic drawing for showing the interactions among S4-S5 loops of domains III and IV and the III-IV linker (G1484-K1495) with KIFMK, which is attached to the D1487-E1492 moiety of the III-IV linker in a sodium channel α-subunit. The S4-S5 loops of domain III (Smith and Goldin, 1997) and domain IV (Tang et al., 1996; Lerche et al., 1997; McPhee et al., 1998) are considered to be forming a receptor for the I1488-F1489-M1490 motif. The KIFMK accelerates intrinsic fast inactivation to the wild-type sodium channels or restores the fast inactivation to the channels having a defective gating machinery owing to the mutations at the III-IV linker (F1489Q) (Eaholtz et al., 1994) or at the S4-S5 loop of domains IV (MM/QQ, MM/AA) (Tang et al., 1996; McPhee et al., 1998). The IIA in parentheses means that the numbering for the amino acid residue is for the rat brain type IIA sodium channels (Noda et al., 1986), and hH1 means that it is for the human heart sodium channels (Gellens et al., 1992).

KIFMT, and KDIFMTK, which is found from electrophysiological experiments (Eaholtz et al., 1994), paralleled their helix-stabilizing action on the model inactivation gate peptide MP-1A. The two lysine residues of KIFMK were concluded to interact electrostatically with D1487 and E1492 of MP-1A (Fig. 8). These electrostatic interactions between MP-1A and KIFMK may have stabilized the α-helical conformation of MP-1A. Marqusee and Baldwin showed that an $(i) \rightarrow (i + 4)$ Glu^{-}–Lys^{+} salt bridge (i.e., a hydrogen-bonded ion pair) in an alanine-based short peptide stabilizes its α-helix conformation (Marqusee and Baldwin, 1987). The helix-stabilization effect of the salt bridge originates from the interactions between the charges of Glu^{-} and Lys^{+} and the helix dipole (Daggett et al., 1989). More recently, the helix-stabilizing action of a lysine side chain in alanine- and lysine-based oligopeptides has also been reported by Groebke et al. (1996) and Vila et al. (1998). They found that an ϵ-NH_3^{+} ion of the lysine residue at the C-terminal side is interacting with the carbonyl oxygen of an $(i - 4)$ amino acid residue. The increased helix stability arises from the electrostatic interaction between the NH_3^{+} ion and the helix dipole through a π-type hydrogen bond between the NH_3^{+} ion and a carbonyl oxygen of the helix core (Groebke et al., 1996). All of these examples for increased stability of the helical conformation by a lysine side chain are "intramolecular" interactions in origin. However, in our present cases, "intermolecular" salt bridges have stabilized an α-helix conformation. It is not certain whether the observed increase in the helical content of MP-1A by KIFMK is due simply to the helix-anchoring effect caused by the salt bridges.

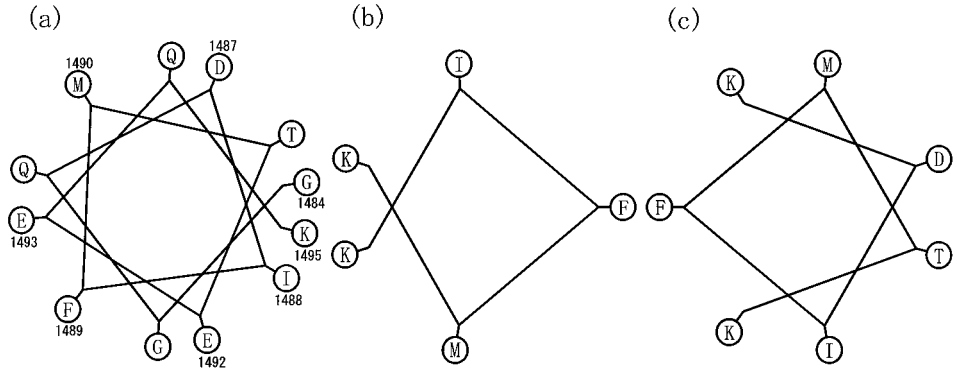

FIGURE 9 Helical wheels of (*a*) MP-1A, (*b*) KIFMK, and (*c*) KDIFMTK.

In the hinged-lid model, because the IFM motif of the III-IV linker is supposed to bind to its receptor by hydrophobic interactions, the environment surrounding the IFM motif and its receptor should thus be hydrophobic in nature. Moreover, an electrostatic interaction is reinforced in the hydrophobic environment (Israelachvili, 1992). It might thus be considered that KIFMK, diffusing near the cytoplasmic III-IV linker in a sodium channel, is attracted to the IFM motif by electrostatic interactions between its positively charged side chains and the negatively charged ones of D1487 and E1492 (Fig. 8) and stabilizes the α-helical conformation around the IFM motif. In the hinged-lid model, the lid should behave more like a rigid body than a flexible loop (Joseph et al., 1990; West et al., 1992); therefore, the stabilization of the helical conformation around the IFM motif is expected to assist in moving the "lid" toward its receptor site and consequently to stabilize the lid to allow it occlude the intracellular mouth of the sodium channels. Moreover, the lid bears two IFM motifs, and the hydrophobicity would be increased. This increased hydrophobicity of the lid may accelerate the competitive binding of one of the IFM motifs to the inactivation gate receptor. Thus it can be explained why fast inactivation of wild-type sodium channels was accelerated (Eaholtz et al., 1994) or why fast inactivation of inactivation defective sodium channels caused by MM/QQ or MM/AA mutations (Tang et al., 1996) was restored.

Fig. 9 shows the helical wheels for MP-1A (Fig. 9 *a*), KIFMK (Fig. 9 *b*), and KDIFMTK (Fig. 9 *c*). Evidently, if both MP-1A and KIFMK are assuming α-helical conformations, the phenylalanine residue of KIFMK points oppositely to MP-1A. Lawrence et al. have shown that rat skeletal muscle (μ1) F1304Q mutant sodium channels, which are equivalent to the rat brain type IIA F1489Q mutants, can still enter the inactivated state but do so reversibly, that is, they return rapidly to the open state (Lawrence et al., 1996). Thus, for the case of F1489Q-mutated sodium channels, the phenylalanine residue of KIFMK, which is binding to the III-IV linker, may have interacted stably with the hydrophobic receptor formed by the S4-S5 loops of both domains III and IV, together with its isoleucine and methionine residues, and restored fast inactivation. In contrast, in the case of KDIFMTK, its phenylalanine residue points toward MP-1A (Fig. 9). This binding situation can be the reason why it interacted weakly with MP-1A (Fig. 2), because the bulky aromatic ring may prevent KDIFMTK from approaching MP-1A. Furthermore, as a consequence of this, it can be explained why it was ineffective as an open channel blocker. In the case of KIFMT, instead of an electrostatic interaction, the OH group of the threonine residue can form a hydrogen bond with a carbonyl oxygen of MP-1A. The electrostatic interaction of the N-terminal lysine residue and this hydrogen bonding may have stabilized the α-helical conformation of MP-1A, but less effectively than did KIFMK.

In conclusion, the synthetic pentapeptide KIFMK, which binds to the D1487 and E1492 in the III-IV linker of a sodium channel α-subunit, stabilized an α-helical conformation around the IFM motif of the III-IV linker and assisted in its hinged-lid motion to stably occlude the channel pore during the fast inactivation process.

Recently, Ghatpande and Sikdar have reported that KIFMK and an alkaloid veratridine, which is classified to bind to the pharmacological site 2 (Catterall, 1992), compete for binding to the rat brain type IIA sodium channel (Ghatpande and Sikdar, 1997). It is also reported that local anesthetics and batrachotoxin, which is another alkaloid that is considered to bind to the pharmacological site 2, compete for their binding sites allosterically (Strichartz and Ritchie, 1987). The present results can be a promising clue to the investigation of the binding sites of local anesthetics and neurotoxins, leading to a clarification of the structure and gating mechanisms of sodium channels.

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