Secondary Structure and Membrane Localization of Synthetic Segments and a Truncated Form of the IsK (minK) Protein†

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ABSTRACT: IsK, also referred to as minK, is a membrane protein consisting of 130 amino acids and localized mainly in epithelial cells but also in human T lymphocytes. Depending on the cRNA concentration that was injected into *Xenopus* oocytes, IsK and its truncated forms can induce either a K^+ current alone or both K^+ and Cl^- currents [Attali et al. (1993) *Nature 365*, 850–852]. To obtain information on the secondary structure and the topology of IsK in a membrane-bound state, the synthesis, fluorescent-labeling, and structural and functional characterization of five polypeptides of 20–63 amino acids within the rat IsK protein were examined. The α-helical content of the segments, assessed in methanol using circular dichroism, suggests that both the N-terminal and transmembrane segments of IsK adopt α-helical structures. Binding experiments and the blue shift of 7-nitrobenz-2-oxa-1,3-diazol-4-yl (NBD)-labeled peptides suggest that while both the α-helical transmembrane segment and the N-terminal of IsK are located within the lipid bilayer, the linking segment between the two segments lies on the surface of the membrane. The fluorescence energy transfer, between donor and acceptor-labeled truncated IsK, suggests that it aggregates within phospholipid membranes. Although a protein whose sequence is similar to that of truncated IsK can induce K^+ channel activity when expressed in *Xenopus* oocytes, the inability of a truncated IsK to form functional K^+ channels in planar lipid membranes supports increasing evidence that the protein alone cannot form a K^+ channel.

IsK, also referred to as minK, a membrane protein consisting of 130 amino acids, is encoded by a gene that has been cloned in mammalian kidney, uterus, heart, and genomic DNA (Takumi et al., 1988; Folander et al., 1990; Pragnell et al., 1990; Murai, 1990). The IsK protein is located mainly in epithelial cells, such as those found in the kidney, duodenum, stomach, pancreas, and submandibular gland, but not in brain or liver (Sugimoto et al., 1990). Recently, the protein was also demonstrated in human T lymphocytes (Attali et al., 1992).

Accumulated data suggested that IsK is a K^+ channel or at least an integral constituent of one. This was mainly based on the finding that one mutation in the transmembrane domain of the protein altered the gating of the K^+ current expressed in *Xenopus* oocytes (Takumi et al., 1991) and that one mutation in the same region altered the relative permeability of the oocytes to different cations (Goldstein et al., 1991). However, IsK induces K^+ currents when expressed in *Xenopus* oocytes, but not when expressed in a variety of other host cells (Lesage et al., 1993). Moreover, membranes highly enriched in the IsK protein do not produce K^+ channel activity after bilayer reconstitution (Lesage et al., 1993). Recently, IsK was shown to be able to induce either a K^+ current or both K^+ and Cl^- currents, depending on the cRNA concentration that was injected into *Xenopus* oocytes (Attali et al., 1993). Thus, it was concluded that IsK is actually not a channel protein itself, but rather an activator or regulator of endogenous and dormant K^+ and Cl^- channels. To explain the effect of one mutation in the transmembrane region of IsK on gating and modest changes of K^+ channel selectivity, Attali et al. suggested that IsK can act as a regulator that defines at least partially the properties of the K^+ pore. By combining, in an oocyte expression system, site-directed and deletion mutations of IsK with electrophysiological measurements of the resultant proteins, Takumi and co-workers (1991) identified a IsK sequence of 63 amino acid residues that elicited a voltage-dependent K^+ channel activity in oocytes indistinguishable from that elicited by wild-type IsK. In similar experiments, Attali and co-workers (1993) identified, by mutagenesis of IsK, that its amino- and carboxy-terminal domains are required for the induction of Cl^- and K^+ channel activities, respectively.

Most studies of IsK focused on its functional activities; therefore, the information on its membranal localization and its structure in a membrane-bound state is yet to be determined. Hydrophobicity analysis according to a Kyte and Doolittle (1982) plot of the IsK protein predicts a single putative transmembrane α-helical domain (amino acids 45–67) (Takumi et al., 1988).

Herein synthetic peptides were utilized to gain information on the structure, topology, and function of IsK in its membrane-bound state. Five segments and truncated forms of IsK were synthesized, fluorescently-labeled, and characterized both structurally and functionally. The secondary structures of the segments were characterized using circular dichroism (CD),[1] and their ability to bind to phospholipid vesicles was monitored with 7-nitrobenz-2-oxa-1,3-diazol-4-yl (NBD)-labeled peptides. Fluorescence resonance energy transfer (FET) experiments were performed with truncated IsK labeled at its N-terminal amino acid residue with the fluorophores carboxyfluorescein (Flu; energy donor) or tetramethylrhodamine (Rho; energy acceptor) (Rapaport & Shai, 1992).

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The ability of the truncated IsK to form ion channels in planar lipid membranes (PLM) was also tested.

The findings indicate that the IsK protein can self-associate, probably at different aggregation levels, in its membrane-bound state, with both its N-terminal and the transmembrane segment embedded within the membrane and adopting α-helical structures. Moreover, IsK by itself is probably not a K^+ channel, although its truncated form can aggregate within phospholipid membranes and can form single channels that are not characteristic of K^+ channels.

EXPERIMENTAL PROCEDURES

Materials. BOC–(amino acid)–Pam resins were purchased from Applied Biosystems (Foster City, CA), and BOC amino acids were obtained from Peninsula Laboratories (Belmont, CA). Other reagents for peptide synthesis were obtained from Sigma. Egg phosphatidylcholine (PC) was purchased from Lipid Products (South Nutfield, U.K). Diphytanoylphosphatidylcholine, 1-palmitoyl-2-oleoylphosphatidylcholine, and 1-palmitoyl-2-oleoylphosphatidylethanolamine were purchased from Avanti Polar Lipid (Alabaster, AL). Cholesterol (extra pure), purchased from Merck (Darmstadt, Germany), was recrystallized twice from ethanol. 5- (and-6-) carboxyflurescein, succinimidyl ester (Flu-Su), and 5- (and-6-) carboxytetramethylrhodamine, succinimidyl ester (Rho-Su), were obtained from Molecular Probes (Eugene, OR). NBD-F (4-fluoro-7-nitrobenz-2-oxa-1,3-diazole) was obtained from Sigma. All other reagents were of analytical grade. Buffers were prepared using double glass-distilled water.

Peptide Synthesis, Fluorescent-Labeling and Purification. Peptides were synthesized by a solid-phase method on Pam-amino acid resin (0.15 mequiv) (Merrifield et al., 1982), as previously described (Shai et al., 1990). Double coupling was carried out with freshly prepared hydroxybenzotriazole (HOBT) active esters of BOC amino acids. The synthetic polypeptides were purified to a chromatographic homogeneity of >98% by reverse-phase HPLC on an analytical C_{18} Vydac column 4.6 mm × 250 mm (pore size of 300 Å), apart from N-20-IsK which was purified on C_4 Vydac column 4.6 mm × 250 mm (pore size of 300 Å). The column was eluted in 40 min using linear gradients of acetonitrile in water in the presence of 0.1% trifluoroacetic acid (TFA) (v/v). The flow rate was 0.6 mL/min, i.e., 10–80% for [10–43]-IsK, 25–80% for N-43-IsK, 40–65% for truncated-IsK, 10–70% for C-27-IsK, and 25–80% for N-20-IsK. The peptides were subjected to amino acid analysis to confirm their composition.

Labeling of the polypeptide's N-terminus was achieved by labeling resin-bound polypeptide in its fully protected form as follows: Resin-bound peptide (30–40 mg, i.e., 10–25 μmol) was treated with TFA (50% v/v in methylene chloride) to remove the BOC protecting group from the N-terminal amino group of the attached peptide (Rappaport & Shai, 1991). The resin-bound peptides were then reacted with either (i) 2 equiv of 5- (and-6-) carboxyflurescein, succinimidyl ester (Flu-Su), (ii) 5–7 equiv of 5- (and-6-) carboxytetramethylrhodamine, succinimidyl ester (Rho-Su), in DMF containing 3% v/v triethylamine, or (iii) 5–7 equiv of 4-fluoro-7-nitrobenz-2-oxa-1,3-diazole (NBD-F) in DMF. These three reactions led to the formation of resin-bound N^1-Flu-, N^1-Rho-, or N^1-NBD-peptides, respectively. After 24 h, the resins were washed thoroughly with DMF and then with methylene chloride wash. After removing the dinitrophenyl and formyl protecting groups from the histidine and tryptophan residues, the peptides were cleaved from the resin with HF. The cleaved peptides were precipitated with ether and purified by using reverse-phase HPLC as described above.

Preparation of Small Unilamellar Vesicles. Small unilamellar vesicles (SUV) were prepared from PC by sonication. Briefly, dry lipid and cholesterol were dissolved in $CHCl_3$/MeOH (2:1 v/v) to yield mixtures that contained 10% w/w of cholesterol. The solvents were evaporated under a stream of nitrogen, and the lipids (at a concentration of 7.2 mg/mL) were resuspended in buffer (50 mM Na_2SO_4, 25 mM HEPES–SO_4^{-2}, pH 6.8) by vortex mixing. The resulting lipid dispersion was sonicated (10–30 min) in a bath-type sonicator (G1125SP1 sonicator, Laboratory Supplies Co. Inc., New York) until turbidity had cleared. The lipid concentration of the solution was determined by phosphorus analysis (Bartlett et al., 1959). Vesicles were visualized after negative staining with uranyl acetate, using a JEOL JEM 100B electron microscope (Japan Electron Optics Laboratory Co., Tokyo, Japan). Vesicles were shown to be unilamellar, with an average diameter of 20–40 nm (Papahadjopoulos & Miller, 1967; Rapaport & Shai, 1992).

CD Spectroscopy. The CD spectra of the peptides were measured in methanol using a Jasco J-500A spectropolarimeter that had been calibrated with (+)-10-camphorsulfonic acid. The spectra were scanned at 23 °C in a capped, quartz optical cell with a 0.5 mm path length. Spectra were obtained at wavelengths of 250 to 200–190 nm. Four scans were performed at a scan rate of 20 nm/min, a sampling interval of 0.2 nm, and a peptide concentration ranging from 1.0×10^{-5} to 1.6×10^{-5} M.

Fractional helicities (Wu et al., 1981) were calculated as follows:

$$f_h = \frac{([\theta]_{222} - [\theta]^0_{222})}{[\theta]^{100}_{222}}$$

where $[\theta]_{222}$ is the experimentally observed mean residue ellipticity at 222 nm, and values for $[\theta]^0_{222}$, and $[\theta]^{100}_{222}$, corresponding to 0% and 100% helix content at 222 nm, were estimated to be 2000 and 30 000 deg·cm²/dmol, respectively (Chen et al., 1974; Wu et al., 1981).

NBD Fluorescence Measurements. NBD-labeled peptides (0.2 nmol) were added to 2 mL of buffer (50 mM Na_2SO_4, 25 mM HEPES–SO_4^{-2}, pH 6.8) containing 75 μL (550 nmol) of PC-SUV, thus establishing a lipid/peptide ratio of 2700:1. Since the fluorescence of the NBD moiety reached its maximum intensity at this lipid/peptide molar ratio, it is assumed that all the peptide is bound to the vesicles. After a 2-min incubation, the emission spectrum of the NBD group was recorded using a Perkin-Elmer LS-50B spectrofluorometer, with excitation set at 470 nm (5-nm slit), in 3 separate experiments.

Binding Experiments. Peptide association with lipid vesicles was measured by adding lipid vesicles to NBD-labeled peptides (0.1 μM) at 27 °C. The resulting fluorescence intensity was measured as a function of the lipid/peptide molar ratio with a Perkin-Elmer LS-50B spectrofluorometer and excitation set at 470 nm (5-nm slit), and with emission set at 530 nm

[1] Abbreviations: NBD-F, 4-fluoro-7-nitrobenz-2-oxa-1,3-diazole; CD, circular dichroism; PLM, planar lipid membrane; Flu-Su, 5- (and-6-) carboxyfluorescein, succinimidyl ester; Rho-Su, 5- (and-6-) carboxytetramethylrhodamine, succinimidyl ester; BOC, butyloxycarbonyl; Pam, (phenylacetamido)methyl; HOBT, hydroxybenzotriazole; HPLC, high-performance liquid chromatography; TFA, trifluoroacetic acid; DMF, *N,N*-dimethylformamide; SUV, small unilamellar vesicles; PC, egg phosphatidylcholine; diph-PC, diphytanoylphosphatidylcholine; FET, fluorescence resonance energy transfer.

(10-nm slit). The fluorescence values were corrected by subtracting the corresponding fluorescence obtained with blanks (buffer containing the same amount of vesicles). The binding isotherms were analyzed as partition equilibria (Schwarz et al., 1986; Rizzo et al., 1987), as previously described in detail (Rapaport & Shai, 1991).

Resonance Energy Transfer Experiments. Peptides were incorporated into PC vesicles as follows. To solutions of PC SUV (10:1 lipid/cholesterol molar ratio and 220 nmol of phospholipids in 50 μL of buffer) in separate Eppendorf tubes were added methanolic solutions containing 0.028 nmol of Flu-truncated-IsK (donor), either (i) alone or (ii) followed by the addition of 0.056 nmol of Rh-truncated-IsK (acceptor) or (iii) followed by the addition of 0.11 nmol of Rho-truncated-IsK. Three to five experiments were repeated for each concentration, with standard deviation being ~3%. Prior to adding the acceptor molecule, the vesicle solutions containing donor molecules were vortexed thoroughly. After addition of acceptor molecules, the mixtures were diluted to 2 mL with 50 mM Na_2SO_4 and 25 mM HEPES–SO_4^{-2}, pH 6.8. Fluorescence spectra were obtained at room temperature in a SLM-8000 spectrofluorometer, with the excitation monochromator set at 470 nm and a 5-nm slit width. Measurements were performed in a 1-cm path length quartz cuvette in a final reaction volume of 2 mL. Although the excitation maximum for fluorescein is 490 nm, a lower wavelength was chosen in order to minimize the excitation of tetramethylrhodamine (Harris et al., 1991).

The efficiency of energy transfer (E) was determined by calculating the decrease in the quantum yield of the donor due to the addition of an acceptor. E was obtained experimentally from the ratio of the fluorescence intensities of the donor in the presence (I_{da}) and in the absence (I_d) of the acceptor at the emission wavelength of the donor, after correcting for membrane light scattering and the contribution of the acceptor's emission. The percentage value of E is evaluated by the following equation:

$$E = (1 - I_{da}/I_d) \times 100$$

The correction for light scattering was made by subtracting the signal obtained when unlabeled analogues were incorporated into vesicles containing the donor molecules. Correction for the contribution of the acceptor's emission was made by subtracting the signal produced by the acceptor-labeled analogue alone.

Single-Channel Measurements on Planar Lipid Membranes (PLM). Planar bilayers were formed on the tip of glass pipets using the method of Coronado and Latorre (1983). Micropipets were pulled from borosilicate glass capillaries (A-M Systems, Inc., Everett, WA) on a Kopf Model 700C puller (Tujunga, CA). A calomel electrode in 3 M KCl or an Ag/AgCl electrode in the same salt as in the dish was used as reference electrode. The lipids used were either diphytanoylphosphatidylcholine plus cholesterol (50% w/w) or palmitoyloleoylphosphatidylcholine/palmitoyloleoylphosphatidylethanolamine plus cholesterol (2:1 + 20% w/w). Monolayers were formed either (i) from the lipids alone in *n*-hexane or (ii) from mixtures of lipids in *n*-hexane (1 mg/mL) and purified truncated IsK, at phospholipid/peptide molar ratios of 10 000 to 100 000:1. The lipid or lipid–polypeptide solutions (1–2 μL) were introduced onto the surface (0.5 cm^2) of 300–600 μL of a salt solution in a glass dish with the micropipet tip placed in the solution. The salt solutions used were as follows: 0.5 M KCl plus 2 mM $CaCl_2$ in 5 mM HEPES buffer, pH 7.4; 0.5 M NaCl plus 2 mM $CaCl_2$ in HEPES buffer; or a mixture of the two salts at a ratio of 10:1 (biionic conditions). After evaporation of the solvent, the pipet was passed through the interface, allowing the formation of a bilayer, and channel appearance was monitored at several applied potentials (40 experiments). In control experiments, bilayers were formed from lipid solutions without the peptide (the electrical resistance was 5–20 GΩ), and their stability was monitored for about 40 min at several potentials to confirm that the lipids were free from contaminants. Electrical measurements were performed on an Axopatch-1D patch clamp amplifier apparatus (Axon Instruments Inc., Foster City, CA). Current flowing through the bilayers was recorded on a FM magnetic tape, Hewlett Packard, Model 3694A. Experimental data were analyzed off-line by replaying the tape recordings through a low-pass filter (Frequency Devices, Series 902, Haverhill, MA) into an IBM PC AT computer and analyzed with the Axotape and p clamp (version 5.5.1) programs (Axon Instruments).

RESULTS

Five polypeptides corresponding to five different regions within the rat IsK protein were synthesized, fluorescently-labeled, and characterized structurally and functionally. One was a truncated IsK of 63 amino acids, comprised of residues 1–9 linked to residues 41–94 (Table 1). A truncated IsK with a similar sequence, when expressed in Xenopus oocytes, elicited a voltage-dependent K^+ channel activity indistinguishable from that of wild-type IsK (Takumi et al., 1991). The second polypeptide was a 27-mer C-terminal (C-27-IsK, amino acids 68–94 of IsK), the sequence of the third ([10–43]-IsK) resembled amino acids 10–43 that were deleted in the truncated IsK, the fourth (N-43-IsK) was the 43-mer N-terminal of the IsK, and the fifth (N-20-IsK) was the 20-mer N-terminal of the IsK (Table 1).

Fluorescently-labeled analogues were prepared by modifying the five polypeptides selectively at their N-terminal amino acids with one of the following fluorescent probes: NBD, Flu (an energy donor), or Rho (an energy acceptor).

CD Spectroscopy. The extent of α-helical secondary structure of the peptides was estimated from their CD spectra, as measured in methanol (Figure 1). All the peptides, except for C-27-IsK, exhibited significant signals. Their mean residual ellipticities at $[\theta]_{222}$ were −11 390, −12 350, −8290, and −16 920 deg·cm^2/dmol, which correspond to fractional helicity values of 0.31, 0.34, 0.2, and 0.5, for the truncated IsK, the N-43-IsK, the [10–43]-IsK, and the N-20-IsK segments, respectively (Wu et al., 1981). The observed α-helical content of the truncated IsK is probably due mainly to the hydrophobic putative transmembrane domain (amino acids 45–67) of IsK, since a synthetic peptide containing this transmembrane domain plus 4–5 amino acids from the flanking regions revealed ~20 amino acids in an α-helical structure (Ben-Efraim et al., 1993). Hydrophobic moment analysis (Eisenberg et al., 1982) revealed that this α-helix is not amphiphilic. However, the data reveal that the N-terminal part of IsK also adopts a significant percentage of α-helix structure. Since the CD spectra of the C-27-IsK did not show a significant signal, its spectra could not be interpreted to a defined structure.

Fluorescence Measurements of NBD-Labeled Peptides. The NBD moiety can facilitate determination of the environment of N-termini of segments in their membrane-bound state, since its fluorescence intensity is sensitive to the dielectric constant of its surroundings. This probe has been already used in polarity and binding experiments (Kenner & Aboderin,

Table 1: Sequences and Designations of the Peptides Investigated[a]

Peptide No.	Peptide Designation	Peptide Modification	Peptide Sequence
1	trucated-IsK	X=H	X-NH-M A L S N S T T V S K L E A L Y I L M V L G
2	NBD-trucated-IsK	X=NBD	F F G F F T L G I M L S Y I R S K K L E H S H
3	Rho-trucated-IsK	X=Rho	D P F N V Y I E S D A W Q E K G K A-COOH
4	Flu-truncated-IsK	X=Flu	
5	C-27-IsK	X=H	X-NH-R S K K L E H S H D P F N V Y I E S D A W Q
6	NBD-C-27-IsK	X=NBD	E K G K A-COOH
7	[10-->43]IsK	X=H	X-NH-L P F L A S L W Q E T D E P G G N M S A D L
8	NBD-[10-->43]IsK	X=NBD	A R R S Q L R D D S K L-COOH
9	N-43-IsK	X=H	X-NH-M A L S N S T T V L P F L A S L W Q E T D E
10	NBD-N-43-IsK	X=NBD	P G G N M S A D L A S Q L R D D S K L-COOH
11	N-20-IsK	X=H	X-NH-M A L S N S T T V L P F L A S L W Q E T-COOH
12	NBD-N-20-IsK	X=NBD	

[a] The following abbreviations are used: NBD, 7-nitrobenz-2-oxa-1,3-diazol-4-yl; Rho, tetramethylrhodamine; Flu, carboxyfluorescein.

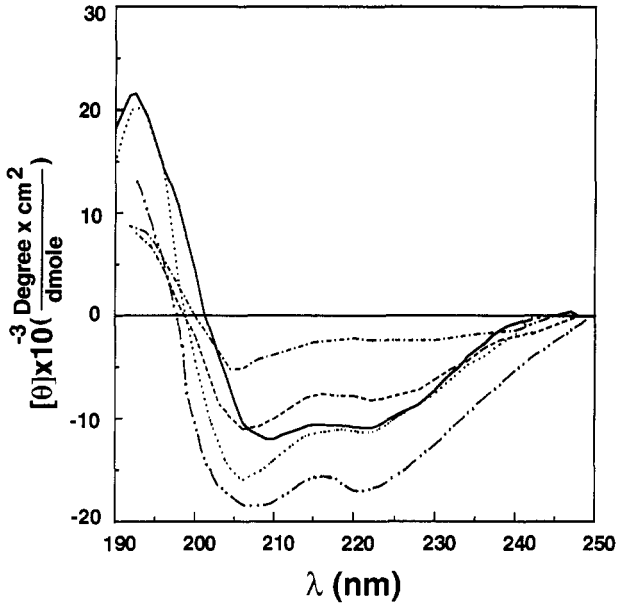

FIGURE 1: CD spectra of IsK peptides in methanol. Spectra were taken at peptide concentrations of 1.5×10^{-5} to 2.0×10^{-5} M. Symbols: truncated IsK (—); [10–43]-IsK (- - -); N-43-IsK (···); C-27-IsK (– · –) ; N-20-IsK (–··–).

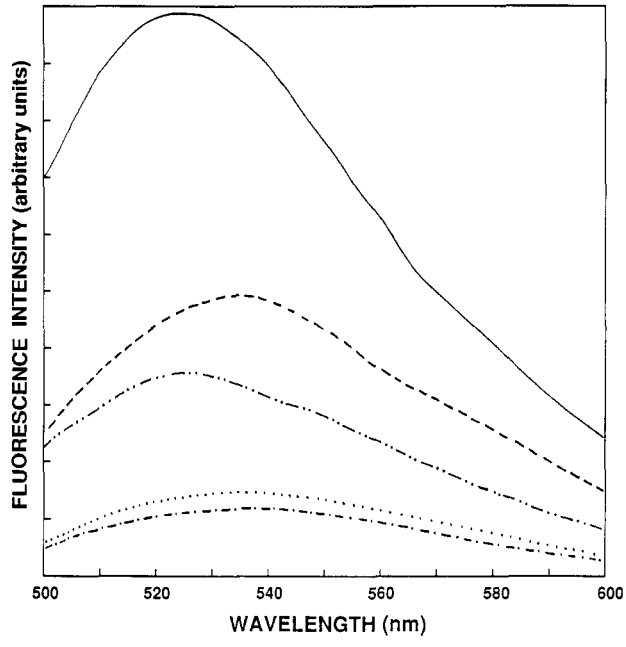

FIGURE 2: Fluorescence emission spectra of NBD-labeled peptides. Spectra were determined in the presence or absence of PC-SUV (270 μM phospholipids) in buffer composed of 50 mM Na_2SO_4 and 25 mM HEPES-SO_4^{-2}, pH 6.8. The excitation wavelength was set at 467 nm. Symbols: (···) NBD-C-27-IsK; (–··–) NBD-truncated IsK; (- - -) NBD-[10–43]-IsK, all in the presence of vesicles; (—) N-43-IsK; and (– · –) all five NBD-peptides in buffer. The results for N-20-IsK were similar to those obtained for N-43-IsK and as such are not given.

1971; Frey & Tamm, 1990; Baidin & Huang, 1990; Rapaport & Shai, 1991; Pouny & Shai, 1992). The fluorescence emission spectra of the five NBD-labeled IsK polypeptides were measured either in aqueous solutions or in the presence of PC vesicles (Figure 2). All five NBD-labeled segments exhibited fluorescence emission maxima around 540 nm in buffer (Figure 2, dashed–dotted line), which agrees with previously reported emission wavelength maxima for NBD derivatives in hydrophilic environments (Rajarathnam et al., 1989; Rapaport & Shai, 1991; Pouny & Shai, 1992; Gazit & Shai, 1993). However, in solutions of PC vesicles at pH 6.8, the fluorescence emission maxima of NBD-truncated IsK, NBD-[10–43]-IsK, NBD-N-43-IsK, and N-20-IsK exhibited blue shifts concomitant with enhanced increases in their fluorescence intensities (Figure 2). The shift was significantly higher for the NBD-truncated IsK, NBD-N-43-IsK, and N-20-IsK (maximum 525 ± 1 nm), than for NBD-[10–43]-IsK (maximum 534 ± 1 nm). Similar magnitudes of blue shifts are observed when surface-active NBD-labeled peptides interact with lipid membranes (Frey & Tam, 1990; Rapaport & Shai, 1991; Pouny et al., 1992) and are consistent with the NBD probe being located (Rajarathnam et al., 1989) within (in the case of NBD-truncated IsK, NBD-N-43-IsK, and N-20-IsK) or on the surface of (in the case of NBD-[10–43]-IsK) the membrane. However, the emission maxima of NBD-C-27 were identical in the buffer alone and in the presence of vesicles (λ_{max} = 540 nm, Figure 2). In these

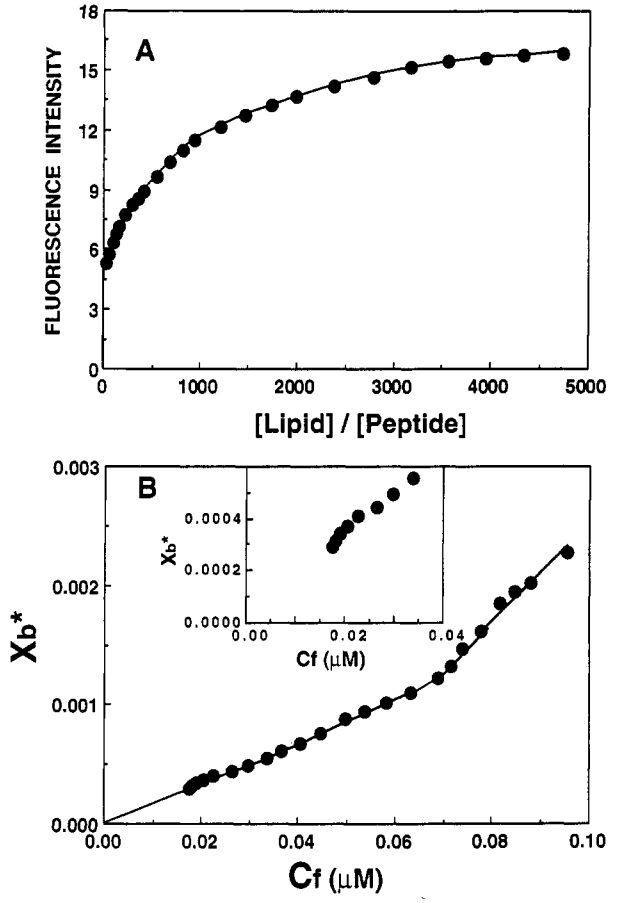

FIGURE 3: Increases in the fluorescence of NBD-N-43-IsK upon titration with PC vesicles (A), and the resulting binding isotherm (B). NBD-N-43-IsK (0.1 μM) was titrated with PC-SUV with excitation set at 467 nm and emission recorded at 530 nm. The experiment was performed at room temperature in 50 mM Na_2SO_4 and 25 mM HEPES-SO_4^{-2}, pH 6.8. The binding isotherm was derived from (A) by plotting X_b* (molar ratio of bound peptide per 60% lipid) versus C_f (equilibrium concentration of free peptide in the solution). The inset depicts the low concentration range of the main figure.

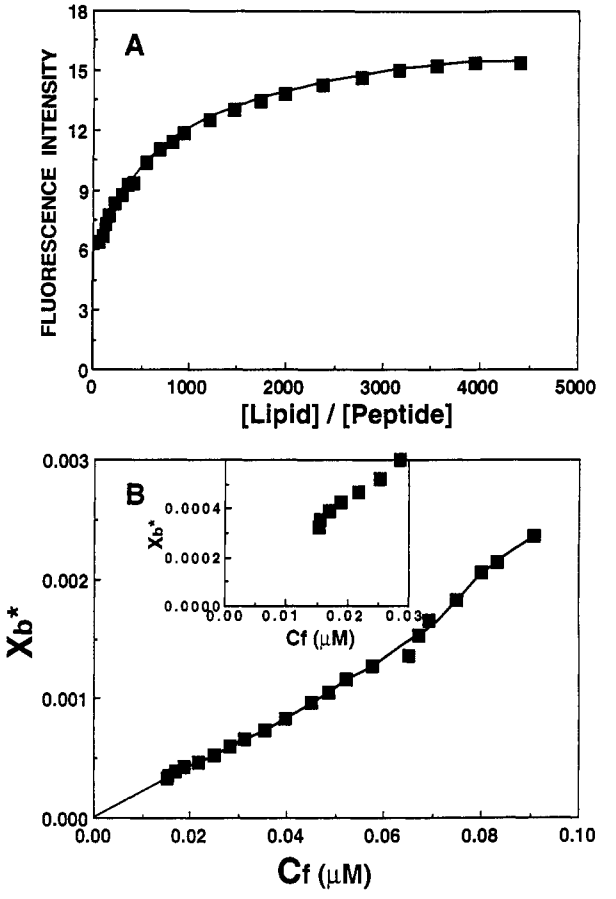

FIGURE 4: Increases in the fluorescence of NBD-[10–43]-IsK upon titration with PC vesicles (A), and the resulting binding isotherm (B). NBD-[10–43]-IsK (0.1 μM) was titrated with PC-SUV with excitation set at 467 nm and emission recorded at 530 nm. See legend to Figure 3.

experiments, the lipid/peptide molar ratio was consistently maintained at an elevated level (2700:1), so that spectral contribution of free peptide would be negligible.

Binding Experiments. To determine the affinity of the segments to phospholipid membranes, fixed concentrations (0.1 μM) of NBD-N-43-IsK and NBD-[10–43]-IsK at pH 6.8 were titrated with vesicles. The resulting increases in the fluorescence intensities of the NBD-labeled peptides were plotted as a function of the lipid/peptide molar ratios (Figure 3A depicts this plot for NBD-N-43-IsK and Figure 4A for NBD-[10–43]-IsK). Due to the low solubility of the NBD-truncated IsK in aqueous solution, and the fact that its long C-terminal does not contribute to its binding properties, truncated IsK's partition coefficient was assumed to be that previously calculated for the transmembrane segment alone (Ben-Efrain et al., 1993). Binding isotherms were analyzed as partition equilibriums (Schwarz et al., 1986, 1987; Rizzo et al., 1987), as previously described in detail (Rapaport & Shai, 1991). The following formula was used:

$$X_b^* = K_p^* C_f$$

where X_b* is defined as the molar ratio of bound peptide per 60% of the total lipid, assuming that the peptides were initially partitioned only over the outer leaflet of the SUV, as had previously been suggested (Beschiaschvili & Seelig, 1990). K_p* corresponds to the partition coefficient, while C_f represents the equilibrium concentration of free peptide in the solution. The curve that results from plotting X_b* versus free peptide, C_f, is referred to as the conventional binding isotherm and is presented in Figures 3B and 4B for NBD-N-43-IsK or NBD-[10–43]-IsK, respectively. The surface partition coefficient of each peptide, K_p*, was estimated by extrapolating the initial slope of its binding curve to a zero C_f value. The estimated surface partition coefficients, K_p*, were calculated to be 1.8 (±0.2) × 10^4 M^{-1} for NBD-N-43-IsK and 2.0 (±0.2) × 10^4 M^{-1} for NBD-[10–43]-IsK. These values are high and are typical of the K_p*s of surface-active peptides (Stankowski & Schwarz, 1990; Thiaudière et al., 1991; Rizo et al., 1987; Rapaport & Shai, 1991; Pouny et al., 1992).

Fluorescence Energy Transfer Studies. Whether the 63-mer, truncated IsK can self-associate within phospholipid membranes was investigated by intermolecular fluorescence energy transfer studies as described previously (Rapaport & Shai, 1992). For these studies, the truncated IsK was selectively labeled at its N-terminal amino acid with either Flu (an energy donor) or Rho (an energy acceptor, Table 1). Profiles of the energy transfer from Flu-truncated IsK to Rho-truncated IsK, in the presence of PC phospholipid vesicles, are depicted in Figure 5. Addition of the acceptor Rho-truncated IsK (final concentration of 0.028 or 0.056 μM) to the donor Flu-truncated IsK (0.014 μM) in the presence of PC phospholipid vesicles (110 μM), quenched the donor's emission and increased the acceptor's emission, which is consistent with energy transfer. The energy transfer was calculated and plotted versus the acceptor/lipid molar ratio

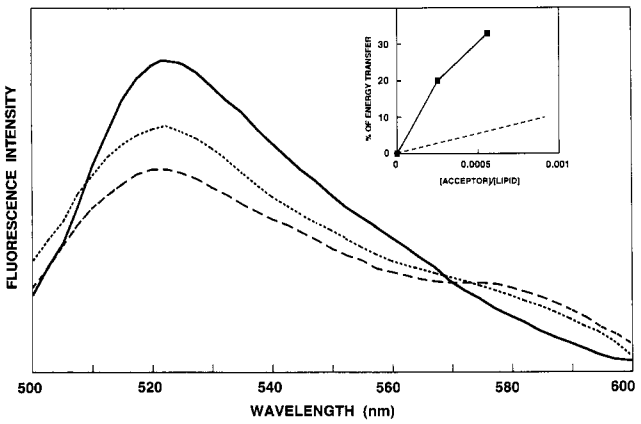

FIGURE 5: Fluorescence energy transfer dependence on Rho-peptide (acceptor) concentrations using PC vesicles. The spectrum of Flu-truncated IsK (0.014 μM), the donor peptide, was determined in the absence (—) or presence of 0.028 μM (···) or 0.056 μM Rho-truncated IsK (– –) (the acceptor molecule). Each spectrum was recorded in the presence of PC vesicles (100 μM) in 50 mM Na_2SO_4 and 25 mM HEPES–SO_4^{-2}, at pH 6.8. The excitation wavelength was set at 470 nm. The spectra of Rho-truncated IsK in the presence of vesicles and unlabeled truncated IsK were subtracted from all the corresponding spectra. Inset: Calculated percentage of energy transfer for a random distribution (dashed line) and for truncated IsK (filled squares). The average of three measurements with an uncertainty of ~3% is depicted.

(inset of Figure 5). In control experiments, no change in the emission spectrum of fluorescein was observed when an equal amount of unlabeled truncated IsK was added instead of the acceptor Rho-truncated IsK (data not shown), or when the acceptor probe was attached to ethanolamine, which does not interact with the donor-labeled peptide. This demonstrates that the observed decrease in the donor emission at 520 nm was caused by an association of the donor and the acceptor-labeled peptides. The lipid/peptide molar ratios in these experiments were kept high (3900:1 and 1780:1), (i) to ensure that practically all the peptides were in their membrane-bound state and (ii) to ensure a low surface density of donors and acceptors, which would reduce energy transfer between unassociated peptide monomers. Adding the acceptor-peptide only after the donor-peptide was already bound to the membrane prevented any association of the peptides in solution. To confirm that the observed energy transfer is due to aggregation, the transfer efficiencies observed for the truncated IsK were compared with those expected for randomly-distributed, membrane-bound donors and acceptors (dashed line, inset of Figure 5). The random distribution was calculated using eq 17 of Wolber and Hudson (1979), and assuming that 45 Å is the R_0 value for the Flu/Rho donor/acceptor pair (Rapaport & Shai, 1992). The energy transfer observed with the truncated IsK donor/acceptor pair is clearly higher than the value expected for a random distribution (inset of Figure 5). This suggests that the truncated IsK is associated rather than randomly distributed throughout the membrane. It also reveals that a long hydrophilic tail on the transmembrane segment of IsK does not affect the segment's ability to associate within the membrane. Unfortunately, these experiments do not allow us to determine the size of the aggregates formed.

Single-Channel Formation by the Truncated IsK in Planar Bilayers (PLM). Single-channel experiments were performed with the truncated IsK to assess its ability to form channels in PLM. Two lipid mixtures were used, rendering similar results. Truncated IsK, dissolved in methanol, was added to the lipids' solution in *n*-hexane. Representative single-channel traces formed by the truncated IsK in diphytanoyl-PC at an applied potential of –50 mV are depicted in Figure 6. Both the lipids and the synthetic peptides were devoid of contaminants; therefore, the single-channel traces reflect the potential of the truncated IsK to form channels. The variability of the current amplitude, as seen in the current amplitude histograms of the three major conductance levels (γ values of 17, 38, and 50 pS, Figure 6B), can be explained by the formation of aggregates, composed of a variable number of monomers, that associate and dissociate at various rates. When experiments were performed with NaCl solution or under biionic conditions, the truncated IsK did not discriminate between K^+ and Na^+. Therefore, further characterization of these channels was not pursued.

DISCUSSION

Although the IsK protein forms K^+ channels when expressed in *Xenopus* oocytes, the protein has both primary and hypothesized secondary structures that differ markedly from those of the well-known family of the *Shaker* type, voltage-dependent K^+ channels. IsK is composed of only 130 amino acids and is thought to contain a single putative transmembrane α-helical domain, as suggested by hydropathy analysis (Takumi et al., 1988). Recent data suggest that IsK is not actually a channel protein itself, but rather an activator or regulator of endogenous and dormant K^+ and Cl^- channels (Attali et al., 1993). However, the protein contributes to the K^+ pore properties, since the substitution of Phe at position 55 with Thr changes the selectivity of the channel (Goldstein et al., 1991). Despite a wealth of information on the functional activities of IsK, only limited data are available on its topology and its structure within the membrane. Hydropathic analysis and immunogold electron microscopy, with polyclonal antibodies directed to the carboxy-terminal and to amino acids 29–44 that precede the transmembrane domain, suggested the following two structural features for the protein (Sugimoto et al, 1990): that the protein contains only one transmembrane domain, and that the C-terminal and the sequence that is close to the N-terminal are probably located on the cytoplasmic and extracellular sides of the membrane.

The major objective of the study presented here was to obtain information on the topology and secondary structure of the IsK protein in a membrane-bound state. Single-channel experiments were then performed with the truncated 63-mer, which has a sequence similar to that of a truncated IsK that has been expressed in oocytes and induced K^+ channels (Takumi et al., 1991).

That two regions of IsK are embedded within the membrane is suggested by the present data and previous studies on a synthetic 32 amino acid segment of the protein (Ben-Efraim et al., 1993). One region is the hypothesized transmembrane segment [amino acids 45–67 (Takumi et al., 1988)], and the other is the N-terminal domain of IsK. The linker between the N-terminal and the transmembrane segment is probably located on the surface of the membrane, since the environment encountered by an NBD group located at the N-terminus of either truncated IsK, N-43-IsK, or N-20-IsK was hydrophobic (emission maximum of 525 ± 1 nm; Figure 2). In contrast, the environment encountered by an NBD group located at the N-terminus of [10–43]-IsK was identical to that detected with an NBD probe located at the surface of the membrane (emission maximum of 534 nm; Figure 2). The relatively low fluorescence intensity of the truncated IsK observed in the presence of vesicles might result from self-quenching of the NBD probe, since truncated Isk self-associates in its membrane-bound state (see below).

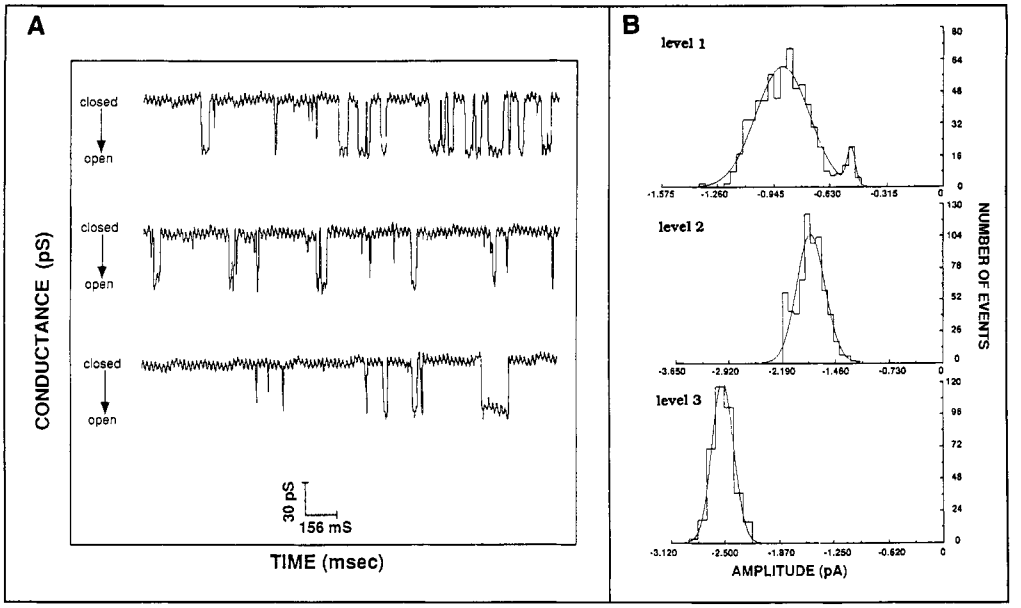

FIGURE 6: (A) Single-channel traces for the truncated IsK. The applied potential was −50 mV and the filtration was 100 Hz. (B) Histograms of current amplitudes. The histograms were constructed from traces of panel A. Recording time was 126 s.

Determination of the secondary structure of the segments in aprotic solvent indicated that, in addition to the transmembrane α-helix (Ben-Efraim et al., 1993), the N-terminal of IsK also adopts an α-helical structure. This is based on the following: (i) The [10–43]-IsK segment adopts 20% α-helix (Figure 1), (ii) the addition of 9 amino acids to the N-terminal of [10–43]-IsK resulted in an increase to 34% in the α-helical content of the resulting N-43-IsK segment, and (iii) N-20-IsK segment adopts 50% α-helical structure. This may be due to the formation of an α-helical segment that contains 10–15 amino acids at the N-terminal of IsK. Since the CD spectrum of the C-27-IsK displayed a low signal, no specific structure could be assigned to this peptide.

The resonance energy transfer studies between donor- and acceptor-labeled truncated IsK at two acceptor/lipid molar ratios (Figure 5) demonstrated that the percentage of energy transfer between membrane-embedded monomers of truncated IsK is higher than that calculated for randomly distributed monomers. For example, a high energy transfer (20%) was already detected with the Flu-truncated IsK/Rho-truncated IsK pair at a peptide/lipid molar ratio of 1:3900. If random distribution had occurred, 3% energy transfer was expected to occur (inset of Figure 5). Furthermore, if each SUV vesicle of 20–40-nm diameter is composed of 4000–10 000 lipid molecules, and if the peptides are equally distributed between the vesicles, then apparently as few as 2–3 molecules within one vesicle can associate. Thus, aggregation of truncated IsK appears to occur within membranes, which agrees with the recent observation that IsK proteins interact with each other on the surface of oocyte (Blumenthal & Kaczmarek, 1993). Unfortunately, the size of the bundles cannot be predicted by this method.

That the N-terminal of IsK adopts α-helical structure and is also embedded within the membrane might be important for IsK function, since the N-terminal might also participate in the self-aggregation and binding of IsK to other membranal proteins. This assumption is supported by increasing data that indicate that intramembranal α-helices of integral membrane proteins can participate in specific interactions that contribute to specific recognition, association, and oligomerization of the proteins within the lipid environment [see review by Lemmon and Engelman (1992)].

Truncated IsK forms single-channel fluctuations in planar lipid membranes, that do not disturb the membrane structure to cause irregular conductance activity. Although truncated IsK does not exhibit all of the properties of the native channel and does not exhibit the required specificity, its channel-forming activity is not a common property of peptides and suggests that this segment may form part of a complex native ion channel. The inability of the purified, synthetic IsK to form functional K^+ channels agrees with recent data that suggest that currents observed in an oocyte system result from the association of IsK with an endogenous factor or with silent channels (Attali et al., 1993).

The reported property of truncated IsK to form channel fluctuations in phospholipid membranes should not be construed to indicate that the segment by itself is a channel. Rather, these findings agree with the suggestion that the truncated IsK can be stabilized within membranes by forming bundles of helices. In the presence of helices of other proteins, heteroaggregates might also be favored. Such heteroaggregates might contain more than one molecule of IsK.

The heterogeneity of the observed channel conductance (Figure 6) may be due to the association of different numbers of monomers in the peptide aggregates that form the channels. If so, the conductances may reflect a difference in the multimeric state of the IsK peptide forming the channel fluctuations. Alternatively, the heterogeneity of conductance could be due to alternative conformations of the side chains of the truncated IsK monomers. It should be noted, however, that various amphipathic polypetides from natural sources or of segments from ion channels can produce single channels in planar phospholipid membrane, some of which are selective to specific ions (Oiki et al., 1988; Tosteson et al., 1989; Grove et al., 1991; Ghosh & Stroud, 1991; Langosch et al., 1991; Ben-Efraim *et al.*, 1993).

In summary, the results presented herein suggest that the IsK protein is self-associated, at different aggregation levels, in its membrane-bound state. When membrane-bound IsK's N-terminal and its transmembrane domain are embedded within the membrane, they adopt α-helical structures. Moreover, IsK by itself is probably not a K^+ channel, although it has the potential to aggregate within phospholipid membranes and to form single channels, not characteristic of K^+ channels.

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