

Change of Glutamic Acid to Lysine in a 13-Residue Antibacterial and Hemolytic Peptide Results in Enhanced Antibacterial Activity without Increase in Hemolytic Activity

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A 13-residue peptide corresponding to a hydrophobic segment of the antimicrobial 47-residue peptide seminalplasmin, PKLLETFLSKWIG (SPF), has been shown to have antibacterial and hemolytic activities (N. Sitaram and R. Nagaraj, J. Biol. Chem. 265:10438–10442, 1990). In an effort to get an insight into the structural and charge requirements for these biological activities, an analog of SPF in which Glu has been replaced with Lys has been synthesized and its antibacterial and hemolytic properties have been examined. It has been demonstrated that the analog, SPFK, exhibits potent antibacterial activity at concentrations at which hemolysis does not occur.

There has been considerable interest in recent years in structure-function studies with peptides composed of 15 to 30 residues and possessing antibacterial and eukaryotic cell lytic activities (2, 4–9, 13, 15, 16, 18, 20, 22–27). One aspect of these studies has been the evaluation of shorter segments of these peptides for biological activity (1, 6, 12, 14, 21). In addition to defining the minimal requirements for biological activity, these peptides can prove to be helpful in possible therapies, since short peptides are easy to generate by chemical synthesis. We have previously demonstrated that a hydrophobic 13-residue segment, PKLLETFLSKWIG (SPF), of the antimicrobial 47-residue peptide seminalplasmin (SPLN), has antibacterial activity as well as erythrocyte lytic activity (26). SPF was shown to adopt an amphipathic helical structure with two Lys and one Glu residue on the polar face of the helix. Thus, SPF has a net positive charge of +1 at neutral pH. In an effort to get an insight into the structural and charge requirements for biological activity, we have synthesized SPFK, an analog of SPF in which Glu has been replaced by Lys. Thus, SPFK would have a net charge of +3 at neutral pH. In this report, we show that SPFK exhibits potent antibacterial activity at concentrations at which erythrocyte lysis does not occur.

MATERIALS AND METHODS

Peptide synthesis. The synthesis of SPF has been reported previously (26). Peptide SPFK was synthesized on triethyl-ene glycoldimethacrylate-cross-linked (1%) polystyrene resin by using t-Boc chemistry and protocols essentially as described for SPF (26). The peptide was characterized by amino acid analysis and sequencing on an Applied Biosystems 473A instrument.

Antibacterial activities. The MICs of the peptides were determined by incubating a logarithmically growing culture of *Escherichia coli* W160.37 in a minimal synthetic medium [0.5 g of NH₄Cl, 0.5 g of (NH₄)₂SO₄, 13.6 g of KH₂PO₄, 0.02 g of MgSO₄ · 7H₂O, 0.015 g of (NH₄)₂Fe(SO₄)₂ · 6H₂O, 20 g of glucose, 0.1 g of L-arginine hydrochloride in 1 liter of medium (pH 7.2)] diluted to an initial A₆₀₀ of 0.01, at 37°C for

6 h in the absence and presence of various concentrations of the peptides. An A₆₀₀ of 0.5 to 0.6 was observed for the control culture, which contained no peptide, and this value was taken to be 100%. The effect of divalent cations was determined by performing the assay in the presence of various concentrations of cations.

Bacteriolytic activity was monitored by measuring the activity of β-galactosidase released from *E. coli* W160.37 cells grown to logarithmic phase in the above medium containing 5 × 10⁻⁴ M isopropylthiogalactopyranoside (17) and incubated in the absence and presence of the peptides at 37°C. The A₆₀₀ of the culture before the peptide was added was 0.2 to 0.3 (2.0 × 10⁸ to 3.0 × 10⁸ cells per ml). Aliquots of 150 μl were withdrawn at different times, diluted to 1 ml with ice-cold assay buffer (0.06 M Na₂HPO₄, 0.04 M NaH₂PO₄, 0.01 M KCl, 0.001 M MgSO₄, 0.05 M β-mercaptoethanol, pH 7.4) and spun down at 12,000 × g at 4°C. β-Galactosidase activity in the supernatant was measured using *ortho*-nitrophenylgalactopyranoside (ONPG) as substrate. In order to determine the permeability properties of the bacterial inner membrane in the presence of the peptide, the influx of ONPG into sedimented cells described above was determined by incubating the cells with ONPG in 1 ml of assay buffer at 37°C. The total enzyme activity in the sedimented cells in the absence of the peptide was measured after the cells were treated with a few drops of 0.1% sodium dodecyl sulfate (SDS)-chloroform. The effect of cations was determined by incubating the cells with the peptides in the absence and presence of various concentrations of cations.

Hemolytic activity. Rat erythrocytes were isolated by centrifugation of freshly collected blood followed by three washes with 5 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer (pH 7.4) containing 150 mM KCl to remove the buffy coat. Erythrocytes (0.5% [vol/vol]) were incubated at 37°C in the same buffer with different concentrations of the peptide for 15 min at 37°C. Samples were centrifuged, and the A₅₄₀ in the supernatant was measured. The absorbance obtained by treatment of erythrocytes with 1% Triton X-100 was taken to be 100%.

The effect of transmembrane potential on hemolysis was determined by preparing the cells as described above with 5 mM HEPES (pH 7.4)–150 mM KCl but suspending them in

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TABLE 1. Antibacterial activities of peptides

Concn of peptide ($\mu\text{g/ml}$)	% Inhibition of growth of <i>E. coli</i> by:		
	SPFK	SPF	SPLN
4	67		0
6.6	84		0
10	100	0	15
20	100	25	95
30	100	60	100
40	100	90	100
50	100	100	100

5 mM HEPES (pH 7.4)–150 mM NaCl buffer for the assay. Three microliters of 1.6 mM valinomycin solution dissolved in dimethyl sulfoxide was added to 0.75 ml of the erythrocyte suspension. After 20 s, 0.5 ml of this cell suspension was mixed with an equal volume of peptide containing buffer of the same composition. The samples were incubated for 15 min and cells were spun down for estimation of hemolysis. The potential induced was checked by using a potential-sensitive dye, 3,3'-dipropylthiocarbocyanine [$\text{diSC}_3\text{-(5)}$], according to the method of Sims et al. (23). The experiment performed in the absence of valinomycin was taken to be the control.

RESULTS AND DISCUSSION

Antibacterial activity. The percent inhibition of growth of *E. coli* W160.37 cells in logarithmic phase during incubation with different concentrations of SPFK was compared with the effects of SPF and SPLN. The results are summarized in Table 1. The MIC of SPFK is 10 $\mu\text{g/ml}$, compared with 50 $\mu\text{g/ml}$ for SPF and 30 $\mu\text{g/ml}$ for SPLN. SPFK is clearly more potent as an antibacterial agent than SPF or SPLN. The antibacterial activities of SPF and SPLN have been previously shown to arise from their ability to alter the permeability properties of the bacterial inner membrane (25, 26). Hence, the bacteriolytic activity of SPFK and its effect on the permeability properties of the bacterial inner membrane were studied. The influx of ONPG into sedimented *E. coli* cells as a function of time and peptide concentration is shown in Fig. 1. Considerable ONPG influx is discernible, even at peptide concentrations of 20 $\mu\text{g/ml}$. At 40 $\mu\text{g/ml}$ and above, the influx is rapid and in 15 min the inner membrane no longer acts as a permeability barrier. Interestingly, no β -galactosidase activity could be detected in the cell-free supernatant even when the inner membrane was completely permeabilized. This rapid concentration-dependent enhancement of ONPG influx in the presence of SPFK indicates the opening of other pathways for the influx of ONPG through the bacterial inner membrane in addition to the transporter *lac* permease and is the cause of cell death. The concentration of SPFK required to permeabilize the bacterial inner membrane is less than that observed for SPF and SPLN, indicating that increased cationicity may enhance the membrane-perturbatory property of the peptide.

We have previously shown that the divalent cations Ca^{2+} , Mn^{2+} , and Zn^{2+} can inhibit the antibacterial activity of SPLN against *E. coli* by stabilizing the bacterial membrane (25). We have investigated the effect of Ca^{2+} ions on antibacterial activity and on membrane permeability changes brought about by SPF and SPFK. It can be seen from Fig. 2a that 600 μM Ca^{2+} can almost completely abolish the anti-

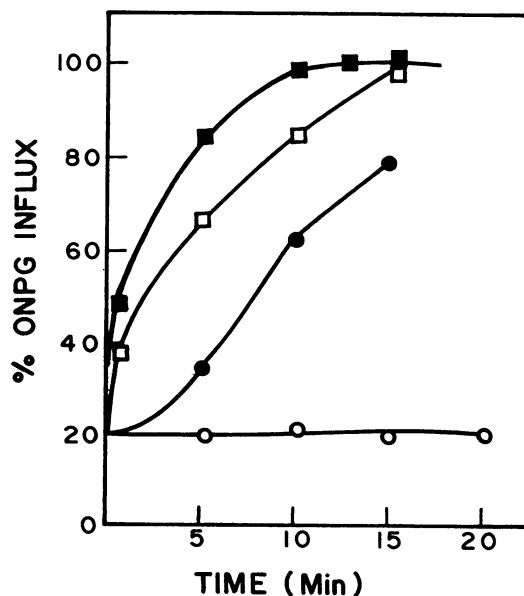


FIG. 1. Effect of SPFK on the influx of ONPG into *E. coli* W160.37. *E. coli* cells in the logarithmic phase of growth with an initial absorbance of 0.3 were incubated with the following concentrations (in micrograms per milliliter) of peptide: 0 (\circ), 20 (\bullet), 40 (\square), and 60 (\blacksquare). Aliquots were withdrawn at different time points, and ONPG influx into cells was determined as discussed in Materials and Methods.

bacterial activity of SPF and SPFK. The enhancement of bacterial inner membrane permeability with ONPG by SPF and SPFK is also inhibited by Ca^{2+} (Fig. 2b). Similar results were obtained with the other divalent cations Mn^{2+} and Zn^{2+} . These results indicate that SPFK perturbs the bacterial outer membrane, enters the cells, and then acts on the inner membrane. The increased cationic nature of SPFK may favor an association with acidic components of the bacterial outer membrane and subsequent entry into the inner membrane. Inhibition of antibacterial activity by cations also rules out receptor-mediated entry through the outer membrane (3) or through porins (19). Although SPLN has four positive charges (24), these are distributed throughout the polypeptide chain and not in the region of SPF (26), which is an important determinant of activity.

Hemolytic activity. The hemolysis of rat erythrocytes by SPFK as a function of peptide concentration was investigated. Hemolysis was discernible above a concentration of 9 μM . The antimicrobial and hemolytic activities of SPF and SPFK are compared in Fig. 3. In the case of SPFK, considerable antibacterial activity is discernible even at a concentration of 3 μM , at which virtually no hemolysis is observed, and at 9 μM 100% antibacterial activity is observed with only 10% hemolytic activity. SPF under similar conditions does not show differences in its hemolytic and antibacterial activities. Thus, the Glu \rightarrow Lys change has resulted in the peptide exhibiting antibacterial activity at concentrations at which erythrocyte lysis does not occur. Thus, by judicious choice of charge replacements it may be possible to alter the cell lytic properties of peptides and obtain selectivity for either prokaryotic or eukaryotic cells.

In addition to hydrophobicity of the peptide, charge on the peptide, and surface charge on the membrane, another important parameter likely to influence the interaction of the

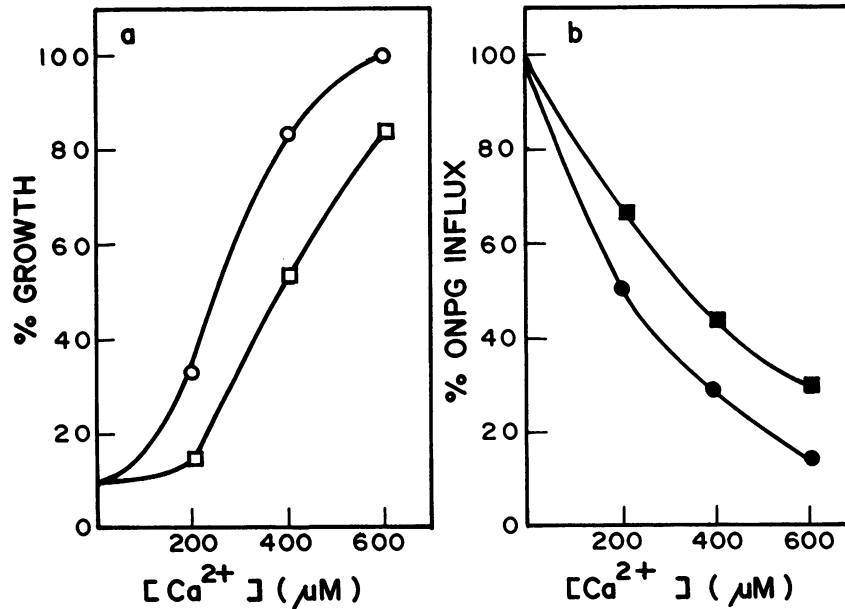


FIG. 2. Effect of cations on antibacterial activity and ONPG influx. (a) Antibacterial activity of 7 µg of SPFK per ml (○) and 40 µg of SPF per ml (□). (b) ONPG influx with 40 µg of SPFK per ml (●) and 80 µg of SPF per ml (■).

peptide with biomembranes is the electrochemical potential existing across the membrane. Transmembrane potentials have been shown to influence the association of hydrophobic peptides with membranes (10, 11). It has also been shown that hemolysis by a 25-residue presequence peptide of subunit IV of the *Saccharomyces cerevisiae* cytochrome oxidase and its effect on the leakage of entrapped carboxyfluorescein from small unilamellar vesicles increase in a potential-dependent manner (8). Hence, we investigated whether the

presence of transmembrane potential has any effect on hemolysis by SPF and SPFK. The hemolytic profiles of both SPF and SPFK, in the presence and absence of valinomycin, are presented in Fig. 4. It is clear from the figure that the presence of transmembrane potential decreases the threshold concentration of the peptide required to cause hemolysis. However, the antibacterial activity of SPFK is appreciably more than its hemolytic activity even in the presence of a membrane potential.

The circular dichroic spectra of SPFK in buffer and SDS

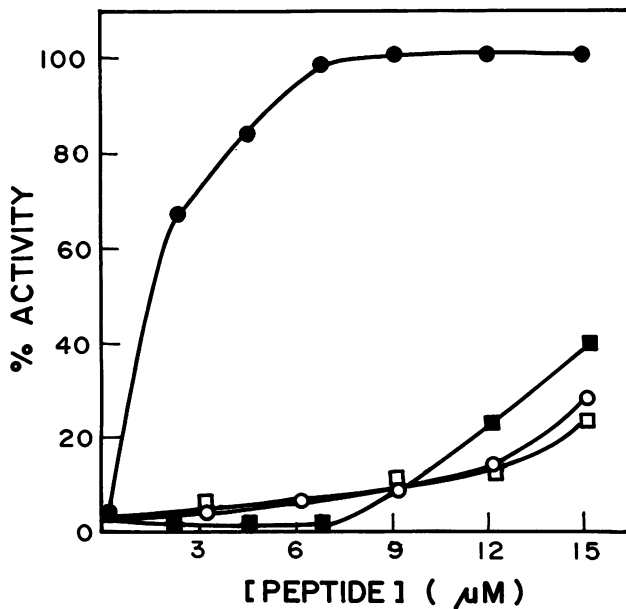


FIG. 3. Comparison of antibacterial and hemolytic activities of SPFK and SPF. Symbols: ●, antibacterial activity SPFK; ○, hemolytic activity of SPFK; ■, antibacterial activity of SPF; □, hemolytic activity of SPF.

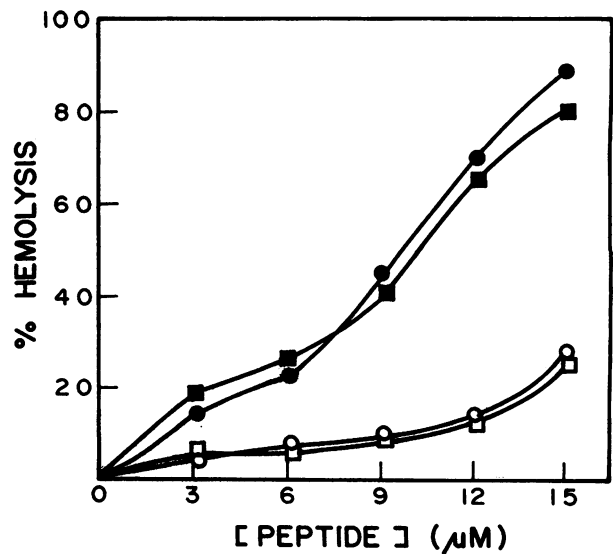


FIG. 4. Effect of transmembrane potential on the hemolytic activities of SPFK and SPF. Symbols: ○ and □, hemolytic activities of SPFK and SPF, respectively, in the absence of valinomycin; ● and ■, hemolytic activities of SPFK and SPF, respectively, in the presence of valinomycin.

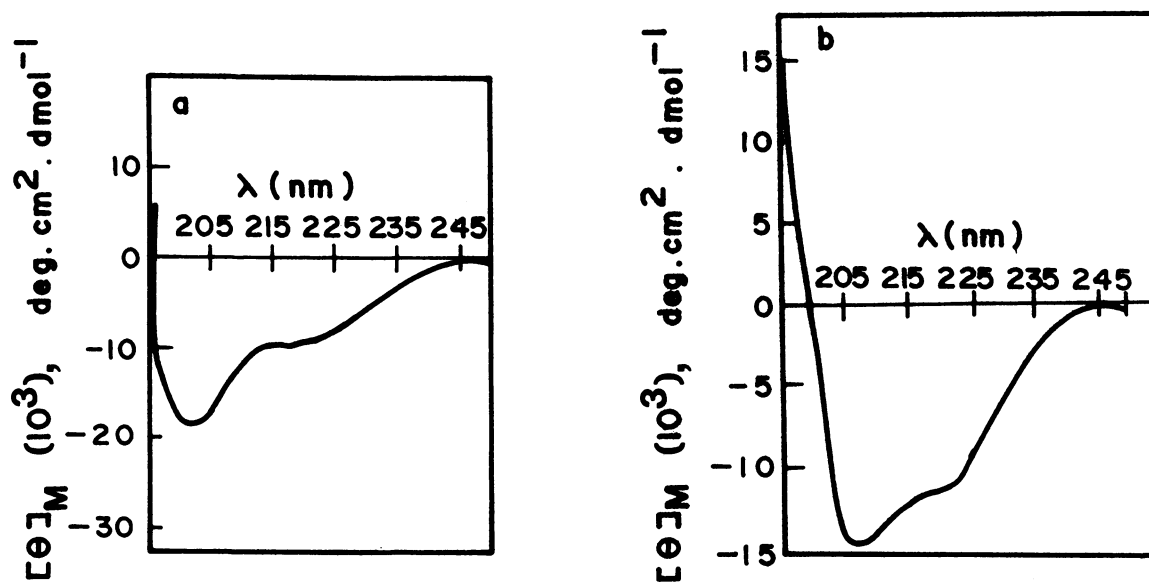


FIG. 5. Circular dichroic spectra of SPFK in (a) buffer at pH 7.4 and (b) micelles of SDS. Spectra were recorded on a Jobin Yvon Dicro-graph V spectropolarimeter at 25°C in cells with a path length of 0.1 cm. $[\theta]_M$ values are mean residue ellipticities.

micelles are shown in Fig. 5. The spectra indicate that SPFK adopts a helical conformation, particularly in a hydrophobic environment. SPF has been shown to adopt a helical conformation in a hydrophobic environment (26). The mean residue ellipticities at 222 nm are comparable for both peptides. The helical wheel projections of SPF and SPFK are shown in Fig. 6. The values for average hydrophobicity and hydrophobic moment for SPF were 0.24 and 0.41 and for SPFK were 0.18 and 0.42. It is evident that the change of Glu→Lys does not result in drastic changes in hydrophobic moment or average hydrophobicity. Hence, the differences in activities of SPFK and SPF are not due to differences in structure.

The data presented in this report demonstrate that replacement of glutamic acid of SPF with lysine results in considerably enhanced antibacterial activity of the peptide without a concomitant increase in erythrocyte lytic activity. This

enhancement of antibacterial activity stems primarily from the increased net positive charge on the peptide, as this modification does not bring about significant changes in its hydrophobicity, hydrophobic moment, or conformation compared with SPF. Interestingly, increased cationicity does not result in the peptide being more hemolytic, as interaction with the negative surface of the erythrocyte membrane would also be favored. Thus, cationic charges clearly play a more important role in interactions with the bacterial membrane. Although cationic antibacterial, nonhemolytic peptides have been isolated from amphibian skin and insects (7, 18, 25, 27, 28), they are considerably longer (25 to 37 residues). Also, in magainins, a reduction in the length of the peptide chain leads to a considerable loss in antimicrobial activity (7, 28). Hence, studies directed toward structure-function correlation with short bioactive peptides such as SPF and SPFK, which are composed of only 13 residues

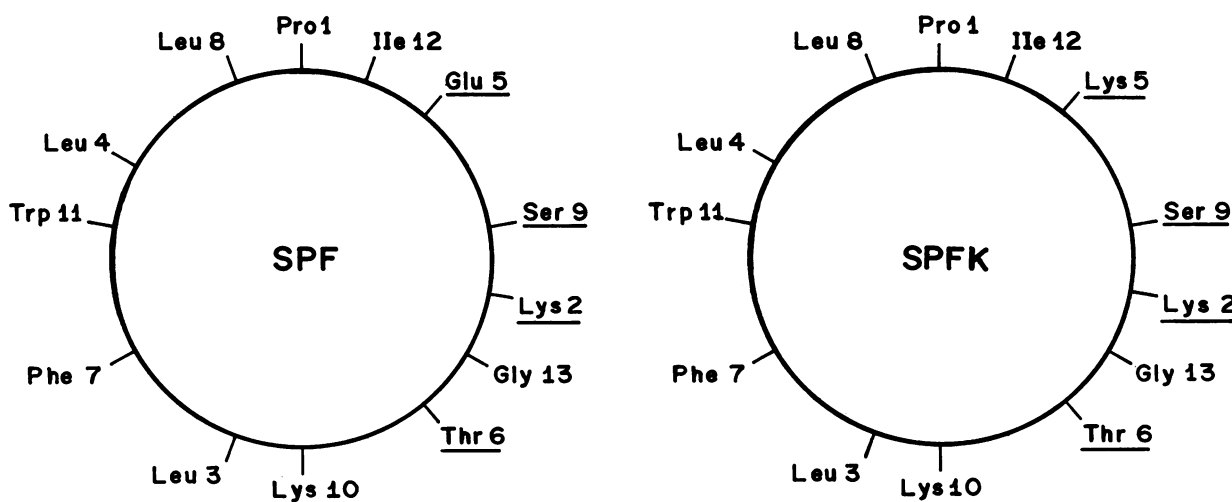


FIG. 6. Helical wheel projections of SPF and SPFK. Polar residues are underlined.

and thus are easily obtainable by chemical synthesis, would be appropriate for possible therapeutic uses.

ACKNOWLEDGMENT

We thank C. Subbalakshmi for purification of SPFK.

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