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Article:

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Biological and Structural Properties of COLINPOWELL, a Synthetic Peptide Amide

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Abstract

A peptide was designed so that the one letter abbreviations for its amino acid sequence corresponded to the joined first and last names of the current United States Secretary of State, Colin Powell. Peptide COLINPOWELL (i.e., Cys-Orn-Leu-Ile-Asn-Pro-Orn-Trp-Glu-Leu-Leu) was synthesized as a C-terminal amide, and found to be inactive against bacteria [methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterococci (VRE)] and herpes simplex virus (HSV-1), and it had no effect on plasma coagulation. However, it was capable of inducing migration of human monocytes and neutrophils, and inhibiting the proliferation of human breast cancer (T47D) cells. Molecular modeling indicated that the peptide has the potential to form intramolecular hydrogen-bonded structures, such as short helices or a small β-sheet, but circular dichroism analysis indicated that the reduced form of the peptide has very little structure in aqueous solutions of 0-90% trifluoroethanol. In such a hydrophobic environment, the structure of COLINPOWELL apparently resembles the relatively nonstructured conformations of similar amino acid sequences that are found in four proteins of known 3D structure (pancreatic kallikrein A, matrix metalloproteinase 8/neutrophil collagenase, Cre recombinase, and hexulose reductase). This study demonstrates the potential for designing bioactive peptides based solely on words or names constructed from the letters of the English alphabet. In addition, the bioactivity test results indicate that peptide, COLINPOWELL, might have potentially interesting biomedical uses.

Introduction

"What's in a name?" (Act II. Scene II. Romeo and Juliet [1]).

In general, biomedical researchers are most comfortable with rational drug design, based on biological and chemical understanding, as the approach to drug discovery [2]. Traditionally, the design of new bioactive peptides has been based upon the amino acid (AA) sequences of naturally occurring peptides or protein fragments. However, the use of unusual or unnatural AAs, and the application of combinatorial synthesis techniques has enabled the creation of many new bioactive peptides with AA sequences that have no basis in nature [3]. Recently, an alternative method for the design of new bioactive peptides was proposed in which words and names composed of letters of the English alphabet are used as the basis for the peptide's AA sequence [4-6]. This method is based upon the internationally accepted convention for abbreviating the names of AAs with single letters of the English alphabet.

The International Union of Pure and Applied Chemistry (IUPAC)-International Union of Biochemistry and Molecular Biology (IUBMB), Joint Commission on Biochemical Nomenclature (JCBN), has selected the letters of the English alphabet as the official one-letter abbreviations for designating AAs, and this nomenclature is in widespread use worldwide for abbreviating the AA sequences of peptides and proteins (Table 1) [7]. Twenty-one of the 26 letters of this alphabet have been officially designated as one-letter abbreviations for AAs, and with minor modifications the IUPAC-IUBMB, JCBN system could produce an abbreviation system that would utilize all 26 letters of the English alphabet and would be recognized by scientists throughout the world. The English word/name-to-peptide method would provide a vast reservoir of new AA sequences to explore for bioactivity, and it might also stimulate interest in amino acids and peptides among the general public. This hypothesis was tested by synthesizing a peptide with an AA sequence for which the one letter abbreviations corresponded to the name of a prominent public figure, the current United States Secretary of State, Colin Powell, and then subjecting to peptide to a variety of biological tests.

Materials and Methods:

a. Peptide design and molecular modeling:

All of the letters of the name, Colin Powell, except O, were assigned to AAs by the IUPAC-IUBMB, JCBN abbreviation system. The letter, O, has been used as a one letter abbreviation for the AA, ornithine (Orn), which occurs only rarely in proteins [8]. Therefore, it was decided that the letter, O, would represent Orn, and the AA sequence for the desired peptide was: Cys-Orn-Leu-Ile-Asn-Pro-Orn-Trp-Glu-Leu-Leu or COLINPOWELL.

(Text of Materials and Methods continues on page 5.)

Table 1. IUPAC-IUBMB, JCBN[a] nomenclature and symbolism for amino acids and peptides.

Amino Acid (A.A.):		**Abbreviations:**	
Trivial Name:	**Systematic Name:**	**3 Letter:**	**1 Letter:**
Alanine	2-Aminopropanoic acid	Ala	A
Aspartic acid or Asparagine	2-Aminobutanedioic acid or 2-Amino-3-carbamoylpropanoic acid	Asx (for Asp/Asn)	B
Cysteine	2-Amino-3-mercaptopropanoic acid	Cys	C
Aspartic acid	2-Aminobutanedioic acid	Asp	D
Glutamic acid	2-Aminopentanedioic acid	Glu	E
Phenylalanine	2-Amino-3-phenylpropanoic acid	Phe	F
Glycine	Aminoethanoic acid	Gly	G
Histidine	2-Amino-3-(1*H*-imidazol-4-yl)-propanoic acid	His	H
Isoleucine	2-Amino-3-methylpentanoic acid	Ile	I
(None)	(None)	(None)	J
Lysine	2,6-Diaminohexanoic acid	Lys	K
Leucine	2-Amino-4-methylpentanoic acid	Leu	L
Methionine	2-Amino-4-(methylthio)butanoic acid	Met	M
Asparagine	2-Amino-3-carbamoylpropanoic acid	Asn	N
(None)	(None)	(None)	O
Proline	Pyrrolidine-2-carboxylic acid	Pro	P
Glutamine	2-Amino-4-carbamoylbutanoic acid	Gln	Q
Arginine	2-Amino-5-guanidinopentanoic acid	Arg	R
Serine	2-Amino-3-hydroxypropanoic acid	Ser	S
Threonine	2-Amino-3-hydroxybutanoic acid	Thr	T
(None or Selenocysteine)	(None or 2-amino-3-selenopropanoic acid)	(None or Sec)	U
Valine	2-Amino-3-methylbutanoic acid	Val	V
Tryptophan	2-Amino-3-(l*H*-indol-3-yl)-propanoic acid	Trp	W
Unknown or other A.A.	Unknown or other A.A.	Xaa	X
Tyrosine	2-Amino-3-(4-hydroxyphenyl)-propanoic acid	Tyr	Y
Glutamic acid or Glutamine	2-Aminopentanedioic acid or 2-Amino-4-carbamoylbutanoic acid	Glx (for Glu/Gln)	Z

[a]Note: International Union of Pure and Applied Chemistry-International Union of Biochemistry and Molecular Biology, Joint Commission on Biochemical Nomenclature.

(**Materials and Methods** continued:)

Many small peptides are capable of forming secondary structures, such as α-helices, in appropriate environments (e.g., in contact with biological membranes), and such structures may influence the biological activity of the peptide. In order to maximize its potential to form α-helices, COLINPOWELL was designed to have an amide group at its carboxyl (C-) terminal end. This modification reduces the helix dipole and stabilizes the helix structure [9].

Predictions of the preference of COLINPOWELL for α-helix and β-sheet conformations were made using affinity values that have been assigned to each type of AA based on a statistical analysis of their frequencies of occurrence in each of the two types of secondary structure among 1,419 protein structures of the PDBSELECT database [10], a subset of the Protein Data Bank (PDB) [11] containing proteins that do not have highly homologous sequences. It was also assumed that the parameters for Orn are the same as those for Lys. This assumption is probably realistic since the side chain of Orn is only one methylene (-CH_2-) group shorter than Lys and has nearly the same pKa value [Orn: -$(CH_2)_3$-NH_3^+, pKa = 10.6; Lys: -$(CH_2)_4$-NH_3^+, pKa = 10.7] [12-13]. The affinity parameters for individual AA's were used to calculate an average (± std. dev.) affinity for the entire COLINPOWELL peptide.

Predictions of the content of α-helix in COLINPOWELL were made using the AGADIR program [14]. Since this program does not accept Orn (O) in the input sequence, Lys (K) was used in its place and the sequence input was CKLINPKWELL. Predictions were made for a default constant temperature of 278 K (5 C), and variable ranges of ionic strength (0.00-1.00) and pH (0-14).

COLINPOWELL was modeled in two dimensions as a fully extended structure with the ISIS™/Draw2.4 program (MDL Information Systems, Inc.) and as an α-helix using a helical wheel diagram [15]. It was modeled in three dimensions (3D) with the Deep View/Swiss Pdb-Viewer v3.7 [16] and RasWin Molecular Graphics, Windows v2.6-ucb [17] molecular modeling programs.

An α-helical model of COLINPOWELL was prepared with the Deep View/Swiss Pdb-Viewer v3.7 program as follows: (1) The starting structure was the 3D crystal structure of melittin (PDB code, 2MLT), a linear peptide comprised of 26 AAs (GIGAVLKVLTTGLPALISWIKRKRQQ), and containing two α-helices separated by a Pro residue. The PDB model of melittin contains four identical copies of the peptide arranged in an overlapping and perpendicular fashion (tetrameric aggregate). Three copies of the peptide were removed leaving a single molecule of melittin with all 3D coordinates preserved. (2) AA residues 1-8 (GIGAVLKV) and 20-26 (IKRKRQQ) of melittin were deleted from the model, leaving the 11 residue 9-19 AA sequence (LTTGLPALISW) with Pro at the center. (3) Residues 9-13 were mutated from LTTGL to CKLIN, and residues 15-19 were mutated from ALISW to KWELL, resulting in the sequence CKLINPKWELL. Lys (K) residues were used in place of Orn (O) residue because the Deep View/Swiss Pdb-Viewer v3.7 modeling program does not

have the Orn option. (4) The two Lys of CKLINPKWELL were converted to Orn by modifying the PDB coordinate file to remove one methylene group from each Lys side chain. (5) Finally, the PDB file was modified to convert the peptide's C-terminal carboxyl group (-COOH) to an amide ($-CONH_2$). Deep View/Swiss Pdb-Viewer v3.7 was also used to generate an electrostatic potential diagram of CKLINPKWELL using the model resulting from step (3) above. Potentials were computed using simple coulomb interactions, and atomic partial charges. Melittin's crystal structure was used as a basis for modeling COLINPOWELL because it probably generates a more realistic 3D model of a peptide containing a central Pro residue than may be possible to obtain using the default α-helix generated by the Deep View/Swiss Pdb-Viewer v3.7 modeling program.

A β-sheet model of COLINPOWELL was prepared by the same general procedure as used for α-helix modeling, except that the starting structure was a hairpin loop/reverse turn that occurs in one of the β-sheets of translation elongation factor P from *Thermus thermophilus* (PDB code 1UEB). (1) The portion of the β-sheet structure containing the four AA residues comprising the turn, plus three residues on its N-terminal side and four residues of its C-terminal side, were extracted from the protein structure and used as the starting structure. (2) Using Deep View/Swiss Pdb-Viewer v3.7, the AA side chains of the starting structure were mutated to the corresponding side chains of the sequence, CKLINPKWELL, while retaining all of the Φ and ψ angles present in the original PDB starting structure. The end result was that the N-terminal three AAs of the peptide, Cys_1-Leu_3, formed the first β-strand of the sheet, AA's Ile_4-Lys_7 formed the turn, and AA's Trp_8-Leu_{11} formed the second, antiparallel strand of the β-sheet. (3) The PDB file for CKLINPKWELL was modified to remove one methylene group from each of the side chains of Lys, thereby converting them to Orn side chains. (4) The Φ and ψ angles from the original PDB starting structure were conserved for the four AA residues, Ile_4-Lys_7, comprising the turn, but all of the other Φ and ψ, and side chain, angles were manipulated to achieve the final β-sheet structure of COLINPOWELL. An electrostatic potential diagram for the COLINPOWELL analog, CKLINPKWELL, was generated using the model resulting from step (3) above. Potentials were computed using simple coulomb interactions, and atomic partial charges.

b. Protein database searching:

BLAST (Basic Local Alignment Search Tool [18]) searches for short, nearly exact matches were done using the protein databases of the National Center for Biotechnology Information (NCBI), and the search sequences, COLINPOWELL and CKLINPKWELL. The latter sequence was used as a substitute for the first sequence because Orn does not occur in any of the AA sequences within the NCBI protein databases, and the structure and physical properties of Lys closely resemble those of Orn (see previous section).

Proteins that were found to contain fragments of the sequence, CKLINPKWELL,

were cross checked with the PDB database to determine if their 3D structures were known, and, if so, what part of the 3D structure contained the CKLINPKWELL sequence fragment.

c. Peptide synthesis, purification, and chemical analysis:

Peptide COLINPOWELL was custom synthesized by Bachem USA (King of Prussia, PA, USA). The purity, AA composition, peptide content, and molecular weight analyses provided by Bachem were verified by AA analysis at the Protein Analysis Center, Karolinska Institutet, Stockholm, Sweden, and by reverse phase high performance liquid chromatography (RP-HPLC) and electrospray ionization mass spectrometry (ESI-MS) at the Department of Chemistry and Chemical Biology, Rutgers University, Piscataway, New Jersey, USA.

d. Preliminary crystallization studies:

Crystallization experiments were done at the Hauptman Woodward Medical Research Institute, Buffalo, N.Y., USA. A volume of 0.2 µl of crystallization cocktail was added to each of the 1,536 wells of 16, 96-well plates, followed by 0.2 µl of 10 mg/ml solution of COLINPOWELL in H_2O. The cocktail was different for each of the 1,536 wells. The progress of crystallization was monitored by making digital photographs of each of the 1,536 wells at 0, 1 3-5, 7, 14, 21, and 28 days.

e. Circular dichroism analysis:

Spectra were recorded on an Aviv model 202-01 CD spectropolarimeter. Aqueous solutions contained 1mM peptide, 1 mM Tris(2-carboxyethyl)phosphine [TCEP, a water soluble, selective reductant of disulfides that was used to keep the sulfhydryl group (-SH) of Cys in its reduced form], and 0-90% of trifluoroethanol (TFE). Measurements were done in a 1 mm cell from 190-260 nm at 1 nm intervals, and 5 scans were averaged. The resulting CD data were deconvoluted into percentages of α-helix, β-structure, turns and random coil with the SELCON [19], SELCON3 [20, 21], CONTIN [22], CONTIN/LL [20, 21] and K2D [23] programs and by single wavelength analyses at 208 [24] and 222 nm [25]. The SELCON, CONTIN, and K2D programs are available at or through http://www2.umdnj.edu/cdrwjweb/index.html, and SELCON3 and CONTIN/LL are part of the CDPro software package available at http://lamar.colostate.edu/~sreeram/CDPro/main.html.

f. Antibacterial assays:

The antibacterial activities of peptide COLINPOWELL were determined using a broth microdilution assay as previously described [26]. Methicillin-sensitive and -resistant (MRSA) strains of Gram positive *Staphylococcus aureus* and *Enterococcus faecium*, and vancomycin resistant strains of *Enterococcus faecalis* (VRE), were grown in nutrient broth and in the presence of various concentrations of the peptide, in the

wells of 96 well microtitre plates. After overnight incubation at 37 ^{o}C, the concentration of peptide causing inhibition of bacterial growth was determined by visual inspection of the wells.

g. Antiviral assay:

The antiviral activities of COLINPOWELL were determined by using peptide at a final concentration of 100 μg/ml (75 μM) and several dilutions of HSV-1 (herpes simplex virus type 1), the cause of fever blisters or lip sores, as the test virus (unpublished, M. Sällberg, 2004).

h. Anticoagulant activity:

The effects of COLINPOWELL on human blood coagulation were determined as previously described [27]. Anticoagulant activity of the peptide dissolved in isotonic saline was tested by determination of prothrombin time (PT) and activated partial thromboplastin time (PTT) of a Coagulation Reference Plasma (Baxter AG), with reagents from Instrumentation Laboratory (Milano, Italy). PTT measures the clotting time from the activation of clotting factor XII, through the formation of fibrin clot, thereby measuring the integrity of the intrinsic and common pathways of coagulation, whereas PT measures the integrity of the extrinsic and common pathways [28]. The presence of inhibitors may prolong PT and PTT times.

i. Chemotaxis assays:

The ability of peptide COLINPOWELL to induce migration of human monocytes and neutrophils was determined as previously described [29]. Monocytes and neutrophils were isolated from human peripheral blood obtained from individual healthy donors, and isolates had a purity of 95%. Their migration in response to a dilution series of peptide concentrations (0.025-50 μM) was measured in 48-well microchemotaxis chambers (NeuroProbe, Gaithersburg, MD) as follows. The peptide was diluted in chemotaxis medium (CM; RPMI 1640 containing 1% BSA) and placed in the wells of the lower compartment of the chamber. Monocytes or neutrophils suspended in CM were added to the upper compartment. The lower and upper compartments were separated by an uncoated polycarbonate membrane with 5-μm pores (NeuroProbe, Gaithersburg, MD). After incubation at 37 ^{o}C in humidified air with 5% CO_2 for a period of time (60 min for neutrophils, 90 min for monocytes), the membranes were removed, scraped, stained, and the cells migrating across the membrane were determined using a BioQuant semiautomatic counting system. Results are presented as the number of cells per high power field (No./HPF). The peptide was tested at least 3 times and the results were similar. The tripeptide, N-formyl-Met-Leu-Phe, customarily abbreviated fMLP, was used as a positive control in all experiments, and at the single concentration of 0.1 μM.

j. Cell proliferation assays:

Human breast cells (T47D) were plated at an initial density of 25000 cells in 2 ml RPMI 1640 medium with 5% fetal calf serum, 25 mM N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) buffer at 37°C for 24 hours. The medium was changed and the cells were incubated in the presence or absence of the peptide for 3 days followed by incubation with 2 μCi [^{3}H]thymidine for 2 h. Extraction of DNA and determination of radioactivity were performed as previously described [30]. Statistical significance of differences between means was established by analysis of variance and Dunnett's test.

Results:

a. Peptide design and molecular modeling:

A skeletal representation of the structure of peptide, COLINPOWELL, is shown in Figure 1. It is a relatively small peptide containing 45% hydrophobic AAs [31], with a predicted net charge of +2 at pH 7. The presence of a sulfhydryl group in the side chain of cysteine (Cys, C), located at the amino (N-) terminal end of the peptide enables it to undergo oxidation to form disulfide bonds (2 –SH → -S-S-) with another molecule of COLINPOWELL to yield a dimeric compound with a net charge of +4 at pH 7 (Figure 2), or to form disulfide bonds with other thiol containing molecules (e.g., glutathione), or covalent bonds with alkylating agents [32].

Many small peptides have the capability to form secondary structures in the appropriate environments, such as when they are in contact with cellular lipid bilayer membranes or in contact with other proteins. Two common types of protein secondary structure that arise from intramolecular hydrogen (H-) bonding are [33]: (1) the α-helix, a structural entity with an overall length of 4-40 AA residues and H-bonds between the peptide bond carbonyl of residue i and the peptide bond NH of residue i+4; (2) the β-sheet, a multisubunit structural entity composed of β-strand subunits, each of which has an almost fully extended conformation with a flat topology, containing 5-10 AA residues, and which is H-bonded to adjacent β-strands. Alternative types of helices are: (1) the 3_{10}-helix with H-bonds between residues i ↔ i+3, 3 AA residues per turn, 10 atoms between the H-bond donor and acceptor atoms, and a smaller diameter coil than that of the α-helix; (2) the π-helix with H-bonds between AA residues i↔ i+5 and a larger diameter coil than that of the α-helix. The 3_{10}- and π-helices do not occur frequently, and are usually found at the ends of α-helices or in single turn helices.

Predictions of the preference of COLINPOWELL for α-helix and β-strand structures, based upon the frequencies of occurrence of each type of AA in each of the two structure types [10], yielded average (± std. dev.) affinity values of -1.10 (± 0.66, n = 11) for α-helix and -1.03 (± 0.67, n = 11) for β-sheet. These statistically equivalent values indicate that the AA sequence has an equivalent affinity for both types of

(Text of Results continues on page 12.)

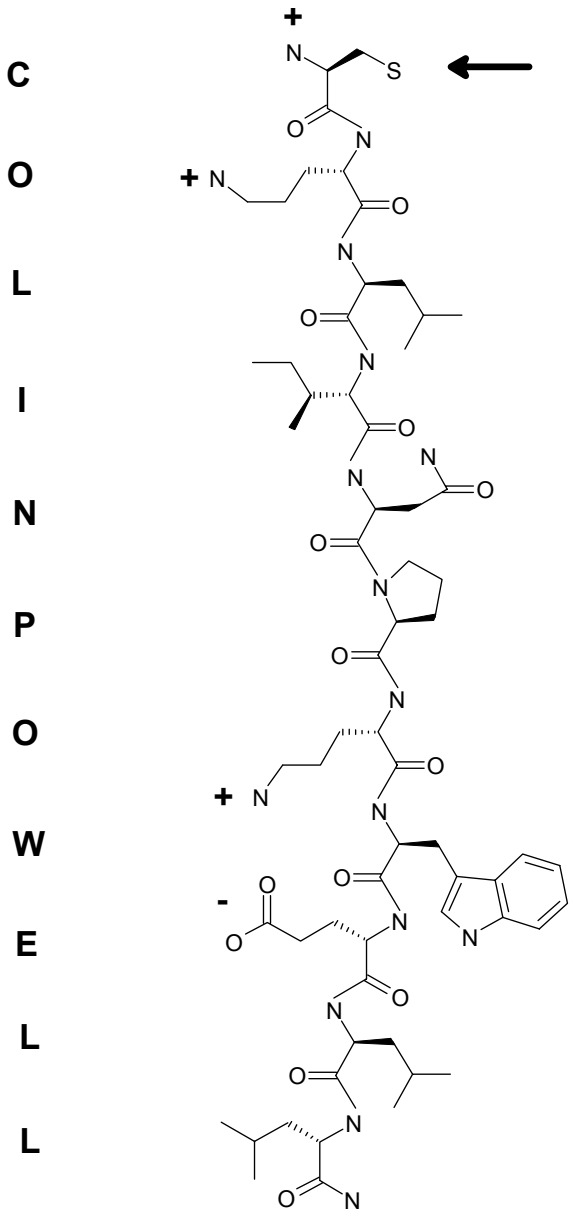


Figure 1. Chemical structure of the peptide amide, COLINPOWELL, in an extended form. One letter abbreviations for the constituent AAs are shown to the left of the structure. Carbon and hydrogen atoms are not shown, but charges on AA side chains at pH 7 are shown, and the net charge on the peptide would be +2. The arrow indicates the position of the sulfhydryl containing side chain of Cys which is capable of undergoing disulfide bond formation with other molecules of COLINPOWELL to form dimeric structures, or with other sulfhydryl/thiol containing compounds.

Figure 2. Use of one letter abbreviations to illustrate the AA sequence of the monomeric [reduced (left)] and dimeric [oxidized (right)] forms of COLINPOWELL. Charges on AA side chains at pH 7 are shown, and the carboxyl terminal end of the peptide is amidated and uncharged. Net charges are +2 for the monomer, and and +4 for the dimer.

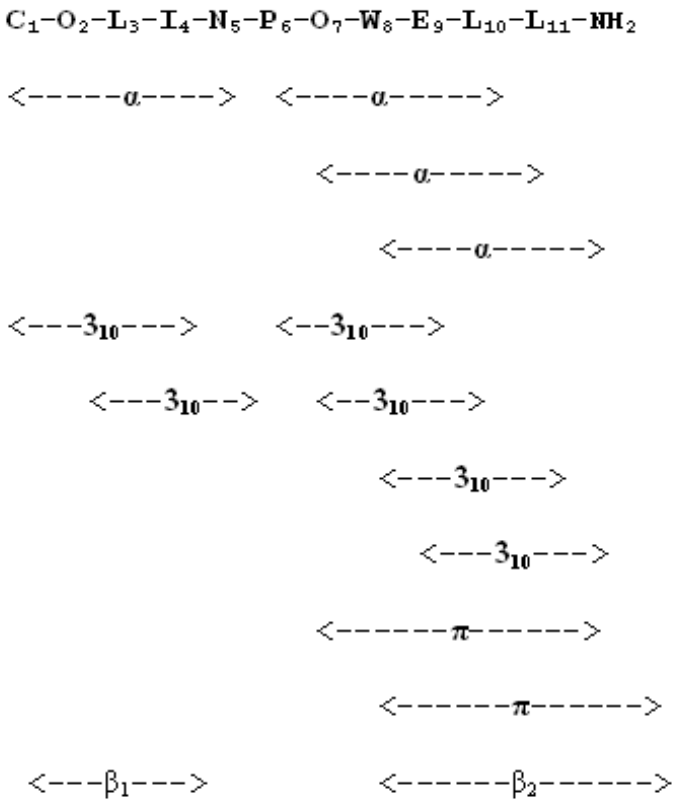


Figure 3. Possible locations of helices (3_{10}, α, and π) and β-strands within COLINPOWELL.

(**Results** continued:)
secondary structure, or, alternatively, no strong preference for either one. Predictions of the α-helix content of COLINPOWELL, as its analog CKLINPKWELL, with the AGADIR program at a temperature of 25 C (298 K), a pH range of 0-14, and an ionic strength range of 0.00-1.00, yielded values of less than 1% (0.13-0.32%). Helical propensity was predicted to be greatest in the Pro_6-Leu_{11} segment of the peptide.

The reduced, or monomeric, form of COLINPOWELL contains only 11 AA residues, and it can form only a small number of intrapeptide H-bonds. This limits the number of structural options for the peptide to (1) no structure (random coil), (2) helix (3_{10}, α-, or π-helix), (3) a small β-sheet, and (4) combinations of helix, random coil, and β-strand. There is a Cys at the N-terminal end of the peptide, and under certain circumstances, α-helices are stabilized by Cys at this position [34]. However, the AA located at the center of the peptide is Pro. Although Pro is found at the N-terminal ends of α-helices, it cannot occur within the interior or at the C-terminal end of an α-helix due to the fact that its nitrogen (N) is an imino N, covalently bound to its side chain and lacking the H needed to form a H-bond [35]. Therefore, the AA sequence of COLINPOWELL that is on the N-terminal side of Pro_6, the Cys_1↔Asn_5 segment, can contain only one turn of α-helix (Cys_1↔Asn_5) or one turn of 3_{10} helix (i.e., Cys_1↔Ile_4 or Orn_2↔Asn_5) (Figure 3). The C-terminal half of the peptide, Pro_6↔C-terminal amide, can contain only one turn of α-helix (i.e., Pro_6↔Leu_{10}, Orn_7↔Leu_{11}, or Trp_8↔C-terminal amide), one or two turns of 3_{10}-helix (i.e., Pro_6↔Glu_9, Orn_7↔Leu_{10}, Trp_8↔Leu_{11}, Glu_9↔C-terminal amide, or Pro_6↔C-terminal amide), or one turn of π-helix (Pro_6↔Leu_{11} or Orn_7↔C-terminal amide) (Figure 3).

If COLINPOWELL contains the maximum possible content of α-helix (i.e., two turns or 91% of the peptide), it will not be a single continuous, coaxial helix, but rather two turns of α-helix separated by a kink between Asn_5 and Pro_6. A similar conformation, two helices separated by a Pro-induced kink, is found in melittin, a peptide toxin found in the venom of the honeybee, *Apis mellifera* (PDB code 2MLT) [36, 37]. α-Helical COLINPOWELL will not be amphipathic/amphiphilic (i.e., having all or most polar and nonpolar AA side chains segregated on opposite sides of the helix), a property that would endow the peptide with particular biological properties, such as the ability to associate in particular orientations with the surfaces of biological lipid membranes (Figures 4-5) [38]. However, the outer surface of a helical COLINPOWELL could have small segregated regions consisting entirely of either nonpolar (hydrophobic) or polar (hydrophilic) AA side chains that might facilitate interactions with other molecules. An electrostatic potential diagram of the COLINPOWELL analog, CKLINPKWELL, in the same helical conformation as COLINPOWELL, shows that the peptide would have a dipolar nature due to a large region of positive potential at its N-terminal end, associated with the N-terminal α-amino nitrogen and the side chain ε-amino nitrogen of Lys_2 and a smaller region of positive potential associated with the ε-amino nitrogen of Lys_7, and a large distinct region of negative electrostatic potential associated with the indole of Trp_8, the side

chain γ-carboxyl group of Glu_9, and oxygen atoms in adjacent peptide bonds (Figure 5E). Another possible intramolecular H-bonded structure that can exist for reduced COLINPOWELL is a small β-sheet composed of two short antiparallel β-strands separated by a reverse turn, or short hairpin loop, and stabilized in this conformation with H-bonds between the two β-strands. For example, $Cys_1 \leftrightarrow Ile_4$ might comprise one β-strand (slightly shorter than the average 5-10 AA residues for a β-strand), Asn_5-Pro_6 the reverse turn, and $Orn_7 \leftrightarrow Leu_{11}$ the second antiparallel β-strand, and with H-bonds between the two β-strands at $Orn_2 \leftrightarrow Glu_9$ and $Ile_4 \leftrightarrow Orn_7$ (Figures 3 and 6). As with helical COLINPOWELL, the β-sheet structure would not be amphipathic/amphiphilic, but it would have regions consisting of mostly nonpolar or polar AA side chains that could facilitate noncovalent interactions with other molecules. Unlike helical COLINPOWELL, the β-sheet structure would not have a dipolar nature (Figure 6E). Both the helical and β-sheet structures for COLINPOWELL would have about the same overall size, with the longest dimension being 20-22 Å, similar in size to the glycopeptide antibiotic, vancomycin (20 Å) (PDB code 1AA5) [39].

b. Protein database searching:

Searches of protein databases for AA sequences that correspond to words or names composed of the letters of the English alphabet will often locate such words, but only if they do not contain the letters B, J, O, U, X, and Z since these letters have not been assigned to single AA's or have no AA assignment at all. For example, an on-line BLAST search of the NCBI protein databases for the letter sequence, COLINPOWELL, revealed no occurrences of this sequence among any of 1,855,543 AA sequences within the databases (Table 2). However, the letter, O, is occasionally used as an abbreviation for the nonnatural AA, Orn [8], a close structural analog of the naturally occurring AA, Lys (K). If O is replaced by K, the AA sequence becomes CKLINPKWELL. Although a BLAST search of the protein databases returned no exact matches for this entire sequence either, several partial sequence matches were found (Table 2). The proteins containing the partial sequence matches where found to come from a variety of organisms including: virus (Ictalurid herpesvirus), bacteria (*Chloroflexus aurantiacus, Listeria monocytogenes*, *Mycoplasma mycoides, Nostoc punctiforme, Pirellula sp., Pasteurella multiocida*, *Wigglesworthia glossinidia*, *Xanthomonas oryzae*), plant (*Arabidopsis thaliana*), insect (*Carabus fiduciarius, Drosophila melanogaster*), nematode (*Caenorhabditis elegans*), frog (*Xenopus laevis*), and human (*Homo sapiens*). This indicates that the combination of AA's comprising the sequence, CKLINPKWELL, occurs in nature and that it probably serves some useful function(s) in the proteins where it is found. Therefore, the closely related AA sequence, COLINPOWELL, might also have biological activities.

Four of the proteins that contain partial sequence matches with CKLINPKWELL have known 3D structures (Table 3): (1) porcine pancreatic kallikrein A (PDB code

(Text of Results continues on page 20.)

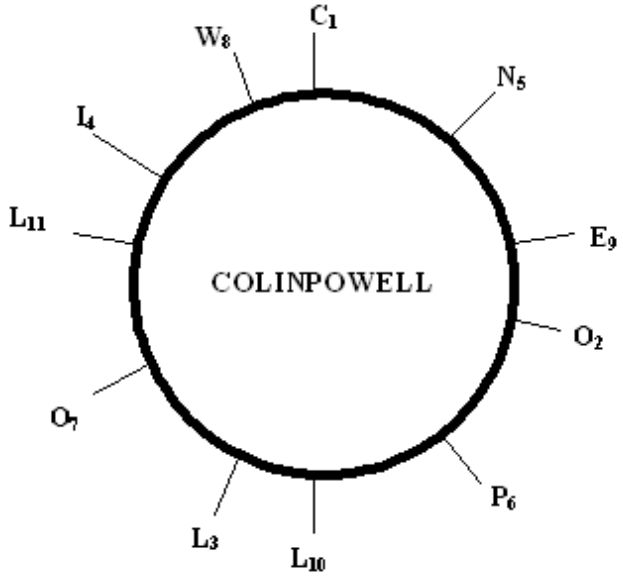
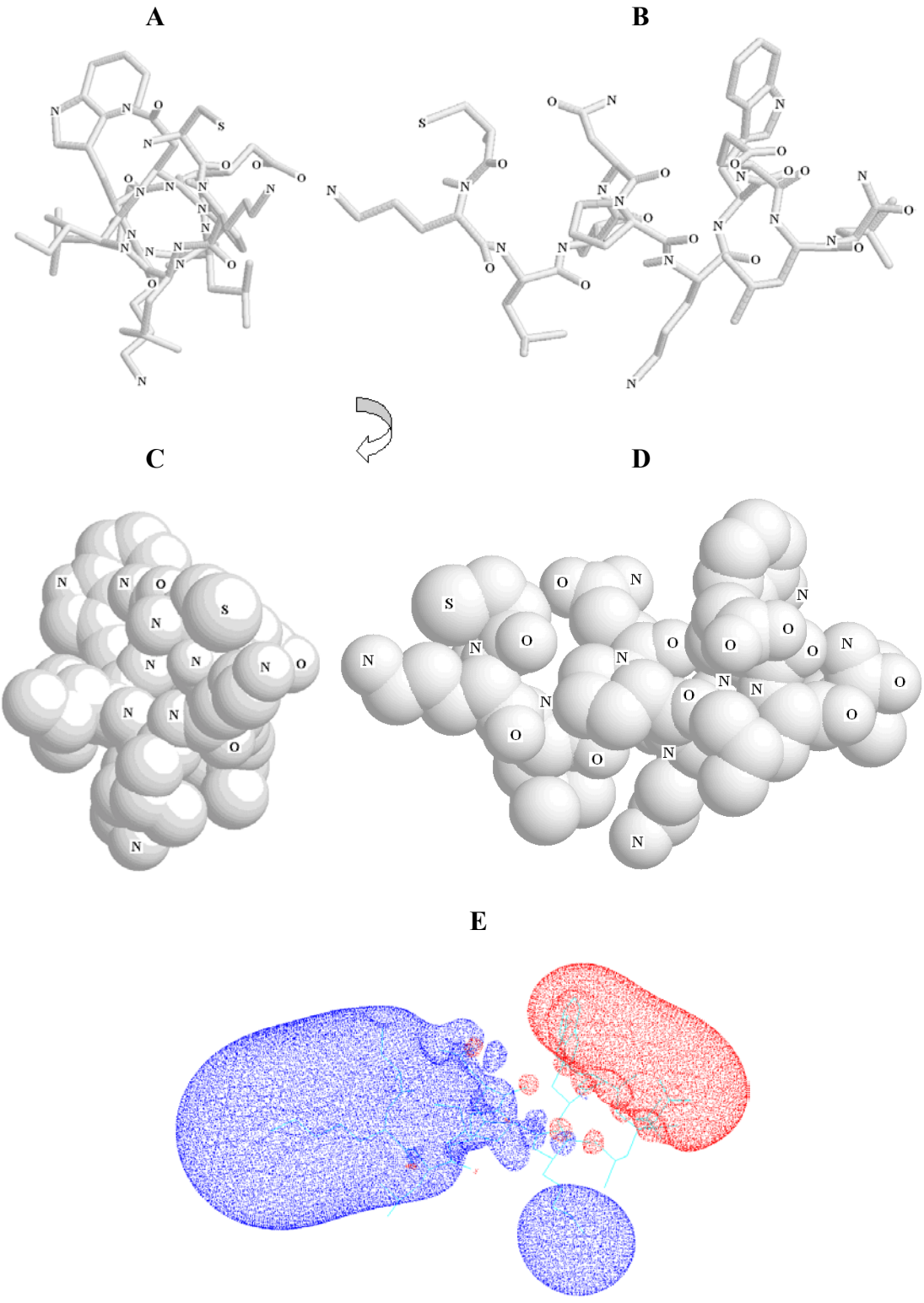


Figure 4. Helical wheel diagram for COLINPOWELL. This model assumes that the entire peptide is in an α-helical conformation, which is not realistic due to the presence of the helix breaking AA, proline (Pro, P) at position 6. However, it would be possible for one turn of α-helix to exist on either side of Pro_6, so that the peptide contained two turns of α-helix. The view of the helix is coaxial, from its N-terminal end toward its C-terminal end. In this model, the peptide is not amphipathic.

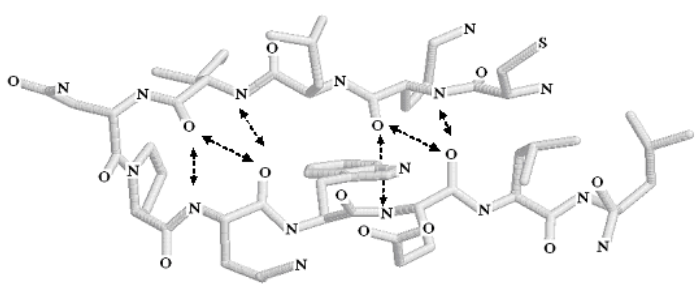
Figure 5 (Next page)**.** α-Helical stick (A and B) and space filling (C and D) models of COLINPOWELL, and an electrostatic potential model (E) of the analog, CKLINPKWELL, in the same helical conformation as COLINPOWELL. Atoms of sulfur (S), oxygen (O), and nitrogen (N) that are visible are labeled, and the unlabeled atoms are carbon. Hydrogen atoms are not shown. The view in A is coaxial and nearly the same as that shown in the helical wheel diagram of Figure 4, with the N-terminal end of the peptide nearest to the viewer. The positions of side chains around the circumference of the core region of view A differs slightly from Figure 4 because the Φ and ψ angles of the 3D crystal structure of melittin were used to construct the model of COLINPOWELL. This resulted in the elimination of all α-helicity from the N-terminal end of the peptide, although the peptide still retains an overall cylindrical topology. The view in B is obtained by rotating the peptide cylinder 90^o so that the N-terminal end of the peptide is on the left and the C-terminal end is on the right. The overall length of the peptide in this conformation is about 20 Å. The views in C and D are the same as in A and B.

(Figure 5 legend continues at top of page 16.)

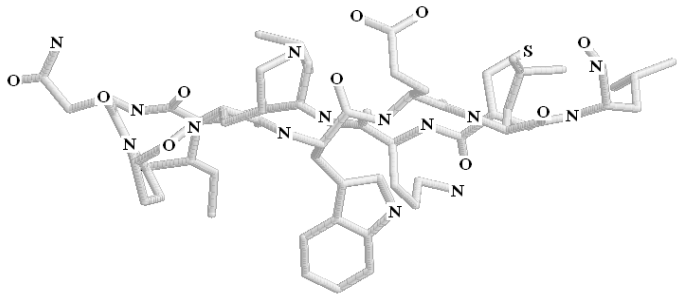


(**Figure 5** legend continued:)
View E, the electrostatic potential diagram of the analog, CKLINPKWELL, is in the same orientation as COLINPOWELL in views B and D. The wireframe skeleton of the molecule in view E is colored light blue/turquoise. The only differences between the molecules in views B, D, and E are that the two Orn side chains of COLINPOWELL have been replaced with Lys side chains, which are only one methylene group (-CH_2-) longer than those of Orn. The reason for the use of Lys rather than Orn is that the Deep View/Swiss Pdb-Viewer v3.7 program used to create the model can generate electrostatic potentials for Lys, but not Orn. A large region of positive potential (blue) is associated with the N-terminal half of the peptide, and a smaller positive region with the Orn_7 side chain. A large region of negative electrostatic potential (red) is associated with much of the C-terminal end of the peptide, in particular the side chains of Trp_8, Glu_9, all peptide bond carbonyl O's from Trp_8-Leu_{11}, and the C-terminal amide carbonyl O.

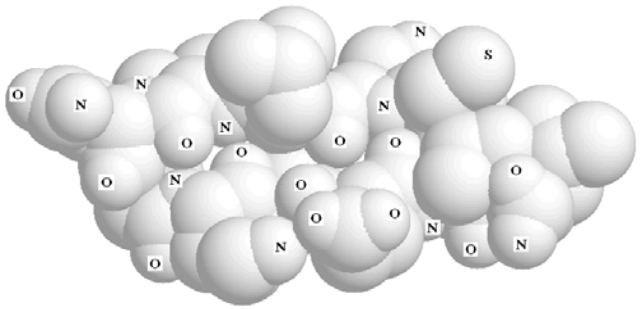
Figure 6 (Next page)**.** β-Sheet stick (A and B) and space filling (C and D) models of COLINPOWELL, and an electrostatic potential model (E) of CKLINPKWELL in the β-sheet conformation. Atoms of sulfur (S), oxygen (O), and nitrogen (N) that are visible are labeled, and the unlabeled atoms are carbon. Hydrogen atoms are not shown. The positions of four intrachain H-bonds (between the peptide bond carbonyl O's and N's of Orn_2 and Glu_9, and Ile_4 and Orn_7) are indicated by the presence of four double headed arrows in A. The views shown in B and D were obtained by rotating the molecules 90° toward the viewer. The view in E is the same orientation shown in B and D. There is a large region of positive electrostatic potential associated with Cys_1-Orn_2, a separate positive region at Orn_7, and two regions of negative potential at Glu_9 and Leu_{11}. The longest dimension of this β-sheet structure is 22 Å.



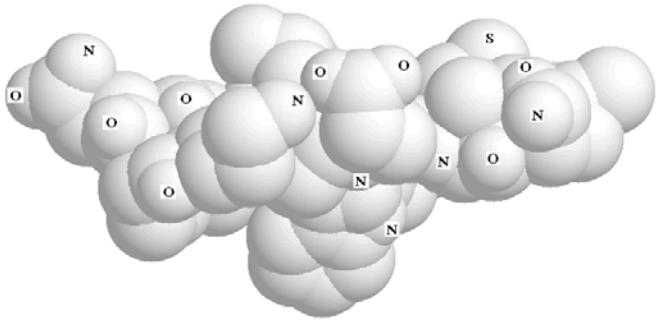
A



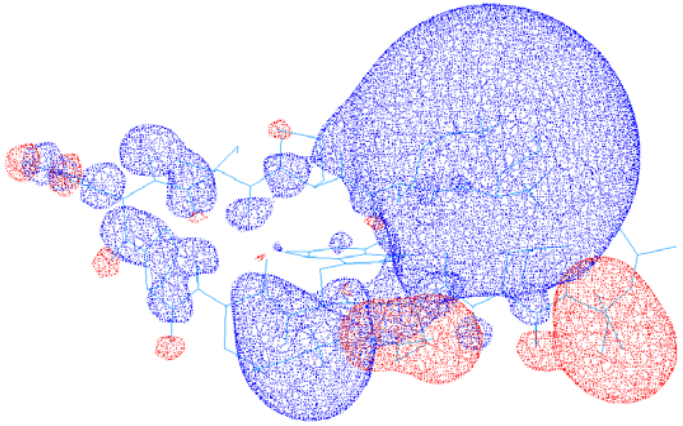
B



C



D



E

Table 2. Representative results from BLAST searches of the NCBI protein databases for AA sequences corresponding to the sequences COLINPOWELL and CKLINPKWELL[a]. Identities between the search and protein sequences are in bold type and underlined.

Search Sequence:	Sequence Found:	Database accession & version codes:	Protein containing sequence:	Location in protein:
COLIN-POWELL	None	--	--	--
CKLIN-PKWELL[b]	**KLI**H**PKWE**	ref|ZP_00234867.1 gi|47097310	Conserved hypothetical protein [*Listeria monocytogenes* str. 1/2a F6854]	477-484
	KLID**P**S**WEL**	ref|NP_508414.1 gi|17548212	G protein-coupled receptor like family member (XC862) [*Caenorhabditis elegans*]	65-73
	LINPKWS**L**	ref|NP_871474.1 gi|32491220	ygjD [*Wigglesworthia glossinidia* endosymbiont of *Glossina brevipalpis*]	325-332
	INE**KWELL**	ref|NP_004265.3 gi|21493045	Protein kinase A anchoring protein 6 [*Homo sapiens*]	1041-1048
	INPKWE	ref|NP_975803.1 gi|42561352	Thymidine phosphorylase [*Mycoplasma mycoides* subsp. mycoides SC str. PG1]	404-409
	KLV**NPKW**	ref|NP_858959.1 gi|32128424	Conserved phage protein [*Xanthomonas oryzae* bacteriophage Xp10]	75-81
	LING**KWEL**	ref|NP_189237.2 gi|42565197	Plastid-lipid associated protein PAP / fibrillin family protein [*Arabidopsis thaliana*]	111-118
	D**PKWELL**	dbj|BAA22281.1 gi|2425188	FGF receptor 3 [*Xenopus laevis*]	452-458

(Table 2 continues on next page.)

Table 2 (Continued). Representative results from BLAST searches of the NCBI protein databases for AA sequences corresponding to the sequences COLINPOWELL and CKLINPKWELL[a]. Identities between the search and protein sequences are in bold type and underlined.

Search Sequence:	Sequence Found:	Database accession & version codes:	Protein containing sequence:	Location in protein:
CKLIN-PKWELL[b]	**KL**V**NPKW**	ref|ZP_00105988.1 gi|23123965	COG1429: Cobalamin biosynthesis protein CobN and related Mg-chelatases [*Nostoc punctiforme*]	1134-1140
	INPKWQ**L**	ref|NP_868571.1 gi|32475577	Probable nucleotide pyrophosphatase homolog [*Pirellula* sp. 1]	329-335
	LINPKW	gb|AAK17903.1 gi|13274356	Glycosyltransferase DcbB [*Pasteurella multocida*]	53-58
	IK**PKWEL**	ref|NP_650999.1 gi|21355841	CG6332-PA [*Drosophila melanogaster*]	218-224
	KLL**NPKW**	gb|AAG15206.1 gi|10198139	BchH [*Chloroflexus aurantiacus*]	1171-1177
	CPI**I**D**PKWE**	gb|AAO32202.1 gi|28207330	Phosphoenolpyruvate carboxykinase [*Carabus fiduciarius*]	114-122
	CKLV**NPK**	pir||C36792 gi|76429	Hypothetical protein ORF56 - Ictalurid herpesvirus 1 (strain auburn 1) (Channel catfish virus)	895-901

Note: [a]Protein BLAST (Basic Local Alignment Search Tool) searches for short, nearly exact matches were done within the NCBI (National Center for Biotechnology Information) databases (http://www.ncbi.nlm.nih.gov/BLAST/): all non-redundant GenBank CDS translations + PDB + SwissProt + PIR + PRF excluding environmental samples. The total number of sequences searched was 1,855,543; [b]K (lysine) is an structural analog of O (ornithine), that occurs in proteins and contains one more methylene (-CH_2-) group in its side chain.

Table 3. Proteins of known 3D structure containing AA sequences similar to COLINPOWELL. Identities in the aligned sequences are in bold type, and other homologies are underlined.

Protein:	Protein Databank Code:	Alignment of AA sequences: CKLINPKWELL (upper sequence), Protein (lower sequence)	Figure 7 Part:
Kallikrein	2PKA	(3) **L**-I-**N-P-K-W**-E-**L** (10) (46) **L**-V-**N-P-K-W**-V-**L** (53)	A
Matrix Metalloproteinase 8 / Human Neutrophil Collagenase	1A85 / 1KBC	(5) **N-P-K-W-E** (9) (85) **N-P-K-W-E** (89)	B
Cre Recombinase	1Q3U	(1) **C-K-L**-I-**N**-P-**K-W** (8) (56) **C-K-L**-N-**N**-R-**K-W** (63)	C
Hexulose Reductase	1N2S	(3) **L-I**-N-**P**-K-**W-E-L** (10) (277)**L-I**-L-**P**-Q-**W-E-L**(284)	D

(**Results** continued:)

2PKA), a specific trypsin-like serine proteinase that cleaves Met-Lys and Arg-Ser bonds in kininogen to release Lys-bradykinin; (2) matrix metalloproteinase 8 / human neutrophil collagenase (PDB codes 1A85 / 1KBC), a collagenase found on the surface of human polymorphonuclear cell (PMN) cells; (3) Cre recombinase (PBD code 1Q3U), an integrase from bacteriophage P1 that catalyzes site-specific recombination between 34-bp DNA repeats (loxP sites) in the absence of any additional cofactors and energy; (4) hexulose reductase (PDB code 1N2S), an enzyme involved in the synthesis of L-Rhamnose, an essential component of the cell wall of many pathogenic bacteria. In the 3D structure of kallikrein A, the 8 AA sequence, L_{46}VNPKWVL_{53} (Figure 7A), is 75% identical to the LINPOWEL segment of COLINPOWELL, or 87.5% identical to the LINPKWEL segment of CKLINPKWELL, assuming K replaces O. This segment lies on the surface of the protein and occurs in a hairpin-like structure composed of two antiparallel β-strands separated by a turn. The two Leu (Leu_{46} and Leu_{53}) at either end of this segment are located in each of the two β–strands, and the NPKW (Asn_{48}-Trp_{51}) portion comprises the turn between the two β-strands. AAs V_{47} and V_{52} link the four residue turn to the two β-strands. The ring of P_{49}, the side chain of K_{50} (one -CH_2- group longer than the side chain of Orn and extending into the solvent environment), and the indole ring of W_{51} are all clearly visible in or near the turn on the left side of figure. There is a H-bond between the carbonyl O of the N_{48}-P_{49} peptide bond and the α-amino N of W_{51} (double headed arrow). The figure demonstrates that the corresponding part of COLINPOWELL has the potential to form a hairpin-like structure as shown in Figure 6. In the 3D structure of matrix metalloproteinase 8/human neutrophil collagenase, the 5 AA sequence, N_{85}PKWE_{89} (Figure 7B), is 80% identical

to the N_5POWE_9 sequence in COLINPOWELL, or 100% identical to the NPKWE segment of CKLINPKWELL, assuming K replaces O. This sequence occurs at the N-terminal end of the protein, as part of a relatively flat and extended, unstructured region and all of the AA side chains are visible in the orientation shown. In the 3D structure of Cre recombinase, a DNA binding protein, the 8 AA segment, $C_{56}KLNNRKW_{63}$ (Figure 7C), has 50% identity with the COLINPOW segment of COLINPOWELL (75% identity with the CKLINPKW segment of CKLINPKWELL). This segment occurs partially within the C-terminal end of an α-helix that is in contact with DNA at its N-terminal end, and partially within a linker sequence to another helix. The side chains of C_{56} (marked with the letter, S), K_{57} (top center), L_{58} (top left), K_{62} (bottom right), and W_{63} (top right) are clearly visible, whereas the other AA side chains are not obvious in the orientation shown. The C_{56}-K_{57} segment is located at the C-terminal end of a helix that binds DNA at its N-terminal end. The sulfhydryl group of Cys_{56} is in its reduced form (-SH), and not bound to the sulfhydryl group of any other Cys in the protein (i.e., not involved in the maintenance of protein structure), or coordinated with any metal or other moiety. The LNNR (Leu_{58}-Arg_{61}) part is extended and unstructured, and the KW (Lys_{62}-Trp_{63}) comprises a short β-strand. In the 3D structure of hexulose reductase, the 8 AA segment, $L_{277}ILPQWEL_{284}$ (Figure 7D), is 75% identical to the $L_3INPOWEL_{10}$ segment of COLINPOWELL (75% identical to the LINPKWEL segment of CKLINPKWELL). The L_{277}-P_{280} segment occurs in an extended (unstructured) region between helices, and the W_{282}-Leu_{284} segment occurs within the N-terminal end of an α-helix that is at the C-terminal end of the protein. In all four proteins, the AA sequences that partially match the COLINPOWELL (or CKLINPKWELL) sequence occur on the surfaces of the proteins, exposed to the solvent environment. It is important to note that in all of the protein structures mentioned above, the AA segments are not free in solution, as is peptide COLINPOWELL, but rather they are constrained on at least their N- and C-terminal ends through covalent linkage to the remainder of the protein, and possibly also by other noncovalent interactions with the rest of the protein.

In regard to predicted physical and biological properties, the free carboxyl form of the Orn → Lys homolog, CKLINPKWELL, is predicted to have a pI of 8.2, and to be relatively stable with an estimated half-life of 1.2 hours in mammalian reticulocytes (*in vitro*), >20 hours in yeast, (*in vivo*), and >10 hours in *Escherichia coli*, (*in vivo*) [40]. The half-life of COLINPOWELL is predicted to exceed that of CKLINPKWELL in mammalian reticulocytes, yeast, and *E. coli* due to the replacement of Lys with Orn. COLINPOWELL is predicted to have a net charge of +2 at neutral pH, a pI of ~9 (Table 4), to be incapable of penetrating cellular lipid membranes [41], and to be susceptible to enzymatic degradation by BNPS skatole, chymotrypsin, glutamyl endopeptidase, iodosobenzoic acid, pepsin, proteinase K, Staphylococcal peptidase I, and thermolysin (Table 5) [42, 43]. It is predicted to not be susceptible to cleavage by trypsin [44, 45].

(Text of Results continues on page 24.)

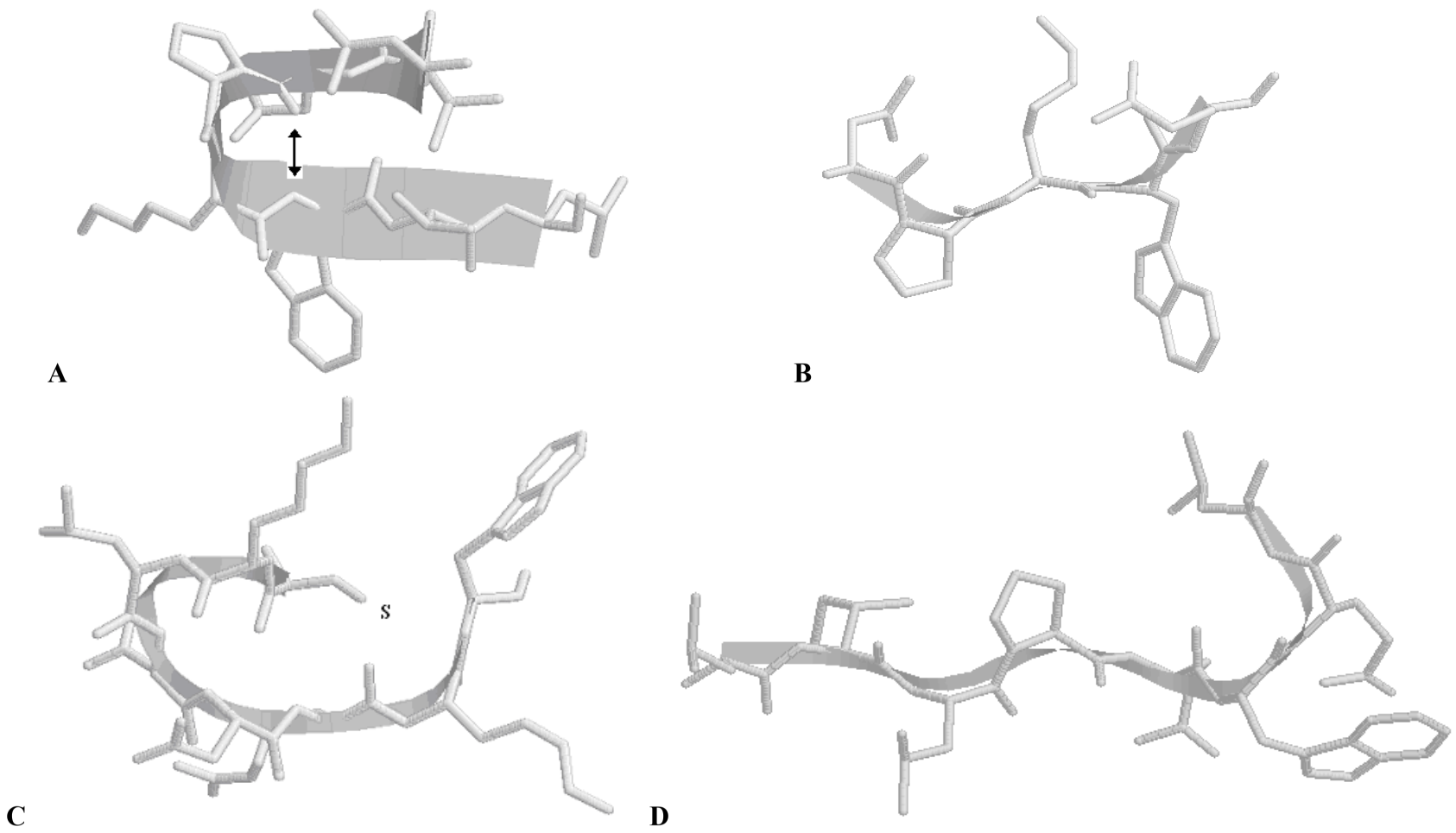


Figure 7. Stick and ribbon diagrams of AA sequences that have homology to COLINPOWELL, and that occur in the three dimensional structures of the Protein Data Bank. (A) Kallikrein (L_{46}VNPKWVL_{53} segment from PDB 2PKA). (B) Matrix metalloproteinase 8/Human neutrophil collagenase (N_{85}PKWE$_{89}$ segment from PDB 1A85). (C) Cre recombinase (C_{56}KLNNRKW$_{63}$ segment from PDB 1Q3U). (D) Hexulose reductase (L_{277}ILPQWEL$_{284}$ segment from PDB 1N2S).

Table 4. Net charge on COLINPOWELL at various pH values. Estimated pI ≈ 9.

		pH value:			
Ionizable group:	pK:	1	7	9	12
N-terminal α-amino ($-NH_3^+ \leftrightarrow -NH_2$)	~8	+1	+1	0	0
Cys_1 thiol ($-SH \leftrightarrow -S^-$)	8.3	0	0	-1	-1
Orn_2 δ-amino ($-NH_3^+ \leftrightarrow -NH_2$)	10.6	+1	+1	+1	0
Orn_7 δ-amino ($-NH_3^+ \leftrightarrow -NH_2$)	10.6	+1	+1	+1	0
Glu_9 β-carboxyl ($-COOH \leftrightarrow -COO^-$)	4.1	+1	-1	-1	-1
Net charge on peptide:	-	+4	+2	0	-2

Table 5. Predicted enzymatic cleavage[a] of peptides CKLINPKWELL[b] and COLINPOWELL.

	C_1-K_2-L_3-I_4-N_5-P_6-K_7-W_8-E_9-L_{10}-L_{11}-OH:[b]		C_1-O_2-L_3-I_4-N_5-P_6-O_7-W_8-E_9-L_{10}-L_{11}-NH_2:	
Enzyme:	Number of cleavages:	Position(s) of cleavage site(s):	Number of cleavages:	Position(s) of cleavage site(s):
BNPS Skatole	1	8↓	1	8↓
Chymotrypsin (high specificity)	1	8↓	1	8↓
Chymotrypsin (low specificity)	4	3↓, 8↓, 10↓, 11↓	4	3↓, 8↓, 10↓, 11↓
Glutamyl endopeptidase	1	9↓	1	9↓
Iodosobenzoic acid	1	8↓	1	8↓
LysC (Lysyl endopeptidase)	2	2↓, 7↓	0	(None)
Pepsin (pH 1.3)	8	↓2↓, ↓3↓, ↓8↓, ↓10↓, ↓11↓	7	↓3↓, ↓8↓, ↓10↓, ↓11↓
Pepsin (pH > 2)	6	↓2↓, ↓3↓, ↓10↓, ↓11↓	7	↓3↓, ↓8↓, ↓10↓, ↓11↓
Proteinase K	5	3↓, 4↓, 8↓, 10↓, 11↓	5	3↓, 4↓, 8↓, 10↓, 11↓
Staphylococcal peptidase I	1	9↓	1	9↓
Thermolysin	3	2↓, 3↓, 10↓	2	3↓, 10↓
Trypsin	2	2↓, 7↓	0	(None)

Note: [a]Predictions for CKLINPKWELL from PeptideCutter program of ExPASy Molecular Biology Server (http://us.expasy.org/tools/peptidecutter). Vertical arrow (↓) indicates side (N- or C-terminal) of AA residue where cleavage is predicted to occur.

(Footnotes for Table 5 continue on next page.)

(**Table 5** footnotes continued:)
[b]CKLINPKWELL is the Orn → Lys analog of COLINPOWELL, and for this prediction, its C-terminal end is in the free carboxyl (-COOH) form rather than amidated (-$CONH_2$) form found in COLINPOWELL. Prediction for COLINPOWELL is based on evidence that replacement of Lys with Orn in proteins/peptides inhibits trypsin hydrolysis at the Orn (formerly Lys) site [44, 45].

(**Results** continued:)
c. Peptide synthesis, purification, and chemical analysis:

The peptide obtained from Bachem was pure, it was in the reduced (monomeric) form, and it had the desired AA composition and MW (1327.7).

d. Preliminary crystallization studies:

Several crystallization solutions produced crystals that may be suitable for further study, most notably: (1) 4 M potassium bromide (KBr), 0.1 M sodium acetate (CH_3COONa), pH 5, produced rectangular crystals after 25 hours that continued to increase in size over the duration of the experiment; (2) 0.1 M sodium bromide (NaBr), 0.1 M 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS), pH 10, 40% (w/v) polyethylene glycol (PEG) 1000 produced many long, thin, needle-like crystals after 25 hours; (3) 3.2 M ammonium sulfate [$(NH_4)_2SO_4$], 0.1 ethyl methyl sulfide (MES), pH 6, produced a cluster of crystals of undefined shapes after 2 weeks.

e. Circular dichroism analysis:

The results of CD analysis of the reduced form of COLINPOWELL, in various concentrations of TFE, are shown in Figure 8. The spectra have maxima at 190 nm, minima at 199-201 nm, and, at high TFE concentrations (50-90%), a second minimum near 222nm. It should be noted that the pH of the peptide containing solutions used for analysis decreased with increasing concentrations of TFE, and was pH 3.1, 2.8, and 1.4 for 0%, 50%, and 90% TFE, respectively. The acidic pH would inhibit any intra- or intermolecular electrostatic interactions (+ ↔ -) {e.g., between the side chains of Orn_7 (+, pKa ~ 10.6) and Glu_9 (-, pKa ~ 4.1 [46])} due to the fact that the side chain carboxyl group of Glu would become protonated and uncharged below pH 4. Consequently, the only types of noncovalent interactions that could be responsible for the spectra of Figure 8 are intramolecular H-bonds (e.g., α-helices and β-sheet), and the aggregation of peptides, with accompanying intermolecular H-bonding and hydrophobic interactions. However, aggregation of the peptide is unlikely due to its moderate hydrophobicity (45% hydrophobic AA residues) and the low peptide concentration (100 μM).

The spectra of ellipticity (θ) versus wavelength do not resemble spectra that are typical for polypeptides [e.g., poly-L-Lysine] in the α-helical (maximum at 191 nm and

(Results continued on page 26.)

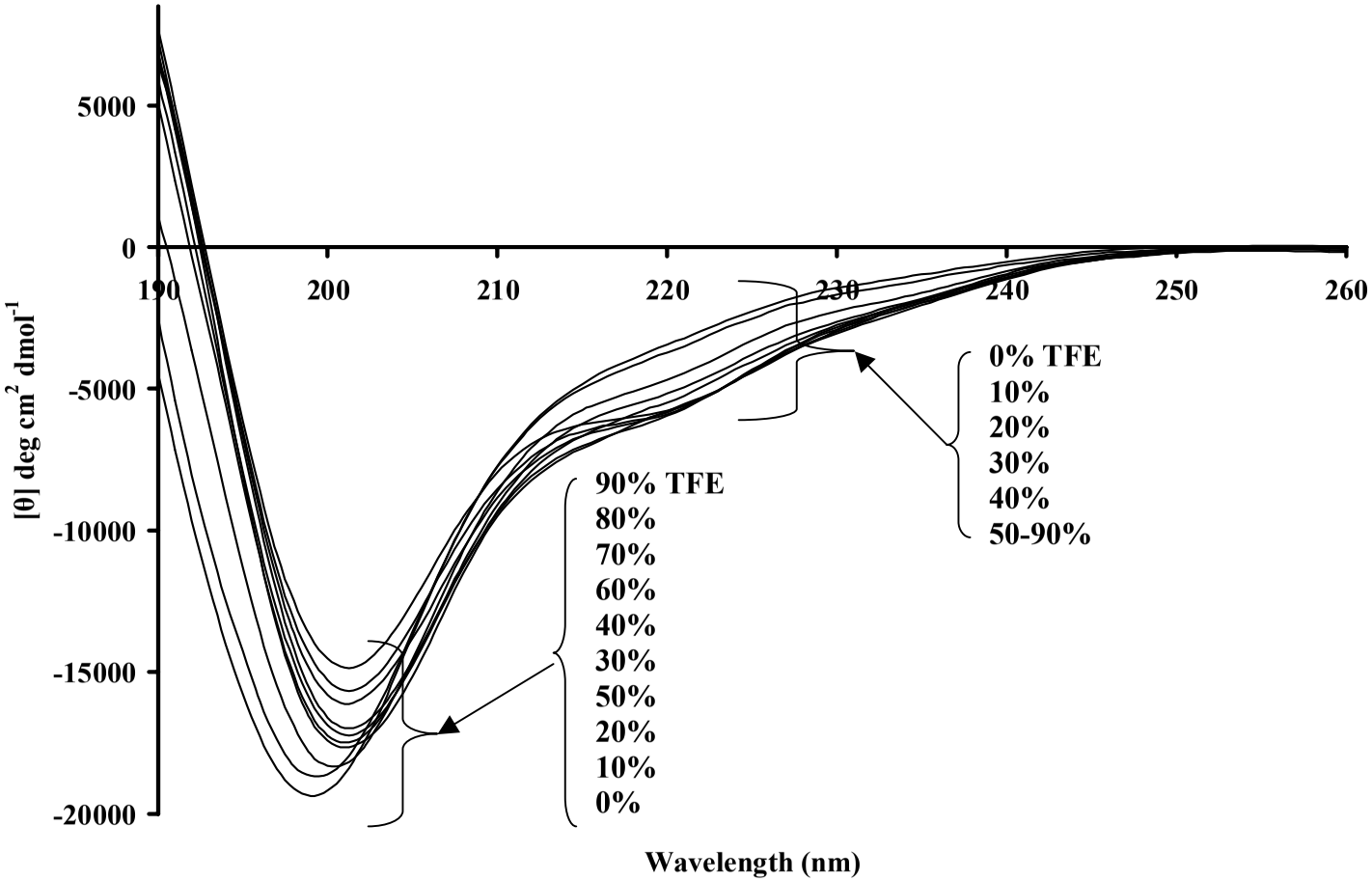


Figure 8. Circular dichroism analysis of COLINPOWELL. The peptide was analyzed at a final concentration of 1 mM in an aqueous solution of 1 mM Tris(2-carboxyethyl)phosphine (TCEP), a water soluble, selective reductant of disulfides that was used to keep the Cys sulfhydryl group in its reduced form, and various concentrations (0-90%) of trifluoroethanol (TFE). Five spectra were collected at each concentration of TFE, over the λ range of 190-260 nm, and averaged. (Spectra courtesy of A. Ng and N.R. Kallenbach.)

Table 6. Results of deconvoluting the CD data for COLINPOWELL in 90% TFE.

Analysis:	% α-Helix:	% β-sheet:	% Turn:	% Other:[a]	% Unordered:[a]
SELCON[b,c]	15.7 ± 2.2 (5)	30.5 ± 4.8 (5)[d]	26.1 ± 2.5 (5)	26.6 ± 8.7 (5)	-
SELCON3[e]	2.8	49.0	22.9	-	25.2
CONTINLL[e]	4.1	41.8	21.5	-	32.6
CONTINLL[f]	3.2	39.7	18.2	-	38.9
K2D	22[g]	30[g]	-	-	49[g]
208 nm[h]	16	-	-	-	-
222 nm[h]	8	-	-	-	-
Mean ± SEM (n):[i]	11.2 ± 2.1 (10)	35.4 ± 3.9 (8)	24.1 ± 1.9 (8)	26.6 ± 3.9 (5)	32.2 ± 4.0 (3)
Number of AAs:	1[j]	4	3	3[k]	3[k]

Notes: [a]Other ≠ unordered; [b]reference protein sets contained 7 or 33 proteins, and there were 5 different secondary structure sets; [c]mean ± std. error (n); [d]24.7% antiparallel β- and 5.6% parallel β-sheet; basis set of 43 soluble proteins and [e]13 membrane proteins or [f]5 denatured proteins; [g]results considered unreliable due to a large amount of error; [h]single wavelength estimate; [i]does not include K2D results; [j]not enough AA's for one turn of α-helix; [k]remaining 3 AAs are either other or unordered; SEM, standard error of the mean; n, number of data values.

(**Results** continued:)
minima at 208 and 222 nm) or β-sheet (maximum at 195 nm and minimum at 217 nm) conformations, but there is some resemblance to spectra typical for the random coil, or unstructured, conformation (minimum at 197 nm and maximum at 218 nm) [24]. The spectra exhibited changes in the wavelength regions of 200-202 nm and 213-230 nm as the concentration of TFE was increased. These changes were relatively continuous with increasing TFE concentration (0-90%) in the low wavelength region (< 205 nm), with the minimum decreasing in the amplitude and a shifting to a higher wavelength (200 → 202 nm). At the higher wavelengths (213-230 nm), the spectral change was the formation of a minimum near 222 nm (i.e., an inflection in all spectra with TFE concentrations greater than 0%) that increased in amplitude with increasing TFE concentration and was maximal by 50% TFE. It is possible that this slight inflection in the spectra near 222 nm could represent the formation of a small amount of α-helix, but there is no minimum at 208 nm to confirm this hypothesis

Although deconvolution of CD data of such a short peptide is unlikely to provide much information, an attempt was made to estimate the maximal content of various types of secondary structures that COLINPOWELL could adopt by deconvoluting the CD data for the peptide in 90% TFE. It has been suggested [47] that the SELCON [19], CONTIN [22], and K2D [23] deconvolution programs be used in conjunction with each other in order to obtain the best overall estimates of polypeptide conformation. The results obtained by use of these deconvolution programs are summarized in Table 6. It

should be noted that two basis sets of reference proteins were used with the CONTINLL program, one containing 5 denatured proteins and one without denatured proteins, and the results obtained for all types of structural elements (α-helix, β-sheet, turn), were not substantially different (Table 6). The inclusion of denatured proteins should make the basis set a better tool for use with proteins that may not contain a substantial amount of secondary structure. In addition, the results obtained for the content of β–sheet and turn were somewhat similar for all deconvolution programs, except SELCON3, which produced a substantially higher than average β–sheet value. There was less agreement among the various methods for the content of α-helix, but they all produced unrealistic values since the maximal helix content corresponded to only 2 AAs involved in an α-helix, a structure that requires a minimum of 4 AAs. In summary, the results of CD spectral deconvolution indicate that the peptide contains 11% α-helix (1 AA), 35% β-sheet (4 AAs), 24% turn (3 AAs), and 27-32% (3 AAs) of either other or unordered structure. The content of β-sheet, turns, and other/unordered structure is not substantially different from that shown in the β-sheet model of Figure 6, where 4 AAs comprise the turn, 6 AA's are shown in H-bonded β-sheet, and the remaining AAs are in somewhat extended conformations. The results of CD deconvolution are also not too different from the 3D protein segments shown in Figure 7A (pancreatic kallirein A) and 7C (Cre recombinase).

f. and g. Antibacterial and antiviral assays:

COLINPOWELL at concentrations of ≤ 25 μM had no activity in antibacterial assays, and at concentrations of ≤ 75 μM had no effect against HSV-1 in the antiviral assays.

h. Blood coagulation assays:

COLINPOWELL had no effect in PT or PTT assays in the range of 3-150 μM.

i. Chemotaxis assays:

COLINPOWELL induced migration of both human monocytes and neutrophils within a concentration range of 0.25-50 μM for monocytes and 0.025-50 μM for neutrophils (Figure 9). Graphs of the peptide concentration versus number of migrating cells were bell shaped, which is characteristic for a chemotactic response, and maximal migration was obtained at 25 μM with each cell type. The numbers of cells induced to migrate was about 2-fold greater with monocytes than with neutrophils.

j. Effects on human cancer cells:

COLINPOWELL caused a significant, 43% inhibition of ^{3}H-thymidine uptake in (i.e., proliferation of) T47D breast cancer cells (Table 7).

In similar experiments, no consistent significant effect was observed on the proliferation of DS19 mouse erythroleukemia cells or on the incorporation of thymidine into DNA of Caco-2 human colon cancer cells after incubation with 50 μM COLINPOWELL (data not shown).

Table 7. Effect of COLINPOWELL (CP) on the incorporation of ^{3}H-thymidine into DNA of T47D human breast cancer cells.

Concentration	cpm/well ± S.D.	N	% Control	P
Control	4026±1241	8	100	-
20 μM CP	2584±826	4	64	N.S.
50 μM CP	2296±194	4	57	<0.05

Note: N.S., not significant.

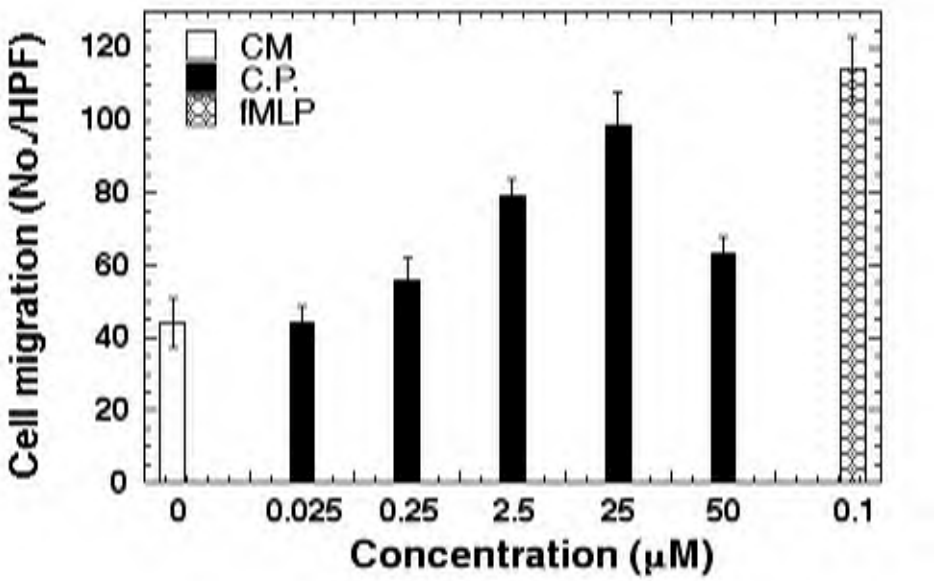
Discussion:

Most new bioactive peptides are discovered through investigations of the individual components of naturally occurring mixtures of molecules that exhibit bioactivity, or by defined chemical modifications of naturally occurring peptides (rational drug design), or by combinatorial methods in which mixtures of peptides with random AA sequences (peptide libraries) are generated systematically and then investigated for biological activity. This report described the design of a bioactive peptide using a novel principle; use of the English alphabet letters of a personal name as the one-letter abbreviations for an AA sequence of the new peptide. This technique is possible only because the internationally recognized convention for describing AA sequences assigns individual letters of the English alphabet to represent each of the naturally occurring types of AAs. Since a very large percentage of the molecules of life are proteins (e.g., 17% of the chemical composition of a 65 kg adult male [48]), it is highly probable that almost any peptide could be found to exhibit some sort of bioactivity depending upon the type of assay used to investigate it. The new approach to bioactive peptide design may seem illogical, but it is scientifically valid and it has the potential for generating enormous numbers of new bioactive AA sequences analogous to telephone catalogs or dictionaries.

In regard to the test peptide used for this new method, COLINPOWELL, a substantial portion of its AA sequence can be found among naturally occurring proteins, including some proteins with known 3D structures. This indicates that the peptide has an AA sequence that is useful in nature, and one that might exhibit biological activities if in its isolated, 11 AA, form. The prediction of a possible β-sheet, or β-strand(s) plus hairpin turn, structure for COLINPOWELL does not seem unreasonable since a similar sequence occurs in a protein of known 3D structure that has just such a β-sheet structure (pancreatic kallirein A). Additional support for this hypothesis is provided by the deconvolution of CD spectral data which indicates that the peptide has substantial β-structure in the reduced, monomeric, form in TFE, a solvent commonly used as a model for the hydrophobic membrane environment [49]. This does not exclude the possibility that COLINPOWELL could assume at least a partial α-helical, or other type of helical, secondary structure in the appropriate environment. Since the electronic transitions

(Discussion continues on page 30.)

A. Human monocytes.



B. Human neutrophils.

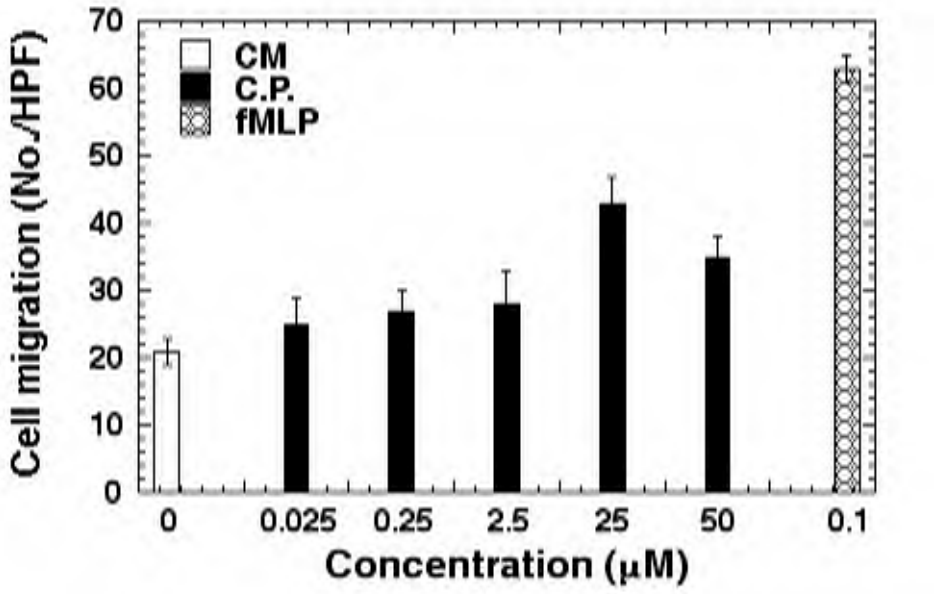


Figure 9. Effect of COLINPOWELL on the migration of human monocytes (A) and neutrophils (B). Results are presented as the number of cells per high power field (No/HPF). CM is chemotaxis medium, CP is COLINPOWELL, and fMLP is the positive control peptide, formylMet-Leu-Phe.

(**Discussion** continued:)
measured by CD occur on the femtosecond time scale, CD measurements will provide an average of all possible conformations in which the peptide spends time. A more informative technique for structure analysis would be a NOSEY NMR spectrum of the peptide in TFE. Such an analysis would provide information about any structures that persist on the millisecond time scale, long enough to develop NOEs characteristic for various types of structure, such as NH-NH NOEs characteristic of α-helices.

In regard to bioactivities, COLINPOWELL was found to have chemotactic activity for human monocytes and neutrophils, antiproliferative activity for human breast cancer cells, and to have no effect the plasma coagulation reaction. Although it had no antibacterial or antiviral activity in the few tests to which it was subjected, the peptide has several chemical and physical features that make it highly probable that additional biological activities will be found for it as the testing program is expanded. These features include: (1) the presence of Cys with a reactive thiol/thiolate group (-SH $\leftrightarrow$ $-S^-$), in its side chain, enabling the participation of the peptide in oxido-reduction reactions (e.g., the formation of disulfide bonded dimers), the coordination of transition metal ions, and reaction with electrophiles; (2) the presence of reactive primary amine ($-NH_2$) groups at one end of the peptide and in the side chains of two Orn residues; (3) the presence of a reactive carboxyl (-COOH) group in the side chain of Glu; (4) a net charge of +2 on the peptide at neutral pH; (5) a pI of ~9; (6) the presence of an indole moiety in the side chain of Trp (e.g., fluorescence); (7) the ability of the peptide to assume secondary structures such as α-helices and β-strands or combinations thereof; (8) the presence of Pro at its center enabling more than one type of secondary structure to exist within the same peptide (e.g., extended region-Pro-helical region); (9) the electrostatically polar nature of the peptide when it is in a nearly α-helical conformation; (10) the relatively small size of the peptide in any conformation. The reactive properties the Cys thiol/thiolate group are particularly notable and have been used to develop novel types of pharmacological agents that have different mechanisms of action than classical agents, including antiviral agents for HIV and human papilloma virus, and new anticancer therapeutics [32].

Several small Cys-containing peptides, have been found to exhibit antiproliferative/anticancer activities, such as Cys-Val-2-Nal-Met [where 2-Nal is β-(2-naphthyl)-L-alanyl]. This synthetic peptidomimetic is a potent inhibitor of p21 Ras farnesyltransferase (Ftase) that prevents farnesylation of the Ras protein, and its association with other cell signaling components on the cytoplasmic side of the cellular plasma membrane [50]. Although COLINPOWELL is predicted to be incapable of penetrating the cell membrane [41], it tempting to speculate that its antiproliferative activities with breast cancer cells might also somehow be due to the inhibition of Ftase.

Its small size, the presence of two basic AAs (Orn) separated by 4 AAs, and a net positive charge (+2) at physiological pH make COLINPOWELL similar in structure to other cationic antimicrobial peptides that have been discovered recently. For example,

the cyclic glycolipodepsipeptide antibiotic family, ramoplanins, which are currently in clinical trials for treatment of Gram positive VRE and MRSA infections, disrupt bacterial cell wall synthesis by binding to the peptidoglycan intermediate Lipid II and blocking its polymerization to form the carbohydrate chains of peptidoglycan [51]. Ramoplanins consists of 17 AA's, 5 of which are nonstandard AA's, including two D-Orn residues that are separated by 5 AA's, and they have net charges of +2 at physiological pH. The function of the two D-Orn of ramoplanins is recognition of the bacterial peptidoglycan monomer, via electrostatic or hydrogen-bonding interactions between the positively charged Orn δ-amino groups of ramoplanins and the pyrophosphate and terminal carboxylate of Lipid II. Ramoplanins are 54% larger than COLINPOWELL and cyclic, but their peptidic nature plus the presence and distance of separation of two Orn residues make them similar in structure to COLINPOWELL. Although COLINPOWELL did not exhibit direct antibacterial activity in the few tests to which it was subjected, it did induce chemotaxis of human monocytes and neutrophils which, in turn, can elicit an antibacterial response [29]. Like temporin A, a 13-residue frog skin antimicrobial peptide, COLINPOWELL may utilize the human FPRL1 receptor to chemoattract phagocytes [52]. It is highly probable that more extensive testing of COLINPOWELL against a larger number of microbial targets will result in the discovery of susceptible organisms. It is also possible that cyclization of COLINPOWELL would improve its antimicrobial activity.

COLINPOWELL might also be capable of acting as an inhibitor of the urea cycle enzyme, ornithine transcarbamoylase (OTC). In the urea cycle, Orn is converted to citrulline by liver mitochondrial OTC: Orn + NH_3 + CO_2 → Citrulline + H_2O. The 3D crystal structure of human OTC complexed with the substrate analog, N-(phosphonoacetyl)-L-Orn (PAO), is known (PDB code 1OTH) [53]. The enzyme's active site is located in a cleft between domains in the protein. Binding of PAO to the enzyme promotes domain closure, with the result that PAO is located near the surface of the protein, with the Orn side chain fully extended and parallel to the protein surface, but partially buried and probably only accessible to molecules of solvent in the protein's environment. Consequently, it might be possible for one of the Orn side chains of COLINPOWELL to fit into the substrate binding site of this enzyme and for it to act as an enzyme inhibitor.

The properties and activities of the oxidized (disulfide bonded or dimeric) form of COLINPOWELL were not investigated, but will be the subject of future studies.

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