Purification, Characterization, and Amino Acid Sequence of the Mating Pheromone Er-10 of the Ciliate *Euplotes raikovi*†

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ABSTRACT: The mating pheromone Er-10 from *mat-10* homozygous *Euplotes raikovi* was purified by a three-step purification procedure with an overall yield of 62%. It was identified as a protein of molecular weight 8000 having an isoelectic point of 3.9. Its complete primary structure was determined by automated Edman degradation of the whole protein after performic acid oxidation and of peptides generated by cyanogen bromide and *Staphylococcus aureus* V8 protease. The proposed sequence is Asp[1]-Leu-Cys-Glu-Gln-Ser-Ala-Leu-Gln-Cys[10]-Asn-Glu-Gln-Gly-Cys-His-Asn-Phe-Cys-Ser[20]-Pro-Glu-Asp-Lys-Pro-Gly-Cys-Leu-Gly-Met[30]-Val-Trp-Asn-Pro-Glu-Leu-Cys-Pro[38]. The calculated molecular weight of 4191.7, which is in good agreement with the value of m/z 4190.7 obtained by fission fragment ionization mass spectrometry, suggests that the native structure is a dimer with three intrachain disulfide bonds in each subunit. The amino acid sequence is 43% identical with that of the *E. raikovi* mating pheromone Er-1, with the identities concentrated in the amino-terminal half. The half-cystine locations are conserved, but Er-10 is two residues shorter than Er-1. Prediction of the secondary structure suggests that Er-10 may also contain a helical structure at the amino terminus. These results indicate that the mating pheromones of *E. raikovi* form a homologous family.

In ciliated protozoa, mating-type products control self-recognition phenomena that are revealed by formation of mating pairs. These products may be diffused as "mating pheromones" into the environment, as in some species of *Blepharisma* (Miyake, 1981), *Euplotes* (Luporini & Miceli, 1986), and *Dileptus* (Afon'kin & Yudin, 1987), or carried as intrinsic membrane proteins as in *Paramecium* (Kitamura, 1988).

The synthesis of *Euplotes raikovi* mating pheromones (termed euplomones and abbreviated as Er, where the *r* stands for the specific name *raikovi*) (Miceli et al., 1983) is at the Mendelian *mat*[1] locus through a series of multiple codominant alleles, most of which remain to be identified (Luporini et al., 1986). Several of these pheromones, designated numerically as members of the Er family, have been prepared from the corresponding *mat* homozygotes, generated as offspring clones from the naturally occurring heterozygotes found in the wild, and shown to be proteins of ~11 000–12 000 Da (Concetti et al., 1986; Raffioni et al., 1987) with subunit masses of 4000–5000 Da (Raffioni et al., 1988). One of these, Er-1, has been sequenced (Raffioni et al., 1988) and the precursor structure predicted from the sequence of the corresponding cDNA (Miceli et al., 1989). In this paper, we described the isolation and characterization of the pheromone Er-10, isolated from cells homozygous for this trait, including its complete amino acid sequence. It is clearly homologous to Er-1 but shows several interesting differences.

Experimental Procedures

Materials. Sep-Pak C_{18} cartridges were purchased from Millipore; Sephadex G-50 (fine grade) and Pharmalyte 2.5-5 were from Pharmacia; Bio-Gel P-10 (fine grade) and electrophoresis reagents were from Bio-Rad; sea salts, β-alanine, Bis-Tris, gel filtration molecular weight standards, CNBr, guanidine hydrochloride, iodoacetic acid (recrystallized before use), and carboxypeptidase Y were from Sigma Chemical Co.; the Gelcode silver stain protein kit, *S. aureus* V8 protease, and TFA were from Pierce Chemical Co.; formic acid was from Mallinkrodt Chemicals; the silver staining detection system and iodo[1-^{14}C]acetic acid (17.9 mCi/mmol) were from New England Nuclear; HPLC-grade solvents were from Fisher; and gas-phase sequencer chemicals were from Applied Biosystems. All other chemicals were of reagent grade.

Clone $1bF_11N$ (*mat-10/mat-10*) was used as the source for Er-10 and clone $1aF_113$ (*mat-1/mat-1*) for the bioassay of Er-10 activity. Both clones were maintained and fed as described previously (Concetti et al., 1986). The cell-free medium used for the pheromone isolation was recovered from 16–24-h-starved cells suspended at a final density of 2×10^4/mL. It was immediately sterilized through filters of 0.2-μm pore size and stored at 4 °C.

Biological Assay. The Er-10 mating-promoting activity was estimated in arbitrary units defined as the minimum amount of a sample that induces tester cells (10^4 in 1 mL) to form at least one mating pair (Miyake & Beyer, 1973). The bioassay was performed by the addition of serially diluted samples (20 μL) to 2-day-starved assay cells (2×10^3 in 200 μL) as previously described (Concetti et al., 1986). The cells were concentrated, washed, and shifted to a new starvation medium

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[1] Abbreviations: mt, mating type; Er-10, euplomone *r*-10; *mat*, mating-type locus designation; CM-Er-10, S-[^{14}C]carboxymethylated Er-10; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; Bis-Tris, [bis(2-hydroxyethyl)amino]tris(hydroxymethyl)methane; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; FPLC, fast protein liquid chromatography; PAO, performic acid oxidation; PMSF, phenylmethanesulfonyl fluoride; TFA, trifluoroacetic acid; HPLC, high-performance liquid chromatography; PTC, phenylthiocarbamyl; PTH, phenylthiohydantoin.

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before use and were scored for mate-pair formation within 4–5 h. Protein concentration was determined by the method of Lowry et al. (1951), with bovine serum albumin as a standard.

Purification of the Pheromone. Er-10 was isolated in homogeneous form essentially by the three-step procedure of Concetti et al. (1986). This method employs Sep-Pak C_{18} concentration, Sephadex G-50 gel filtration, and FPLC ion-exchange chromatography on a column of Mono Q HR5/5.

Molecular Weight Determination. Estimates of molecular weight were made by gel filtration on a Bio-Gel P-10 column (1.6 × 85 cm), equilibrated in 0.2 M Tris-HCl at pH 8.2 and run at a constant flow rate of 12 mL/h at 4 °C, and on a prepacked Superose 12 HR 10/30 column (FPLC) in 0.1 M Tris-HCl and 0.15 M KCl at pH 7.5, developed at a flow rate of 0.5 mL/min. The columns were standardized with mixtures of proteins and polypeptides of known mass. Mass spectrometric analyses of Er-10 were performed on a ^{252}Cf fission fragment ionization time-of-flight mass spectrometer, constructed at the Rockefeller University (Chait et al., 1981), as described previously (Raffioni et al., 1988). SDS–PAGE was carried out according to Laemmli (1970). Samples, reduced with 2-mercaptoethanol and/or carboxymethylated and then boiled for 6 min, were loaded on a mini slab gel (7.3 × 8.3 cm) and run at 200 V for 40 min. The gel was eventually stained with the Gelcode silver stain protein kit.

Gel Electrophoresis and Isoelectric Focusing. Nondenaturing PAGE was performed at pH 4.5 according to Reisfeld et al. (1962), and isolectric focusing was performed on 5% polyacrylamide rod gels containing Pharmalyte in the pH range 2.5–5, at 500 V for 6 h, essentially by the method of Wringley (1981). After electrofocusing the gel was cut into 2-mm slices to measure pH and biological activity and eventually stained with Coomassie blue G-250 by the procedure of Blakesley and Boezi (1977).

Protein Modification. Er-10 (20 μg) was subjected to performic acid oxidation according to Hirs (1967). After 4 h the reaction was stopped with 10 volumes of distilled water followed by lyophilization. S-Carboxymethylation was performed essentially according to Angeletti et al. (1971) as described previously (Raffioni et al., 1988).

Cleavage and Purification of Peptides. CM-Er-10 (0.2 mg) was dissolved in 70% acid formic, and a 50-fold molar excess of CNBr over total estimated methionine residues was added. After 24 h under nitrogen the reaction mixture was diluted with 15 volumes of distilled water and lyophylized (Gross & Witkop, 1961).

CM-Er-10 (0.3 mg) was digested with *S. aureus* V8 protease (2% w/w) in 0.05 M NH_4HCO_3 at pH 7.8 (Houmard & Drapeau, 1972). After 16 h at room temperature, the reaction was stopped by the addition of excess of PMSF. The resulting peptides were separated by reverse-phase HPLC on a Vydac C_{18} column (4.6 × 250 mm) equilibrated with 0.1% TFA, an acetonitrile gradient being used.

CM-Er-10 (70 μg) was dissolved in 0.02 M sodium phosphate at pH 6.0, and carboxypeptidase Y was added to a final ratio of 1:100 (Hayashi et al., 1973). Aliquots were removed at designated times, acidified with 1 N HCl, and centrifuged, and the recovered supernatant was dried prior to derivatization and amino acid analysis.

Amino Acid Analysis. The amino acid composition was determined after hydrolysis of the protein and peptides in 6 N HCl for 22 h at 110 °C, under reduced pressure. The PTC derivatives were analyzed by reverse-phase HPLC on a Hewlett-Packard 1090 instrument (Heinrikson & Meredith, 1984).

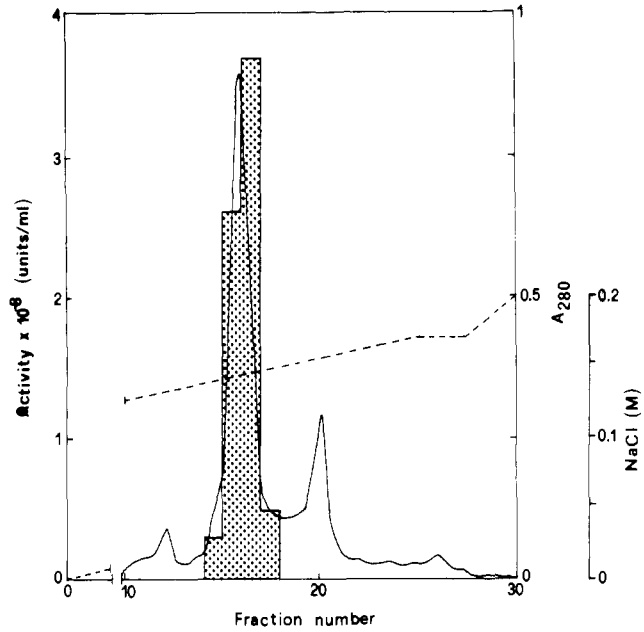

FIGURE 1: Elution profile for ion-exchange chromatography of the active fraction recovered from gel filtration and applied to a Mono Q column in the FPLC system. Fractions of 1 mL were collected. Biological activity was assayed on aliquots removed from every fraction (stippled area). Absorbance at 280 nm (—). Applied salt gradient (– –).

Amino Acid Sequence Determination. Sequence analysis was performed on an Applied Biosystem 477A pulsed-liquid sequencer with an on-line 120A PTH-amino acid analyzer. When radioactive derivatives were used, aliquots from each cycle were subjected to scintillation counting to determine the position of the S-[^{14}C]carboxymethylated cysteinyl derivatives.

Prediction of Secondary Structure. The PEPPLOT program (Gribskov et al., 1986) was employed to analyze the secondary structure of Er-10 according to the parameters of Chou and Fasman (1978) and the hydropathy profile as recommended by Kyte and Doolittle (1982) using a nine-residue window.

RESULTS

Purification of the Pheromone Er-10. Er-10 was purified according to the recently published procedure (Concetti et al., 1986) from the homozygous clone $1bF_11N$ (*mat-10/mat-10*) of *E. raikovi* by a three-step protocol involving separations by Sep-Pak C_{18}, Sephadex G-50, and Mono Q FPLC. The specific activities, recoveries, and *x*-fold purifications were essentially the same as those found for other Er's (Concetti et al., 1986; Raffioni et al., 1987). During the final step of purification, achieved by ion-exchange chromatography (Figure 1), the principle active component emerged as a symmetrical peak although small amounts of leading and trailing shoulders were usually observed. These may correspond to minor allotypic variants as has been identified for pheromone Er-1 (C. Miceli and A. La Terza, unpublished observations). Typically, the amount of Er-10 recovered was 1.5 mg, starting from a 10-L volume of cell-free medium (variations were within limits of ±0.2 mg), with a 3.4-fold purification and a yield of 62%. It was stored dry at −20 °C. The biologically effective concentration of the protein corresponded to 2.5×10^{-13} M, estimated with $1aF_113$ cells.[2]

As shown in Figure 2, Er-10 was judged to be homogeneous by PAGE carried out either under nondenaturing conditions

[2] Pheromone activity may vary if cells of other mating types are used.

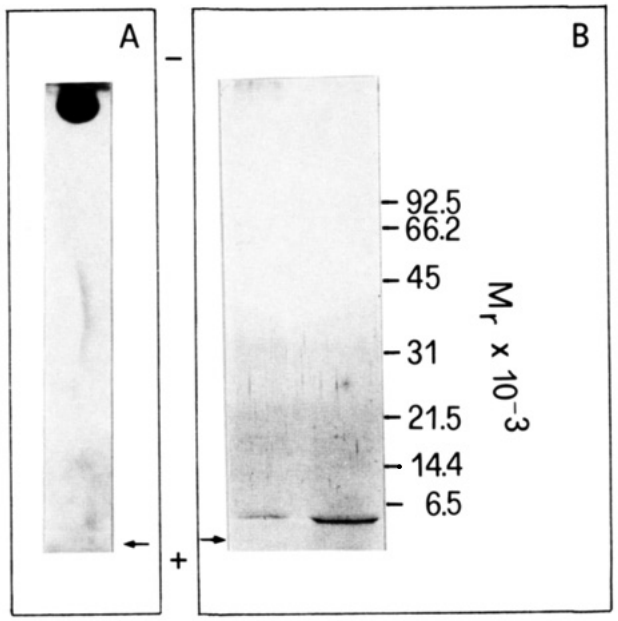

FIGURE 2: Purity of E*r*-10 samples assessed by nondenaturing PAGE (A) and denaturing SDS–PAGE (B). In (A), a 5-μg sample was run on a 7.5% gel at pH 4.5. In (B), a 2.5-μg sample reduced with 2-mercaptoethanol (lane 1) and a 3-μg S-carboxymethylated sample (lane 2) were run on a 5–20% gradient gel. Sample migration was from top to bottom. Arrows indicate the dye front.

at pH 4.5 or in SDS under reducing conditions (Laemmli, 1970). Amino-terminal sequence analyses, as discussed below, also indicated a single protein to be present. In both PAGE systems, a single protein band, which was visualized by silver staining, was shown to contain the pheromone activity, albeit that it was somewhat reduced from that of the SDS experiment.

The single band observed on isoelectric focusing migrates in a position corresponding to pH 3.9, which was also shown by gel slicing to contain the biological activity (data not shown).

Estimation of Molecular Weight. The determination of the molecular weight of E*r*-10 utilized gel filtration, SDS-PAGE, and mass spectrometry. Gel filtration was carried out either on a calibrated Bio-Gel P-10 column or on a calibrated Superose 12 column; the molecular weight values estimated were 11 000 and 8000, respectively. On SDS–PAGE, samples either reduced (with 2-mercaptoethanol) or reduced and carboxymethylated migrated as single bands corresponding to a molecular weight of 4000–5000 (Figure 2). Mass spectrometry of a sample of native E*r*-10, kindly performed by Dr. Brian Chait, Rockefeller University, on a ^{252}Cf fission fragment ionization time-of-flight mass spectrometer, gave a strong protonated quasi-molecular ion $[(M + H)^+]$ appearing in the positive spectrum at m/z 4190.7, corresponding to an isotopically averaged mass of 4190.2. In addition, a weaker doubly protonated quasi-molecular ion $[(M + 2H)^{2+}]$ appears at m/z 2095.1, corresponding to an isotopically averaged mass of 4190.2. These values are in excellent agreement with those obtained from SDS–PAGE. The spectrum of E*r*-10 also contains a secondary peak at m/z 8380, which may arise from the presence of homodimers of noncovalently associated subunits in the analyzed sample.

Amino Acid Analysis. The amino acid composition of E*r*-10 determined after acid hydrolysis is reported in Table I. The protein was calculated to contain 37 amino acids (with no estimation of tryptophan), in excellent agreement with the composition predicted by sequence analysis (see below). As expected from the estimated p*I* value, the molecule is rich in aspartic and glutamic acids and poor in basic residues. It is devoid of threonine, tyrosine, isoleucine, and arginine.

Table I: Amino Acid Composition of the *E. raikovi* Mating Pheromone E*r*-10

amino acid	residues/mol: from acid hydrolysates[a]	residues/mol: from amino acid sequence
aspartic acid	4.3 (4)	5
glutamic acid	7.2 (7)	7
serine	1.9 (2)	2
glycine	2.9 (3)	3
histidine	0.9 (1)	1
alanine	1.1 (1)	1
proline	4.4 (4)	4
valine	0.8 (1)	1
methionine	0.9 (1)	1
half-cystine[b]	6.6 (7)	6
leucine	4.0 (4)	4
phenylalanine	0.9 (1)	1
lysine	0.9 (1)	1
tryptophan	ND[c]	1
total	36.8 (37)	38

[a] Average of four determinations. Calculated by assuming a molecular weight of 4200. Integral values are given in parentheses. [b] Determined as cysteic acid (Hirs, 1967) and as *S*-(carboxymethyl)-cysteine. [c] Not determined.

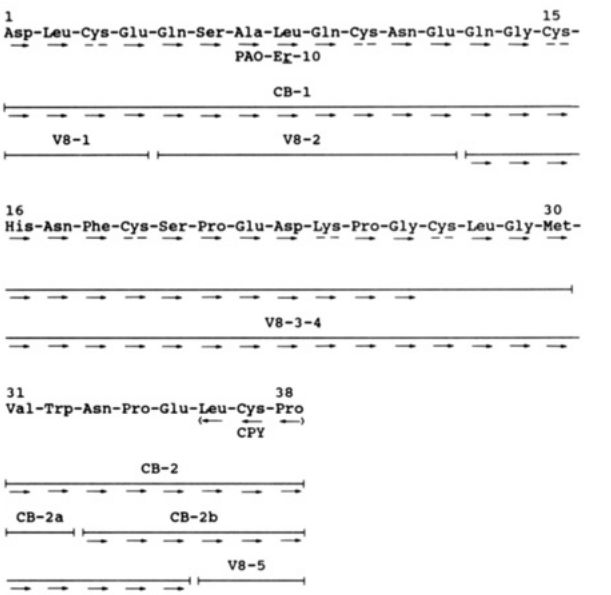

FIGURE 3: Summary of the complete amino acid sequence determination of E*r*-10 mating pheromone. Peptides are numbered in the order expected for the anticipated (or observed) cleavage sites. Designations: CB, cyanogen bromide, V8, *S. aureus* V8 protease. The peptides generated in the cyanogen bromide digest by a cleavage at tryptophan are indicated by lower-case letters. Peptides indicated by two numbers connected with a hyphen result from partial cleavage. Residues directly identified by automated Edman degradation and carboxypeptidase Y digestion (CPY) are marked by right and left arrows, respectively. Residues identified by amino acid composition are indicated by dashed lines.

Amino Acid Sequence Determination. The complete amino acid sequence of E*r*-10 and the strategy used for its determination are presented in Figure 3. The sequence was determined by automated Edman degradation of the entire E*r*-10 after performic acid oxidation and of chemically and enzymatically derived peptide fragments of carboxymethylated E*r*-10.

The amino-terminal analysis of a performic acid oxidized sample (1.5 nmol) of E*r*-10 gave identification of the first 30 residues except for cycles 3, 10, 15, 19, 24, and 27 (Table II).

Table II: Amino Acid Sequence Data for *E. raikovi* Mating Pheromone Er-10

position	residue	cycle yield (pmol)[a] PAO Er-10	CB-1	CB-2	CB-2b	V8-3-4
1	Asp	(1) 220	(1) 1970			
2	Leu	(2) 530	(2) 3530			
3	Cys	(3) *b*	(3) *d*			
4	Glu	(4) 315	(4) 1320			
5	Gln	(5) 365	(5) 1170			
6	Ser	(6) 125	(6) 490			
7	Ala	(7) 370	(7) 2065			
8	Leu	(8) 390	(8) 2130			
9	Gln	(9) 270	(9) 1090			
10	Cys	(10) *b*	(10) *d*			
11	Asn	(11) 210	(11) 740			
12	Glu	(12) 175	(12) 540			
13	Gln	(13) 220	(13) 700			(1) 2030
14	Gly	(14) 200	(14) 770			(2) 2640
15	Cys	(15) *b*	(15) *d*			(3) *d*
16	His	(16) 40	(16) 105			(4) 1180
17	Asn	(17) 120	(17) 410			(5) 2640
18	Phe	(18) 115	(18) 710			(6) 2940
19	Cys	(19) *b*	(19) *d*			(7) *d*
20	Ser	(20) 30	(20) 110			(8) 450
21	Pro	(21) 60	(21) 290			(9) 870
22	Glu	(22) 35	(22) 55			(10) 500
23	Asp	(23) 30	(23) 50			(11) 350
24	Lys	(24) *b*	(24) 25			(12) 670
25	Pro	(25) 50	(25) 35			(13) 520
26	Gly	(26) 45				(14) 710
27	Cys	(27) *b*				(15) *d*
28	Leu	(28) 35				(16) 540
29	Gly	(29) 45				(17) 610
30	Met	(30) *c*				(18) 600
31	Val			(1) 2690		(19) 610
32	Trp			(2) 160		(20) 265
33	Asn			(3) 530	(1) 1320	(21) 450
34	Pro			(4) 810	(2) 1370	(22) 340
35	Glu			(5) 800	(3) 540	(23) 65
36	Leu			(6) 930	(4) 1275	
37	Cys			(7) *d*	(5) *d*	
38	Pro			(8) 170	(6) 300	

[a] Load samples: PAO Er-10, 1500 pmol; CB-1/CB-2/CB-2b, 5000 pmol; V8-3-4, 3000 pmol. Numbers in parentheses are Edman cycle identifications. [b] Not identified. [c] Identified qualitatively as methionine sulfone. [d] Identified as PTH-*S*-([^{14}C]carboxymethyl)cysteine from HPLC and radioactive analysis.

All but residue 24 were subsequently established to be half-cystines on the basis of the ^{14}C isotope released during degradation of CNBr and *S. aureus* V8 protease peptides (see below). Cycle 30 was qualitatively identified as methionine sulfone.

Given the amino-terminal assignments and the presence of one residue of methionine (Table I), CM-Er-10 was subjected to CNBr cleavage and automatic Edman degradation without further fractionation. Two major sequences, in approximately equimolar amounts, and one minor sequence were observed. The sequence of the longest one, corresponding to peptide CB-1, confirmed the assignments made for positions 1–26 in the amino-terminal degradation of intact Er-10 and established the identification of four half-cystine residues (positions 3, 10, 15, and 19). The amino acid sequence for the carboxyl-terminal region corresponding to CB-2 was derived by subtracting the sequence obtained from undigested oxidized Er-10 at each cycle. The additional minor peptide, CB-2b, corresponded to residues 33–38. This peptide was formed by cleavage at Trp-32, as has been reported to occur at tryptophan bonds during CNBr fragmentation in other proteins (Blumenthal et al., 1975). As peptide CB-2b was present in lesser amounts, it was also possible to identify its minor sequence by subtracting the two major sequences corresponding to peptides CB-1 and CB-2, respectively.

To complete the Er-10 sequence, CM-Er-10 was digested with *S. aureus* V8 protease, and the resulting peptides were separated by reverse-phase HPLC on a C_{18} Vydac column (Figure 4). Only peptide V8-3-4, shown by amino acid analysis to contain the single lysine residue, was subjected to automated Edman degradation. It was sequenced for all 23 residues (Table II). This peptide, which resulted from incomplete cleavage at Glu-22, provided the overlap information between Met-30 and Val-31 as well as the identification of the half-cystine residue at position 27 (Table II, Figure 3).

Treatment of CM-Er-10 with carboxypeptidase Y for 30 min released proline, *S*-(carboxymethyl)cysteine, and leucine, in essentially equal amounts (approximately 0.5 residue). Longer time points showed the appearance of a second residue of proline along with glutamic acid, asparagine, valine, methionine, tryptophan, and glycine. These data are consistent with the C-terminal sequence obtained from the automatic sequencing of CB-2.

Discussion

The mating pheromone Er-10 has been purified to homogeneity by the same simple and rapid purification procedure developed by Concetti et al. (1986) that has been used to purify the *E. raikovi* pheromones Er-1, Er-2, Er-3, and Er-11 (Raffioni et al., 1987). Homogeneity of the final preparation was assessed by the presence of a single protein band which comigrated with the biological activity in different polyacrylamide gel systems and by the amino-terminal sequence of whole Er-10.

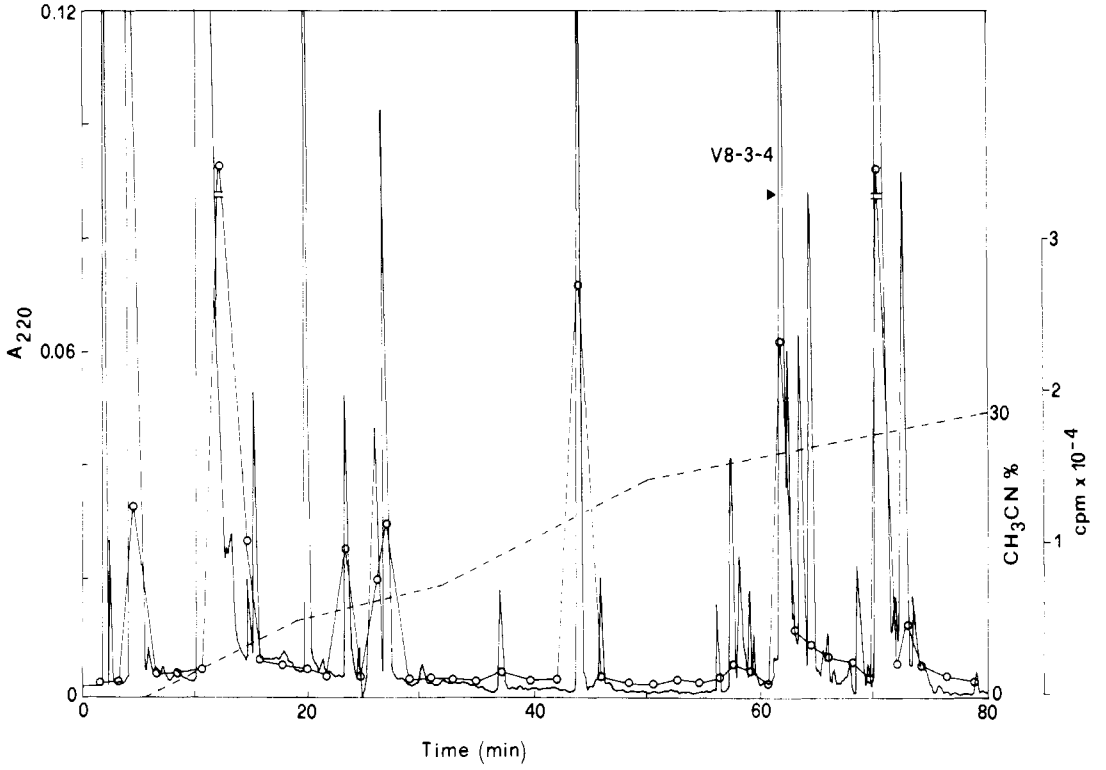

FIGURE 4: Separation of the peptides obtained from an *S. aureus* V8 protease digestion of CM-E*r*-10 by reverse-phase HPLC. The peptides were eluted with a gradient of acetonitrile (--) at a flow rate of 1 mL/min. Absorbance at 220 nm (—). Radioactivity was counted on 50-μL aliquots taken from every other fraction (O). The only peptide subjected to sequence analysis, V8-3-4, is indicated.

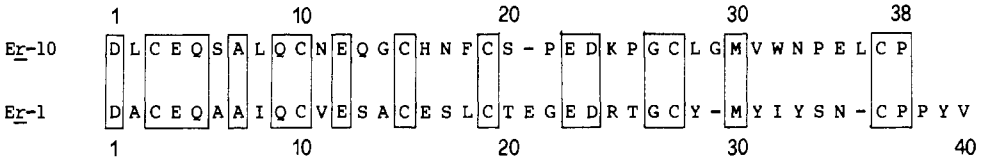

FIGURE 5: Comparison of the amino acid sequences of *E. raikovi* pheromones E*r*-1 and E*r*-10. Identical sequences are enclosed. E*r*-1 data taken from Raffioni et al. (1988).

The sequence analysis yielded a self-consistent structure of 38 amino acids that is in excellent agreement with the composition determined from acid hydrolysates and the molecular weight given by the mass spectrometer. The sequence assignments are primarily provided by the analysis of the whole protein and the carboxyl-terminal CNBr fragment (CB-2). Identification of the five half-cystines found in the first 30 residues (as well as the lysine at position 24) was given by the sequences of CB-1 and the *S. aureus* V8 protease peptide V8-3-4. The latter also provided the overlap for the two CNBr fragments. The carboxypeptide Y digestion provided confirmation that the C-terminal sequence ends with the residues -Leu-Cys-Pro. However, the essentially perfect agreement between the calculated and determined molecular weights (arising from the mass spectrometer) clearly indicates that all residues are accounted for by the proposed sequence (Figure 3).

E*r*-10 is similar to the other *E. raikovi* pheromones that have been isolated with respect to biological activity, molecular weight, p*I*, and amino acid composition. However, there are some notable differences. The estimated concentration of purified E*r*-10 required for the minimum induction of activity is only 2.5×10^{-13} M. This is the lowest value yet reported for a mating pheromone; the corresponding figures for the other E*r* pheromones are as follows: E*r*-1, 3.4×10^{-12} M (Concetti et al., 1986); E*r*-2, 4.7×10^{-12} M; E*r*-3, 2.9×10^{-12} M; E*r*-11, 1.2×10^{-11} M (Raffioni et al., 1987). Further, amino acid analyses showed that although it is acidic (p*I* = 3.9 compared to the range 3.7–4.0 estimated for other pheromones) and rich in half-cystines, it lacks threonine, tyrosine, and isoleucine that are found in the other pheromones. These differences extend to the sequence as well. As shown in Figure 5, when the sequence of E*r*-10 is compared to that of E*r*-1 (Raffioni et al., 1988), there is an overall identity of 43% (17/39) although three gaps are required. Furthermore, six of the identities are provided by half-cystine residues; nonetheless, the two sequences are unmistakably homologous although the similarity is clearly concentrated in the amino-terminal half. In the carboxyl-terminal half, there are only seven identities (including all the gaps), and E*r*-10 lacks three residues at the end (in the alignment used). When compared to the amino-terminal sequences of E*r*-2 and E*r*-9 (Raffioni et al., 1988), E*r*-10 is clearly more closely related to E*r*-1. The conserved motif Cys-Glu-Gln-Ala-X-X-X-Cys, found in the amino-terminal portion of all of the pheromones examined so far, is now reduced to Cys-Glu-Gln-X-X-X-X-Cys.

On the basis of both SDS–PAGE and mass spectrometer measurements, the minimal covalent unit is comprised of the 38 amino acid polypeptide, suggesting all of the half-cystine residues are present as intrachain disulfide bonds. This is consistent with independent analyses that are in progress to determine these pairings (S. Raffioni, T. Lee, J. Shively, and R. A. Bradshaw, unpublished observations). The native molecular weight has been determined to be 8000–11 000 by various gel filtration experiments and suggests that the pheromone exists as a dimer or higher aggregrate of nonco-

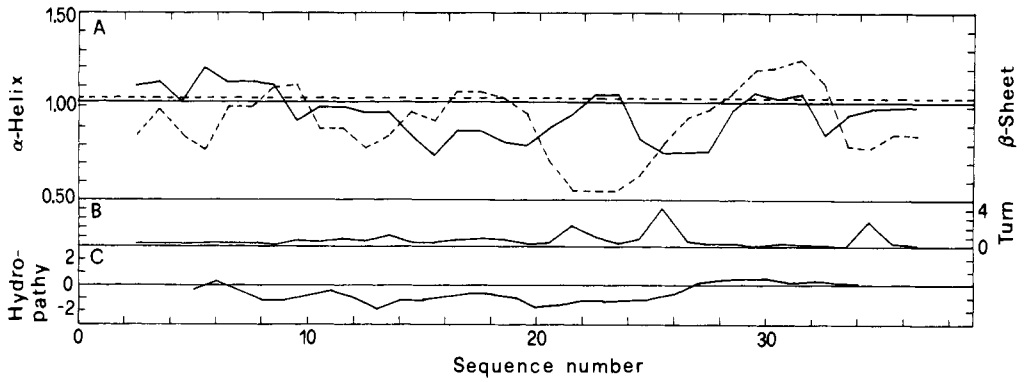

FIGURE 6: PEPPLOT graphic of secondary structure prediction (A and B) and hydropathy profile (C) of Er-10 mating pheromone. In (A), dotted lines are used to distinguish the curves for β-structures from the curves for α-structures. The horizontal dotted and solid lines indicate the mininum levels for predicting β- and α-structures. In (C), values above and below the horizontal line indicate hydrophobic and hydrophilic regions, respectively.

valently associated subunits in solution.

The predicted secondary structure of Er-10 using the parameters of Chou and Fasman (1974) is shown in Figure 6. The amino terminus may contain a short helical segment (Figure 6A), as has been found for Er-1 (Raffioni et al., 1988), and three β-turns are indicated at residues 22, 26, and 34 (Figure 6B).

An evaluation of the hydropathy profile (Kyte & Doolittle, 1982) is presented in Figure 6C. Er-10 can be regarded as a hydrophilic molecule with such a domain spanning residues 8–26. In particular, three charged amino acids are grouped at the region between residues 22–24, which lies in the largest hydrophilic segment. Only the carboxyl terminus shows any hydrophobic character. Although these analyses are of limited value for such a small protein (especially one containing three intrachain disulfide bonds), they do emphasize the similarity in three-dimensional structure that can be expected for Er-1 and Er-10, and quite possibly for other Er molecules as well.

The mating pheromones of *E. raikovi* are released from normally heterozygous (with regard to mating type) cells by an as yet uncharacterized mechanism to induce conjugation through the formation of mating pairs. Although the details of this process are not understood, it is presumed that the mature pheromone binds to a cell surface receptor resulting in the production of an intracellular signal that is a prerequisite for the mating event. Since there are demonstrably many pheromone molecules (with unique sequences as judged by their compositions) (Raffioni et al., 1987), it follows that there must also be many receptors as well. In fact, genetic evidence (Luporini & Miceli, 1986) suggests that each pheromone has its own receptor with which it can form a selective, high-affinity complex. Such a situation can explain the differences observed in mating efficiency by assuming variations in binding affinities. However, crossover in binding, as has been observed in other cases of homologous ligands, may well occur (Straus, 1984). Since the cells that produce Er-1 and Er-10 show an extensive mating response, it may be assumed that there is very little receptor cross-reaction.

An examination of the sequences of Er-1 and Er-10 clearly shows the amino-terminal halves of these molecules are quite similar, including a likely helical segment. This suggests a function for this part of the protein common to both; an opposite conclusion, namely, that it contains a function unique to each pheromone, applies for the carboxyl-terminal half. This is consistent with the view that the receptor binding site is concentrated in this region. The homodimer contacts, which could be generally expected to have similar structures in both Er-1 and Er-10, would then be contributed by the amino-terminal sequences. The elucidation of the three-dimensional structure, now in progress, as well as additional Er sequences should help to clarify such predictions.

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Persistence of the α-Helix Stop Signal in the S-Peptide in Trifluoroethanol Solutions†

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ABSTRACT: α-Helix formation in the S-peptide (residues 1-19 of ribonuclease A) was studied in detail by use of two-dimensional ^{1}H nuclear magnetic resonance to monitor the effects of 2,2,2-trifluoroethanol (TFE) at 0 °C and pH* 2.07. TFE stabilizes the S-peptide α-helix. Helix formation by a particular amino acid was monitored by the chemical shifts of the C^α, C^β, and C^γ protons while increasing the concentration of TFE: large changes in chemical shift of a particular residue indicate that it is induced to go helical, whereas small chemical shift changes indicate little helix formation. Residues Thr-3 to Met-13 undergo chemical shift changes consistent with helix formation, whereas the other residues do not. Earlier work [Kim, P. S., & Baldwin, R. L. (1984) *Nature 307*, 329-334] reported that residues Thr-3 to His-12 become helical in aqueous solution. The existence of a "helix stop signal" was inferred from this behavior. We thus conclude that this helix stop signal persists in TFE solutions.

The S-peptide, residues 1-19 or 1-20 of RNase A,[1] forms an unusually stable α-helix in aqueous solution (Brown & Klee, 1971). Extensive studies on analogues of the C-peptide, residues 1-13 of RNase A, have shown that the stability depends on the charges of the amino acid side chains and helix termini (Shoemaker et al., 1987, 1985). This has been termed the "charged-group effect" and is determined by measuring the helix stability as the charges of the titratable side chains are altered by changing the pH, as well as by chemically introducing or removing charged side chains.

An NMR study indicated that only residues 3-12 of S-peptide-(1-20) become helical in aqueous solution (Kim & Baldwin, 1984). Because the last several residues of the S-peptide fail to become helical, they hypothesized the existence of a "helix stop signal" that terminates the helix near His-12. Standard helix-coil transition theory predicts that short helical segments are strongly cooperative and that, once nucleation is achieved, propagation of helix is facile.

It is well-known that the cosolvent 2,2,2-trifluoroethanol (TFE) stabilizes the S-peptide α-helix (Filippi et al., 1976, 1978). A circular dichroism study of the effects of pH*, temperature, and TFE concentration was reported by us previously (Nelson & Kallenbach, 1986). We discovered that the magnitude of the charged-group effect does not increase in TFE solutions, so that the difference in stability vs pH* is nearly the same at TFE concentrations up to 40 mol %. One difference is that in TFE solutions the helix does not become less stable as the pH* is reduced below about 3.8, as it does in aqueous solution. The titration curve of helix stability vs TFE concentration is cooperative at 0 °C, meaning that the helix stability increases greatly as the TFE concentration increases up to 10 mol % and then levels off. However, the cooperativity is much lower at temperatures between 50 and

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[1] Abbreviations: TFE, 2,2,2-trifluoroethanol; TFE-d_3, fully deuterated TFE; RNase A, ribonuclease A; S-peptide-(1-19), residues 1-19 of RNase A; S-peptide-(1-20), residues 1-20 of RNase A; pH*, pH in solutions containing organic cosolvent; TSP, sodium 3-(trimethylsilyl)-propionate-*2,2,3,3-d*$_4$; NMR, nuclear magnetic resonance; 1D NMR, one-dimensional NMR; 2D NMR, two-dimensional NMR; FID, free induction decay; ppm, parts per million; DQF-COSY, double-quantum-filtered correlation spectroscopy; NOESY, nuclear Overhauser effect spectroscopy; RELAY, relayed coherence transfer spectroscopy; TPPI, time-proportional phase increments; $[\theta]_{222}$, mean residue ellipticity at 222 nm; Ala_3, tripeptide alanylalanylalanine.

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