Formyl Peptide Chemoattractants: A Model of the Receptor on Rabbit Neutrophils†

Richard J. Freer,* Alan R. Day, Natesa Muthukumaraswamy, Delia Pinon, Arthur Wu, Henry J. Showell, and Elmer L. Becker

ABSTRACT: Twenty small molecular weight peptides related to the chemotactic peptide *N*-formylmethionylleucylphenylalanine (CHO-Met-Leu-Phe-OH) have been prepared by classical peptide synthesis. Compounds were prepared to evaluate the requirements in position 2 (leucine) and the C-terminal carboxyl group. Each analogue was tested for its ability to induce lysosomal enzyme release from cytochalasin B treated rabbit polymorphonuclear leukocytes. In addition representative samples were also tested for their ability to inhibit specific binding of a ^{3}H-labeled chemotactic peptide (CHO-Nle-Leu-[^{3}H]Phe-OH). The results indicate that both linear aliphatic and branched aliphatic residues in position 2 result in potent chemoattractants. Analysis of these data as well as previously published data for position 1 (methionine) indicates that hydrophobicity is a major determinant of biological activity. So that the role of the C-terminal area of the molecule could be probed, a number of tripeptide benzyl esters and the benzylamide derivative of CHO-Met-Leu-Phe-OH were prepared. These were uniformly more active (12–533-fold) than their free acid counterparts. A similar relationship was not seen with a limited number of tetrapeptides and their benzyl esters, although the free acids were highly active (ED_{50} lysozyme release of CHO-Met-Leu-Ile-OH = 4.6×10^{-11} M and CHO-Met-Leu-Phe-Phe-OH = 2.7×10^{-11} M). When 11 analogues were compared with respect to biological potency vs. inhibition of specific binding, some analogues were found to deviate from the close correlation noted in previous studies. Several analogues modified in the carboxyl area had higher ID_{50}'s in the binding assay than would be predicted based on their biological activity. These data as well as previously published data have been analyzed, and a hypothetical model of the chemotactic peptide receptor of rabbit neutrophil is presented.

The chemotactic response of the rabbit neutrophil to the synthetic tripeptide N^{α}-formylmethionylleucylphenylalanine (CHO-Met-Leu-Phe-OH) is well established to be mediated via interaction of the peptide with a specific receptor (Becker, 1979). This receptor resides in the plasma membrane and has a dissociation constant virtually identical with its biological potency (Sha'afi et al., 1978). In addition to stimulating chemotaxis, this peptide/receptor interaction initiates a number of other events in the neutrophil including lysosomal enzyme release (Showell et al., 1976), superoxide formation (Becker, 1979), and redistribution of cations, especially Ca^{2+} (Naccache et al., 1979). Without a doubt the chemically defined nature of the ligand and the ease with which the rabbit neutrophil can be obtained and studied have made this an important model of chemotaxis.

Even in the earliest studies of Schiffmann (Schiffmann et al., 1975), it was apparent that for chemotaxis, at least, there was high degree of selectivity among a variety of simple formylated amino acids and dipeptides. Further work extended these observations to the tri- and tetrapeptide level (Showell et al., 1976), and most recently the synthesis and evaluation of 30 tripeptides delineated the structural requirements of position 1 for optimal activity (Freer et al., 1980). The N^{α}-formyl group is essential for good activity since N-acetylation, removal of α-amino group, or replacement by an ethyl group results in a 1000–10 000-fold loss in activity. In addition, the sulfur-containing side chain of position 1 methionine produces optimum activity of the tripeptide. Moreover, extensions of position 1 side chains with linear aliphatic groups produced marked increases of activity up to norleucine (Nle). However, position 2 (Leu) and the carboxy terminus of the CHO-Met-Leu-Phe-OH molecule have not been studied in sufficient detail to define their role in biological activity. The lack of these data has precluded any speculation on the overall topography of the receptor and the kinds of potential interactions which might occur between ligand and receptor. To this end we have prepared 20 new analogues modified at these positions and, with these data in hand, have developed a working model of the chemotactic peptide receptor of the rabbit neutrophil.

Materials and Methods

Peptides were prepared by a rapid mixed anhydride method as described previously (Freer et al., 1980) with phenylalanine benzyl ester (H-Phe-OBzl) as the starting material. The benzylamide analogues were prepared in the same way by using benzylamine as the starting material. The α-amino group of the amino acids was protected as the *tert*-butyloxycarbonyl (*t*-Boc) derivative. The *t*-Boc amino acids were prepared in this laboratory by the method of Anderson & McGregor (1957). The formyl group was introduced through a conventional anhydride coupling as recently described by Day et al. (1980). Removal of the benzyl ester was affected by treatment with anhydrous HF (45 min at 4 °C with 1 mL of anisole/g of peptide). The synthesis strategy is given in Figure 1.

Peptides were purified by crystallization and were considered homogeneous when a single spot was observed (100-μg load) in three thin-layer chromatography (TLC) systems (Freer et al., 1980) using two different methods of detection (i.e., UV, ninhydrin, or *o*-tolidine) in each system. Each compound was also subjected to quantitative amino acid analysis (Beckman 119C amino acid analyzer), elemental analysis (Galbraith Laboratories, Knoxville, TN), and melting point determina-

† From the Department of Pharmacology, Medical College of Virginia, Richmond, Virginia 23298 (R.J.F., A.R.D., N.M., D.P., and A.W.), and the Department of Pathology, University of Connecticut Health Center, Farmington, Connecticut 06032 (H.J.S. and E.L.B.). *Received June 18, 1981.* This work was supported by National Institute of Dental Research Contract DE-62494, U.S. Public Health Service Grant AI-09648, and the Clinical Research Center for Periodontal Disease, School of Dentistry, Medical College of Virginia.

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Table I: Analytical Data

compd	structure[a]	thin-layer chromatography (R_f)[b] sys A	sys B	sys C	amino acid analysis (molar ratio)	mp (°C)	C, H, N calcd (obsd)
II	CHO-Met-Gly-Phe-OH	0.51	0.50	0.44	Phe:Gly:Met (1.00:1.06:0.93)	165–167	53.53, 6.08, 11.02 (53.77, 6.11, 10.89)
III	CHO-Met-Gly-Phe-OBzl	0.58	0.79	0.63	Phe:Gly:Met (0.99:1.00:1.01)	166–167	61.13, 6.19, 8.91 (61.20, 6.42, 8.90)
IV	CHO-Met-Ala-Phe-OH	0.57	0.61	0.45	Phe:Ala:Met (0.99:1.00:1.04)	203–205	54.67, 6.37, 10.62 (54.74, 6.50, 10.47)
V	CHO-Met-Ala-Phe-OBzl	0.63	0.85	0.67	Phe:Ala:Met (0.99:1.00:1.03)	164–165	61.83, 6.43, 8.65 (61.67, 6.40, 8.60)
VI	CHO-Met-Abu-Phe-OH	0.64	0.74	0.62	Phe:Abu:Met (1.00:1.06:1.04)	210–212	55.72, 6.65, 10.26 (55.53, 6.67, 10.08)
VII	CHO-Met-Abu-Phe-OBzl	0.67	0.88	0.77	Phe:Abu:Met (0.99:1.00:1.09)	170	62.63, 6.47, 8.47 (62.54, 6.47, 8.33)
VIII	CHO-Met-Nva-Phe-OH	0.70	0.75	0.74	Phe:Nva:Met* (1.00:0.91:1.05)	217	56.72, 6.90, 9.92 (56.90, 7.09, 9.64)
IX	CHO-Met-Nva-Phe-OBzl	0.74	0.90	0.81	Phe:Nva:Met* (1.05:0.94:1.00)	185	63.14, 6.86, 8.18 (63.22, 6.84, 8.09)
X	CHO-Met-Val-Phe-OH	0.67	0.78	0.45	Phe:Val:Met (1.00:1.03:0.92)	209–210 dec	56.72, 6.90, 9.92 (56.86, 7.04, 10.01)
XI	CHO-Met-Val-Phe-OBzl	0.71	0.95	0.88	Phe:Val:Met (1.00:0.96:1.09)	195–197	63.13, 6.87, 8.18 (62.28, 6.85, 7.94)
XII	CHO-Met-Ile-Phe-OH	0.70	0.80	0.67	Phe:Ile:Met (1.00:0.97:1.00)	220	57.65, 7.14, 9.60 (58.2, 7.48, 9.42)
XIII	CHO-Met-Ile-Phe-OBzl	0.74	0.90	0.82	Phe:Ile:Met (0.94:1.002:1.00)	191	63.73, 7.07, 7.96 (64.03, 7.08, 8.11)
XIV	CHO-Met-Cys(Me)-Phe-OH	0.61	0.73	0.43	Phe:Cys(Me):Met (1.00:1.090:1.04)	184	51.52, 6.16, 9.51 (51.32, 6.30, 9.37)
XV	CHO-Met-Cys(Me)-Phe-OBzl	0.80	0.92	0.78	Phe:Cys(Me):Met (1.06:0.93:1.00)	167	58.73, 6.25, 7.91 (58.95, 6.35, 7.91)
XVI	CHO-Met-Leu-Phe-OBzl	0.75	0.90	0.76	Phe:Leu:Met (1.00:1.05:0.99)	133–134	63.73, 7.03, 7.96 (63.80, 7.22, 7.97)
XVII	CHO-Met-Leu-Phe-NHBzl	0.77	0.88	0.68	Phe:Leu:Met (1.00:1.01:1.03)	228–230	63.85, 7.27, 10.63 (64.05, 7.32, 10.60)
XVIII	CHO-Met-Leu-Phe-Phe-OH	0.73	0.82	0.64	Phe:Leu:Met (2.00:1.02:1.00)	218–222	61.62, 6.89, 9.58 (61.63, 6.87, 9.40)
XIX	CHO-Met-Leu-Phe-Phe-OBzl	0.76	0.99	0.80	Phe:Leu:Met (2.04:0.97:1.00)	179–181	65.85, 6.87, 8.30 (66.05, 6.91, 8.25)
XX	CHO-Met-Leu-Phe-Ile-OH	0.70	0.88	0.76	Phe:Leu:Ile:Met (1.00:1.02:0.97:0.98)	230–244 dec	58.88, 7.68, 10.17 (59.06, 7.73, 9.92)
XXI	CHO-Met-Leu-Phe-Ile-OBzl	0.77	0.94	0.76	Phe:Leu:Ile:Met (1.00:1.06:0.98:1.04)	206–208.5	63.72, 7.55, 8.74 (63.58, 7.48, 8.62)

[a] Abu = α-aminobutyric acid, Nva = norvaline, and Cys(Me) = *S*-methylcysteine. Met*, analyzed as methionine sulfone. [b] Thin-layer chromatography on silica gel (Merck, G60 F254): sys A = 1-butanol–acetic acid–water (4:1:1), sys B = chloroform–methanol–acetic acid–water (60:30:4:1), and sys C = benzene–water–acetic acid (9:1:9).

tions. The analytical data are included in Table I.

Each compound was tested for its ability to induce the release of the lysosomal enzymes β-glucuronidase and lysozyme from cytochalasin B treated rabbit neutrophils. Because the release of the two systems paralleled each other, only the results for lysozyme are reported. Second, in all instances the cells were tested for LDH leakage. As before no increased leakage was obtained, and these data were not reported. Details of these assays have been published previously (Showell et al., 1976).

The radioreceptor assay was carried out essentially as described by Aswanikumar et al. (1977a). Each tube contained 70 000–90 000 cpm of CHO-Nle-Leu-*p*-[^{3}H]Phe-OH and increasing concentrations (10^{-10}–10^{-5} M) of unlabeled CHO-Nle-Leu-Phe-OH. The incubation was initiated by addition of 2 mL of cell suspension and carried out at 4 °C for 60 min. The incubation was terminated by rapid filtration (Hoeffer filtration apparatus) onto Whatman GF/F glass-fiber filters. Each tube was rapidly washed with 2 mL of ice-cold Grey's balance salt solution, and the filters were washed with 2 × 10 mL of ice-cold phosphate-buffered saline (pH 7.4, 0.02 M). The filters were then suspended in 10 mL of Aquasol (New England Nuclear), and scintillation spectrometry was carried out in a Beckman LS100C scintillation spectrometer. ID_{50} concentrations of peptides were determined from complete displacement curves (done in duplicate) which varied less than 10%.

Results

Previous work (Schiffmann et al., 1975; Showell et al., 1976) demonstrated that in CHO-Met-X dipeptides the nature of the second residue, as long as it was neutral, was not critical for chemotactic activity. It was, however, essential that some residue be present as evidenced by the fact that CHO-Met-OH itself was only weakly chemotactic. The peptides shown in Table II were prepared in order to determine, at the tripeptide level, the contribution of the position 2 residue to the biological activity. To this end a series of analogues were prepared in which the Leu side chain is replaced by H (i.e., Gly^2; compound II), CH_3 (Ala^2; compound IV), or linear aliphatic side chains of increasing length up to norvaline (compound VIII). The latter is the unbranched equivalent of Leu. As is clear, increasing side chain length increases biological activity (Table II). The increase is most notable between the Gly^2 (II) and Ala^2 (IV) analogues where a 50-fold change is seen. Overall, the increase in activity between Gly^2 (II) and Nva^2 (VIII) analogues is greater than 1000-fold. None of the latter aliphatics, however, were as active as the parent Leu^2 analogue

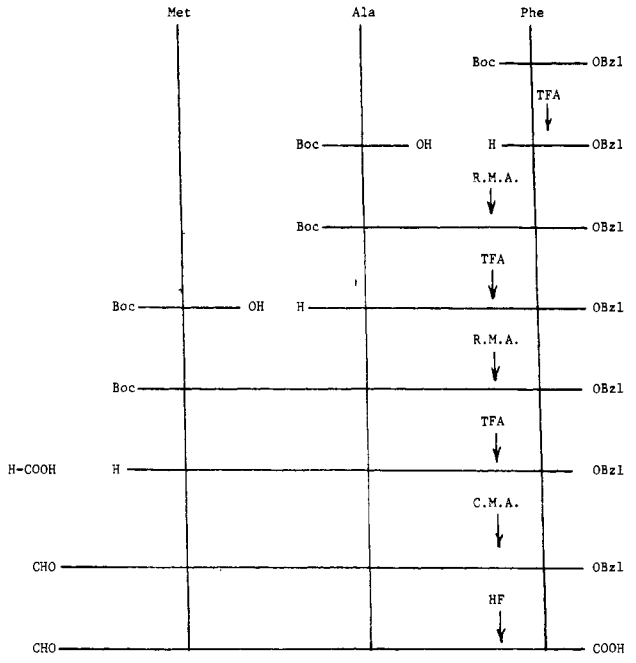

FIGURE 1: General synthetic scheme for formyl free acids and benzyl esters with Met-Ala-Phe as the example. TFA = trifluoroacetic acid, R.M.A. = rapid mixed anhydride method of Tilak (1970), C.M.A. = conventional mixed anhydride method of Day et al. (1980), and HF = anhydrous hydrofluoric acid. Details of synthesis are given in the text.

Table II: Biological Activity of Position 2 Analogues

compd[a]	structure[b]	lysozyme release, ED_{50} (M)[c]
I	CHO-Met-Leu-Phe-OH	$(5.5 \pm 2.2) \times 10^{-10}$
II	CHO-Met-Gly-Phe-OH	$(2.9 \pm 1.7) \times 10^{-6}$
IV	CHO-Met-Ala-Phe-OH	$(6.4 \pm 0.9) \times 10^{-8}$
VI	CHO-Met-Abu-Phe-OH	$(6.9 \pm 3.0) \times 10^{-9}$
VIII	CHO-Met-Nva-Phe-OH	$(1.7 \pm 1.0) \times 10^{-9}$
X	CHO-Met-Val-Phe-OH	$(1.2 \pm 1.6) \times 10^{-9}$
XII	CHO-Met-Ile-Phe-OH	$(1.6 \pm 0.7) \times 10^{-9}$
XIV	CHO-Met-Cys(Me)-Phe-OH	$(1.7 \pm 0.9) \times 10^{-9}$
XXII	CHO-Met-Met-Phe-OH[d]	$(1.9 \pm 0.4) \times 10^{-9}$

[a] Numerals refer to compounds as listed in Table I. [b] Abu = α-aminobutyric acid and Cys(Me) = *S*-methylcysteine. [c] ED_{50} = concentration of peptide required to produce 50% of the maximum effect as determined by the concentration–effect curve. Each value is the mean ± SEM of at least five determinations. [d] Value taken from Showell et al. (1976).

(I). Similarly other branched amino acids [i.e., Val (compound X) and Ile (compound XII)] produced highly active analogues. However, introduction of a sulfur-containing linear aliphatic derivative [i.e., Cys(Me); compound XIV] significantly reduces biological activity vs. the parent CHO-Met-Leu-Phe-OH (I), and it is, in fact, only equipotent with its non-sulfur-containing counterpart (Nva; compound VIII).

Effect of Carboxyl Modification on Biological Activity. Previous studies from this group (Freer et al., 1980) have produced somewhat anomalous results with respect to carboxyl modifications. On the one hand, the decarboxy analogue (CHO-Met-Leu-β-phenethylamine) showed a 60-fold reduction in activity compared to CHO-Met-Leu-Phe-OH. This might suggest a possible interaction of the negatively charged carboxyl with a positively charged area of the receptor. This explanation, however, is not compatible with the finding that CHO-Met-Leu-Phe-Lys-OH, an analogue containing a positively charged side chain, showed only a 4-fold reduction in the same comparison. If we assume that the conformation of the Lys tetrapeptide would permit the ε-amino group to approach the same area of the receptor normally interacting with the carboxylate anion, one might expect this analogue to be repelled and hence show a much greater reduction in biological potency. In an attempt to clarify the functional role of the carboxyl group, the benzyl ester analogue of CHO-Met-Leu-Phe-OH (CHO-Met-Leu-Phe-OBzl, Table III, compound XVI) was prepared and found to be a full order of magnitude more active than the free acid. Since the route chosen for synthesis allowed for isolation of the benzyl ester of each primary sequence, all of these were isolated and tested for biological potency (Table III). They too showed enhanced activity (24–533-fold) vs. their equivalent free acid. Similarly an analogue in which the benzyl group is present in amide linkage (i.e., CHO-Met-Leu-Phe-benzylamide, Table III, compound XVII) exhibits a further 2–3-fold increase in activity over CHO-Met-Leu-Phe-OBzl and a 30-fold increase over CHO-Met-Leu-Phe-OH itself.

Two tetrapeptides and their benzyl esters were also prepared to determine if the enhancing effect of the benzyl ester derivatives of the tripeptide might reflect a need for an additional amino acid at the carboxy terminus rather than a simple charge neutralization effect of increased resistance to proteolysis. The amino acids chosen were Phe and Ile, with the former being chosen to approximate the aromatic ring of the benzyl ester in the tripeptide series. As can be seen in Table III, both of the tetrapeptide free acids [i.e., CHO-Met-Leu-

Table III: Biological Activity of Position 3 Carboxyl-Modified Analogues

compd[a]	structure[b]	lysozyme release, ED_{50} (M)[c]	potency ratio[d] (acid vs. carboxyl modified)
I	CHO-Met-Leu-Phe-OH	$(5.5 \pm 2.2) \times 10^{-10}$	
III	CHO-Met-Gly-Phe-OBzl	$(6.7 \pm 3.3) \times 10^{-8}$	43.3
V	CHO-Met-Ala-Phe-OBzl	$(1.2 \pm 0.5) \times 10^{-10}$	533.3
VII	CHO-Met-Abu-Phe-OBzl	$(5.5 \pm 3.2) \times 10^{-11}$	125.4
IX	CHO-Met-Nva-Phe-OBzl	$(5.2 \pm 1.8) \times 10^{-11}$	32.7
XI	CHO-Met-Val-Phe-OBzl	$(4.5 \pm 1.4) \times 10^{-11}$	26.7
XIII	CHO-Met-Ile-Phe-OBzl	$(6.5 \pm 2.1) \times 10^{-11}$	24.6
XV	CHO-Met-Cys(Me)-Phe-OBzl	$(9.6 \pm 6.2) \times 10^{-11}$	17.7
XVI	CHO-Met-Leu-Phe-OBzl	$(4.6 \pm 1.2) \times 10^{-11}$	11.9
XVII	CHO-Met-Leu-Phe-NHBzl	$(1.8 \pm 0.6) \times 10^{-11}$	30.6
XVIII	CHO-Met-Leu-Phe-Phe-OH	$(2.7 \pm 0.9) \times 10^{-11}$	
XIX	CHO-Met-Leu-Phe-Phe-OBzl	$(9.3 \pm 1.9) \times 10^{-11}$	0.29
XX	CHO-Met-Leu-Phe-Ile-OH	$(4.6 \pm 0.6) \times 10^{-11}$	
XXI	CHO-Met-Leu-Phe-Ile-OBzl	$(8.1 \pm 3.6) \times 10^{-11}$	0.57
XXIII	CHO-Nle-Leu-Tyr-OH	$(6.5 \pm 0.9) \times 10^{-8}$	

[a] Numerals refer to compounds as listed in Table I. [b] Abu = α-aminobutyric acid, Nva = norvaline, and Cys(Me) = *S*-methylcysteine. [c] ED_{50} = concentration of peptide required to produce 50% of the maximum effect as determined by the concentration–effect curve. Each value is the mean ± SEM of at least five determinations. [d] Potency ratio is defined as ED_{50} of free acid/ED_{50} of carboxyl modified.

Table IV: Effect of Selected Peptides on Specific Binding[a]

compd[b]	structure[c]	binding inhibition, ID_{50} (M)
I	CHO-Met-Leu-Phe-OH[d]	3.3×10^{-10}
II	CHO-Met-Gly-Phe-OH	$(4.1 \pm 1.1) \times 10^{-7}$
III	CHO-Met-Gly-Phe-OBzl	$(1.1 \pm 0.17) \times 10^{-8}$
VIII	CHO-Met-Nva-Phe-OH	$(2.8 \pm 1.2) \times 10^{-9}$
XII	CHO-Met-Ile-Phe-OH	$(5.7 \pm 1.2) \times 10^{-9}$
XIII	CHO-Met-Ile-Phe-OBzl	$(2.8 \pm 0.7) \times 10^{-9}$
XVII	CHO-Met-Leu-Phe-NHBzl	$(9.3 \pm 2.2) \times 10^{-10}$
XVIII	CHO-Met-Leu-Phe-Phe-OH	$(3.7 \pm 1.8) \times 10^{-9}$
XXII	CHO-Nle-Leu-Phe-OH[d]	3.4×10^{-9}

[a] CHO-Nle-Leu-*p*-[^{3}H]Phe-OH was incubated with six concentrations of nonradioactive peptides and the concentration required to displace 50% of the specifically bound peptide estimated from the plots of binding inhibition vs. concentration. Each value is the mean ± SEM of five determinations done in duplicate. [b] Numerals refer to compounds as listed in Table I. [c] Nva = norvaline and Nle = norleucine. [d] Values taken from Freer et al. (1980).

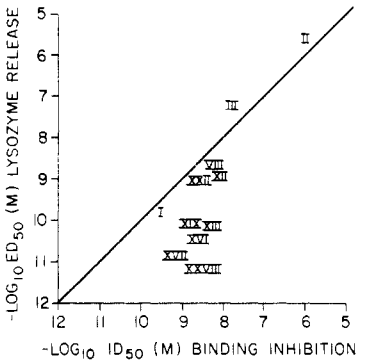

FIGURE 2: Correlation diagram comparing ED_{50} for lysozyme release and ID_{50} for binding inhibition of 11 peptides. II, CHO-Met-Gly-Phe-OH; III, CHO-Met-Gly-Phe-OBzl; VIII, CHO-Met-Nva-Phe-OH; XXII, CHO-Nle-Leu-Phe-OH; XII, CHO-Met-Ile-Phe-OH; I, CHO-Met-Leu-Phe-OH; XIX, CHO-Met-Leu-Phe-Phe-OBzl; XIII, CHO-Met-Ile-Phe-OBzl; XVI, CHO-Met-Phe-OBzl; XVII, CHO-Met-Leu-Phe-Phe-OH. Biological data are the average of at least five determinations. Binding data are the averages of at least six determinations done in duplicate. Data for compounds XXII and I were taken from Freer et al. (1980).

Phe-Phe-OH (compound XVIII) and CHO-Met-Leu-Phe-Ile-OH (compound XX)] were highly active. Respectively they were 20- and 10-fold more active than CHO-Met-Leu-Phe-OH (I) and equipotent with CHO-Met-Leu-Phe-OBzl (XVI). In contrast to what was seen with tripeptides, the tetrapeptide benzyl esters were less effective than their free acid counterparts (Table III).

Correlation of Binding Inhibition and Biological Response. In all instances where it has been tested, the ability of a given analogue to produce lysosomal enzyme release from rabbit neutrophils was directly proportional to its ability to bind to the specific receptor in these cells (Aswanikumar et al., 1977a,b; Day et al., 1977; Freer et al., 1980). So that the continuing validity of this relationship could be tested, binding inhibition curves were determined for a selected group of peptides. The binding inhibition data are given in Table IV, and the correlation diagram for lysozyme release vs. binding inhibition is given in Figure 2. It is evident, especially in the correlation diagram, that the peptides fall into two categories. The first are those compounds (compounds I–III, VIII, XII, and XXII, Figure 2) with relatively low to moderate biological activity which show a strong correlation between biological response and binding inhibition. The second group of compounds which show high biological activity (ED_{50}'s $< 10^{-10}$ M) (compounds XIII, XVI, XVII, XIX, and XXVIII, Figure

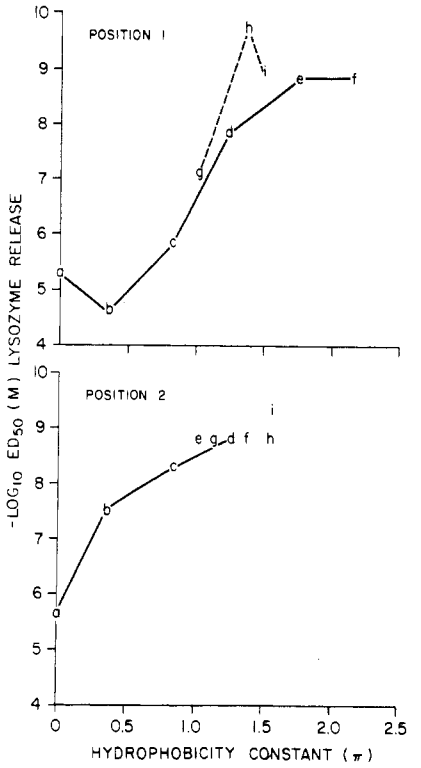

FIGURE 3: Comparison of lysozyme releasing activity and hydrophobic nature of various side-chain modifications. The hydrophobicity constant (π) was calculated for each side chain as per Pliska & Fauchère (1979). CHO-Met-Leu-Phe-OH is the reference compound and the π represents only the contribution of the side chain of the modified residue. (Upper) Comparison of lysozyme releasing activity vs. π for position 1 analogues of CHO-Met-Leu-Phe-OH. a, Gly[1]; b, Ala[1]; d, Nva[1]; e, Nle[1]; f, aminoheptanoyl[1]; g, [Cys(Me)][1]; h, Met[1]; i, ethionyl[1]. Biological data are taken from Freer et al. (1980). (Lower) Comparison of lysozyme releasing activity vs. π for position 2 analogues of CHO-Met-Leu-Phe-OH. a, Gly[2]; b, Ala[2]; c, Abu[2]; d, Nva[2]; e, [Cys(Me)][2]; f, Met[2]; g, Val[2]; h, Ile[2]; i, Leu[2]. Biological data are the average of at least five determinations.

2) deviates very significantly from this relationship and displays much higher ID_{50}'s for inhibition (lower apparent binding; than one would predict on the basis of the ED_{50} for lysozyme release. It is worth noting that all of these are not only highly active compounds, but they also have in common that they are modified in the region of the Phe[3] carboxyl.

Correlation of Hydrophobicity and Biological Potency of Selected Analogues. It is clear that many of the substitutions reported previously for Met in position 1 (Freer et al., 1980) as well as those reported herein for position 2 (Table II) would have marked effects on the hydrophobic nature of the peptide analogue. Since in both sets of analogues single side-chain modifications were introduced, it is possible to calculate, using the method of Pliska & Fauchère (1979), the contribution of each substitution of the overall hydrophobicity of the analogue. The data are expressed as a hydrophobicity constant (π) and in Figure 3 are plotted vs. the ED_{50} for lysozyme release. For position 1 (Met), nine analogues are shown (Figure 3, upper), and it is clear that for the linear aliphatic (b–e), the Cys(Me) (g) and the ethionyl (i) analogues there is an extremely good correlation between the calculated contribution of that side chain to hydrophobicity and the potency of the peptide. The Gly (a), Met (h), and α-aminoheptanoyl (Hep, f) analogues do not, however, conform to this general relationship.

Similarly for position 2 (Leu) there is a reasonable correlation for most analogues (Figure 3, lower). The most notable discrepancies here are compounds d–h of Figure 3 which are equipotent but have markedly different π values and between

the Ile (h) and Leu (i) analogues for which the calculated π constant is identical but biological activity differs by nearly 3-fold. The major difference between the latter is that Ile is β branched while Leu is γ branched.

Discussion

Exposure of rabbit neutrophils to the formylated tripeptide CHO-Met-Leu-OH is known to initiate many physiological and biochemical functions of the cell (Becker, 1979). It does so by interaction with a specific receptor as evidenced by direct binding studies (Answanikumar et al., 1977a; Day et al., 1977; Freer et al., 1980). Similar receptors have also been observed in human neutrophils (Williams et al., 1977), guinea pig macrophages (Synderman & Fudman, 1980), human mononuclear cells (Ho et al., 1978), the human macrophage derived U-937 cell line (Pike et al., 1980), and the promyelocytic leukemia HL-60 cell (Neidel et al., 1980).

Previous studies have demonstrated that the receptor on rabbit neutrophils is highly selective with respect to binding of ligand and the binding with the apparent exception of some of the analogues reported herein exactly reflects the biological activity. Several years ago Showell et al. (1976) outlined the gross structure–activity requirements. The result was identification of the highly active peptide CHO-Met-Leu-Phe-OH (ED_{50} for chemotaxis of 7×10^{-11} M) which has become the prototype of this family of peptides. When CHO-Met-Leu-Phe-OH was taken as the prototype compound, subsequent studies (Freer et al., 1980) probed, via more detailed modifications, the role of the formyl group and the Met in position 1. The data obtained with these analogues clearly showed that the formyl group was essential and that the presence of Met in the first position resulted in the most potent analogue. With the analogues reported in this paper, we have focused our attention on two other areas of the molecule. These are the position 2 (Leu) and the carboxyl of the position 3 (Phe). Again an attempt has been made to gain some insight into the fine structure of a receptor on the basis of a series of selected minor modifications of the groups in question.

As with a similar group of position 1 analogues (Freer et al., 1980), replacement of the Leu in position 2 by a series of linear aliphatics of increasing chain lengths results in compounds with increased biological activity. Most remarkable is the 50-fold increase in activity on going from Gly to Ala (Table I). This is in contrast to the 5-fold drop in activity with the same change in position 1 (Freer et al., 1980). An additional methylene group (Abu) improves activity by another log, but the Nva analogue is only slightly more active than the Abu. It is interesting that comparison of biological activity vs. chain length (Table II) and biological activity vs. hydrophobicity (Figure 3) reveals a virtually identical relationship. One might speculate therefore that for this group of analogues at least, the hydrophobicity of the substituent at position 2 is a major determinant of biological potency. That this is not the only determinant is clear when one compares the peptide with branched aliphatics in position 2, Val (X) and Ile (XII), or sulfur-containing linear side chains, Cys(Me) (XIV) and Met (XXII). These four analogues have quite different hydrophobicity constants (1.0–1.6) but are equipotent (ED_{50}'s between 1.2×10^{-9} and 1.9×10^{-9} M) as in the Nva analogue (VIII). Also noteworthy is the 3-fold increase in biological activity seen with the Leu (I) vs. Ile (XII) analogues, even though there is no difference in calculated hydrophobicity between these closely related side chains. It therefore seems that hydrophobicity, although a major factor, is not the only property of a position 2 residue which influences biological activity. Clearly, branched aliphatics are readily accepted by the receptor but branching at the γ carbon (Leu) seems to be preferred. However, the possibility that modifications at this position can influence conformation cannot be ruled out.

Previous studies (Freer et al., 1980) have produced somewhat conflicting results concerning the C terminus of CHO-Met-Leu-Phe-OH. While the decarboxy analogue (CHO-Met-Leu-β-phenethylamine) was quite inactive (ED_{50} lysozyme release = 2×10^{-8} M), the tetrapeptide CHO-Met-Leu-Phe-Lys-OH retained good activity ($ED_{50} = 1.7 \times 10^{-9}$ M). Although an obvious interpretation of the former result is that the carboxylate anion might interact with an area of relative positivity on the receptor, this was difficult to reconcile with the relatively high activity of CHO-Met-Leu-Phe-Lys-OH. This analogue with its positively charged side chain, if brought into juxtaposition to such an area, would be expected to repel and hence cause a further reduction in activity. The analogues reported in this paper may resolve this apparent contradiction if one considers that the critical area is the Phe carbonyl oxygen rather than the carboxylate anion. This hypothesis is compatible with the consistently enhanced activity of the benzyl esters and the benzylamide which clearly demonstrates that the carboxylate anion is not required.

In addition to providing information about the carboxyl function itself, the esters and the benzylamide suggest that this area of receptor has sufficient "room" to accommodate an additional residue. In retrospect, the surprisingly good activity of the CHO-Met-Leu-Phe-Lys-OH referred to earlier and the finding that CHO-$(Met)_4$-OH (Showell et al., 1976) was highly active also hinted at the same thing. The approximately 100-fold increase in activity of the two tetrapeptides CHO-Met-Leu-Phe-Phe-OH ($ED_{50} = 2.7 \times 10^{-11}$ M) and CHO-Met-Leu-Phe-Ile-OH ($ED_{50} = 4.6 \times 10^{-11}$ M) has confirmed these suspicions. Interestingly the benzyl esters of these tetrapeptides did not show enhanced activity but rather a slightly decreased potency. This suggests that the primary effect of the benzyl ester group in the tripeptide series is to mimic the presence of an additional hydrophilic amino acid residue and, once the additional residue is present, benzylation causes no further increase.

A final point worthy of note is the observation that carboxyl-modified analogues which are active in the 10^{-11} M range do not show a good correlation between biological activity and binding inhibition. At present we have no well-founded explanation for this discrepancy. One might speculate, however, that (a) there is a second, very high-affinity binding site which is not detectable by using our standard binding conditions or (b) the efficacy of these analogues is such that only a small portion of the total receptor population is necessary for full biological effectiveness. Synthesis of ligands related to the carboxyl-modified analogues capable of being labeled with high specific activity is currently under way and should allow us to address these possibilities.

The structure–activity data presented herein as well as in previous publications from our laboratories (Showell et al., 1976; Freer et al., 1980) and others (Schiffmann et al., 1975; Aswanikumar et al., 1977a; Neidel et al., 1979, 1980; Wilkinson, 1979) are, we believe, now sufficient to allow speculation on receptor binding mechanisms and receptor topography. Our working model is shown in Figure 4. In this model we tentatively define a receptor conformation and five critical areas of interaction between the peptide and the receptor in rabbit neutrophils.

We propose that the peptide exists on the receptor in a β-pleated sheet (antiparallel) conformation. There are a number of pieces of evidence which support this notion.

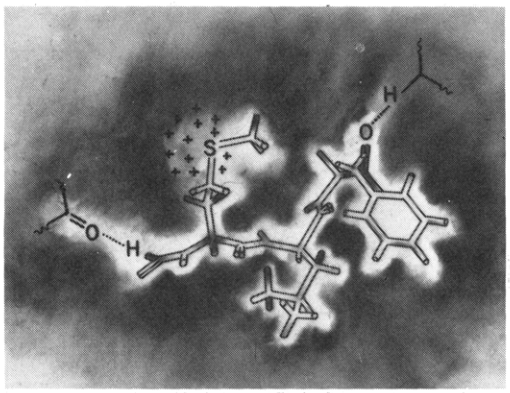

FIGURE 4: Hypothetical interaction of CHO-Met-Leu-Phe-OH with the receptor in rabbit neutrophils. Details are given in the text.

Nuclear magnetic resonance (NMR) spectroscopy showed that in solution the peptide CHO-Met-Leu-Phe-OH has an antiparallel β-pleated sheet conformation (Becker et al., 1979). Furthermore, although the side chains were relatively free to move in space, the peptide backbone was quite rigid. This might indicate that an induced conformational change of the molecule on interaction with the receptor could be energetically unfavorable. As clear-cut as these results are, it should be pointed out that they refer to the conformation of a 1 mM solution in Me_2SO. The fact that CHO-Met-Leu-Phe exhibits such a stable conformation suggests that this could be the conformation present at much lower concentrations in aqueous medium or on the receptor where the peptide exerts biological activity. Consistent with this idea is the observation that each of the amino acids of this tripeptide is found with a high degree of frequency in peptides and proteins exhibiting a β-pleated sheet conformation (Lifson & Sander, 1980).

Quite indirect, but still supportive, is the finding that antibody directed against CHO-Met-Leu-Phe-OH is apparently homogeneous and shows virtually the same structure–activity requirements as the neutrophil receptor (W. A. Marasco et al., unpublished results). This suggests that the molecule is presented to the antigen recognizing cells in much the same conformation present on the receptor as could be expected if the molecule has a rigid conformation. If so, then the remarkable similarity between the antibody binding site and the receptor would be predicted. Taken together then the data are quite consistent with the notion that the peptide exists in an extended β-pleated sheet conformation on the receptor.

Allowing this kind of extended conformation and the apparent exquisite spatial sensitivity of the receptor system, one might predict multiple points of interaction between peptide and receptor. If true, this should be reflected as major changes in biological activity following chemical modification at a number of sites in the molecule. The structure–activity data bear out this prediction, and we therefore are proposing five critical areas of interaction. These are in a vicinity of (1) the formyl group, (2) the methionine side chain, (3) the leucine side chain, (4) the phenylalanine side chain, and (5) the phenylalanine carbonyl function and are as follows:

Specifics. (*1*) *Formyl Group.* The requirement for the formyl group is for both binding (Freer et al., 1980) and biological activity (Showell et al., 1976). It appears to be unique in that even an acetyl group is not an acceptable substitute. One major difference between the formyl and acetyl moieties is that the formyl group is able to H bond (albeit weak) while the acetyl group cannot. We therefore tentatively suggest that the formyl group may participate in H bonding to a H-bond acceptor in this area of the receptor. This suggestion is depicted in Figure 4.

(*2*) *Methionine Side Chain.* On the basis of our previous studies (Freer et al., 1980) (see also Figure 3), we propose that the methionine side chain of position 1 occupies a hydrophobic pocket in the receptor. This pocket is of limited depth since derivatives with side chain lengths longer than four carbons show no further increase in activity. Similarly this pocket may be of limited capacity in the area of the δ carbon since branching at that point is not well tolerated [e.g., CHO-Leu-Leu-Phe, $ED_{50} = 5 \times 10^{-8}$ M; CHO-Nle-Leu-Phe, $ED_{50} = 1.5 \times 10^{-9}$ M; CHO-Val-Leu-Phe-OH, $ED_{50} = 1.3 \times 10^{-8}$ M; CHO-Nva-Leu-Phe, $ED_{50} = 1.3 \times 10^{-8}$ M (Freer et al., 1980)]. In contrast, there is no restriction in the area of the β carbon since residues branched at this point still produced highly potent analogues [e.g., CHO-Ile-Leu-Phe, $ED_{50} = 1.9 \times 10^{-9}$ M; CHO-Nle-Leu-Phe, $ED_{50} = 1.5 \times 10^{-9}$ M (Freer et al., 1980)]. Finally, we propose a discrete area of positive charge around the relatively electron-rich sulfur atom. Interaction of these two areas is, we believe, responsible for the enhanced activity of CHO-Met-Leu-Phe-OH over its non-sulfur-containing counterpart, CHO-Nle-Leu-Phe-OH. The precise positioning of the sulfur atom is of paramount importance, as evidenced by the fact that incorporation of a *S*-methylcysteine residue with its side chain one carbon shorter than Met produces an analogue with less activity than either CHO-Met-Leu-Phe-OH or the non-sulfur-containing CHO-Nva-Leu-Phe-OH.

(*3*) *Leucine Side Chain.* The large increases in biological activity (i.e., 1000-fold) seen on going from the Gly^2 analogue to the longer aliphatic side chains suggest that this side chain also interacts with a hydrophobic area of the receptor (see also Figure 4). The capacity of the area of the receptor at the β carbon would appear less restricted than for the methionine side chain, with there being no difference in activity between the peptides containing Nva, Val, or Ile. However, the branching at the γ carbon may be of importance.

(*4*) *Phenylalanine Side Chain.* The phenylalanine side chain also would appear to reside in a hydrophilic area. This is evidenced by the fact that charged residues at this position are not compatible with good activity (Showell et al., 1976) whereas neutral residues are acceptable. Although it is true that the Phe residue is the most desirable at this position, other properties in addition are important in this area of the receptor. The hydrophobic pocket may be of limited depth since para substitutions, i.e., –Cl (Day et al., 1977) or –OH on the phenylalanine ring (Table III), markedly reduce biological activity.

(*5*) *Phenylalanine Carbonyl.* Of all the carboxyl-modified analogues tested, only those which contain the C=O function of the Phe display good biological activity. For example, the analogue in which the carboxyl group was eliminated (i.e., CHO-Mey-Leu-β-phenethylamine) was only slightly active whereas the benzylamide and the benzyl ester of CHO-Met-Leu-Phe-OH are even more potent than the parent compound. Furthermore, CHO-Met-Leu-Phe-Lys-OH is surprisingly active (~15% of CHO-Met-Met-Leu-Phe-OH), considering the presence of the charged side chain. We propose therefore a critical interaction of the Phe C=O with the receptor, possibly via hydrogen bonding.

We emphasize that this is a *working model* and is certainly much oversimplified, as it does not take into account the location of the receptor in the membrane. For example, our observation that alcohols expose more receptors (Liao & Freer, 1980) and the observation of Schiffmann (Schiffmann et al., 1980) that the receptor is resistant to a number of proteases

may mean the receptor exists in a relatively inaccessible area of the membrane. Nonetheless, this model should provide a useful basis for future studies.

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Accumulation of 6-Deoxyerythronolide B in a Normal Strain of *Streptomyces erythreus* and Hydroxylation at Carbon 6 of the Erythranolide Ring System by a Soluble Noninduced Cell-Free Enzyme System†

John W. Corcoran* and Auste M. Vygantas

ABSTRACT: Erythronolide B, a presumed intermediate in the biosynthesis of the erythromycins, has been shown to be formed from 6-deoxyerythronolide B by hydroxylation at C-6. The substrate, a metabolite of a blocked mutant of *Streptomyces erythreus* and postulated to be an intermediate in the biosynthesis of the erythromycins, is found also in wild-type cultures of *S. erythreus* CA340 either normally or in increased amount when an inhibitor of NADPH function is present. The hydroxylation of 6-deoxyerythronolide B is catalyzed by a stable and soluble cell-free enzyme preparation obtained from noninduced *S. erythreus* CA340, and the maximal specific activity of the hydroxylase system is found with the protein fraction precipitating between 50% and 90% of saturation with ammonium sulfate. The hydroxylase activity correlates well with the specific content of a cytochrome P-450 moiety present in the system and is inhibited by anaerobiosis and carbon monoxide.

The macrocyclic lactones (Figure 1) present in the known erythromycins A, B, C, and D each possess hydroxyl group functions that cannot be derived directly from the substrates believed used for their biosynthesis. The lactones are presumably formed from a "starter" molecule of activated propionate and six chain-extending units of activated 2-methylmalonate (Masamune et al., 1977; Corcoran, 1981). The mechanism proposed is that of a general fatty acid synthase (GFAS) in which chain growth does not depend on prior or "lock-step" removal of the β-oxo function introduced at each stage of elongation. The assembly of the "primative" and as yet unknown 14-member macrolide ring, with all substrate-derived oxo functions still present, may take place on a polyenzyme template since no partial structures or prelactonic intermediates have been detected. The simplest structure yet

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