sequences with ribonucleases. The majority of poly(A) tracts in ct-mRNAs have been found to range from 50 to 150 adenosine residues.

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Isolation and Partial Characterization of Membrane Protein Constituents of Human Neutrophil Receptors for Chemotactic Formylmethionyl Peptides†

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ABSTRACT: Plasma membranes of human neutrophils were solubilized in buffer containing a nonionic detergent and applied to a formylmethionylleucylphenylalanine (fMet-Leu-Phe)–Sepharose column that was washed and eluted with the chemotactic peptide fMet-Leu-Phe. Analysis of the eluate by filtration on Bio-Gel P150 in sodium dodecyl sulfate ($NaDodSO_4$) buffer and by $NaDodSO_4$–polyacrylamide gel electrophoresis revealed three predominant membrane proteins of approximate molecular weight 94 000 (MP-1), 68 000 (MP-2), and 40 000 (MP-3), of which MP-2 accounted for 74–93% of the total protein. Purified MP-1 and MP-2 contained an above average content of hydrophobic amino acids, while MP-2 and MP-3 had an above average content of acid and/or amide amino acids and a below average content of basic amino acids. MP-2 and MP-3, but not MP-1, bound [^{3}H]fMet-Leu-Phe in equilibrium dialysis chambers. Both MP-2 and MP-3 exhibited high-affinity sites with a valence of 0.2–0.3 and mean K_A values of 9×10^8 and 2×10^7 M^{-1}, respectively, and low-affinity sites with a valence of 0.3–0.5 and mean K_A values of 3×10^7 and 2×10^6 M^{-1} ($n = 3$). The specificity of the binding of fMet-Leu-Phe was suggested by the failure of MP-2 and MP-3 to bind lipid chemotactic factors and to adhere to a Sepharose column to which had been coupled chemotactic fragments of the fifth component of complement. A series of synthetic formylmethionyl peptides exhibited the same rank order of potency as inhibitors of the binding of [^{3}H]fMet-Leu-Phe by MP-2 and as stimuli of neutrophil chemotaxis. Membrane proteins isolated by fMet-Leu-Phe–Sepharose affinity chromatography may represent constituents of specific human neutrophil receptors for chemotactic peptides.

The chemotactic migration of leukocytes in response to a concentration gradient of a formylmethionyl peptide is initiated by the binding of the peptide stimulus to plasma membrane receptors on rabbit and human polymorphonuclear (PMN)[1] leukocytes and human monocytes (Aswanikumar et al., 1977; Pike et al., 1980; Williams et al., 1977). The relationship of the induction of chemotaxis to the specificity of the leukocyte receptors for formylmethionyl peptides initially was suggested by the close correlation between binding affinity and chemotactic potency for a variety of structurally distinct formylmethionyl peptides (Showell et al., 1976). Chemotactically inactive analogues inhibit the leukocyte chemotactic response to formylmethionyl peptides but not to the chemotactic peptide fragment of the fifth component of complement, and the in-

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[1] Abbreviations used: fMet-Leu-Phe, formylmethionylleucylphenylalanine; MP, membrane protein; PMN, polymorphonuclear; DNase, deoxyribonuclease; PMSF, phenylmethanesulfonyl fluoride; TLCK, *N*-tosyl-L-lysyl chloromethyl ketone; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride; NP40, Nonidet P40; $NaDodSO_4$, sodium dodecyl sulfate; 5-HETE, 5-hydroxyeicosatetraenoic acid; LTB, leukotriene B, 5,12-dihydroxyeicosatetraenoic acid; C5fr, chemotactic fragments of the fifth component of complement; Me_2SO, dimethyl sulfoxide; EDTA, ethylenediaminetetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; Bicine, *N,N*-bis(2-hydroxyethyl)glycine.

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hibitory potency of the analogues is determined directly by their affinity for the receptors (O'Flaherty et al., 1978). The inhibition of chemotaxis to formylmethionyl peptides by covalent chemical modification of human neutrophil receptors with impermeant reagents (Goetzl & Hoe, 1979) and the results of microscopic analyses of human neutrophil receptors by the introduction of fluorescent derivatives of formylmethionyl peptides (Niedel et al., 1979) have confirmed the plasma membrane localization of the receptors. Affinity labeling of the receptors on human neutrophils with several covalently reactive derivatives of a chemotactic formylnorleucyl peptide led to the identification of a membrane protein of molecular weight 55000–70000, the selective labeling of which was suppressed by the native peptide at concentrations that permitted protection of the receptor (Niedel et al., 1980). The application of formylmethionyl peptide–Sepharose affinity chromatography now has permitted the isolation of soluble membrane protein constituents of specific neutrophil receptors, which retain binding activity for the formylmethionyl peptides.

Materials and Methods

Hanks' balanced salt solution with or without calcium and magnesium (Microbiological Associates, Bethesda, MD), ovalbumin that had been recrystallized 5 times (Miles Laboratories, Inc., Elkhart, IN), macromolecular dextran, Ficoll-Hypaque, and AH-Sepharose 4B (Pharmacia Fine Chemicals, Inc., Piscataway, NJ), deoxyribonuclease (DNase) and calcium ionophore A23187 (Calbiochem-Behring Corp., La Jolla, CA), synthetic *N*-formylmethionylleucylphenylalanine (fMet-Leu-Phe), zymosan, reagents for the quantitation of lactic acid dehydrogenase and β-glucuronidase, diisopropyl fluorophosphate, phenylmethane sulfonyl fluoride (PMSF), and *N*-tosyl-L-lysyl chloromethyl ketone (TLCK) (Sigma Chemical Co., St Louis, MO), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) and 3 M mercaptoethanesulfonic acid (Pierce Chemical Co., Rockford, IL), Nonidet P40 (NP40) (Gallard-Schlesinger Chemical Manufacturing Corp., Carle Place, NY), SM-2 Bio-Beads, electrophoresis grade acrylamide, *N,N'*-methylenebis(acrylamide), β-mercaptoethanol, Coomassie brilliant blue, sodium dodecyl sulfate ($NaDodSO_4$), bromophenol blue, protein molecular weight standards, and Bio-Gel P150 (Bio-Rad Laboratories, Richmond, CA), and [^{3}H]arachidonic acid and [^{3}H]formylmethionylleucylphenylalanine (46.4 Ci/mmol) (New England Nuclear Corp., Boston, MA) were obtained as noted. 5-Hydroxy[^{3}H]eicosatetraenoic acid (5-[^{3}H]HETE, 6.8 Ci/mmol) and 5,12-dihydroxy[^{3}H]eicosatetraenoic acid ([^{3}H]leukotriene B, [^{3}H]LTB, 5.1 Ci/mmol) were extracted from neutrophils that had been preincubated with [^{3}H]arachidonic acid and challenged with calcium ionophore A23187; the products were purified by sequential silicic acid and high-performance liquid chromotography and quantified as described (Goetzl & Pickett, 1980, 1981). Chemotactic fragments of the fifth component of complement (C5fr) were purified from zymosan-activated human serum (Goetzl & Hoe, 1979). The chemotactic peptides fMet-Phe-Leu, fMet-Leu-Arg, and fMet-Leu-Glu were synthesized and purified by Dr. Richard J. Freer (Showell et al., 1976) (Medical College of Virginia, Richmond, VA).

Preparation of Human Neutrophil Membranes. Mixed leukocytes were harvested from citrate-anticoagulated venous blood of normal subjects as described (Goetzl & Austen, 1972; Goetzl & Hoe, 1979) and were subjected to centrifugation on Ficoll-Hypaque cushions to obtain neutrophils of over 96% purity (Boyum, 1968). Purified neutrophils [$(1–3) \times 10^9$] were washed twice and resuspended at a concentration of 1×10^7/mL in calcium-free Hanks' solution containing 2.5 mM $MgCl_2$, 0.1 g of ovalbumin/100 mL, 100 units of DNase/mL, 10^{-4} M PMSF, and 10^{-3} M TLCK (pH 7.4). Half of each of two preparations was preincubated for 30 min at 37 °C in calcium- and magnesium-free Hanks' solution containing 10^{-3} M diisopropyl fluorophosphate and washed twice in calcium- and magnesium-free Hanks' solution prior to disruption by nitrogen cavitation. The suspension of neutrophils was subjected to 400 psi of nitrogen in a pressure chamber (Parr Instrument Co., Moline, IL) for 20 min at 4 °C and was collected by discharge of 10-mL portions into 14-mL plastic test tubes containing 1.1 mL of 25 mM EDTA in 0.5 M $NaHCO_3$ (pH 8.0). The suspension was centrifuged at 400*g* for 20 min at 4 °C to remove unbroken neutrophils and nuclei, and the resultant supernate was centrifuged at 10000*g* for 40 min at 4 °C to sediment the lysosomal granules. The membranes were pelleted by centrifugation of the supernate at 225000*g* for 30 min at 4 °C and were washed sequentially in 10 mM Hepes (pH 7.5) and 1 mM Hepes (pH 7.5) by resuspension and recentrifugation (Klempner et al., 1980).

The extent of disruption of the neutrophils by nitrogen cavitation was 92–98% for five consecutive preparations, as assessed by the ratio of lactic acid dehydrogenase activity in the 400*g* supernate to the mean of that in duplicate sonicates of a concomitant percentage of the initial suspension of neutrophils. For the same preparations, assays of sonicates of portions of the membranes suspended in 1 mM Hepes indicated a recovery of 59–73% of the plasma membrane marker alkaline *p*-nitrophenylphosphatase and contamination with only 1–6% of the lysosomal marker β-glucuronidase (Bretz & Baggiolini, 1974).

fMet-Leu-Phe–Sepharose Affinity Chromatography of Neutrophil Membrane Proteins. AH-Sepharose 4B (12 mL) that had been washed sequentially with 600 mL of 0.5 M NaCl and 300 mL of distilled water was incubated for 6 h at 37 °C with 36 μmol of fMet-Leu-Phe dissolved in 120 μL of dimethyl sulfoxide and 120 mg of EDC in 30 mL of distilled water (pH 4.5). A second equal amount of EDC was added, and the mixture was incubated at room temperature for 10 h. The fMet-Leu-Phe–Sepharose was washed with 200 mL of 0.5 M NaCl and 100 mL of distilled water and stored at 4 °C under a N_2 atmosphere for up to 1 week. The extent of derivatization was 2.6–2.8 μmol/mL of Sepharose for three consecutive preparations based on the binding of [^{3}H]fMet-Leu-Phe.

Membranes prepared from $(1–3) \times 10^9$ neutrophils were suspended in 1.5 mL of 0.1 M NaCl–0.02 M Hepes (pH 7.2) containing 0.3 mL of NP40/100 mL (chromatography buffer) and sonicated at 150 W for 2 min at 4 °C by utilizing a pulsed mode (Model 350, Branson Sonic Power Co., Danbury, CT). The membrane sonicate was applied to an fMet-Leu-Phe–Sepharose column of 1.5-mL bed volume that had been prewashed with 20 mL of chromatography buffer at room temperature. After the column was washed with an additional 20 mL of chromatography buffer, it was eluted with 2 mL of chromatography buffer containing 10 mg of fMet-Leu-Phe that had been dissolved in 100 μL of Me_2SO and then with 2 mL of chromatography buffer. The eluate was dialyzed against 1 L of chromatography buffer for 24 h at 4 °C and then 1 L of 0.1 M NaCl–0.05 M Tris-HCl (pH 8.0) containing 0.02 mL of NP40/100 mL for 48 h at 4 °C. After concentration of the eluate to 1 mL by vacuum ultrafiltration in a collodion bag (molecular weight cutoff 10000; Schleicher & Schuell, Inc., Keene, NH), it was dialyzed against 1 L of 0.05 M NaCl–0.05 M Tris-HCl (pH 7.2) containing 0.02 mL of NP40 for 48 h at 4 °C. The protein content of the eluate was

assessed by determining the absorbance at 280 nm and by a Lowry method (Lowry et al., 1951). For resolution of the membrane proteins recovered from the fMet-Leu-Phe–Sepharose column, some eluates were filtered on a 1.6 × 50 cm column (bed volume = 100 mL) of Bio-Gel P150 that was equilibrated and developed with 0.05 M Tris-HCl (pH 8.0) containing 0.5 g of $NaDodSO_4$/100 mL. The flow rate was 4 mL/h at room temperature, and 1-mL fractions were collected. The fractions comprising individual protein peaks were pooled, and the pools were concentrated, refiltered on Bio-Gel P150, pooled, reconcentrated, dialyzed against distilled water for 24 h at 4 °C, incubated with an equal volume of SM-2 Bio-Beads for 1 h at room temperature, and dialyzed against 0.05 M NaCl–0.05 M Tris-HCl–0.02 mL of NP40/L (pH 7.2) prior to structural or functional studies.

Characterization of Neutrophil Membrane Proteins Isolated by Affinity Chromatography. $NaDodSO_4$ (0.1% w/v)–polyacrylamide (10% w/v) slab gel electrophoresis was performed as described (Laemmli, 1970) by utilizing an apparatus that holds a 10 × 14 cm slab in a vertical position (Model 220, Bio-Rad Laboratories, Richmond, CA). Samples (100 μL) containing 2% (w/v) $NaDodSO_4$, 2% (v/v) β-mercaptoethanol, and 5% (w/v) sucrose were boiled for 5 min prior to application to the slab gel and electrophoresis at a constant 120 V for 3–4 h at room temperature; bromophenol blue (0.01 g/100 mL) served as the tracking dye. Some samples were analyzed by electrophoresis in 0.1% (w/v) $NaDodSO_4$ tube gels that contained 12% (w/v) acrylamide and 0.4% (w/v) bis(acrylamide) by utilizing a buffer system composed of 0.1 M Tris–Bicine and 0.1% (w/v) $NaDodSO_4$ (pH 8.3) (Goldman et al., 1979). Slab gels and tube gels were stained with Coomassie Blue, and the bands in some tube gels were quantified by densitometry (Gel Scanner Model 252OH, Gilford Instrument Laboratories, Inc., Oberlin, OH).

Membrane proteins that had been purified by filtration on Bio-Gel P150 were desalted by dialysis against distilled water for 10 h at 4 °C, lyophilized in 9 × 150 mm glass test tubes, and resuspended in 0.4 mL of 6 M HCl containing 0.001 M phenol. The tubes were evacuated and heated at 110 °C for 24 h (Hare, 1975). Half of two preparations of each of the membrane proteins was oxidized with performic acid prior to hydrolysis in 6 M HCl, and the other half was hydrolyzed in 3 M mercaptoethanesulfonic acid in order to permit analyses of cysteine, as cysteic acid, and of tryptophan, respectively. The amino acids in the hydrolysates were quantitated on a Durrum D-500 analyzer by employing norleucine as an internal standard (Dionex Co., Sunnyvale, CA).

Equilibrium dialysis was carried out in a rotary microdialyzer with multiple eight-chamber modules (Model EMD 101, Hoeffer Scientific Instruments, San Francisco, CA). The dialysis membranes separating the two 250-μL compartments of each chamber were boiled for 5 min in 5 g of Na_2CO_3/100 mL of 50 mM EDTA and washed in distilled water prior to assembly of the modules. One-hundred microliter portions of membrane protein and of [^{3}H]fMet-Leu-Phe, at concentrations of 10^{-9}–10^{-6} M, were added to the compartments, and the modules were rotated for 16 h at room temperature, after which time equilibrium was achieved as assessed by the distribution of [^{3}H]fMet-Leu-Phe in control chambers containing buffer alone in the protein compartment. The samples were removed from the compartments, and 30-μL portions were transferred with precalibrated glass micropipets (Arthur H. Thomas Co., Philadelphia, PA) to scintillation vials for quantitation of radioactivity. The concentration of membrane protein was determined from the results of amino acid analyses.

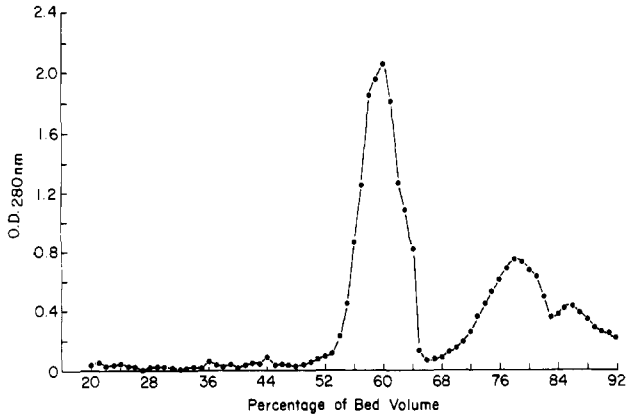

FIGURE 1: Bio-Gel P150 filtration of neutrophil membrane proteins eluted from an fMet-Leu-Phe–Sepharose affinity column. An affinity chromatography eluate of membrane proteins from 2.9 × 10^9 neutrophils was applied to the Bio-Gel P150 column in 1 mL of 0.05 M NaCl–0.05 M Tris-HCl containing 0.5 g of $NaDodSO_4$/100 mL (pH 8.0). The peaks of optical density at 280 nm represent MP-1, MP-2, and MP-3, in order of descending molecular weight.

The data were analyzed and presented as standard Scatchard plots.

Results

The application of a sonicate of neutrophil membranes to an fMet-Leu-Phe–Sepharose column equilibrated in buffer containing 0.3 g of NP40/100 mL resulted in the retention of 7.6–14.9% of the total protein in the sonicate (range, n = 8), as assessed by Lowry assays of the protein in the effluent and in the sonicate. Specific elution of the adherent proteins with fMet-Leu-Phe yielded 2.7–4.1% of the quantity of proteins in the initial sonicate (range, n = 6). Filtration of the eluted membrane proteins on Bio-Gel P150 in a buffer containing 0.5 g of $NaDodSO_4$/100 mL resolved three distinct constituents (Figure 1). The absorbance values at 280 nm of the fractions did not reflect accurately the relative quantities of the individual membrane proteins, since Lowry assays indicated that the constituent which was intermediate in molecular weight accounted for 74–93% (range, n = 6) of the total protein. Each of the constituents was refiltered separately on a Bio-Gel P150 column to achieve a purity of over 92%, as assessed by comparing the protein content of the peak to that of a pool of the remaining effluent. After the purified proteins were dialyzed against distilled water and incubated with SM-2 beads to remove free and loosely bound detergent, the absorbancy at 280 nm correlated closely with the protein content as quantified by the Lowry assay. $NaDodSO_4$–polyacrylamide gel electrophoretic analyses of eluates from fMet-Leu-Phe–Sepharose columns confirmed the quantitative predominance of the ~68 000-dalton membrane protein (MP-2) over the approximately 94 000-dalton (MP-1) and 40 000-dalton (MP-3) membrane proteins in the eluates (Figure 2). Densitometric analyses of $NaDodSO_4$ tube gel patterns indicated that the 68 000-dalton band comprised 90% or more of the total protein in purified MP-2 preparations. Of the three other bands detected, the next most prominent comprised only 4% or less of the protein. When half of each of two preparations of neutrophils was incubated in 10^{-3} M diisopropyl fluorophosphate for 30 min at 37 °C prior to nitrogen cavitation, the MP-2 recovered accounted for 71% and 84% of the total protein, as assessed by Lowry assays of Bio-Gel P150 pools, compared to 79% and 90%, respectively, for MP-2 isolated from the untreated half of the preparations.

Replicate preparations of the membrane proteins that had been purified by sequential chromatography on fMet-Leu-

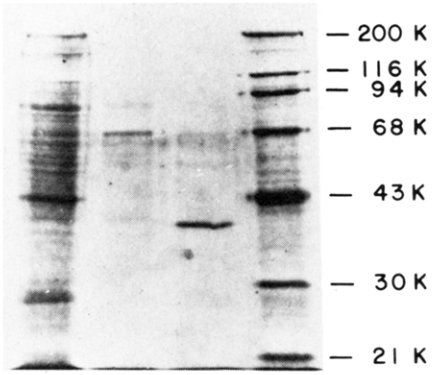

FIGURE 2: $NaDodSO_4$–polyacrylamide gel electrophoresis of human neutrophil membrane proteins purified by fMet-Leu-Phe–Sepharose affinity chromatography. Samples applied to the wells in each track were (from left to right) as follows: sonicate of membrane preparation prior to chromatography, fMet-Leu-Phe eluate from affinity column, pool of 40 000 molecular weight protein (MP-3) of six eluates that had been filtered on Bio-Gel P150 in 0.05 M Tris-HCl containing 0.5 g of $NaDodSO_4$/100 mL (pH 8.0), and molecular weight standards. The molecular weight standards were myosin (M_r = 200 000), β-galactosidase (M_r = 116 500), phosphorylase *b* (M_r = 94 000), bovine serum albumin (M_r = 68 000), ovalbumin (M_r = 43 000), carbonic anhydrase (M_r = 30 000), and soybean trypsin inhibitor (M_r = 21 000).

Phe–Sepharose and Bio-Gel P150 were subjected to acid hydrolysis to analyze the amino acid composition and to provide an additional approach to quantification. The MP-2 recovered ranged in quantity from 0.039 to 0.062 nmol/10^9 neutrophils (n = 6) as quantified by amino acid analyses of pools of 7–12 preparations, each of which was derived from (1.9–3.0) × 10^9 neutrophils. MP-1 and MP-2 possessed an above-average content of hydrophobic residues that accounted for 36.8% and 40.1% of the total amino acids, respectively, while MP-2 and MP-3 contained an above-average content of acidic and/or acid amide amino acids and a below average content of basic amino acids (Table I) (Dayhoff et al., 1978). The approximate mean number of cysteine and tryptophan residues determined by amino acid analyses of two additional preparations of each protein was 7.9 and 2.8, respectively, for MP-1, 5.7 and 1.9 for MP-2, and 1.8 and 0.7 for MP-3. As assessed by amino acid analyses, MP-1, MP-2, and MP-3 comprised 5–12%, 79–92% and 3–9%, respectively, of the total protein recovered from the Bio-Gel P150 columns. Although the MP-1 and MP-3 together accounted for only 10% or less of the protein in many preparations, pooling and refiltration on Bio-Gel P150 resulted in substantial purification of the minor constituents (Figure 2).

The binding of [^{3}H]fMet-Leu-Phe to preparations of MP-2 containing <10% of the other constituents and to Bio-Gel P150 purified MP-3 was assessed by equilibrium dialysis in microchambers. Both MP-2 and MP-3 bound [^{3}H]fMet-Leu-Phe with two or more apparent affinities, as determined by standard Scatchard plots (Figure 3). The binding sites of MP-2 and MP-3 with high apparent affinities possessed a mean K_A of approximately 9 × 10^8 and 2 × 10^7 M^{-1}, respectively, and a valence of approximately 0.2–0.3. The binding sites of MP-2 and MP-3 with low apparent affinities exhibited respective mean K_A values of approximately 3 × 10^7 and 2 × 10^6 M^{-1} and a valence of 0.3–0.5, as determined by subtracting the valence of the high-affinity sites from the apparent maximum values of R at saturation (Figure 3). The high-affinity and low-affinity K_A for three preparations of MP-2 that had been purified further by filtration on Bio-Gel P150 were (8.6 ± 0.29) × 10^8 and (2.2 ± 0.6) × 10^7 M^{-1} (mean ± SD), respectively. Binding of [^{3}H]fMet-Leu-Phe to

Table I: Amino Acid Compositions of Human Neutrophil Membrane Proteins Isolated by Formylmethionyl Peptide Affinity Chromatography

	MP-1[a]	MP-2	MP-3
Asx	81.8 ± 6.7[b]	61.9 ± 4.9	48.7 ± 3.6
Thr	46.2 ± 2.5	34.8 ± 1.2	16.9 ± 2.2
Ser	58.9 ± 5.1	29.1 ± 6.4	25.8 ± 3.3
Glx	99.1 ± 8.4	73.0 ± 5.1	57.1 ± 6.5
Pro	48.9 ± 9.6	30.9 ± 6.7	22.8 ± 3.4
Gly	93.0 ± 3.8	56.0 ± 1.6	28.0 ± 2.7
Ala	114.8 ± 4.1	75.8 ± 1.0	45.0 ± 1.7
Val	54.7 ± 7.8	41.1 ± 5.9	20.1 ± 4.4
Met	13.9 ± 2.4	10.7 ± 1.8	5.1 ± 1.6
Ile	26.8 ± 4.3	29.0 ± 2.7	14.6 ± 1.8
Leu	70.0 ± 6.4	60.2 ± 4.2	27.2 ± 1.5
Tyr	26.7 ± 5.0	19.8 ± 3.9	6.9 ± 0.9
Phe	33.1 ± 2.6	26.1 ± 2.0	13.8 ± 1.0
His	19.1 ± 3.5	11.0 ± 2.5	6.1 ± 1.3
Lys	54.0 ± 6.2	36.2 ± 5.4	15.9 ± 1.7
Arg	43.8 ± 7.7	32.1 ± 6.1	11.2 ± 1.1
total no. of residues (mean)	885[c]	628	365
calcd M_r (mean)	93 963	67 965	39 078
% of hydrophobic residues[d]	36.8	40.1	34.7
no. of determinations	3	5	3

[a] The quantities of Bio-Gel P150 purified membrane proteins that were subjected to hydrolysis and amino acid analysis ranged from 0.29 to 0.61 nmol for MP-1, 1.37 to 2.46 nmol for MP-2, and 0.43 to 1.07 nmol for MP-3. [b] Each value is the mean ± SD of the results of amino acid analyses of the specified number of preparations. [c] The total number of amino acid residues per molecule does not include cysteine and tryptophan. [d] Hydrophobic residues were defined as Ala, Val, Ile, Leu, Tyr, and Phe. Mean values for the amino acid compositions of 314 diverse proteins (Dayhoff et al., 1978) included 13.5% basic amino acids, 19.8% acidic and/or amide amino acids, and 34.1% hydrophobic amino acids. MP-1, MP-2, and MP-3 contained 20.4%, 21.5%, and 29.0% acidic and/or acid amide amino acids, respectively, and 13.2%, 12.6%, and 9.1% basic amino acids.

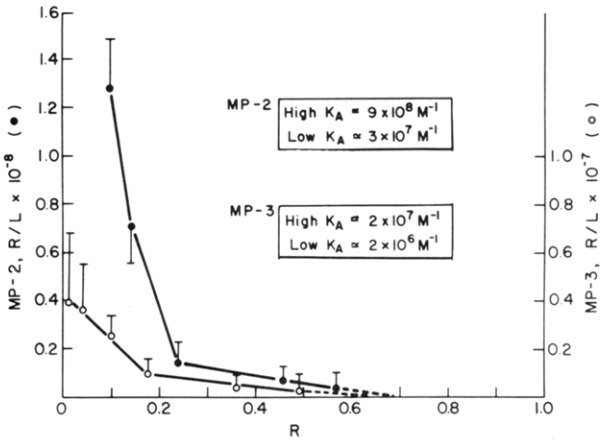

FIGURE 3: Binding of [^{3}H]fMet-Leu-Phe by neutrophil membrane proteins purified by affinity chromatography. Each point with error bar depicts the mean ± SD, respectively, of the results of three experiments. The preparations of MP-2 were obtained from three separate affinity columns and contained <10% of the other membrane proteins. The three preparations of MP-3 were obtained from pools of five to seven affinity column eluates that were filtered on Bio-Gel P150. The protein concentrations in the dialysis chambers ranged from 0.4 × 10^{-8} to 1.9 × 10^{-8} M for MP-2 and 4.2 × 10^{-7} to 7.4 × 10^{-7} M for MP-3, as assessed from amino acid analyses. R is the ratio of the concentration of bound fMet-Leu-Phe to that of protein, and L is the concentration of unbound fMet-Leu-Phe.

MP-1 could not be detected in three separate experiments at protein concentrations ranging from 2.0 × 10^{-8} to 1.3 × 10^{-6} M, although a mean of 84% of MP-1 (n = 4) bound to

Table II: Inhibition of Binding of [^{3}H]fMet-Leu-Phe to MP-2 by Other Formylmethionyl Peptides

peptide	IC_{50} for inhibition of binding to MP-2[a] (M)	mean rel binding inhibitory act.	mean EC_{50} for neutrophil chemotactic act.[b] (M)	mean rel neutrophil chemotactic act.[b]
fMet-Leu-Phe	$(2.7\text{–}3.2) \times 10^{-8}$	133.9	7.0×10^{-11}	5050
fMet-Phe-Leu	$(1.2\text{–}1.5) \times 10^{-6}$	2.9	5.4×10^{-8}	10
fMet-Leu	$(3.1\text{–}4.8) \times 10^{-6}$	1	4.0×10^{-7}	1
fMet-Leu-Arg	$(3.5\text{–}5.3) \times 10^{-6}$	0.9	3.6×10^{-7}	1
fMet-Leu-Glu	$(0.9\text{–}1.4) \times 10^{-5}$	0.3	1.3×10^{-6}	0.4

[a] Various concentrations of each peptide were added to duplicate chambers containing 3×10^{-8} M [^{3}H]fMet-Leu-Phe and 0.5×10^{-8} M MP-2. Two different preparations of MP-2 were employed to study the inhibitory activity of each peptide, and the values presented for the 50% inhibitory concentrations (IC_{50}) are the results of the two studies; the means of the IC_{50} values were used to calculate the inhibitory activities relative to 1 for fMet-Leu. [b] The neutrophil chemotactic results are from Showell et al. (1976). EC_{50} is the abbreviation for the concentration which achieves a response equal to 50% of the maximal chemotaxis evoked by each peptide.

fMet-Leu-Phe–Sepharose, as assessed by Lowry assays of protein in the column effluents. The addition of various concentrations of unlabeled synthetic formylmethionyl peptides to a mixture of [^{3}H]fMet-Leu-Phe and MP-2 that alone exhibited an R value of ~0.25 resulted in inhibition of binding of [^{3}H]fMet-Leu-Phe (Table II). An equimolar concentration of fMet-Leu-Phe and higher concentrations of the chemotactically less potent peptides inhibited the binding of [^{3}H]-fMet-Leu-Phe by 50%. The relative activities of the synthetic peptides in inhibiting binding and in stimulating neutrophil chemotaxis displayed a similar rank order.

The specificity of binding of [^{3}H]fMet-Leu-Phe to MP-2 and MP-3 was examined in affinity chromatography and equilibrium dialysis experiments. Portions of purified MP-2 and MP-3 were applied to columns of fMet-Leu-Phe–Sepharose and C5fr–Sepharose in 0.1 M NaCl–0.02 M Hepes buffer (pH 7.2) containing 0.3 mL of NP40/100 mL. The concentration of protein in the initial preparations and in the effluents was assessed by the Lowry method. In two separate experiments, the fMet-Leu-Phe–Sepharose retained 96% and >98% of MP-2 and 91% and 93% of MP-3. In contrast, the C5fr–Sepharose bound only 15% and 19% of MP-2 and 11% and 16% of MP-3. Equilibrium dialysis studies of the binding of 5-[^{3}H]HETE and [^{3}H]LTB to the membrane proteins utilized the same technique as studies with [^{3}H]fMet-Leu-Phe, except that the lipid chemotactic factors were suspended in ethanol prior to dilution in buffer to achieve an ethanol concentration of 2% (v/v) and the buffers were saturated with argon gas to preserve the lipids. The incubation period at room temperature was shortened to 5 h, at which time the concentration of 5-[^{3}H]HETE had reached the same level in both compartments of chambers that contained buffer alone in the protein compartment. MP-2 and MP-3 failed to bind either 5-[^{3}H]HETE or [^{3}H]LTB at concentrations of 1.4×10^{-8}–1.1×10^{-6} M, indicating that the K_A values were at least 2 orders of magnitude lower than those for [^{3}H]fMet-Leu-Phe.

Discussion

Affinity chromatography of a sonicate of membranes from 10^9 neutrophils on an fMet-Leu-Phe–Sepharose column in buffer containing the nonionic detergent NP40 and the elution of the column with a 10-fold molar excess of the chemotactic peptide fMet-Leu-Phe yielded nanomole quantities of three proteins. The three membrane proteins recovered by affinity chromatography were resolved both by $NaDodSO_4$–polyacrylamide gel electrophoresis (Figure 2) and by filtration on Bio-Gel P150 in the presence of $NaDodSO_4$ (Figure 1) and were designated MP-1, MP-2, and MP-3 in the order of descending molecular weight. Both Lowry protein assays and amino acid analyses of the membrane proteins purified by Bio-Gel P150 filtration indicated that MP-2 (68 000 daltons) accounted for 74–93% of the total protein, while MP-1 (94 000 daltons) and MP-3 (40 000 daltons) were quantitatively minor components. The recovery of 0.039–0.062 nmol of MP-2 from 10^9 neutrophils, as assessed by amino acid analyses, represents a yield of $\sim 1/3$ of the calculated total of 0.17 nmol of receptor on 10^9 neutrophils, based on the specific binding of radiolabeled fMet peptides by a maximum of 10^5 receptors per neutrophil (Showell et al., 1976; Williams et al., 1977; Niedel et al., 1980). MP-2 exhibited an above-average content of hydrophobic amino acids and of acidic and/or acid amide amino acids and a below average content of basic amino acids relative to nonmembrane proteins (Table I). While intrinsic membrane proteins generally have some hydrophobic sequences, the overall content of hydrophobic amino acids is variable. A high content of 42.5% and 37.5% hydrophobic amino acids, respectively, has been found in the anion-transport protein of human erythrocytes (Ho & Guidotti, 1975) and the calcium ATPase of sarcoplasmic reticulum (Stewart et al., 1976), while a low content of 32.5% was detected in glycophorin A of human erythrocytes (Furthmay et al., 1975). MP-2 resembles in size the neutrophil membrane protein that was affinity labeled by covalently reactive derivatives of chemotactic formylnorleucyl peptides, but the latter protein has not been characterized further (Niedel et al., 1980).

The complex Scatchard plots of the results of binding of [^{3}H]fMet-Leu-Phe to MP-2 and MP-3 are consistent either with the presence of multiple independent sites of different affinities or with binding sites that interact cooperatively (Figure 3). Assuming the existence of two classes of independent binding sites, the mean K_A values of the high-affinity sites of MP-2 and MP-3 were 9×10^8 and 2×10^7 M^{-1}, respectively, and of the low-affinity sites were 3×10^7 and 2×10^6 M^{-1}. MP-2 prepared by sequential affinity chromatography and filtration on Bio-Gel P150 in $NaDodSO_4$ bound [^{3}H]fMet-Leu-Phe with K_A values indistinguishable from those of MP-2 that had not been purified by gel filtration in ionic detergent and contained quantities of the minor constituents which together amounted to 10% or less of the total protein. The association constant of the MP-2 high-affinity site was similar in magnitude to that of the receptors on intact neutrophils, while the association constants of the low-affinity sites of MP-2 and MP-3 were approximately $1/30$ and $1/450$, respectively, of that of the native receptors (Aswanikumar et al., 1977; Williams et al., 1977). The binding specificity of MP-2 and MP-3 was demonstrated both by their failure to adhere to Sepharose affinity columns bearing chemotactic peptide fragments of the fifth component of complement and by the absence of detectable binding activity for the lipid chemotactic factors 5-HETE and LTB. In addition, a series of synthetic formylmethionyl peptides inhibited the binding of [^{3}H]fMet-Leu-Phe by MP-2 with relative inhibitory activities that paralleled the chemotactic potencies of the peptides for neutrophils (Table II). Although differences in affinity

were found among the binding sites on MP-2 and MP-3, it is not clear at present whether such differences are attributable to multiple independent sites on one or more membrane proteins or to cooperative interactions between sites on either the same membrane protein or the predominant protein and minor contaminants. The apparent increases in the net binding activity of receptors for formylmethionyl peptides on intact neutrophils treated with membrane perturbants (Liao & Freer, 1980) or on monocytes exposed to immunological stimuli (Pike et al., 1980) may be explained by the exposure of latent high-affinity receptors. The solubilization and purification of constituents of leukocyte chemotactic factor receptors that retain specific binding activity for the homologous stimulus may facilitate an understanding of the coupling of such receptors to a variety of distinct leukocyte functions.

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Roles of Arginyl Residues in Pyridoxamine-5′-phosphate Oxidase from Rabbit Liver†

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ABSTRACT: Pyridoxamine-5′-phosphate oxidase (pyridoxine-5′-phosphate oxidase) is inactivated by arginine-specific reagents. Inactivation by phenylglyoxal follows pseudo-first-order kinetics and is first order with respect to modifier. The substrate-competitive inhibitors pyridoxal 5′-phosphate oxime and 4′-deoxypyridoxime 5′-phosphate and product pyridoxal 5′-phosphate protect holoenzyme against inactivation but have no significant effect on the inactivation of apoenzyme. The extent of protection is dependent on their respective binding constants. Extrapolation to complete inactivation shows modification of ~4 out of the 40 total arginyl residues in the native enzyme, with ~1 residue protected by pyridoxal 5′-phosphate, as determined by incorporation of [7-^{14}C]-phenylglyoxal. Binding of coenzyme flavin mononucleotide increases the rate of inactivation 3-fold by enhancing reactivity of an essential arginyl residue toward reagent. This and the fact that substrates and product do not bind well to apoenzyme indicate that an arginyl residue essential for substrate/product binding is exposed by formation of functional holoenzyme. The oxidase is also inactivated by either 2,3-butanedione in borate buffer or 2,4-pentanedione in phosphate buffer and is similarly protected by pyridoxal 5′-phosphate. Inactivation by butanedione is fully reversible on removal of excess butanedione and borate. Inability of hydroxylamine to restore activity of pentanedione-modified oxidase suggests that inactivation is due to modification of arginyl, not lysyl, residues. Unlike native enzyme, modified enzyme is not able to bind product pyridoxal 5′-phosphate. These results provide evidence for the essential role of an arginyl residue in the binding of substrate/product to the enzyme and suggest the possible shielding of many arginyls in ionic association of the two polypeptide chains of the monomer.

Pyridoxamine-5′-phosphate oxidase (pyridoxine-5′phosphate oxidase) (EC 1.4.3.5) catalyzes the conversion of the phosphorylated B_6 vitamers to coenzyme pyridoxal 5′-phosphate (McCormick & Merrill, 1980). It has been postulated to be one of the regulating steps in vitamin B_6 metabolism, since product pyridoxal 5′-phosphate is strongly inhibitory (Snell & Haskell, 1971; Merrill et al., 1978). The oxidase from rabbit liver contains one FMN per 54 000 molecular weight

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