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ENZYMATIC REDUCTION OF OXIDIZED CHEMOTACTIC PEPTIDE N-FORMYL-L-METHIONYL-SULFOXIDE-L-LEUCYL-L-PHENYLALANINE

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The oxidized chemotactic peptide N-formyl-L-methionyl-sulfoxide-L-leucyl-L-phenylalanine can be reduced using either a partially purified methionine sulfoxide peptide reductase from Escherichia coli or a neutrophil extract. The product of the enzymatic reduction shows chemotactic activity.

We have recently purified an enzyme (Met(O)-peptide reductase) from E. coli extracts that catalyzes the reduction of methionine sulfoxide (Met(O)) residues in peptides and proteins (1). The initial studies used oxidized ribosomal protein L12 (Met(O)-L12) as substrate but the enzyme has been shown to also reduce Met(O) residues in oxidized Met-enkephalin (1), oxidized α-1-proteinase inhibitor (α-1-PI) (2) and in a simple methionine analog, N-acetylmethionine sulfoxide (N-AcetylMet(O)). The reduction of the latter compound has been used to develop a rapid assay for the enzyme (3). Methionine sulfoxide, itself, is not reduced by this enzyme although another enzyme system in E. coli has been described (Met(O) reductase) that reduces this oxidized amino acid (4).

ABBREVIATIONS

fMet-Leu-Phe, n-formyl-L-methionyl-L-leucyl-L-phenylalanine; fMet(O)-Leu-Phe, sulfoxide derivative of fMet-Leu-Phe; Met(O), methionine sulfoxide; α-1-PI, α-1-proteinase inhibitor; DTT, dithiothreitol.

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It is now apparent that methionine plays an essential role in the biological activity of many proteins including ribosomal protein L12, lysozyme, ribonuclease, pepsin, and calmodulin (5). In all of the above cases, oxidation of a methionine residue(s) causes a loss of biological activity. With some proteins there is good evidence that oxidation can occur in vivo. For example, emphysema in cigarette smokers has been linked to the oxidation of α-1-proteinase inhibitor (6,7). Moreover, neutrophils, when exposed to a chemotactic peptide (fMet-Leu-Phe), produce oxidants (e.g. O_2^-, H_2O_2, OCl^-) that can inactivate the peptide by oxidation of the formylmethionine to the sulfoxide derivative (8). Because of the unusually high oxidative ability of neutrophils these cells were of special interest. In this report we show that neutrophil extracts have a high Met(O)-peptide reductase activity and can reduce and thereby restore activity to the chemically oxidized chemotactic peptide.

MATERIALS AND METHODS

Synthesis of fMet(O)-Leu-Phe. To 400 µL (400 µCi) of fMet-Leu[^{3}H]Phe (46.7 Ci/mmol, in ethanol, New England Nuclear) were added 40 µL of fMet-Leu-Phe (10 mM, in ethanol, Sigma) and 44 µL of 0.5 N HCl. Twenty µL of H_2O_2 (30%) were added and the tube was kept at room temperature for 60 min. The solution was taken to dryness under vacuum, and the residue dissolved in 50 µL of ethanol and applied to a Silica Gel G-precoated glass TLC plate (20 x 20 cm, Analtech, Newark, Delaware). Chromatography was carried out in butanol, acetic acid, water (60:15:25) for 2.5 hr. The location of the radioactive oxidized peptide was determined by comparison with standard unlabelled fMet(O)-Leu-Phe which was detected by spraying the plate with a hypochlorite, potassium iodide, starch solution (9). The area on the plate containing the radiolabelled peptide was scraped off, extracted several times with 0.5 mL aliquots of water and centrifuged to remove any silica gel particles. The clear supernatant was taken to dryness under vacuum. The residue was dissolved in water and stored at -20°C. The final yield was about 65%.

Enzymatic assay for reduction of fMet-Leu-Phe. The assay was based on a previously described procedure in which N-acetyl-Met(O) served as substrate (3). The incubations (30 µL) contained fMet(O)-Leu[^{3}H]Phe, 540 pmol (523 cpm/pmol); Tris-HCl, pH 7.5, 25 mM; $MgCl_2$, 10 mM; DTT, 15 mM; and enzyme. The reaction mixture was incubated at 37° for 60 min and was terminated by the addition of 1.5 mL of 0.5 N HCl. Three mL of ethyl acetate were added, the tubes were thoroughly mixed and centrifuged briefly at room temperature to separate the phases. A 2 mL aliquot of the ethyl acetate phase was removed and the radioactivity determined. Under the conditions used, about 67% of the fMet-Leu-Phe was extracted into the ethyl acetate, whereas only 25% of the substrate was extracted. The data are corrected for the extraction efficiency of both the substrate and product in calculating the amount of fMet-Leu-Phe formed. The ethyl acetate extracts were also analyzed for fMet(O)-Leu-Phe by thin layer chromatography. After evaporation of the ethyl acetate under a stream of oxygen-

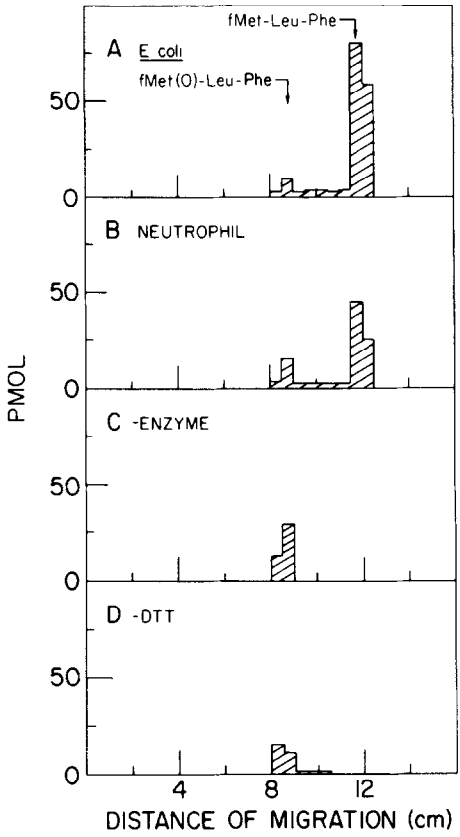

Fig. 1: Thin layer chromatographic separation and identification of products after enzymatic reduction of fMet(O)-Leu-Phe. Panel A: complete system with 6.4 μg purified of E. coli enzyme. Panel B: complete system with 12.6 μg of neutrophil extract. Panel C: No enzyme added. Panel D: Same as Panel A minus DTT. See text for details.

free nitrogen, the residues were redissolved in a small volume of ethanol and applied to Silica Gel G precoated glass TLC plates for analysis. Chromatographic conditions were as described above.

Preparation of cell extracts. The preparation of a 100-fold purified Met(O)-peptide reductase from E. coli has been described previously (1). Human neutrophil crude extracts were prepared as described elsewhere (3).

Assay for chemotaxis. Activated rabbit polymorphonuclear leukocytes were obtained 14-16 hours after the intraperitoneal injection of 0.2% oyster glycogen (Sigma) (10). The preparation contained about 90% neutrophils. The chemotatic assay was based upon the modified Boyden chamber procedure (10). Cells were added to the upper well of the chamber at a concentration of 2.2 x 10^6/mL in Gey's balanced salt solution containing 2% bovine serum albumin. Materials to be tested as attractants were added in Gey's solution to the lower well. The compartments were separated by a nitrocellulose filter (5 μm, Millipore Corp., Bedford, MA) and the chambers were incubated for 2 hr at 37°C in an atmosphere of humidified 5% CO_2 in air. The filters were then removed, stained with hematoxylin, and examined microscopically (900 x) for cells that had migrated to the underside of the filter. Using a grid in the ocular, the cells in five fields were counted for each filter, and the average computed. The S.E.M.s did not vary by more than 10%.

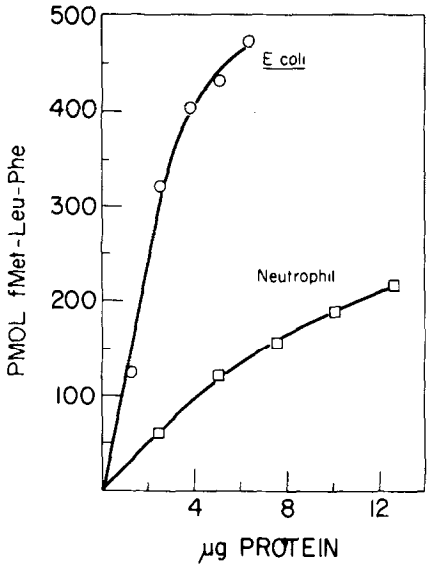

Fig. 2: Effect of protein concentration on the reduction of fMet(O)-Leu-Phe by neutrophil extracts and the E. coli purified enzyme. The assay was performed as described in Materials and Methods.

RESULTS

A partially purified E. coli Met(O)-peptide reductase preparation and a crude neutrophil extract both reduced fMet(O)-Leu-Phe in the presence of DTT. Initial identification of the product, fMet-Leu-Phe, was obtained using thin layer chromatography. Typical results are seen in Fig. 1. Panel A shows that in the complete system, using the E. coli reductase, a major radioactive peak is obtained that co-migrates with authentic fMet-Leu-Phe. A small peak of substrate is also seen due to the extraction of some of the fMet(O)-Leu-Phe into ethyl acetate. A similar chromatographic pattern is obtained using the neutrophil extract as the source of enzyme (Panel B). In the absence of either enzyme or DTT (Panels C and D) no fMet-Leu-Phe is observed and only the radioactive substrate is present on the plate.

Although the thin layer chromatographic procedure provided direct evidence that fMet-Leu-Phe was formed, an extraction procedure was used for routine assays. As described in Methods, under the conditions used, the selective extraction of the reduced chemotactic peptide into ethyl acetate

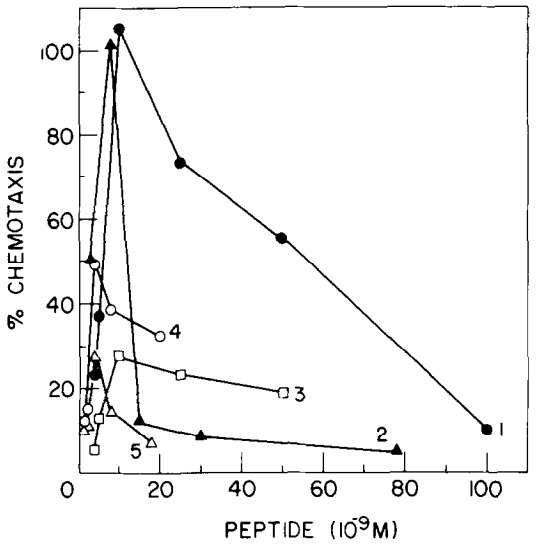

Fig. 3: Effect of increasing concentrations of fMet-Leu-Phe, produced by enzymatic reduction, on neutrophil chemotaxis. Chemotaxis was assayed as described in Methods. Each point on the curves is the average of three determinations, the S.E.M. of which did not exceed 10%. (1) complete system, E. coli enzyme; (2) complete system, neutrophil preparation; (3) minus enzyme; (4) complete E. coli system minus DTT; (5) complete neutrophil system minus DTT.

provided the basis for a rapid assay. The effect of different amounts of the E. coli enzyme and the neutrophil extract on the rate of reduction of fMet(O)-Leu-Phe is seen in Fig. 2. The reaction with the neutrophil extract was found to be linear for up to 120 min (data not shown). It should be noted that under the conditions of the assay the crude neutrophil extract showed about 1/4 the activity of the 100 fold purified E. coli enzyme. Of all the biological sources thus far examined, neutrophils appear to be the most active source of this enzyme.

It was also of interest to ascertain whether the enzymatically reduced fMet(O)-Leu-Phe was biologically active. Since it is known that the oxidized peptide, fMet(O)-Leu-Phe, is much less active than fMet-Leu-Phe as a chemotactic agent, the restoration of chemotactic activity was also determined in these studies. The results of one such dose response experiment, in which the chemotactic activity was assayed after incubating fMet(O)-Leu-Phe with either E. coli or neutrophil reductase preparations, are shown in Figure 3. A maximal chemotactic response to the fMet-Leu-Phe that was

generated by both the E. coli enzyme and the neutrophil extract, occurs at a concentration of about 1 x 10^{-8} M (curves 1 and 2). In the absence of enzyme or DTT (curves 3-5), the response ranges between 15-40% of the maximum due primarily to the weak chemotactic activity of the substrate.

The experiments in Figure 3 are only a qualitative measure of chemotactic activity since it was noted that the E. coli enzyme and neutrophil extracts as well as the oxidized chemotactic peptide all significantly inhibited the chemotactic activity of fMet-Leu-Phe. In fact, whereas standard fMet-Leu-Phe gave a maximal chemotactic response under the conditions used in Fig. 3 at 2 x 10^{-9} M, the enzymatically formed material gave a maximal response at only 1 x 10^{-8} M probably because of the inhibitory components in the incubations.

DISCUSSION

These studies demonstrate that human neutrophil extracts and a partially purified Met(O)-peptide reductase preparation from E. coli are capable of reducing the methionine sulfoxide residue in the peptide fMet(O)-Leu-Phe. The assay used in these studies was a modification of an extraction procedure developed recently in our laboratory to measure Met(O)-peptide reductase activity (3). As with other protein and peptide substrates (1), there was a requirement for DTT with both the E. coli enzyme and the neutrophil extract. The product of the reaction was shown to be fMet-Leu-Phe by both its chromatographic behavior and chemotactic activity.

The chemotactic activity of n-formyl-methionyl peptides with polymorphonuclear cells was first noted by Schiffmann et al. (11). Of the peptides examined, fMet-Leu-Phe was subsequently shown to be the most potent (12). This peptide is thought to mimic the action of the natural prokaryotic chemotactic peptides, and functions by attracting neutrophils to sites of infection, where the cells produce a high level of oxidizing agents (oxygen burst pheonmenon) as part of the body's defense mechanism (13,14). It has been shown recently that the chemotactic peptide fMet-Leu-Phe can be oxi-

dized under these conditions to the inactive fMet(O)-Leu-Phe (8,15). It is unclear whether or not the oxidation of the chemotactic peptide serves any biological function. It is conceivable that this oxidative inactivation of the peptide may actually serve to sharpen the concentration gradient of the chemotactic peptide and thus increase the ability of the cell to detect differences in concentrations of attractant. It appears that the Met(O)-peptide reductase is a cytoplasmic enzyme (data not shown), and as yet, there is no evidence that the reductase functions in intact neutrophils to regenerate the methionine-containing attractant. However, the apparently indiscriminate release of oxidizing agents by the neutrophils may also have a deleterious effect on the neutrophil and surrounding tissues (16). One form of damage may be the oxidation of essential methionine residues in proteins since numerous instances of protein inactivation by oxidation of methionine residues have been cited (5). The high level of Met(O)-peptide reductase activity in neutrophils may be required to repair some of the damage to proteins resulting from the oxygen burst.

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