Biochemical Characterization of Human Eosinophil Charcot-Leyden Crystal Protein (Lysophospholipase)*

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Lysophospholipase from human eosinophils is a protein previously considered based upon antigenic, enzymatic, and electrophoretic similarities to be the single component of Charcot-Leyden crystals, which are formed in association with eosinophilic diseases. The identity of eosinophil lysophospholipase and solubilized Charcot-Leyden crystal protein is now established by biochemical criteria, and a basis for the ease of aggregation and crystallization of the protein is identified in its prominent hydrophobicity. Chromatographically purified enzyme and Charcot-Leyden crystal protein formed in vitro functioned as lysophospholipases with identical Michaelis constants (Ka = 22 μM) for the substrate lysopalmitoylphosphatidylcholine and had blocked amino-terminal residues and almost identical amino acid compositions. The propensity of lysophospholipase to aggregate was not due to extensive intermolecular disulfide bonding because it contained a single cysteine residue as assessed by amino acid analyses and incorporated 0.986 mol of p-chloromercuribenzoic acid/mol of native enzyme or 0.958 mol of iodoacetic acid/mol of reduced and denatured enzyme. By equilibrium dialysis, lysophospholipase bound 3.820 g of detergent/g of protein in 1% sodium dodecyl sulfate and 0.506 g of detergent/g of protein in 10 mm sodium deoxycholate. In addition, monomeric protein demonstrated enhanced binding of detergent as evidenced by its aberrantly rapid electrophoretic mobility in 1%, but not 0.1%, sodium dodecyl sulfate. The hydrophobic nature of this protein, which accounts for 10% of the protein of the eosinophil, may contribute to its unique propensity for crystallization in vivo.

RESULTS

Purification of Human Eosinophil Lysophospholipase—The chromatographic purification of lysophospholipase activity from sonicates of 2 × 10⁹ leukocytes yielded a protein that was homogeneous as evidenced by single Coomassie Blue staining bands on alkaline, acidic, and SDS-polyacrylamide gel electrophoretic analyses of 10–40 pg of protein. The overall yield was 38% with a 10.7-fold purification and a specific activity of 23.4 units/mg (Table I). In two additional purifications, the yields were 30% and 22%, the purifications 10.2-fold and 14.7-fold, and the specific activities 42.7 and 69.8 units/mg, respectively. Purified enzyme (170 μg/ml), after concentration 4-fold by ultrafiltration in the presence of 0.3 M dithiothreitol and standing overnight at 4 °C, formed di- CALLYAMIDED-shaped Charcot-Leyden crystals, as assessed by light microscopy.

Amino Acid Composition and Molecular Weight Determination—The amino acid compositions of eosinophil lysophospholipase obtained by chromatographic purification and by solubilization of Charcot-Leyden crystals were essentially the same (Table II). The calculated residues per mol normalized to integral numbers of arginine residues and based on the Mₙ of the enzyme estimated as 17,400 from SDS-polyacrylamide

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2 The abbreviations used are: SDS, sodium dodecyl sulfate; pCMB, p-chloromercuribenzoic acid.
gel electrophoresis yielded a minimum $M_c$ of 16,720 for the chromatographically purified preparation and 16,027 for the crystal-derived preparation of lysophospholipase. Because of limited amounts of protein, 48-h and 72-h hydrolysates were not performed, nor were separate analyses conducted for tryptophan.

The sulfhydryl residues in lysophospholipase were determined by two additional methods. Titration of free sulfhydryl groups was assessed by quantitation of binding of $[14C]$pCMB. Separation of protein-bound ligand from unbound ligand was achieved by centrifugation over Sephadex G-25 columns, which also allowed aggregated protein to be retained. Lyso-

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Chromatographically purified</th>
<th>Crystal-derived</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mol amino acid/mol lysophospholipase</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Observed</td>
<td>Integer</td>
</tr>
<tr>
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<td>His</td>
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<tr>
<td>Arg</td>
<td>8.00</td>
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</table>

* Based on integral number of arginine residues to approximate $M_c$ of 17,400.

$^*$ S-Carboxymethylcysteine.

1.12 and 1.0 in two determinations; and for tetrameric enzyme was 1.0 in a single determination.

No amino-terminal amino acids were found when 3.5 and 10.1 nmol of the two different chromatographic lysophospholipase preparations were analyzed by automatic Edman degradation. Digestion of crystal-derived lysophospholipase with pyroglutamate aminopeptidase failed to unblock the amino terminus.

Both the chromatographically derived and the crystal-de-

FIG. 1. Ferguson plots of the electrophoretic migration of purified eosinophil lysophospholipase and six reference proteins in 0.1% (bottom) and 1% (top) SDS with polyacrylamide gels of different acrylamide concentrations. $R_c$ was determined relative to the distance of migration of bromphenol blue. The migration of lysophospholipase (LPLase) was aberrantly fast in 1% SDS and not in 0.1% SDS, relative to the six reference proteins (1, lactalbumin ($M_c = 14,400$); 2, soybean trypsin inhibitor ($M_c = 20,100$); 3, carbonic anhydrase ($M_c = 30,000$); 4, ovalbumin ($M_c = 43,000$); 5, bovine serum albumin ($M_c = 67,000$); 6, phosphorylase $b$ ($M_c = 94,000$).
**Detergent Binding of Eosinophil Lysophospholipase**—The hydrophobic nature of lysophospholipase, suggested by avid binding to hydrophobic chromatographic media and by the aberrant electrophoretic migration in 1% SDS, prompted an analysis of its binding of detergents. As determined by equilibrium dialysis (Table III), at 0.1% SDS, lysophospholipase bound 0.427 g of SDS/g of protein and this binding increased markedly in 1% SDS to 3.820 g of SDS/g of protein. Similarly, the binding of deoxycholate to lysophospholipase increased progressively with increasing concentrations of deoxycholate from 0.1 to 10.0 mM. Interaction of lysophospholipase with both detergents led to the formation of insoluble protein-detergent complexes which prevented further attempts to assess detergent binding by gel filtration.

**Enzymatic Properties of Lysophospholipase**—Both the chromatographically purified protein and the protein solubilized from Charcot-Leyden crystals functioned as lysophospholipases with $K_m$ values of 23.7 ± 1.2 $\mu$M (mean ± S.E.) and 21.9 ± 1.2 $\mu$M (mean ± S.E.), respectively, that did not differ significantly ($p > 0.05$) (Fig. 2). The $V_{\max}$ was 31.8 nmol/h/mg for chromatographically purified enzyme and 46.8 nmol/h/mg for the crystal-derived enzyme.

The reactivity of eosinophil lysophospholipase varied with its state of purity (Table IV). In eosinophil sonicates, the enzyme was inhibited in a dose-related fashion to about 90% by sulfhydryl group reactants (mercuric ions, $p$-hydroxymercuribenzoate, $p$-chloromercuribenzen sulfonate, N-ethylmaleimide, and iodoacetic acid), indicating that the single cysteine residue of lysophospholipase was necessary for enzymatic function. The enzyme also had properties of a serine esterase and was inhibited in the unpurified state in a dose-dependent fashion to about 90% by diisopropyl fluorophosphate and $N_\alpha,p$-tosyl-L-lysine chloromethyl ketone. Enzymatic activity was not dependent on divalent cations as it was uninhibited by up to 50 mM EDTA. In contrast, three purified lysophospholipase preparations were minimally inhibitable at the maximum concentrations of serine esterase or sulfhydryl reactants (Table IV). To demonstrate that the inhibitable lysophospholipase activity in human eosinophil extracts was due to the lysophospholipase/Charcot-Leyden crystal protein and not to other esterases, eosinophil extracts were subjected to sequential immunoadsorptions with anti-lysophospholipase or nonimmune rabbit IgG. In two immunoadsorptions, 86 and 98% of enzymatic activity was immunoprecipitated by anti-lysophospholipase IgG relative to control nonimmune IgG.

**Discussion**

Charcot-Leyden crystals are composed of a single protein which is detectable immunohistochemically in cell sonicates and in solubilized crystals derived from human eosinophils and basophils (2, 4, 5, 17, 18). We have previously provided initial evidence that the enzyme lysophospholipase from human eosinophils is the component of Charcot-Leyden crystals formed in vitro or in vivo in that both eosinophil lysophospholipase and Charcot-Leyden crystal protein function as lysophospholipases, co-migrate on SDS-gel electrophoresis, and exhibit antigenic identity (2, 4). That chromatographically purified lysophospholipase and the crystal-derived protein both express lysophospholipase activity with Michaelis constants of 25.7 $\mu$M and 21.9 $\mu$M, respectively, which do not differ statistically (Fig. 2), further indicates that the lysophospholipase activities are mediated by the same protein. In addition, both the chromatographically purified and crystal-derived proteins had almost identical amino acid compositions (Table II) and had blocked $\text{NH}_2$-terminal amino acids. These findings indicate that the amino acid composition of Charcot-Leyden crystal protein may be identical with that of native lysophospholipase or may differ in having a few less residues at the carboxyl terminus.

Although rodent (19, 20) and porcine (21) eosinophils express appreciable lysophospholipase activity, Charcot-Leyden crystal formation has not been demonstrated with non-primate eosinophils (22). The reasons that crystallization occurs with the human protein are identified as quantitative and qualitative in the present study. The eosinophil enzyme is quantitatively a prominent constituent of this cell. In the chromatographic purification (Table I), only a 10.2- to 14.7-fold purification is required to yield homogeneous protein from solubilized eosinophil sonicates which contain two-thirds of the protein of the eosinophil-enriched cells. Since eosinophils constitute 90% of the original cells and only a small proportion of enzyme remains in the cell pellet after sonication, about 10% of eosinophil protein is lysophospholipase. Radioimmunoassay measurements of the amount of Charcot-Leyden crystal protein per eosinophil (8.5 pg) (17) compared to the amount of total protein per eosinophil (91.7 pg) (23) also indicate that lysophospholipase represents about 10% of the protein of human eosinophils.

Unreduced eosinophil lysophospholipase contains only a single free sulfhydryl group/mol as determined by reaction with 5,5'-dithiobis-(2-nitrobenzoic acid) (17) and by $[^3]C\text{PMB}$ binding. After treatment with dithiothreitol and carboxymethylation to reduce possible disulfide bonds, the find-
ing of a single cysteine residue/mol by amino acid analyses (Table II) and by the incorporation of 0.958 mol of [3H]iodoacetic acid/mol of denatured protein indicates the absence of additional intrachain disulfide bonding within lysophospholipase. Thus, any interchain disulfide bonding would be limited to dimeric complexes and could not account for polymeric aggregation and crystallization. The occurrence of crystallization in the presence of a molar excess of dithiothreitol and the equimolar incorporations of [3H]iodoacetic acid into the quantity of SDS binding to soluble proteins. This enhanced binding (Table III) approximates the amount of SDS bound by membrane proteins such as glycoporin A (26) and the hydrophobic portion of cytochrome b (27), the terminal complement components (28), human low-density lipoprotein, Semliki Forest virus envelope, and human erythrocyte stroma (29). At all concentrations of both anionic detergents, eosinophil lysophospholipase formed insoluble complexes and the binding ratios reflect the presence of detergent in these insoluble complexes. Because the binding studies were performed at pH 7.0 and 8.9 for SDS and deoxycholate, respectively, which are above the isoelectric points of 5.5-5.7 for this protein (17), electrostatic binding of the anionic detergents would not contribute to the precipitation of the detergent complexes, as hypothesized for myelin basic protein (30).

The prominent amounts of SDS bound by eosinophil lysophospholipase at 1% detergent concentration are also evidenced by the aberrantly rapid migration of this protein demonstrated on Ferguson analysis (Fig. 1) of SDS-gel electrophoresis in 1% SDS. The augmented charge contributed by the increased bound SDS caused the protein to migrate with an apparent M, less than the valid M, estimate of about 17,400 in 0.1% SDS and would account for the M, estimate of about 13,000 determined for Charcot-Leyden crystal protein in 8% polyacrylamide gels in 1% SDS (5). While the augmented binding of SDS and deoxycholate found by equilibrium dialysis might represent detergent binding to polymeric aggregates of this protein, the results of SDS-gel electrophoresis in 1% SDS confirm the augmented hydropiobiolytic of monomeric, nonaggregated lysophospholipase. From its amino acid composition (Table II), 34% of the residues of the protein are hydrophobic (Leu, Val, Ile, Met) and aromatic (Phe, Tyr) amino acids, an amount in excess of the 27% average for 314 diverse proteins (31). Definition of specific hydrophobic domains within the enzyme, which account for its augmented detergent binding, await determination of the amino acid sequence.

In its unpurified state, eosinophil lysophospholipase is inhibited by sulphydryl group reactive reagents and by diisopropyl fluorophosphate (Table IV). In contrast, the purified enzyme, in aqueous solution, either purified chromatographically or obtained by crystallization, is not as susceptible to inhibition by these reactants (Table IV). That the predominant lysophospholipase activity of human eosinophils is attributable to the enzymatic activity of this purified enzyme is supported by finding only a single peak of enzyme activity on each of the three chromatographic purification steps. Further, the lysophospholipase activity of eosinophil sonicates is fully immunoprecipitable with the IgG fraction of monospecific rabbit anti-lysophospholipase antibody.

These results indicate that the inhibitors are reacting with lysophospholipase in the eosinophil sonicates and that this enzyme has features of a serine- and sulphydryl-dependent

### Human Eosinophil Lysophospholipase

#### TABLE IV

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration</th>
<th>M</th>
<th>% activity</th>
<th>Purified lysophospholipase*</th>
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<tr>
<td>Iodoacetic acid</td>
<td>10^-3</td>
<td>74</td>
<td>97 (94-101)</td>
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<td></td>
<td>10^-2</td>
<td>39</td>
<td>91 (73-102)</td>
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<td></td>
<td>10^-1</td>
<td>11</td>
<td>80 (51-97)</td>
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<tr>
<td>N-Ethylmaleimide</td>
<td>10^-4</td>
<td>34</td>
<td>96 (79-107)</td>
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<td>4</td>
<td>87 (67-103)</td>
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<tr>
<td>p-Chloromercuribenzenesulfonate</td>
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<td>19</td>
<td>101</td>
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<tr>
<td></td>
<td>10^-3</td>
<td>6</td>
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<td></td>
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<td>p-Hydroxymercuribenzoate</td>
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<td>86 (68-103)</td>
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<td></td>
<td>10^-4</td>
<td>19</td>
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<td></td>
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<td>100</td>
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<td></td>
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<td>Disopropyl fluorophosphate</td>
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<td>Na\textsubscript{a}-p-Toylyl-L-lysine chloromethyl ketone</td>
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<td></td>
<td>10^-2</td>
<td>12</td>
<td>89 (72-106)</td>
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*Three different preparations, one chromatographically purified and two derived from Charcot-Leyden crystals, were assayed; only one was assayed for p-chloromercuribenzenesulfonate.
esterase. An intact sulfhydryl residue is accessible during purification of the enzyme as judged by binding and elution from the organomercurial column. Further, the cysteine within denatured and reduced enzyme can react quantitatively with iodoacetic acid and iodoacetamide. The binding of 0.986 mol of $[^14C]$pCMB per mol of undenatured lysophospholipase probably reflects binding to unaggregated enzyme since between 65 and 96% of lysophospholipase was not recovered from the Sephadex columns. Although large aggregates of enzyme were removed by centrifugation at 32,000 × g before enzyme assay, hydrophobic aggregation of enzyme molecules or some related conformational change may account for the resistance of purified lysophospholipase to inhibition. The unique amphipathic, detergent-like properties of lysophospholipids may allow these compounds access to the active site within the purified enzyme.

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REFERENCES
HUMAN EOSINOPHIL LYSOPHOSPHOLIPASE

SUBLATURAL MATERIAL TO

HUMAN EOSINOPHIL LYSOPHOSPHOLIPASE PROTEIN (LYSOPHOSPHOLIPASE)  

PEER F. KOLLER, SARV S. RAVAT, AND ERNKA KRAWETZ  

EXPERIMENTAL PROCEDURES

Materials: 

- Synthetic phosphatidylcholine, egg-yolk phosphatidylcholine, and phosphatidylethanolamine were obtained from Avanti Polar Lipids, Inc., Alabaster, AL. 
- N-alkylphosphatidylcholines were obtained from Lipoid, GmbH, Veltheim, Germany. 
- N-alkylglycerol-3-phosphate and other lipids were obtained from Sigma-Aldrich. 
- All other reagents were of analytical grade.

Methods: 

- Preparation of lysosomes: Human eosinophils were isolated from healthy donors by centrifugation at 32,000 rpm and subsequently washed and resuspended in PBS containing 1% bovine serum albumin (BSA) and 0.1% sodium azide.
- Isolation of lysosomes: Lysosomes were isolated by differential centrifugation and subjected to density gradient centrifugation.
- Enzyme activity: Lysophospholipase activity was measured by monitoring the release of fatty acid from phosphatidylcholine.
- Western blot analysis: Protein bands were detected using specific antibodies and visualized using an enhanced chemiluminescence system.

RESULTS

- Lysosomes from human eosinophils were isolated and characterized. 
- Lysophospholipase activity was detected in lysosomes from human eosinophils.
- Western blot analysis revealed the presence of a specific protein band that was used for further studies.

DISCUSSION

- The results provide evidence for the involvement of lysophospholipase in human eosinophils.
- Further studies are needed to understand the role of lysophospholipase in eosinophil function.

ACKNOWLEDGMENTS

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REFERENCES