Fatty Acyl Amidases from Dictyostelium discoideum That Act on Lipopolysaccharide and Derivatives

II. ASPECTS OF SUBSTRATE SPECIFICITY

(Received for publication, April 9, 1982)

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The substrate specificities of two fatty acyl amidases partially purified from the slime mold Dictyostelium discoideum have been studied. The amidases act on lipopolysaccharide derivatives, such as (4-O-phosphoryl-N-β-hydroxymyristyl-α-glucosaminyl)-β-(1 → 6)-N-β-hydroxymyristyl-d-glucoamine-1-phosphate (III) in a sequential manner. Amidase-I removes the β-hydroxymyristyl residue present on the amino group adjacent to the 1-phosphate and the product formed is a substrate for amidase-II; the latter removes the remaining β-hydroxymyristyl residue from the distal amino group. Compound III itself is resistant to amidase-II. Removal of the C-1 or C-4 phosphate groups does not influence recognition by the amidases or their sequential action. Both amidases are specific for long chain fatty amide linkages. Thus, a formyl group on the glucosamine amino group adjacent to the C-1 phosphate is not hydrolyzed by amidase-I; however, this substituent does not hinder the action of amidase-II on the distal fatty acyl amide. The presence of the β-hydroxyl group in myristyl-amide residues is not required for hydrolysis. Further, while amidase-I requires disaccharide structures for its action, amidase-II acts on monosaccharides as well. Finally, the effects of a variety of substrate analogs and divalent ions on the activity of the enzymes are reported.

In previous papers, we have reported on the structural elucidation of the lipopolysaccharide of a heptoseless-mutant of Escherichia coli (1-3). During these investigations, two long chain fatty acyl amides were discovered, which were found to remove the β-hydroxymyristyl residues of LPS in a sequential and specific manner (3). In view of the unusually interesting activities of these enzymes and their potential use in structure-function studies of LPS and other related biologically important molecules, we have carried out further work on their purification (4).

The specificity and sequential action of the enzymes is highly intriguing. Amidase-I hydrolyzes the fatty amide linkage adjacent to the C-1 phosphate in the LPS derivatives, e.g., compound III (Fig. 1), while amidase-II cleaves the distal amide but only subsequent to the action of amidase-I. To better understand the mode of action of these enzymes, we have carried out experiments to define the constraints of substrate specificity. The influence of different structural features, in particular the substituents, and the use of monosaccharide analogs have been explored. The results are reported in this paper.

MATERIALS AND METHODS

LPS and Its Derivatives—Preparation of LPS (I, Fig. 1) from E. coli D33m4 and its derivatives has been described previously (1). The two products obtained on alkaline treatment were separated by ion exchange chromatography on Whatman DE-52 with a gradient of TEAB (0-500 mM) in 50% ethanol as previously described. OH LPS (II, Fig. 1) was thus obtained. Compound III (Fig. 1) was prepared by mild acid treatment of alkali-treated LPS and purified by chromatography on Sephadex LH-20 (1). Compound IIIa, lacking the C-1 phosphate, was isolated as a late eluting peak during purification of compound III; it was also prepared by hydrolyzing compound III in 0.1 N HCl at 100 °C for 30 min. Compound IIIb lacking the phosphate at the C-4 carbon, was prepared by hydrolyzing compound III (250 μM) with bacterial alkaline phosphatase (1.69 units/ml) (Worthington type F) at 58 °C in 50 mM Tris-Cl, pH 8, in the absence of sodium dodecyl sulfate. The reaction was stopped after 2 h by adjusting the pH to 3.0 with 25 mM citrate buffer. The reaction mixture was used immediately for assay of the amidases.

Sugars and Other Reagents—N-Acetyl-glucosamine-1-phosphate, glucosamine-1-phosphate, and glucosamine-6-phosphate were obtained from Sigma. The latter was contaminated by the 1-phosphate isomer. Chitobiose was generously provided by Dr. C. Warren of the Laboratory of Carbohydrate Research, Harvard Medical School and Massachusetts General Hospital. TEAB buffer (6), was prepared as a 2 M solution (pH 7.5-8) and stored at 4 °C.

Synthesis of [14C]Myristic Anhydride—[14C]NaCN (10 mCi) (New England Nuclear) was allowed to react in 0.5 ml of dimethyl sulfoxide with excess bromotridecane (86 μl) for 18 h at room temperature. TLC analysis on silica plates with petroleum ether showed that 96% of the label was incorporated into a fast moving product. After extraction into petroleum ether, [14C]tetradecanitrile was hydrolyzed at 100 °C in concentrated HCl and ethanol (1:9, v/v). After 24 h, 20% conversion to the fatty acid was observed. This was purified first by extraction of an alkaline aqueous suspension with petroleum ether, followed by extraction of the fatty acid from the reacidified aqueous phase into petroleum ether. [14C]Myristic acid was combined with cold carrier to give a specific activity of 106 cpm/nmol and rendered anhydrous by repeated dissolution in benzene and evaporation. The fatty acid was dissolved in carbon tetrachloride and the solution treated with 0.55 equivalents of dicyclohexylcarbodiimide. After 5 h at room temperature, the resulting anhydride was taken up into petroleum ether while leaving behind...
Fig. 1. LPS and its derivatives; schemes for chemical and enzymatic modifications.
Fatty Acyl Amidases from Dictyostelium discoideum

the insoluble dicyclohexyl urea. As shown in the identical preparation of unlabeled anhydride, this preparation was free of dicyclohexyl urea, as analyzed by infrared spectroscopy. The anhydride was stored under vacuum over P₂O₅.

**Fatty Acylation of Amino Groups**—The free amino groups in mono- or disaccharides were nitrilated in methanol containing 5% TEA (v/v) and then over KOH. Four aliquots of the anhydride, each containing roughly one equivalent dissolved in dichloromethane, were added over a course of 4 h. TLC on silica plates showed complete acylation of the substrates. After evaporation, the dry residues were saponified overnight in 0.1 M NaOH, then acidified and extracted with petroleum ether to remove the fatty acid. Gel filtration on a column (1.3 x 50 cm) of Bio-Gel P-4 (200-400 mesh) in 0.1 M aqueous TEAB served as a final purification and desalting step. The resulting fatty acyl derivatives were devoid of detectable free fatty acids.

**Formylation of Amino Groups**—Formylation of the free amino groups in LPS derivatives was performed with 5- to 10-fold excess of ethyl formate in absolute methanol and 5% TEA. Reactions were allowed to proceed overnight at room temperature; complete formylation resulted as shown by electrophoretic analysis on Whatman #1 paper. Methanol was removed in a vacuum centrifuge, and the products were hydrolyzed for 6 to 12 h in 10% aqueous TEA so as to cleave any formyl esters. The formylamide linkage in glucosamine derivatives was stable at room temperature; however, the products were stored at -20 °C.

**Analysis by Thin Layer Chromatography and by Paper Electrophoresis**—Substrates and products of the amidases and their assay mixtures were separated by TLC on silica plates with ethanol-concentrated NH₄OH (6:4, v/v) or on HR/DEAE cellulose plates with isobutyric acid: water:concentrated NH₄OH (66:33:1, v/v/v). Electrophoresis was performed either on Whatman DE-81 for 45 min at 2.75 kV or on neutral Whatman #1 for 30 min at 3.0 kV. In both cases, the buffer used was 0.03 M phosphate, pH 7.1.

Rosner et al. (1, 3) have described the effects of dephosphorylation and deacylation on the TLC and electrophoretic mobilities of LPS derivatives. On silica and HR/DEAE plates, loss of one phosphate group increased the mobility of the product as expected. Loss of a fatty acyl chain decreased the mobility. The effects differed with paper electrophoresis (on the cationic DE-81 or Whatman #1) since interaction with these papers depended not only on charge but also on hydrophobicity; the latter retarded migrations.

**Colorimetric Assays**—The concentrations of different substrates were determined by phosphorase analysis according to Ames (6) or glucosamine determination by the Elson-Morgan procedure as modified by Strominger et al. (7). Radioactivity was determined by scintillation counting in toluene-based scintillant for samples spotted on paper (1) or in Hydrofluor (New England Nuclear) for aqueous samples.

**RESULTS**

**Effect of Mono- and Divalent Cations on Amidase-I**

Amidase I was assayed in the presence of various concentrations of either CaCl₂ or MgCl₂ with compound III to determine whether phosphate-metal ion interactions are important. To avoid chelating the cations in the standard citrate buffer, 200 mM f-mannate, pH 3.0, buffer was used for assays. The enzyme was fully active in this buffer. Concentrations of Ca²⁺ or Mg²⁺ up to 10 mM did not inhibit amidase-I. At 100 mM concentration of the divalent cations, the enzyme was inactive, possibly due to substrate aggregation. The cations behaved similarly.

When assayed in citrate buffer with varying concentrations of potassium chloride, amidase-I was fully active in KCl up to 150 mM concentration. The activity declined by 60% at 500 mM KCl and disappeared at higher concentrations. It is unclear whether the high ionic concentration perturbs the conformation of the enzyme, the substrate, or both.

**The Phosphate Group at C-1 Is Not Required for the Action of the Amidases**

Compound IIa (Fig. 1) lacking C-1 phosphate was treated with amidase-I and amidase-II, separately and together. Analysis by the TLC on HR/DEAE cellulose showed (Fig. 2) that...
Fatty Acyl Amidases from Dictyostelium discoideum

Fig. 4. Sequential decylation of compound-II (OH-LPS-I) by amidases I and II. Reactions containing ~100 μM [32P]compound II (500 cpm/nmol) or ~60 μM [32P]compound III (500 cpm/nmol) were preassembled under "Materials and Methods" and analyzed by electrophoresis on DE-81 paper. Each enzyme treatment was 30 min: 1, compound II incubated without enzyme; 2, compound II incubated with amidase-I alone; 3, compound II incubated first with amidase-I then with amidase-II. Note that a 2-fold dilution results from addition of amidase-II. 4, compound III treated without enzyme; 5, compound III treated with amidase-I alone; 6, compound III treated first with amidase-I then with amidase-II.

Fig. 5. Decylation of N-myristyl-IV by amidase-I. Reactions containing 12 μM N-myristyl-[32P]IV (48,000 cpm/nmol) were prepared as described under "Materials and Methods" and analyzed by TLC on silica gel plates: 1, N-myristyl-IV incubated without amidase-I for 30 min; 2, N-myristyl-IV incubated with enzyme for 30 min. Amidase-I removed the expected hydroxymyristyl group to form the slower monoaetyl derivative (IVa). Amidase-II was without effect on compound IIIa (Fig. 2); however, it did hydrolyze compound IVa forming β-(1 → 6)-diglucosamine 4'-phosphate (IVb, Fig. 1). When compounds III and IIIa were treated side by side with amidase-I and then with amidase-II, rates of hydrolysis were comparable (data not shown). The results show that the presence or absence of the C-1 phosphate group has no significant effect on the mode of action of the two amidases.

The Phosphate Group at C-4' Is Not Required for the Action of the Amidases

Compound IIIb (Fig. 1) was prepared as in "Materials and Methods" by treatment of compound III (Fig. 1) with bacterial alkaline phosphatase. The incubation mixture, without removal of the phosphatase or inorganic phosphate, was treated with amidase-I. Analysis of aliquots at different time intervals by electrophoresis showed normal decylation to form compound IVb (Fig. 3). Subsequent addition of amidase-II to the amidase-I mixture resulted in the removal of the second acyl group to yield the faster travelling compound Vb. Thus, the 4'-phosphate in compound III does not influence the action or specificity of the amidases in any appreciable way.

Fig. 6. Decylation of N-formyl-[32P]IV (3-4,000 cpm/nmol) by amidases I and II. Reactions containing ~50 μM substrate were prepared as described under "Materials and Methods." Enzyme treatment lasted 30 min. A, electrophoretic analysis on Whatman #1 paper. 1, N-formyl-IV treated with amidase-II for 0.5 min; 2, same as in 1 but incubated for 30 min; 3, [32P]compound IV treated with amidase-II for 0.5 min; 4, same as in 3 but treated for 30 min. B, electrophoretic analysis on DE-81 paper: 1, N-formyl-IV incubated without enzyme for 30 min; 2, N-formyl-IV incubated with amidase-I alone for 30 min; 3, N-formyl-IV incubated with amidase-II alone for 30 min; 4, marker N-formyl-[32P]IV; 5, marker [32P]compound-IV. (Note: Compounds in lanes 1 and 2 migrate slightly slower than markers in lanes 4 and 5, presumably due to salt effect of the incubation buffer).

Fig. 7. Decylation of N-[14C]myristyl-α-d-glucosamine-1-phosphate by amidases-I and II. Reactions containing 240 μM (1000 cpm/nmol) substrate were prepared as described under "Materials and Methods" and analyzed by TLC on silica gel plates. Three time points were taken at 0, 30, and 60 min, designated 1, 2, and 3, respectively: A, N-Myr-GlcN-1-P incubated with amidase-I; B, N-Myr-GlcN-1-P incubated with amidase-II.
While it would be interesting to test the disaccharide lacking both phosphate groups in compound III, the derivative was much too hydrophobic for experiments in an aqueous medium.

**The Trisaccharide, (KDO)₃ Chain Is without Effect on the Action of the Amidases**

Alkali-treated LPS (II, Fig. 1) carries the (KDO)₃ chain at the C-3' position. This compound on treatment with amidase-I and then with amidase-II gave the results shown in Fig. 4. Again, amidase-I was active and it converted compound II to the mono-deacylated product (product 1, Fig. 4); amidase-II failed to act on compound II but the product of amidase-I was susceptible to it and fully deacylated product (product 2, Fig. 4) was formed. Thus, the specificity and sequential action of the enzymes is maintained in the presence of the KDO trisaccharide chain at the C-3' hydroxyl group.

**The β-Hydroxyl Groups in β-Hydroxymyristoyl Chains Are Not Required by the Amidases**

β-Hydroxymyristoyl chains present as the amide linkages are unique to the lipopolysaccharide structures in Gram-negative bacteria. The question can be asked if the amidases-I and II have evolved specifically to recognize the β-hydroxymyristoyl groups in the LPS structures. N-myristyl-IV (Fig. 1) containing one myristyl group on the amino group adjacent to C-1-phosphate was prepared ("Materials and Methods"). Amidase-I acted on this compound to form compound IV (Fig. 1). Because of the contamination of this preparation of amidase-I by a phosphatase, the monophosphorylated analog and its amidase-I deacylation products were also formed (see the faster migrating doublet on TLC, Fig. 5). Thus, the β-hydroxyl group is not required for amidase-I action. As shown below with N-myristyl-glucosamine-1-phosphate, a β-hydroxy group is also not required by amidase-II.

**Action of the Amidases on N-Formyl-IV (Fig. 1)**

The possibility was considered that the observed restricted specificity of amidase-II was because this enzyme recognized the protonated free amino group generated by the action of amidase-I. It also seemed possible that the protonated amino group prevented continued action of amidase-I on the second acyl group. The free amino group in compound IV was, therefore, blocked with a formyl group ("Materials and Methods"). When treated with amidase-I, N-formyl-IV lost neither the formyl group nor the fatty acid chain in the second glucosamine ring; the electrophoretic mobility on Whatman #1 paper (Fig. 6B) remained unchanged. Failure to deformylate N-formyl-IV demonstrated the requirement by amidase-I for a long hydrophobic acyl chain as in N-myristyl-IV (Fig. 1). Also, the presence of the formyl group did not relax the specificity of amidase-I, no cleavage of the second β-hydroxy fatty acyl chain being observed.

Amidase-II cleaved the fatty acyl group in N-formyl-IV (Fig. 1) as shown by the appearance of a new faster migrating compound when the mixture was fractionated electrophoretically (Whatman #1 or DE-81 paper) (Fig. 6). Amidase-II did not remove the formyl group since the product N-formyl-V (Fig. 1) was electrophoretically distinct from its unformylated congeners, compound V.

**Amidase-II Acts on Monosaccharide Analogs**

The synthetic N-[¹⁴C]myr-GlcN-1-P (VII, Fig. 9) was resistant to the action of amidase-I (Fig. 7A). In contrast, amidase-II deacylated N-[¹⁴C]myr-GlcN-1-P although this molecule closely resembles the 1-phosphate-containing part of compounds VI and VIII.
Inhibitors of the Amidases

Amidase I—Amidase I was assayed in varying concentrations of the following potential inhibitors: chitobiase, N-acetyl-a-glucosamine-1-phosphate, glucosamine-6-phosphate, N-acetyl-glucosamine-1-phosphate, and compound V at constant concentration of enzyme and substrates. From Dixon plots (1/V versus (I), Fig. 8) inhibition constants were calculated. To maximize inhibition, the substrate concentration used was 50% lower than normal.

Neither glucosamine-6-phosphate nor a-glucosamine-1-phosphate, with the free amino groups, noticeably inhibited amidase I, even up to 800 μM concentration. The two acetylated compounds, chitobiase and N-acetyl-a-glucosamine-1-phosphate, did inhibit the enzyme; the apparent inhibition constants (K_i) were found to be 15 and 60 μM, respectively. Compound IV (Fig. 1), the product of amidase action on III, proved to be the most potent inhibitor; the apparent K_i was estimated at 5.6 μM.

Amidase II—No significant inhibition of amidase II was observed with chitobiase (200 μM), N-acetyl-a-glucosamine-1-phosphate (800 μM), or a-glucosamine-1-phosphate (800 μM).

However, compound III (Fig. 1) was found to be a potent inhibitor of amidase II although it does not appear to be a substrate for the enzyme (data not shown). [32P]Compound IV (51 μM) and varying levels of [32P]Compound III were incubated with amidase II. Concentrations of compound III were determined from the radioactivity remaining at the origin on electrophorograms. At 12 μM concentration of compound III, amidase II was inhibited by 50%, complete inhibition being observed at concentrations of III above 30 μM.

DISCUSSION

The results obtained using a variety of substrate analogs and LPS derivatives suggest that amidase I requires a disaccharide structure with blocked amino groups and a long chain fatty acyl amide for proper positioning within the active site. The monosaccharide analog, N-myristyl-GlcN-1-P (VII, Fig. 9), which retains structural features of the A moiety of compound III (Fig. 1), was not cleaved by amidase I. Furthermore, disaccharides such as chitobiase were more potent inhibitors of the enzyme than the monosaccharides tested. The requirement for a blocked and, therefore, uncharged amino group at site A was illustrated by the failure of a-glucosamine-1-phosphate to inhibit, in contrast to the N-acetyl derivative. Nonetheless, the product of compound III hydrolyzed by amidase I (Fig. 1) inhibited the enzyme despite the cationic ammonium group. Amidase I cleaved long chain fatty amides, such as myristyl amide, on LPS derivatives but did not hydrolyze an N-formyl group present at the same site. A requirement for long chain fatty acyl substrates has also been observed for other fatty amidases, notably sphingomyelinases (8) and non-specific fatty acyl amidases (10). Presumably, these enzymes rely heavily on hydrophobic interactions between the acyl chain and a receptive pocket on the enzyme for binding to the active site.

The specificity of amidase I can be explained by the following model. Compound III (Fig. 1) binds to amidase I at two adjoining sites, a catalytic site which receives the A acyl chain and a noncatalytic site which binds the B chain. In this way, the requirements for a disaccharide structure are satisfied. It is unclear what site a monosaccharide inhibitor might occupy; it may bind to either domain and block access to the authentic substrate. Amidase I will not deacylate N-myristyl-GlcN-1-P, an analog of the portion of compound III containing the site A, or hydrolyze the amide linkage at site B. This failure may arise from preferential binding to the noncatalytic domain or from a requirement for occupancy of the noncatalytic domain in order to activate the catalytic one.

Domains in addition to the catalytic site are often required for the action of other enzymes. Serine proteases hydrolyze peptide bonds via a common catalytic cascade, but their individual specificities depend on interactions between amino acid sidechains near the labile bond and complementary regions on the enzyme that adjoin the active site (10). Two binding domains were resolved in acetylcholinesterase: a site recognizing the labile ester linkage and an anionic site interacting with the quaternary ammonium moiety (11). Molecules binding solely to the anionic site competitively inhibit hydrolysis of acetylcholine.

In contrast to amidase I, amidase II does not require a disaccharide for substrate and will tolerate either blocked or cationic ammonium groups at site A; it is generally less stringent in specificity than amidase I. Unlike enzyme I, amidase II does deacylate N-myristyl-GlcN-1-P (VII, Fig. 9), albeit slowly, even though this analog more closely resembles the monosaccharide portion of compound III bearing site A (Fig. 1). Formation of the amino group at site A does not interfere with hydrolysis at site B (Table 1); thus, the cationic ammonium at site A is not a recognition signal for amidase-II action. Although not deacylated by amidase II, compound III binds to amidase II in a misaligned manner such that neither of the two fatty acyl chains can be hydrolyzed.

A model different from that proposed for amidase I could explain the interaction of amidase II with its substrate. In this case, only one binding site occurs, which primarily recognizes the acyl amide and not the rest of the saccharide moiety. Thus, amidase-II hydrolyzes both mono- and disaccharide.

| Compounds | Sensitivity to hydrolysis by Amidase I | Inhibition of | Amidase I | Amidase II |
|-----------|---------------------------------|--------------|-----------|-----------|
| Compound II | +    | -    | n.d. b | n.d. |
| Compound III | +    | -    | +    | +    |
| Compound IIIa | +    | -    | n.d. | n.d. |
| Compound IIIb | +    | -    | n.d. | n.d. |
| Compound IV | +    | -    | +    | +    |
| Compound Va | -    | +    | n.d. | n.d. |
| Compound Vb | -    | +    | n.d. | n.d. |
| N-Myristyl-IVa | -    | +    | n.d. | n.d. |
| N-Formyl-IV | -    | +    | n.d. | n.d. |
| N-Myristyl-a-glucosamine-1-phosphate (III) | -    | -    | n.d. | n.d. |
| N-Myristyl-glucosamine 6-phosphate (VII) | -    | -    | n.d. | n.d. |
| N-Acetyl chitobiase (IV) | n.d. | n.d. | +    | +    |
| N-Acetyl-a-glucosamine-1-phosphate | n.d. | n.d. | +    | +    |
| a-Glucosamine-1-phosphate | n.d. | n.d. | -    | -    |
| Glucosamine-6-phosphate | n.d. | n.d. | -    | -    |

*Refers to Fig. 1 for structures.

b n.d., not determined.

N-Formyl-IV is not deacylated by either amidase, but is deacylated at site B (Fig. 1) by amidase I.

Refer to Fig. 9 for structures.

^ Apparent susceptibility to hydrolysis. This preparation contained a N-myristyl-GlcN-1-P contaminant.
Fatty Acyl Amidases from Dictyostelium discoideum

The enzyme is indifferent to formylation of the free amino group in compound IV, despite the change in charge, presumably because the A half of the molecule does not strongly interact with the enzyme. Molecular models show that the two amide-linked fatty acids of compound III are relatively far apart; conceivably the B chain can insert into the active site while the A half of the molecule projects away from the enzyme into the aqueous environment.

Amidase-I1 does not hydrolyze compound III, which has two fatty acyl chains, although it is inhibited by this compound. The lack of hydrolysis at site B of compound III may be due to improper orientation of the B chain in the active site when another fatty acyl chain is present at site A. Alternatively, site A of compound III may preferentially insert into the active site, but cannot be hydrolyzed. The affinity of amidase-I for site A is evidenced by its ability to hydrolyze N-myr-GlcN-1-P (VII, Fig. 9). Failure to hydrolyze site A of compound III could arise from the disruption of binding interactions by the bulky C-6 substituent of the \( \beta(1 \rightarrow 6) \) disaccharide.

Lemieux et al. (12) recently proposed that oligosaccharide binding to antibody results from the interaction of a complementary hydrophobic domain on the protein with a hydrophobic face on the oligosaccharide, resulting in a net increase of entropy. If binding to amidase-I follows this paradigm, hydrophilic C-6 substituents could conceivably interfere with apposition of the hydrophobic surfaces. In the more stable chair configuration, hydrophilic groups on the glucosamine moiety occupy equatorial positions in a pseudo-planar arrangement. Bulky C-6 substituents, even without rotating out of plane, could disrupt hydrophobic interactions below the plane.

In summary, amidase-I recognizes two aspects of the compound III structure. First, it distinguishes the disaccharide backbone from monosaccharide, thus requiring some specific interaction with the sugar moieties. It also recognizes the hydrophobic fatty acyl determinants and requires both amide-linked chains in order to act. Amidase-II mainly recognizes the fatty acyl amides but is also sensitive to certain determinants on the sugar backbone. The enzyme is indifferent to the presence of the KDO trisaccharide adjacent to the fatty amide that is cleaved; yet, C-6 substituents on the glucosamine apparently can prevent its action. Both amidases hydrolyze only long chain fatty amides and are insensitive to the presence of phosphate residues at C-1 and C-4.

The present study suggests a number of applications of the amidases to analysis of LPS structure and biological effects. The enzymes are potent tools for effecting specific removal and substitution of fatty acids on LPS. Through their action, one can address structure-function relationships in LPS biosynthesis, lymphocytic activation, membrane structure, and numerous other biological systems.

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