Human Lysosomal Sphingomyelinase: Substrate Efficacy of Apolipoprotein/Sphingomyelin Complexes†

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ABSTRACT: Human apolipoprotein stimulation of sphingomyelin (SM) hydrolysis by sphingomyelinase from human skin fibroblasts has been studied. Apolipoproteins A-I, A-II, B, C-I, and E do not enhance sphingomyelin hydrolysis above control levels. In contrast, apoC-II stimulates sphingomyelin hydrolysis by ~2.5-fold. ApoC-III, the most potent apoprotein activator, stimulates hydrolysis by 3–4-fold. ApoC-III stimulation is not significantly different for the three different isoforms which carry 0, 1, or 2 sialic acid residues. The amino-terminal half of this apoprotein, C-III(1–40), which does not bind to phospholipid surfaces, does not activate sphingomyelinase. In contrast, the carboxyl-terminal half, C-III(41–79), which strongly binds to phospholipid surfaces, stimulates sphingomyelin hydrolysis to the same level as that produced by the intact, full-length apoprotein. Incubation of sphingomyelin vesicles with increasing proportions of apoC-III results in the formation of complexes of increasing apoC-III:SM ratio and decreasing radius. The hydrolysis of sphingomyelin in the 1:50 (mol/mol) complex was more than 2-fold greater than that of the 1:200 (mol/mol) complex. The rate of hydrolysis of egg yolk sphingomyelin in the 1:50 complex was maximal [0.9 μmol h^{-1} (mg of protein)$^{-1}$] at the gel → liquid-crystalline phase transition temperature (T_m) of the complex (40 °C). The rate of hydrolysis fell markedly at either higher or lower temperature. Determination of the apparent K_m and V_{max} values below, at, and above T_m indicated that the temperature dependence of sphingomyelin hydrolysis was attributable primarily to changes in V_{max}. These data suggest that apoC-III enhances the hydrolysis of sphingomyelin by breaking down the organized lipid matrix into much smaller units which can be more easily accommodated at the active site of the enzyme.

Sphingomyelin (SM)[1] is an important phospholipid component of plasma lipoproteins, cell membranes, and arterial tissues. It is hydrolyzed by the enzyme sphingomyelinase (EC 3.1.4.12) to water-soluble phosphorylcholine and ceramide. A genetic deficiency of lysosomal sphingomyelinase causes Niemann–Pick disease, which is characterized by an excessive accumulation of sphingomyelin in the tissues (Brady, 1983). Several proteins which stimulate this enzyme have been studied. Christomanou (1980) has suggested that some patients with the juvenile form of Niemann–Pick disease may be missing a required activator protein. However, Fujibayashi and Wenger (1985) recently reported that the fibroblasts from 46 cases of Niemann–Pick disease type C as well as types A, B, and D had normal concentrations of a sphingolipid activator protein (SAP-2) which they have isolated. ApoC-III, an apolipoprotein from human plasma very low density lipoproteins, has been shown to enhance the activity of lysosomal sphingomyelinase from human skin fibroblasts in vitro (Alpert & Beaudet, 1981). Recent studies in our laboratory have been directed toward understanding the mechanism of this enhanced lipolysis. In the preceding paper (Ahmad et al., 1986), we describe the capacity of apoC-III to convert large SM vesicles to much smaller structures. In this report, we present data which demonstrate a correlation between the size of the sphingomyelin-containing substrate particle and the rate at which its phospholipid is hydrolyzed by lysosomal sphingomyelinase.

Materials and Methods

Egg yolk sphingomyelin and Triton X-100 were purchased from Sigma Chemical Co. (St. Louis, MO). [^{3}H]Methyl iodide was obtained from Amersham (Arlington, IL). Protein assay reagent was purchased from Pierce (Rockford, IL). 1,4-Diazabicyclo[2.2.2]octane was obtained from Aldrich Chemical Co. (Milwaukee, WI). Diethylmalonic acid and its potassium salt were purchased from Calbiochem (LaJolla, CA). Unless otherwised specified, all experiments were conducted in 10 mM DEMA, 1 mM NaN_3, 150 mM NaCl, and 1 mM EDTA, pH 7.2 (specified as DEMA buffer).

[*choline*-(C^3H_3)$_3$]Sphingomyelin was prepared by demethylation of EYSM with 1,4-diazabicyclo[2.2.2]octane (Stoffel, 1975) and remethylation with [$^3H_3$]methyl iodide (Patel et al., 1979). EYSM and ^{3}H-EYSM were dissolved in chloro-

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[1] Abbreviations: EYSM, egg yolk sphingomyelin; TO, triolein; EYPC, egg yolk phosphatidylcholine; DMPC, dimyristoylphosphatidylcholine; LCAT, lecithin–cholesterol acyltransferase; apoC-I, -C-II, -C-III, -A-I, -A-II, -B, and -E, apolipoproteins isolated from human plasma lipoproteins; apoC-III-0, -1, and -2, apoC-III bearing either zero, one, or two residues of sialic acid at Thr-74; LAP-20, a 20-residue lipid-associating peptide with the sequence VSSLLSSLKEYWSSLKESFS (Pownall et al., 1980); LAP-20(11-Pro) and LAP-20(8,15-Pro), lipid-associating peptides in which proline has replaced the normally occurring residue at position 11 or at positions 8 and 15, respectively; DEMA, diethylmalonic acid; T_c, transition temperature; R_s, Stokes radius; DSC, differential scanning calorimetry; EDTA, ethylenediaminetetraacetic acid; HPLC, high-pressure liquid chromatography; LDL, low-density lipoprotein(s); SDS, sodium dodecyl sulfate.

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form/methanol (2:1) in a 15-mL Corex tube, and the solvent was evaporated under a stream of dry nitrogen. Residual traces of solvent were removed under reduced pressure in a vacuum desiccator overnight. Five milliliters of DEMA buffer was added to the dry phospholipid film. Unilamellar vesicles of the above mixture were obtained by sonication using a Heat Systems sonifier (W-350) equipped with a microtip as described previously (Ahmad et al., 1986). The working stock solution was adjusted to 0.7–1.2 mg of sphingomyelin/mL (~600000 cpm/mL) as determined by the phosphorus content (Bartlett, 1959). Following the same procedure, unilamellar vesicles of EYPC and EYPC/EYSM (5 g/g) were prepared. EYSM/TO microemulsions with various initial ratios were prepared by sonication also (Tajima et al., 1983). Apolipoproteins were obtained from human plasma (Morrisett et al., 1977) and purified by HPLC (Hancock & Sparrow, 1984). ApoC-III-O was obtained by treatment of apoC-III-1 with neuraminidase (Brown et al., 1970). ApoC-III(1–40) and apoC-III(41–79) fragments were obtained by thrombin digestion of apoC-III-1 (Sparrow et al., 1977). LAP-20, LAP-20(11-Pro), and LAP-20(8,15-Pro) were prepared by solid-phase peptide synthesis (Pownall et al., 1980). Protein and peptide concentrations were determined by the method of Bradford (1976) or by the fluorescamine assay (Weigele et al., 1972) using bovine serum albumin as a standard.

Substrates were prepared by incubation of each apolipoprotein with EYSM in the desired ratio at 40 °C for 2 h. These mixtures were either used directly as crude or purified by gel filtration on a 1.6 × 90 cm column of Sepharose CL-4B (Ahmad et al., 1986). The desired fractions were combined and then concentrated in a collodion bag (Schleicher & Schuell) under reduced pressure for use in the assay.

Human lysosomal sphingomyelinase was partially purified from skin fibroblasts as described previously (Alpert & Beaudet, 1981) except that the cell sonicate was centrifuged under different conditions (48000*g* for 0.5 h).

The hydrolytic release of [^{3}H]phosphorylcholine from [*choline*-(C^3H_3)$_3$]sphingomyelin was assayed as described earlier (Alpert & Beaudet, 1981). Prior to addition of enzyme, the pH of the substrate solution was brought from 7.2 to 5.0 by addition of 10 μL of 1 M sodium acetate. The total assay reaction volume was 0.1 mL, and substrate concentration was 0.3 mM for most studies. Apparent kinetic parameters (K_m and V_{max}) were determined by using nonlinear least-squares analysis as described previously (Cleland, 1967). Simple Michaelis–Menten kinetics have been used to describe the lipolysis of sphingomyelin. This treatment must be considered nonrigorous because it does not consider each individual protein/lipid recombinant type in terms of the concentration of substrate sphingomyelin at its lipid/water interface. Although values of the kinetic parameters obtained may not be accurate in an absolute sense, these values are still useful for making valid comparisons.

Stokes radii of the various vesicles, microemulsions, and complexes were determined by gel filtration on the same calibrated Sepharose CL-4B column used to estimate the size of apoC-III/EYSM complexes (Ahmad et al., 1986).

RESULTS

To determine the stability of lysosomal sphingomyelinase under assay conditions, the time-dependent hydrolysis of sphingomyelin in EYSM vesicles and in apoC-III-1/EYSM complexes (1:50) was studied (Figure 1). Hydrolysis proceeded linearly over a 48-h time period, regardless of whether hydrolysis was enhanced by apoC-III or not. Hence, the enzyme preparation was considered sufficiently stable to perform assays requiring up to 1 day of incubation.

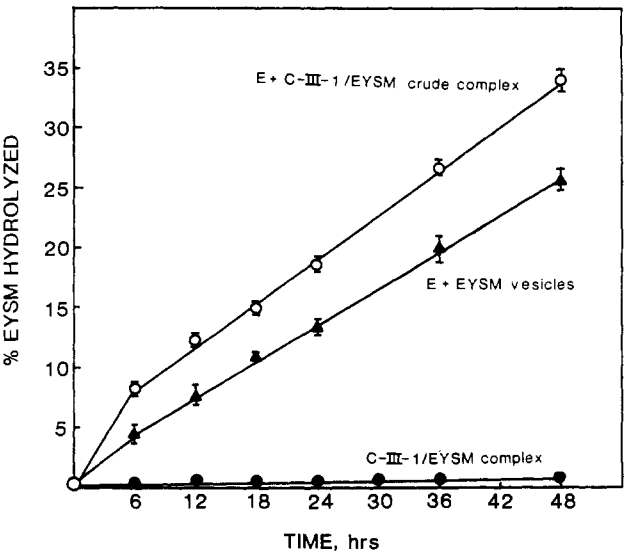

FIGURE 1: Time-dependent lipolysis of EYSM in vesicles and C-III-1/EYSM (1:50 mol/mol) complexes by lysosomal sphingomyelinase. Assays were conducted as described under Materials and Methods, using 9.5 μg of protein from the lysed cell supernatant and 0.3 mM substrate.

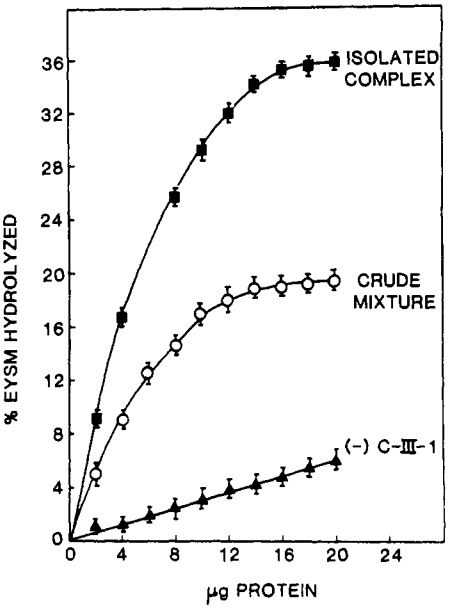

FIGURE 2: Dependence of sphingomyelin lipolysis on amount of lysosomal sphingomyelinase. Three forms of the substrate were assayed: sonicated EYSM vesicles (closed triangles); unfractionated apoC-III-1/EYSM complexes (open circles); and fractionated apoC-III-1/EYSM complexes (closed squares). Assays were conducted as described under Materials and Methods and in the Figure 1 legend. An apoC-III-1:EYSM molar ratio of 1:50 was used for the crude mixture and the complex isolated by gel filtration. The values shown are the average of duplicate measurements of hydrolysis occurring over 24 h.

To identify conditions which allowed reasonable but not maximal hydrolysis of sphingomyelin, activity was measured as a function of enzyme concentration. In the absence of apoC-III-1, sphingomyelin hydrolysis proceeded at a low rate that was linearly dependent on the amount of enzyme present in the assay mixture. In contrast, when apoC-III-1 was present in the crude mixture, sphingomyelin hydrolysis proceeded much more rapidly and displayed a curvilinear dependence on the amount of enzyme present, reaching a maximum rate at ~14 μg of protein (Figure 2). When isolated apoC-III-

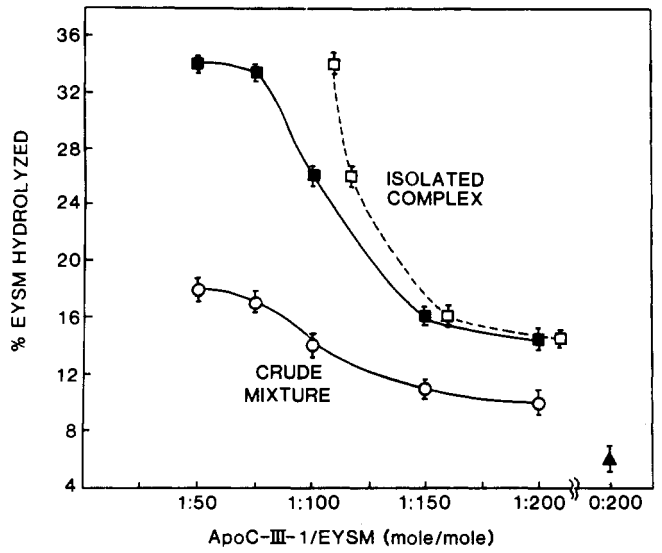

FIGURE 3: Dependence of sphingomyelin lipolysis on protein:phospholipid molar ratio in substrate particles. The enzyme (9.5 μg of cell lysate supernate protein) was incubated with substrate for 24 h. Open circles indicate rates of hydrolysis for unfractionated apoC-III-1/EYSM complexes; closed squares represent *mixing* molar ratios for gel filtered complexes whose *final* molar ratios are indicated by open squares.

1/EYSM complexes were used as the substrate, the rate of hydrolysis more than doubled but continued to show the same curvilinear dependence on enzyme, reaching a maximum rate at ~16 μg of protein. On the basis of these measurements, we selected 8 μg as an appropriate compromise level of enzyme protein for obtaining sufficiently high levels of substrate hydrolysis for meaningful measurements, yet still using assay conditions where maximum hydrolysis was avoided.

To determine the effect of substrate particle composition and size on sphingomyelinase activity, a series of unfractionated and of gel filtered apoC-III-1/EYSM complexes were assayed. Sphingomyelin in unilamellar vesicles (380-Å diameter) was hydrolyzed at a very low rate (Figure 3). When apoC-III-1 was added to these vesicles in a molar ratio of 1:200, a discoidal complex of 305-Å diameter was formed, and sphingomyelin within it was hydrolyzed 2 times faster than that in vesicles. The mixtures containing increasing proportions of apoC-III-1 produced particles of decreasing size and increasing substrate efficacy until a molar ratio of 1:50 was reached, where the rate of sphingomyelin hydrolysis reached 3 times that of EYSM vesicles alone. This dependence of sphingomyelin hydrolysis on substrate particle size became more striking when the complexes used for substrate were isolated by gel filtration. The apoprotein:phospholipid ratios of these isolated complexes are somewhat less than those of the initial incubation mixtures. This is especially true for the 1:50 incubation mixture which, upon purification by gel filtration, had a protein:lipid ratio of 1:110, similar to that formed by the 1:100 incubation mixture (1:110) (Ahmad et al., 1986). Even though the 1:50 incubation mixture produced particles with a stoichiometry (1:110), similar to that of the 1:100 incubation mixture, the diameter of particles formed under the first conditions (204 Å) was significantly less than that formed under the latter (231 Å). Hence, the smallest apoC-III/EYSM complex contained sphingomyelin in a form which was hydrolyzed the most rapidly.

The temperature dependence of sphingomyelin hydrolysis in the apoC-III-1/EYSM isolated complex (1:110) was studied over the temperature range 25–50 °C. At 25 °C, the rate of hydrolysis was ~0.25 μmol h^{-1} mg^{-1} (Figure 4). As the

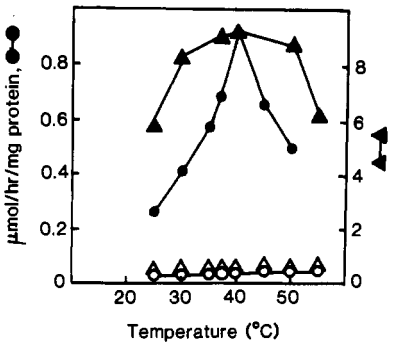

FIGURE 4: Effect of temperature on lipolysis of sphingomyelin by lysosomal sphingomyelinase. For the enzyme, 9.5 μg of cell lysate supernatant protein was used. Closed circles represent fractionated apoC-III-1/EYSM complexes which had a 1:50 mixing molar ratio and a final molar ratio of 1:110 (0.12 g/g); closed triangles represent Triton X-100/EYSM mixed micelles (1 mol:0.6 mol = 1.48 g/g); open circles and triangles represent controls without enzyme. In all cases, 0.3 mM EYSM was used. In a separate control experiment, duplicate aliquots of the enzyme were preincubated for 1 h at 30, 40, and 50 °C before addition of substrate and then assayed at 40 °C. These aliquots exhibited 115, 106, and 101% of the activity shown by the non-preincubated control.

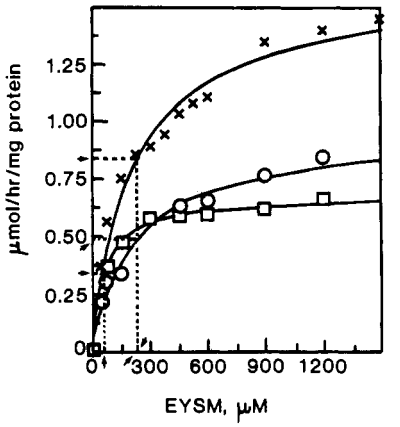

FIGURE 5: Effect of substrate concentration on lipolysis of sphingomyelin by lysosomal sphingomyelinase. The fractionated complex apoC-III/EYSM (1:50 mixing molar ratio) was used as substrate. The incubation time was 12 h, and the temperature was 35 (open squares), 40 (crosses), and 45 °C (open circles). Horizontal arrows indicate $V_{max}/2$, and vertical arrows indicate K_m for each of the three curves.

temperature increased, there was a concomitant increase in hydrolytic rate until the level of ~0.9 μmol h^{-1} mg^{-1} was reached at 40 °C. At higher temperatures, the rate of hydrolysis dropped abruptly; at 50 °C, it decreased to 0.5 μmol h^{-1} mg^{-1}. To evaluate the possibility that the decreased hydrolysis above 40 °C was due to enzyme denaturation, the enzyme was preincubated at 30, 40, and 50 °C before substrate addition; this had little effect on the extent of lipolysis (see legend to Figure 4). We also measured the hydrolysis of sphingomyelin in the Triton X-100/EYSM mixed micelle (1 mol/0.6 mol). At 30 °C, sphingomyelin in this micelle was hydrolyzed at the rate of 8 μmol h^{-1} mg^{-1}. This rose to 9 μmol h^{-1} mg^{-1} at 40 °C and did not diminish significantly at 50 °C. However, at 55 °C, the activity decreased to 6 μmol h^{-1} mg^{-1}. Hence, it appears that under the assay conditions, the enzyme is resistant to thermal inactivation up to 50 °C. This suggests that the peak in rate of sphingomyelin hydrolysis for the apoC-III-1/EYSM complex is a real effect and that the abrupt decrease in hydrolysis above this temperature is not due to enzyme denaturation.

To determine whether the occurrence of maximal hydrolysis rate at the thermotropic transition temperature of sphingo-

Table I: Kinetic Parameters for Lysosomal Sphingomyelinase Hydrolysis of Sphingomyelin in an ApoC-III/EYSM Recombinant (1:50 Mixing Molar Ratio) as a Function of Temperature

temp (°C)	app K_m (μM)	app V_{max} (μmol h^{-1} mg^{-1})
35	62	0.679
40	228	1.608
45	232	0.934

Table II: Human Apolipoprotein and Synthetic Peptide Enhancement of Sphingomyelin Hydrolysis by Lysosomal Sphingomyelinase

additive[a]	sp act. [μmol h^{-1} (mg of protein)$^{-1}$]
none	0.084 ± 0.001
A-I, A-II, B, E, C-I, C-III(1–40), LAP-20(11-Pro), or LAP-20(8,15-Pro)	0.074 ± 0.02
C-II	0.211 ± 0.02
LAP-20	0.263 ± 0.01
C-III(41–79)	0.268 ± 0.02
C-III-2	0.289 ± 0.01
C-III-0	0.301 ± 0.02
C-III-1	0.316 ± 0.03
Triton X-100	9.158 ± 0.1

[a] Mixing molar ratios of 1:50 were used except in the case of Triton X-100 where a 1:0.6 molar ratio was used.

myelin was due to a change in K_m or V_{max}, a series of enzyme saturation experiments was performed at temperatures below (35 °C), at (40 °C), and above (45 °C) the transition temperature of the substrate (Figure 5). The kinetic parameters obtained from these experiments are presented in Table I. On the basis of these data, it appears that the abrupt increase in sphingomyelin hydrolysis at 40 °C can be attributed in large part to a change in V_{max}.

A number of apoproteins in addition to apoC-III-1 are known for their capacity to bind sphingomyelin, suggesting that they might also enhance its lipolysis by lysosomal sphingomyelinase. When tested under standard assay conditions, A-I, A-II, B, E, and C-I did not enhance sphingomyelin hydrolysis above control levels (Table II). ApoC-II enhanced hydrolysis by ~2.5-fold. ApoC-III was clearly the most potent apoprotein activator, stimulating lipolysis by 3.4–4.3-fold. Enhanced hydrolysis by apoC-III was not significantly different for the three different isoforms which carried zero, one, or two sialic acid residues. The amino-terminal half of the apoprotein, C-III(1–40), which does not bind to phospholipid surfaces, does not enhance lipolysis. In contrast, the carboxyl-terminal half, C-III(41–79), which strongly binds to phospholipid surfaces, stimulates sphingomyelin hydrolysis almost to the same level as that produced by the intact, full-length apoprotein. The lipid-associating peptide LAP-20, which exhibits a high affinity for phospholipid surfaces, enhances lipolysis to a level comparable to that of apoC-III-1. However, when the helix-forming potential of this peptide is blocked by inclusion of proline residues at position 11 or at positions 8 and 15, the capacity of this peptide to enhance lipolysis is lost (Table II).

The observation that a number of the apoproteins did not enhance sphingomyelin lipolysis raised the question of whether they actually bind to the lipid substrate and, if so, what the structure of the resulting apoprotein/sphingomyelin complex might be. Accordingly, apoproteins C-I or A-II were mixed with EYSM vesicles and resulting complexes purified by gel filtration chromatography. The complexes formed by apoC-I and apoA-II eluted from the column at significantly lesser volumes (110 and 116 mL) than the volume at which the apoC-III-1/EYSM complex eluted (124 mL). These larger

Table III: Hydrolysis of Sphingomyelin in Crude Apolipoprotein/Sphingomyelin Mixtures and in Fractionated Complexes

apoprotein[a] added	elution vol of complex (mL)	R_s (Å)	% EYSM hydrolyzed in 24 h: crude mixture	% EYSM hydrolyzed in 24 h: fractionated complex
none	102	175	6.0 ± 0.2	
C-III-1	124	80	17.0 ± 0.5	36.0 ± 1.1
C-I	110	133	6.0 ± 0.3	9.8 ± 0.3
A-II	116	110	6.0 ± 0.2	6.6 ± 0.1

[a] All mixing protein:lipid molar ratios were 1:50.

Table IV: Sphingomyelin Hydrolysis as a Function of Size and Composition of the Substrate Particle

substrate	elution vol (mL)	R_s (Å)	% hydrolysis in 24 h
Mixtures[a]			
EYSM/TO (1:3)[b] + C-III	62[e]	[g]	2.4
EYSM + C-III	102[e]	175	18.5
EYSM/TO (1:1)[c] + C-III	102[e]	175	6.8
EYPC	118[e]	106	
EYPC/EYSM (5:1) + C-III	118[e]	106	4.1
Isolated Complexes[a]			
C-III-1/EYPC–EYSM (5:1)	114[f]	108	4.5
C-III-1/EYSM–TO (1:1)[c]	104[f]	173	8.1
C-III-1/EYSM	124[f]	80	35.6
Triton X-100/EYSM[d]	138[f]	45	70–75

[a] Initial protein:lipid molar ratios = 1:50; this was the lowest ratio that produced maximum hydrolysis. [b] Initial lipid:lipid molar ratio = 1:3; final ratio after sonication and centrifugation = 1:(3–4). [c] Initial lipid:lipid molar ratio = 1:1; final ratio after sonication and centrifugation = 1:(0.5–0.7). [d] Triton X-100/EYSM molar ratio = 1:0.6; this was the lowest ratio that produced maximum hydrolysis. [e] Refers to elution volume of lipid particles alone. [f] Refers to elution volume of the isolated complexes. [g] Void volume.

complexes contained sphingomyelin which was lipolyzed only to the extent of 9.8% and 6.6%, respectively, whereas sphingomyelin in the apoC-III/EYSM complex was hydrolyzed to the extent of 36% (Table III).

The relationship of substrate particle size and efficacy was further examined with a series of substrate particles not capable of collapsing to small particles comparable in size to that of the apoC-III-1/EYSM complex. For example, addition of apoC-III-1 to the EYSM/TO (1:3) microemulsion did not reduce the size of this particle which normally elutes in the void volume of the column. Sphingomyelin in this microemulsion was lipolyzed at a rate no greater than that of control values. Previous studies have shown that when apoC-III is added to vesicles of EYPC, apoprotein binds to the surface of the vesicles but does not cause them to collapse to a structure of smaller size (Morrisett et al., 1974). In similar fashion, when apoC-III was added to EYPC vesicles containing 16% sphingomyelin, these vesicles did not collapse to smaller structures either (Table IV). Lipolysis of sphingomyelin in these mixed vesicles proceeded at a rate only slightly above control values, regardless of whether the crude mixture or the isolated complex was used in the assay. Somewhat surprisingly, the SM/TO (1:1) emulsion treated with apoC-III collapses to a complex of exactly the same size as that produced from sphingomyelin vesicles alone. In each of these mixed lipid systems, the smaller the resulting apoprotein/lipid complex, the more extensive the lipolysis of sphingomyelin within it. This phenomenon is aptly illustrated by the Triton X-100/EYSM system which elutes from the gel filtration column in 138 mL corresponding to a Stokes radius of 45 Å. This was the smallest particle examined in the study and by

far the best substrate for sphingomyelinase.

Discussion

The observation by Alpert and Beaudet (1981) that apoC-III stimulated the lipolysis of sphingomyelin by lysosomal sphingomyelinase in vitro and that it did so more effectively than other apoproteins tested prompted us to explore the mechanism of this activation process. Toward this end, a series of apoC-III-1/EYSM complexes spanning a range of protein:lipid ratios from 1:250 to 1:50 were prepared and carefully characterized (Ahmad et al., 1986). Complexes which were richer in apoprotein were also smaller in size, until a limiting diameter of 204 Å was reached for the 1:50 complex. Significantly, as the size of the complex decreased, its efficacy as a substrate for sphingomyelinase increased (Figure 3). Gel filtration chromatography of unfractionated mixtures of apoC-III-1 and EYSM always resulted in the isolation of apoC-III-1/EYSM complexes which were somewhat poorer in apoprotein than the original mixture. These chromatographed complexes were about 2 times more effective as sphingomyelinase substrates than were the crude mixtures (Figure 3). Apparently, excess apoC-III-1 inhibits the lipolytic action of the enzyme; this might occur by apoprotein binding to the polar head groups, thereby blocking them from the enzyme, by apoprotein binding to/blocking the enzyme's active site, or by apoprotein binding to an allosteric site so as to alter the enzyme's catalytic efficiency. The correlation of substrate particle size with substrate efficacy is not unique to lysosomal sphingomyelinase. Other investigators have shown that the activity of plasma lecithin–cholesterol acyltransferase also increases with decreasing size of the substrate particle (Marcel et al., 1980; Jahani & Lacko, 1981; Jonas & McHugh, 1984). Apparently, the steric access of the enzyme to substrate molecules is related to the fraction of those molecules which exist on the curved surface of the discoidal particle [see Discussion of preceding paper (Ahmad et al., 1986)].

The rate of sphingomyelin hydrolysis is dependent not only on the physical form of the substrate as described above but also on its phase state. Maximum hydrolysis of sphingomyelin in the apoC-III-1/EYSM complex was observed at 40 °C, its thermotropic transition temperature (Figure 4). Since T_m is the temperature at which the gel and liquid-crystalline phases coexist, packing defects are therefore maximal, allowing maximum steric access of the enzyme to substrate molecules. A number of other lipolytic enzymes are greatly influenced by the physical state of their lipid substrates. The activities of sphingomyelinase from *Staphylococcus aureus* (Cohen & Barenholz, 1978), phospholipases A_2 from pancreas (Op den Kamp et al., 1975; Goormaghtigh et al., 1981) or cobra venom (Kensil & Dennis, 1979), and lysosomal acid lipases (Burrier & Brecher, 1984) all sharply increase at the T_m of the substrate. It is significant that no sharp maximum in lipolysis rate for sphingomyelinase is observed when the Triton X-100/EYSM complex is used as substrate (Figure 4). On the basis of its gel filtration elution volume, this substrate particle had a Stokes radius of ~45 Å (Table IV). We attribute the lack of a sharp increase in hydrolytic rate for this complex at 40 °C to the small cooperative units allowed by the small particle size and/or the disruption of efficient packing of sphingomyelin molecules in these complexes. These same effects would also be expected to enhance hydrolysis, which is indeed observed, to a level almost twice that of the smallest apoC-III/EYSM complex (Table IV).

Why are apoC-II and apoC-III good activators of lysosomal sphingomyelinase but other apoproteins are not? The answer to this question clearly does not lie only in the sphingomyelin-associating capacity of the apoproteins. ApoC-I and apoA-II bind to sphingomyelin vesicles and even cause them to collapse to somewhat smaller particles; however, these are not as small as those formed by apoC-III (Table III). Space-filling CPK models of segments of these apoproteins predicted to form amphipathic helices revealed that the fraction of hydrophobic surface for the amphipathic helix of apoC-III is significantly greater than that of apoC-I or apoA-II (Sparrow et al., 1980). Apparently, the fraction of hydrophobic helical surface is directly related to the capacity of an apoprotein to bind to the surface of the sphingomyelin bilayer and convert it to smaller complexes.

Whether apoC-III stimulates lysosomal sphingomyelinase in vivo is not certain at the present time. Intermediate density lipoproteins (IDL) and hypertriglyceridemic very low density lipoproteins (HTG-VLDL) both contain apoC-III and are taken up by the specific LDL (apoB/E) receptor of fibroblasts (Gianturco et al., 1978). Internalization and subsequent fusion with the lysosome deliver this apoprotein to the interior of this organelle. It is not known how well apoC-III survives the acidic and proteolytic environment of the lysosome. However, it is known that when this apoprotein is bound to a phospholipid surface, its carboxyl-terminal (lipid binding) half is protected from trypsinolysis while the amino-terminal (non lipid binding) half is fragmented (Sparrow et al., 1977). Hence, if apoC-III is bound to sphingomyelin within the lysosome, at least the segment that enhances sphingomyelin hydrolysis may be protected from degradation. Recently, Fujibayashi and Wenger (1985) reported the isolation from fibroblasts of a sphingomyelin activator protein (SAP-2) which stimulates the hydrolysis of glucosylceramide, galactosylceramide, and sphingomyelin. SDS–polyacrylamide electrophoresis of fibroblast extracts followed by electroblotting and immunochemical staining gave two major bands of molecular weights of 9000 and 10 000. Isoelectric focusing followed by the same visualization method also gave two bands; these had p*I*'s in the range 4.4–4.6. These properties of SAP-2 are very similar to those of apoC-III which exists mostly in two polymorphic forms having molecular weights of about 9300 and 9600 corresponding to forms carrying one or two sialic acid residues. However, apoC-III maps to chromosome 11 (Karathanasis et al., 1983), and SAP-2 maps to chromosome 10 (Fujibayashi et al., 1985). Hence, the two proteins cannot be identical. Nevertheless, it will be instructive to compare the amino acid sequence of SAP-2 (when it becomes available) to that of apoC-III to determine if these proteins contain common secondary structural elements.

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Alternative Carbon Monoxide Binding Modes for Horseradish Peroxidase Studied by Resonance Raman Spectroscopy†

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ABSTRACT: Resonance Raman (RR) spectroscopy and infrared spectroscopy have been used to characterize the three vibrational modes, CO and FeC stretching and FeCO bending, for carbon monoxide bound to reduced horseradish peroxidase, with the aid of ^{13}CO and $C^{18}O$ isotope shifts. At high pH, one species, I, is observed, with ν_{FeC} = 490 cm^{-1} and ν_{CO} = 1932 cm^{-1}. The absence of a band attributable to δ_{FeCO} suggests a linear FeCO unit normal to the heme plane. The data were consistent with I having a strongly H-bonded proximal histidine, as shown by a comparison with imidazole and imidazolate adducts of Fe^{II}PPDME(CO) (PPDME = protoporphyrin IX dimethyl ester), with ν_{FeC} = 497 and 492 cm^{-1} and ν_{CO} = 1960 and 1942 cm^{-1}. At low pH an additional species, II, is observed, with ν_{FeC} = 537 cm^{-1}, ν_{CO} = 1904 cm^{-1}, and δ_{FeCO} = 587 cm^{-1}; it is attributed to FeCO that is H bonded to a protonated distal histidine, the H bond strongly lowering ν_{CO} and raising ν_{FeC}. The appearance of δ_{FeCO} in the RR spectrum suggests that the FeCO unit in II is tilted with respect to the heme plane. At low pH, the population of I and II depends on the CO concentration. I dominates at low CO/protein levels but is replaced by II as the amount of CO is increased. This behavior is suggested to arise from secondary binding of CO, which induces a conformation change involving the distal residues of the heme pocket.

The peroxidases are of much interest in connection with ideas about how O_2 is activated toward O–O bond scission. They contain the same prosthetic group as hemoglobin (Hb) and myoglobin (Mb), noncovalently bound heme coordinated to a histidine residue. Peroxidases do not form stable O_2 adducts, however. Instead, they catalyze the oxidation of organic substrates by peroxide via the agency of highly oxidized intermediates containing the Fe^{IV}=O unit (Roberts et al., 1981; LaMar et al., 1983; Terner et al., 1985). The crystal structure of cytochrome *c* peroxidase (CCP) (Poulos & Kraut, 1980; Poulos & Finzel, 1984; Finzel et al., 1984) has focused attention on differences among heme proteins, particularly with

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