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Mechanism of the Hepatic Lipase Induced Accumulation of High-Density Lipoprotein Cholesterol by Cells in Culture†

Mark Bamberger,‡ Sissel Lund-Katz,* Michael C. Phillips, and George H. Rothblat
Department of Physiology and Biochemistry, Medical College of Pennsylvania, Philadelphia, Pennsylvania 19129
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ABSTRACT: Hepatic lipase can enhance the delivery of high-density lipoprotein (HDL) cholesterol to cells by a process which does not involve apoprotein catabolism. The incorporation of HDL-free (unesterified) cholesterol, phospholipid, and cholesteryl ester by cells has been compared to establish the mechanism of this delivery process. Human HDL was reconstituted with ^{3}H-free cholesterol and [^{14}C]sphingomyelin, treated with hepatic lipase in the presence of albumin to remove the products of lipolysis, reisolated, and then incubated with cultured rat hepatoma cells. Relative to control HDL, modification of HDL with hepatic lipase stimulated both the amount of HDL-free cholesterol taken up by the cell and the esterification of HDL-free cholesterol but did not affect the delivery of sphingomyelin. Experiments utilizing HDL reconstituted with ^{14}C-free cholesterol and [^{3}H]cholesteryl oleoyl ether suggest that hepatic lipase enhances the incorporation of HDL-esterified cholesterol. However, the amount of free cholesterol delivered as a result of treatment with hepatic lipase was 4-fold that of esterified cholesterol. On the basis of HDL composition, the cellular incorporation of free cholesterol was about 10 times that which would occur by the uptake and degradation of intact particles. The preferential incorporation of HDL-free cholesterol did not require the presence of lysophosphatidylcholine. To correlate the events observed at the cellular level with alterations in lipoprotein structure, high-resolution, proton-decoupled ^{13}C nuclear magnetic resonance spectroscopy (90.55 MHz) was performed on HDL_3 in which the cholesterol molecules were replaced with [4-^{13}C]cholesterol by particle reconstitution. The loss of HDL phospholipid following incubation with hepatic lipase or phospholipase A_2 in the presence of albumin caused cholesterol molecules at the surface of the HDL particle to exhibit greater segmental motion as indicated by a decrease in line width of the 4-^{13}C resonance at a chemical shift of 41.70 ppm. Removal of phospholipid molecules was also associated with some redistribution of cholesterol molecules from the core to the surface of the HDL particle and an increased polar group segmental motion of the phospholipid molecules remaining in the surface. It follows that phospholipid and cholesterol molecules are apparently more widely spaced in the surface of modified HDL. These results support the hypothesis that hepatic lipase, via its phospholipase activity, shifts the equilibrium of free cholesterol between HDL and the plasma membrane, resulting in a net delivery of free cholesterol to the cell by a surface transfer process.

The role of high-density lipoproteins (HDL)[1] in lipid metabolism has not been clearly defined. The most popularly held concept is that HDL participates in the removal of cholesterol from peripheral cells to the liver for biliary excretion, a process often referred to as reverse cholesterol transport. Although this proposal is consonant with a number of reports implicating HDL in the removal of cholesterol from cells (Bates & Rothblat, 1974; Stein et al., 1976; Oram et al., 1981), the mechanism involved in the delivery of this cholesterol to the liver has not been established. Uptake may occur by a receptor-mediated endocytosis, with subsequent catabolism of the lipoprotein as a single unit (Roheim et al., 1972; Sigurdsson et al., 1979). Such a process would result in the

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‡ Present address: Department of Biological Chemistry, The Johns Hopkins University School of Medicine, Baltimore, MD 21205.

[1] Abbreviations: apo, apolipoprotein; EC, esterified cholesterol; FC, free (unesterified) cholesterol; HDL, high-density lipoprotein(s); lyso-PC, lysophosphatidylcholine; MEM, Eagle's minimal essential medium; NMR, nuclear magnetic resonance; PBS, phosphate-buffered saline; PC, phosphatidylcholine; PL, phospholipid; SM, sphingomyelin; TC, total (free plus esterified) cholesterol; δ, chemical shift; $\Delta\nu_{1/2}$, line width; TLC, thin-layer chromatography; BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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stoichiometric delivery of HDL components to the liver. There is a growing body of evidence which indicates, however, that, at least in the rat, the uptake of HDL lipid by tissues can occur by an alternate method. For example, Van't Hooft et al. (1981) reported that partial hepatectomy in the rat decreased the removal rates of HDL phospholipid and cholesteryl ester in vivo but did not affect the turnover of HDL apoproteins (Van Tol et al., 1978), suggesting a divergence in the hepatic metabolism of HDL apoprotein and lipid. This observation is supported by several recent studies in vivo which demonstrate a preferential hepatic uptake of HDL cholesteryl ester relative to apoprotein (Stein Y., et al., 1983; Glass et al., 1983). In addition to liver, the delivery of HDL cholesterol to steroidogenic tissues, such as the adrenal and ovary, does not require a corresponding degradation of apoprotein (Gwynne & Hess, 1980; Schuler et al., 1981; Glass et al., 1983).

Recently, there has been an increased interest in the role of the liver enzyme hepatic lipase and similar enzymes located in the rat adrenal and ovary (Jansen & DeGreef, 1981) in the uptake of HDL cholesterol. The activity of hepatic lipase, which is capable of hydrolyzing both lipoprotein phospholipid (PL) and triacylglycerol, has been demonstrated to be inversely correlated with plasma HDL levels (Kuusi et al., 1980; Tikkanen et al., 1981, 1982). There is also evidence from studies in vivo which suggest that hepatic lipase is involved in the delivery of HDL phospholipid and cholesterol (ester) to the liver (Kuusi et al., 1979a; Jansen et al., 1980). This relationship between hepatic lipase and HDL metabolism is strengthened by a previous report from this laboratory (Bamberger et al., 1983) which demonstrated that hepatic lipase can stimulate the delivery of HDL-free (unesterified) cholesterol (FC) to cells in culture. An interesting observation was that the enhanced uptake of HDL-free cholesterol by the cell did not involve a parallel increase in apoprotein degradation, suggesting that hepatic lipase(s) is (are) involved in a selective metabolism of HDL components. In the present study, we further investigate the effect of hepatic lipase on the uptake of HDL lipid by cells in culture. In addition, we examine the changes which occur in lipoprotein structure after the action of this enzyme in an attempt to establish the molecular mechanisms whereby cells accumulate HDL cholesterol.

Experimental Procedures

Materials

Tissue culture supplies were obtained from Flow Laboratories (McLean, VA). [7-^{3}H(N)]Cholesterol (34.6 Ci/mmol), [4-^{14}C]cholesterol (60 mCi/mmol), and [*methyl*-^{14}C]choline chloride (50.5 mCi/mmol) were purchased from New England Nuclear (Boston, MA). The radiolabeled sterols were repurified immediately prior to use by thin-layer chromatography (TLC) on silica gel G plates developed in diethyl ether. Crystalline [4-^{13}C]cholesterol with 90 atom % ^{13}C enrichment was obtained from Merck Co. (Montreal, Canada). Delipidized serum protein was prepared as previously described (Rothblat et al., 1976). Bovine serum albumin (BSA) (fraction V, essentially fatty acid free) was obtained from Sigma Chemical Co. (St. Louis, MO). Silica gel plates were purchased from Analtech (Newark, DE). Plastic plates coated with silica gel IB2 were purchased from Baker Chemicals (Phillipsburg, NJ). All organic solvents were purchased from Fisher Chemical Co. (King of Prussia, PA).

Methods

Lipoproteins. Human HDL ($1.063 < d < 1.21$ g/mL) and HDL_3 ($1.125 < d < 1.21$ g/mL) were isolated from pooled sera by ultracentrifugation in KBr as described by Havel (1955) and modified by Marsh (1976). The lipoproteins were extensively dialyzed against a phosphate-buffered saline (PBS) solution containing 0.15 M NaCl and 0.002 M sodium phosphate, pH 7.4, before use. To remove contaminating low-density lipoproteins, the HDL isolated in the density range of 1.063–1.21 g/mL was applied to a column of heparin–Sepharose CL-6B equilibrated with 0.05 M NaCl and 0.002 M sodium phosphate, pH 7.4 (Quarfordt et al., 1978), and the nonretained fraction was collected and concentrated on a PM-10 ultrafiltration membrane (Amicon Corp., Lexington, MA). The purity of the lipoproteins was assessed by electrophoresis (Noble, 1968) on precast agarose slides (Bio-Rad Laboratories, Rockville Center, NY) at pH 8.6, before use.

Synthesis of Radiolabeled Lipids. [^{3}H]Cholesteryl oleoyl ether was synthesized by the method of Morton & Zilversmit (1982) using [7-^{3}H(N)]cholesterol, unlabeled cholesterol, oleoyl methanesulfonate (NuChek Prep., Elysian MN), and potassium metal in dry benzene. The product was purified by preparative TLC on silica gel G plates developed in petroleum ether/diethyl ether/acetic acid (80:20:1). The final specific activity was approximately 33 mCi/mmol. The ether compound comigrated with cholesteryl ester in the same system. The labeled cholesteryl ether was greater than 98% pure by radioactivity and resisted saponification in alcoholic KOH under conditions which resulted in the hydrolysis of greater than 99% of [^{3}H]cholesteryl ester. To obtain labeled sphingomyelin, Fu5AH rat hepatoma monolayers were incubated with [*methyl*-^{14}C]choline chloride for 72 h in Eagle's minimal essential medium (MEM) containing 0.8 μCi/mL and 10 mg/mL delipidized serum protein. The cells were harvested by scraping with a rubber policeman, and the total lipids were extracted by the procedure of Folch et al. (1957). [^{14}C]Sphingomyelin was isolated by preparative TLC on silica gel H in chloroform/methanol/acetic acid/water (50:30:8:4) as described by Skipski et al. (1964). The purity of the [^{14}C]sphingomyelin was greater than 97% by radioactivity, as determined by TLC using either chloroform/methanol/water (65:25:4) or chloroform/methanol/7 N ammonium hydroxide (60:35:5). The final specific activity was approximately 14 mCi/mmol.

Preparation of Labeled HDL. HDL was radiolabeled with free cholesterol by exchange from Celite (Harleco, Philadelphia, PA) by the method of Avigan (1959). Analysis of the HDL by TLC indicated that greater than 99.5% of the label was present as free cholesterol. Reconstitution of HDL was accomplished by the procedure of Hirtz & Scanu (1970). To obtain lipid-free protein, HDL was extracted with 3:2 (v/v) ethanol/diethyl ether at 0 °C (Scanu & Edelstein, 1971). The ethanol/diethyl ether extracts containing the HDL lipids were brought to dryness by rotary evaporation and stored in 2:1 chloroform/methanol under N_2. To reassemble doubly labeled HDL with [^{14}C]cholesterol and [^{3}H]cholesteryl oleoyl ether, the labeled compounds were added directly to the HDL lipid extract. When reconstituting with [^{3}H]cholesteryl ether, the final specific activity was approximately 550 μCi of [^{3}H]-cholesteryl ether per millimole of HDL cholesteryl ester. To form HDL labeled with both [^{3}H]cholesterol and [^{14}C]-sphingomyelin, the HDL polar lipids were separated by TLC and extracted. After quantitation, an appropriate amount of HDL sphingomyelin was replaced with [^{14}C]sphingomyelin, [^{3}H]cholesterol was added, and the HDL was reconstituted. This additional step was necessary due to the low specific activity of the synthesized [^{14}C]sphingomyelin. The final specific activity of the HDL sphingomyelin after reconstitution

was approximately 800 μCi/mmol. For NMR experiments, the HDL nonpolar lipids were separated by TLC on silica gel G plates using benzene/ethyl acetate (20:1). The cholesterol was removed, quantitated by gas–liquid chromatography, and then replaced with [4-^{13}C]cholesterol. Reconstituted HDL used for experiments in vitro was isolated by ultracentrifugation in the density range of 1.063–1.21 g/mL. HDL that was reconstituted for NMR studies was recovered at a density of 1.125–1.21 g/mL. Prior to NMR experiments, reconstituted HDL was concentrated to about 100 mg/mL by dialysis against "Water-Lock" superabsorbent polymer (Grain Processing Corp., Muscatine, IA). After a final dialysis against a solution containing 0.15 M NaCl, 0.001 M EDTA, and 0.02% NaN_3, pH 7.6, 20 μL of 1,4-dioxane was added, and then D_2O was added as the NMR lock compound to increase the volume by 20%. The lipoproteins were transferred to 10-mm NMR tubes under N_2 and used within 24 h.

Isolation of Hepatic Lipase. Rat hepatic lipase was obtained by liver perfusion and partially purified by heparin–Sepharose chromatography as described by Kuusi et al. (1979b). The triacylglycerol hydrolase activity of the enzyme was monitored by the method of Nilsson-Ehle & Shotz (1976) except that 1 M NaCl was present and serum was omitted. Under these conditions, a unit of hepatic lipase is defined as that amount of enzyme activity needed to generate 1 μmol of fatty acid from triolein per hour at 37 °C. Following column chromatography, the enzyme was concentrated by using a YM-30 membrane (Amicon).

Modification of HDL. To modify doubly labeled lipoproteins for cell culture studies, HDL, at a concentration of 0.9 mM phospholipid, was incubated with 100 units/mL rat hepatic lipase for 4 h at 37 °C. The incubation medium contained 0.15 M NaCl, 0.002 M sodium phosphate, 0.20 M Tris-HCl, pH 7.4, 1% glycerol (v/v), and 1% BSA (w/v) [BSA:HDL phospholipid (mol/mol) = 0.15]. Under these conditions, approximately 25% of the lysophosphatidylcholine (lyso-PC) formed by the action of hepatic lipase remains associated with the HDL particle. After 4 h, no additional hydrolysis of phospholipid occurred. For the control HDL preparation, heat-inactivated hepatic lipase (20 min, 60 °C) was used. For ^{13}C NMR studies, reconstituted HDL_3 was modified with either hepatic lipase or snake venom phospholipase A_2 from *Crotalus durissus* (Boehringer Mannheim, Indianapolis, IN). Treatment of HDL with hepatic lipase was as described above, except that after 4 h an additional amount of albumin was added to a final concentration of 0.35 mol of BSA/mol of phospholipid and the mixture incubated for 1 h at 37 °C. This level of albumin was sufficient to remove the remaining lyso-PC from the HDL. Modification of HDL with phospholipase A_2 was performed at 37 °C under the following conditions: 1.8 mM HDL phospholipid, 0.006 M $CaCl_2$, 0.15 M NaCl, 0.02 M Tris-HCl, pH 7.4, and 1 μg of phospholipase A_2/mg of phospholipid. After the desired level of hydrolysis was obtained, EDTA was added to a final concentration of 0.012 M. Since albumin was not present, all of the lyso-PC generated by the hydrolysis of phosphatidylcholine (PC) remained on the HDL particle. For the control, EDTA was included before the addition of the enzyme. To remove lysophospholipids, the HDL was postincubated for 1 h at 37 °C with 1 mol of BSA/mol of phospholipid. It was determined from preliminary experiments that this higher level of albumin in the postincubation mixture was necessary to remove the greater amounts of lyso-PC generated in the experiments with phospholipase A_2 compared to those which used hepatic lipase and reconstituted HDL_3. To investigate the effect of lyso-PC on the cellular uptake of cholesterol, HDL labeled with ^{3}H-free cholesterol was modified with hepatic lipase in the presence of 1% BSA for 4 h, after which an aliquot of the incubation mixture was postincubated with either a buffered saline solution or a saline solution containing albumin to a final concentration of 1 mol of BSA/mol of phospholipid. In all cases, HDL was reisolated by ultracentrifugation at a density of 1.21 g/mL. It was routinely observed that the removal of a large portion (>80%) of the phospholipid from reconstituted HDL_3 did not affect the stability of the lipoprotein, as determined by electron microscopy, or otherwise alter its composition. Native HDL_3 has been reported to behave in a similar fashion (Pattnaik et al., 1976).

Cell Culture Experiments. Use of the Fu5AH rat hepatoma cell line in studies regarding cholesterol and cholesteryl ester metabolism has been described elsewhere (Rothblat, 1974; Rothblat & Phillips, 1982). Stock cultures were maintained in MEM supplemented with basal-modified Eagle's vitamins and 5% (v/v) bovine serum. Cells were seeded in 35-mm plastic petri dishes, and after 24 h, the medium was replaced with MEM containing 2.5 mg/mL delipidized serum protein. When confluency was reached, the hepatoma monolayers were washed and HDL added in MEM buffered with 27 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.4, containing 2% (w/v) BSA at 37 °C. At the end of the incubation, the monolayers were washed 3 times with cold PBS and the cells harvested with trypsin. The cell pellet was disrupted by sonication, and the lipids were extracted. Aliquots were taken to quantitate cholesterol mass, the total cell-associated radioactivity, and the distribution of the labeled sterol between the cellular free and esterified cholesterol pools.

^{13}C NMR Analysis of HDL. ^{13}C nuclear magnetic resonance (NMR) measurements were made on a Brucker WH 360 spectrometer at 90.55 MHz as described by Lund-Katz & Phillips (1984). The spectra were obtained at 37 ± 1.5 °C. Chemical shifts are given in parts per million downfield from $(CH_3)_4Si$ with internal, aqueous 1,4-dioxane (66.55 ppm) as reference and are accurate to ±0.05 ppm. Line widths ($\Delta\nu_{1/2}$) were measured as the peak width at half-height from expanded printouts of the resonances, with an estimated error of ±10%. All spectra were processed with 2.0-Hz exponential filtering. When it was necessary to compare integrated intensities of selected resonances, the proton decoupler was gated to eliminate the nuclear Overhauser effect. In these experiments, the irradiation for proton decoupling was switched off for a period of at least 5 times the longest spin–lattice relaxation time (T_1) of the resonances compared. The areas from expanded printouts were determined either digitally by a DISNMR peak-picking routine, graphically by planimetry, or gravimetrically by weighing tracings of peaks resolved by assuming Lorentzian line shapes: all methods gave comparable results.

Analytical Techniques. Cellular lipids were extracted by the procedure of Bligh & Dyer (1959) and separated by TLC on silica gel IB2 using petroleum ether/diethyl ether/acetic acid (70:30:1). Lipoprotein lipids were extracted and washed by the procedure of Folch et al. (1957), and the phospholipid species were separated by TLC on silica gel G plates as described by Skipski et al. (1964). Phospholipid phosphorus was determined by the method of Sokoloff & Rothblat (1972), and the amount of phospholipid was calculated by using a conversion factor of 25 for phospholipids and 16.5 for lysophospholipids. After TLC, individual phospholipid classes were extracted from silica gel by the addition of chloroform/methanol/acetic acid/water (50:50:5:10). The gel was pelleted by centrifugation and the supernatant removed. The silica gel

Table I: Effect of Hepatic Lipase on the Uptake of HDL Cholesterol and Sphingomyelin by Fu5AH Rat Hepatoma Cells[a]

	esterified cholesterol			sphingomyelin		
	EC incorpd[b]	FC incorpd[c]	FC delivered[d] (calcd)	SM incorpd[e]	FC incorpd[c]	FC delivered[f] (calcd)
control	6.6 (6.4–6.9)	21.3 (20.4–22.4)	2.6	2.7 (2.7–2.8)	24.8 (24.2–25.6)	1.8
hepatic lipase modified	8.0 (7.8–8.2)	27.1 (26.2–27.6)	3.2	3.0 (2.7–3.2)	31.0 (29.6–32.3)	2.0
modified minus control	1.4	5.8	0.6	0.3	6.2	0.2

[a] Values are the mean (range) of triplicate dishes expressed as micrograms per milligram of cell protein. To determine cholesteryl ester delivery, HDL was reconstituted with [^{3}H]cholesteryl oleoyl ether and ^{14}C-free cholesterol. SM uptake was quantitated by using HDL reconstituted with [^{14}C]SM and ^{3}H-free cholesterol. In either case, cells were incubated with control or hepatic lipase modified HDL at a concentration of 75 μg of HDL-free cholesterol/mL. After 24 h, the uptake of the labeled HDL components by cells was determined. Hydrolysis of HDL phosphatidylcholine was approximately 20%. The results are representative of several experiments performed with doubly labeled, reconstituted HDL. [b] This value is determined from the cellular content of ^{3}H label and the cpm of [^{3}H]cholesteryl ether per microgram of HDL cholesteryl ester. [c] Based on the uptake of labeled HDL-free cholesterol. [d] Calculated from the amount of HDL-esterified cholesterol metabolized and the FC:EC ratio of the HDL (FC:EC = 0.40), assuming stoichiometric delivery. [e] Based on the uptake of [^{14}C]sphingomyelin. [f] Calculated from the uptake of HDL sphingomyelin by the cell and the HDL FC:SM ratio (w/w) = 0.66, assuming stoichiometric delivery via uptake of intact HDL particles.

pellet was washed once with the same solvent mixture. The supernatants were pooled, and water (0.26 × volume) was added to produce a phase separation. Phospholipids were removed from the lower chloroform phase. Protein was assayed by a modification of the Lowry technique (Markwell et al., 1978). Cholesterol mass was determined by gas–liquid chromatography using coprostanol as an internal standard. ^{3}H and ^{14}C were quantitated by liquid scintillation techniques in a Beckman LS7500 counter. To calculate molar ratios, the following molecular weights were used: phospholipid, 750; lysophospholipid, 520; cholesterol, 386; esterified cholesterol, 386; and apolipoprotein (apo) A-1, 28 000. Throughout this paper, the term "esterified cholesterol" refers solely to the cholesterol moiety of this sterol ester.

Results

Incorporation of HDL Cholesteryl Ester and Phospholipid. In an earlier report (Bamberger et al., 1983), we established that in vitro modification of human HDL with rat hepatic lipase resulted in the hydrolysis of HDL phospholipid without affecting the total apoprotein, free cholesterol, or esterified cholesterol levels. When incubated with hepatic lipase modified HDL, rat hepatoma cells typically incorporated 1.5–2 times more HDL-free cholesterol, compared to cells exposed to unmodified HDL, resulting in a corresponding increase in cellular cholesterol mass. Since this effect occurred without an increase in the amount of apoprotein degraded, it appeared that hepatic lipase was stimulating a selective uptake of HDL cholesterol by the cell. Whether this involved an increased delivery of HDL-free cholesterol alone or both free and esterified cholesterol (EC) was not known. Initial attempts to answer this question utilized HDL doubly labeled with ^{14}C-free cholesterol and [^{3}H]cholesteryl ester. The data from several experiments (not shown) indicated that while hepatic lipase modification stimulated the incorporation of both of these HDL components by the cell, the uptake of HDL-free cholesterol predominated over that of esterified cholesterol. The analysis of these experiments is complicated, however, by the fact that [^{3}H]cholesteryl ester can be hydrolyzed by the cell. To circumvent this problem, HDL was reconstituted with labeled cholesteryl ether. Once internalized, this compound is not degraded by the cell and is therefore a useful probe of cholesteryl ester metabolism (Chajek-Shaul et al., 1981). Modification of HDL containing ^{14}C-free cholesterol and [^{3}H]cholesteryl oleoyl ether with hepatic lipase did not alter the esterified to free cholesterol ratio in the HDL or the ^{3}H to ^{14}C ratio. In addition, incubation with hepatoma monolayers for 24 h did not result in the generation of ^{3}H-free cholesterol, indicating that the ether compound was resistant to intracellular hydrolases.

When the delivery of HDL-free and esterified cholesterol to hepatoma cells was quantitated by using reconstituted lipoprotein, it was found that more than 75% of the cholesterol delivered to the cell from unmodified HDL was due to the uptake of lipoprotein-free cholesterol (21.3 compared to 6.6 μg/mg of cell protein) (Table I). Consistent with our previous results, treatment of HDL with hepatic lipase stimulated the amount of HDL-free cholesterol taken up by the cell. Cells incubated with modified HDL also appeared to incorporate more HDL cholesteryl ester. However, when compared on the basis of mass, the stimulated uptake of HDL-free cholesterol was greater than 4 times that of esterified cholesterol. Furthermore, on the basis of the ratio of free to esterified cholesterol in the lipoprotein, it can be calculated that the amount of free cholesterol taken up by the cell as a result of hepatic lipase modification (5.8 μg) is about 10-fold that which would accompany the internalization of esterified cholesterol as intact HDL particles.

These data imply that hepatic lipase selectively augments a transfer to the cell of HDL surface lipid (free cholesterol) compared to core components (cholesteryl ester). To determine if this effect was specific for free cholesterol, hepatoma cells were incubated with HDL containing labeled phospholipid. We chose to use labeled sphingomyelin (SM), since Van Tol et al. (1980) reported that this compound is not a substrate of hepatic lipase. Indeed, we found that modification of HDL containing ^{3}H-free cholesterol and [^{14}C]sphingomyelin did not change the free cholesterol to sphingomyelin ratio in the HDL or the ^{3}H to ^{14}C ratio. The results indicate that while hepatic lipase modification generates a significant increase in the cellular uptake of HDL-free cholesterol, there was no difference in sphingomyelin delivery (Table I).

Effect of Lysophosphatidylcholine. Studies of the various enzymatic activities of hepatic lipase have focused on its ability to degrade lipoprotein phospholipid. By using HDL which had been modified with snake venom phospholipase A_2, we demonstrated that the ability of hepatic lipase to stimulate the uptake of HDL-free cholesterol by hepatoma cells was linked to its phospholipase activity (Bamberger et al., 1983). Furthermore, a high degree of correlation was observed between the extent of phospholipid removal and the accumulation of cellular cholesterol. We wanted to verify that this effect was due to a loss of phosphatidylcholine (PC) from the HDL and not to the presence of lysophosphatidylcholine (lyso-PC), one of the products of phospholipid hydrolysis. To address this question, we took advantage of the fact that albumin is capable

Table II: Effect of Lysophosphatidylcholine on the Uptake of HDL Cholesterol[a]

		lipoprotein			cellular response	
	BSA:HDL PL[b]	FC:PL[b]	FC:PC[b]	FC:lyso-PC[b]	μg of EC/mg of protein	% esterification[c]
control HDL	0.15	0.25	0.33	8.7	6.4	32.9
	1.0	0.25	0.38	*d*	9.5	38.8
hepatic lipase modified HDL	0.15	0.34	0.65	2.2	26.7	57.1
	1.0	0.43	0.63	*d*	23.1	53.0

[a] HDL, labeled with ^{3}H-free cholesterol, was incubated with active or heat-inactivated hepatic lipase for 4 h at 37 °C in the presence of 1% BSA [BSA:HDL PL ratio (mol/mol) = 0.15]. The HDL was then incubated for an additional hour with either a buffered saline solution or a buffered saline solution containing an appropriate amount of BSA so that the BSA:HDL PL ratio (mol/mol) = 1.0. HDL was reisolated at a density of 1.21 g/mL and incubated with Fu5AH hepatoma cells for 24 h at a concentration of 100 μg of free cholesterol/mL in the presence of 2% albumin. Values are the mean of duplicate plates. [b] Molar ratio. [c] This value represents the amount of HDL-free cholesterol taken up and esterified by the cell. [d] No lyso-PC detectable by mass analysis.

of binding lyso-PC and removing it from HDL (Pattnaik et al., 1976). When HDL was modified with hepatic lipase under conditions used in our previous experiments, i.e., in the presence of 1% albumin [BSA:phospholipid (mol/mol) = 0.15], approximately 30% of the lyso-PC generated remained on the HDL after reisolation by ultracentrifugation (Table II). Postincubation of lipase-modified HDL in the presence of 1 mol of albumin per mole of HDL phospholipid did not change the amount of phosphatidylcholine relative to free cholesterol but resulted in a removal of lyso-PC from the lipoprotein. The absence of lyso-PC did not significantly affect the response of the cell to hepatic lipase modified HDL, as judged by the accumulation of cellular cholesteryl ester or the esterification of HDL-free cholesterol. The results in Table II indicate that, under the conditions which these experiments were performed, the presence of lyso-PC is not required to stimulate the delivery of cholesterol to cells.

^{13}C NMR Analysis of HDL. To more fully understand how hepatic lipase stimulates a selective transfer of free cholesterol to cells, the effect of phospholipid hydrolysis on the structure of HDL was examined by nuclear magnetic resonance (NMR) spectroscopy. Figure 1 shows the NMR spectra from control and phospholipid-depleted HDL in which cholesterol was replaced with [4-^{13}C]cholesterol to enhance the signal from this compound. The chemical shifts (δ) are similar for both the control and modified HDL and approximate those reported in the literature for native HDL_3 (Hamilton & Cordes, 1978). As has been previously reported from this laboratory (Lund-Katz & Phillips, 1984), expansion of the [4-^{13}C]cholesterol resonance obtained with control HDL reveals two separate peaks, one corresponding to cholesterol associated with phospholipid on the surface of HDL (δ = 41.7) and the other due to cholesterol not exposed to the aqueous phase and located in the nonpolar core (δ = 42.2) (Figure 1, panel A). Two peaks can also be identified in the samples which had been modified with hepatic lipase (panel B) or phospholipase A_2 (panel C). It is apparent from Figure 1 that lipolysis leads to a change in the line widths and relative intensities of the two cholesterol resonances. This implies that there is an alteration in the motion and packing of cholesterol molecules in modified HDL compared to control HDL.

To further investigate if a loss of phospholipid causes changes in the molecular packing and motions in the HDL particle, the line widths for selected resonances of phospholipid and cholesterol from both control and lipase-modified particles were measured (Table III). The depletion of phospholipid from the HDL surface by the action of hepatic lipase or phospholipase A_2 results in a decreased line width, and therefore increased segmental motion of the *N*-trimethyl region of the phospholipid moiety and both surface and core cholesterol pools. This suggests that these molecules are more

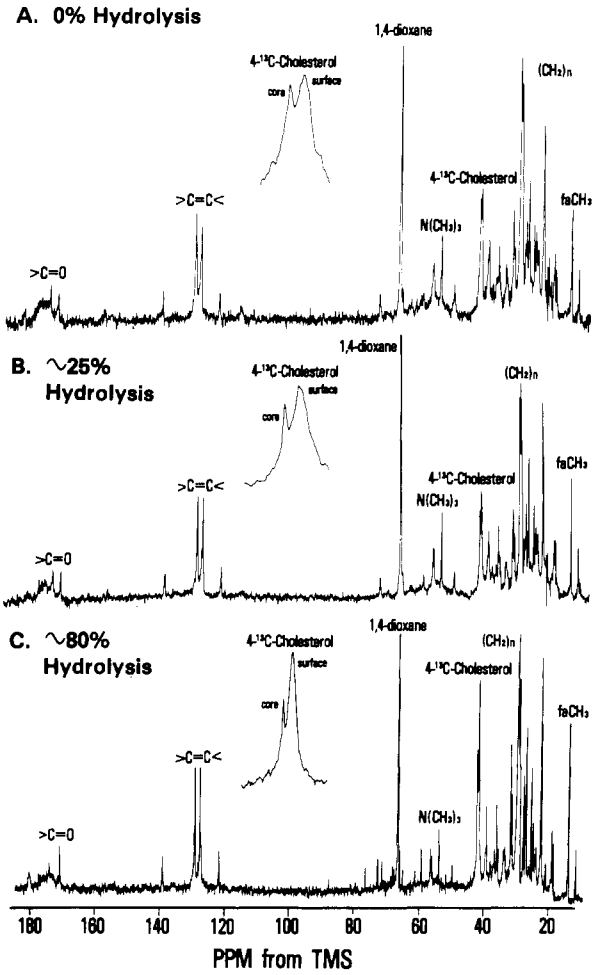

FIGURE 1: Proton-decoupled ^{13}C NMR spectra (90.55 MHz) at 37 °C of human HDL_3 reconstituted with [4-^{13}C]cholesterol. (A) Unmodified HDL_3, 1500 accumulations; (B) hepatic lipase modified HDL_3, 1275 accumulations, ~25% depletion of phospholipid; (C) phospholipase A_2 modified HDL_3, 3700 accumulations, 80% depletion of phospholipid. Spectra were obtained with gated decoupling and a recycling time of 2.9 s so that the nuclear Overhauser effect was suppressed. The expanded regions have the horizontal axis increased by a factor of roughly 20. All spectra were processed with 2.0-Hz exponential filtering.

loosely packed in modified HDL. The line width of the resonances of the phospholipid, cholesteryl ester, and triacylglycerol fatty acid terminal methyl group remains unchanged.

Comparison of the relative intensities of the two resonances originating from free cholesterol in HDL (see inset spectra in Figure 1) suggests that increased removal of phospholipid from HDL causes a progressive movement of cholesterol molecules from the core to the lipoprotein surface. Since the nuclear Overhauser effect is suppressed in the spectra of

Table III: Effect of Phospholipid Removal on the Line Widths ($\Delta\nu_{1/2}$) of Selected Resonances of HDL_3[a]

sample	% FC on surface[b]	$\Delta\nu_{1/2}$[c] $N(CH_3)_3$	surface FC	core FC	fatty acid terminal CH_3
control HDL_3	60	20	56	27	14
hepatic lipase modified HDL_3 (20 ± 5% hydrolysis)[d]	70	13	40	14	14
phospholipase A_2 modified HDL_3 (80% hydrolysis)[e]	80	8	31	9	14

[a] Reported values include 2.0-Hz exponential line broadening applied during processing. For control and hepatic lipase modified HDL_3, the FC:PL molar ratios are 0.3 and 0.4, respectively; the equivalent FC:PC ratios are 0.4 and 0.5. For HDL_3 treated with phospholipase A_2, FC:PC = 1.7 and FC:PC = 7.0. [b] Integration of the NMR resonances was usually reproducible to ±5%. [c] $\Delta\nu_{1/2}$ in hertz (line widths are normalized to that of the 1,4-dioxane internal standard). [d] Mean of five experiments. [e] Mean of two experiments.

control and modified HDL (Figure 1), the intensities of the 41.7 and 42.2 ppm resonances reflect the number of cholesterol molecules residing in either the surface or the core of HDL, respectively (Wehrli & Wirthlin, 1978). Integration of these peaks indicates that approximately 60% of the cholesterol in unmodified HDL (Figure 1A) is located on the surface (Lund-Katz & Phillips, 1984; Table III). Hydrolysis and removal of 20–25% of the phospholipid by hepatic lipase increase this amount to about 70% (Table III). If all the free cholesterol molecules in HDL were located on the surface, the loss of one-fourth of the phospholipid would raise the free cholesterol to phospholipid molar ratio from 0.32 to 0.42, or an increase of 31%. However, when the distribution of cholesterol between the surface and the core is taken into account, the cholesterol to phospholipid molar ratios for the surface monolayers of the control and lipase-modified HDL are 0.20 and 0.32, respectively, or an increase of 60%. Thus, the redistribution of cholesterol potentiates the effect of phospholipid removal on the surface-free cholesterol to phospholipid molar ratio. To confirm that the movement of free sterol from core to surface is a result of the removal of phospholipid and not simply phosphatidylcholine hydrolysis, the postincubation of phospholipase A_2 modified HDL with albumin was omitted. In this situation, all of the lyso-PC formed remains associated with the HDL_3 particle; an earlier ^{31}P NMR study (Brasure et al., 1978) showed that in this situation the microenvironment of the phosphorus in the polar groups of HDL_3 phospholipids probably does not change as a result of lipolysis. Examination of these lipoproteins by ^{13}C NMR spectroscopy indicates that a redistribution of cholesterol does not occur, even when greater than 90% of the phosphatidylcholine has been hydrolyzed (data not shown).

Discussion

There are at least two distinct processes by which cells in culture can obtain cholesterol from lipoproteins in the extracellular medium. In the first case, uptake occurs through a specific binding of the lipoprotein to a membrane receptor, with subsequent internalization and degradation of the entire particle (Goldstein & Brown, 1977). This process results in the stoichiometric delivery of all constituents of the lipoprotein particle. Another means of delivery, not involving macromolecular uptake, is by a surface translocation of sterol between the lipoprotein and the cell membrane [for a review, see Bell (1978)]. In this situation, the rate of movement of lipoprotein cholesterol to the plasma membrane (influx) could be balanced by the transfer of another molecule in the opposite direction (efflux), resulting in no accumulation. Alternatively, a net transfer of lipid can occur if the movement of cholesterol is unidirectional or, as is more likely to occur in vivo, bidirectional but unbalanced. Lipid exchange and transfer are physicochemical processes and do not involve a stoichiometric uptake of lipoprotein components by the cell or the expenditure of metabolic energy. The relative contribution made by these two modes of delivery would depend upon the nature of the cell and the physical properties and composition of the lipoproteins.

Relative Uptake of HDL Lipid by Cells in Culture. In an earlier study (Bamberger et al., 1983), it was established that the hydrolysis of HDL phospholipid by either hepatic lipase or phospholipase A_2 resulted in a lipoprotein with an enhanced ability to deliver cholesterol to cells in culture. This was demonstrated by several criteria, including cellular cholesterol mass and the uptake and esterification of HDL-free cholesterol. Initial investigations into the mechanism of this response indicated that the lipase-mediated delivery of HDL cholesterol was not accompanied by a corresponding increase in apoprotein degradation, suggesting that hepatic lipase was stimulating a physicochemical process. The data given in the present study are consistent with this concept. For example, hepatic lipase had a negligible effect on the delivery of HDL phospholipid to the cell, as monitored by sphingomyelin (Table I). Although it appeared that hepatic lipase modification stimulated the incorporation of HDL cholesteryl ester, this response was minor when compared to the amount of HDL-free cholesterol that was transported into the cell and esterified (Table I). Only one-tenth of the HDL-free cholesterol incorporated by the cell could be attributed to the increased uptake of intact particles (Table I). Integration of these results provides convincing evidence that hepatic lipase is not stimulating the internalization and degradation of the lipoprotein particle, for if it were, the uptake of HDL-free cholesterol would be accompanied by the delivery of 10 and 25 times more esterified cholesterol and sphingomyelin, respectively, than what was was observed (Table I). Instead, the data support the hypothesis, proposed by Jansen & Hulsmann (1980), that hepatic lipase functions by altering the equilibrium of free cholesterol between the HDL and the plasma membrane, resulting in a net influx of free cholesterol by a surface transfer process. The finding that the preferential transport of free cholesterol to the cell is not dependent upon lyso-PC (Table II), coupled with the previously described direct correlation between the removal of HDL phospholipid and the cellular uptake of HDL-free cholesterol, strongly suggests that the flux of cholesterol in this system is determined by the amount of free cholesterol relative to phospholipid. On the basis of available evidence, it is reasonable to assume that this transfer involves the desorption of cholesterol into the aqueous phase, which then acts as an intermediate in the movement of sterol between the lipoprotein and cell membrane (Rothblat & Phillips, 1982; Bojesen, 1982; Lange et al., 1983). The half-time for cholesterol desorption from HDL_3 at 37 °C has been estimated to be 3 min (Lund-Katz et al., 1982).

HDL Structural Changes. The enhanced ability of lipase-modified HDL to transfer cholesterol to cells in culture must be the result of some alteration(s) in the structure and/or physical properties of the lipoprotein molecule. Using ^{13}C NMR spectroscopy, Lund-Katz & Phillips (1984) previously

reported that free cholesterol in reconstituted HDL exists in a fluid state and is present in two physically distinct environments. On the basis of their data, approximately 40% of the cholesterol is located in the core, and 60% is associated with phospholipid at the surface and exposed to the aqueous phase. The present study shows that, as a result of the removal of phospholipid, this distribution is altered. Free cholesterol moves from the core of the lipoprotein to the surface, thereby providing a secondary means, in addition to the initial loss of phospholipid, to increase the free cholesterol to phospholipid ratio in the surface monolayer (Figure 1). Concurrent with the redistribution of cholesterol, there is an increase in the molecular motion of phospholipid molecules, as well as free cholesterol molecules located both in the core and on the surface (Table III). Furthermore, a reorganization of free cholesterol within the HDL particle was found to be dependent upon the removal of lyso-PC. These changes in lipoprotein structure are consonant with the model, depicted from the studies in vitro, in which hepatic lipase raises the chemical potential of cholesterol in HDL via the hydrolysis and depletion of lipoprotein phospholipid.

It is plausible that phospholipid removal leads to a movement of cholesterol to the surface of HDL because there is a need to replace one amphiphilic molecule, phosphatidylcholine, with another amphiphilic molecule, cholesterol. The surface of a human HDL_3 particle is composed of apoproteins (963 amino acid residues per particle at 16 $Å^2$/residue), which is consistent with the α-helices lying in the plane of the lipid–water interface (Phillips & Sparks, 1980), phospholipid (51 molecules at about 65 $Å^2$/residue), and free cholesterol (8 molecules at 40 $Å^2$/residue) (Shen et al., 1977). It can be calculated from these numbers that the major portion (approximately 81%) of the surface area of HDL is taken up by apoprotein, with phospholipid and free cholesterol occupying 17% and 2%, respectively. Given the relatively minor contribution in area by phospholipid molecules on the surface, it is evident that the hydrolysis and removal of a large percentage of phospholipid would not have a significant effect on the total surface area. For example, the depletion of 80% of the phospholipid present would result in the loss of only 14% of the surface area. This space can be easily filled by a molecular expansion or decreased packing density of the remaining phospholipid and apoprotein molecules. Such an effect was observed in these studies (Table III). The expansion of the remaining phospholipid molecules may explain why the loss of large portions of this surface component from reconstituted or native HDL (Pattnaik et al., 1976) does not destroy the integrity or cause aggregation of the lipoprotein. The contribution made by the rearrangement of cholesterol would be minor. It can be estimated that the movement of three molecules of free cholesterol to the surface, such as would result from the loss of 80% of the phospholipid, would fill only about 4% of the total area exposed. This movement of free cholesterol would occur on the time scale of 10 ms to approximately 300 s (Lund-Katz & Phillips, 1984). It would appear, therefore, that although free cholesterol may move from the core to the surface to occupy available space, this redistribution does not play a significant role in replacing phospholipid.

Physiological Significance. The current studies are relevant to several reports which have appeared in the literature demonstrating that, in the rat, there is a preferential uptake of HDL cholesteryl ester compared to apoprotein by certain organs, namely, the liver, adrenal, and ovary (Stein, Y., et al., 1983; Glass et al., 1983). The delivery of HDL-free cholesterol was not determined. A dissociation in the delivery of chylomicron cholesteryl ester and apoprotein has also been observed in the perfused rat heart (Fielding, 1978) and rat heart mesenchymal cells in culture (Friedman et al., 1981; Chajek-Shaul et al., 1981). In these cases, the selective uptake of cholesteryl ester appears to require the presence of lipoprotein lipase (Chajek-Shaul et al., 1982; Stein, O., et al., 1983). Since hepatic lipase or a hepatic lipase-like enzyme has been isolated from the liver, adrenal, and ovary in the rat (Jansen & DeGreef, 1981), it is possible that the reported selectivity in HDL metabolism is due to the presence of this enzyme. Van't Hooft and colleagues (Van't Hooft et al., 1981) have suggested that in vivo, the generation of lyso-PC by hepatic lipase may mediate a preferential incorporation of HDL cholesterol (ester) by cells. The present data imply that lyso-PC does not play a role in the delivery of cholesterol. Furthermore, preliminary experiments in which cells are incubated with HDL containing lyso-PC in the absence of albumin suggest that unless lyso-PC is removed from the HDL particle, there is no hepatic lipase stimulated cholesterol transfer.[2] However, the generation of lyso-PC and fatty acid in the proximity of the plasma membrane may in some way facilitate the uptake of HDL cholesteryl ester by cells. Regarding this possibility, it should be noted that in none of our experiments was hepatic lipase present in combination with HDL and cells. In any event, the present investigation would predict that, for those tissues that contain hepatic lipase(s), free cholesterol, not cholesteryl ester, would be the major contributor to the total amount of HDL sterol delivered to the cell. Although our data suggest that this delivery is via a surface transfer process, we cannot dismiss the possibility that the lipoprotein is internalized by the cell and cholesterol is selectively removed before the particle is returned to the exterior (retroendocytosis) (Aulinskas et al., 1981; Kinnunen & Virtanen, 1979). These two processes (surface transfer and retroendocytosis) are not mutually exclusive.

Whether the binding of HDL to the cell surface is a prerequisite for the delivery of cholesterol is not known [cf. Oram (1983), Tabas & Tall (1984), and Chacko (1984)]. One explanation for our results is that the removal of phospholipid increases the affinity of HDL for a receptor located on the plasma membrane, thereby resulting in an enhanced delivery of sterol to the cell. Preliminary experiments which have quantitated the binding of both control and lipase-modified HDL to rat liver membranes, along with our results using ^{125}I-labeled HDL and Fu5AH hepatoma cells [Table III in Bamberger et al. (1983)], suggest that this is not the case. If an HDL receptor is involved, it is possible that its function may be rather nonspecific, i.e., to increase the local concentration of HDL particles near the plasma membrane, with the net effect on cellular cholesterol levels being governed by the physical properties (e.g., the free cholesterol to phospholipid ratio) of the lipoprotein.

One factor which must be accounted for in any model explaining hepatic lipase function is the localization of this enzyme. Although hepatic lipase is synthesized and secreted by liver parenchymal cells, it appears to be bound on the extracellular surface of the hepatic endothelial cells which line the venous sinusoids (Jansen et al., 1979; Kuusi et al., 1979c). Since these two cell types are separated by the space of Disse, this arrangement might appear to be inconsistent with a hepatic lipase mediated delivery of HDL cholesterol to the hepatocyte, and, therefore, a role for this enzyme in reverse

[2] M. Bamberger and G. H. Rothblat, unpublished observations.

cholesterol transport. It is conceivable, however, that within the space of Disse, a localized concentration gradient of modified HDL is established in the proximity of the hepatocyte, with the outcome being that free cholesterol is delivered to that cell. A similar scheme has been given to explain how free fatty acids, which are generated at the surface of one cell type, the capillary endothelium, by lipoprotein lipase, are delivered to another cell type, the adipocyte (Dietschy, 1978). It is equally possible that after the action of hepatic lipase, HDL-free cholesterol is transferred to another lipoprotein, e.g., a very low-density lipoprotein remnant, which is then taken up by the liver.

In summary, data from both physical studies and experiments in vitro support a role for hepatic lipase in the delivery of cholesterol to the liver or other lipase-containing tissue. How the changes in HDL structure, which are brought about by the removal of phospholipid, stimulate the delivery of cholesterol to cells is not known. This process undoubtedly involves a reciprocal movement of cholesterol molecules between the lipoprotein and the cell membrane. Net cellular accumulation may be the result of either an increased influx of HDL sterol to the cell or a decreased efflux of cellular cholesterol to the medium. Studies are currently under way to investigate this topic.

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Stereochemical Analysis of Peptide Bond Hydrolysis Catalyzed by the Aspartic Proteinase Penicillopepsin†

Michael N. G. James* and Anita R. Sielecki

Medical Research Council of Canada Group in Protein Structure and Function, Department of Biochemistry, University of Alberta, Edmonton, Alberta, Canada T6G 2H7

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ABSTRACT: The X-ray crystal structures of native penicillopepsin and of its complex with a synthetic analogue of the inhibitor pepstatin have been refined recently at 1.8-Å resolution. These highly refined structures permit a detailed examination of peptide hydrolysis in the aspartic proteinases. Complexes of penicillopepsin with substrate and catalytic intermediates were modeled, by using computer graphics, with minimal perturbation of the observed inhibitor complex. A thallium ion binding experiment shows that the position of solvent molecule O39, between Asp-33(32) and Asp-213(215) in the native structure, is favorable for cations, a fact that places constraints on possible mechanisms. A mechanism for hydrolysis is proposed in which (1) Asp-213(215) acts as an electrophile by protonating the carbonyl oxygen of the substrate, thereby polarizing the carbon–oxygen bond, (2) a water molecule bound to Asp-33(32) (O284 in the native structure) attacks the carbonyl carbon as the nucleophile in a general-base mechanism, (3) the newly pyramidal peptide nitrogen is protonated, either from the solvent after nitrogen inversion or by an internal proton transfer via Asp-213(215) from a hydroxyl of the tetrahedral carbon, and (4) the tetrahedral intermediate breaks down in a manner consistent with the stereoelectronic hypothesis. The models permit the rationalization of observed subsite preferences for substrates and may be useful in predicting subsite preferences of other aspartic proteinases.

An understanding of the hydrolytic mechanism of the aspartic proteinase family is of paramount importance, due to the roles that some of its members have in the control of a variety of biological processes (e.g., renin in hypertension, cathepsins D and E in protein turnover). Toward this end, the tertiary structures of three microbial aspartic proteinases have been determined (Hsu et al., 1977a,b; Subramanian et al., 1977a,b; Jenkins et al., 1977). The structure of porcine pepsin (Andreeva et al., 1984) is highly homologous to these enzymes. Inhibitor binding to *Rhizopus chinensis* pepsin (Bott et al., 1982) and to penicillopepsin (James et al., 1982) has also been analyzed by crystallographic techniques. On the basis of these structures, possible binding modes of substrates and catalytic mechanisms have been suggested (James, 1980; James et al., 1977, 1981; Blundell et al., 1980; Andreeva et al., 1981; Foltmann, 1981; Bott et al., 1982). Unfortunately, none of the proposals have been based on refined crystal structures and therefore require further examination in light of the more accurate atomic coordinates now available.

The crystal-structure refinement of penicillopepsin at 1.8-Å resolution has recently been completed (James & Sielecki, 1983). This refinement showed a solvent peak, O39, bound between the carboxyl groups of Asp-33(32)[1] and Asp-213(215) in the final electron density map. The size of this peak is consistent with a neutral water molecule or with an ion (NH_4^+ or H_3O^+). Further experiments reported in this paper indicate that this site is a favorable cationic binding site. This fact imposes important limitations on the hydrolytic pathway.

Additionally, the synthetic analogue of pepstatin Iva-Val-Val-Sta-OEt [Iva = isovaleryl; Sta-OEt = ethyl ester of statine [(4*S*,3*S*)-4-amino-3-hydroxy-6-methylheptanoic acid]] has been refined at 1.8-Å resolution (James et al., 1983). The results of these higher resolution structural studies have given rise to more concrete proposals for the productive mode of substrate binding to penicillopepsin (Hofmann et al., 1984).

The earlier extensive chemical and kinetic data on pepsin and synthetic substrates [see reviews by Clement (1973), Fruton (1970, 1976, 1977), Hofmann (1974), and Knowles

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[1] The sequential numbering of penicillopepsin (James & Sielecki, 1983) is used throughout; the corresponding residue numbering of porcine pepsin (Tang et al., 1973) is in parentheses.

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