CHOLESTEROL ESTERASE - A POLYMERIC ENZYME

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SUMMARY

A direct interaction of cholic acid and pancreatic cholesterol esterase has been shown using labeled bile salt of high specific activity and Sephadex G-50 chromatography. This interaction results in the formation of a protein of M.W. 400,000 which appears to be a hexamer of the protein in pancreatic juice (M.W. 65,000 - 69,000). Rechromatography of the polymer on Sephadex G-200 results in dissociation to the monomeric form. Evidence is provided, suggesting that the polymeric form of cholesterol esterase represents the active enzyme responsible for sterol ester synthesis and hydrolysis.

It has been well documented that there is an absolute requirement for bile salt for the catalytic activity of cholesterol esterase (1-4). Among the bile salts tested, only 3α,7α,12α-trihydroxycholanic acid (cholic acid) and its conjugates, glycocholic acid and taurocholic acid, are effective in enzyme activation. The cofactor function of these bile salts for cholesterol esterase has been shown to be distinct from the detergent effect of these compounds (4). It has also been reported that taurocholate specifically protects pancreatic cholesterol esterase against proteolytic inactivation (5), and there is no effect of the bile salt on the activation or stability of other enzymes of pancreatic juice, e.g., proteases, lipase, amylase and ribonuclease (5). Furthermore, bile salts protect against but cannot reverse inhibition by p-chloromercuribenzoate (6). The possibility of the formation of enzyme-bile salt complex had been considered on the basis of the available data.

In the present report, data are presented which indicate that there is a molecular interaction between the enzyme and bile salts which results in polymerization of the enzyme monomer.

Studies on the direct interaction of bile salt and cholesterol esterase were carried out by Sephadex G-50 column (0.6 cm x 40 cm) chromatography. Sephadex was equilibrated with 0.05 M sodium phosphate buffer, pH 6.2, containing cholic acid-carboxyl-^{14}C (I.C.N.; Sp. act. 52.6 uc/umole; 10^5 dpm/ml). Twenty units of purified cholesterol esterase (6) were applied to the column in 0.2 ml of the buffer, and the elution of enzyme activity was carried out with the same buffer containing labeled bile salt. The flow rate was maintained at 0.5 ml/min, and 0.5 ml fractions were collected for counting and assay of enzymatic activity (7). Figure 1 shows that elution of enzyme activity was accompanied by an increase in the elution of radioactive cholic acid (fractions 6-9); this was followed by a trough in the level of radioactivity in the eluants (fractions 11-14) and then a return to a constant level of elution of labeled cholate. Similar complexing could be accomplished by mixing the enzyme and labeled cholate prior to application on a Sephadex G-50 column equilibrated and eluted with buffer alone. In this case, there was a coincidental elution of bile salt radioactivity and enzyme activity in fractions 6-9, followed by the major radioactive peak of free cholic acid in fractions 11-20.

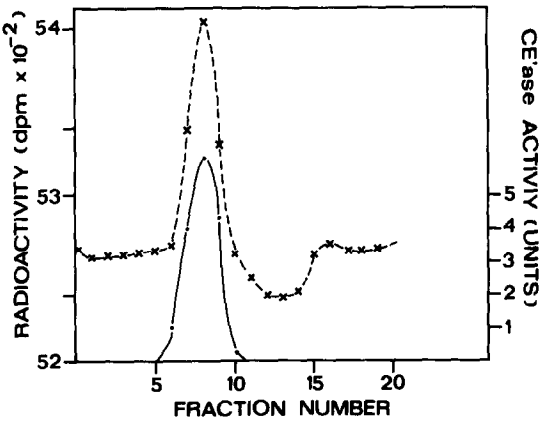

Fig. 1. - Elution of cholesterol esterase (o) and cholic-carboxy-^{14}C-acid (x) from Sephadex G-50 gels equilibrated with 0.05 M phosphate buffer, containing labeled cholic acid. The column was eluted with the same buffer containing labeled cholic acid until the elution of radioactivity was constant. Twenty units of purified cholesterol esterase (CE'ase) were applied to the column and the elution was continued with buffer-labeled cholic acid solution.

Evidence for "polymerization" of the enzyme, due to bile salt complexing, was obtained by Sephadex G-200 column chromatography. Two identical Sephadex G-200 columns (2.5 cm x 100 cm), equipped with flow adoptors at both ends, were prepared for the isolation of the pancreatic cholesterol esterase and the "enzyme-bile salt complex". One of these columns was equilibrated with a 0.05 M sodium phosphate buffer, pH 6.2 (Column I), and the other with a 0.05 M sodium phosphate buffer, pH 6.2, containing 0.05% sodium taurocholate (Column II). The corresponding buffers were also used for the elution of the respective columns.

To obtain the partition coefficient of the original enzyme present in the pancreatic juice, 4 ml of fresh rat pancreatic juice (80-100 mg protein), free of bile, was chromatographed on Column I. Another 4 ml of the pancreatic juice was incubated with sodium taurocholate (2.2 mg/ml, Maybridge Chem. Co.) at 25°C for 5 minutes and was chromatographed on Column II. All chromatographic procedures were carried out at 2-5°C., employing the techniques of ascending chromatography with a hydrostatic pressure head. Ten ml fractions were collected in an automatic fraction collector (LKB) at a flow rate of 20 ml per hour. Each fraction was assayed for cholesterol esterase activity (7) and protein (8). The partition coefficients (K_{av}) and Stokes Radii were calculated (see Fig. 2),

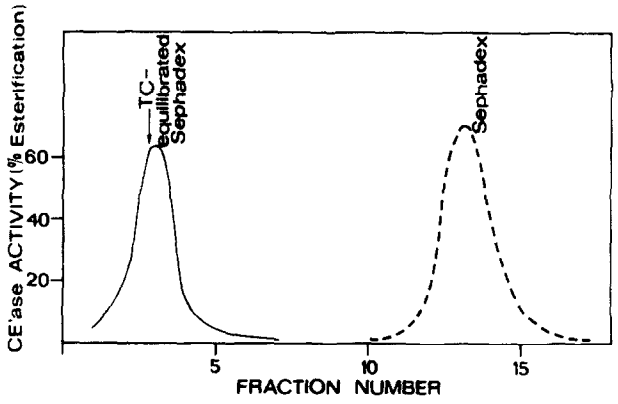

Fig. 2. - Elution profiles of cholesterol esterase activity from Sephadex G-200 equilibrated and eluted with either 0.05 M phosphate buffer, pH 6.2 (———), or buffer containing 0.05% sodium taurocholate (------).

and these values for several standard proteins (shown in Fig. 3) were not affected when bile salt was present during equilibration and elution of the Sephadex column.

Figure 2 shows the elution profile of cholesterol esterase on Sephadex G-200 eluted with buffer (Column I) and on Sephadex G-200 equilibrated with taurocholate and eluted with buffer containing taurocholate (Column II). Cholesterol esterase activity is eluted close to the void volume on a bile salt-equilibrated Sephadex G-200 column (K_{av} = 0.103), compared to the much delayed elution of activity in a non-bile salt environment (K_{av} = 0.43).

From the data in Figure 3, the Stokes radius and the molecular weights of the enzyme were calculated; the M.W. of the enzyme eluted with buffer on Column I was estimated to be 65,000 and that eluted by bile salt-buffer on Column II was 400,000. The molecular weight of the monomer was also determined by electrophoresis of the enzyme and standard proteins of known molecular weights on

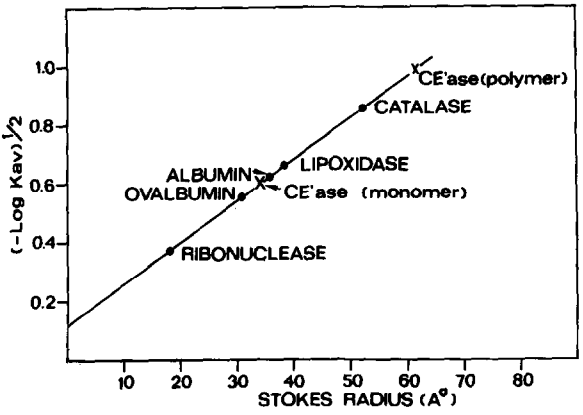

Fig. 3. - Correlation curve for Stokes values (10) and K_{av}. The K_{av} values were determined from Sephadex G-200 elution volumes of proteins of known molecular radii (11). The data are plotted according to Laurent and Killander (12).

$$K_{av} = \frac{Ve - Vo}{Vt - Vo}$$

Ve = elution volume
Vt = total bed volume
Vo = void volume

sodium dodecylsulfate (SDS)-polyacrylamide gel (9), and the M.W. of the cholesterol esterase monomer was calculated to be 69,000.

The enzymatically-active fractions from the taurocholate buffer-eluted Sephadex column were pooled, concentrated and rechromatographed on Sephadex

TABLE I

REVERSIBILITY OF POLYMERIZATION OF CHOLESTEROL ESTERASE

Enzyme Source	Molecular Weight Calculated from Sephadex G-200 Filtration		
	Buffer Elution Column I	Bile Salt-Buffer Elution Column II	Rechromatographed* Column I
Fresh pancreatic juice	65,000	400,000	65,000
35% Acetone precipitate of pancreatic juice	135,000	400,000	65,000

*The active fractions from Column II (bile-salt equilibrated sephadex G-200) were concentrated and rechromatographed on Column I (sephadex without bile salt.)

G-200 without bile salt (Column I). As shown in Table I, the polymeric enzyme (M.W. 400,000) placed on the column was depolymerized and elution of enzyme activity was associated with a protein of M.W. 65,000.

It was reported previously that the M.W. of purified pancreatic juice cholesterol esterase was approximately 136,000 (6). However, it has been found that the initial step in the purification of pancreatic juice cholesterol esterase, which involves fractionation with cold acetone (35%), caused formation of a dimer consisting of identical monomers of M.W. 65,000. The interaction of sodium taurocholate with this dimeric form of the enzyme results in polymerization (Table I). The reversal of this process, i.e. depolymerization, produces the monomer of M.W. 65,000. These observations suggest that the dimeric species of the enzyme, produced by dehydration during organic solvent fractionation, is still capable of forming the active enzyme polymer with the proper conformation.

Evidence that the polymeric form of the enzyme is the active cholesterol esterase was obtained by studies with a serine-group reagent, phenylmethylsulfonylfluoride (PMSF). Using fresh pancreatic juice as the enzyme source, PMSF, added at 10^{-4}M or 10^{-3}M, resulted in little inhibition of enzyme activity (Table II). At 5×10^{-3}M PMSF, inhibition of cholesterol esterase was more

TABLE II

EFFECT OF TAUROCHOLATE ON INHIBITION OF CHOLESTEROL ESTERASE ACTIVITY BY PHENYL METHYL SULFONYL FLUORIDE (PMSF)

Additions to Enzyme*	% Inhibition
PMSF, 10^{-4}M	12
PMSF, 10^{-3}M	16
PMSF, 10^{-3}M + taurocholate**	15
Taurocholate + PMSF, 10^{-3}M	73
PMSF, 5 x 10^{-3}M	64

*The enzyme was incubated for 15 min. with bile salt and/or PMSF, added in the order shown, prior to assay for activity.

**2.2 mg/ml enzyme solution.

apparent. This higher level of PMSF was apparently required due to the levels of proteins other than cholesterol esterase in the juice.

The addition of taurocholate to pancreatic juice containing 10^{-3}M PMSF did not alter the level of enzyme inhibition from that seen with PMSF alone. However, when bile salt was added to the juice prior to PMSF (10^{-3}M) addition, enzyme activity was markedly inhibited. Similar to other esterases, the most probable group for the acyl transfer reaction during the synthesis or hydrolysis of cholesterol esters is the serine hydroxyl group(s) of the enzyme protein. From the inhibition studies with PMSF, it appears that the active serine moiety only becomes fully exposed and highly sensitive to 10^{-3}M PMSF in the presence of bile salt, which has induced the formation of the polymeric form.

It has previously been shown that there is no cholesterol esterase activity in the absence of trihydroxycholanic acid (4), which has now been shown to induce formation of a high molecular weight polymer. Furthermore, in the presence of these bile salts, the esterase is resistant to proteolytic enzymes, denaturation, and inhibition by p-chloromercuribenzoate (6). These findings, together with

those on PMSF inhibition, suggest that the polymeric form of cholesterol esterase is the active enzyme in synthesis and hydrolysis of sterol esters.

ACKNOWLEDGMENTS

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