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Glycosyl-phosphatidylinositol-specific phospholipase D

Interaction with and stimulation by apolipoprotein A-I

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Glycosyl-phosphatidylinositol-specific phospholipase D (GPI-PLD) is an amphiphilic protein which, in serum, is associated with high-density lipoproteins (HDL). It is shown that the major component of the HDL fraction, apolipoprotein A-I (apo A-I), is responsible for this association. In the absence of apo A-I, purified GPI-PLD occurred as virtually inactive aggregates which became disaggregated by apo A-I. The enzyme/apo A-I complex efficiently hydrolyzed the solubilized GPI-anchored substrate, acetylcholinesterase. Triton X-100 was also able to dissociate aggregated GPI-PLD, however, it strongly inhibited enzyme activity at detergent concentrations above the critical micellar concentration.

Glycosyl-phosphatidylinositol; Phospholipase D; Acetylcholinesterase; High-density lipoprotein; Apolipoprotein A-I

1. INTRODUCTION

A large number of cell surface proteins have been shown to be attached to cell membranes by a glycosyl-phosphatidylinositol (GPI)- anchor [1] from where they may be hydrolyzed by GPI-specific phospholipases. In mammals, the only purified and well characterized GPI-specific phospholipase is of the D-type (GPI-PLD) producing phosphatidic acid as one of the products. GPI-PLD was originally discovered in serum [2,3] and later in whole brain [4], neurons [5], and cerebrospinal fluid [6], in the islets of Langerhans [7], liver [8,9], liver mast cells [10], and milk [6]. GPI-PLD has been purified and characterized from serum [6,11–13], and its primary sequence is known from bovine liver cDNA [8].

GPI-PLD is an amphiphilic protein which, in the absence of a detergent, forms high molecular weight aggregates, and in serum, is associated with high-density lipoproteins (HDL) [6]. We now report that GPI-PLD is able to associate with the major component of the HDL fraction, apolipoprotein A-I (apo A-I) which disaggregates the high molecular weight forms of GPI-PLD. While aggregated GPI-PLD is virtually inactive, the enzyme/apo A-I complex efficiently hydrolyzes the solubilized GPI-anchored substrate, acetylcholinesterase (AChE). Triton X-100 was also able to dissociate aggregated GPI-PLD, however, it inhibited enzyme activity at detergent concentrations above the critical micellar concentration (CMC). A preliminary account of the present results has been presented previously [14].

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Abbreviations. GPI, glycosyl-phosphatidylinositol; PLD, phospholipase D, HDL, high-density lipoprotein; apo, apolipoprotein; AChE, acetylcholinesterase; mf-AChE, membrane-form of AChE; s-AChE, soluble AChE; CMC, critical micellar concentration.

2. MATERIALS AND METHODS

2.1. *Biological material*

GPI-PLD was purified from bovine serum essentially as described by Hoener and Brodbeck [6]. The membrane form of acetylcholinesterase (mf-AChE) was purified by affinity chromatography from bovine erythrocytes as described by Brodbeck et al. [15]. Apo A-I, A-II, and A-IV from bovine serum were isolated and purified essentially as described by Brewer et al. [16], HDL were obtained by sodium phosphotungstate precipitation as described by Mills et al. [17].

2.2. *Assays*

GPI-PLD activity was assayed using purified mf-AChE from bovine erythrocytes as substrate. Erythrocyte AChE is membrane-bound through covalently linked PI-glycan, and its conversion from the amphiphilic mf-AChE to the soluble enzyme (s-AChE) serves as measure for anchor-degrading activity [18]. Samples containing GPI-PLD were placed into a 1 ml tube and adjusted to 21 μl with 20 mM Tris-HCl, pH 7.4. To this 4 μl of substrate solution containing 0.60 pmol (0.24 IU) mf-AChE in 10 mM Tris-HCl, pH 7.4, 144 mM NaCl, 6 mM $CaCl_2$ and 0.1% Triton X-100, was added and incubated at 37°C for varying lengths of time. The product of the reaction, i.e. s-AChE, was separated from the substrate by phase separation in Triton X-114 [19] as described by Hoener and Brodbeck [6]. GPI-PLD activity was expressed as U/ml where 1 U equals 1 nmol of mf-AChE converted per min at 37°C.

2.3. *Density gradient centrifugation*

Purified GPI-PLD (100 μl) was dialyzed against 20 mM Tris-HCl, pH 7.4, and layered on top of 11.0 ml linear sucrose density gradients (5–30%) in the absence and presence of 0.1% Triton X-100. The gradi-

ents were centrifuged at 4°C and 195,000 × g_{av} for 15 h in a Centrikon/2070 centrifuge equipped with TST 4114 rotor. The gradients were emptied from the bottom with a glass capillary by means of a peristaltic pump Fractions of about 0.3 ml were collected in microtiter plates and assayed for GPI-PLD activity under standard assay conditions

3. RESULTS

In serum, GPI-PLD occurs in association with the HDL fraction [6]. In order to characterize this association further, we studied the interaction of GPI-PLD with purified bovine apo A-I, the major protein constituent of the HDL fraction. For this purpose, GPI-PLD was purified from bovine serum by separation in Triton X-114 followed by column chromatography on DEAE–cellulose, octyl–Sepharose, concanavalin-A–Sepharose and hydroxyapatite, resulting in a more than 90% pure enzyme as estimated by SDS-PAGE [6]. Then, we compared the sedimentation behaviour of the purified and aggregated GPI-PLD in the absence and presence of increasing amounts of bovine apo A-I to that of GPI-PLD associated with HDL particles. As seen from Fig. 1A, the majority of HDL-associated GPI-PLD sedimented at 7.6 S in the absence of detergent, while in the presence of 0.1% Triton X-100, it sedimented at 6.0 S. Pure GPI-PLD sedimented in the absence of apo A-I as a broad peak with a maximum at around 14.5 S (Fig. 1B). Increasing amounts of apo A-I in the gradient shifted the sedimentation pattern to lower S values. (Fig. 1B and C). In the presence of a 10-fold molar excess of apo A-I, a bimodal distribution of GPI-PLD activity was obtained with peaks at around 13.3 S and 10.8 S. In the presence of a 40-fold molar excess of apo A-I, a further shift to lower S values resulted with a major peak at around 8.9 S (Fig. 1B). At a 125-fold molar excess of apo A-I, the main activity sedimented at 7.3 S (Fig. 1C). At this molar excess, the sedimentation profile of GPI-PLD was similar to that of HDL-associated GPI-PLD. However, it clearly differed from that obtained in the presence of 0.1% Triton X-100 (compare Fig. 1A and C). As seen from Fig. 1D, the presence of bovine serum albumin in up to 40-times molar excess had virtually no effect on the sedimentation behaviour of GPI-PLD, whereas at a 125-fold molar excess of bovine serum albumin, a partial shift towards lower sedimentation coefficients was also observed.

In order to study a possible effect of apo A-I on GPI-PLD activity, purified enzyme was incubated with increasing amounts of apo A-I at different concentrations of Triton X-100 in the assay. As seen from Fig. 2A, increasing amounts of apo A-I stimulated GPI-PLD activity up to 5-fold. Maximal activity was measured at Triton X-100 concentrations of 0.016 to 0.018% which correspond to the CMC of Triton X-100 [20]. Below and above the CMC, lower activities resulted. From the saturation curve obtained at 0.018% Triton

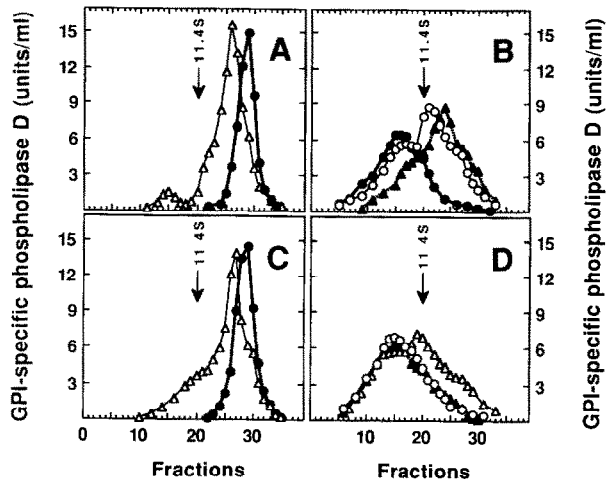

Fig. 1. Sedimentation analysis of bovine HDL associated GPI-PLD and of purified GPI-PLD in the absence and presence of apo A-I or bovine serum albumin. (A) Sedimentation profiles of HDL-associated GPI-PLD in the absence (△) or presence of 0.1% Triton X-100 (●). (B) Sedimentation profiles of GPI-PLD in the absence of apo A-I (●), and in the presence of a 10- (○) and 40-fold (▲) molar excess of apo A-I as compared to the concentration of GPI-PLD. (C) GPI-PLD in the presence of a 125-fold molar excess of apo A-I over GPI-PLD in the absence (△) and presence of 0.1% Triton X-100 (●) in the gradient. (D) GPI-PLD in the presence of a 10- (○), 40- (▲), and 125-fold (△) molar excess of bovine serum albumin as compared to the concentration of GPI-PLD. The arrow indicates the position of catalase at 11.4 S

X-100, the apparent half saturation was estimated at an apo A-I to GPI-PLD molar ratio of 15. As seen from Fig. 2B, GPI-PLD activity was also stimulated with increasing amounts of Triton X-100 up to detergent concentrations corresponding to the CMC of Triton X-100. At Triton X-100 concentrations above the CMC, GPI-PLD was inhibited. In control assays, it was verified that the apo A-I preparation contained no measurable GPI-PLD activity.

4. DISCUSSION

Our present results demonstrate that the previously observed association of GPI-PLD with HDL [6] may be mediated by binding of the enzyme to apo A-I. High molecular mass aggregates of pure GPI-PLD could be dispersed either by the addition of detergent or with apo A-I. As shown by density gradient centrifugation, apo A-I forms a complex with GPI-PLD which sedimented with a sedimentation coefficient clearly different from GPI-PLD in the presence of detergent. Moreover, GPI-PLD was retained by immobilized apo A-I on Sepharose-4B from where it could be eluted by Triton X-100 (unpublished observation). Disaggregation of GPI-PLD aggregates could also be achieved by purified apo A-II and A-IV (unpublished result). In contrast, only a slight effect was observed with bovine serum albumin.

Our studies further showed that aggregated GPI-PLD displayed practically no activity, which, however,

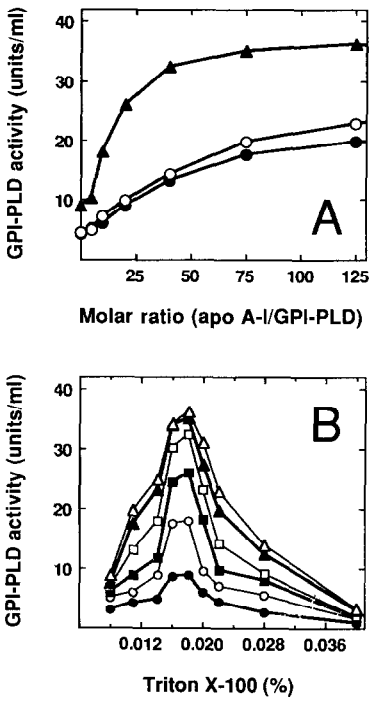

Fig. 2. (A) Stimulation of GPI-PLD activity by apo A-I. GPI-PLD activity was measured at the CMC (0.018%) of Triton X-100 (▲), above (0.022%, ○) and below (0.011%, ●) the CMC of the detergent in the assay. The assay contained 0.6 pmol substrate (mf-AChE), 0.01 pmol GPI-PLD, and increasing amounts of apo A-I. On the *x*-axis, the molar ratio of apo A-I to GPI-PLD is plotted. (B) GPI-PLD activity as a function of the Triton X-100 concentration in the assay in the presence of different amounts of apo A-I. The substrate (0.6 pmol mf-AChE) was diluted into assay solution to yield the indicated Triton X-100 concentrations. GPI-PLD activity was measured in the absence (●) and in the presence of a 10- (○), 20- (■), 40- (□), 75- (▲), and 125-fold (△) molar excess of apo A-I over GPI-PLD.

could be restored upon addition of detergent and/or apo A-I. This suggests that apo A-I not only disaggregated GPI-PLD but also stimulated GPI-PLD activity. From the results presented in Fig. 2, it is evident that, regardless of the detergent concentration in the assay, apo A-I stimulated GPI-PLD activity up to 5-fold. Again, apo A-II and A-IV showed a similar effect as apo A-I, whereas bovine serum albumin only slightly stimulated the GPI-PLD (unpublished results). The assay of GPI-PLD is complicated by the fact that in the absence of detergents, the amphiphilic substrate mf-AChE itself aggregates by self micellarization [21], and in this physical state, the anchor is not hydrolyzed by GPI-PLD (unpublished result). It is reasonable to assume that in the protein micelle, the hydrophobic anchor of the substrate is in the interior and, thus, inaccessible to GPI-PLD. In the presence of detergent, the substrate micelle is dispersed, and in this physical state, GPI-PLD is able to bind substrate and to convert it to products. In the assay of GPI-PLD, Triton X-100 as well as a number of other detergents have a dual effect. They are needed at micellar amounts to disaggregate both enzyme and substrate, but as shown previously [6], they strongly inhibit GPI-PLD activity at detergent concentrations above the CMC. As shown now, the inhibition by Triton X-100 is largely reduced by apo A-I.

The stimulation of GPI-PLD by apo A-I parallels observations on a number of enzymes acting on lipid substrates. A well documented example is lecithin–cholesterol acyltransferase which is also stimulated by apo A-I [22]. Apo A-II [23], as well as apo E [24,25] increased the activity of hepatic lipase, while lipoprotein lipase is activated by apo CII [26,27]. Plasma apolipoproteins are known to consist of amphipathic alpha-helices [28]. This, together with the observation that full activation of GPI-PLD took place when apo A-I was present in large excess over GPI-PLD, suggests that apo A-I exerts a detergent-like action on GPI-PLD. This explains the dissociation of pure, aggregated GPI-PLD by apo A-I and the observed non-stochiometric complex formation between the two proteins. Additionally, apo A-I may serve as protein cofactor to optimally present the substrate to the enzyme. This is indeed the case with lecithin–cholesterol acyltransferase, where apo A-I was shown to serve as a substrate binding cofactor [22].

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