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MOLECULAR INTERACTIONS IN THE HYDROGEN BELTS OF MEMBRANES

GLUCOSE-6-PHOSPHATASE, LYSOPHOSPHATIDYLCHOLINE, AND CHOLESTEROL

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Microsomal glucose-6-phosphatase from rat liver is activated by phosphatidylcholine but inhibited by lysophosphatidylcholine. Inhibition occurs not by membrane lysis but in an intact bilayer; it is reversible; and it is overcome by addition of cholesterol but not if the cholesterol-hydroxyl group is blocked. An analog of lysophosphatidylcholine deprived of hydrogen bonding sites, 1-ether-2-deoxylysophosphatidylcholine, is a partial activator, and its effect on the enzyme in a phosphatidylcholine bilayer is not modulated by cholesterol. It appears to be one of the functions of cholesterol to buffer the lysophospholipids in membranes by complexing with them through hydrogen bonding in the hydrogen belt region. Lysophosphatidylcholine/cholesterol association is favored over phosphatidylcholine/cholesterol association.

Introduction

Cholesterol and lysophosphatidylcholine (lysoPC) associate in 1:1 molar ratio [1–3]. Evidence has been presented that the transient complex of the two lipids is held together not by hydrophobic bonding alone but also by hydrogen bonding, between the hydroxyl group of cholesterol and hydroxyl and carbonyl groups of the lysolipid [3,4]. Lipid-lipid hydrogen bonding of such kind has been postulated to take place in those regions of bilayer membranes, between the hydrophobic core and polar zone, which we like to call 'hydrogen belts' because they are neither hydrophobic nor polar but are occupied by hydrogen bond acceptors (the CO groups of phospho- and sphingolipids) and hydrogen bond donors (the OH groups of cholesterol, sphingosine, water) [5,6]. It has also been suggested that membrane proteins participate in the hydrogen bond pattern of the hydrogen belts [5–7]. It might then be expected that some membrane proteins will change their conformations in response to changes in the hydrogen bond donor/acceptor profiles of the belts. For an enzymic protein residing in the bilayer, the result might be a measurable change in its activity. We find that glucose-6-phosphatase exhibits such an effect: its activity responds sharply to changes in the lysoPC/cholesterol balance, but this sensitivity is lost when the hydrogen bonding sites on either the lysolipid or the sterol are removed. The data also support a previous suggestion [3,4] that it may be one of the functions of cholesterol to protect membranes from the effects of lysophospholipids, by binding these in a complex. The preference of cholesterol for lysoPC over PC appears to be roughly of one order of magnitude.

It should be mentioned here that although lipid-protein interactions in membranes are conventionally discussed in terms of membrane 'fluidity' or 'perturbation' there appeared to be no reason to do so here. These concepts are not to be negated; but their explanatory power is not very great. We have discussed 'fluidity', and what it can explain, elsewhere [7].

Materials

Lysophosphatidylcholine and glucose-6-phosphate were from Sigma (St. Louis, MO); 1-ether-2-deoxylysophosphatidylcholine (1-*O*-hexadecanyl-1,3-dioxypropane-3-phosphocholine) was synthesized [8–10]; so was cholesterol-PEG (3-*O*-methoxyethoxyethoxyethylcholesterol) [11]. All lipids were pure as judged by thin-layer chromatography.

Methods

Livers were excised from fasted male rats weighing 200–250 g. Glucose-6-phosphatase was partially purified from microsomes and delipidated, essentially by the method of Garland and Cori [12]. Microsomal specific activity was 0.033 μmol/min per mg, activity of the 'M_1' fraction [12] was 0.79. This preparation was delipidated with sodium deoxycholate and Sepharose 4B chromatography to fraction M_2 [12], spec. act. 0.388 μmol/min per mg protein (with PC, phosphatidylcholine, added), and the deoxycholate was then removed by passage over an Amberlite XAD-4 column (fraction M_3). There is a drop of specific activity at this step, to 0.066 μmol/min per mg protein, which has to be accepted because for our reconstitution experiments it is essential that the detergent be removed. Residual detergent (quantitated with [^{14}C]deoxycholate) was 0.02% of the 0.1% solution applied to the column; this amounts to 0.004% deoxycholate in the assay solution. Residual organic phosphorus, in the M_3 fraction, was 0.08 μmol/mg protein, i.e., the preparation contained approx. 6.4% (w/w) of phospholipid (assuming an M_r of 800). This residue of phospholipid may be responsible for the residual activity of the delipidated preparation (Table I, 1).

Monolamellar liposomes were prepared as described earlier [3]. For measurement of the glucose-6-phosphatase activity, fraction M_3, 0.1 ml (6 μg protein) was mixed with 0.1 ml of liposome (0.8 mg lipid) by vortexing for 30 s, and left at room temperature for 10 min. The reaction was then started at 37°C by adding 100 μl of 0.1 M glucose 6-phosphate (pH 6.8) in a total volume of 0.5 ml. The reaction was found to be linear up to 75 min; for maximal sensitivity we chose 60 min incubation. Doubling the concentration of M_3 protein doubled the release of phosphate. The reaction was terminated by adding 0.5 ml of cold 4% perchloric acid solution in 10% trichloroacetic acid; 0.1 ml of 10% albumin had been added, just before the reaction was stopped, to coprecipitate protein and phospholipids. Inorganic phosphorus was estimated by the method of Ames [13], total phosphorus by the method of Marinetti [14].

Since glucose-6-phosphatase has not been isolated, our experiments have been performed with a mixture of microsomal proteins. This might be thought to complicate the results beyond the possibility of interpretation. However, the recombinant system consists of a monolamellar lipid in 133-fold weight excess of protein, and there is no reason to assume that the enzyme would not, with its hydrophobic part, dissolve in the bilayer as a single molecule. Thus, in spite of a great number of other proteins possibly inbedded in the bilayer (but at large intervals) the system is, in fact, not at all complicated. If glucose-6-phosphatase should occur as a polymer, or be associated with some other protein, this possibility could be incorporated in our otherwise unchanged interpretation of the results.

Results

The glucose-6-phosphatase activity of the delipidated microsomal protein is restored on incubation with monolamellar egg PC liposomes (Table I, 2). Incubation with lysoPC, on the other hand, reduces the activity of the delipidated preparation even below its residual value (Table I, 3). The inhibition is equally pronounced in a PC bilayer containing 20 mol% lysoPC (Table I, 4). Cholesterol when included in a PC bilayer without lysoPC does not interfere with the restoration of the enzymic activity in either direction (Table I, 5). Cholesterol in 1 : 1 mixture with lysoPC blocks the inhibitory effect of the lysophospholipid completely (Table I, 6). An *O*-substituted derivative, cholesterol-PEG, which is not itself an inhibitor (Table I, 7), has no influence on the inhibition of the enzyme of lysoPC (Table I, 8). If the bilayer consists of equal parts of PC, lysoPC, and cholesterol, activity is restored not fully, but partially (Table I, 9).

TABLE I

EFFECT OF PHOSPHATIDYLCHOLINE, LYSOPHOSPHATIDYLCHOLINE, AND CHOLESTEROL ON GLUCOSE-6-PHOSPHATASE

Values are means ± S.D. 6–8 μg of fraction M_3 were incubated with 20 mM glucose-6-phosphate (pH 6.8) and 1 μmol of lipid in a total volume of 0.5 ml at 37°C for 60 min. Then, inorganic phosphorus was estimated as described in Methods. Net activity: activity over lipid-free system. Number of experiments in parenthesis.

Lipid	Activity (nmol P_i/mg protein per min)	Net activity	% Restoration of activity
1. None	11 ± 2(9)	0	0
2. Egg PC	66 ± 12(6)	55	100
3. LysoPC	6 ± 1(4)	−5	−9
4. LysoPC/PC (1 : 4)	8 ± 1(3)	−3	−5
5. Cholesterol/PC (1 : 1)	71 ± 10(3)	60	109
6. LysoPC/cholesterol (1 : 1)	69 ± 11(4)	58	105
7. PC/cholesterol-PEG (1 : 1)	61 ± 5(2)	50	91
8. LysoPC/Cholesterol-PEG (1 : 1)	10 ± 1(3)	−1	−2
9. LysoPC/cholesterol/PC (1 : 1 : 1)	33 ± 9(3)	22	40

TABLE II

EFFECT OF PHOSPHATIDYLCHOLINE, ETHER-DEOXY-LYSOPHOSPHATIDYLCHOLINE, AND CHOLESTEROL ON GLUCOSE-6-PHOSPHATASE ACTIVITY

Values are means ± S.D. Glucose-6-phosphatase was assayed as described in Table I. Net activity: activity over lipid-free system. Number of experiments in parentheses.

Lipid	Activity (nmol P_i/mg protein per min)	Net activity	% Restoration of activity
1. None	11 ± 2(9)	0	0
2. Egg PC	66 ± 12(6)	55	100
3. EtherdeoxylysoPC	22 ± 3(3)	11	20
4. EtherdeoxylysoPC/PC (1 : 4)	29 ± 9(3)	18	33
5. EtherdeoxylysoPC/cholesterol/PC (1 : 1 : 1)	39 ± 10(3)	28	51
6. EtherdeoxylysoPC/cholesterol (1 : 1)	44 ± 8(3)	33	60

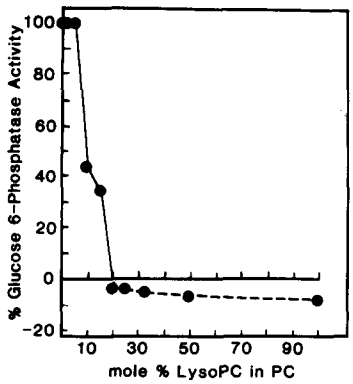

Fig. 1. Activity of glucose-6-phosphatase in phosphatidylcholine bilayers with different contents of lysoPC. Glucose-6-phosphatase was estimated as described in Table II.

LysoPC in the PC bilayer at 5 mol% or lower does not inhibit the enzyme (Fig. 1). At 20 mol%, inhibition is complete. The midpoint is around 10 mol%.

The lysoPC analog in which the carbonyl-O and the secondary glycerol hydroxyl have been removed, i.e., etherdeoxylysoPC, is a partial activator, rather than an inhibitor, of the enzyme, by itself (Table II, 3). At 20 mol% concentration in a PC bilayer, etherdeoxylysoPC allows one-third of the enzymic activity to be expressed (Table II, 4). Inclusion of cholesterol in an etherdeoxylysoPC/PC bilayer (Table II, 5) does not cause significant further activation, nor does an etherdeoxylysoPC/

cholesterol bilayer display fully restored enzymic activity (Table II, 6).

Discussion

LysoPC is known to lyse membranes and liposomes [15], and cholesterol to prevent such lysis [16], but lysis is not the cause of deactivation of the glucose-6-phosphatase, since the PC bilayer structure is intact at inhibitory lysoPC concentrations: only above 50 mol% lysoPC will the bilayer disintegrate [17]. Also, the inhibition is reversible. This cannot be demonstrated directly because the enzyme lacks the stability needed for inhibition-reactivation experiments, but it can be deduced from the experiment of Table I, 8 which displays partial inhibition: if the process were irreversible, for example due to protein denaturation by lysoPC acting as a detergent, there should be complete inactivation, or none at all.

The mechanism of inhibition, then, appears to be more selective than denaturation or destruction of the bilayer. The major functional difference of lysoPC from PC as well as from etherdeoxylysoPC being the free glycerol 2-hydroxyl group, it stands to reason that this group is responsible for the inhibition of the enzyme. We suggest that lysoPC hydroxyls bond to one or more hydrogen bond acceptors in the hydrogen belt region of the protein, and thereby reversibly change its conformation; in other words, that the enzyme has hydrogen bonding allosteric sites which can be occupied by lysoPC hydroxyl groups. Support for this suggestion is furnished by the action of cholesterol. Evidence has previously been presented that lysoPC and cholesterol bond together by complexing in the hydrogen belt of bilayers [3,4]. Such bonding would pre-occupy the hydroxyl group of lysoPC; and addition of one mole of cholesterol to lysoPC does, in fact, restore the activity of the enzyme fully (Table I, 5). Restoration also occurs when lysoPC, cholesterol, and PC are combined in a bilayer (Table I, 8), but it is not complete. The activity found, about 40%, is comparable to the activity found when the ratio of lysoPC to PC is about 1 to 10 (Fig. 1). It would appear, therefore, that in the ternary system one-tenth of the lysoPC is still free to react with the protein, while nine-tenths are engaged by cholesterol. Consequently, the ratio of lysoPC/cholesterol to PC/cholesterol will be around ten, or the affinity of cholesterol to lysoPC ten-times stronger than that to PC. Because of the variance of the assays, this value of preference, ten, is possibly not very accurate; but, clearly, a preference does exist.

Cholesterol-PEG, a derivative in which the sterol-OH group has been blocked, behaves physically very much like cholesterol, in a bilayer [11,18]. This steroid does not inhibit the glucose-6-phosphatase (Table I, 7), and it should, if our interpretation is correct, not be able to overcome the inhibition of the enzyme by lysoPC; and this is what is found (Table I, 8).

In contrast to lysoPC, etherdeoxylysoPC is not an inhibitor but a mild activator of the enzyme. At the concentration of 20 mol%, where lysoPC is a total inhibitor (Table I, 4), deoxylysoPC in PC allows one-third of the maximal enzymic activity to be restored (Table II, 4). There is some reactivation even if the analog is presented without PC (Table II, 3), i.e., presumably, in micellar form rather than in a bilayer [19]. It may be noticed, though, that none of the lipid mixtures containing etherdeoxylysoPC achieves the level of restoration reached by a PC bilayer (Table II, 2) or by the lysoPC/cholesterol bilayer (Table I, 6). The reason is not clear; perhaps a subtle change in PC bilayer geometry, introduced by the mono-chain lipid, prevents the enzyme from attaining its optimal conformation. Despite this unexplained incongruity, the difference in action between lysoPC and etherdeoxylysoPC is clearcut, and it must be based on the presence or absence of the carbonyl and hydroxyl groups of the lysophospholipids. If this is so, and the etherdeoxylysoPC molecule cannot, like lysoPC, form a complex with cholesterol in the hydrogen belt [4], then we would not expect cholesterol to have any appreciable effect on the enzyme in a PC bilayer containing that lysolipid. This is, in fact, the experimental result: there is no significant difference in enzymic activity between the etherdeoxylysoPC/PC (Table II, 4) and the etherdeoxylysoPC/PC/cholesterol (Table II, 5) systems.

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