A CONTINUOUS SPECTROPHOTOMETRIC ASSAY FOR MEMBRANE-BOUND LYSOPHOSPHOLIPASES USING A THIOESTER SUBSTRATE ANALOG

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1. Introduction

The turnover of membrane phosphoglycerides requires their deacylation and this is catalyzed by phospholipases A (EC 3.1.1.4 and EC 3.1.1.34) and lysophospholipases (EC 3.1.1.5) (see ref. [1] for review). In general these lipolytic enzymes are assayed by titrimetric [2,3] or radiochemical procedures [4]. The former method is rather insensitive and can not usually be applied to intracellular phospholipases, whereas radiochemical methods suffer from the disadvantages inherent to fixed-time assays. By using substrate analogs of lysolecithin, in which the fatty acid is linked in thioester linkage to the backbone of the substrate, we have developed a continuous spectrophotometric assay for purified lysophospholipases [5]. The sensitivity of the method, (hydrolysis rates of 1 $nmol.min^{-1}$ can easily be detected) should allow the determination of lysophospholipase activity in crude systems such as subcellular fractions. Lands and Hart [6] were the first to determine the activity of an enzyme involved in phospholipid metabolism by the continuous measurement of released thiol groups in the presence of 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB). They studied acyl-CoA:lysophosphoglyceride acyltransferases (EC 2.3.1.23) but this method has since been used for acyl-CoA:*sn*-glycero-3-phosphate acyltransferase (EC 2.3.1.15) (ref. [7]). Unfortunately, this method has been unsuccessful for the assay of acyl-CoA:monoacylglycerol acyltransferase (EC 2.3.1.22) in intestinal mucosa [8] or fat-body [9] microsomes owing to the fact that the CoA remained bound to the membrane in a form which was unreactive with DTNB. The present study was undertaken to see whether the activity of membrane-bound lysophospholipases could be measured by our continuous spectrophotometric assay. The thioester substrate appeared to be hydrolyzed more rapidly than the corresponding [^{14}C]acyl-oxyester substrate and no indications were obtained for binding of the released thiol groups in a form unreactive to DTNB.

2. Materials and methods

2-Hexadecanoylthio-1-ethylphosphorylcholine (thioglycollecithin) was synthesized as described previously [5]. The corresponding ^{14}C-labelled oxyester analog, 2-[1-^{14}C]hexadecanoyloxy-1-ethylphosphorylcholine ([^{14}C]glycollecithin, specific activity 120 dpm/nmol) was synthesized by similar procedures from glycol and [1-^{14}C]palmitoylchloride was prepared by chemical synthesis as described by Van den Bosch et al. [10].

Fresh beef liver was obtained from the local slaughterhouse. A 10% homogenate was prepared in 0.25 M sucrose containing 5 mM Tris–HCl buffer, pH 7.2 and 1 mM EDTA. Centrifugation was done as described previously [11]. Pellets were resuspended in homogenization medium. A membrane fraction of *Acholeplasma laidlawii* strain B, isolated according to the procedure of Van Golde et al. [12], was obtained from Dr E. M. Bevers of this laboratory. Lysophospholipase activities were measured in 20 mM potassium phosphate buffer, pH 7.4, in the presence of 1 mM DTNB at saturating substrate concentrations. Absorbance changes were recorded with a double-beam Varian spectrophotometer Model 635 equipped with a thermostated cuvette. A molar extinction coeffi-

cient of 12 800 liters.mol^{-1}.cm^{-1} was used for the thionitrobenzoic acid anion. Hydrolysis of radioactive oxyester substrates was determined by extraction of the labelled fatty acids as described previously [11]. For comparison DTNB was included also in these assays. Protein was determined according to Lowry et al. [13].

3. Results and discussion

The initial rates of hydrolysis of thioglycollecithin as measured spectrophotometrically by the release of thiol groups was a linear function of the amount of *Acholeplasma laidlawii* membrane protein (fig.1). The specific activity amounted to 140 nmol.min^{-1}.mg protein^{-1}. The enzymatic activity towards thioester substrates was not affected by the addition of Ca^{2+} or EDTA, however, it was completely inhibited by sodium deoxycholate at a concentration of 1.25 mg/ml. These results are in accord with previous findings [12] using the oxyester substrates lysophosphatidylcholine or lysophosphatidylglycerol. The behaviour of the *A. laidlawii* lysophospholipase towards Ca^{2+}, EDTA and deoxycholate both with oxyester and thioester substrates is thus very similar to that found for lysophospholipases from other sources [5,14]. Maximal hydrolysis rates for [1-^{14}C]-palmitoyl-oxyglycollecithin and [1-^{14}C]palmitoyl-lysophosphatidylcholine with the *A. laidlawii* lysophospholipase were found to be 85 and 115 nmol.min^{-1}.mg^{-1}, respectively. Thus, the thioglycollecithin was hydrolyzed 1.6-fold more rapidly than the corresponding oxyglycollecithin. Owing to the rather high activity of the membrane-bound lysophospholipase from *A. laidlawii* the spectrophotometric assays were very straightforward. In contrast, several problems were encountered in initial attempts to determine the lysophospholipase activity of beef liver microsomes by the spectrophotometric method. The lysophospholipase activity of these microsomes with lysolecithin as substrate amounted to about 7 nmol.min^{-1}.mg protein^{-1} [11]. Consequently, more protein had to be used which gave rise to higher turbidity and higher absorbance values due to the presence of free thiol groups in the added protein. Although these effects can easily be corrected for in a double-beam spectrophotometer the disturbing observation was made that addition of the thioglycollecithin to the sample cuvette caused considerable changes in both the turbidity and the amount of thiol groups reactive to DTNB. These effects are most likely attributed to membrane solubilization owing to the detergent properties of the lysolecithin substrate analog [15]. The problems could be overcome nicely by the simultaneous addition of thioglycollecithin to the sample cuvette and lysolecithin to the reference cuvette. Figure 2 represents recorder tracings obtained by using this procedure for different amounts of beef liver microsomes. There was a linear relationship between the initial velocity of thioglycollecithin hydrolysis and the amount of microsomal protein, at least in the range of 40–200 μg protein investigated (data not shown). The specific activity of hydrolysis was 13 nmoles.min^{-1}.mg^{-1}, which compares favourably to the value of 6.8 nmoles.min^{-1}.mg^{-1} found for [^{14}C]oxyglycollecithin. Moreover, this ratio of 1.9 for the hydrolysis rates of thio- and oxyglycollecithin is the same as that found for a 500-fold purified preparation of the microsomal enzyme (Aarsman, Hille and Van den Bosch, unpublished observations). This shows that the method is valid for the assay of membrane-bound enzymes; furthermore all thiol groups released by the action of the enzyme can be detected by reaction with DTNB.

Next, a subcellular fractionation of a beef liver

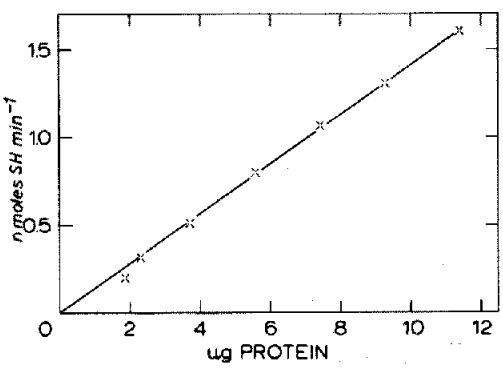

Fig.1. Initial rates of thioglycollecithin hydrolysis as a function of the amount of *A. laidlawii* membrane protein. The sample cuvette contained 200 nmol. thioglycollecithin and the indicated amounts of protein in total vol. 1.0 ml, 1 mM DTNB in 20 mM potassium phosphate buffer, pH 7.4. The reference cuvette contained no substrate.

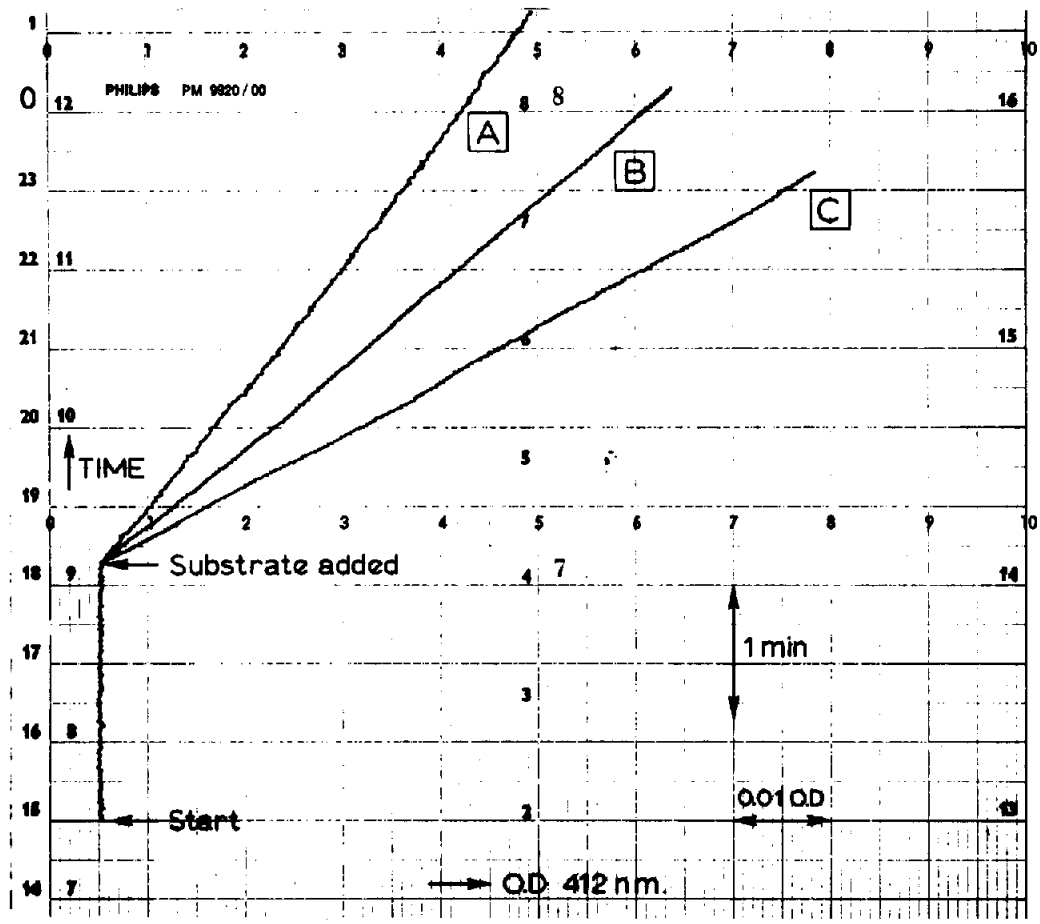

Fig.2. Spectrophotometric assay of beef liver microsomal lysophospholipase. The cuvettes contained the microsomal protein in 0.9 ml 1 mM DTNB in 20 mM potassium phosphate buffer, pH 7.4. At the time indicated by an arrow 200 nmol thioglycollecithin in 0.1 ml water were added to the sample cuvette and 200 nmol egg lysophosphatidylcholine was similarly added to the reference cuvette. A, B and C represent experiments using 60 μg, 100 μg and 160 μg microsomal protein, respectively.

homogenate was carried out as described previously [11]. The distribution of protein and lysophospholipase activity over the various subcellular fractions are plotted as recommended by De Duve et al.[16], in fig.3. Lysophospholipase activity in each subcellular fraction was determined with [^{14}C]oxyglycollecithin and with thioglycollecithin. The distribution of lysophospholipase activity over the subcellular fractions as determined with [^{14}C]oxyglycollecithin as substrate is similar to that found by Van den Bosch and De Jong with the natural lysophosphatidylcholine substrate [11]. However, the distribution of activity towards thioglycollecithin as substrate is somewhat different; relatively more lysophospholipase activity is present in the cytoplasmic fraction and relatively less in the lysosomal and microsomal fractions. This can be explained by the fact that beef liver contains two lysophospholipases with a different subcellular localization [11]. The cytoplasmic fraction contains mainly lysophospholipase I whereas the lysosomal and microsomal fractions contain almost exclusively lysophospholipase II which originates from the endoplasmic reticulum [11]. As shown in fig.3 both enzymes have a preference for the hydrolysis of thioglycollecithin as compared to oxyglycollecithin, but this preference is more pronounced for the cytoplasmic enzyme.

A comparison of oxyglycollecithin hydrolysis in

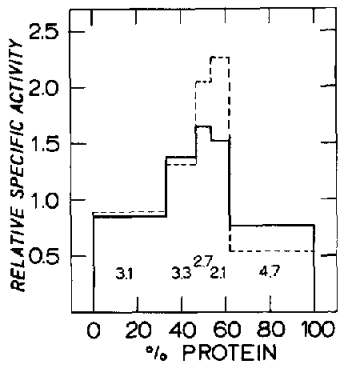

Fig.3. Relative specific activities of total lysophospholipase versus percentage of total recovered protein in subcellular fractions from beef liver. Subcellular fractions from left to right: nuclear and debris, mitochondrial, lysosomal, microsomal and cytoplasmic fraction. Enzymatic activities were determined either spectrophotometrically with thioglycollecithin (drawn line; total recovery 100%) or radiochemically with [^{14}C]oxyglycollecithin (dotted line; total recovery 112%). Protein recovery was 98%. Numbers indicate the ratio of the specific activity of thioester and oxyester hydrolysis.

the presence or absence of 1 mM DTNB showed the lysophospholipase activity of beef liver subcellular fractions to be inhibited by about 20–30% at this concentration of DTNB. Much higher inhibitions were found for mouse liver (40–60%) or rat liver (70–90%) subcellular fractions, which indicate some limitations of the method.

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