Fluorimetric coupled enzyme assay for lysoplasmalogenase activity in liver

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We developed a continuous spectrofluorimetric assay of lysoplasmalogenase activity with the use of horse liver alcohol dehydrogenase as a coupling enzyme. In this method the disappearance of NADH is measured spectrofluorimetrically. The excitation and emission monochromators were set at 340 and 460 nm respectively. The assay is 10 times as sensitive as the previous u.v. spectrophotometric method. We could detect approx. 0.02 nmol of aldehyde produced/min per ml.

INTRODUCTION

Plasmalogens, one type of ether-linked glycerophospholipids, are major phospholipid constituents of mammalian membranes (Horrocks & Sun, 1972). They are widely distributed in various mammalian tissues and may act as a reservoir of prostaglandin and thromboxane precursors (Horrocks *et al.*, 1986; Swenson *et al.*, 1983). These lipids may also be involved in the transport of ions across membranes (Gross, 1985). The degradation of plasmalogens may be a receptor-mediated process that is closely related to signal transduction, indicating that the plasmalogen-hydrolysing enzymes may be very important metabolically. The hydrolysis of plasmalogens is catalysed by several hydrolytic enzymes, including plasmalogenase, lysoplasmalogenase and plasmalogen-specific phospholipase A₂ (Horrocks *et al.*, 1986). Lysoplasmalogenase (EC 3.3.2.2) catalyses the hydrolysis of the alkenyl ether bond of 1-alk-1'-enyl-GroPCho or 1-alk-1'-enyl-GroPEtn (lysocholine plasmalogen or lysoethanolamine plasmalogen) and was found in microsomal fractions (referred to below simply as microsomes) by the use of potentiometric and spectrophotometric procedures (Warner & Lands, 1961; Ansell & Spanner, 1965). Both procedures are relatively insensitive and require assays at every desired time point. Gunawan & Debuch (1981, 1982) characterized the lysoplasmalogenase activity of rat liver and brain microsomes by the use of a radiochemical procedure. This procedure is sensitive but also discontinuous. Spectrophotometric continuous assay procedures have been described with the use of auxiliary enzymes (Freeman & Carey, 1980; Arthur *et al.*, 1986). A modified coupled-enzyme assay was used during the partial purification of lysoplasmalogenase from rat liver microsomes (Jurkowitz-Alexander *et al.*, 1989).

In the present paper, we report a continuous and more sensitive fluorimetric assay procedure for lysoplasmalogenase in rat liver microsomes.

MATERIALS AND METHODS

Materials

Choline glycerophospholipid from bovine heart was purchased from Serdary Co., Toronto, Ont., Canada. Horse liver alcohol dehydrogenase, NADH, dithiothreitol, fatty acid-free bovine serum albumin, tetramethylammonium iodide and glycerophosphocholine were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Other chemicals and solvents in this study were of reagent grade.

Preparation of choline lysoplasmalogen and lysoplasmalogenase

LysoPlsCho was prepared from bovine heart choline glycerophospholipid by the use of mild base hydrolysis (Gunawan *et al.*, 1979). Lysoplasmalogenase was partially purified from adult rat liver microsomes by the use of DEAE-cellulose chromatography (Jurkowitz-Alexander *et al.*, 1989).

Spectrofluorimetric measurement of NADH

Spectrofluorimetric measurements were performed at 37 °C with a Perkin-Elmer L 55 spectrofluorimeter. The excitation and emission monochromators were set at 340 and 460 nm respectively. After 3 min preincubation, the difference of relative fluorescence ($y - x$) before and after addition of 10 nmol of NADH (Fig. 1a, traces A and B) to the reaction mixture was measured. After initiation of reaction (Fig. 1a, trace C), the decrease of fluorescence (Δz) was recorded on a Perkin-Elmer R 100A recorder. The NADH change was calculated from the following formula:

$$\Delta \text{NADH (nmol)} = \Delta z / (y - x) \times 10 \text{ (nmol)}$$

The change of fluorescence and incubation time were measured from the recording chart with a ruler.

Abbreviations used: GroPCho, *sn*-glycero-3-phosphocholine; GroPEtn, *sn*-glycero-3-phosphoethanolamine; PlsCho, 1-alk-1'-enyl-2-acyl-GroPCho; PlsEtn, 1-alk-1'-enyl-2-acyl-GroPEtn.

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Fig. 1. Representation of the decrease in the fluorescence in the spectrofluorimetric lysoplasmalogenase assay

LysoPlsCho ($50 \mu\text{M}$) was incubated with $10 \mu\text{M}$ -NADH, 1 mM -dithiothreitol, fatty acid-free bovine serum albumin ($0.35 \mu\text{g}$ of protein), horse liver alcohol dehydrogenase ($38.5 \mu\text{g}$ of protein) and lysoplasmalogenase ($3.56 \mu\text{g}$ of protein) in 0.2 M -Mops buffer, pH 7.4, at 37°C (1.0 ml final volume). Even-numbered traces include alcohol dehydrogenase, whereas odd-numbered traces did not include the enzyme. Additionally, traces 1 and 2 were without lysoPlsCho and curves 3 and 4 were without lysoplasmalogenase. Excitation was at 340 nm and the emission was monitored at 460 nm . A, Initiation of fluorescence measurement without NADH; B, addition of NADH; C, addition of lysoplasmalogenase. x , y and Δz indicate the relative fluorescence or the change of fluorescence.

Assay of alcohol dehydrogenase activity

The assay mixture contained 0.2 M -Mops buffer, pH 7.4, 1 mM -dithiothreitol, $10 \mu\text{M}$ -NADH, fatty acid-free bovine albumin (0.35 mg of protein/ml) and an appropriate concentration of acetaldehyde. The change of fluorescence was monitored after addition of the appropriate amount of alcohol dehydrogenase.

Assay of lysoplasmalogenase activity

The lysoplasmalogenase assay was performed according to the following reaction scheme:

1-Alk-1'-enyl-GroPCho was hydrolysed by lysoplasmalogenase to free aldehyde and GroPCho. The free aldehyde was quantitatively converted into the alcohol by alcohol dehydrogenase with the oxidation of NADH. The disappearance of NADH was measured spectrofluorimetrically.

LysoPlsCho ($50 \mu\text{M}$) was incubated in a reaction mixture containing 1 mM -dithiothreitol, $10 \mu\text{M}$ -NADH, fatty acid-free albumin (0.35 mg), alcohol dehydrogenase ($38.5 \mu\text{g}$ of protein) and an appropriate amount of lysoplasmalogenase in 0.2 M -Mops buffer, pH 7.4. The volume of the final reaction mixture was 1 ml . The necessary amount of alcohol dehydrogenase ($38.5 \mu\text{g}$ of protein) was determined by the titration of alcohol dehydrogenase concentration with the use of 0.5 nmol of acetaldehyde. The substrate, dithiothreitol, NADH and alcohol dehydrogenase solution were prepared fresh for each series of assays.

Control incubations were critical for this assay. The standard experiment included the following incubations according to the method of Jurkowitz-Alexander *et al.* (1989):

Tube no.	1	2	3	4	5	6
Alcohol dehydrogenase	—	+	—	+	—	+
Substrate	—	—	+	+	+	+
Lysoplasmalogenase	+	+	—	—	+	+

Fluorescence changes in incubation tubes 6 and 5 were measured after monitoring changes in tubes 4 and 3 respectively. The decreases in NADH in incubation tubes 2 and 1 were measured 3 min after addition of NADH. The volume of NADH solution was $5 \mu\text{l}$ and the volumes of enzyme source were less than $5 \mu\text{l}$ in order to minimize the dilution effect (Fig. 1).

Determination of lysoplasmalogenase activity by measuring GroPCho in the reaction mixture

The activity of lysoplasmalogenase was also determined by the measurement of GroPCho, product of lysoplasmalogenase, according to the method of Schmidt & Speth (1975).

RESULTS

The addition of lysoplasmalogenase to the reaction mixture did not shift the wavelength of the emission maximum (460 nm) and there was a time-dependent decrease in NADH fluorescence with a linear rate for 3 min (Figs. 2a and 2b).

The rate of lysoplasmalogenase activity was obtained from the following equation:

$$\text{Rate of lysoplasmalogenase} = \frac{\text{rate}(6-5) - \text{rate}(4-3+2-1)}{3 \text{ min}}$$

We confirmed that rates of tubes 3 and 4 with and without waiting 3 min after addition of NADH were in agreement (results not shown). Rates of tubes 3 and 4

Fig. 2. Effect of lysoplasmalogenase on NADH fluorescence

(a) Fluorescence spectra: A, before the addition of NADH; B, 30 min after the beginning of reaction; C, immediately after the addition of 10 nmol of NADH. (b) Fluorescence decreased in the reaction mixture with time. The conditions of reaction mixture were the same as in Fig. 1. The arrow indicates the time of addition of lysoplasmalogenase.

without waiting 3 min were used in this procedure, for saving ingredients and time (Figs. 1a and 1b).

The present coupled assay procedure requires an excess of the auxiliary enzyme, alcohol dehydrogenase, so that the rate of NAD^+ formation becomes proportional to the lysoplasmalogenase activity. Determination of kinetic parameters for horse liver alcohol dehydrogenase indicated that this enzyme followed simple Michaelis-Menten kinetics. From a direct linear plot (Cornish-Bowden & Eisenthal, 1974), the apparent K_m and V_{max} values for acetaldehyde were $75 \mu\text{M}$ and $7.1 \mu\text{mol/min per mg}$ of protein. The optimal substrate concentration was $200 \mu\text{M}$, which is 200–40 000 times higher than the concentration of aldehyde produced during 2.5 min in the linear range with respect to the amount of lysoplasmalogenase. The variation of alcohol dehydrogenase concentration demonstrated that $3.85 \mu\text{g}$ of alcohol dehydrogenase was sufficient to reduce 0.5 nmol of acetaldehyde within a few seconds. The decrease of the fluorescence of NADH almost corresponded to the amount of aldehyde added. Because some reaction conditions are different, a 10-fold greater amount of enzyme, $38.5 \mu\text{g}$, was employed in this coupled assay. With this amount of alcohol dehydrogenase 0.5 nmol of acetaldehyde was reduced quickly under the experimental conditions used in this study, but with more than 1.0 nmol of acetaldehyde a time lag was observed.

The results obtained for lysoplasmalogenase activity with the fluorimetric assay were compared with the results from direct measurement of the amount of GroPCho formed. The aqueous phase was freeze-dried and the residue was subjected to t.l.c. (Yavin, 1976). With I_2 -vapour staining, only one spot, which corresponded to standard GroPCho, was detected. No spot except this one was detected by staining for choline (Vaskovsky & Suppes, 1971). The phosphorus of this spot was 95 % of the total phosphorus subjected to t.l.c. After confirming that almost all water-soluble choline compounds in the reaction mixture were GroPCho, we measured the phosphorus of the aqueous phase (Rouser

et al., 1970). Recovery of standard GroPCho was 96.2 %. By using this ratio, the mean lysoplasmalogenase activity was $108 \text{ nmol/min per mg}$ of protein ($n = 2$). We measured the activities by the fluorimetric method during the same day. The value was $121 \pm 20.2 \text{ nmol/min per mg}$ of protein ($n = 3$).

The effect of variation of amount of lysoplasmalogenase on its activity is shown in Fig. 3. The assay was linear up to $0.367 \text{ nmol/min per ml}$.

DISCUSSION

In the present procedure we have determined the oxidation of NADH by a fluorimetric procedure. During our assay, lysoPlsCho is hydrolysed by lysoplasmalogenase to GroPCho and free aldehyde. The free aldehyde is quantitatively converted into the alcohol by

Fig. 3. Effect of increasing lysoplasmalogenase amount on the activity

The assay procedure was the same as in Fig. 1 except for the amount of lysoplasmalogenase.

alcohol dehydrogenase with the oxidation of NADH. NADH is fluorescent, whereas NAD⁺ is not. We used the spectrofluorimeter instead of a spectrophotometer for the measurement of NADH decrease in the present method. The fluorimetric determination of the enzyme activity is about 10 times more sensitive than with the spectrophotometric method (Jurkowitz-Alexander *et al.*, 1989). The lower limit of the detection was 0.02 nmol/min per ml.

The coupled reaction of our assay procedure can be represented by the equation:

where A is lysoPlsCho, S is long-chain aldehyde, P is long-chain alcohol, E₁ is lysoplasmalogenase and E₂ is alcohol dehydrogenase. For a valid coupled assay, (a) the primary reaction must be zero-order with respect to [A] over the assay time and (b) the second-stage reaction must be first-order with respect to [S]. Condition (a) is easily met if only a small fraction of [A]₀, the initial concentration of A, is utilized over the assay period. Condition (b) is met if [S]_{ss}, the steady-state concentration of S, is $\ll K_m$ of E₂ for S. This condition can be maintained by using sufficient excess of E₂. Under these conditions, the rate of P formation will be constant and proportional to the concentration of E₁ and independent of the concentration of E₂ (McClure, 1969).

In the present assay, the utilization of 0.01–1.84% of substrate in 2.5 min was in the linear range with respect to the amount of lysoplasmalogenase (Fig. 3). The [S]_{ss} of aldehyde is therefore assumed to be sufficiently lower than the K_m of alcohol dehydrogenase. Further, the amount of alcohol dehydrogenase in the reaction mixture (1 ml) is 38.5 µg of protein, which is a sufficient excess to reduce the aldehyde produced in the coupled-enzyme assay and the [S]_{ss} $\ll K_m$ for S can be maintained throughout the incubation period.

The spectrofluorimetric assay is more sensitive than the spectrophotometric assay procedures described by previous investigators (Freeman & Carey, 1983; Arthur *et al.*, 1986; Hirashima *et al.*, 1989; Jurkowitz-Alexander *et al.*, 1989). Fluorescence changes in control cuvettes are very small and stable compared with absorbance changes monitored spectrophotometrically at 340 nm. In this assay, 10 µM-NADH was employed instead of 150 µM-NADH for spectrophotometric assay (Jurkowitz-Alexander *et al.*, 1989). Therefore the decrease in NADH concentration allows the detection of small changes in emission fluorimetrically. This accounts for part of the increased sensitivity of this fluorimetric procedure.

On the other hand, this method has some disadvantages. Many spectrophotometers allow monitoring of several cuvettes simultaneously and the rate of absorbance change for each cuvette can be easily determined

with kinetics software. Such a convenience is not yet available with a spectrofluorimeter.

Development of rapid and sensitive assay procedures for plasmalogen-degrading enzymes such as plasmalogenase, lysoplasmalogenase and plasmalogen-specific phospholipase A₂ will be very useful in understanding the role of these phospholipids in signal transduction.

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