

Identification of lysophosphatidic acid acyltransferase in the microsomal membranes of developing castor endosperm

Ajay W. Tumaney, S. Narkunaraja and Ram Rajasekharan*

Department of Biochemistry, Indian Institute of Science, Bangalore 560 012, India.

A photoreactive substrate analog of acyl-CoA, 12-[(4-azidosalicyl)amino]dodecanoyl-CoA (ASD-CoA) acts as a competitive inhibitor of acyl-CoA : lysophosphatidic acid acyltransferase in the microsomal membranes of developing castor endosperm (*Ricinus communis* L.). The acyltransferase was irreversibly inactivated when the microsomal membranes were exposed to ultraviolet light in the presence of ASD-CoA. The substrate, oleoyl-CoA was able to protect the enzyme against photoinactivation. Analysis by SDS-PAGE followed by autoradiography of microsomal membrane photolysed with ^{125}I -labelled ASD-CoA revealed major labelling of a single protein with the molecular mass of 29 kDa, suggesting that this protein could be the putative LPA acyltransferase.

Acyl-CoA plays an important role in the metabolism of carbohydrates and lipids as an acyl group activator¹. Acyl-CoA is a primary substrate for many membrane-bound enzymes involved in lipid and wax biosynthesis². Attempts to solubilize and purify these enzymes from plants have been largely unsuccessful. While there are a variety of methodologies that may be employed to isolate membrane-bound enzymes, an alternative approach involves covalent labelling of proteins with site-directed ligands bearing photoreactive groups. The nitrene generating analogs of fatty acid³ and acyl-CoA (ref. 4) have been successfully used in the identification and characterization of membrane-bound proteins^{5,6}.

In this paper, we report the use of 12-[(4-azidosalicyl)amino]dodecanoyl-CoA (ASD-CoA), a photoreactive acyl-CoA analog on the activity of lysophosphatidic acid (LPA) acyltransferase (EC 2.3.1.51) in microsomal membranes of developing castor endosperm. ASD-CoA acts as a reversible inhibitor in the dark, and as an irreversible inactivator on exposure to ultraviolet light. Upon photolysis, the photoprobe was selectively incorporated into a 29 kDa polypeptide among the microsomal membrane proteins.

Materials and methods

Materials

[1- ^{14}C]Oleoyl-CoA (58.3 Ci/mmol) and [^{125}I]NaI (17 Ci/mg/0.1 ml) were obtained from New England Nuclear, Boston, USA. *N*-hydroxysuccinimidyl-4-azidosalicylic acid (NHS-ASA) and bicinchoninic acid protein assay reagents were obtained from Pierce, Rockford, USA. Prep-sep C18 reverse phase column (1 ml) was obtained from Fisher Scientific, USA. All other chemicals and enzymes were purchased from Sigma Chemical Company, St. Louis, USA. Seedpods were harvested from field grown castor (*Ricinus communis* L.) plants at 35–44 days after flowering. Endosperms were collected and stored at -80°C until further use.

Preparation of castor microsomal membranes

Developing castor endosperm (5 g) was ground in a pre-chilled mortar and pestle for 5 min with 150 mM Hepes (pH 7.6), 10 mM KCl, 1 mM MgCl_2 , 1 mM EDTA, 1000 U/ml catalase and 0.25 M sucrose (10 ml). All the operations were performed at 4°C . The crude homogenate was passed through two layers of miracloth and centrifuged at 270 g for 10 min to remove debris. Sucrose step gradient was prepared according to the procedure of Lord⁷ with minor modifications. The gradient consisted of 10 ml each of 40, 30 and 20% (w/v) sucrose layered over 5 ml of 50% sucrose in a 40 ml centrifuge tube. The 270 g supernatant (4 ml) was directly layered on the sucrose gradient and centrifuged at 82,000 g for 3 h in a Beckman SW 28 rotor. The fraction at the 20–30% sucrose interface was collected by puncturing the tube on the side with an 18-gauge needle attached to a syringe, and was diluted 5-fold with the grinding medium containing no sucrose. This was again centrifuged at 100,000 g for 60 min and the pellet obtained was resuspended in a small volume of buffer containing 50 mM Hepes (pH 7.6) and 1 mM EDTA with the aid of a glass homogenizer. Microsomal membrane fraction was either used immediately or frozen in

*For correspondence. (e-mail: lipid@biochem.iisc.ernet.in)

small aliquots in liquid nitrogen and stored at -80°C . The activity of LPA acyltransferase in such preparations was stable at -80°C for a minimum of 3 months.

LPA acyltransferase assay

The assay mixture consisted of 50 mM Hepes (pH 7.6), 1 mM EDTA, 5 mM NaF, 20 μM ^{14}C -labelled oleoyl-CoA (55,000 cpm), 20 μM oleoyl-LPA and 20–30 μg of membrane proteins in a total volume of 100 μl . After incubation at 30°C for 10 min, the reaction was stopped by the addition of 0.7 ml chloroform/methanol (1:2 v/v) followed by 0.2 ml 1% acetic acid. The lipids were extracted and separated on precoated 250 μm silica gel G plates (Merck) using a chloroform/methanol/acetic acid/water (170:25:35:4 v/v) solvent system⁸. After visualizing the lipids with iodine vapour, phosphatidic acid was scraped and radioactivity determined in a liquid scintillation counter. Membrane preparations kept in a boiling water bath (10 min) were used as controls and these values were subtracted from the values measured in the presence of active membranes. Protein concentrations were determined by the bicinchoninic acid method⁹ using bovine serum albumin as the standard.

Synthesis of ^{125}I -labelled ASD-CoA

12-[(4-azidosalicyl)amino]dodecanoic acid was synthesized by reacting 12-aminododecanoic acid with NHS-ASA. ASD-CoA was synthesized by coupling CoA with 12-[(4-azidosalicyl)amino]dodecanoic acid and purified using Prep-Sep C18 reverse phase column. Iodination of ASD-CoA was performed by using oxidative chloramine-T method. The iodinated product, 12-[(3-iodo-4-azidosalicyl)amino]dodecanoyl-CoA, was also purified by reverse phase column chromatography¹⁰. Initially, we studied the incorporation of ^{125}I -labelled ASD-CoA into LPA and found no radioiodinated phosphatidic acid suggesting that this photoprobe does not act as a substrate for LPA acyltransferase.

Photoaffinity labelling of microsomal membranes

The photolabelling reaction was carried out in the cap of microcentrifuge tube and a final volume of 50 μl containing 60 μg of microsomal membrane proteins in 50 mM Hepes (pH 7.6), 1 mM β -mercaptoethanol and 0.5 μM of the labelled photoprobe (0.5 μCi). The reaction mixture was kept in an ice bath and irradiated with a hand-held UV lamp with the filter removed (model UVG-54, Ultraviolet Products) at a distance of 7 cm for 2 min. The photoprobe first exposed to UV light before addition to the microsomal membranes served as a control. Proteins were precipitated with 10% trichloro-

acetic acid and the mixture was kept in the ice bath for 15 min and centrifuged in a microfuge for 15 min. The resulting pellets were washed with cold acetone, solubilized with 50 μl of loading buffer and analysed by 10% SDS-PAGE¹¹. The gel was stained with Coomassie blue R-250, dried, and labelled polypeptides were identified by autoradiography. The stained radio-labelled bands were excised and radioactivity determined by scintillation counting. The experiments were repeated four times with different microsomal membrane preparations.

Results

Reversible inhibition of LPA acyltransferase activity by ASD-CoA

We examined the effect of ASD-CoA on the LPA acyltransferase activity. The enzyme was assayed in the presence of varying concentrations (0–10 μM) of ASD-CoA using castor microsomal membranes. The incorporation of ^{14}C -oleoyl-CoA to LPA was inhibited by ASD-CoA in a concentration-dependent manner (Figure 1). Plots of $1/v$ vs $1/x$ from these data yielded a family of lines that intersected to the left of the y-axis. The apparent K_i was calculated to be 13 μM .

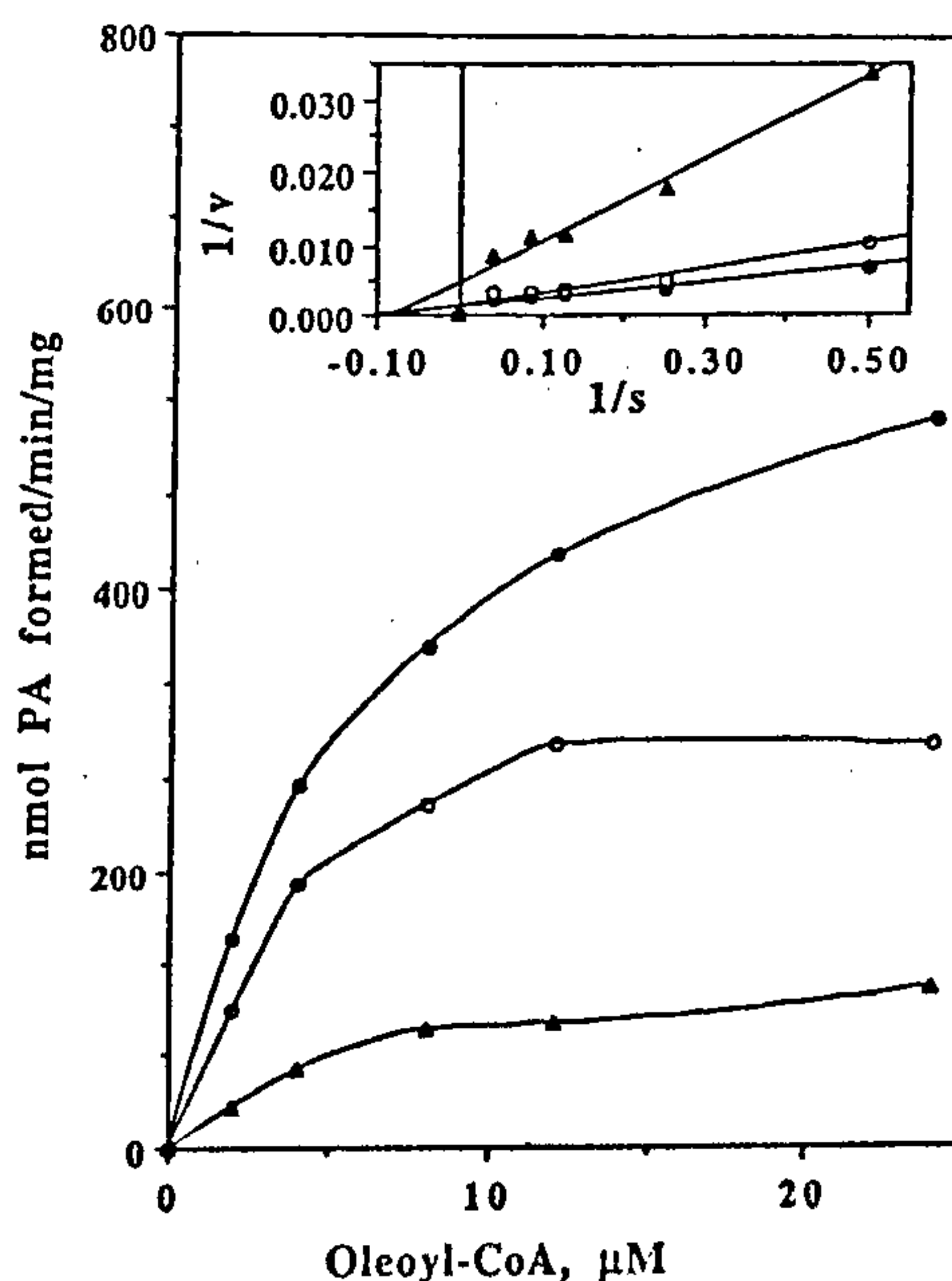


Figure 1. Inhibition of LPA acyltransferase activity by ASD-CoA, 0 (\bullet), 5 (\circ) or 10 (Δ) μM was added and the enzyme assayed in the dark containing oleoyl-CoA (0–24 μM) and 35 μg of microsomal membrane proteins in the dark. Inset shows the Lineweaver-Burk analysis of the same data. Values are expressed as mean of three determinations. The variation among individual experiments was < 13%.

(Figure 1, inset). After incubation with ASD-CoA (25 μ M), the castor microsomal membranes were diluted 100-fold with 50 mM Hepes (pH 7.6) and then washed. This resulted in 80% recovery of activity (Table 1). Initial experiments showed that the above washing procedure removed all the 125 I-labelled ASD-CoA incubated with castor microsomal membranes.

Irreversible inactivation of LPA acyltransferase by ASD-CoA

In another set of experiments, ASD-CoA (25 μ M) was photolysed by exposure to UV light in the presence of castor microsomal membranes. The membranes were then washed to remove the unbound photoprobe and assayed for LPA acyltransferase activity under standard conditions. The enzyme activity was inhibited (73%) as compared to unphotolysed samples. This indicated that the photolysis of the substrate analog resulted in an irreversible inhibition of the enzyme. In the next set of experiments, the castor microsomal membranes were incubated with increasing concentrations of oleoyl-CoA along with ASD-CoA during photolysis. The unbound photoprobe was removed by washing and the membranes were then assayed for the activity. Addition of oleoyl-CoA in these reaction mixtures protected the enzyme against the inactivation with complete protection at 0.1 mM (Table 1). Implicit in this finding of irreversible inactivation are interaction of the photoprobe at the acyl-CoA-binding site of the enzyme and its covalent linking to the protein during photolysis.

Photolabelling of castor microsomal membrane proteins with ASD-CoA

Photolysis-dependent transfer of the photoprobe to the

Table 1. Photoinactivation of LPA acyltransferase activity by ASD-CoA

Addition (μ M)		Irradiation	Activity (%)
Oleoyl-CoA	ASD-CoA		
25	—	+	100
—	25	—	81
—	25	+	22
25	25	+	43
50	25	+	64
100	25	+	78

Microsomal membranes (186 μ g protein) were photolysed in a total volume of 0.1 ml containing 25 μ M ASD-CoA, followed by 100-fold diluted with 50 mM Hepes buffer (pH 7.6). The mixture was pelleted at 100,000 g for 1 h and assayed under standard conditions using 37.2 μ g protein. The presence or absence of UV irradiation is indicated by + and —, respectively. 100% activity represents 0.618 nmol of phosphatidic acid formed/min/mg. Values are mean per cent of three determinations.

protein was confirmed by the incorporation of 125 I-labelled ASD-CoA. Castor microsomal membranes were added to 125 I-labelled ASD-CoA during and after photolysis and the membrane proteins were separated by SDS-PAGE. Autoradiography of the gel showed that a 29 kDa polypeptide is the single major labelled product in the sample when the protein and the photoprobe were presented together during exposure to UV light. Traces of radioactivity also appeared in other polypeptides with molecular masses of 63, 55, 52 and 33 kDa (Figures 2 a and 3 a). A significant incorporation occurred even at 1 min of irradiation and the maximal incorporation was at 2 min (Figures 2 a and 2 b), indicating the rapidity

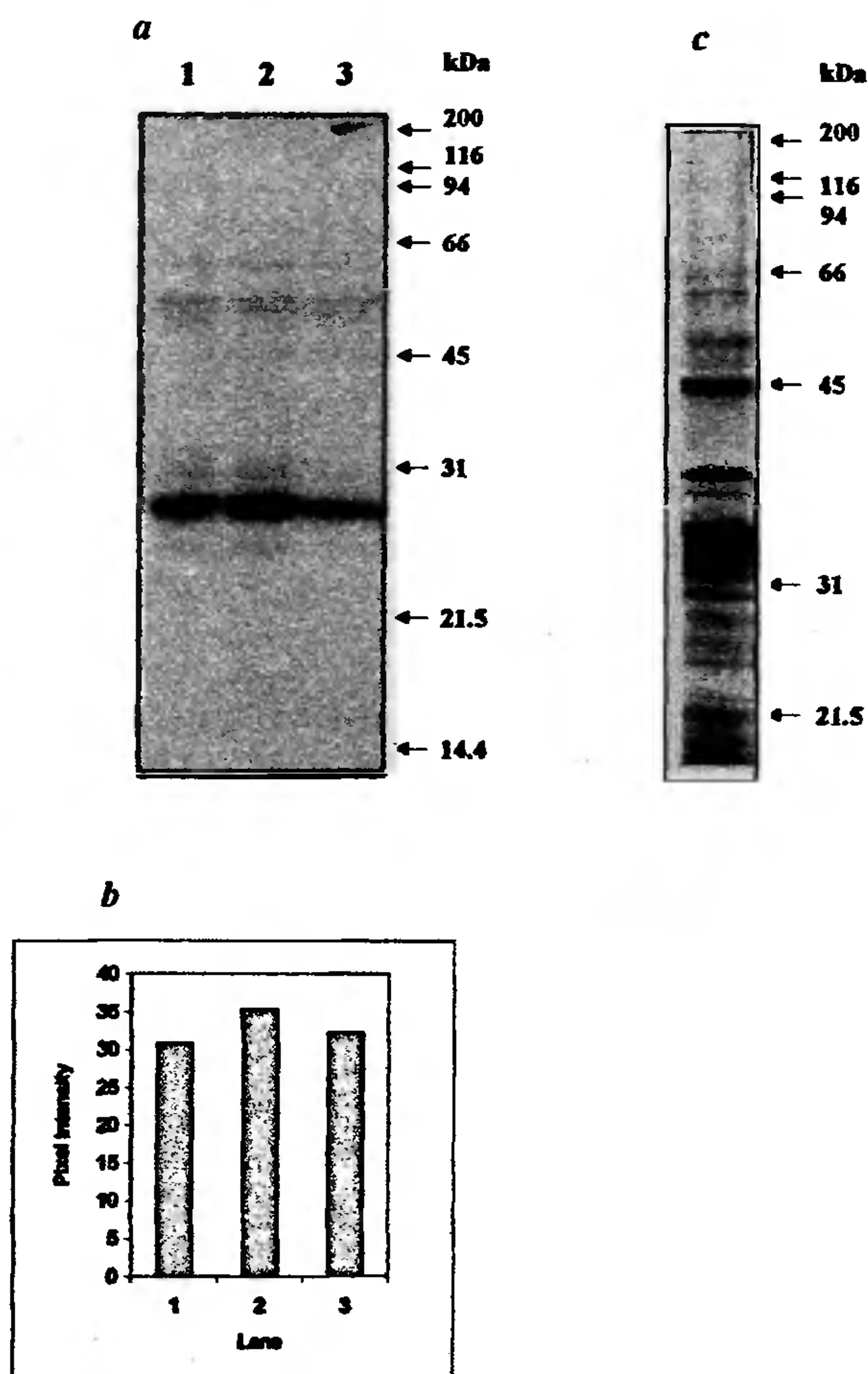


Figure 2 a-c. Time course of photolabelling of microsomal membranes with ASD-CoA. *a*, Microsomal membranes (60 μ g) were photolysed with 125 I-labelled ASD-CoA under standard conditions, for 1 min (lane 1), 2 min (lane 2) and 3 min (lane 3); *b*, Densitometric scanning data of the 29-kDa polypeptide; *c*, Coomassie blue stained microsomal membrane proteins (60 μ g) profile. The photolysed samples were analysed on 10% SDS-PAGE and autoradiography. Molecular weight markers (kDa) are indicated along the side.

of the reaction. 2 min irradiation brought about 17% increase in incorporation compared to 1 min irradiation (Figure 2 *b*). No labelling was observed without exposure to UV light. The labelled 29 kDa polypeptide is not an abundant protein in the microsomal membranes as evident from the total protein pattern observed with Coomassie blue staining (Figure 2 *c*). Nevertheless, it accounted for 85% of the radioactivity recovered, by excising the band on the dried gel and counting.

As expected from the foregoing experiments on enzyme activity, protection against covalent incorporation of ASD-CoA by oleoyl-CoA was also observed (Figure 3 *a*). The labelling of the 29 kDa polypeptide decreased in a concentration-dependent manner with ~60% protection observed at 50 μ M oleoyl-CoA (Figure 3 *b*).

Discussion

A photoreactive, radioiodinatable acyl-CoA analog of high specific activity synthesized in our laboratory proved to be a useful tool in identifying LPA acyltransferase from microsomal membranes of developing castor endosperm. The photoprobe apparently interacts with the LPA acyltransferase activity with high avidity as indicated by low K_i . The specificity of the interaction with ASD-CoA was demonstrated by the protection of the

enzyme from photoinactivation by oleoyl-CoA (Table 1). The analog therefore can be used as a specific photo-affinity reagent for this enzyme.

To obtain specific affinity labelling, we have done all the photolabelling experiments with a low concentration of ASD-CoA (0.5 μ M) and in the presence β -mercaptoethanol. Monothiols act as good nucleophiles and hydrogen atom donors to quench the unbound reactive intermediates such as nitrenes and anilinyll radicals, thereby reducing non-specific labelling of proteins¹². Photolysis of ASD-CoA prior to the addition of microsomal membranes did not label the protein, indicating that long-lived chemically reactive intermediates are not involved in this labelling (Figure 3 *a*, lane 1). The specificity of labelling of the protein was also shown by the protection experiments (Figure 3 *a*). The substrate, oleoyl-CoA prevented photolysis-dependent incorporation of ASD-CoA into proteins, indicating that ASD-CoA interacts with the acyl-CoA-binding site of the acyl-transferase. Put together, these results show that it is highly unlikely that the labelling of proteins had resulted from non-specific cross-linking of the photoprobe.

Bulk of the radioactivity in the labelled membrane proteins was associated with the 29-kDa polypeptide suggesting that the chemically reactive intermediate has high reactivity towards this protein. In addition to the

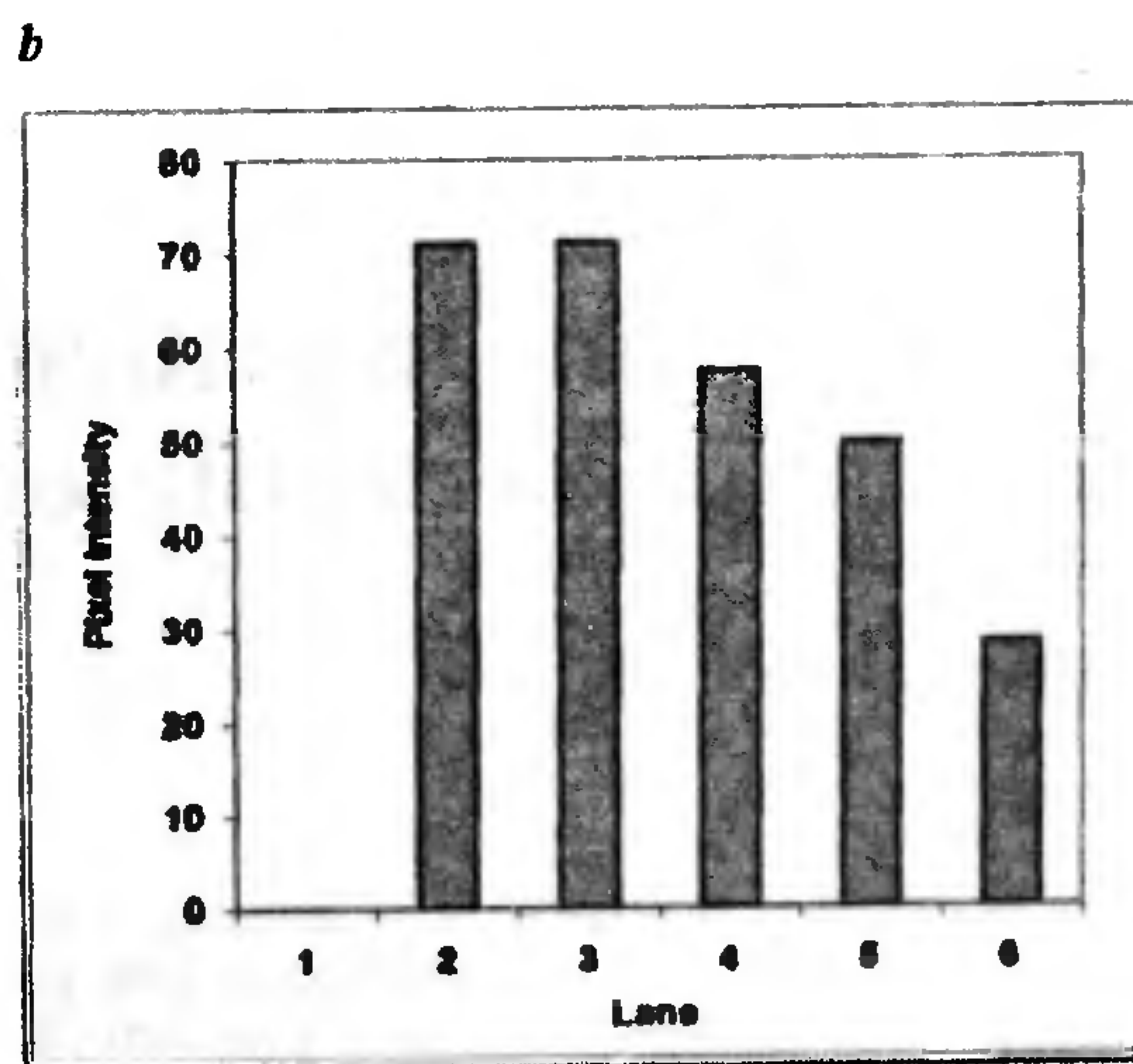
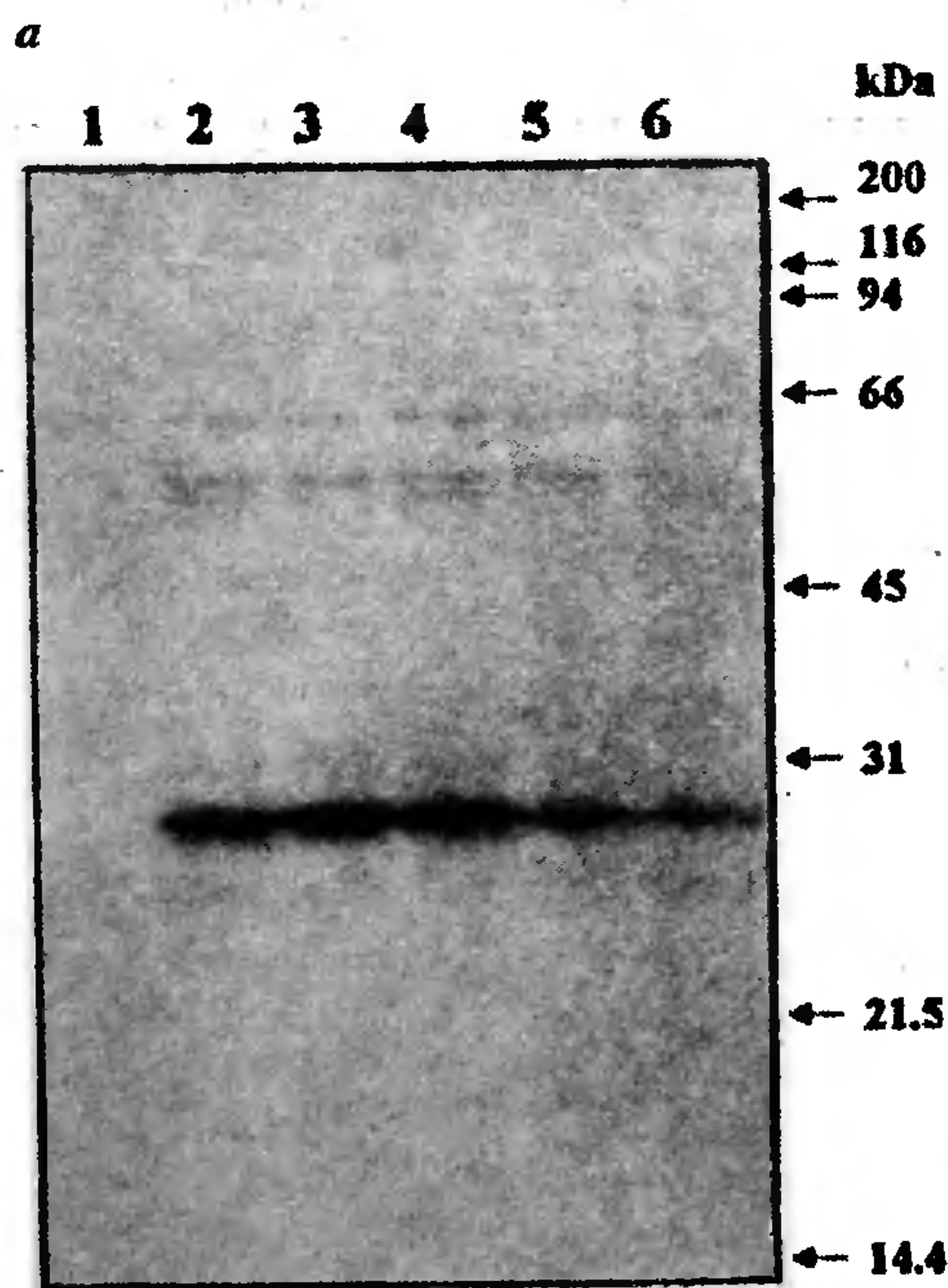


Figure 3 *a, b*. Protection against photolabelling of ASD-CoA by oleoyl-CoA. *a*, Microsomal membranes were photolabelled for 2 min with ASD-CoA in the presence of increasing concentrations of oleoyl-CoA: lane 2-6, 0, 5, 10, 25, 50 μ M. As a control, the photoprobe was irradiated with UV for 2 min prior to the addition of microsomal membrane (lane 1). The photolysed samples were analysed on 10% SDS-PAGE and autoradiography. Molecular weight markers (kDa) are indicated along the side; *b*, Densitometric scanning data of the 29-kDa polypeptide. The photolysed samples were analysed on 10% SDS-PAGE and autoradiography. Molecular weight markers (kDa) are indicated along the side.