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A sensitive spectrophotometric assay for peroxisomal acyl-CoA oxidase

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A simple spectrophotometric assay was developed for peroxisomal fatty acyl-CoA oxidase activity. The assay, based on the H_2O_2-dependent oxidation of leuco-dichlorofluorescein catalysed by exogenous peroxidase, is more sensitive than methods previously described. By using mouse liver samples, cofactor requirements were assessed and a linear relationship was demonstrated between dye oxidation and enzyme concentration. By using this assay on subcellular fractions, palmitoyl-CoA oxidase activity was localized for the first time in microperoxisomes of rat intestine. The assay was also adapted to measure D-amino acid oxidase activity, demonstrating the versatility of this method for measuring activity of other H_2O_2-producing oxidases.

There is now much evidence that peroxisomal fatty acid oxidation occurs in the liver of a wide variety of animal species (Lazarow, 1978; Murphy *et al.*, 1978; Bronfman *et al.*, 1979; Small *et al.*, 1980). A smaller organelle, microperoxisomes, is ubiquitous in animal tissues, and the presence of β-oxidation enzymes has been established for several tissues (Kramar *et al.*, 1978; Small *et al.*, 1980; Silcox *et al.*, 1982; Le Hir & Dubach, 1982; Connock & Perry, 1983).

Of utility in surveying for peroxisomal fatty acid oxidation is the H_2O_2-producing acyl-CoA oxidase (EC 1.3.99.3), which is characteristic only of the peroxisomal system, whereas the other catalytic activities of the β-oxidation sequence may be shared with the mitochondria. Several assays have been developed for measuring this oxidase, including the method of Lazarow & de Duve (1976), which measures the cyanide-insensitive acyl-CoA-dependent reduction of NAD^+, the O_2-electrode measurement of oxygen consumption (Inestrosa *et al.*, 1979), estimation of H_2O_2 production by coupling with the peroxidation of methanol to formaldehyde, which is then measured (Inestrosa *et al.*, 1979), and a spectrophotometric assay measuring the peroxidase-coupled oxidation of a chromogen (*p*-hydroxybenzoic acid + 4-amino-antipyrine) (Hyrb & Hogg, 1979).

In order to detect peroxisomal β-oxidation in extrahepatic tissues as well as in liver, we developed a highly sensitive spectrophotometric assay for fatty acyl-CoA oxidase and reported finding activity in a wide range of guinea-pig tissues, in a publication which included a brief description of the assay (Small *et al.*, 1980).

Here we describe the development and characteristics of the spectrophotometric assay, which is more sensitive than previous methods, owing to the large absorption coefficient of the dye leuco-DCF, and also the favourable stoichiometry of the peroxidation reaction. We also report the presence and subcellular localization of the oxidase in rat intestinal mucosa.

Abbreviation used: DCF, 2,7-dichlorofluorescein.

Materials and methods

Chemicals

Leuco-DCF diacetate was from Eastman Kodak, Rochester, NY, U.S.A. 3-Amino-1,2,4-triazole was from Fluka A.G., Buchs, Switzerland. Palmitoyl-CoA, horseradish peroxidase, bovine liver catalase, cofactors and enzyme substrates were purchased from Sigma, Poole, Dorset, U.K. All other chemicals used were of highest analytical grade, from BDH Chemicals, Poole, Dorset, U.K.

Animals used were Sprague–Dawley rats, weighing 250–300g, and albino mice, weighing 40–60g.

Tissue preparation

All homogenates were prepared in 10% (w/w) sucrose containing 3mM-imidazole, pH7.4 (SI

medium). The animals were killed and the tissues removed into ice-cold SI medium.

To prepare a peroxisome-enriched fraction, the liver was minced with a razor blade and homogenized with three strokes by hand in a Potter–Elvehjem homogenizer. The resulting homogenate was centrifuged at 6000*g* for 10min at 6°C; the pellet was resuspended in a small volume of SI medium and re-centrifuged as above. The supernatants were combined and centrifuged for 30min at 30000*g*; the resulting pellet was resuspended in SI medium.

Rat intestinal fraction was obtained by preparing a mucosal scrape, which was then homogenized vigorously (15 strokes with motor-driven pestle). The resulting homogenate was centrifuged (30000*g* for 30min) and the pellet was resuspended in an appropriate volume of SI medium.

Subcellular fractionation

Homogenate from rat intestine was obtained as above. The homogenate was centrifuged (2000*g* for 3min), and the pellet was washed and re-centrifuged at 3500*g* for 3min to obtain a supernatant, which could easily be poured from the mucous pellet. Then 30ml of this post-nuclear supernatant was loaded into a B XIV zonal rotor containing a 450ml linear sucrose gradient (13–35%, w/w, in 3mM-imidazole, pH7.4) resting on a cushion of 52% sucrose. An overlay of SI medium was added, and centrifugation was at 16000 rev./min for 45min at 6°C. Fractions were collected and assayed for enzyme activities and protein. Enzyme distributions between subcellular fractions are given in the form of frequency histograms (de Duve, 1967).

Fatty acyl-CoA oxidase assay

The spectrophotometric assay of acyl-CoA oxidase was based on the determination of H_2O_2 production, which was coupled to the oxidation of leuco-DCF in a reaction catalysed by exogenous peroxidase. For the latter the method of Kochli & Von Wartburg (1978) for measuring monoamine oxidase was adapted.

The reaction was carried out in a semi-micro cuvette at 30°C in a final volume of 1ml. The assay mixture contained 0.05mM-leuco-DCF (prepared daily at 2.6mM in 1 vol. of *NN*-dimethylformamide and 9vol. of 0.01M-NaOH, stored in a light-tight container under N_2 gas), 0.08mg of horseradish peroxidase, 40mM-aminotriazole, 0.02% Triton X-100, 11mM-potassium phosphate buffer, pH7.4, and an appropriate volume of tissue homogenate or subcellular fraction and SI medium. This mixture was preincubated in the dark for 5min, as some impurities in the peroxidase cause a small amount of oxidation of leuco-DCF (Kochli & Von Wartburg, 1978). After this time there is a slow rate of autoxidation of the dye, which was determined by measuring the change in A_{502} in a Unicam split-beam recording spectrophotometer for approx. 2min. The reaction was then started with the addition of 30μM-palmitoyl-CoA, and the enzymic reaction rate was determined. Rates were then corrected for substrate blank.

Other assays

Cyanide-insensitive palmitoyl-CoA-dependent reduction of NAD^+ was assayed as described by Lazarow & de Duve (1976). Fluorimetric determination of palmitoyl-CoA oxidase was as described by Walusimbi-Kisitu & Harrison (1983).

D-Amino acid oxidase activity was measured by a modification of the leuco-DCF method, with 45mM-D-alanine as substrate.

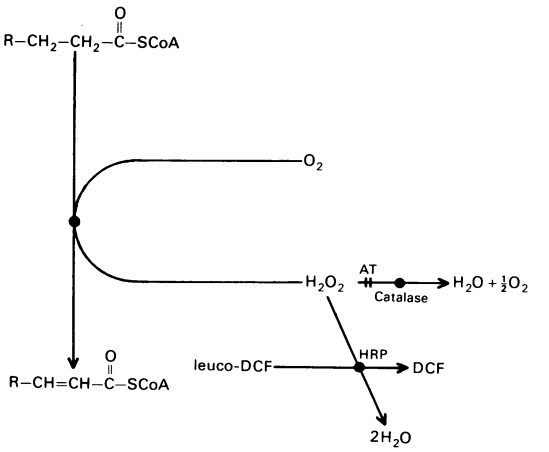
Assays for marker enzymes of other subcellular organelles were as previously described (Small *et al.*, 1980). Protein was measured as described by Lowry *et al.* (1951), with bovine serum albumin as standard.

Results and discussion

The properties of leuco-DCF have been described by Kochli & Von Wartburg (1978). Our modifications to their assay include the use of aminotriazole to prevent the destruction of H_2O_2 by endogenous catalase (Scheme 1).

Effect of aminotriazole

The effects of aminotriazole were determined by two methods. Firstly the concentration of amino-



Scheme 1. *Method for the spectrophotometric measurement of acyl-CoA oxidase*

Horseradish peroxidase (HRP) is added to catalyse the H_2O_2-dependent oxidation of leuco-DCF to DCF, a green dye. Aminotriazole (AT) is included to inhibit interference by catalase.

triazole required to prevent interference by catalase in a peroxisome-enriched fraction of mouse liver was investigated by assaying catalase activity in the presence of aminotriazole. Fig. 1 indicates that the degree of catalase inactivation depends on the concentration and duration of preincubation with aminotriazole.

The second method used was to add pure bovine catalase to samples, which were then assayed for palmitoyl-CoA oxidase activity. In the absence of aminotriazole, a 2.5-fold increase in catalase activity decreased the apparent oxidase activity by 20–30%. However, a 5 min incubation with 40 mM-aminotriazole allowed up to a 10-fold increase in catalase activity without effect on the measured oxidase activity.

Aminotriazole (40 mM) was included in all subsequent assays of acyl-CoA oxidase; this concentration is likely to be in excess of that necessary to inactivate the catalase present in most tissue samples.

Stoichiometry of H_2O_2 production and leuco-DCF oxidation

Kochli & Von Wartburg (1978) quoted a stoichiometry of 5.3 mol of leuco-DCF oxidized/mol of H_2O_2 produced in their assay for monoamine oxidase. To determine this ratio under our assay conditions for palmitoyl-CoA oxidase we attempted to produce a standard curve by adding known amounts of H_2O_2 to mock reaction mixtures containing all reagents except tissue homogenate. However, the results of such experiments were variable, probably because of the instability of the micromolar concentrations of H_2O_2 at 30°C. Therefore we adopted two indirect methods for estimating the stoichiometry of this reaction.

Firstly we used the leuco-DCF-based assay to measure the palmitoyl-CoA oxidase activity of a peroxisome-enriched fraction of mouse liver and compared this with the activity measured by the NAD^+-based assay of Lazarow & de Duve (1976), in which the ratio of H_2O_2 production and NAD^+ reduction is thought to be 1:1 (Inestrosa *et al.*, 1979). The medium for the NAD^+-based assay was modified slightly from that published, so that the conditions corresponded as closely as possible to those in the leuco-DCF-based assay. Aminotriazole had no effect when tested in this assay; however, the inclusion of horseradish peroxidase inhibited NADH production, and it was therefore excluded from the medium. The same sample was used for the two assays, but had to be much diluted for use in the leuco-DCF method.

The relative stoichiometry is defined as:

$$\frac{\text{mol of leuco-DCF oxidized} \cdot \text{min}^{-1}}{\text{mol of NAD}^+ \text{ reduced} \cdot \text{min}^{-1}}$$

assuming absorption coefficients of $91000\,\text{M}^{-1} \cdot \text{cm}^{-1}$ and $6200\,\text{M}^{-1} \cdot \text{cm}^{-1}$ for leuco-DCF and NADH respectively (Kochli & Von Wartburg, 1978; Dawson *et al.*, 1974).

The results of six such experiments produced a relative stoichiometry of 3.5 ± 0.3.

The relative sensitivity of the assays, defined as:

$$\frac{\Delta A_{502}\ (\text{DCF})}{\Delta A_{340}\ (\text{NAD}^+)}$$

was 51.3 ± 4.4 for the same six experiments.

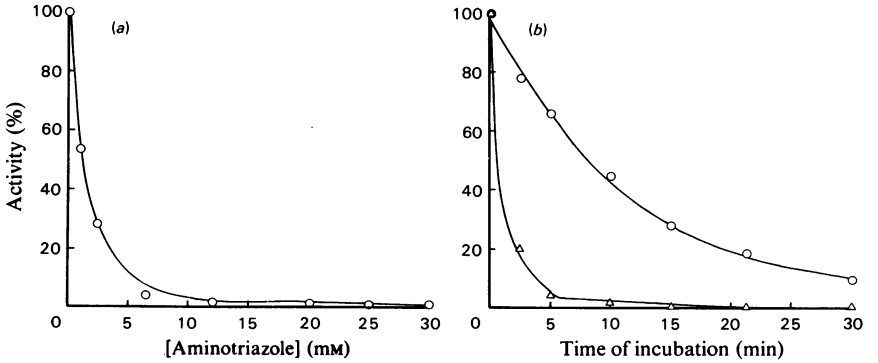


Fig. 1. *Effect of aminotriazole on catalase activity*

(*a*) Dependence of catalase activity of a mouse liver fraction (0.19 mg of protein/ml) on aminotriazole concentration in the incubation. Samples were incubated at room temperature for 20 min at a similar concentration to that found suitable for measuring acyl-CoA oxidase activity by the leuco-DCF assay. (*b*) Dependence on time of incubation with (○) 20 mM- and (△) 40 mM-aminotriazole on catalase activity of a mouse liver fraction containing approx. 0.3 mg of protein/ml.

The second method used to determine the stoichiometry of this reaction involved the use of the fluorimetric assay of Walusimbi-Kisitu & Harrison (1983) for measuring acyl-CoA oxidase activity. The effect of including different concentrations of aminotriazole in this assay medium was tested. In the presence of increasing amounts of aminotriazole, the H_2O_2 was progressively less able to decrease the fluorescence of scopoletin; therefore aminotriazole could not be included in the medium for the fluorescence-based assay. This assay was first used to produce a standard curve for H_2O_2 solutions, which could be kept on ice and were therefore relatively stable. The relationship was linear in the range used (0–0.7 nmol of H_2O_2). The assay was then employed to measure palmitoyl-CoA oxidase activity in a peroxisome-enriched liver fraction. The result, expressed in nmol of H_2O_2 released, was deduced from the standard curve. The same enzyme sample was then used in the leuco-DCF assay to measure nmol of DCF oxidized. As the H_2O_2 production should be identical in both assays, the ratio of leuco-DCF oxidized to H_2O_2 produced could be calculated. With peroxisome-enriched liver fractions from rat and mouse, the H_2O_2 production was 4.64 and 2.46 nmol of H_2O_2/min per mg of protein respectively, and the leuco-DCF oxidation was 14.08 and 8.25 nmol of DCF oxidized/min per mg of protein for the same samples. This indicates a stoichiometry of approx. 3.2 mol of leuco-DCF oxidized/mol of H_2O_2 produced, and confirms the results above.

The results of the latter experiment indicate that our assay is approx. 3 times more sensitive than the fluorimetric assay of Walusimbi-Kisitu & Harrison (1983), a conclusion which was confirmed by continuous dilution of the enzyme sample until activity was no longer detectable fluorimetrically over 20 min (the maximum time for which this assay is linear), but could still be measured by the spectrophotometric assay.

A further advantage of the leuco-DCF assay is its linearity over a wide range of protein concentration, whereas the fluorimetric method has a very limited linearity range (Fig. 2).

Effect of cofactors

The effect of various cofactors was studied in order to determine optimum assay conditions. Addition of CoA and NAD^+ had no effect on the measured activity. Similarly, there was no benefit

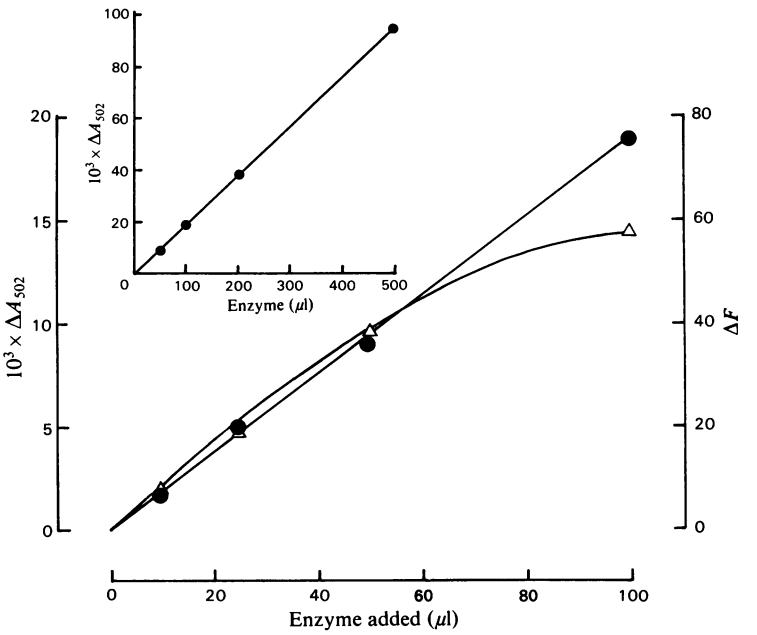


Fig. 2. *Effect of enzyme concentration on spectrophotometric and fluorimetric assays for measuring acyl-CoA oxidase*
The spectrophotometric assay was carried out as in the Materials and methods section, with various amounts of mouse liver fraction (0.3 mg of protein/ml). The results are expressed as change in A_{502} of DCF/min (●). The fluorimetric assay of Walusimbi-Kisitu & Harrison (1983) was used to measure acyl-CoA oxidase activity in the same enzyme sample. Incubations were at 37°C for 20 min, and results are expressed as change in fluorescence (ΔF) of scopoletin/20 min (△). Inset shows change in A_{502} of DCF on a condensed scale with greater amounts of enzyme sample.

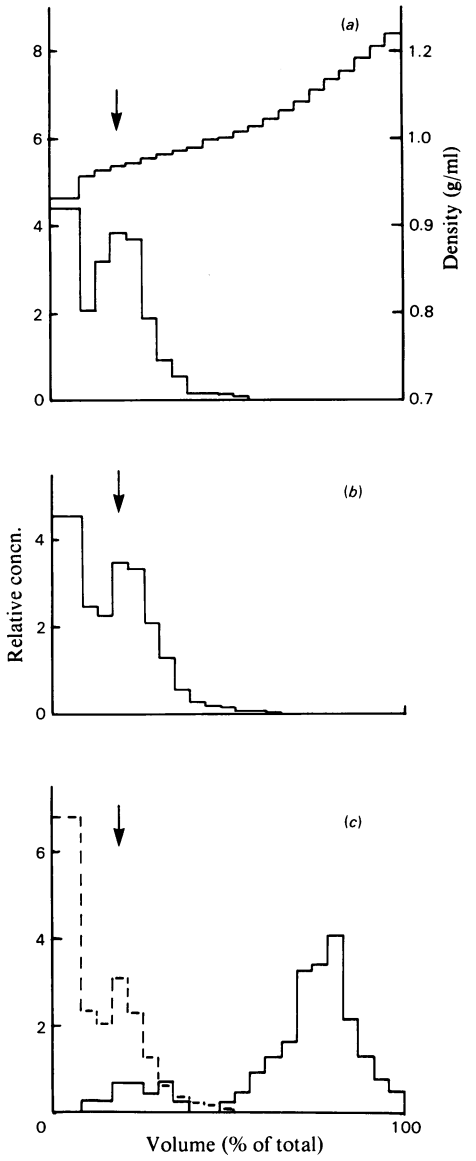


Fig. 3. *Distribution of enzyme activities in subcellular fractions of rat intestinal mucosa*

Rate-dependent centrifugation was carried out in a B XIV zonal rotor at 16000 rev./min for 45min at 6°C. (*a*) Lower line shows distribution of catalase (peroxisomes); higher line shows density (g/ml). (*b*) Palmitoyl-CoA oxidase. (*c*) Continuous line shows distribution of succinate dehydrogenase (mitochondria); broken line shows that of D-amino acid oxidase (peroxisomes). The arrow marks the peak peroxisome fraction. The total recoveries of the constituents in the fractions, compared with post-nuclear supernatant, were (%); catalase, 119; palmitoyl-CoA oxidase, 78; succinate dehydrogenase, 79; D-amino acid oxidase, 70; protein (not shown), 99.

from inclusion of FAD in the reaction mixture, although the oxidase is a flavoprotein; furthermore the rate of DCF oxidation in the absence of substrate was increased in the presence of FAD, which therefore was omitted from further assays.

Subcellular fractionation

Using their fluorimetric assay for measuring fatty acyl-CoA oxidase, Walusimbi-Kisitu & Harrison (1983) were unable to detect activity in several tissues, including homogenates of rat intestine. We have previously shown that with the leuco-DCF assay acyl-CoA oxidase is localized in the microperoxisomes of guinea-pig and mouse small intestine (Small *et al.*, 1980, 1981). In rat intestinal homogenates we have found that measurement of this enzyme is hindered by high blank rates in the absence of substrate; however, the enzyme activity can readily be measured in a particulate fraction prepared from such homogenates.

To confirm that we were measuring peroxisomal palmitoyl-CoA oxidase activity, we compared the subcellular distribution of the oxidase with that of organelle marker enzymes in fractions prepared from a rat intestinal-mucosa post-nuclear supernatant (Fig. 3).

The similar distribution of palmitoyl-CoA oxidase activity and that of catalase (peroxisome marker) implies that both enzymes are localized in the same organelle. Furthermore, the distribution of D-amino acid oxidase as assayed by the leuco-DCF method confirms the single previous report of the enzyme's peroxisomal localization in this tissue (Peters & Shio, 1976).

In conclusion, the assay reported here for measuring peroxisomal acyl-CoA oxidase activity is approx. 50 times more sensitive than the first spectrophotometric method used for measuring this enzyme (Lazarow & de Duve, 1976). It also has advantages of greater sensitivity and linearity over the fluorimetric assay more recently reported (Walusimbi-Kisitu & Harrison, 1983). Using our assay we have been able to detect palmitoyl-CoA oxidase activity in rat small-intestine microperoxisomes for the first time.

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