The activity and subcellular distribution of the peroxisomal enzyme acyl-CoA oxidase in human blood platelets

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The peroxisomal enzyme acyl-CoA oxidase is localized in the 'dense-tubular-system-enriched fraction', probably identical with the endoplasmic reticulum, in human blood platelets. This localization is strongly different from the localization of catalase which seems to be a cytosolic enzyme, in agreement with Marcus, Zucker-Franklin, Safir & Ullman [(1966) *J. Clin. Invest.* **45**, 14–28]. A localization of acyl-CoA oxidase in the endoplasmic reticulum seems to be in good accordance with the important role of peroxisomes in the metabolism of prostaglandins, as recently demonstrated by Diczfalussy, Kase, Alexson & Björkhem [(1991) *J. Clin. Invest.* **88**, 978–984].

INTRODUCTION

Very few studies on peroxisomes in human blood platelets have been published. The existence of a subcellular organelle termed 'microperoxisomes' in human platelets was described by Breton-Gorius & Guichard (1975). White (1979) found peroxidase-positive small granules in platelets from patients with the Gray platelet syndrome. Decreased activity of the peroxisomal enzyme acyl-CoA: dihydroxyacetone phosphate acyltransferase in human blood platelets has been described in Zellweger-syndrome patients (Wanders *et al.*, 1985) and in infantile Refsums disease (Poll-The *et al.*, 1986).

Previously we have described that the subcellular distribution of several fatty-acid-metabolizing enzymes in human platelets is similar to that in most other cells and tissues studied. Long-chain acyl-CoA synthetase (Farstad *et al.*, 1973; Bakken & Farstad, 1989) was found in the mitochondria and the 'dense tubular system', acyl-CoA hydrolase (Berge *et al.*, 1980) was found in the mitochondrial matrix and the cytosolic fraction, and acyl-CoA:2-lysophosphatidyl acyltransferase(s) (A. M. Bakken & M. Farstad, unpublished work) in mitochondria and the 'dense tubular system'.

We now describe the subcellular distribution of the peroxisomal enzyme acyl-CoA oxidase in human platelets. The results indicate that the peroxisomes on human platelets are located in, or closely connected to, the 'dense tubular system'.

MATERIALS AND METHODS

Reagents

Palmitic acid, oleic acid, palmitoyl-CoA, L-carnitine, FAD, BSA and peroxidase (type II) were from Sigma, St. Louis, MO, U.S.A. L-[methyl-¹⁴C]Carnitine hydrochloride, [1-¹⁴C]palmitic acid and [1-¹⁴C]oleic acid were from Amersham International, Amersham, Bucks., U.K. [1-¹⁴C]Tyramine (*p*-hydroxyphenethylamine) hydrochloride was from New England Nuclear, Boston, MA, U.S.A. All other reagents were of the highest purity commercially available.

Preparation of platelets and platelet subcellular fractions

Platelets were prepared from 450 ml portions of blood from healthy donors in ACD solution, and centrifuged and washed as previously described (Bakken & Farstad, 1989). Subcellular fractions were obtained after equilibration with nitrogen at 4.14 kPa, followed by decompression and differential centrifug-

ation as described by Bakken & Farstad (1989). The fractions were immediately frozen, and kept at –80 °C until analysed.

Platelet concentrates were stored for 7 days in blood bags (Travenol Laboratories, Castlebar, Ireland) as described by Hervig *et al.* (1990).

Protein determination

Protein concentration was determined in all fractions by the Bradford (1976) method, with BSA as standard.

Subcellular markers

Amine oxidase (EC 1.4.3.4) was determined as described by Aas (1971), carnitine palmitoyltransferase (EC 2.3.1.21), measuring total activity, as described by Bird *et al.* (1985), NADH dehydrogenase (EC 1.6.99.3) as described by Wallach & Kamat (1966), as modified by Record *et al.* (1982), and lactate dehydrogenase (EC 1.1.1.27) as described by the Scandinavian Committee on Enzymes (1981).

Catalase (EC 1.11.1.6) was determined as described by Aeby (1974), and acyl-CoA oxidase (EC 1.3.99.3) as described by Small *et al.* (1985). Palmitoyl-CoA dependent dehydrogenase (usually termed peroxisomal β -oxidation) was assayed by a procedure described by Berge *et al.* (1984).

Oxidation of [1-¹⁴C]palmitate or [1-¹⁴C]oleate by intact platelets, giving one [1-¹⁴C]acetyl unit per molecule of [1-¹⁴C]fatty acid, was determined as 1-¹⁴C-labelled acid-soluble products as described by Bremer & Wojtczak (1972) and Holmsen & Farstad (1987), with the following modifications: modified Tyrode buffer (dextrose omitted), 400 μ M-[1-¹⁴C]palmitate or [1-¹⁴C]oleate, 37 °C and incubation time 20 min, and terminating the reaction with 200 μ l of 24 % (w/v) HClO₄. The KCN-insensitive fatty acid oxidation was assayed by the same method in the presence of 1 mM-KCN.

RESULTS

Table 1 shows that the oxidation of both [1-¹⁴C]oleate and [1-¹⁴C]palmitate was lower in platelets stored for 7 days than in fresh platelets. In fresh platelets the oxidation of both oleate and palmitate was about the same as previously reported (Holmsen & Farstad, 1987). In the presence of KCN, both oleate and palmitate oxidation, in fresh as well as in stored platelets, was inhibited by about 85–90 %. This indicated that some of the fatty acid oxidation took place in peroxisomes or an organelle similar to peroxisomes.

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Table 1. Formation of $1\text{-}^{14}\text{C}$ -labelled acid-soluble products ($[1\text{-}^{14}\text{C}]\text{-ACP}$) from $[1\text{-}^{14}\text{C}]\text{palmitic acid}$ and $[1\text{-}^{14}\text{C}]\text{oleic acid}$ by intact human platelets from concentrates prepared from three donors, and by platelets from the same concentrates after 7 days of storage

Platelets (2×10^8) were incubated with $400 \mu\text{M}$ - $[1\text{-}^{14}\text{C}]\text{palmitate}$ or $[1\text{-}^{14}\text{C}]\text{oleate}$ in 1.0 ml of a modified Tyrode buffer (dextrose omitted) containing 8 mg of BSA/ml (pH 7.4). Incubation time was 20 min, and incubation temperature 37°C . The reaction was stopped by $200 \mu\text{l}$ of 24% HClO_4 , and the radioactivity was counted in a Packard Tri-Carb model 2450 instrument, by using Ultima Gold from Packard Instr. (Netherlands) as scintillator. The KCN-insensitive oxidation was assayed in the presence of 1 mM-KCN.

Platelets	Substrate	$[1\text{-}^{14}\text{C}]\text{ACP}$ formation (nmol/min per 10^8 platelets)		Inhibition (%)
		Without KCN	+ 1 mM KCN	
Fresh	$[1\text{-}^{14}\text{C}]\text{Oleate}$	0.130 ± 0.044	0.015 ± 0.009	88
	$[1\text{-}^{14}\text{C}]\text{Palmitate}$	0.137 ± 0.053	0.020 ± 0.010	85
Stored	$[1\text{-}^{14}\text{C}]\text{Oleate}$	0.079 ± 0.009	0.016 ± 0.004	80
	$[1\text{-}^{14}\text{C}]\text{Palmitate}$	0.061 ± 0.017	0.006 ± 0.003	90

Table 2. Specific activity of acyl-CoA oxidase in fractions of human blood platelets, prepared from three blood donors

The activity was assayed with palmitoyl-CoA as substrate in the presence of peroxidase, FAD and dichlorofluorescein as chromophore as described by Small *et al.* (1985). The 'M' fraction is the 12000 g, or 'mitochondria-enriched', fraction, the 'L' fraction is the 24000 g fraction, the 'P' fraction is the 100000 g, or 'dense-tubular-system-enriched' fraction, and the 'S' fraction the particle-free supernatant.

Fraction	Specific activity (nmol/min per mg of protein)		
M	3.6	2.1	2.3
L	3.3	2.5	2.7
P	9.6	3.7	5.4
S	0.3	0.4	0.3

Table 2 shows the specific activity of acyl-CoA oxidase in the various fractions of human platelets. In all preparations the specific activity was highest in the 'P' or 'dense-tubular-system-enriched' fraction, although a considerable variation was noted. The results indicate that the activity of acyl-CoA oxidase is from about 10% up to about 30% of the activity of acyl-CoA synthetase in fractions of human platelets, with $[1\text{-}^{14}\text{C}]\text{palmitic acid}$ as substrate (Bakken & Farstad, 1989).

To ensure that the acyl-CoA oxidase was due to a peroxisomal activity, the palmitoyl-CoA-dependent dehydrogenase (peroxisomal β -oxidation) was also assayed. This activity was about 1–2 nmol/min per mg of protein when whole homogenates of human platelets were used as a source of the enzyme.

Table 3 shows an experiment with a representative distribution of the peroxisomal enzyme acyl-CoA oxidase in relation to marker enzymes. The distribution indicates that the 'M' ('mitochondria-enriched') fraction was to some extent contaminated with the 'P' ('dense-tubular-system-enriched') fraction. Also, the 'P' fraction was to some extent contaminated with mitochondria, indicated by the presence of amine oxidase in this fraction. The distribution is not, however, very different from that found in other attempts to isolate subcellular fractions of blood platelets (Gerrard *et al.*, 1976; Fukami *et al.*, 1978; Gogstad, 1980; Bakken & Farstad, 1989). The distribution of acyl-CoA oxidase is similar to that of NADH dehydrogenase, and different from those of catalase and the mitochondrial marker carnitine palmitoyltransferase.

Fig. 1 shows the relative specific activities of acyl-CoA oxidase and catalase in relation to marker enzymes, calculated from the data presented in Table 3. The relative specific activity of acyl-CoA oxidase is grossly different from that of catalase. The

Table 3. Total activities and distributions of acyl-CoA oxidase, catalase and some marker enzymes in fractions isolated by differential centrifugation of homogenates of blood platelets from 0.5 litre of blood from each of two donors.

The enzyme activities and the protein contents of the fractions are expressed as percentages of those in the 'E' fraction (i.e. the cytoplasmic extract). M, L, P and S are defined in Table 2. All enzymes were assayed as stated in the Materials and methods section.

Enzyme	Total activity (nmol/min)	Recovery (%) in fractions				Recovery (%)
		M	L	P	S	
Acyl-CoA oxidase	123	28	18	22	35	103
Catalase	430	11	3	2	68	84
Carnitine palmitoyl-transferase	80	72	21	6	8	107
Amine oxidase	18	60	26	15	0	101
NADH dehydrogenase	35	37	19	22	20	98
Lactate dehydrogenase	497	7	3	1	86	97
Protein (mg)	167	9	5	3	83	100

distribution of the latter enzyme is in fair agreement with that found by Marcus *et al.* (1966). Thus Fig. 1 indicates that peroxisomes of human platelets either are destroyed to a great extent during preparation of subcellular fractions, or are too small to be sedimented with the 'L' fraction. No amine oxidase and little fatty acyl-CoA oxidase are found in the particle-free supernatant. Although soluble proteins of platelet organelles leak to the cytosolic space during preparation of subcellular fractions of human platelets (Berge *et al.*, 1980), it is unlikely that the present distribution of acyl-CoA oxidase is solely due to destruction of peroxisomes.

No structures typical of peroxisomes have been demonstrated by transmission electron microscopy in available literature in the field, except for two publications (Breton-Gorius & Guichard, 1975; White, 1979). In agreement with these findings, the present results indicate that peroxisomes of human platelets are so small, or so closely attached to the dense tubular system, that they are sedimented with the 'P' fraction during ultracentrifugation.

DISCUSSION

Little attention has been paid to the role of peroxisomes in human blood platelets. This is surprising, since peroxisomes seem to be strongly involved in the metabolism of biologically

Fig. 1. Relative specific activities of marker enzymes, carnitine palmitoyltransferase (CPT I + II) and amine oxidase (MAO) for mitochondria, NADH dehydrogenase (NADH DH) for the 'dense tubular system', and lactate dehydrogenase (LD) for the particle-free fraction, and of acyl-CoA oxidase (Ac-CoA Ox) and catalase

M is the mitochondrial or 12000 g fraction, L is the light mitochondrial or 24000 g fraction, P is the 'dense tubular system' or 100000 g fraction, and S is the particle-free fraction. Incubation conditions for the assays of enzyme activities were as described in the Materials and methods section.

active peroxides such as prostaglandins and thromboxanes (Diczfalusy *et al.*, 1991). Although the existence of peroxisomes was demonstrated in 1975 (Breton-Gorius & Guichard, 1975), peroxisomal activities have been studied only in relation to diseases due to defects in peroxisomal function (Wanders *et al.*, 1985; Poll-The *et al.*, 1986). Breton-Gorius & Guichard (1975) indicated that the peroxisomes of platelets were 'microperoxisomes', in close connection with the endoplasmic reticulum or the dense tubular system. This assumption is in good agreement with the present finding that the activity of the peroxisomal enzyme acyl-CoA oxidase sedimented with the 'P' fraction, or 'dense-tubular-system-enriched fraction'. It is noteworthy that the activity of this enzyme in human platelets is high, about 10% of the activity of long-chain acyl-CoA synthetase (Bakken & Farstad, 1989). Also, the peroxisomal β -oxidation is remarkably high, about one-third to one-half of the activity reported for rat liver (Aarsland & Berge, 1991).

We express our gratitude to Knut Halvorsen, M.D., Head of the Blood Bank, University Hospital, for providing us with blood platelet concentrates, and to Randi Sandvik, engineer, and Svein Kruger, M.Sc., for their assistance.

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