Substrate selectivities differ for hepatic mitochondrial and peroxisomal β -oxidation in an Antarctic fish, *Notothenia gibberifrons*

Elizabeth L. CROCKETT* and Bruce D. SIDELL

Department of Zoology and Center for Marine Studies, University of Maine, Orono, ME 04469, U.S.A.

Hepatic mitochondrial and peroxisomal β -oxidation were examined in an Antarctic marine teleost, *Notothenia gibberifrons*. Enzymic profiles and rates of β -oxidation by intact organelles were determined by using a range of fatty acyl-CoA substrates to evaluate substrate preferences. Partitioning of β -oxidation between organelles was estimated. Substrate selectivities are broader for peroxisomal β -oxidation than for mitochondrial β -oxidation. Mitochondria show marked preference for the oxidation of a monounsaturated substrate, palmitoleoyl-CoA ($C_{16:1}$), and two polyunsaturates, eicosapentaenoyl-CoA ($C_{20:5}$) and docosahexaenoyl-CoA ($C_{22:6}$). Carnitine palmitoyltransferase activities with

palmitoleoyl-CoA ($C_{16:1}$) are 2.4-fold higher than activities with palmitoyl-CoA ($C_{16:0}$). Most polyunsaturated acyl-CoA esters measured appear to inhibit by over 40% the oxidation of palmitoyl-CoA by peroxisomes. Our findings suggest that the polyunsaturates, eicosapentaenoic acid ($C_{20:5}$) and docosahexaenoic acid ($C_{22:6}$), found in high concentrations in Antarctic fishes [Lund and Sidell (1992) *Mar. Biol.* **112**, 377–382], are utilized as fuels to support aerobic energy metabolism. Metabolic capacities of rate-limiting enzymes and β -oxidation rates by intact organelles indicate that up to 30% of hepatic β -oxidation in *N. gibberifrons* can be initiated by the peroxisomal pathway.

INTRODUCTION

β -Oxidation of fatty acids occurs in two metabolic compartments, the mitochondrion and the peroxisome (Lazarow and de Duve, 1976; Lazarow, 1978). The peroxisome is thought to chain-shorten long-chain fatty acids, including polyunsaturated fatty acids, that are otherwise poor substrates for mitochondrial β -oxidation (reviewed by Osmundsen et al., 1987, 1991).

Antarctic fishes possess two characteristics that suggest that they may rely on peroxisomal catabolism of fatty fuels to supplement mitochondrial β -oxidation. First, these animals have high capacities to utilize lipid fuels relative to carbohydrate fuels, in marked contrast with temperate-zone marine fishes, which do not display such strong preferences for lipid fuels (Crockett and Sidell, 1990). Second, tissues from Antarctic fishes have high concentrations of polyunsaturated fatty acids in neutral-lipid stores. The predominant polyunsaturates are eicosapentaenoic acid ($C_{20:5}$) and docosahexaenoic acid ($C_{22:6}$), which range from 8 to 26% of total fatty acid pools from skeletal muscle and serum (Lund and Sidell, 1992). These polyunsaturates occur, at least in part, as a result of diet in this group of fishes. Many Antarctic marine invertebrates, prominent prey of South Polar fishes, are rich in these polyunsaturates (e.g. Clarke, 1980; Fricke and Oehlenschläger, 1988).

The physiological significance of abundant polyunsaturated fatty acids in neutral lipids of Antarctic fishes is unresolved. Polyunsaturates serve as potential fuels for energy metabolism in Antarctic fishes, or alternatively, these lipids may accumulate in tissues if little or no means exists for their catabolism.

Purposes of this study are (1) to characterize β -oxidation of polyunsaturated fats and (2) to estimate partitioning of β -oxidation between mitochondria and peroxisomes in an Antarctic fish, *Notothenia gibberifrons*. We utilize two approaches in addressing these questions. The first approach measures enzymes from the rate-limiting steps in mitochondrial and peroxisomal β -

oxidation to determine substrate selectivities and to evaluate capacities for β -oxidation. Activities of mitochondrial carnitine palmitoyltransferase (CPT; EC 2.3.1.21) and peroxisomal acyl-CoA oxidase (ACO; EC 1.3.99.3) are measured with a range of substrates varying in chain length and degree of unsaturation. The second approach measures metabolism in a more integrated preparation. β -Oxidation of a radiolabelled substrate by isolated intact organelles is monitored in the presence of a second unlabelled substrate. The effects of different unlabelled substrates on the oxidation of the labelled substrate are compared to evaluate substrate selectivities of the organelles and inhibition of their β -oxidative pathways.

Our results indicate that substrate preferences of mitochondria and peroxisomes differ. Peroxisomes appear capable of β -oxidizing all substrates examined at relatively high rates. In contrast, mitochondria show marked substrate preference for a monounsaturate and two polyunsaturates. Peroxisomal β -oxidation in *Notothenia gibberifrons* can initiate as much as 30% of total hepatic β -oxidation.

EXPERIMENTAL

Materials

[1- 14 C]Palmitoyl CoA and [1- 14 C]eicosapentaenoic acid were purchased from New England Nuclear (Boston, MA, U.S.A.). All unlabelled substrates (except eicosapentaenoyl-CoA and docosahexaenoyl-CoA), other biochemicals, including acyl-CoA synthetase (from *Pseudomonas* spp.), Percoll and sucrose, were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Eicosapentaenoyl-CoA (unlabelled and 1- 14 C-labelled) and docosahexaenoyl-CoA were synthesized as described below. 2',7'-Dichlorofluorescein diacetate was from Eastman Kodak Co. (Rochester, NY, U.S.A.). All other chemicals used were of reagent grade or better.

Abbreviations used: CPT, carnitine palmitoyltransferase; ACO, acyl-CoA oxidase; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid); CMC, critical micelle concentration.

*To whom correspondence should be sent. Present address: Department of Zoology, Arizona State University, AZ 85287, U.S.A.

Synthesis of eicosapentaenoyl- and docosahexaenoyl-CoA

Acyl-CoA esters were synthesized enzymically by a procedure similar to that described in Frentzen et al. (1983). Fatty acids (eicosapentaenoic and docosahexaenoic) were converted into potassium salts. Butylated hydroxytoluene was included, at 0.005% (w/v), to prevent autooxidation of substrates. Reaction mixtures for syntheses contained 0.62 mM potassium salts of the fatty acids, 0.7 mM CoA, 5 mM ATP, 20 mM MgCl_2 , 1 unit of acyl-CoA synthetase/ml (unit = μmol of product/min) in 150 mM Tris/HCl (pH 7.8 at 25 °C). The extent of CoA esterification was monitored periodically by following the reduction of 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) by free CoA at 412 nm in sub-samples of reaction mixtures as described by Bar-Tana et al. (1971). Acyl-CoA substrates (including synthesis mixture) were diluted approx. 20-fold into β -oxidation and enzymic reaction mixtures.

Animals

Specimens of *Notothenia gibberifrons* were collected off the Antarctic Peninsula in 1989, 1990 and 1991. Fish used in experiments ranged in size from 300 g (32 cm length) to 600 g (40 cm length). Fish were maintained in running-seawater aquaria at ambient temperatures (-1.5 °C to 0 °C) at the U.S. Research Station, Palmer Station. Fish were used within 2 weeks of capture and were fed on items that are similar to their natural diet. This included commercial shellfish.

Determination of enzyme activities

Enzyme activities were measured in livers which had been frozen in liquid N_2 (stability of frozen enzymes was confirmed). Livers were homogenized (10%, w/v) with 40 mM Hepes/1 mM EDTA/2 mM MgCl_2 (pH 7.8 at 1 °C) with Tenbroeck ground-glass homogenizers. All samples were sonicated in two 15 s bursts with a 15 s cooling interval at 35% maximal power (Artek-Sonic 300 Dismembrator). Enzyme activities were measured at physiological temperature (1 ± 0.5 °C) with a Perkin-Elmer Lambda 4B spectrophotometer. Dry N_2 gas was used to prevent condensation on cuvettes.

CPT was assayed by a procedure modified from Bieber et al. (1972). Reduction of DTNB was followed by measuring the increase in A_{412} for 10 min. Assay medium contained 40 mM Hepes, 1.5 mM EDTA, 0.25 mM DTNB, 1.25 mM carnitine (synthetic) and 0.035 mM acyl-CoA (pH 8.2 at 1 °C). Reactions were initiated by addition of carnitine. Enzyme samples were supernatants prepared by centrifugation of crude homogenates (700 g_{av} for 5 min at 2 °C).

ACO was measured by the method described by Small et al. (1985). Oxidation of 2',7'-dichlorofluorescein diacetate (DCF) was monitored by following the increase in A_{502} for 10 min. Assay medium consisted of 11 mM potassium phosphate buffer, 0.05 mM DCF, 40 mM 3-amino-1,2,4-triazole, 0.02% (v/v) Triton X-100, 1700 units of horseradish peroxidase (Type II)/100 ml and 0.030 mM acyl-CoA (pH 7.8 at 1 °C). DCF was dissolved separately in 7.2 ml of 0.01 M NaOH and 0.8 ml of *NN*-dimethylformamide, and exposure to air and light was minimized until time of assay. Final concentrations of these solvents in the reaction medium were 0.2 μM and 0.2% (v/v) respectively. Reactions were initiated by addition of acyl-CoA ester. Enzyme activity was estimated in crude homogenates.

Determination of kinetic constants

Apparent K_m and apparent K_{cat} values were determined by measuring ACO activities at seven substrate concentrations from

2.5 to 30 μM . A sample size of three fish was used for each substrate. Kinetic constants (apparent K_m and K_{cat}) were calculated with the non-linear-regression analysis program ENZFITTER (Elsevier-Biosoft, Cambridge, U.K.).

Preparation of organelles

Solutions and protocol for centrifugation were adapted from the procedures described by Neat et al. (1980) for rat liver. All solutions were made iso-osmotic to *Notothenia gibberifrons* sera as measured by a vapour-pressure osmometer, with addition of either mannitol or sucrose. Fish were killed by a blow to the head, and livers were removed. Livers were minced finely with a razor blade on an ice-cold glass stage. A 10% (w/v) tissue homogenate was made by using a loose-fitting (130–180 μm clearance) Potter-Elvehjem homogenizer powered by a slow-speed drill. Normally about two passes were required for homogenization. Homogenization medium contained 400 mM mannitol, 10 mM Hepes and 1 mM EGTA (pH 7.8 at 1 °C).

Homogenates were centrifuged at 500 g_{av} for 3 min (4 °C). Supernatants were collected and centrifuged at 9000 g_{av} for 22 min (4 °C). Pellets were resuspended in 350 mM sucrose (grade I)/10 mM Hepes/5 mM EGTA (pH 7.8 at 1 °C) and passed through a tight-fitting (100–150 μm clearance) Potter-Elvehjem homogenizer.

Density-gradient medium was 30% (v/v) Percoll solution made iso-osmotic with 350 mM sucrose, and buffered by 10 mM Hepes/2 mM EGTA (pH 7.8 at 1 °C). A 2 ml portion of resuspended material was layered on 27 ml of density-gradient medium. A 3 ml cushion of 60% (w/v) sucrose and a 2 ml overlay of 1.1% (w/v) NaCl were used. Self-generated Percoll gradients were formed in a Beckman 50.2 Ti fixed-angle rotor at 71000 g_{av} for 25 min (acceleration/deceleration times not inclusive) in a Beckman L5-50E ultracentrifuge (4 °C).

Tubes were pierced with a Hoefer FS 101 gradient tube fractionator, and fractions (1.6 ml) were collected with a fraction collector. Marker enzymes for mitochondria (cytochrome *c* oxidase) and peroxisomes (catalase) were measured at 5 °C. Cytochrome *c* oxidase was assayed by the method described in Wharton and Tzagoloff (1967). The catalase assay was adapted from Beers and Sizer (1952).

Once the position of organelles in the gradient had been confirmed, they were collected for β -oxidation experiments by direct tube puncture with an 18-gauge needle and withdrawal of the band characteristic of that organelle. Percoll was removed from these samples by diluting fractions in 2 vol. of 350 mM sucrose/10 mM Hepes (pH 7.8 at 1 °C), followed by centrifugation at 9000 g_{av} for 30 min (4 °C). Enriched organellar fractions were pelleted by this procedure.

β -Oxidation experiments

The oxidation rate of [$1\text{-}^{14}\text{C}$]palmitoyl-CoA was measured in the presence of a second, unlabelled, substrate. Various unsaturated substrates were used. Total acyl-CoA concentration was 25 μM . The concentration of [$1\text{-}^{14}\text{C}$]palmitoyl CoA was 12.5 μM (0.5 $\mu\text{Ci}/\mu\text{mol}$), and the concentration of unlabelled substrate was also 12.5 μM . Palmitoyl-CoA ($1\text{-}^{14}\text{C}$; 12.5 μM) with 12.5 μM unlabelled palmitoyl-CoA served as reference to which the effects on the oxidation of [$1\text{-}^{14}\text{C}$]palmitoyl-CoA, by other unlabelled substrates, were compared.

β -Oxidation rates were determined for intact mitochondria and peroxisomes, isolated as described above. Incubation media contained 25 μM acyl-CoA, 0.5 mM CoA (sodium salt), 0.75 mM ATP (disodium salt), 2 mM carnitine (synthetic) (mitochondria

only), 2 mM NAD⁺ (grade IIIC) (peroxisomes only) in 350 mM sucrose/10 mM Hepes/1 mM EDTA/2 mM MgCl₂/0.5 mM dithiothreitol (pH 7.8 at 1 °C). Total volume of the incubation mixture was 2.5 ml, and incubations were carried out in sealed 25 ml Erlenmeyer flasks. CO₂ traps (200 μ l of methylbenzethonium hydroxide in plastic wells) were affixed to rubber serum sleeves, which sealed the flasks. Incubations were carried out for 1.5 h at a physiological temperature of 1 °C in a shaking water bath. Linearity and proportionality between oxidation rate and amount of organelle used were established. All reactions were carried out in duplicate.

Reactions were stopped by addition of 0.5 ml of ice-cold 20% (v/v) HClO₄. A zero-time control was used in which HClO₄ was added to the reaction mixture before addition of organelle. This control established the acid-stability of [1-¹⁴C]palmitoyl-CoA, as virtually all unchanged substrate was precipitated by acid. At 1 h after stopping the reaction, CO₂ traps were placed in mini scintillation vials. A 1 ml portion of reaction mixture from each flask was centrifuged for 10 min in a micro-centrifuge (10000 g_{av}). Acid-soluble supernatant was withdrawn and counted for radioactivity in an LKB Wallac 1217 Rackbeta scintillation counter, with Aquasol 2 (New England Nuclear) as scintillant.

No ¹⁴CO₂ above background was detected in the CO₂ traps in any metabolic experiments. With mitochondria from human muscle, Veerkamp et al. (1986) found ¹⁴CO₂ contribution to total oxidation (¹⁴CO₂ plus ¹⁴C-labelled acid-soluble products) to be less than 2%. Because the ¹⁴CO₂ evolution was negligible in our experiments, only acid-soluble products were considered for calculations, although CO₂ traps were monitored throughout. Although the absence of any ¹⁴CO₂ suggests that β -oxidation products may form ketone bodies, it is unlikely that this occurs. Teleosts, including *Notothenia gibberifrons*, display poor capacities to catabolize ketone fuels in peripheral tissues (Zammit and Newsholme, 1979; E. L. Crockett and B. D. Sidell, unpublished work).

Protein concentrations were determined for mitochondrial and peroxisomal fractions by the bicinchoninic acid method of Smith et al. (1985).

Experimental design and data analysis

The oxidation rate of [1-¹⁴C]palmitoyl-CoA, in the presence of an alternative unlabelled acyl-CoA, was measured with three unlabelled substrates for each fish sampled. One of the unlabelled substrates was always palmitoyl-CoA. Nine substrates of different chain length and degree of unsaturation were used. Experiments were grouped by combinations of substrate (e.g. group 1 was [1-¹⁴C]palmitoyl-CoA with palmitoyl-CoA, palmitoleoyl-CoA or oleoyl-CoA). Each group had a sample size of four. Analysis of variance (ANOVA) using a randomized block design was performed on absolute oxidation rates within each experimental group. For each organelle, Tukey's HSD method was used to analyse substrate differences within an experimental group. Student's *t* tests were used to determine substrate differences between experimental groups. Data were logarithmically transformed when necessary.

Enzymic data also were analysed by ANOVA using a randomized block design. Tukey's HSD tests were used to evaluate substrate differences for a particular enzyme. A sample size of 12 animals was used for substrate-selectivity determinations for each enzyme, except for the substrates which had to be synthesized. In these cases sample sizes between two and four individuals were used.

RESULTS

Separation of mitochondria and peroxisomes

Marker enzymes [cytochrome *c* oxidase for mitochondria and catalase for peroxisomes] indicate a clear separation of peak organelle distributions after density-gradient centrifugation (Figure 1). Cytochrome *c* oxidase activity in the peroxisomal region of the gradient represents mitochondrial fragments incapable of β -oxidation. Three lines of evidence support this conclusion (results not shown). First, calculated oxidation rate of [1-¹⁴C]palmitoyl-CoA per unit of cytochrome *c* oxidase in the mitochondrial fraction exceeds the rate observed per unit of cytochrome *c* oxidase in the peroxisomal fraction by several-fold. Second, our experiments with Triton X-100, an inhibitor of mitochondrial β -oxidation (Mannaerts et al., 1979; Hovik and Osmundsen, 1988), show Triton X-100 inhibition of oxidation in the mitochondrial fraction, whereas β -oxidation in the peroxisomal fraction is slightly enhanced. Finally, β -oxidation experiments yield markedly different substrate profiles for mitochondrially and peroxisomally enriched fractions (see below).

Enzymic substrate specificities

CPT shows marked preference for particular substrates, whereas ACO has comparable rates of catalysis with a broad range of substrates (Table 1). CPT activities with palmitoleoyl-CoA (C_{16:1}) are 2-fold greater than activities with palmitoyl-CoA (C_{16:0}), oleoyl-CoA (C_{18:1}) or linoleoyl-CoA (C_{18:2}). Two of the polyunsaturates, linolenoyl-CoA (C_{18:3}) and arachidonoyl-CoA (C_{20:4}), yield activities that are 10% or less of the activity with palmitoyl-CoA (C_{16:0}), whereas the two most unsaturated substrates, eicosapentaenoyl-CoA (C_{20:5}) and docosahexaenoyl-CoA (C_{22:6}), yield activities that are comparable or even higher than with palmitoyl-CoA (C_{16:0}).

By contrast, ACO activities with all the polyunsaturated substrates are $\geq 50\%$ of that with palmitoyl-CoA (C_{16:0}). In addition, the activity with the monounsaturate, palmitoleoyl-CoA (C_{16:1}), is not significantly different from that with palmitoyl-CoA (C_{16:0}).

β -Oxidation substrate profiles

Increased oxidation of [1-¹⁴C]palmitoyl-CoA in the presence of a second unlabelled substrate (compared with oxidation rates with

Figure 1 Typical distribution of mitochondria and peroxisomes in a self-generated Percoll gradient

Marker enzymes are cytochrome *c* oxidase (●) for mitochondria and catalase (○) for peroxisomes. Fractions (1.6 ml each) were collected from the bottom of the tube.

Table 1 Enzymic substrate selectivities for mitochondrial and peroxisomal β -oxidation

CPT is used as a mitochondrial indicator, and ACO as a peroxisomal indicator. Data are expressed as means ($\pm$ S.E.M.; $n = 12$), in units ($\mu\text{mol}/\text{min}/\text{g}$ wet wt. Letters indicate results of Tukey's HSD analysis, with ^a not significantly different from $\text{C}_{16:0}$ -CoA. Acyl-CoA esters with asterisks are those synthesized by the authors ($n = 2, 3$ or 4). The lower estimate of CPT activity with $\text{C}_{16:0}$ -CoA synthesized compared with the commercial preparation may be due to the presence of residual precursors (e.g. CoA, ATP) used in synthesis. Rates among the synthesized substrates should reflect relative capacities for β -oxidation.

Substrate	Enzyme	
	CPT	ACO
$\text{C}_{16:0}$ -CoA	0.035 (0.002) ^a	0.014 (0.001) ^a
$\text{C}_{16:1}$ -CoA	0.084 (0.006) ^b	0.016 (0.001) ^a
$\text{C}_{18:1}$ -CoA	0.037 (0.003) ^a	0.011 (0.001) ^b
$\text{C}_{18:2}$ -CoA	0.037 (0.003) ^a	0.014 (0.001) ^a
$\text{C}_{18:3}$ -CoA	0.001 (0.000) ^c	0.009 (0.001) ^b
$\text{C}_{20:2}$ -CoA	0.022 (0.002) ^d	0.009 (0.001) ^b
$\text{C}_{20:4}$ -CoA	0.004 (0.001) ^c	0.007 (0.001) ^b
$\text{C}_{16:0}$ -CoA*	0.014 (0.002)	0.017 (0.002)
$\text{C}_{20:5}$ -CoA*	0.046 (0.006)	0.013 (0.001)
$\text{C}_{22:6}$ -CoA*	0.035 (0.003)	0.015 (0.001)

Figure 2 Substrate profiles of β -oxidation for intact mitochondria and peroxisomes

Oxidation rates of [$1\text{-}^{14}\text{C}$]palmitoyl-CoA with unlabelled acyl-CoA esters are expressed relative to rates with unlabelled palmitoyl-CoA. Data shown are means $\pm$ S.E.M. ($n = 4$) for each unlabelled substrate. Letters above bars denote membership in groups according to Tukey's HSD analysis within each organelle. Bars with the symbol 'a' are not significantly different from unlabelled palmitoyl-CoA ($\text{C}_{16:0}$). Substrates are palmitoyl-CoA ($\text{C}_{16:0}$), palmitoleoyl-CoA ($\text{C}_{16:1}$), oleoyl-CoA ($\text{C}_{18:1}$), linoleoyl-CoA ($\text{C}_{18:2}$), linolenoyl-CoA ($\text{C}_{18:3}$), eicosadienoyl-CoA ($\text{C}_{20:2}$), arachidonoyl-CoA ($\text{C}_{20:4}$), eicosapentaenoyl-CoA ($\text{C}_{20:5}$) and docosahexaenoyl-CoA ($\text{C}_{22:6}$).

Table 2 Reciprocal competition experiments of mitochondrial β -oxidation with palmitoyl-CoA ($\text{C}_{16:0}$) and eicosapentaenoyl-CoA ($\text{C}_{20:5}$)

Experiments were performed as described in the Experimental section with $12.5 \mu\text{M}$ labelled substrate plus $12.5 \mu\text{M}$ unlabelled substrate. Values shown are means ($\pm$ S.E.M.; $n = 2$) for each unlabelled substrate and are expressed as nmol/h per mg of protein.

[$1\text{-}^{14}\text{C}$]Acyl-CoA	Unlabelled acyl-CoA	
	$\text{C}_{16:0}$ -CoA	$\text{C}_{20:5}$ -CoA
$\text{C}_{16:0}$ -CoA	6.9 (0.9)	1.4 (0.1)
$\text{C}_{20:5}$ -CoA	2.8 (0.2)	1.0 (0.1)

Table 3 Inhibition of ACO by a polyunsaturated acyl-CoA ester

Data are means ($\pm$ S.E.M.; $n = 3$) in units ($\mu\text{mol}/\text{min}/\text{g}$ wet wt. Activity with the two substrates combined is significantly lower than activity with $12.5 \mu\text{M}$ $\text{C}_{16:0}$ -CoA alone ($P < 0.001$).

Substrate	Concn.	ACO activity
$\text{C}_{16:0}$ -CoA	$25 \mu\text{M}$	0.018 (0.002)
$\text{C}_{16:0}$ -CoA	$12.5 \mu\text{M}$	0.017 (0.002)
$\text{C}_{16:0}$ -CoA	$12.5 \mu\text{M}$	0.012 (0.001)
+ $\text{C}_{18:3}$ -CoA	$12.5 \mu\text{M}$	
$\text{C}_{18:3}$ -CoA	$25 \mu\text{M}$	0.007 (0.001)
$\text{C}_{18:3}$ -CoA	$12.5 \mu\text{M}$	0.005 (0.001)

palmitoyl-CoA as unlabelled competitor) suggests preference for oxidation of palmitoyl-CoA. The metabolic system treats [$1\text{-}^{14}\text{C}$]palmitoyl-CoA and the second unlabelled substrate as two separate pools and preferentially oxidizes the former. Conversely, decreased oxidation of [$1\text{-}^{14}\text{C}$]palmitoyl-CoA in the presence of a second unlabelled substrate suggests preference for the oxidation of the unlabelled substrate or inhibition by the unlabelled substrate, or perhaps a combination of these effects. Inhibition or preference may be discerned when results of β -oxidation experiments are viewed with those from enzymic analyses.

The patterns of substrate preferences or inhibition are clearly dissimilar for mitochondrial and peroxisomal β -oxidation, depending on the specific unlabelled acyl-CoA ester that is competing with [$1\text{-}^{14}\text{C}$]palmitoyl-CoA (Figure 2). All polyunsaturated acyl-CoA esters, except eicosadienoyl-CoA ($\text{C}_{20:2}$), decrease the peroxisomal β -oxidation rate of [$1\text{-}^{14}\text{C}$]palmitoyl-CoA ($\text{C}_{16:0}$) by over 40%, whereas some of the same substrates significantly increase the mitochondrial β -oxidation of [$1\text{-}^{14}\text{C}$]palmitoyl-CoA ($\text{C}_{16:0}$). Eicosapentaenoyl-CoA ($\text{C}_{20:5}$) and docosahexaenoyl-CoA ($\text{C}_{22:6}$) depress the mitochondrial β -oxidation rate of [$1\text{-}^{14}\text{C}$]palmitoyl-CoA ($\text{C}_{16:0}$) by 30–40%, whereas linolenoyl-CoA ($\text{C}_{18:3}$) and arachidonoyl-CoA ($\text{C}_{20:4}$) elevate the oxidation rate of [$1\text{-}^{14}\text{C}$]palmitoyl-CoA ($\text{C}_{16:0}$) by over 35%. A monounsaturate, palmitoleoyl-CoA ($\text{C}_{16:1}$), significantly depresses the oxidation rate of [$1\text{-}^{14}\text{C}$]palmitoyl-CoA ($\text{C}_{16:0}$) in mitochondria, but not in peroxisomes.

Reciprocal competition experiments further indicate that palmitoyl-CoA ($\text{C}_{16:0}$) and eicosapentaenoyl-CoA ($\text{C}_{20:5}$) are not equivalent substrates for mitochondrial β -oxidation (Table 2). The presence of unlabelled eicosapentaenoyl-CoA ($\text{C}_{20:5}$) relative to unlabelled palmitoyl-CoA ($\text{C}_{16:0}$) depresses the oxidation of [$1\text{-}^{14}\text{C}$]palmitoyl-CoA ($\text{C}_{16:0}$) by nearly 80%, whereas unlabelled palmitoyl-CoA ($\text{C}_{16:0}$) relative to unlabelled eicosa-

Table 4 Kinetic constants for ACO with saturated and polyunsaturated acyl-CoA substrates

Values given are means ($\pm$ S.E.M.; $n = 3$); * $P < 0.05$. Activities of ACO were measured at seven substrate concentrations for each acyl-CoA ester and analysed as described in the Experimental section.

Substrate	Apparent K_m (μ M)	Apparent K_{cat} (nmol/min)
C _{16:0} -CoA	7.8 (2.2)	25 (2.7)
C _{18:3} -CoA	8.8 (3.3)	9.3 (3.3)*

Table 5 Partitioning of β -oxidation between mitochondrial and peroxisomal compartments by (a) enzymic and (b) metabolic estimates

Enzymic estimates are from maximal CPT and ACO activities for mitochondria and peroxisomes respectively, as shown in Table 1. Metabolic estimates are from [1^{14} C]palmitoyl-CoA (C_{16:0}-CoA) oxidation rates, corrected for marker-enzyme yields ($n = 12$). For each substrate the percentage decrease or increase in palmitoyl-CoA oxidation (from Figure 2) was used to calculate an estimate for palmitoyl-CoA oxidation in the presence of alternative acyl CoA esters.

Substrate	Distribution (%)	
	Mitochondria	Peroxisomes
(a) Enzymic estimates for the substrates shown		
C _{16:0} -CoA	71	29
C _{16:1} -CoA	84	16
C _{18:1} -CoA	77	23
C _{18:2} -CoA	73	27
C _{18:3} -CoA	10	90
C _{20:2} -CoA	71	29
C _{20:4} -CoA	36	64
C _{20:5} -CoA	78	22
C _{22:6} -CoA	70	30
(b) Metabolic estimates for C _{16:0} -CoA oxidation in the presence of the substrates shown		
C _{16:0} -CoA	73	27
C _{16:1} -CoA	67	33
C _{18:1} -CoA	71	29
C _{18:2} -CoA	84	16
C _{18:3} -CoA	88	12
C _{20:2} -CoA	78	22
C _{20:4} -CoA	87	13
C _{20:5} -CoA	82	18
C _{22:6} -CoA	77	23

pentaenoyl-CoA (C_{20:5}) elevates the oxidation of [1^{14} C]eicosapentaenoyl-CoA (C_{20:5}) by 280%. The absolute oxidation rate of palmitoyl-CoA (C_{16:0}), however, is about 7-fold higher than the oxidation rate of eicosapentaenoyl-CoA (C_{20:5}) in mitochondria from *Notothenia gibberifrons*.

ACO substrate inhibition and kinetics

The presence of linolenoyl-CoA (C_{18:3}) strongly inhibits palmitoyl-CoA (C_{16:0}) oxidation by ACO (Table 3). ACO activity with 12.5 μ M palmitoyl-CoA (C_{16:0}) plus 12.5 μ M linolenoyl-CoA (C_{18:3}) is significantly lower than activities with either 25 μ M or 12.5 μ M palmitoyl-CoA (C_{16:0}) alone ($P < 0.001$).

Apparent K_m values of ACO oxidation for palmitoyl-CoA (C_{16:0}) and linolenoyl-CoA (C_{18:3}) are not significantly different,

whereas the K_{cat} for palmitoyl-CoA (C_{16:0}) is nearly 3-fold higher than the K_{cat} for linolenoyl-CoA (C_{18:3}) (Table 4).

Estimates of β -oxidation partitioning

Estimates from both enzymic and β -oxidation analyses suggest that peroxisomes may initiate up to 30% of total hepatic β -oxidation of palmitoyl-CoA (C_{16:0}) in *Notothenia gibberifrons* (Table 5). The percentage of maximal capacity of β -oxidation attributable to either mitochondria or peroxisomes varies with substrate. Based on enzymic estimates, 16% of capacity for β -oxidation of palmitoleoyl-CoA (C_{16:1}) may be peroxisomal, whereas 90% of oxidation of linolenoyl-CoA (C_{18:3}) may be peroxisomal. The extent of partitioning of palmitoyl-CoA oxidation between organelles also varies with the alternative substrate present. For example, our estimates suggest that when equimolar concentrations of palmitoyl-CoA (C_{16:0}) and palmitoleoyl-CoA (C_{16:1}) are present, 33% of the first turn of β -oxidation with the saturated substrate is peroxisomal. However, with linolenoyl-CoA (C_{18:3}) or arachidonoyl-CoA (C_{20:4}) present, less than 15% of palmitoyl-CoA (C_{16:0}) oxidation may be attributed to the peroxisomal compartment.

β -Oxidation rates of 25 μ M palmitoyl-CoA are 0.53 ± 0.07 (S.E.M.; $n = 12$) by mitochondria and 0.20 ± 0.03 (S.E.M.; $n = 12$) by peroxisomes, both expressed as nmol/min per g of liver. Oxidation rates expressed per g of liver should be used for comparison, because estimates expressed per mg of protein will vary depending on the degree of contamination by protein from non- β -oxidizing organelles. This contamination may differ between mitochondrial and peroxisomal fractions and between species.

DISCUSSION

Our present paper characterizes capacities and substrate selectivities *in vitro* for mitochondrial and peroxisomal β -oxidation in an Antarctic fish, *Notothenia gibberifrons*. Our β -oxidation experiments measure the oxidation rate of a radiolabelled substrate in the presence of an unlabelled competing substrate. Unlike single-substrate oxidation, presenting organelles with a mixture of alternative substrates is more similar to conditions *in vivo*, where a variety of acyl-CoA esters that vary in chain length and degree of unsaturation will be present. All acyl-CoA substrates used in the present study are found in trout hepatocytes (Hazel and Livermore, 1990), albeit at different concentrations. In addition, measuring the oxidation rate of one substrate in the presence of a second substrate reveals inhibition characteristics that would otherwise go unnoticed.

Substrate selectivities differ for mitochondrial and peroxisomal β -oxidation

Substrate selectivities for mitochondrial and peroxisomal β -oxidation in *Notothenia gibberifrons* are generally similar to those reported from mammals (reviewed by Osmundsen et al., 1987, 1991) and another teleost, *Myoxocephalus octodecimsipinosus* (Crockett and Sidell, 1993). Peroxisomes appear capable of oxidizing a broad range of acyl-CoA substrates at relatively high rates, whereas more defined substrate preferences are displayed by mitochondria. Our observations are in contrast with the range of suitable acylcarnitine substrates found for mitochondrial β -oxidation with rainbow trout, *Oncorhynchus mykiss* (Henderson and Sargent, 1985). Either substrate selectivity for mitochondrial β -oxidation varies among species or selectivity of the intact pathway is imposed at the level of CPT.

Eicosapentaenoyl-CoA ($C_{20:5}$) and docosahexaenoyl-CoA ($C_{22:6}$) are preferred substrates for mitochondrial β -oxidation, whereas two other polyunsaturates, linolenoyl-CoA ($C_{18:3}$) and arachidonoyl-CoA ($C_{20:4}$), are oxidized poorly by mitochondria from *Notothenia gibberifrons*. Compelling data for preference of eicosapentaenoyl-CoA ($C_{20:5}$) and docosahexaenoyl-CoA ($C_{22:6}$) are (1) high activities of CPT with these substrates (Table 1), (2) decrease in the oxidation rate of [$1-^{14}C$]palmitoyl-CoA in the presence of these substrates (Figure 2), and (3) results of reciprocal competition experiments with palmitoyl-CoA ($C_{16:0}$) and eicosapentaenoyl-CoA ($C_{20:5}$) (Table 2). On the other hand, linolenoyl-CoA ($C_{18:3}$) and arachidonoyl-CoA ($C_{20:4}$) appear to be poor substrates for mitochondria, on the basis of low activities of CPT with these acyl-CoAs and the increase in [$1-^{14}C$]palmitoyl-CoA ($C_{16:0}$) oxidation in the presence of these substrates.

High capacities for mitochondrial β -oxidation of some polyunsaturated substrates in *Notothenia gibberifrons* are contrary to trends observed for mammalian mitochondria. Mitochondria from mammalian (rat) liver generally are considered to be poor oxidizers of substrates with increasing degree of unsaturation (summarized in Osmundsen et al., 1987). Osmundsen and Bjornstad (1985) found rates of O_2 utilization by rat liver mitochondria with linolenoyl- ($C_{18:3}$), arachidonoyl- ($C_{20:4}$) and docosahexaenoyl- ($C_{22:6}$) carnitine to be only 20 % of the rates observed with oleoylcarnitine ($C_{18:1}$). In addition, these authors found docosahexaenoylcarnitine ($C_{22:6}$) to be a potent inhibitor of the mitochondrial oxidation of oleoylcarnitine ($C_{18:1}$). However, Christensen et al. (1986) observed total (mitochondrial and peroxisomal) β -oxidation rates with arachidonoyl- ($C_{20:4}$) and eicosapentaenoyl- ($C_{20:5}$) carnitine similar to that with oleoylcarnitine ($C_{18:1}$), and concluded that both polyunsaturated substrates are oxidized primarily by mitochondria.

The monounsaturate palmitoleoyl-CoA ($C_{16:1}$) clearly is a preferred substrate for mitochondrial β -oxidation in *Notothenia gibberifrons* liver. This interpretation also is consistent with reports that monounsaturates are preferred substrates for mitochondrial β -oxidation in *N. gibberifrons* muscle (Crockett et al., 1988) and in carp muscle (Murata and Higashi, 1979).

Peroxisomes from *Notothenia gibberifrons* liver are capable of β -oxidizing a wide range of fatty acyl substrates. ACO activities with various substrates have been shown to predict accurately substrate selectivities for peroxisomal β -oxidation in rat liver (Hryb and Hogg, 1979; Handler and Thurman, 1988). It is therefore likely that ACO in *N. gibberifrons* should reflect capacities for peroxisomal β -oxidation.

Although capable of utilizing a broad range of substrates, peroxisomes from teleost fishes may catabolize polyunsaturates at lower rates (relative to palmitoyl-CoA) than peroxisomes from mammals. Hepatic peroxisomes from mammals are capable of β -oxidizing polyunsaturated substrates, often at rates higher than their more saturated counterparts (reviewed by Osmundsen et al., 1987, 1991). Compared with other substrates, linolenoyl-CoA ($C_{18:3}$) is oxidized at the highest rates by rat liver peroxisomes, although its apparent K_m is similar to those of linoleoyl-CoA ($C_{18:2}$) and oleoyl-CoA ($C_{18:1}$) (Hiltunen et al., 1986). For the C_{20} and C_{22} carbon substrates, the trend is for higher oxidation rates with greater degree of unsaturation (Hovik and Osmundsen, 1987).

Site of substrate inhibition of peroxisomal β -oxidation

Substrate inhibition of peroxisomal β -oxidation occurs at ACO. Our observations that linolenoyl-CoA ($C_{18:3}$) depresses the

peroxisomal oxidation rate of [$1-^{14}C$]palmitoyl-CoA ($C_{16:0}$) by 50 %, together with linolenoyl-CoA ($C_{18:3}$) inhibition of palmitoyl-CoA oxidation by ACO (nearly 65 %), are consistent in supporting this conclusion. The same trend also occurs in the temperate teleost *Myoxocephalus octodecimspinosus* (E. L. Crockett, unpublished work). Single-substrate inhibition of both ACO and peroxisomal β -oxidation has been widely reported (Osumi et al., 1980; Alexson and Cannon, 1984; Hovik and Osmundsen, 1987). Hovik and Osmundsen (1987) proposed that the site of inhibition of peroxisomal β -oxidation is at ACO. These authors observed substrate inhibition of β -oxidation, using solubilized peroxisomal fractions similar to that reported with purified ACO (Osumi et al., 1980).

Inhibition of peroxisomal β -oxidation of palmitoyl-CoA by polyunsaturated acyl-CoA esters is probably due to competition for the active site of ACO and not to detergent effects from acyl-CoA micelles. The critical micelle concentration (CMC) of palmitoyl-CoA is 42 μM (Smith and Powell, 1986). Temperature has a small effect on CMC (Tanford, 1980). Also, unsaturation raises CMC (Smith and Powell, 1986). All these factors combined suggest that 25 μM acyl-CoA used in our experiments is below CMC.

Partitioning of β -oxidation

Peroxisomes probably initiate almost 30 % of total hepatic β -oxidation of palmitoyl-CoA in *Notothenia gibberifrons*. Metabolic estimates of peroxisomal contribution to hepatic palmitoyl-CoA β -oxidation range from 18 to 33 % in the presence of acyl-CoA esters likely to be found in significant concentrations (Table 5). These fatty acyl compounds are palmitoleoyl-CoA ($C_{16:1}$), oleoyl-CoA ($C_{18:1}$), eicosapentaenoyl-CoA ($C_{20:5}$) and docosahexaenoyl-CoA ($C_{22:6}$), based on the fatty acid compositional analysis of *N. gibberifrons* performed by Lund and Sidell (1992). Enzymic estimates also reflect significant peroxisomal capacity for β -oxidation for all substrates measured. CPT and ACO are reported to catalyse the rate-limiting steps in mitochondrial and peroxisomal β -oxidation respectively (Crabtree and Newsholme, 1972; Inestrosa et al., 1979; Osumi and Hashimoto, 1979), and thus provide reliable estimates of organellar capacities for β -oxidation.

A prominent role for hepatic peroxisomes in β -oxidation of fats may be widespread among marine teleosts and not be unique to the polar *N. gibberifrons*. In a temperate teleost, *Myoxocephalus octodecimspinosus*, β -oxidation by peroxisomes represents 50 % of total hepatic β -oxidative capacity for palmitoyl-CoA (Crockett and Sidell, 1993). However, our estimates for peroxisomal contribution to total β -oxidation in marine fishes are in contrast with the low estimates for peroxisomal relative to mitochondrial oxidative capacities based on enzyme activities in the herring *Clupea harengus pallasii* (Moyes et al., 1991). Although generally lower than estimates for teleosts described here, estimates for peroxisomal β -oxidative partitioning in mammalian hepatocytes have ranged widely (reviewed by Osmundsen et al., 1991). Estimates for the peroxisomal contribution to total β -oxidation have ranged from less than 10 % (Mannaerts et al., 1979), 32 % (Kondrup and Lazarow, 1985), to as high as 50 % in animals which have been administered the peroxisome proliferator clofibrate (Rognstad, 1991).

Utilization of polyunsaturated fatty substrates

Notothenia gibberifrons probably utilizes eicosapentaenoic acid ($C_{20:5}$) and docosahexaenoic acid ($C_{22:6}$) as fuels for energy metabolism. *N. gibberifrons* liver has the capacity to oxidize these

polyunsaturates (as fatty acyl-CoA esters) by either the mitochondrial or the peroxisomal pathways. It also is possible that oxidative muscle mitochondria in *N. gibberifrons* may utilize these polyunsaturates, although this remains to be resolved.

Eicosapentaenoic acid ($C_{20:5}$) and docosahexaenoic acid ($C_{22:6}$) also are likely to be important metabolic fuels in other teleosts. It is observed commonly that marine teleosts are rich in $n-3$ fatty acids, particularly eicosapentaenoic and docosahexaenoic acids (reviewed by Sargent, 1976). Zooplankton, a food for many fishes, are a rich dietary source of these polyunsaturates (Sargent, 1976). Although a more limited scan of substrate selectivities was performed in *Myoxocephalus octodecimspinosus*, a temperate marine teleost, substrate preferences for β -oxidation appear similar to those of *Notothenia gibberifrons* (Crockett and Sidell, 1993). It is then likely that the temperate species also utilizes eicosapentaenoic ($C_{20:5}$) and docosahexaenoic ($C_{22:6}$) acids for energy metabolism.

In summary, we have established that substrate selectivities for mitochondrial and peroxisomal β -oxidation are different in *Notothenia gibberifrons*. Hepatic mitochondria have preferences for utilizing a monounsaturate, palmitoleoyl-CoA ($C_{16:1}$), and two polyunsaturates, eicosapentaenoyl-CoA ($C_{20:5}$) and docosahexaenoyl-CoA ($C_{22:6}$), whereas peroxisomes do not show clear preferences. Instead, peroxisomes appear capable of β -oxidizing a wide array of substrates. In addition, polyunsaturates inhibit the β -oxidation of palmitoyl-CoA by peroxisomes and the inhibition site is ACO. Finally, peroxisomal β -oxidation can initiate almost 30% of hepatic β -oxidation in *N. gibberifrons*.

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REFERENCES

- Alexson, S. E. H. and Cannon, B. (1984) *Biochim. Biophys. Acta* **796**, 1–10
- Bar-Tana, J., Rose, G. and Shapiro, B. (1971) *Biochem. J.* **122**, 353–362
- Beers, R. F. and Sizer, I. W. (1952) *J. Biol. Chem.* **195**, 133–140
- Bieber, L. L., Abraham, T. and Helmrath, T. (1972) *Anal. Biochem.* **50**, 509–518
- Christensen, E., Hagve, T.-A. and Christophersen, B. O. (1986) *Biochim. Biophys. Acta* **879**, 313–321
- Clarke, A. (1980) *J. Exp. Mar. Biol. Ecol.* **43**, 221–236
- Crabtree, B. and Newsholme, E. A. (1972) *Biochem. J.* **130**, 697–705
- Crockett, E. L. and Sidell, B. D. (1990) *Physiol. Zool.* **63**, 472–488
- Crockett, E. L. and Sidell, B. D. (1993) *Am. J. Physiol.*, in the press
- Crockett, E. L., Sidell, B. D. and Driedzic, W. R. (1988) *Am. Zool.* **28**, 42A
- Frentzen, M., Heinz, E., McKeon, T. A. and Stumpf, P. K. (1983) *Eur. J. Biochem.* **129**, 629–636
- Fricke, H. and Oehlenschläger, J. (1988) *Comp. Biochem. Physiol.* **89B**, 39–42
- Handler, J. A. and Thurman, R. G. (1988) *Eur. J. Biochem.* **176**, 477–484
- Hazel, J. R. and Livermore, R. G. (1990) *J. Exp. Zool.* **256**, 31–37
- Henderson, R. J. and Sargent, J. R. (1985) *Comp. Biochem. Physiol.* **82B**, 79–85
- Hiltunen, J. K., Karki, T., Hassinen, I. E. and Osmundsen, H. (1986) *J. Biol. Chem.* **261**, 16484–16493
- Hovik, R. and Osmundsen, H. (1987) *Biochem. J.* **247**, 531–535
- Hovik, R. and Osmundsen, H. (1988) *Biochem. J.* **255**, 745–751
- Hryb, D. J. and Hogg, J. F. (1979) *Biochem. Biophys. Res. Commun.* **87**, 1200–1206
- Inestrosa, N. C., Bronfman, M. and Leighton, F. (1979) *Biochem. J.* **182**, 779–788
- Kondrup, J. and Lazarow, P. B. (1985) *Biochim. Biophys. Acta* **835**, 147–153
- Lazarow, P. B. (1978) *J. Biol. Chem.* **253**, 1522–1528
- Lazarow, P. B. and de Duve, C. (1976) *Proc. Natl. Acad. Sci. U.S.A.* **73**, 2043–2046
- Lund, E. D. and Sidell, B. D. (1992) *Mar. Biol.* **112**, 377–382
- Mannaerts, G. P., Debeer, L. J., Thomas, J. and DeSchepper, P. J. (1979) *J. Biol. Chem.* **254**, 4585–4595
- Moyes, C. D., Suarez, R. K., Brown, G. S. and Hochachka, P. W. (1991) *J. Exp. Zool.* **260**, 267–273
- Murata, H. and Higashi, T. (1979) *Bull. Jpn. Soc. Sci. Fish.* **45**, 211–217
- Neat, C. E., Thomassen, M. S. and Osmundsen, H. (1980) *Biochem. J.* **186**, 369–371
- Osmundsen, H. and Bjornstad, K. (1985) *Biochem. J.* **230**, 329–337
- Osmundsen, H., Thomassen, M. S., Hiltunen, J. K. and Berge, R. K. (1987) in *Peroxisomes in Biology and Medicine* (Fahimi, H. D. and Sies, H., eds.), pp. 152–165, Springer-Verlag, Berlin
- Osmundsen, H., Bremer, J. and Pedersen, J. I. (1991) *Biochim. Biophys. Acta* **1085**, 141–158
- Osumi, T. and Hashimoto, T. (1979) *J. Biochem. (Tokyo)* **85**, 131–139
- Osumi, T., Hashimoto, T. and Ui, N. (1980) *J. Biochem. (Tokyo)* **87**, 1735–1746
- Rognstad, R. (1991) *Biochem. J.* **279**, 147–150
- Sargent, J. R. (1976) in *Biochemical and Biophysical Perspectives in Marine Biology*, vol. 3 (Malins, D. C. and Sargent, J. R., eds.), pp. 149–212, Academic Press, New York
- Small, G. M., Burdett, K. and Connock, M. J. (1985) *Biochem. J.* **227**, 205–210
- Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J. and Klenk, D. C. (1985) *Anal. Biochem.* **150**, 76–85
- Smith, R. H. and Powell, G. L. (1986) *Arch. Biochem. Biophys.* **244**, 357–360
- Tanford, C. (1980) *The Hydrophobic Effect: Formation of Micelles and Biological Membranes*, pp. 1–233, John Wiley and Sons, New York
- Veerkamp, J. H., van Moerkerk, H. T. B., Glatz, J. F. C., Zuurveld, J. G. E. M., Jacobs, A. E. M. and Wagenmakers, A. J. M. (1986) *Biochem. Med. Metab. Biol.* **35**, 248–259
- Wharton, D. C. and Tzagoloff, A. (1967) *Methods Enzymol.* **10**, 245–250
- Zammit, V. A. and Newsholme, E. A. (1979) *Biochem. J.* **184**, 313–322