The Journal of Experimental Biology 207, 897-903
Published by The Company of Biologists 2004
doi:10.1242/jeb.00823

The substrate specificity of hormone-sensitive lipase from adipose tissue of the Antarctic fish *Trematomus newnesi*

J. R. Hazel[1] and B. D. Sidell[2,*]

[1]*Department of Biology, Arizona State University, Tempe, AZ 85287-1501, USA* and [2]*School of Marine Sciences, University of Maine, Orono, ME 04469-5751, USA*

*Author for correspondence (e-mail: bsidell@maine.edu)

Accepted 3 December 2003

Summary

Antarctic fishes of the suborder Notothenioidei characteristically possess large stores of neutral lipids that have been shown to be important both in conferring buoyant lift and as a caloric resource for energy metabolism. Previous work has established that the aerobic energy metabolism of Antarctic fish is fueled predominantly by the catabolism of fatty acids, with the catabolic machinery displaying a preference for the oxidation of unsaturated fatty acids. The composition of the fatty acids released from adipose tissue of Antarctic fish during lipolysis, however, has not previously been demonstrated. Employing a substrate competition assay, we have characterized the substrate specificity of hormone-sensitive lipase (HSL) from adipose tissue of the Antarctic fish *Trematomus newnesi*. Rates of oleic acid release from radiolabeled triolein were quantified in the presence and absence of a nonradiolabeled cosubstrate. Polyunsaturated species of triacylglycerols (TAGs) containing 18:2 or 20:4 depressed rates of oleate release by 70–75% below control values. Most of the molecular species of TAG containing monoenoic fatty acids (i.e. those containing 14:1, 16:1 or 20:1) had no significant effect on rates of oleate release. By contrast, oleate release from triolein was actually stimulated (by 2–4-fold) by both saturated species of TAG (i.e. those containing 14:0, 16:0 and 18:0) and those possessing long-chain (22:1 and 24:1) monoenes (by 1.2–1.5-fold). Thus, the rank order of substrate preference for adipose tissue HSL was: polyunsaturates > monoenes > saturates. Degree of fatty acid unsaturation had a more marked effect on rates of hydrolysis than did fatty acid chain length. In addition, the enzyme displayed a preference for the hydrolysis of *sn*-1,2 rather than *sn*-1,3 diacylglycerols. These data indicate that the substrate specificity of adipose tissue HSL may be an important factor in determining which fatty acids are mobilized during stimulated lipolysis and which are made available for catabolism by other tissues of Antarctic fishes. Our data further suggest that TAGs containing some saturated fatty acids may be sufficiently poor substrates for catabolism by HSL to explain their disproportionate accumulation in adipose tissue. Such a mechanism could also contribute to the ontogenetic accumulation of fats that has been reported as an underlying basis for the positive correlation of buoyancy with increasing body mass in this group.

Key words: hormone-sensitive lipase, triacylglycerol lipase, Antarctic fish, *Trematomus newnesi*, adipose tissue, substrate specificity.

Introduction

Notothenioid fishes are endemic to the chronically cold waters of the Southern Oceans surrounding Antarctica. These fishes display numerous adaptations in morphology, cardiac, circulatory and respiratory physiology, freezing avoidance and metabolism to maintain physiological function at near-freezing temperatures (Eastman, 1993). High levels of tissue lipids, primarily triacylglycerols (TAGs), are a characteristic and conspicuous feature of these fishes (Clarke et al., 1984). It has recently become clear that proportional corporeal lipid content of at least four different Antarctic notothenioids increases ontogenetically with increasing body size (Friedrich and Hagen, 1994; Hagen et al., 2000; Near et al., 2003), suggesting that this phenomenon may be a widespread characteristic of fishes in this group. High lipid contents of tissues in Antarctic fishes have been postulated to contribute to their physiology in a variety of ways: (1) by increasing the buoyancy of these swimbladderless fishes in seawater, thus permitting some species to exploit pelagic niches (Eastman, 1988); (2) by increasing the diffusing capacity of oxygen (because oxygen is more soluble in lipid than in the aqueous cellular milieu) and thereby the delivery of oxygen to sites of oxidative phosphorylation (Desaulniers et al., 1996; Londraville and Sidell, 1990) and (3) by providing an important source of stored calories to support aerobic metabolism (Sidell et al., 1995). How a single chemical class of compound, TAG, can be managed to meet the two apparently conflicting imperatives

of accumulation for buoyancy and mobilization for energy metabolism has remained unresolved. In the present paper, we examine the nature of fatty acids that are released *via* lipolysis from adipose tissue stores through the action of hormone-sensitive TAG lipase (HSL) and whether the behavior of this system may provide insight into the regulation of TAGs for purposes of buoyancy and energy metabolism.

A considerable body of evidence suggests that the obligately aerobic energy metabolism of Antarctic fishes is fueled predominantly by the catabolism of fatty acids derived from TAG stores in liver and adipose tissue. For example, the maximal activity of carnitine palmitoyl transferase (CPT; a marker enzyme for fatty acid oxidation) assayed at 1°C is elevated to a greater degree than activities of citrate synthase and cytochrome oxidase in tissues of polar compared with temperate fish species (Crockett and Sidell, 1990). Furthermore, in the Antarctic fish *Gobionotothen gibberifrons*, fatty acids were more effective substrates of energy metabolism in oxidative skeletal muscle and heart than either glucose or lactate (Sidell et al., 1995). Not only is there a high capacity for fatty acid catabolism in polar species but also, in several instances, the biochemical machinery is best able to metabolize unsaturated fatty acids. For example, aerobic skeletal and ventricular muscle of *G. gibberifrons* displays a clear preference for the catabolism of monounsaturated fatty acids (particularly palmitoleic acid), a preference that is mirrored by that of CPT, the rate-limiting enzyme in the β-oxidation pathway (Sidell et al., 1995). Similarly, in *G. gibberifrons*, mitochondria showed a marked preference for the oxidation of palmitoleoyl-CoA and two polyunsaturated fatty acids (PUFAs; 20:5 and 22:6), suggesting that in this species both monoenes and PUFAs may support aerobic energy metabolism.

Metabolism of fats by vertebrates requires a complex suite of integrated processes that encompass dietary acquisition, assimilation, synthesis of storage depots (TAGs), mobilization of stored fats and, ultimately, catabolism. Although catabolism of fats has been partially characterized in Antarctic fish, processes of fat mobilization have not been characterized. The rate-limiting enzyme responsible for initiating the mobilization of stored fats from adipose tissue reserves is HSL (alternatively called TAG lipase). A limited survey of Antarctic fish has previously demonstrated that, even when measured at physiological temperature (0°C), the activity of adipose tissue HSL is comparable to that observed in tissues of temperate-zone fishes at their much higher body temperatures (Sidell and Hazel, 2002). Some limited data suggest that the process of lipolysis in Antarctic fish tissues is nonrandom so that some fatty acids (perhaps those that are not preferentially oxidized) are over represented in their abundance in storage TAG compared with the composition of fats transported to other tissues for oxidation. For example, the percentage of 14:0 in neutral lipid deposits of adipose tissue exceeds that in pools of serum or oxidative skeletal muscles in both *G. gibberifrons* and *Trematomus newnesi* (Lund and Sidell, 1992). Likewise, in mammals, the mobilization of fatty acids from adipose tissue is nonrandom, and chemically different fatty acids are mobilized at different rates: rapidly mobilized fatty acids include $C_{16–20}$ fatty acids with 4–5 double bonds, whereas slowly mobilized fatty acids include $C_{20–24}$ fatty acids with 0–1 double bonds (Raclot et al., 2001).

We undertook the present study to characterize partially the substrate specificity of HSL isolated from adipose tissue of the Antarctic fish *Trematomus newnesi* and to determine whether this enzyme biases the composition of fatty acids released from adipose tissue and made available to other tissues for catabolism.

Materials and methods

Animals

The Antarctic fish *Trematomus newnesi* Boulenger 1902 was captured from depths of 80–150 m by 6 m otter trawl deployed from the *R/V Polar Duke* at sites near Low Island (63°25′ S, 62°10′ W) and Dallmann Bay in the vicinity of Astrolabe Needle (64°10′ S, 62°35′ W) off the Antarctic Peninsula. Fish were held in running seawater tanks aboard the vessel and transported to the United States' research station, Palmer Station, Antarctica. At Palmer Station, animals were maintained in running seawater tanks (–1.0° to +1°C) under ambient photoperiod for up to two weeks prior to experiments. During this time, animals were not fed.

Just prior to experiments, fish were killed by a sharp blow to the head, followed by severance of the spinal cord posterior to the braincase. Body mass and standard length of all animals were measured and recorded. The fish used in these experiments averaged 79.3±3.46 g (mean ± S.E.M.) in mass, were 18.5±0.23 cm in length and possessed 0.22±0.03 g of mesenteric adipose tissue.

Tissue and enzyme preparation

Mesenteric adipose tissue was finely diced using a single-edged razor blade and homogenized (20% w/v) in 0.35 mol l^{-1} sucrose, 1 mmol l^{-1} EDTA, 25 mmol l^{-1} Tris-HCl, pH 7.5 at 0°C employing a hand-held, ground-glass Tenbroeck tissue grinder (Wheaton, Millville, NJ, USA). The homogenate was centrifuged at 10 000 ***g*** for 30 min and the supernatant brought to 0.5 mol l^{-1} NaCl by the addition of an appropriate mass of solid NaCl. HSL activity (which does not bind to heparin under the conditions employed) was separated from lipoprotein lipase (LPL) activity (which does bind to heparin) by heparin–Sepharose affinity chromatography as described by Sheridan and Allen (1984). The 10 000 ***g*** supernatant was loaded onto a 1.5 cm×20 cm heparin–Sepharose affinity column (prepared as described in a technical bulletin provided by Sigma Chemical Company, St Louis, MO, USA) previously equilibrated with homogenizing medium containing 0.5 mol l^{-1} NaCl. Adipose tissue HSL eluted immediately after the void volume of the column (Fig. 1); fractions containing this activity were pooled and concentrated from 15–25 ml to approximately 5 ml by membrane ultrafiltration (Amicon, Millipore Corp., Billerica, MA, USA; 10 kDa cutoff) under a

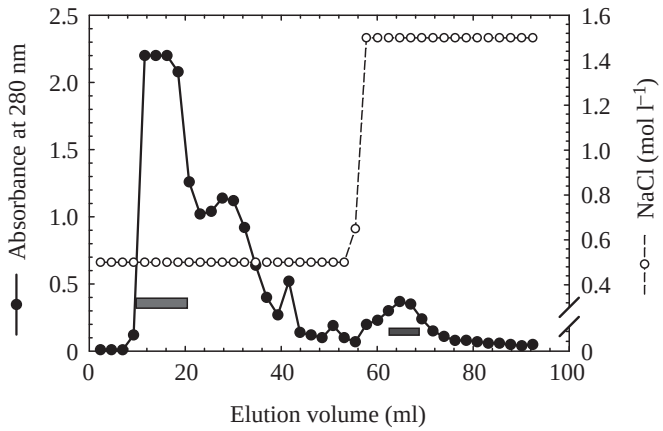

Fig. 1. Elution profile of heparin–Sepharose affinity chromatography column illustrating the elution of protein (absorbance at 280 nm; solid symbols) and the concentration of NaCl in the eluate (open symbols). Typical results from a single experiment. Horizontal bars indicate those fractions with detectable lipase activity against TAG substrates. Activity eluting early in the column is HSL, which is not retarded by the heparin affinity column. Activity eluting later in the chromatogram at high salt concentration is LPL, which is retarded by the heparin affinity column.

head of nitrogen pressure. Enzyme activity was determined immediately after concentration, without prior freezing.

Measurement of HSL activity and assessment of substrate specificity

We employed a modification of the radiometric assay of Sheridan and coworkers (Sheridan and Allen, 1984; Sheridan et al., 1985) to measure HSL activity. This assay employed ^{14}C-labeled triolein (isotope was present at the carboxyl carbon of the oleic acid esterified to each of the three carbons of glycerol) as a substrate, and activity was measured as radioactivity recovered in free fatty acid after exposure to lipase.

A stock solution of radiolabeled triolein was prepared by drying solvent (ethanol) from 14.8 MBq of carboxyl-[^{14}C]triolein [New England Nuclear, Perkin-Elmer, Boston, MA, USA; catologue no. NEC-674 (4.22 GBq mmol^{-1})] under a gentle stream of nitrogen gas, followed by dissolving the radiolabel and 48 μmoles of nonradiolabeled triolein in 4 ml of absolute ethanol. This stock solution was stored at 0–4°C in an amber vial prior to use in experiments. The substrate specificity of the enzyme was inferred from the effects of the addition of nonradiolabeled competing substrates on the rate of hydrolysis of radiolabeled triolein; substrates more effective than triolein are expected to reduce the amount of radioactivity recovered in the assay. The final reaction mixture was prepared by diluting the above stock solution in buffer so that the final reaction mixture contained: 125 μmol l^{-1} [^{14}C]triolein, 1% (w/v) fatty-acid-free bovine serum albumin, 25 mmol l^{-1} Tris-HCl, pH 7.5 at 0°C, and 25 μmol l^{-1} nonradiolabeled competing substrate [the total triacylglycerol concentration in the assay was 150 μmol l^{-1}, with 83.3% of this amount comprised of radiolabeled triolein and 16.6% comprised of nonradioactive TAG (triolein in the case of control experiments or other species of TAG in the case of competition experiments)].

Execution of the experimental series, in some cases, required more than a single preparation of post-column enzyme. We observed insignificant differences in specific activity among the preparations of enzyme when measured with reference triolein substrate. Measurements with each preparation were made on all substrates within an experimental series (e.g. monoenoic, saturated or polyunsaturated TAGs) and, as indicated below, referenced to a corresponding estimate of activity in the presence of triolein. This method of normalization should ensure correction for even small variance in specific activities between preparations.

Reactions were initiated by the addition of 1 ml of post-column, concentrated, enzyme preparation to 3.075 ml of otherwise complete assay buffer. Immediately after addition of enzyme, a small aliquot (50 μl) of the reaction mixture was sampled for the determination of specific radioactivity. The assay temperature was 0°C. Reactions were stopped at 240 min (previous experiments had established linearity of product release over this time period; Sidell and Hazel, 2002) by the addition of organic solvents [chloroform:methanol:benzene (1:2.4:2, v/v)], and free fatty acids were extracted as described previously (Sidell and Hazel, 2002). Radioactivity was determined by the method of external standard quench correction employing an LKB-Wallac 1409 liquid scintillation spectrometer (Perkin-Elmer, Boston, MA, USA). HSL activities were expressed in units of pmoles [^{14}C]oleate released min g^{-1} wet mass. For assessment of substrate specificity, rates of oleate release in the presence of 25 μmol l^{-1} of the competitive substrate being tested were compared with those induced by the addition of 25 μmol l^{-1} triolein.

Statistical analyses

Comparisons between substrates were performed by one-way analysis of variance (ANOVA) using the Statistica™ (Statsoft, Tulsa, OK, USA) software package. To capture the natural variance associated with the addition of nonradiolabeled triolein as a control (which is lost when results are expressed as a ratio relative to triolein addition), statistical comparisons were made for all TAG additions relative to the addition of a comparable volume of assay buffer (devoid of TAG). *Post-hoc* tests for significance between substrates employed the least significant difference test for planned comparisons.

Chemicals and reagents

Sepharose 6MB, heparin and all species of both tri- and diacylglycerols were from Sigma Chemical Company. All other chemicals were of reagent grade or better.

Results

Separation of adipose tissue HSL from LPL

Since both adipose tissue HSL and LPL catalyze the hydrolysis of TAGs, it was first necessary to remove LPL

activity from the high-speed tissue supernatant. Affinity chromatography employing heparin coupled to Sepharose beads resolved two distinct populations of lipase activity (Fig. 1): the fraction eluting at low NaCl concentration (0.5 mol l^{-1}) displayed low affinity for heparin and corresponded to adipose tissue HSL; the fraction eluting at high salt concentration (1.5 mol l^{-1}) displayed a higher affinity for heparin, consistent with the properties of LPL. No loss of activity occurred during affinity chromatography (based on pre- and post-column assays) and, on average, ~75% of the total lipase activity applied to the column was recovered in the adipose tissue HSL fraction and ~25% in the LPL fraction.

Substrate specificity of adipose tissue HSL activity

Inferences about relative substrate specificity were made by determining the effects of adding nonradiolabeled TAGs of varying chain length and degree of fatty acyl unsaturation, or in some cases by the addition of defined molecular species of diacylglycerols (DAGs), on lipase-catalyzed liberation of oleic acid from triolein. All additions of competitive substrates were compared to the effects of adding a similar concentration of nonradiolabeled triolein (in each case, the nonradiolabeled TAG is in excess concentration to the labeled compound). A significant reduction in HSL activity in the presence of a competitive substrate (compared with triolein) indicates that the substrate competes more effectively for the enzyme than triolein. ANOVA indicated a highly significant effect (P<0.00001) of competitive substrate addition on rates of triolein hydrolysis.

Saturated species of TAGs

Saturated species of TAGs significantly stimulated rather than inhibited rates of triolein hydrolysis (Fig. 2). The stimulatory effect of trisaturated species of TAG on rates of triolein hydrolysis was inversely related to chain length: in the presence of tri-14:0, tri-16:0 and tri-18:0 species of TAG, rates of triolein hydrolysis were 3.7-fold (P<0.0001), 2.1-fold (P<0.001) and 1.9-fold (P<0.002) greater, respectively, than that observed with triolein as the sole substrate. The particularly pronounced stimulation of triolein hydrolysis in the presence of tri-14:0 suggests that this compound is a particularly poor substrate for HSL, thus effectively elevating the specific radioactivity of the triolein pool in the assay.

Monounsaturated species of TAGs

Rates of triolein hydrolysis were significantly influenced by only two of the tri-monounsaturated species of TAG varying in chain length from 14 to 24 carbons (Fig. 3). The longer chain (C_{22} and C_{24}) monoenoic species of TAG, similar to saturated species, significantly (P<0.04) stimulated rather than inhibited rates of triolein hydrolysis. Although other differences were not significant, tri-16:1 and tri-18:1 molecular species yielded the lowest rate ratios.

Polyunsaturated species of TAGs

Rates of triolein hydrolysis were significantly (P<0.04) depressed in the presence of polyunsaturated (tri-20:4 and tri-18:2) molecular species of TAG (Fig. 4).

Positional isomers of DAGs

DAGs are intermediate products in the hydrolysis of TAGs to monoglycerides or glycerol and fatty acids. None of the positional isomers of either 18:1 or 16:0 DAG significantly influenced the rates of triolein hydrolysis (Fig. 5). Two trends in these data are, however, noteworthy: (1) rates of triolein hydrolysis in the presence of 16:0 DAGs were significantly lower than those observed with tri-16:0 TAG (compare Figs 5

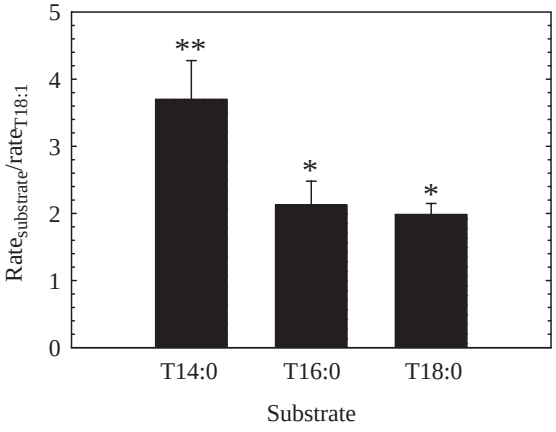

Fig. 2. The effects of various totally saturated species of triacylglycerol (TAG; e.g. T14:0 indicates the addition of trimyristoyl TAG) on rates of oleate release from triolein by hormone-sensitive lipase (HSL). Results are presented as means ± S.E.M. (N=4) for the ratio of the rate of oleate released in the presence of the substrate indicated on the abscissa compared with the rate measured with triolein. **P<0.0001; *0.0001<P<0.002, indicating significant differences compared with the addition of triolein.

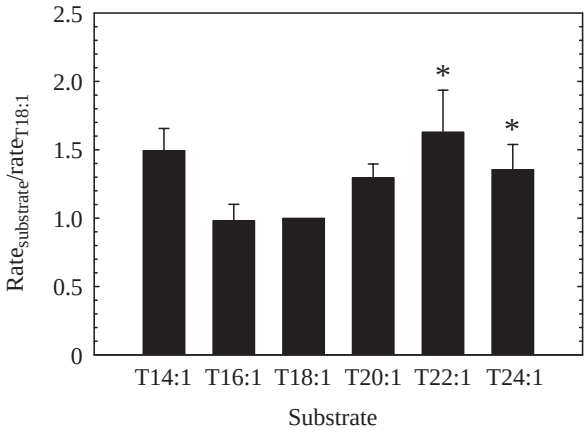

Fig. 3. The effects of various monoenoic species of triacylglycerol (TAG; e.g. T16:1 indicates the addition of tripalmitoleoyl TAG) on rates of oleate release from triolein by hormone-sensitive lipase (HSL). Results are presented as means ± S.E.M. (N=4) for the ratio of the rate of oleate released in the presence of the substrate indicated on the abscissa compared with the rate measured with triolein. *P<0.04, indicating significant differences compared with the addition of triolein.

and 2) and (2), although not significantly different, for DAG species of both 18:1 and 16:0, the 1,2-molecular species consistently depressed rates of triolein hydrolysis to a greater extent than the 1,3-molecular species.

Discussion

Fatty acids stored in the form of TAGs are a major fuel for energy metabolism. The hydrolysis of TAGs to fatty acids and glycerol requires the concerted action of two enzymes, HSL – the initial and rate-limiting step in the process – and monoglyceride lipase (MGL). HSL is one member of the triglyceride lipase gene family that also includes pancreatic lipase, LPL, hepatic lipase and endothelial lipase (McCoy et al., 2002). HSL is distinguished, in part, from other members of the gene family by its intracellular location and relatively broad substrate specificity. In addition, HSL is subject to reversible phosphorylation and activation, primarily in response to catecholamine stimulation of adipose tissue mediated *via* β-adrenergic receptors and the downstream activation of phosphorylase kinase (PKA) by cAMP. Stimulation of adipocytes with catecholamines also triggers translocation of HSL from the cytoplasmic compartment to the surface of lipid droplets (Holm et al., 2000). Activation by catecholamines is reversed by insulin, which stimulates a cAMP phosphodiesterase (Østerlund, 2001). Thus, HSL catalyzes the key regulated step in lipolysis.

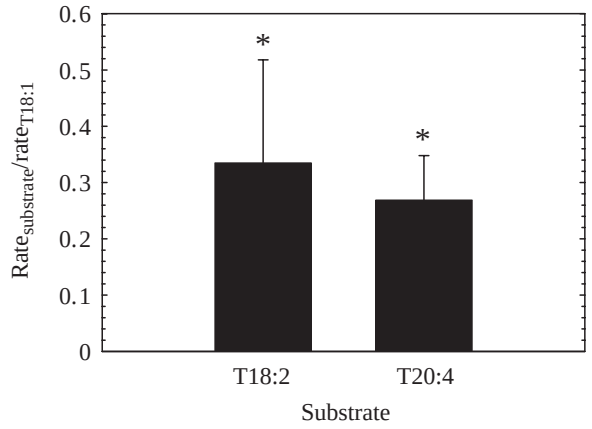

Fig. 4. The effects of polyenoic species of triacylglycerol (TAG; e.g. T20:4 indicates the addition of triarachidonyl TAG) on rates of oleate release from triolein by hormone-sensitive lipase (HSL). Results are presented as means ± S.E.M. (N=4) for the ratio of the rate of oleate released in the presence of the substrate indicated on the abscissa compared with the rate measured with triolein. *P<0.04, indicating significant differences compared with the addition of triolein.

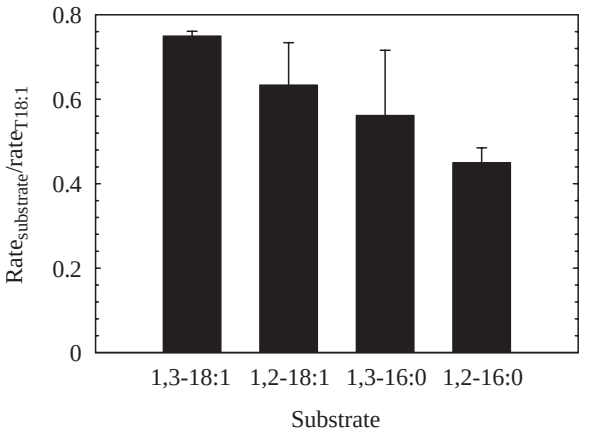

Fig. 5. The effects of differing positional isomers of diacylglycerol (DAG) species on rates of oleate release from triolein by hormone-sensitive lipase (HSL). Results are presented as means ± range (N=2) for the ratio of the rate of oleate released in the presence of the substrate indicated on the abscissa compared with the rate measured with triolein.

In mammals, it is clear that the release of fatty acids from adipose tissue is not random. For example, in rabbit adipose tissue, mobilization of fatty acids into the plasma was not proportional to their abundance but rather was determined by their molecular structure; rates of mobilization varied over a range of 46-fold and correlated positively with degree of unsaturation and negatively with chain length (Connor et al., 1996). Indeed, eicosapentaenoic acid (20:5*n*3) and arachidonic acid (20:4*n*6), important precursors in prostaglandin biosynthesis, were mobilized at the highest rates. Since HSL is a key and rate-limiting enzyme in lipolysis, we hypothesized that the substrate specificity of this enzyme in Antarctic fish might result in the hydrolysis of selected fatty acids from storage reserves. In particular, we wished to determine whether the previously established preferences in the capacities for fatty acid catabolism by muscle and liver tissues (Crockett and Sidell, 1990, 1993a,b; Sidell et al., 1995) were matched by a corresponding pattern of fatty acid release from adipose tissue reserves.

We have previously reported that mass-specific activities of HSL in Antarctic fish, when measured at 0°C, are roughly comparable to those of temperate zone species assayed at their physiological temperatures (Sidell and Hazel, 2002). This observation most probably arises from one, or a combination of, unexplored adaptations that might include: (1) evolution of a lipase that is more efficient at low temperatures, as has commonly been reported for a variety of other enzymes (Somero, 1995), (2) elevated titers of the enzyme in tissues of Antarctic fish or (3) a reduced temperature sensitivity for HSL compared with other enzymes. With regard to the third possibility, it is interesting to note that human HSL displays significant sequence homology to an Antarctic bacterial lipase and displays significant hydrolytic capacity even at low temperatures (<10°C), causing this enzyme to be classified as psychrotolerant (Laurell et al., 2000). However, aside from demonstration of significant catalytic activity at low temperature, the potential role of this enzyme in determining the nature of the fatty acids released for catabolism in Antarctic fish has not been studied previously.

To characterize the substrate specificity of HSL from the adipose tissue of Antarctic fish, it was first necessary to separate HSL from other members of the same gene family with different substrate preferences and kinetic properties. The other activity

of concern is LPL, which functions to degrade serum lipids destined for deposition in adipose tissue and possesses a heparin-binding domain not present in HSL. Heparin–Sepharose affinity chromatography (Fig. 1) clearly resolved HSL and LPL activities present in total tissue extracts of adipose tissue from *T. newnesi*. Of these two activities, HSL accounted for approximately 75% of the total activity (LPL 25%).

To assess the substrate specificity of HSL, a competition assay was employed. Control assays were used to define the maximal release of oleic acid from radiolabeled triolein. Substrate specificity was assessed by adding a second non-radiolabeled substrate to the reaction mixture and determining the extent to which the liberation of oleic acid was inhibited. The degree to which the release of oleic acid is decreased in the presence of the non-radiolabeled substrate reflects the ability of HSL to hydrolyze one species of TAG preferentially over another. The substrate causing the greatest degree of inhibition is presumably the one most readily hydrolyzed (Crecelius and Longmore, 1984). Conversely, elevation in the rate of radiolabeled release from triolein by the added substrate would indicate either a true stimulation of HSL activity or, alternatively, that the added TAG was such a poor substrate for HSL that it was effectively 'transparent' to the enzyme, thus elevating the specific radioactivity of the labeled triolein pool.

To our surprise, totally saturated molecular species of TAG caused an apparent stimulation rather than inhibition in rates of oleate release from triolein (Fig. 2). The magnitude of this activation varied between 2- and 4-fold and decreased as a function of increasing chain length for species of TAG bearing fatty acids between 14 and 18 carbons in length. By contrast, molecular species of TAG containing monoenoic fatty acids varying between 14 and 20 carbons in length had no significant effect on rates of oleic acid release (Fig. 3) compared with triolein. The observation that activity ratios were somewhat lower for tri-16:1 and tri-18:1 TAG than tri-14:1 or tri-20:1 is suggestive of a slight preference for monoenoic species of TAG with chain lengths of 16–18 carbons. Longer chain ($C_{22–24}$) monoenoic species of TAG resembled totally saturated species more than shorter chain monoenoic species since they significantly stimulated rather than inhibited oleate release (Fig. 3). More highly unsaturated species of TAG (containing di- and tetraenoic fatty acids) significantly depressed rates of oleate release (Fig. 4), indicating that these species are preferentially hydrolyzed compared with triolein.

Taken collectively, the above data support the following rank order of preference for the hydrolysis of molecular species of TAG by adipose tissue HSL from the Antarctic fish *T. newnesi*: polyenoic species > monoenoic species > saturated species. This order corresponds roughly with that established for the rates of fatty acid release from adipose tissue in mammals (Connor et al., 1996). Among monoenes, the preferential release of oleic acid also resembles the substrate selectivity of rat and human HSL (Raclot et al., 2001); however, the preferential release of PUFAs is a unique feature of the *T. newnesi* HSL that is not shared by either the rat or human enzymes. Thus, in contrast to the situation for human and rat HSL, for which fatty acid chain length is the major determinant of rates of hydrolysis, the degree of fatty acid unsaturation appears to be the primary determinant of HSL activity in *T. newnesi*. The substrate specificity of *T. newnesi* HSL also meshes well with the pattern of differential peripheral utilization of fatty acids by liver and muscle (Crockett and Sidell, 1993a,b; Sidell et al., 1995).

The failure of fully saturated and long-chain monounsaturated species of TAG to inhibit the hydrolysis of triolein was unanticipated and a novel feature of the present data set. The pronounced 3.7-fold increase in triolein hydrolysis in the presence of trimyristin (tri-14:0) is particularly noteworthy, given the disproportionate representation of this TAG in adipose tissues of Antarctic fish species (Lund and Sidell, 1992). As indicated above, this observation could suggest that tri-14:0 either specifically stimulates HSL activity or, alternatively, is a poor substrate for this enzyme. The percentage of 14:0 in adipose tissue TAG of *T. newnesi* (15.1±1.0%) is vastly greater than in pools of TAG from either oxidative skeletal muscle (5.7±0.6%) or combined serum TAG and cholesterol esters (4.5±0.4%; Lund and Sidell, 1992). Since fully saturated fatty acids possess significantly higher melting points than oleic acid (Gurr and Harwood, 1991), one possibility is that TAG species containing these acids may phase-separate from triolein at 0°C and, thus, might not be readily accessible to the enzyme. However, our data indicate significant differences among saturated fatty acids in their abilities to stimulate triolein hydrolysis, which is not consistent with a phase separation argument (i.e. the ability to stimulate triolein hydrolysis is inversely related to fatty acid melting point, exactly the opposite trend predicted by a phase-separation argument; Fig. 2). Thus, it seems most likely that the differences in rates of triolein hydrolysis observed in the presence of competing saturated and other species of TAG reflect the true substrate specificity of the enzyme rather than a biophysical effect of low temperature on the state of the substrate. In light of the above observations, we favor the interpretation that tri-14:0 (and, by inference, the other saturated TAGs) is a particularly poor substrate for lipolysis by HSL when more suitable alternative TAG substrates are available to the enzyme. It is worth noting, however, that regardless of the mechanisms in play, the physiological implications remain the same.

Although HSL is the most likely determinant of the mix of fatty acids released from adipose tissue, it should be acknowledged that two other proteins may play a secondary role in regulating this process: (1) perilipins are proteins located on the surface of lipid droplets in unstimulated adipocytes that presumably block the access of HSL to substrate in unstimulated adipocytes (perilipins, like HSL, are phosphorylated by PKA as a consequence of adipocyte activation, causing them to redistribute away from the lipid droplet, thus permitting HSL to bind to the droplet surface; Østerlund, 2001) and (2) the adipocyte lipid binding protein (ALBP) specifically interacts with HSL and most likely removes the fatty acid product of lipolysis (Holm et al., 2000).

Activated HSL displays a preference (3–4-fold) for

hydrolysis of the 1- or 3-ester bond of its acylglycerol substrate compared with the 2-ester linkage (Holm et al., 2000). Interestingly, the *T. newnesi* enzyme displays a slight preference for DAG compared with TAG and for the *sn*-1,2 over the *sn*-1,3 isomer of DAG (Fig. 5), implying that initial cleavage of the *sn*-3 linkage increases the likelihood of the resulting *sn*-1,2 species being converted to a 2-monoglyceride. Thus, *sn*-2 monoacylglycerols are expected to be the primary substrates for monoacylglycerol lipase.

An interesting regulatory aspect of lipid mobilization from adipose tissue of Antarctic fish is how this storage depot is managed to meet the potentially differing and conflicting demands of buoyancy regulation and as an energy source to fuel aerobic metabolism. Our results do offer some possible insight into this process. Our present data suggest that saturated species of TAG (especially short-chain species such as tri-14:0) are poorly hydrolyzed by HSL. Previous results likewise show that especially 14:0 accounts for a disproportionate fraction of stored neutral lipid in these species (Lund and Sidell, 1992) and, consequently, may be among the primary species of fatty acid involved in buoyancy regulation. Evidence has also been accumulating that the corporeal stores of lipid in Antarctic fishes increase with increasing body size, conferring greater buoyant advantage as the animals become larger (Friedrich and Hagen, 1994; Hagen et al., 2000; Near et al., 2003). In combination, these observations make it possible to envision that the spectrum of dietary lipid in excess of immediate needs is readily partitioned into storage by lipogenic machinery but that subsequent hormone-activated mobilization of these stores favors the breakdown of TAGs substituted with more unsaturated fatty acids, leaving a gradually increasing component of TAGs substituted with saturated fatty acids, especially 14:0, over time. Thus, a pool of relatively metabolically inert neutral lipids would be accumulated and contribute to whole animal buoyancy.

In summary, our results indicate that adipose tissue HSL of *T. newnesi* releases fatty acids from a TAG emulsion in a nonrandom fashion. PUFAs are preferentially released, followed by monoenes; saturated species of TAG are hydrolyzed most slowly. Degree of fatty unsaturation, more than chain length, is the primary determinant of hydrolytic rates. These data indicate that HSL is a potentially important determinant of the specific fatty acids released from the storage depots of Antarctic fish. In addition, there is a reasonable correspondence between the fatty acids released due to the action of adipose HSL and the patterns of peripheral fatty acid catabolism by liver and muscle in these fish and an equivalent correspondence between those most poorly released and the composition of accumulated storage TAG in adipose tissues.

The authors gratefully acknowledge the support personnel of Palmer Station, Antarctica and the masters and crew of the *R/V Polar Duke* who were essential to the success of both the field and laboratory work. This research was supported by U.S. National Science Foundation grants DPP 88-19469 and OPP 01-25890 to B.D.S.

References

Clarke, A., Doherty, N., De Vries, A. L. and Eastman, J. T. (1984). Lipid content and composition of three species of Antarctic fish in relation to buoyancy. *Polar Biol.* **3**, 77-83.

Connor, W. E., Lin, D. S. and Colvis, C. (1996). Differential mobilization of fatty acids from adipose tissue. *J. Lipid Res.* **37**, 290-298.

Crecelius, C. A. and Longmore, W. J. (1984). A study of the molecular species of diacylglycerol, phosphatidylcholine, and phosphatidylethanolamine and of choline phosphotransferase and ethanolamine phosphotransferase activities in type II pneumocytes. *Biochim. Biophys. Acta* **795**, 247-256.

Crockett, E. L. and Sidell, B. D. (1990). Some pathways of energy metabolism are cold adapted in Antarctic fishes. *Physiol. Zool.* **63**, 472-488.

Crockett, E. L. and Sidell, B. D. (1993a). Peroxisomal β-oxidation is a significant pathway for catabolism of fatty acids in a marine teleost. *Am. J. Physiol.* **264**, R1004-R1009.

Crockett, E. L. and Sidell, B. D. (1993b). Substrate selectivities differ for hepatic mitochondrial and peroxisomal β-oxidation in an Antarctic fish, *Notothenia gibberifrons*. *Biochem. J.* **289**, 427-433.

Desaulniers, N., Moerland, T. S. and Sidell, B. D. (1996). High lipid content enhances the rate of oxygen diffusion through fish skeletal muscle. *Am. J. Physiol. Reg. Integ. C* **40**, R42-R47.

Eastman, J. T. (1993). *Antarctic Fish Biology*. New York: Academic Press.

Eastman, J. T. (1988). Lipid storage systems and the biology of two neutrally buoyant Antarctic notothenioid fishes. *Comp. Biochem. Physiol. B* **90**, 529-537.

Friedrich, C. and Hagen, W. (1994). Lipid contents of five species of notothenioid fish from high-Antarctic waters and ecological implications. *Polar Biol.* **14**, 359-369.

Gurr, M. I. and Harwood, J. L. (1991). *Lipid Biochemistry*. London: Chapman and Hall.

Hagen, W., Kattner, G. and Friedrich, C. (2000). The lipid compositions of high-Antarctic notothenioid fish species with different life strategies. *Polar Biol*. **23**, 785-791.

Holm, C., Osterlund, T., Laurell, H. and Contreras, J. A. (2000). Molecular mechanisms regulating hormone-sensitive lipase and lipolysis. *Annu. Rev. Nutr.* **20**, 365-393.

Laurell, H., Contreras, J. A., Castan, I., Langin, D. and Holm, C. (2000). Analysis of the psychrotolerant property of hormone-sensitive lipase through site-directed mutagenesis. *J. Lipid Res.* **13**, 711-717.

Londraville, R. L. and Sidell, B. D. (1990). Ultrastructure of aerobic muscle in Antarctic fishes may contribute to maintenance of diffusive fluxes. *J. Exp. Biol.* **150**, 205-220.

Lund, E. D. and Sidell, B. D. (1992). Neutral lipid compositions of Antarctic fish tissues may reflect use of fatty acyl substrates by catabolic systems. *Mar. Biol.* **112**, 377-382.

McCoy, M. G., Sun, G. S., Marchadier, D., Maugeais, C., Glick, J. M. and Rader, D. J. (2002). Characterization of the lipolytic activity of endothelial lipase. *J. Lipid Res.* **43**, 921-929.

Near, T. J., Russo, S. E., Jones, C. D. and De Vries, A. L. (2003). Ontogenetic shift in buoyancy and habitat in the Antarctic toothfish, *Dissostichus mawsoni* (Perciformes: Nototheniidae). *Polar Biol.* **26**, 124-128.

Østerlund, T. (2001). Structure-function relationships of hormone-sensitive lipase. *Eur. J. Biochem.* **268**, 1899-1907.

Raclot, T., Holm, C. and Langin, D. (2001). Fatty acid specificity of hormone-sensitive lipase: implication in the selective hydrolysis of triacylglycerols. *J. Lipid Res.* **42**, 2049-2057.

Sheridan, M. A. and Allen, W. V. (1984). Partial purification of a triacylglycerol lipase isolated from steelhead trout (*Salmo gairdneri*) adipose tissue. *Lipids* **19**, 347-352.

Sheridan, M. A., Woo, N. Y. and Bern, H. A. (1985). Changes in the rates of glycogenesis, glycogenolysis, lipogenesis, and lipolysis in selected tissues of the coho salmon (*Oncorhynchus kisutch*) associated with parr-smolt transformation. *J. Exp. Zool.* **236**, 35-44.

Sidell, B. D., Crockett, E. L. and Driedzic, W. R. (1995). Antarctic fish tissues preferentially catabolize monoenoic fatty acids. *J. Exp. Zool.* **271**, 73-81.

Sidell, B. D. and Hazel, J. R. (2002). Triacylglycerol lipase activities in tissues of Antarctic fishes. *Polar Biol.* **25**, 517-522.

Somero, G. N. (1995). Proteins and temperature. *Annu. Rev. Physiol.* **57**, 43-68.