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The Antarctic toothfish (*Dissostichus mawsoni*) lacks plasma albumin and utilises high density lipoprotein as its major palmitate binding protein

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Abstract

Plasma from the Antarctic toothfish, *Dissostichus mawsoni*, a member of the advanced teleost Nototheniidae family, was analysed. Agarose gel electrophoresis showed a major diffuse anionic protein that bound [^{14}C]palmitic acid but not $^{63}Ni^{2+}$, and two more cationic proteins that bound $^{63}Ni^{2+}$ but not palmitate. Oil Red O staining following cellulose acetate electrophoresis indicated that the palmitate binding protein was a lipoprotein. Two-dimensional electrophoresis showed that this palmitate binding band was composed of three proteins with M_r of 11, 30, and 42 kDa, without any trace of material at $\sim$65 kDa, the mass of albumin. N-terminal sequencing of the palmitate binding band gave a major sequence of DAAQPSQELR-, indicating a high degree of homology to apolipoprotein A-I (apo-AI), the major apolipoprotein of high density lipoprotein (HDL). N-terminal sequencing of the major nickel binding band produced a sequence with no homology to albumin. When ultracentrifugation was used to isolate the lipoproteins from Antarctic toothfish plasma, the palmitate binding protein was found solely in the lipoprotein fraction. In competitive binding experiments, added human albumin did not prevent palmitate binding to toothfish HDL. In conclusion, there is no evidence for albumin in Antarctic toothfish plasma and HDL assumes the role of fatty acid transport.

Keywords: Albumin; Antarctic toothfish; Apo-AI; Apo-AIV; Fatty acid; High density lipoprotein (HDL); Palmitate; Protein sequence

1. Introduction

The waters around Antarctica are constantly at subzero temperatures and have been since the formation of the continental ice sheet 10–15 mya (million years ago) [29]. The Antarctic Ocean is characterised by little temperature fluctuation, high water viscosity, and increased oxygen solubility. Despite this inhospitable marine environment, over 1% of the world's fish species are found in Antarctic waters [19,20], with more than 50% of Antarctic fish species belonging to the Perciformes suborder Notothenioidei [4]. The Order Perciformes was the most recent teleost order to evolve, making species of the suborder Notothenioidei among the most phyletically derived of fish. The formation of the Antarctic Convergence current resulted in the isolation of the southern ocean 23–30 mya [21]. The fishes of the suborder Notothenioidei are thought to have evolved in situ as the Antarctic continent moved south and the surrounding ocean cooled.

The notothenioids found in the Antarctic Ocean are characterised by the presence of antifreeze glycopeptides (AFGPs), while those species with habitats outside the Convergence current, such as the earliest notothenioids, the Bovichtidae family, do not possess AFGPs [4,20]. The AFGP genes evolved from a pancreatic trypsinogen gene [13], with the multiple isoforms collectively maintained at an extremely high plasma concentration of 30–35 mg ml^{-1} [15,16]. Another notable characteristic of the notothenioids is a decrease in

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blood viscosity due to a lowering of the hematocrit (ratio of red cell volume to total blood volume) compared to other teleost species [57]. The icefishes of the Channichthyidae family have further reduced blood viscosity through a complete absence of haemoglobin [26,50]. Notothenioids also lack a swim bladder [20]. The current hypothesis of notothenioid evolution, using mtDNA divergence rates, is that after the evolution of the Bovichtidae family 10–15 mya, rapid speciation of the other families occurred [4,49]. This recent divergence time is consistent with the divergence time of 5–14 my between the trypsinogen and AFGP gene [13], and the estimated 10–15 mya time frame of the freezing of the Antarctic Ocean [29].

The notothenioid genus *Dissostichus* is composed of just two species; *D. mawsoni* and *D. eleginoides* [3], with the former endemic to the seas around Antarctica and possessing AFGP, and the latter found outside the Antarctic Convergence and lacking AFGP [20]. The Antarctic toothfish, *D. mawsoni*, is a relatively inactive piscivorous epibenthic species that attains a length of 175 cm and a weight of 80 kg or more [20,25]. *D. mawsoni* maintains neutral buoyancy in the absence of a swim bladder by decreasing the extent of bone and scale mineralisation, substituting cartilage for bone, and also by the deposition of large amounts of lipid in the form of triacylglycerols in its muscles [18,20]. Usually teleosts utilise wax esters to maintain neutral buoyancy [39,40,46], and it is possible that the choice of triacylglycerols in *D. mawsoni* may reflect dual use as both buoyancy lipids and as an energy reserve during the winter months [51]. Its hepatocytes also contain numerous lipid droplets, with the liver being unique in containing many perisinusoidal cells specialised for protein synthesis [17]. It is postulated that the product of these cells transports lipids in plasma.

Due to the inhospitable environment *D. mawsoni* lives in and its requirement for large amounts of lipid to maintain neutral buoyancy, lipid and long-chain fatty acid (LCFA) transport is of major importance. Highly insoluble by themselves [54], LCFA are transported by albumin in most vertebrates [48]. Albumin is typically the major anionic protein in vertebrate plasma, and has roles in maintaining blood oncotic pressure as well as acting as a transport molecule for a variety of ligands. Albumin binds long-chain fatty acids freed from lipids through enzymatic action, resulting in the stimulation of lipoprotein lipase activity in adipose tissue [10], as well as stimulating LCAT (lecithin–cholesterol acyltransferase) activity [56].

The presence of albumin in the Salmonidae family has been demonstrated by cDNA sequencing in Atlantic salmon [9], and N-terminal sequencing of albumin proteins from chinook salmon and brown trout [36]. There has been doubt however, as to whether albumin is present in all teleost fish [14]. The plasma lipoproteins HDL (high density lipoprotein), LDL (low density lipoprotein) and VLDL (very low density lipoprotein) also play important roles in lipid transport and metabolism. The HDL apolipoprotein, apo-AI (apolipoprotein A-I), like albumin, functions as an activator of LCAT [24]. HDL is found in low concentrations in mammals (~ 2 mg ml^{-1} in humans), but occurs in higher concentrations in the plasma of teleost fish (2.4–33 mg ml^{-1}) [12]. The wide range of HDL concentrations suggests that it may have varying importance as a fatty acid transporter in teleost species. It is the major plasma protein in carp [30], and HDL concentrations in rainbow trout were found to be very high at ~ 15 mg ml^{-1} [11,53]. The authors recently reported that New Zealand eels lack albumin in their plasma, and that HDL acts to transport LCFA in these species [37]. As in higher vertebrates, apo-AI and apo-AII (apolipoprotein A-II) are the major HDL apolipoproteins in teleost fish [2,11,37,53].

Thus, while albumin is the major LCFA transporter in salmonids, at least two eel species lack albumin and use HDL to transport LCFA. This raises the question, how do other teleost families transport LCFA? The Antarctic toothfish is a member of the most phyletically derived teleost order, the Perciformes, and like other Antarctic notothenioid species it has extremely high blood AFGP concentrations, has a high demand on lipid transport and metabolism, and possesses unusually large lipid muscle stores to maintain neutral buoyancy. In this study the authors report that the major acidic protein in *D. mawsoni* plasma binds palmitic acid with high affinity and that this protein is HDL and not albumin.

2. Materials and methods

Freeze-dried plasma from the Antarctic toothfish, *D. mawsoni*, was reconstituted to its original volume with distilled water and was stored at -80°C until required.

Agarose gel electrophoresis was performed in 1% agarose in 38 mM Tris, 46 mM Na-barbitone, 16 mM diethylbarbituric acid (pH 8.6) for 40 min [6], and ^{63}Ni binding and autoradiography was performed as previously described [6,7]. Binding of [^{14}C]palmitic acid and autoradiography was carried out at room temperature as in [36].

Reducing SDS-PAGE (sodium dodecyl sulfate-polyacrylamide electrophoresis) at pH 8.8 was performed in 12% polyacrylamide gels with a 3% stacking gel as

per the method of Laemmli [31]. Samples were incubated in SDS reducing buffer; 4% SDS, 10% 2-mercaptoethanol, 125 mM Tris, 40% glycerol, 0.002% bromophenol blue at 100°C for 5 min before electrophoresis. Gels were stained with 0.1% Coomassie Brilliant Blue R.

Two-dimensional electrophoresis was performed as in [36,37]. Plasma samples (2.6 μl) were electrophoresed as above on agarose gels, half of each lane was cut out and reduced with SDS reducing buffer for 15 min at room temperature. This gel strip was then placed on top of a 1.5 mm SDS-PAGE gel (12%) with 5 mm of stacking gel (3%) and electrophoresed and stained as above.

Cellulose acetate electrophoresis was performed on plasma samples as described in [32] and [37]. Plates (76×90 mm) were wetted in 38 mM Tris, 46 mM Na-barbitone, 16 mM diethylbarbituric acid (pH 8.6) buffer before sample application. Electrophoresis was performed in this same buffer at 170 V for 15–30 min. Plates were either stained in: (a) 0.1% amido black in 10% acetic acid in 50% methanol for 10 min, followed by destaining in 10% acetic acid in 50% methanol; or (b) 30 ml 1.4% (w/v) Oil Red O in methanol/7 ml 1 M NaOH for 2 h, followed by gentle rinsing and wiping using distilled water.

Competitive palmitate-binding experiments were carried out at room temperature as follows. A mixture was made of Antarctic toothfish plasma (~ 5 μg HDL protein) and ~ 5 μg freeze-dried human albumin (purified in the laboratory by ion exchange chromatography). This was added to 1.25 μl of [^{14}C]palmitic acid diluted to 5 μCi ml^{-1}, mixed, and then applied to an agarose gel, before electrophoresis and autoradiography as in [36].

Ultracentrifugation was performed using a variation [37] of the methods described for the isolation of rainbow trout lipoproteins [11,53]. Reconstituted Antarctic toothfish plasma (2 ml) and fresh human plasma (2 ml) were diluted to 5 ml with 1.015 g ml^{-1} NaCl, and the density of the solution adjusted to 1.215 g ml^{-1} with the addition of 1.6 g KBr. Samples (5 ml) were transferred to 6.5 ml ultracentrifuge tubes and spun in a Beckman TYP TFT 45.6 rotor in a Beckman L8-70M Ultracentrifuge at 37 000 rpm ($175\,000 \times g$) for 17 h at 15°C. Remaining material was analysed for total protein (g l^{-1}), and cholesterol (mmol l^{-1}) on a Hitachi Boehringer Mannheim 717 Automatic Analyser. After ultracentrifugation tubes were cut into two fractions; a top fraction of 1.5 ml, and a bottom fraction of 3.5 ml. Each Antarctic toothfish fraction was re-diluted to 5 ml with 1.215 g ml^{-1} KBr density solution and 100 μl of each was analysed as above for total protein and cholesterol. Fractions were then recentrifuged, and re-cut as before. The top fraction contained the purified lipoproteins, while the bottom fraction (infranatant) contained the plasma proteins. The second ultracentrifugation step was not performed on the human plasma control. Fractions were dialysed exhaustively at 4°C against 0.02% sodium-azide.

Antarctic toothfish protein samples were transferred to ProBlott membrane directly from agarose gels, with N-terminal sequence analysis performed by the Protein Microchemistry Facility, University of Otago, using established methods [28].

3. Results

Reconstituted plasma from the Antarctic toothfish, *D. mawsoni*, was analysed using agarose gel electrophoresis (pH 8.6). Albumin is usually the predominant anionic plasma band under these conditions, as is the case with human plasma (Fig. 1A, lane 1). While plasma from *D. mawsoni* also had a major anionic band

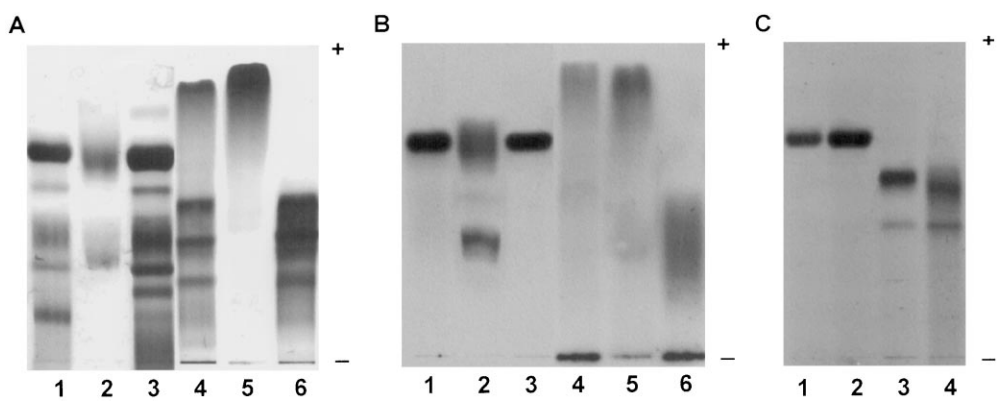

Fig. 1. Agarose gel electrophoresis (pH 8.6) showing (A) Coomassie-blue stained gel, (B) [^{14}C]palmitic acid autoradiograph, and (C) $^{63}Ni^{2+}$ autoradiograph of plasma samples and ultracentrifugation fractions. (A) and (B): lane 1, 3 μl of a 1 in 10 dilution of human plasma; lane 2, 4 μl human lipoprotein fraction; lane 3, 6 μl human infranatant fraction; lane 4, 1.3 μl (A) and 3 μl (B) *D. mawsoni* plasma; lane 5, 6 μl (A) and 9 μl (B) *D. mawsoni* lipoprotein fraction; lane 6, 10 μl (A) and 6 μl (B) *D. mawsoni* infranatant fraction. (C). Lane 1, 3 μl of a 1 in 10 dilution of human plasma; lane 2, 2 μl of human infranatant fraction; lane 3, 1.6 μl *D. mawsoni* plasma; lane 4, 6 μl *D. mawsoni* infranatant fraction.

(Fig. 1A, lane 4), this was considerably more acidic than human albumin, and other characterised albumins [36,44]. Unlike albumin, this acidic protein was diffuse, and showed variable mobility. On agarose gel electrophoresis lipoproteins appear as broad diffuse bands, suggesting that as for New Zealand eels [37] the major acidic protein in *D. mawsoni* plasma may be a lipoprotein. Amido black and Oil Red O staining following cellulose acetate electrophoreses (pH 8.6) confirmed that the major acidic toothfish protein was a lipoprotein (data not shown).

The ability of a protein to bind palmitate has been used as a principal means of identifying albumin. The major acidic protein in *D. mawsoni* plasma bound [^{14}C]palmitic acid with high affinity (Fig. 1B, lane 4), as did albumin in human plasma (Fig. 1B, lane 1). In each species palmitate bound only to a single plasma protein.

Ultracentrifugation was employed to isolate lipoproteins from human and *D. mawsoni* plasma, and the resulting fractions were analysed for cholesterol and protein concentration. In both cases most (99%) of the cholesterol was found in the lipoprotein fraction, with the majority of the protein associated with the infranatant (data not shown). Agarose gel electrophoresis of the human plasma ultracentrifugation fractions showed that as expected, they contained both alpha (HDL) and beta (LDL/VLDL) lipoproteins (Fig. 1A, lane 2) which in the absence of albumin both bound [^{14}C]palmitic acid (Fig. 1B, lane 2). The infranatant fraction contained the remainder of the plasma proteins, including albumin (Fig. 1A, lane 3), which was the only protein to bind palmitate in the infranatant (Fig. 1B, lane 3). The major band in the lipoprotein fraction of Antarctic toothfish plasma corresponded to the most acidic plasma protein (Fig. 1A, lane 5) and bound palmitate (Fig. 1B, lane 5), establishing it tentatively as HDL. Small amounts of protein more cationic than HDL were observed in the lipoprotein fraction, and may correspond to beta lipoproteins (Fig. 1A, lane 5). The infranatant fraction of Antarctic toothfish plasma shows diffuse palmitate binding spanning several plasma proteins (Fig. 1B, lane 6). When free [^{14}C]palmitic acid was electrophoresed under identical conditions it migrated towards the anode in a very diffuse, erratic manner (data not shown) unlike the binding shown in Fig. 1B, lane 6. Binding in the infranatant may therefore be non-specific or low affinity binding to plasma proteins in the absence of a specific high affinity binding protein such as HDL or albumin.

$^{63}Ni^{2+}$ binding is usually associated with mammalian albumins [45] (Fig. 1C, lane 1), but not fish albumins [36]. As expected the palmitate binding lipoprotein in Antarctic toothfish plasma failed to bind nickel. However, two more cathodal proteins bound $^{63}Ni^{2+}$; one with high affinity(Fig. 1C, lane 3). Like human albumin

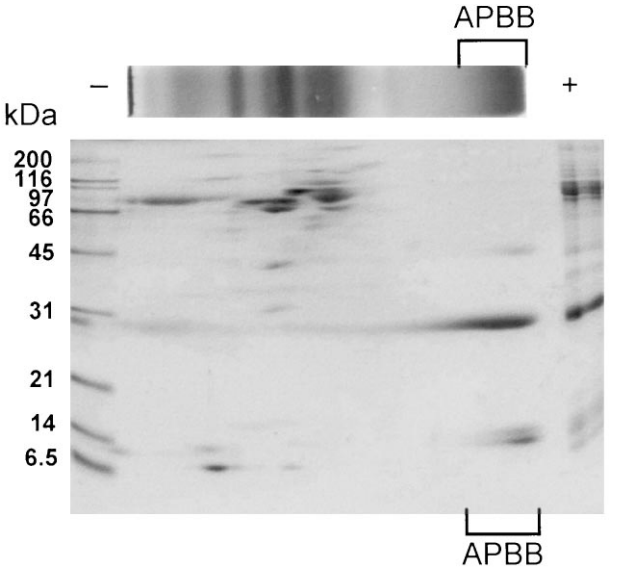

Fig. 2. Two-dimensional electrophoresis of Antarctic toothfish plasma. First dimension, 1% agarose gel electrophoresis; second dimension, 12% SDS-PAGE. The first dimension agarose gel profile is shown aligned along the top, with the position of the acidic palmitate binding band (APBB) indicated. Left lane, molecular mass markers; right lane, 0.4 μl Antarctic toothfish plasma.

(Fig. 1C, lanes 1 and 2) these nickel binding proteins segregated with the infranatant fraction in Antarctic toothfish (Fig. 1C, lane 4).

Albumin typically has a mass of $\approx$65 kDa, and this characteristic M_r is often used to identify albumins. When two-dimensional electrophoresis was used to separate the components of Antarctic toothfish plasma the palmitate binding band was seen to be composed of at least three proteins, but as expected devoid of protein at the mass of albumin, 65 kDa (Fig. 2). The major protein component was at 30 kDa, with other minor proteins at $\approx$9, 11, and 42 kDa. This confirmed that HDL is the palmitate binding band in *D. mawsoni* plasma, as the major apolipoprotein in human HDL is apo-AI with a mass of 28 kDa [12].

The toothfish palmitate binding band was isolated from plasma by transfer to ProBlott from agarose gels, and subsequent N-terminal sequence analysis produced a major protein sequence of DAAQP(S)QEL(R)-, and a minor sequence of EVPSQ(L)(Q)HI(V)-. Comparison of the major sequence with apo-AI N-terminal sequences from a range of species including human and eels established its identity as apo-AI (Fig. 3A). The minor sequence exhibited homology to N-terminal apo-AIV protein sequences from mammalian species (Fig. 3B). These sequences identify the palmitate binding protein in the toothfish as HDL. The N-terminal sequence of the major nickel binding band, ISLGLVNT- showed no homology to albumin from other species.

In competitive binding experiments, [^{14}C]palmitic acid was added to a mixture of toothfish plasma and

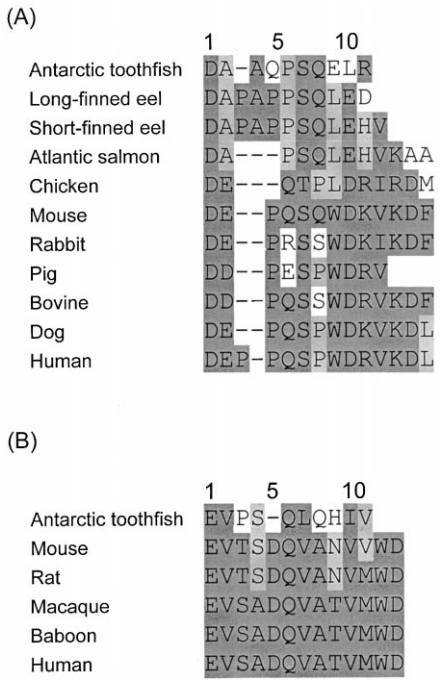

Fig. 3. Mature N-terminal sequences for Antarctic toothfish apo-AI (A) and apo-AIV (B) aligned with mature N-terminal sequences from other species [5,8,22,27,35,37,38,41–43,47,52,55,58]. A dash indicates a position where a gap has been introduced in the sequence to maximise sequence identity between species; identical and chemically conserved residues are highlighted.

purified human albumin, and the distribution of palmitate assessed by agarose gel electrophoresis and autoradiography. The presence of human albumin, which has a high affinity for palmitate, did not interfere with the binding of palmitate to HDL (Fig. 4, lanes 1–4). Indeed palmitate appeared to preferentially bind to the HDL.

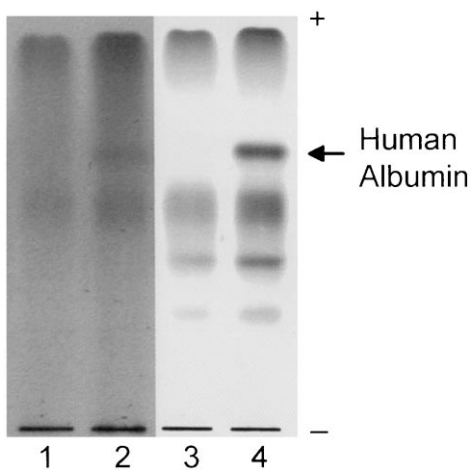

Fig. 4. Competitive binding of palmitate between Antarctic toothfish HDL and human albumin. Lanes 1–2, autoradiograph, 12 days film exposure; lanes 3–4, Coomassie stain. Lanes 1 and 3, 5 μl Antarctic toothfish plasma; lanes 2 and 4, 5 μl Antarctic toothfish plasma plus 0.75 μl human albumin at 5 mg ml^{-1}. The position of the human albumin band is indicated with an arrow.

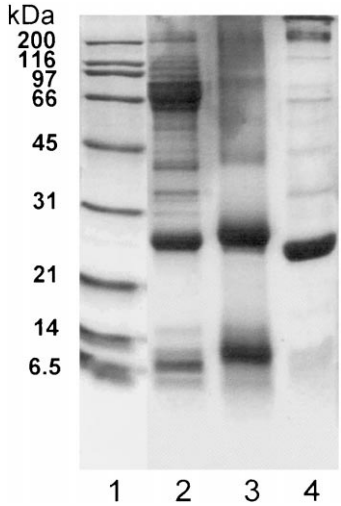

Fig. 5. Twelve percent SDS-PAGE of ultracentrifuge fractions. Lane 1, molecular mass markers; lane 2, 0.4 μl Antarctic toothfish plasma; lane 3, 0.8 μl Antarctic toothfish lipoprotein fraction; lane 4, 6 μl human lipoprotein fraction.

The apolipoprotein components of Antarctic toothfish HDL were examined by reducing SDS-PAGE (12%). The major apolipoprotein had a molecular mass of 30 kDa (Fig. 5, lane 3), and this protein is presumably apo-AI (28 kDa in humans (Fig. 5, lane 4) [12]). An 11 kDa apolipoprotein, presumably apo-AII, was present at an $\approx$1:1 ratio with apo-AI; in humans the apo-AII:apo-AI ratio is 1:20, while in eels it is $\approx$1:5 [37]. A third apolipoprotein was present at $\approx$42 kDa, and this is probably apo-AIV, which has an M_r of 46 kDa in rat HDL [12]. The Antarctic toothfish lipoprotein fraction also contained a small amount of material at $\approx$55, 80, and $>$200 kDa. The latter material may correspond to apo-B, which is the major apolipoprotein of human LDL/VLDL and has an M_r of $>$250 kDa.

4. Discussion

The major acidic plasma protein in Antarctic toothfish bound palmitate (Fig. 1A and B), and Oil Red O staining, ultracentrifugation, and cholesterol analysis showed that it was a lipoprotein. N-terminal sequencing established that two of the components of this band were the apolipoproteins apo-AI and apo-AIV, identifying it as HDL (Fig. 3). LDL and VLDL were at low or negligible levels in *D. mawsoni* plasma, which is similar to the situation in New Zealand eels, rainbow trout, and carp where HDL is also present at high levels, and LDL/VLDL are at very low or non-de-

tectable levels [11,30,37,53]. While a major nickel binding protein was present in Antarctic toothfish plasma, it was not albumin, as determined by its N-terminal protein sequence, and lack of palmitate binding. Albumin appears to be absent from the plasma of *D. mawsoni*, as is also the case with New Zealand eels and probably carp [1,37].

The high negative charge on toothfish HDL is unusual among vertebrate species. HDL runs just cathodal to albumin in human and other mammalian plasmas, and at a position equivalent to that of human albumin in New Zealand eels [37]. This indicates then that Antarctic toothfish HDL may differ considerably in its structure or composition as compared with HDL from other vertebrates. Alternatively, the high negative charge may be a spurious result of using reconstituted lyophilised plasma.

The major apolipoprotein in *D. mawsoni* plasma was apo-AI (Fig. 5) with a molecular weight of 30 kDa. The other major apolipoprotein with a mass of 11 kDa is presumably apo-AII (Fig. 5), and a minor apolipoprotein with a mass of 42 kDa is likely to correspond to apo-AIV (Fig. 5). Both rainbow trout and eel HDL have similar apolipoprotein compositions [11,37,53]. The molecular masses of all three apolipoproteins in *D. mawsoni* HDL show an apparent post-ultracentrifugation increase (Fig. 5, lanes 2 and 3). This probably reflects the irreversible structural and compositional changes in HDL that are known to be induced by repeated ultracentrifugation [12]. In plasma the apo-AII:apo-AI ratio is $\approx$1:5, whilst in the lipoprotein fraction it is $\approx$1:1 (Fig. 5, lanes 2 and 3). The relative amount of apo-AIV decreases following ultracentrifugation (Fig. 5, lanes 2 and 3). In eel HDL the apo-AII:apo-AI ratio was 1:5 [37], while both a 1:5 and a 1:1 ratio have been observed following bioaffinity of carp plasma [1,2]. Loss of apo-AI is known to occur during ultracentrifugation of human HDL [23], but this does not explain the apo-AIV decrease, unless it has also been lost during ultracentrifugation.

The major protein sequence from *D. mawsoni* HDL shows homology to apo-AI from other species, and in particular, other teleosts (Fig. 3). The Ala at residue 2, and Pro at residue 6 are unique to teleosts. The Antarctic toothfish is a more recently evolved teleost than both eels and salmonids. The Pro–Ala–Pro sequence insertion from residues 3–5 in the primitive eels is absent in the more recently evolved salmonids, although the Antarctic toothfish have an Ala at residue 4 and a Gln at residue 5, which could result from a point mutation of Pro. The Ser at residue 7 is conserved in all species except chicken, while the Gln at residue 8 appears to be restricted to lower vertebrates and mouse. A charged residue, Arg is found at residue 11 in *D. mawsoni*, and although dissimilar to the Asp or His found in other teleosts, is similar to the Arg or Lys found in higher vertebrates. It appears as if two point mutations have occurred at residues 9 (Glu) and 10 (Leu) in *D. mawsoni*, as the residues in other teleosts are Leu–Glu.

The minor HDL protein sequence from Antarctic toothfish shows homology to mammalian apo-AIV sequences, with differences at 3 residues (3, Pro/Thr/Ser; 8, Gln/Ala; 9, His/Asn) all explainable by point mutations. This is the first apo-AIV sequence determined for a non-mammalian species. Although more apo-AII than apo-AIV is present in *D. mawsoni* HDL, apo-AII in several species including turkey, rat and human has a blocked N-terminus [12], probably explaining why a N-terminal apo-AII sequence was not detected in Antarctic toothfish HDL.

Palmitate appears to bind preferentially to Antarctic toothfish HDL over added albumin. It appears that rather than just binding LCFAs in the absence of albumin, HDL in *D. mawsoni* has had its high affinity selected for during evolution. Like New Zealand eels, the situation in *D. mawsoni* is analogous to analbuminaemia in humans. People suffering from analbuminaemia have circulating levels of albumin of <1 mg ml^{-1} instead of the normal range of 35–45 mg ml^{-1}. A greatly increased beta lipoprotein concentration in these individuals results in altered lipid and fatty acid metabolism [45], while Antarctic toothfish and eels possess low levels of beta lipoproteins but high HDL levels [37]. In these fish species and analbuminaemic humans, lipoprotein levels are increased, maintaining LCFA transport.

It appears that albumin expression has been lost in at least three teleost species (Antarctic toothfish, two New Zealand eel species, and probably carp). Eels were among the first teleosts to evolve, followed by the orders containing salmonids and carps, with the Antarctic notothenioid suborder being the most recent teleost group. The holostean origin of teleosts is now thought to be polyphyletic, meaning the ancestral eel arose from holosteans independently, and separate from the origin of other teleost orders. Salmonids possess albumin indicating that loss of albumin and the adoption of HDL as the LCFA transport protein may have happened at least two and possibly three times during teleost evolution; in the eel line after divergence from the rest of teleosts, in the carp line, and possibly again in the notothenioid line. Lack of albumin expression in these teleosts may be the result of gene deletion, mutational inactivation, or permanent down-regulation.

Alternatively if all teleosts are derived from ancestral eels, a more parsimonious explanation would be that loss of albumin expression occurred once in the ancestral eel. Thus, the absence of albumin in carp and notothenioids would be due to a phyletic effect, rather than a specific adaptation. Albumin expression in salmonids could then be explained by mutational reac-

tivation of the gene or gene up-regulation sometime after the divergence of salmonids from other teleosts.

It has been proposed that loss of albumin in eels and the adoption of HDL in fatty acid transport may be an adaptation to a predominantly freshwater life [37]. There must be different evolutionary reasons governing the loss of albumin and use of HDL as the fatty acid transporter in *D. mawsoni* when compared with eels. Loss of gene expression can occur in a short evolutionary period if environmental pressure is significant, and the notothenioid suborder shows examples of this. The AFGP family is thought to have evolved in situ in Antarctic notothenioids as the continent moved south and freezing of the ocean occurred. Notothenioid species which lack AFGPs are those of the earliest family, the Bovichtidae, as well as three species including *D. eleginoides* [20]. All these species have habitats outside the subzero waters, and in the latter three species the AFGP absence is a secondary more recent loss. The Antarctic notothenioids have decreased the number of haemoglobin forms in the last 15 my, again through gene deletion. One family, the icefishes, have lost haemoglobin entirely as a method of reducing blood viscosity to combat the subzero water temperatures [57]. The kinematic viscosity of seawater doubles as the temperature decreases from 20 to 2°C [34]. The loss of albumin in *D. mawsoni* may be a means of reducing blood viscosity, and thus will have occurred in this species in the last 5–15 my. Albumin is typically found in high concentrations in plasma, ≈ 40 mg ml^{-1} in mammalian species and 10–15 mg ml^{-1} in teleosts [45]. Antarctic notothenioid blood with AFGPs at high plasma concentrations of 30–35 mg ml^{-1} in plasma will already be viscous without the addition of albumin. In *D. mawsoni*, the HDL is not present at a level comparable with that of albumin in other teleosts, thereby reducing viscosity. This may enable this and potentially also other notothenioid species to survive better at low temperatures.

At subzero temperatures the metabolism of lipids and the transport of fatty acids are important processes in Antarctic fish. Large muscular lipid depositions are also essential in maintaining neutral buoyancy in *D. mawsoni*, and are possibly used as an energy store in winter [18,20,51]. Lipids are stored in the form of triacylglycerols [18], with the major fatty acids being long chain (palmitic and oleic acid). In addition, intracellular lipid droplets in the liver and muscle may serve as an oxygen store enhancing oxygen diffusion. As cold environmental temperatures affect metabolism, this oxygen store may enhance rates of ATP production and metabolite diffusion [20]. The use of HDL as the LCFA transporter of choice rather than albumin in *D. mawsoni* may reflect an enhanced ability to transport fatty acids at low temperatures, thus ensuring that neutral buoyancy, energy stores, and metabolism are all maintained. It is known that several Antarctic fish enzymes are more efficient at low temperatures than their temperate counterparts [33]. The binding of LCFA to HDL is possibly more efficient at low temperatures than the binding of LCFA to albumin.

Regardless of the reasons for this unusual adaptation, it is worthwhile investigating other notothenioid fish, as well as other teleosts to see whether use of HDL to transport LCFA and the loss of albumin are widespread among other species. In particular, the sister species to *D. mawsoni*, *D. eleginoides*, which was originally thought to have been cold-adapted but now lives outside the subzero water temperature zone, and has lost AFGP, warrants study.

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