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Differential expression of adipose- and heart-type fatty acid binding proteins[1] in hibernating ground squirrels

Dustin Hittel, Kenneth B. Storey *

Institute of Biochemistry and Department of Biology, Carleton University, 1125 Colonel By Drive, Ottawa, ON, Canada K1S 5B6

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Abstract

The up-regulation of heart- and adipose-type fatty acid binding proteins (H-FABPs and A-FABPs) was detected during hibernation in brown adipose tissue (BAT) of 13-lined ground squirrels, *Spermophilus tridecemlineatus*, using a commercial rat cDNA array. Full length cDNAs encoding H-FABPs and A-FABPs were subsequently retrieved from a BAT cDNA library. These cDNAs were used to probe Northern blots of total RNA from tissues of euthermic versus hibernating ground squirrels. H-FABP mRNA transcripts increased in BAT, skeletal muscle and heart of hibernating animals whereas A-FABP transcripts, which are normally expressed exclusively in adipose tissue, increased in both BAT and heart during torpor. It is proposed that the increased expression of H-FABPs and A-FABPs during hibernation accelerates the rate at which fatty acids can be transported to the mitochondria for oxidization, particularly in support of the huge increase in thermogenesis by BAT and rapid increase in heart rate that are required during arousal from torpor. Comparison of the deduced polypeptide sequence of ground squirrel H-FABP with that from other mammals also revealed three unique amino acid differences which may be important for protein function at low body temperatures during hibernation.

Keywords: Fatty acid binding protein; cDNA array; Hibernation; Brown adipose tissue; *Spermophilus tridecemlineatus*

Hibernation is a state of profoundly depressed physiological and metabolic activity that is used by many small mammals to survive long, cold winters [1–3]. By hibernating, animals reduce the 'burn rate' of stored fat reserves that would otherwise be used to maintain the steep thermal gradient between their body temperature (T_b) and that of the environment. By spending prolonged periods in torpor with only brief periodic arousals over the winter, small mammals can gain net energy savings of ~90% compared with the cost of remaining euthermic all winter [1,2].

Whereas the organs of most mammals sustain significant metabolic injuries at low T_b (hypothermia) and/or under situations of reduced blood flow (ischemia), hibernators routinely lower T_b, heart rate and breathing to a fraction of the corresponding euthermic rates and maintain a stable, dormant state with no signs of metabolic decay [1,2]. The key to this is a regulated suppression of the rates of all metabolic processes, virtually shutting off many processes and coordinating those that remain so that rates of ATP-producing versus ATP-synthesizing processes remain balanced [4–7]. Hibernation is also supported by selected changes in gene expression that alter the amount or isoform of particular protein products [1]. To date, several examples of increased gene expression during hibernation have been reported including α-2-macroglobulin in the liver [8], pyruvate dehydrogenase kinase isozyme 4 and pancreatic lipase in the heart [9] and myosin light chain 1 and NADH-ubiquinone oxidoreductase subunit 2 in the heart and skeletal muscle [10].

In an attempt to gain an overview of the key changes in gene expression that support entry into hibernation, we turned to DNA array technology. Atlas rat cDNA expression arrays (Clontech, Palo Alto, CA, USA) were chosen; these feature 588 cDNAs representing proteins from a range of key biological processes including stress responses and signal transduction [11]. We chose to analyze changes in gene expression in brown adipose tissue (BAT) of euthermic versus hibernating 13-lined ground squirrels, *Spermophilus tridecemlineatus*. BAT was chosen because of its

Abbreviations: FABP, fatty acid binding protein; BAT, brown adipose tissue; WAT, white adipose tissue; T_b, body temperature

* Corresponding author. Fax: +1-613-520-2569.

E-mail address: kenneth_storey@carleton.ca (K.B. Storey).

[1] The sequences reported in this paper for H-FABP and A-FABP have been deposited in the GenBank database under the accession numbers AF327854 and AF327855, respectively.

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critical role in hibernators in generating much of the heat that is used to warm the animal during arousal [2,12–14].

Ground squirrels (*S. tridecemlineatus*) weighing 120–180 g were captured in Illinois during August by a US Department of Agriculture-licensed trapper (TLC Research, Batlett, IL, USA) and shipped by air to the animal housing facility of the National Institutes of Health (Bethesda, MD, USA). Animals were held in individual shoe-box cages under environmental conditions of 22–24°C, 60% relative humidity and 12/12 h light/dark cycle with free access to food and water. Squirrels were weighed weekly to determine their phase in the annual life cycle, and when a rapid increase in body weight had been achieved (signaling a readiness for hibernation), some squirrels were moved to a chamber at 5–6°C (60% humidity, continuous darkness) to facilitate hibernation. Others were maintained as before and then sacrificed as euthermic controls. In the cold chamber most squirrels entered deep torpor within 3–8 d and they were then sampled while torpid at times ranging from 1–7 d of torpor. All animals were sacrificed as described previously with organ samples rapidly excised and flash frozen in liquid nitrogen [7,15]. Samples were transported to Ottawa on dry ice and stored at −80°C until use.

Atlas rat cDNA expression arrays were purchased from Clontech. ^{32}P-Labeled cDNA probes were synthesized with mRNA purified from BAT of euthermic versus hibernating squirrels as per manufacturer's instructions. Arrays were washed with a low stringency solution (2×SSC, 1% SDS) and, if needed, a high stringency solution (0.1×SSC, 0.5% SDS) at 68°C until counts were reduced to 1000–5000 cpm as measured with a hand-held counter. Wet arrays were immediately covered in plastic wrap (to prevent drying) and exposed to a phosphorimaging (Bio-Rad Laboratories) screen for 2–10 h to optimize exposure time. Array images generated from phosphorimager analysis were analyzed with the EST program [11] followed by comparison of relative levels in control versus stressed states.

Although the use of a rat array to analyze mRNA transcript levels in another species may have some drawbacks, for genes that have high nucleotide sequence identity the rat arrays can provide key initial evidence of the differential expression of genes in control versus stressed states. Indeed, we found that cross-species reactivity was high and ground squirrel probes cross-reacted with at least 90% of the cDNAs present on the array. Differential screening revealed two genes that were prominently up-regulated in BAT of hibernating ground squirrels, when compared with euthermic controls. These were the heart (H) and adipose (A) types of fatty acid binding protein (FABP; also known as FABP3 and FABP4, respectively; [16]) and their up-regulation during hibernation was subsequently confirmed via Northern blotting. Overall, transcript levels of most of the 588 genes represented on the array did not change or significantly decreased during hibernation.

To obtain full length cDNAs, a BAT cDNA library constructed from BAT poly(A)$^+$ RNA isolated from three hibernating individuals, was screened using a polymerase chain reaction (PCR) product with the partial sequence of squirrel H-FABP cDNA and a plasmid containing rat A-FABP cDNA (IMAGE ID# 1771436) [17]. Several candidate clones for each FABP isoform were obtained and then subjected to a second round of screening. Cross-hybridization of the excised clones revealed four that reacted with the H-FABP probe and three with the A-FABP probe. There was no cross-hybridization between the putative H-FABP and A-FABP clones. Isolated clones were sequenced (CMRS, Ottawa, ON, Canada) then loaded into the BLAST program at the NCBI (Bethesda, MD, USA) for a similarity search in GenBank. Processed sequences were submitted to GenBank and accession numbers are given.

Clones pHFAB41 (GenBank accession number AF327854) and pAFAB83 (GenBank accession number AF327855) both contained full length open reading frames for H-FABP and A-FABP, respectively, with start and stop codons, polyadenylation signals and poly(A) tails. The processed cDNA sequences of the two clones were only 47% identical which may explain their lack of cross-hybridization. The open reading frame of pHFAB41 extended from 54 to 452 bp and encoded a 133 amino acid protein that was identified from a GenBank search as mammalian H-FABP. The deduced amino acid sequence of ground squirrel H-FABP shared 91, 90.3 and 89.5% amino acid sequence identity with rat, human and pig H-FABP, respectively (Fig. 1A). The predicted molecular mass of ground squirrel H-FABP was 14 818 Da. The open reading frame of pAFAB83 extended from 47 to 442 bp and encoded a 132 amino acid protein with a predicted molecular mass of 14 730 Da. A search in GenBank identified the cDNA as encoding A-FABP. The translated amino acid sequence of ground squirrel A-FABP shared 96.9% amino acid identity with human FABP, and 88.6% with both rat and pig proteins (Fig. 1B). The deduced polypeptide sequences of ground squirrel A-FABP and H-FABP were 64.6% identical.

Fig. 2 shows a Northern blot analysis of the tissue distribution and relative transcript levels of H-FABP and A-FABP in euthermic versus hibernating squirrels. Of seven tissues tested, H-FABP mRNA was detected in BAT, heart, skeletal muscle and white adipose tissue (WAT) (Fig. 2A). During hibernation, H-FABP transcript levels rose in three of these: by 3.1-fold in BAT, 2.3-fold in skeletal muscle and 1.4-fold in heart (Fig. 3A). A-FABP mRNA was detected only in BAT and WAT of euthermic animals and transcript levels rose only in BAT during hibernation (by 2.3-fold) (Fig. 3B). However, A-FABP, which was undetectable in euthermic heart, was detected

A. H-FABP

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Squirrel    MVDAFVGTWKLVDSKNFDDYMKSLGVGFATRQVASMTKPTTIIEKNGDTIILKTQSTFKNTEISFQLGKE 70
Human       MVDAFLGTWKLVDSKNFDDYMKSLGVGFATRQVASMTKPTTIIEKNGDILTLKTHSTFKNTEISFKLGVE 70
Rat         MADAFVGTWKLVDSKNFDDYMKSLGVGFATRQVASMTKPTTIIEKNGDTITIKTHSTFKNTEISFQLGVE 70
Pig         MVDAFAGTWKLVDSKNFDDYMKSIGVGFATRQVANMTKPTTIIEVNGDTIIIKTQSTFKSTEISFKLGVE 70

Squirrel    FDETTADDRKVKSTVTLDGGKLVHVQKWDGQETTLVRELNDGKLIL.TLTHGSVVCTRTYEKEA      133
Human       FDETTADDRKVKSIVTLDGGKLVHLQKWDGQETTLVRELIDGKLIL.TLTHGTAVCTRTYEKEA      133
Rat         FDEVTADDRKVKSVVTLDGGKLVHVQKWDGQETTLIRELSDGKLILLTLTHGNVVSTRTYEKEA      134
Pig         FDETTADDRKVKSIVTLDGGKLVHLQKWNGQETTLVRELVDGKLIL.TLTHGSAVCTRTYEKEA      133
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B. A-FABP

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Squirrel    MCDAFVGTWKLVSSENFDDYMKEVGVGFATRKVAGMAKPNMIISVNGDVITIKSESTFKNTEISFILGQE 70
Human       MCDAFVGTWKLVSSENFDDYMKEVGVGFATRKVAGMAKPNMIISVNGDVITIRSESTFKNTEISFKLGQE 70
Rat         MCDAFVGTWKLVSSENFDDYMKEVGVGFATRKVAGMAKPNLIISVEGDLVVIRSESTFKNTEISFKLGVE 70
Pig         MCDAFVGTWKLVSSENFDDYMKEVGVGFATRKVAGMAKPNLIITVNGDMITIRSESTFKNTEIAFKLGQE 70

Squirrel    FDEVTADDRKVKSTITLDGGVLVHVQKWDGKSTTIKRKREDDKLVVECVMKGVTSTRVYERA        132
Human       FDEVTADDRKVKSIITLDGGVLVQVQKWDGKSTTIKRKREDDKLVVECVMKGVTSTRVYERA        132
Rat         FDEITPDDRKVKSIITLDGGVLVHVQKWDGKSTTIKKRRDGDKLVVECVMKGVTSTRVYERA        132
Pig         FDEVTADDRKVKSTITLDGGALVQVQKWDGKTTTINRKIVDDKLVVECIMKGVTATRIYERA        132
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Fig. 1. Comparison of the deduced amino acid sequence of 13-lined ground squirrel H-FABP (A) and A-FABP (B) with the proteins from human, rat and pig. Shading shows those amino acids that differ from the ground squirrel sequence; unique amino acid substitutions in the ground squirrel sequences are underlined. GenBank accession numbers are 4096097, 6601400 and 2143386 for human, rat and pig H-FABP and 2144859, 5002228 and 4160392 for human, rat and pig A-FABP. GenBank accession numbers are AF327854 for H-FABP and AF327855 for A-FABP.

in heart of hibernating animals (Fig. 2B,D) with an estimated 6.3-fold rise during hibernation (Fig. 3B).

The use of cDNA array screening technology proved to be an effective way of searching for genes that were up-regulated by hibernation in ground squirrel BAT. A comparison with probe made from hibernating versus euthermic animals indicated that transcript levels of most genes did not change during hibernation but a small number ($<1\%$) were up-regulated in the torpid state. This suggests that the transition to the hibernating state with its low metabolic rate and low T_b requires the up-regulation of very few genes. Other studies have also turned up only a few hibernation responsive genes. For example, screening of a cDNA library made from *Spermophilus lateralis* heart revealed only two genes that were up-regulated during hibernation (myosin light chain 1 and subunit 2 of NADH-ubiquinone oxidoreductase) [10] whereas two others were found using a PCR-based subtractive screen with *S. tridecemlineatus* heart (pyruvate dehydrogenase kinase and pancreatic lipase) [9]. Limited gene up-regulation during hibernation is perhaps not surprising since, intuitively, the torpid state at low T_b is not the time for major biosynthesis of proteins. However, changes in the expression of a few specific genes is clearly important and each up-regulated gene confirmed to date has a defined role in supporting hibernation. The two FABPs identified in the present study also fit this pattern.

FABPs constitute a multi-gene family of small ($\sim$15

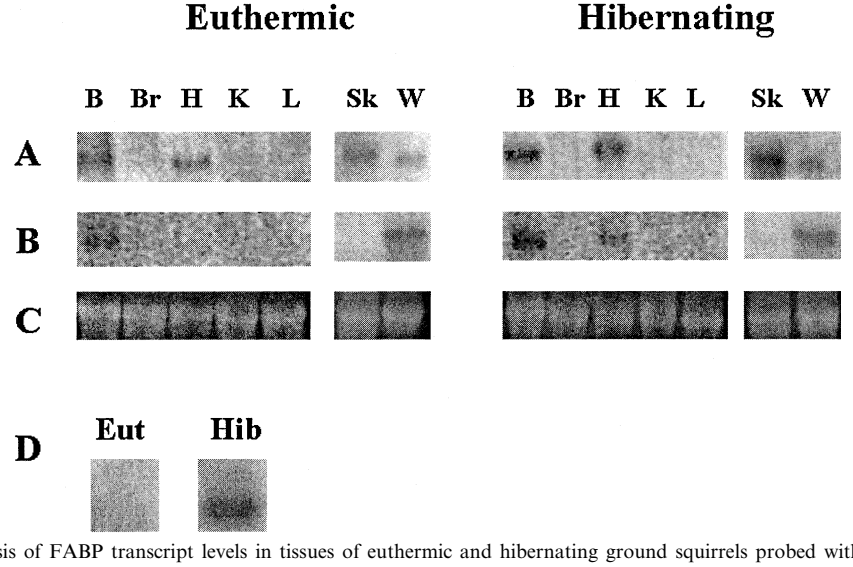

Fig. 2. Northern blot analysis of FABP transcript levels in tissues of euthermic and hibernating ground squirrels probed with ^{32}P-labeled cDNA for (A) H-FABP or (B) A-FABP. The 28S rRNA band of ethidium bromide stained nitrocellulose blots (C) demonstrates equal loading and high quality RNA in each lane. After hybridizing and washing, blots were exposed to imaging screens for 2 h. Hybridizing bands of about 850 nt (H-FABP) and 750 nt (A-FABP) are shown for BAT (B), brain (Br), heart (H), kidney (K), liver (L), skeletal muscle (Sk) and WAT (W). An additional Northern blot (D) is shown to further demonstrate the presence of A-FABP in hibernating heart.

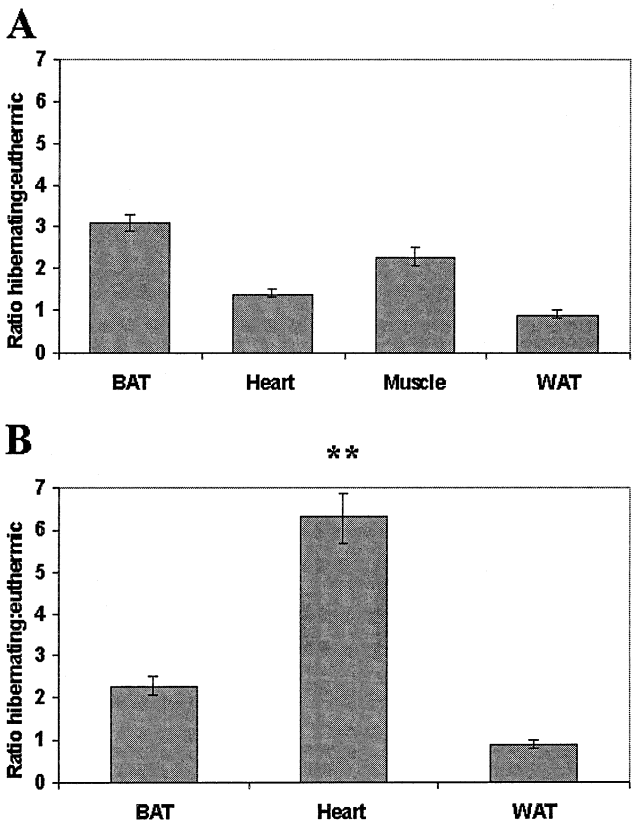

Fig. 3. Histograms depicting the relative amounts of H-FABP (A) and A-FABP (B) mRNA transcripts in hibernating and euthermic ground squirrel tissues. Band intensities were quantified from phosphorimager analysis of Northern blots, normalized to ethidium bromide-stained 28S rRNA band intensities, and ratios for hibernating versus euthermic samples were calculated. For A-FABP induction in heart, the fold increase during hibernation was calculated relative to the background intensity of controls which showed no A-FABP band; therefore, this represents a minimum fold increase. Data are means ± S.E.M., $n = 3$ separate trials, each trial using a separate sample of total RNA isolated from pooled tissue of three animals.

kDa) proteins that reversibly bind hydrophobic ligands such as long chain fatty acids, bile acids, prostaglandins and heme [16,18,19]. In vivo, FABPs are believed to have a primary function in binding fatty acids and transporting them through the cytosol to various compartments within the cell. Nine isoforms are known [16]; these were first named for the organs in which they predominate but it is now known that some cell or tissue types express multiple forms [18–20]. H-FABP (or FABP3) is found in heart, skeletal muscle and mammary but FABP5 (the epidermal isoform) also occurs in heart [16] and, as the present study shows, transcripts of A-FABP (FABP4) also appear in heart during hibernation (Fig. 3). We believe that this is the first report of A-FABP in a mammalian heart and its presence may serve a special need during hibernation. Notably, A-FABP has also been found in the hearts of Antarctic teleost fishes [21] which further supports the idea that this isoform is important for low temperature function. BAT and WAT of ground squirrels contained both A-FABP and H-FABP transcripts. Both isoforms, as well as FABP5 have been reported in rat BAT [16,22] and expression of H-FABP increased in cold-exposed animals [22].

The importance of FABP expression to hibernation is supported by the results from Northern blot studies that show up-regulation of transcript levels for both H-FABP and A-FABP in BAT and heart during hibernation as well as H-FABP up-regulation in skeletal muscle. Notably, the two organs that contribute the most to thermogenesis during arousal, BAT which provides non-shivering thermogenesis (NST) to start the warm-up and skeletal muscle which initiates shivering thermogenesis once T_b rises somewhat, both show elevated transcript levels for FABPs during hibernation. Heart is also a critical organ in the arousal process; it is rapidly warmed by the BAT surrounding it and a rapid rise in heart metabolic rate then allows a rapid distribution of warmed blood to all other organs. Although FABP protein levels were not reported in the present study, a dramatic increase in H-FABP protein has recently been demonstrated in hibernating BAT (Hittel and Storey, unpublished). Furthermore, experiments that both reduced (null mutations) or raised (transfections with FABP cDNA) FABP gene expression have been shown to cause significant changes in cellular fatty acid uptake, implying that changes in FABP protein correlated well with changes in gene expression [16].

Ground squirrels do not eat during the hibernating season and their winter metabolism is based almost exclusively on lipids, slowly consuming the huge reserves of body fat that are laid down during late summer and early autumn feeding [2]. Hence, all components of lipid catabolism including intertissue and intracellular transport, storage and release mechanisms, and oxidative pathways need to be optimized for the challenges of hibernation. FABPs are one of these components. Although there are several suggestions for the in vivo function of FABPs [16], it is generally agreed that they play a role in the intracellular trafficking of free fatty acids. Hence, the up-regulation of FABP genes during hibernation is probably effective in increasing intracellular supplies and movement of long chain fatty acids. This would help to support both basal lipid oxidation during torpor and, perhaps more importantly, the high rates of lipid catabolism that fuel thermogenesis during arousal.

Heat production during arousal is supplied first from NST in BAT. BAT depots surround the major internal organs and arteries of hibernating species, particularly in the thoracic cavity [12]. The mitochondria of BAT contain an uncoupling protein (UCP) which dissipates the proton gradient spanning the inner mitochondrial membrane and releases the energy that is normally captured in the synthesis of ATP as heat instead [13,14,23]. Not surprisingly, the expression of UCP in BAT increases with cold exposure in rats [23] and seasonally in hibernators [14]. BAT relies on both its internal lipid stores and plasma triglycerides (derived from WAT) to fuel the fires of NST [12].

These two lipid sources may account for the presence of both H- and A-FABP in this adipose tissue. One of the unique features of the A isoform of FABP is its ability to form a complex with hormone-sensitive lipase and this suggests that A-FABP functions to carry fatty acids away from intracellular lipid droplets after triglyceride hydrolysis [16]. In BAT, the primary destination of A-FABPs loaded with fatty acids would probably be the mitochondria where they would be used for oxidation and thermogenesis. This function for A-FABP may also be the reason for its induced presence in ground squirrel heart during hibernation. Mammalian heart typically relies on fatty acids for about 70% of its fuel and during hibernation this may shift to almost 100% [20]. Typically, mammalian heart imports fatty acids and it has been proposed that the role of H-FABP is to transport fatty acids from the sarcolemma to intracellular destinations [16]; the up-regulation of H-FABP during hibernation in heart would increase this transport capacity and the delivery of fatty acids to the mitochondria whose capacity for electron transport is similarly enhanced during hibernation [10]. Hearts of hibernating ground squirrels, however, maintain substantial intracellular triglyceride lipid droplets [24] which are probably needed because the demand for lipid fuels by heart during arousal could exceed the capacity for delivery of triglycerides via the blood. The induction of A-FABP in heart during hibernation would support the catabolism of intracellular lipid droplets and the transport of their released fatty acids via A-FABP to the mitochondria for oxidation. Our model, then, is one where A-FABP has a primary role in the transport of fatty acids that are released from intracellular lipid droplets via the action of hormone-sensitive lipase whereas H-FABP is involved in the importation of fatty acids into the cell. The up-regulation of both FABP isoforms in BAT and heart of hibernating animals serves the needs of both organs by giving them the means to exploit two sources of fatty acids from two different triglyceride pools, those in the blood and those in intracellular lipid droplets. The model would also fit the data for skeletal muscle which up-regulates the fatty acid importing isoform, H-FABP, during hibernation to feed its dependence on blood-borne lipid delivery but does not have major intracellular lipid reserves.

Mammalian hibernators allow their core T_b to cool to close to 0°C, a value that is lethal to most humans and most other mammals. Temperature change can have profound deleterious effects on proteins affecting, for example, their conformation, their kinetic/regulatory properties, their binding interactions with subcellular structures, and their integrated functioning within metabolic pathways. Clearly, however, the proteins of hibernators must remain functional over a wide temperature range of low T_b values and it is likely that selected structural differences in hibernator proteins, arising from discrete amino acid substitutions, may allow them to function over a broad temperature range. The amino acid sequence alignments of ground squirrel FABPs with their homologs from human, rat and pig revealed a high degree of sequence similarity between species, about 90% in all cases (Fig. 2). Significantly, however, H-FABP showed three distinct amino acid substitutions as compared with the protein from the other species. These occurred at lysine 69, threonine 84, and asparagine 110 of the cDNA which correspond to residues 68, 83 and 109 of the mature protein after cleavage of the N-terminal methionine. Using the X-ray structure of the human heart/muscle FABP as a model (created with the use of RasMol v2.6 with data from the Brookhaven Protein DataBank, Id# 1 HMS), we determined that two of these substitutions occurred in the turns connecting β-sheets in the protein, Lys 68 between β-sheets D and E, and Asn 109 between β-sheets H and I. Both substitutions replace non-polar amino acids that are present in the other species. The location of both turns covers a 'gap' in the structure of all known FABPs which is believed to confer flexibility to the protein [16,20]. The third unique amino acid substitution in ground squirrel H-FABP, Thr 83, places a polar amino acid where hydrophobic amino acids were previously (Fig. 2A). Although this residue is not located in a turn region, it lies adjacent to Lys 68 in the tertiary structure. The side chains of all three of these amino acids project outwards from the plane of the secondary structure and may serve to stabilize the structure of the protein, allowing it to function effectively at low temperatures. The presence of a lysine residue on the exposed surface of the protein may also enhance the interaction of the H-FABP with membranes at low temperatures where there is perhaps little collisional energy to transfer fatty acids from FABP to (mitochondrial) membranes [19,20]. Stewart et al. have previously demonstrated that the dissociation constant (K_d) for a variety of fatty acids of the liver-type FABP from a hibernating ground squirrel is temperature-insensitive over 5–37°C relative to the FABP purified from rat liver [25]. Interestingly, recent evidence indicates also, a similar temperature insensitivity for H-FABP purified from hibernating ground squirrel skeletal muscle (J.M. Stewart, personal communication) in support of our hypothesis that ground squirrel H-FABPs remain functional at low T_bs. Whereas H-FABP from hibernators showed these very distinct amino acid substitutions, the same could not be said for ground squirrel A-FABP. Two unique substitutions were noted: the lysine at position 53 was arginine in other species and the isoleucine at position 66 was lysine in the others. However, neither of these changes should significantly alter protein functionality.

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