Mitochondrial enzyme content in the muscles of high-performance fish: evolution and variation among fiber types

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Mitochondrial enzyme content in the muscles of high-performance fish: evolution and variation among fiber types. Am J Physiol Regul Integr Comp Physiol 288: R163–R172, 2005. First published September 23, 2004; doi:10.1152/ajpregu.00152.2004.—Muscle mitochondrial content varies widely among fiber types and species. We investigated the origins of variation in the activity of the mitochondrial enzyme citrate synthase (CS), an index of mitochondrial abundance, among fiber types and species of high-performance fish (tunas and billfishes). CS activities varied up to 30-fold among muscles: lowest in billfish white muscle and highest in billfish heater organ. Among species, CS activities of red, white, and cardiac muscles of three tuna species were twofold greater than the homologous muscles of two billfish species. Because comparisons of CS amino acid sequences deduced from a combination of PCR methods argue against clade-specific differences in catalytic properties, CS activity reflects CS content among these five species. To assess the bases of these differences in CS activity, we looked at the relationship between CS activity (U/g muscle), nuclear content (DNA/g muscle), and CS transcript levels (CS mRNA/g RNA). Muscle CS activity differed by 10- to 30-fold when expressed per gram of muscle but only threefold when expressed per milligram of DNA. Thus it is DNA content, not fiber-type differences, in CS gene expression that may be the main determinant of CS activity in muscle. Conversely, evolutionary (tunas vs. billfishes) differences in CS arise from differences in posttranscriptional regulation, based on relationships between CS enzyme levels and CS mRNA assessed by quantitative competitive RT-PCR. These data argue that fiber-type differences can arise without major differences in fiber-type-specific regulation of the CS gene, whereas evolutionary differences may be largely due to posttranscriptional regulation.

Citrate synthase; suborder Scombroidei; nuclear DNA content; posttranscriptional regulation

Mitochondrial content, an important determinant of aerobic capacity, varies widely among muscle types and species (37). Within an individual, muscle mitochondrial content increases with differentiation (40) and then diverges as fiber types are established (30). Environmental and physiological challenges, such as cold exposure (e.g., Ref. 10) and exercise (e.g., Ref. 23), also lead to phenotypic changes in mitochondrial abundance. In addition, differences exist among species, with athletic species possessing higher levels of mitochondria than sedentary species (e.g., Ref. 39). Interestingly, the mechanisms that control evolutionary variations in mitochondrial content remain unstudied. Instead, the origins of differences in mitochondrial enzyme content are best understood in the contexts of developmental and physiological changes in mammalian muscle fiber types (13, 20, 23, 32, 33, 37, 38, 46, 53, 54). Most studies of mitochondrial biogenesis have focused on how transcriptional regulators control and coordinate the expression of nuclear- and mitochondrial-encoded genes (46). The peroxisome proliferator-activated receptor γ-coactivator 1α has emerged as a master regulator (32, 54), acting through a network of transcription factors to alter mitochondrial biogenesis in mammalian muscle fibers (13, 20, 33, 53). Although transcriptional regulation of mitochondrial enzymes is clearly important within an individual, it is not yet known whether it contributes to evolutionary variations in mitochondrial abundance that arise between species.

In this study, we use scombroid fish (order Perciformes, suborder Scombroidei) to address the origins of variations in mitochondrial content among species that arise over evolutionary time and also among fiber types within individuals. Fish are good models in which to investigate fiber-type differences in mitochondrial properties because, in contrast to mammalian fiber types, their skeletal muscles are divided into distinct red (aerobic) and white (glycolytic) muscle fibers with large differences in mitochondrial content (29, 30). In particular, the scombroid fish have many properties that are conducive to the study of evolutionary and developmental variations in mitochondrial abundance. They show marked phenotypic variations in muscle oxidative capacity associated with aerobic activity and thermal physiology (14, 27). The tunas (family Scombridae) have impressive exercise physiology, including high metabolic rates, rapid swimming speeds, long migrations, rapid recovery from burst swimming, endothermy, numerous adaptations for oxygen uptake and delivery, and a unique mode of swimming (9). Also, tunas have a muscle mitochondrial capacity that exceeds that of most fish (39), including the closely related billfishes (family Xiphiidae and Istiophoridae) (6, 14, 27). Billfishes possess a heater organ, which is a modified muscle that warms the brain and eyes to preserve vision in cold water (4). Derived from the superior rectus eye muscle, the heater organ lacks most muscle contractile machinery and contains a very high mitochondrial content (55–70% of cell volume) (4–6, 8). It is not known how this increased mitochondrial content is regulated in the heater tissue, although it is possible that the mechanisms are similar to those found between red and white muscles. This exceptional tissue may be

1 We use the descriptive terms red and white muscle in preference to tetrapod distinctions of slow-oxidative, fast-oxidative/glycolytic, and fast-glycolytic or myosin I, IIa, IIb, or IId/x. The use of the terms red and white avoids implicit links with specific myosin isoform profiles, which in many fish is complicated by early and recent genome duplications.

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unique to the suborder Scombroidei: an analogous tissue has been found only in the butterfly mackerel (Gasterochisma melampus) of the family Scombridae (7).

In this study, we assess the molecular basis for differences in muscle mitochondrial enzyme content found among fiber types (red muscle, white muscle, cardiac muscle, and heater organ) and also arising over evolutionary time, between clades of scombroid fishes (tunas vs. billfishes). Focusing on citrate synthase (CS) as a marker enzyme for mitochondrial content, and also arising over evolutionary time, between clades of scombroid fishes (tunas vs. billfishes). Focusing on citrate synthase (CS) as a marker enzyme for mitochondrial content, we hypothesized that differences among species and tissues would be attributed to differences in CS mRNA levels that are ultimately linked to transcriptional regulators.

MATERIALS AND METHODS

Tissue samples. Tissue samples from two species of billfish [swordfish (Xiphias gladius) and striped marlin (Tetrapturus audax)] and three tuna species [bigeye tuna (Thunnus obesus), yellowfin tuna (Thunnus albacares), and skipjack tuna (Katsuwonus pelamis)] were collected on the National Oceanic and Atmospheric Administration ships Townsend Cromwell and Oscar Elton Sette. Fish were caught in the Pacific Ocean around the Hawaiian Islands at approximate latitudes of 18–35° north and longitudes of 155–170° west by longline and trolling. Tissues (heater organ, red muscle, white muscle, and cardiac muscle) were removed and immediately frozen in liquid nitrogen. Samples were then powdered in a porcelain mortar and pestle in liquid nitrogen and stored at −80°C until analyses. All enzyme assays were performed within 1 mo.

RNA isolation and CS cDNA sequence information. We purified total RNA from guanidine thiocyanate extracts using a standard acid phenol protocol (12). Poly(A) RNA was isolated from total RNA using the poly(A) Purist MAG kit (Ambion).

All scombroid CS sequence data were determined from RT-PCR analyses. First-strand cDNA was synthesized from red muscle total RNA and used as the template for subsequent PCR reactions. Degenerate forward (F251) and reverse (R252) primers, based on CS mRNA sequences from human (GenBank accession number NM 004077), mouse (NM 026444), rat (NM 130755), pig (M21197), and zebrafish (BC045362.1), were used to amplify a 590-bp portion of CS cDNA at 50°C using standard PCR conditions (Table 1). Products were sequenced, and this information was used to design specific primers for use in 3′ and 5′ rapid amplification of cDNA ends (RACE) using the Roche 5′/3′ RACE kit. Poly(A) RNA was used as the template for 5′ RACE, and gene-specific reverse primers 5′-R1, 5′-R2, 5′-R3, and 5′-R4 were used in cDNA synthesis and subsequent nested amplifications (Table 1). Total RNA was used as the template for 3′ RACE and reverse transcribed with an oligo(dT) primer. We performed nested PCR amplifications with this cDNA as the initial template using the specific forward primers 3′-F1, 3′-F2, and 3′-F3, respectively, each with the anchor primer (Table 1). The band from the second PCR for yellowfin and marlin and the third PCR for skipjack were excised and cloned.

Table 1. Primers designed for citrate synthase PCRs, 5′ RACE, 3′ RACE, preparation of CS deletion, and QC-RT-PCR amplifications

<table>
<thead>
<tr>
<th>Product</th>
<th>Primer</th>
<th>Direction</th>
<th>Sequence (5′–3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CS (590 bp)</td>
<td>F251</td>
<td>F</td>
<td>GATGAAAGARCTTCCACACAGCG</td>
</tr>
<tr>
<td>CS (590 bp)</td>
<td>R252</td>
<td>R</td>
<td>GTGGYGAATTAKSGACACCTCC</td>
</tr>
<tr>
<td>5′CS</td>
<td>5′-R1</td>
<td>R</td>
<td>ATCTTGTRGCAATGAGGAC</td>
</tr>
<tr>
<td>5′CS</td>
<td>5′-R2</td>
<td>R</td>
<td>CATGGTACAGGTTGAGGAGG</td>
</tr>
<tr>
<td>5′CS</td>
<td>5′-R3</td>
<td>R</td>
<td>ACGCTGCTCTGAGACAGACAG</td>
</tr>
<tr>
<td>5′CS</td>
<td>5′-R4</td>
<td>R</td>
<td>AAAAGGATGAGCCTTACAG</td>
</tr>
<tr>
<td>5′CS, 3′CS</td>
<td>Oligo(dT)-anchor</td>
<td>F, (5′CS), R, (3′CS)</td>
<td>GACAGCCTCATAGTTGAC(T)16V</td>
</tr>
<tr>
<td>5′CS, 3′CS</td>
<td>Anchor</td>
<td>F, (5′CS), R, (3′CS)</td>
<td>GACACCCGGTATAGTGTCGAC</td>
</tr>
<tr>
<td>3′CS</td>
<td>3′-F1</td>
<td>F</td>
<td>AGACGTCCTGAGAGCAC</td>
</tr>
<tr>
<td>3′CS</td>
<td>3′-F2</td>
<td>F</td>
<td>GAAACGTGCCCAGCATTG</td>
</tr>
<tr>
<td>3′CS</td>
<td>3′-F3</td>
<td>F</td>
<td>ATGCAAGAATTGAGGACAG</td>
</tr>
<tr>
<td>3′CS</td>
<td>3′-F4</td>
<td>F</td>
<td>CCTGCTGACAGAATGTCG</td>
</tr>
<tr>
<td>3′CS</td>
<td>Scomb-R1</td>
<td>F</td>
<td>ATGAAGGGATGAGCCTTACAG</td>
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<td>QC</td>
<td>QC-F1</td>
<td>F</td>
<td>ATGAAAGGGATGAGCCTTACAG</td>
</tr>
<tr>
<td>QC, QC</td>
<td>QC-R1</td>
<td>R</td>
<td>GCCGCTGACAGGAGTTCG</td>
</tr>
</tbody>
</table>

CS (590 bp), internal 590 bp portion of citrate synthase (CS), amplified with degenerate primers; 5′ CS, 5′ region of CS, amplified with consensus primers; 3′ CS, 3′ region of CS, amplified with consensus primers; CS (900 bp), 900 bp portion at 3′ end of CS, amplified with consensus primers; QC: QC-RT-PCR amplification; AJP-Regul Integr Comp Physiol, VOL 288, JANUARY 2005, www.ajpregu.org
represents <2% of total DNA in striated muscles due to its small size (≈16,500 bp) relative to the nuclear genome (2, 36).

We measured total RNA levels per gram of tissue directly from the upper (aqueous) phase of a standard RNA extraction protocol using RiboGreen (Molecular Probes). This organic extraction with acid phenol ensures that contaminating DNA levels are low (<2.5% of total nucleic acid content as determined from a Picogreen assay; data not shown). A mock RNA extraction (no tissue added) was performed to obtain an equivalent aqueous phase to use with the standard curve. Both Picogreen and RiboGreen analyses were conducted with a SpectraMAX GEMINI XS spectrophotometer.

**RESULTS**

**Quantitative competitive RT-PCR.** CS mRNA levels were quantified using quantitative competitive (QC) RT-PCR with an RNA competitor template. The competitor template was constructed by using PCR to delete an internal portion of the endogenous template (11). Swordfish CS cDNA was amplified with the forward primer QC-delete (ATGAGGGGATGAAGGCTCTGTGTA-AAGCTTCAGAAGTGCTAGAGGTGCC), containing a HindIII restriction site, and the reverse primer QC-R1, yielding a product with a 50-bp deletion just after the end of the QC-F1 primer binding site in the CS gene product. The resulting product, ΔCS, was cloned into pCR 2.1 (Invitrogen), analyzed for orientation by restriction enzyme digestion with HindIII, and sequenced.

The plasmid containing ΔCS was linearized with BamHI, purified (QIAquick PCR purification kit, Qiagen), and used to generate sense strand ΔCS RNA using T7 RNA polymerase. The in vitro transcript was DNase treated (1 unit of DNase per μg DNA) and then purified with an RNeasy Mini kit (Qiagen). No contaminating DNA was detectable at the dilution to be used in QC-RT-PCR when tested in negative control PCRs (no RT). The purity and size of the ΔCS transcript were verified by gel electrophoresis, quantified with RiboGreen (Molecular Probes), diluted to 500 pg/μl (4.05 fmol/μl) in DEPC-treated water, and stored in single-use aliquots at −80°C.

Before the reverse transcription procedure, RNA samples from tissues were quantified by absorbance at 260 nm using a quartz plate treated with 1 M sodium hydroxide to destroy RNases. Aliquots were taken directly from the plate well to ensure accurate quantification. A constant amount of sample RNA (500 ng) and various amounts of competitor RNA transcript were added to each RT tube. Samples were reverse transcribed at 42°C for 1 h. The synthesized cDNA was then amplified by PCR as described previously (31), using strict consensus primers (QC-F1 and QC-R1, Table 1) for all five species at an annealing temperature of 65°C. An aliquot of [α-32P]dCTP was added to each reaction for the last five cycles. Only reactions with full-length products within 50% of competitor products (once corrected for deoxyctosine content) were used in quantification (47). Controls with no added RT, as well as a negative PCR control, were run in parallel to detect any genomic DNA contamination. None of these negative controls generated PCR products. The amplified products were size fractionated on a 12% polyacrylamide gel, and the signal strength was quantified with a storage phosphor screen (Molecular Dynamics) and analyzed with Imagequant software (Molecular Dynamics). We converted values from nanograms to femtomoles of CS mRNA using the molecular mass of the competitor transcript (8.10 fmol/μg).

**Statistical analyses.** For all parameters, significant differences (P < 0.05) among tissue types within a species and for a tissue type among species were detected with one-way ANOVAs and identified with the Tukey-Kramer test.

**RESULTS**

**Mitochondrial enzyme content.** CS and COX activities displayed similar trends between the two clades of fish. When expressed per gram of tissue, mean tuna (skipjack, yellowfin, and bigeye) activities were approximately two- to threefold higher than white muscle activities (Fig. 1, A and D). In addition, each tuna species had significantly higher CS and COX activities for a given fiber type than each billfish species, with the exception of COX in bigeye white muscle and yellowfin cardiac muscle (P < 0.05). CS and COX also displayed similar trends across fiber types within a species (Fig. 1, A and C). Red and cardiac muscles had ~6- to 10-fold higher CS activity per gram of tissue than white muscle. The heater organ had the highest activities of all fiber types, with 15- to 30-fold more CS than billfish white muscle.

**CS sequence analyses.** The full coding region of CS was sequenced for the three tuna species (yellowfin tuna (GenBank accession number AY461848), bigeye tuna (AY461849), and skipjack tuna (AY461850)) and the two billfish species (swordfish (AY461851) and striped marlin (AY461852)). The impact of any clade-specific nonsynonymous variation (defined as sites for which all tunas have the same amino acid and all billfishes have another amino acid) was examined to determine whether variations in the enzyme’s catalytic capacity may be responsible for the higher CS activity in tuna striated muscles.

In the 439 amino acid CS monomer, there are five sites (Ser7Thr, Thr19Ser, Ile171Val, Pro222Ala, Leu292Met) with clade-specific variations (Fig. 2). Amino acid hydrophobicity is also conserved in sites with clade-specific differences. Although no crystal structure is available for fish CS, the conservation of structure between mammals (Sus scrofa, Protein Data Bank no. 2CTS) and birds (Gallus gallus, Protein Data Bank no. 3CTS) suggests that the three-dimensional properties of scombroid CS should parallel those of other vertebrates (see Fig. 6).

**Impact of DNA content on enzyme levels.** When investigating the origins of these differences in CS content, the choice of denominator is very important. Differences found when expressed per gram tissue may not reflect gene regulatory differences per se but instead differences in nuclear content. For example, if two tissues differed in nuclear DNA content (and thus CS gene copy number), they could achieve different CS synthesis per gram muscle even if each CS gene was similarly regulated. We measured total DNA content per gram muscle, a measure of nuclei per gram muscle, so that enzyme levels could be expressed relative to DNA content as well as per gram tissue. Nuclear DNA content is a valid measure of the CS gene content of a tissue if, as expected, all nuclei within an individual are similar. This would not be the case in some tissues, such as polytenic salivary glands, where specific genes are amplified in the genome. Among these species of fish, DNA content per haploid genome size is also comparable (21, 22). There were no significant differences in DNA content between homologous tissues in tunas and billfishes (P < 0.05), but there were large differences between fiber types (Fig. 3A). Red muscle, cardiac muscle, and heater organ had, respectively, 2.5- to 4-fold, 2- to 6-fold, and 7- to 9-fold more DNA per gram tissue than white muscle. These differences are likely due to major differences in myonuclear domain, although the potential im-
pact of nonmuscle cells (e.g., fibroblasts, endothelial cells, and vascular smooth muscle) cannot be ruled out. It is important to acknowledge that all whole muscle analyses, including enzyme, RNA, and nuclear run-off analyses, are subject to potential concerns about the relative activities and levels of myonuclei in the muscle.

When expressed per milligram DNA, mean tuna CS activities remained ∼2-fold higher than mean billfish values in red and white muscle and increased to ∼3.5-fold higher in tuna cardiac muscle (Fig. 3C). Each tuna species also had a significantly higher CS activity per milligram DNA than each billfish species in all homologous tissues (P < 0.05). Conversely, the large differences in CS activity seen among fiber types are greatly reduced when enzyme levels are expressed relative to nuclear content (Fig. 3B). For example, the ∼30-fold difference in enzyme activity between the swordfish heater organ and white muscle is reduced to a threefold difference when expressed per milligram DNA (Fig. 1A vs. Fig. 3B). In addition, the 6- to 10-fold difference in CS enzyme activity between the red and cardiac muscle compared with the white muscle is reduced to an approximate threefold difference (Fig. 1A vs. Fig. 3B). These results argue for the large role of constitutive expression in combination with differences in nuclear content in determining fiber-type mitochondrial phenotype, even within the exceptional heater organ.

Role of transcriptional and posttranscriptional regulation.

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Cardiac muscle, and the billfish white and red muscle are found to have 40% and 3% more CS mRNA than tuna muscles, opposite to CS enzyme trends (Fig. 4D). Each billfish species also contained as much or more CS transcript per milligram DNA than each tuna species in all homologous muscles, with the exception of the swordfish red and cardiac muscles compared with the skipjack (significant, $P < 0.05$). Therefore, neither intertissue nor interspecies variations in CS enzyme activity (Fig. 3, B and C) are reflected in CS mRNA levels (Fig. 4, C and D), arguing against major differences in transcriptional regulation and suggesting that differences in CS activity found when DNA content is accounted arise from posttranscriptional regulation. Within a species, red and cardiac muscle produce approximately two- to threefold more CS enzyme per femtomole of CS mRNA, and the heater organ produces four- to eightfold more CS enzyme than white muscle (Fig. 5A). Between clades, mean CS enzyme production per femtomole CS mRNA for tunas is about two- to threefold greater than homologous billfish tissues (Fig. 5B). Each tuna species also produces significantly more CS enzyme per femtomole CS mRNA than each billfish species in all homologous muscles, with the exception of the bigeye and skipjack red muscle compared with the swordfish ($P < 0.05$).

**DISCUSSION**

In this study, we assessed the relative importance of gene dosage and transcriptional and posttranscriptional regulation in determining evolutionary and fiber-type differences in mitochondrial enzyme content in muscles of scombroid fish. Studies on mammalian muscle fiber types suggest that transcriptional regulators play an important role in controlling differences in mitochondrial enzymes between muscles and with physiological challenges (23, 32, 33, 37, 38, 46, 53, 54). However, the genetic bases for differences in mitochondrial content between animals are virtually unknown, although mitochondrial abundance is a heritable trait that can be selected for on population-level time scales (24).

We have compared the mitochondrial capacity of selected tunas and billfishes using CS, a citric acid cycle enzyme located in the mitochondrial matrix, as an index of mitochondrial content. The differences we found in CS activity among fiber types and species are consistent with previous studies (6, 15, 27, 50). Within each clade (i.e., tunas and billfishes), homologous muscles displayed similar CS activities (Fig. 1A). Between clades, tunas had about twofold higher CS activity than billfishes in each homologous muscle (Fig. 1B). Within each species, red muscle and cardiac muscle had similar enzyme activities, both ~6- to 10-fold greater than white muscle (Fig. 1A). Billfish heater organ CS activities were about 3-fold greater vs. cardiac and red muscle and 15- to 30-fold higher than activities of white muscle of the same species (Fig. 1A).

**CS activity** is a good marker for mitochondrial content because it correlates with morphometric measures of mitochondria volume density (42) and milligrams of mitochondrial protein (39). In this study, we also found good correlation between the activities of CS and the inner mitochondrial membrane enzyme COX or complex IV of the electron transport chain (Fig. 1). Although COX is probably a more accurate predictor of maximal mitochondrial respiration, its complex structure and regulation make such functional and regulatory variations in transcriptional control of the CS gene. CS mRNA levels were determined by QC-RT-PCR using primers designed in areas of exact consensus for all five species. CS transcript levels per gram total RNA calculated by QC-RT-PCR (Fig. 4B) were expressed per milligram DNA by correcting for both total DNA (Fig. 3A) and RNA (Fig. 4A) content of the tissue. Within a species, white muscle contained as much or more CS transcript per milligram DNA than the red, cardiac, and heater muscle in 10 of 12 comparisons (Fig. 4C) (significant, $P < 0.05$). Among clades, mean CS mRNA per milligram DNA values vary $<15\%$ between tuna and billfish.

![Fig. 3. DNA content per gram tissue (A) and CS activity per milligram DNA (B) in scombroid muscles. In A and B, tissues with different superscripts are significantly different from other tissues ($P < 0.05$). In A, all tissues and species are compared. In B, comparisons are made within species. Data are means ± SE. C: CS activity per milligram DNA for each clade expressed relative to billfish. Data are means for each clade, with circles (○) representing the mean for each species within the clade. For A–C, $n = 8$ for all tuna and swordfish tissues and $n = 4$ for all marlin tissues, except the heater organ where $n = 2$.](http://ajpregu.physiology.org/doi/10.1152/ajpregu.01005.2004)

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investigations difficult. The genetics and enzymology of CS are well suited to study the evolution of mitochondrial properties. Metazoan CS is a homodimer encoded by a single copy nuclear gene with no known isoforms (17), no allosteric regulators (44), and no known covalent regulators. Thus any difference in CS catalytic activity between tissues also reflects a difference in CS protein content. However, when comparing across species, it is important to assess the potential for genetic differences that could affect the relationship between CS catalytic activity and CS protein content. Changes in enzyme catalytic efficiency could, in principle, occur in CS or other mitochondrial enzymes as an evolutionary mechanism to increase catalytic activity. However, this is the least parsimonious mechanism by which a global increase in mitochondrial capacity could arise because of the complexity of mitochondrial metabolism. For example, many changes in primate COX subunits have structural implications that necessitate coevolution of binding partners (19). We sequenced the full-length coding region of CS from all five species and found no clade-specific mutations that affected functionally relevant sites in the protein. There are no variations in catalytic, substrate binding, or dimerization sites, arguing that the catalytic efficiency of CS is similar between the tunas and billfishes (Figs. 2 and 6). Thus we conclude that CS activity patterns reflect CS content in comparisons between these species as well as among tissues within each species. To assess the regulatory origins of differences in CS content among species and tissues, we considered the importance of CS gene dosage (nuclei per gram tissue), transcriptional regulation (CS mRNA per CS gene), and posttranscriptional regulation (CS activity per CS mRNA).

Importance of muscle DNA content. Myonuclear domain and muscle morphology are important considerations when comparing muscles in terms of gene expression and protein levels. Consider two muscles that differ in myonuclei per gram and therefore CS gene copy number per gram. The CS genes in both tissues could be regulated identically, but a tissue with more nuclei per gram (e.g., red muscle) would be expected to synthesize more CS per gram muscle. Because mitochondrial DNA contributes <2% to total DNA content, total DNA per gram tissue is a valid measure of nuclear DNA per gram tissue (2, 36). Nuclear DNA content should also be a valid measure of the CS gene content of a tissue if all nuclei within an individual are the same. This would not be the case in some tissues, such as polytenic salivary glands, where specific genes are amplified in the genome. This measure should also be valid for our evolutionary comparisons since haploid genome size is comparable among the tunas and billfishes (21, 22). Thus expressing CS per milligram DNA provides a better indication of potential differences in CS gene expression between tissues. Although this may be less important in mammals, where myonuclear domain differs only two- to threefold between
skeletal fiber types (49), it might be very important in fish, which display a much greater divergence in metabolic properties (30). The scombroid fish are of particular interest because differences seen in CS activity among scombroid muscles after acclimation. Instead, it is the amount of CS enzyme produced per femtomole of CS mRNA (Fig. 5A) that accounts for the majority of differences in CS enzyme content between clades and threefold among muscles.

After developing a QC-RT-PCR approach that took interspecies differences in CS sequence into consideration, we assessed the relationship between CS transcript levels and CS enzyme activity. CS is transcriptionally regulated during the mitochondrial biogenesis that accompanies electric stimulation in mammalian striated muscle (1). Under steady-state conditions, CS mRNA correlates with CS enzyme levels (1). Thus transcriptional regulation is a potential site for evolutionary differences in CS content among species. For example, variations in enzyme levels could arise from mutations in 1) cis-regulatory regions of genes (altering transcription factor binding), 2) cis-regulatory regions of the transcriptional regulators, or 3) transcription factor coding regions, causing structural changes that influence DNA binding or catalytic properties (25, 45). Although transcriptional regulation is increasingly implicated in evolutionary variations, in other nonmitochondrial proteins (25, 45, 52), it is not yet known whether evolutionary differences in mitochondrial protein levels arise through effects on transcriptional regulators. If the differences in CS activity between species and tissues arose by transcriptional regulation, we would also predict a correlation between CS mRNA and CS enzyme levels. However, there was little correlation between CS activity and CS mRNA levels when compared between clades of fish (Fig. 3C vs. Fig. 4D, note differences in scale). Instead, it is the amount of CS enzyme produced per femtomole of CS mRNA, a measure of posttranscriptional regulation, that accounts for the majority of differences in CS enzyme content between tunas and billfishes (Fig. 5B). There is also very little correspondence between CS enzyme and CS mRNA values when compared across fiber types within an individual (Fig. 3B vs. Fig. 4C). Again, it is the amount of CS enzyme produced per femtomole of CS mRNA (Fig. 5A) that accounts for a higher CS enzyme content per milligram DNA in the red, cardiac, and heater muscles (Fig. 3B). These data argue that the differences seen in CS activity among scombroid muscles after DNA content is accounted for likely arise through variations in posttranscriptional processes, rather than transcriptional control.

Regulation of CS content. Although gene dosage accounted for most of the differences between fiber types, it did not influence the differences in CS activities between tunas and billfishes (Fig. 3C). Most studies that assess the determinants of mitochondrial gene expression in mammalian striated muscles focus on transcriptional regulators, particularly peroxisome proliferator-activated receptor γ-coactivator-1α (33, 37, 38, 46). However, it is important to recognize that many mitochondrial proteins are also regulated by posttranscriptional controls [e.g., ω-subunit of the F1-ATPase complex (26), mitohormonal protein S12 (35), subunit VIII-L of COX (41), aconitase (18)].

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Fig. 5. Posttranscriptional regulation of CS. A: CS enzyme activity/fmol CS mRNA. Data are means ± SE. Tissues with different superscripts are significantly different from other tissues within the given species (P < 0.05). B: CS enzyme activity/fmol CS mRNA for each clade expressed relative to billfish. Data are means for each clade, with circles (○) representing the mean for each species within the clade. For A and B, n = 5 for all tuna and swordfish tissues and n = 4 for all marlin tissues, except the heater organ where n = 2.
Posttranscriptional regulation of CS during mitochondrial biogenesis also occurs in the livers of North Sea eelpout (*Zoarces viviparous*) during cold acclimation (34). The mechanism by which the posttranscriptional regulation of CS occurs in North Sea eelpout liver and in scombroid muscles, both within and among species, is unknown. It is important to note that both studies have measured steady-state levels of CS transcripts and not transcription per se. CS mRNA levels may also be influenced by mRNA stability. However, even if this is a contributing factor, it would not affect the calculations of CS enzyme produced per CS mRNA, and the importance of posttranscriptional controls remains.

As mentioned previously, several mitochondrial enzymes are known to experience characteristic differences in posttranscriptional processing. Furthermore, declines in muscle energetics during aging and disease can also arise through posttranscriptional defects [e.g., protein import (48)]. Disease models, involving loss of function, provide insight into possible sites of posttranscriptional regulation, but they may not be directly applicable to evolutionary or developmental comparisions. Tunas are exceptional athletes among fish; it is likely that they possess unusual regulatory features that enable them to maintain higher CS levels in relation to CS mRNA. The most likely points of differential regulation are enhanced translation initiation, protein stability, or mitochondrial stability.

This is the first work to examine the genetic determinants of evolutionary variations in muscle mitochondrial content. Surprisingly, we found evidence for a substantial role of posttranscriptional differences in determining CS activity patterns among species. Posttranscriptional processes also contribute to fiber-type differences in CS content within scombroid fishes. However, we identify nuclear DNA content as the main determinant of phenotypic differences in mitochondrial content among muscle fiber types. Elucidating the posttranscriptional control mechanisms by which CS is regulated in these two contexts may have broad implications for the regulation of mitochondrial content and will represent an important step in unraveling the possible molecular mechanisms by which phys-
ologically important traits, such as mitochondrial content, may evolve.

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REFERENCES


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