ISSN 1068-1620, Russian Journal of Bioorganic Chemistry, 2016, Vol. 42, No. 2, pp. 162–169. © Pleiades Publishing, Ltd., 2016.
Original Russian Text © O.V. Meshcheryakova, M.V. Churova, A.E. Veselov, N.N. Nemova, 2016, published in Bioorganicheskaya Khimiya, 2016, Vol. 42, No. 2, pp. 182–190.

Activities of Cytochrome *c* Oxidase and Mitochondrial Lactate Dehydrogenase Isozymes and *Cox1*, *Cox2*, *Cox4*, and *Cox6* Gene Subunit Expression in Cold Adaptation of *Salmo trutta* L.

O. V. Meshcheryakova[1], M. V. Churova, A. E. Veselov, and N. N. Nemova

Institute of Biology, Karelian Research Centre, Russian Academy of Science, ul. Pushkinskaya 11, Petrozavodsk, 185910 Russia

Received April 16, 2015; in final form, July 7, 2015

Abstract—Characteristic changes in some parameters of white muscle mitochondria (mitochondrial volume, activity of cytochrome *c* oxidase (COX, EC 1.9.3.1) and the level of *Cox1*, *Cox2*, *Cox4*, and *Cox6* subunit gene expression and activity and kinetic characteristics of mitochondrial lactate dehydrogenase isozymes (mtLDH, EC 1.1.1.27)) in adaptation to seasonal decrease in temperature from 16 to 6°C of one-year juvenile brown trout *Salmo trutta* L. from rivers of Lake Onega basin were investigated. A 1.5-fold increase in the activity of COX, and the increase in the levels of gene expression of both Cox4 and Cox6 nuclear subunits and increased activity of mitochondrial LDH isozymes, which have a low affinity towards lactate, has been shown. The possible role of nuclear and mitochondrial subunits of cytochrome *c* oxidase in improving the efficiency of the enzyme in molecule biogenesis and further modulation of its activity, as well as regulation of pyruvate formation to maintain the required rate of oxidative phosphorylation in mitochondria under low temperature were discussed.

Keywords: cytochrome c oxidase, gene expression, mitochondrial lactate dehydrogenase, Michaelis–Menten constant, cold adaptation, brown trout

DOI: 10.1134/S1068162016010106

INTRODUCTION

It is known that mitochondrial biogenesis with simultaneous changes in a number of mitochondrial physicochemical, biochemical and molecular genetic parameters, is activated to a large extent in many animal species at low environmental temperatures. This effect aimed to compensate for the metabolic level is particularly evident for ectothermic organisms, in particular, fish, whose body temperature depends on the temperature of the environment [1]. A decrease in environmental temperature leads to an increase in capillarization in fish muscle tissue, an enhancement of oxygen diffusion into cells and an increase in mitochondrial density. This, in turn, compensates for the reduction of the rate of oxidative phosphorylation and ATP formation.

Coordination of mitochondrial cold functional activity is caused by a range of mechanisms, among them two main mechanisms can be noted: firstly, activation of mitochondrial protein synthesis, which provides an increase in the concentration of aerobic metabolic enzymes with the required activity, and, secondly, alteration of structural and functional properties of mitochondrial membranes (increasing fluidity) due to modulation of phospholipid and fatty acid composition, which regulates membrane enzyme activity, oxygen and metabolite transport [3].

Cytochrome *c* oxidase (COX, EC 1.9.3.1) is a key enzyme of the mitochondrial respiratory chain, which includes subunits encoded by nuclear and mitochondrial genomes. The number of polypeptide chains in a COX molecule depends on the evolutionary stage of species [4, 5]. Fish COX, like mammals COX, consists of 13 subunits: three subunits are encoded by the mitochondrial genome (COX 1, 2, 3), and ten minor subunits (COX 4, 5a, 5b, 6a, 6b, 7, 7a, 7b, 7c, 8) are encoded by the nuclear genome. It is well known that subunits encoded by the mitochondrial genome, are directly involved in catalysis. Nuclear subunits are formed around the catalytic site of the molecule and involved in the regulation of enzyme activity and stabilization of its structure [6–8]. The regulation of enzyme activity due to alteration of expression level of different type subunits was studied in detail in models of various physiological conditions in mammals. In contrast to mammals, strong consistency and stoichiometry in the expression level of mitochondrial and nuclear genomes and clear correlation with alteration

Abbreviations: COX, cytochrome *c* oxidase; mtLDH, mitochondrial lactate dehydrogenase; *a*3-CuB, binuclear center heme *a*3 of cytochrome *c* oxidase and copper atom; CuA, copper atom in the active site of cytochrome *c* oxidase.

[1] Corresponding author: phone: +7 (8142) 77-27-51; fax: +7 (8142) 76-98-10; e-mail: o-mesch@yandex.ru.

of enzyme activity is not observed in fish, presumably due to the direct effect of temperature on stability of transcripts, polypeptides and posttranslational processes. In the case of thermophilic species, like zebrafish, goldfish and dace it was found that an increase in COX activity at low temperatures is not associated with increased expression of mitochondrial catalytic subunits, and is regulated at the level of transcription of some nuclear subunits involved in modulation of enzyme activity [9, 10]. In cold-tolerant fish species found in subarctic and arctic regions, the problem of COX subunits gene expression has been barely investigated. Also, the mechanism of regulation of mitochondrial reactions at low temperatures due to alteration of catalytic properties of isozyme systems is not completely understood. For example, dependence of the Michaelis–Menten constant on temperature for mitochondrial malate dehydrogenase (EC 1.1.1.37) has been studied chiefly for thermophilic fish. Herein, this dependence is defined only for a mixture of malate dehydrogenase isozymes from the mitochondrial fraction without isolation of its individual isoforms and differential determination of their properties [11]. Kinetic properties of isozymes of recently discovered mitochondrial lactate dehydrogenase (mtLDH, EC 1.1.1.27) have not been studied. The dependence on temperature was investigated only for cytoplasmic LDH [12].

The representatives of Salmonidae salmon family are cold-loving species and are adapted to a wide range of annual and daily temperatures. Optimal regulation of mitochondrial metabolism supports the needs in the required amount of ATP and determines the adaptive capacity and survival of salmonids, especially at low temperatures. In view of the foregoing, it is interesting and important to investigate the alteration of COX activity and gene expression level of its subunits *Cox1*, *Cox2*, *Cox4* and *Cox6*, as well as kinetic parameters of mtLDH isozymes in white muscle of one-year juvenile brown trout *Salmo trutta* L. from from rivers of Lake Onega basin in adaptation to the seasonal decrease in water temperature from 16 to 6°C.

RESULTS AND DISCUSSION

1. Alteration of Cytochrome c Oxidase Activity and Mitochondrial Volume

Cytochrome *c* oxidase (COX, EC 1.9.3.1.) is the most important enzyme of the mitochondrial respiratory chain (complex IV); it catalyzes the final step of electron transfer from cytochrome *c* to oxygen in the process of oxidative phosphorylation. COX is a complicated protein in terms of its structure and regulation. Cytochrome *c* oxidase usually exists in a dimeric form and is tightly associated with phospholipid molecules of membranes.

The results of the study on enzyme activity in white muscles of brown trout at water temperatures of 16, 10 and 6°C show 1.5-fold increase in COX activity in muscles at 10°C and also the maintenance of this parameter at 6°C (Fig. 1). At the same time, an increase in the mitochondrial volume by 24 and 28% was noted at 10 and 6°C, respectively, compared to 16°C.

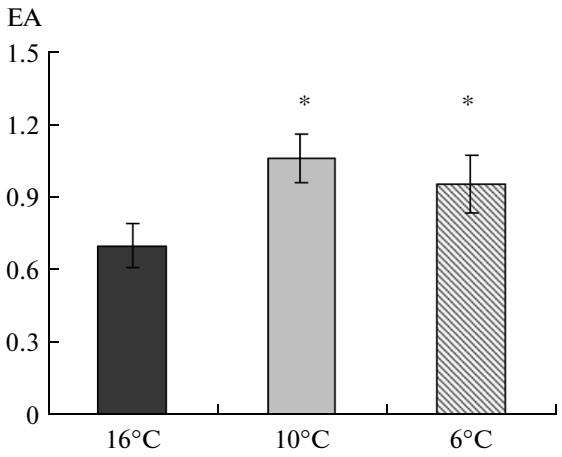

Fig. 1. The activity of cytochrome *c* oxidase (EA, *k*/g) in white muscle of brown trout caught at water temperature 16°C (July), 10°C (October) and 6°C (November), $M \pm m$. *Differences compared with the value at 16°C are significant at $p < 0.05$

COX activity differs in different fish species due to their biological, ecological features and phylogenetic position [13]. However, a reduction in the environmental temperature causes a common effect—an increase in COX activity, which is demonstrated for many fish species, irrespective of their temperature preferences [14–16]. The higher enzyme activity allows the compensation of the direct effect of low temperatures on the rate of metabolism in fish. An increase in the enzyme activity may occur due to alteration of the reaction rate, or changes in amount of the enzyme per gram of tissue. Cold adaptation in fish of arctic and temperate latitudes is accompanied by an increase in a number of mitochondria and the protein concentration per gram of tissue [15, 17, 18]. Since increased enzyme activity is associated with increased volume of mitochondria in brown trout, in this case one can say that the adaptive increase in enzyme activity is achieved in part by an increase in COX concentration.

2. Alteration of Gene Expression Level of Certain Cytochrome c Oxidase Subunits

Due to the functional differentiation of COX subunits, the study of the regulation of enzyme activity at the level of gene expression of different subunits is of great interest.

Analysis of COX1 and COX2 subunit gene expression, encoded by mitochondrial genome in muscle of

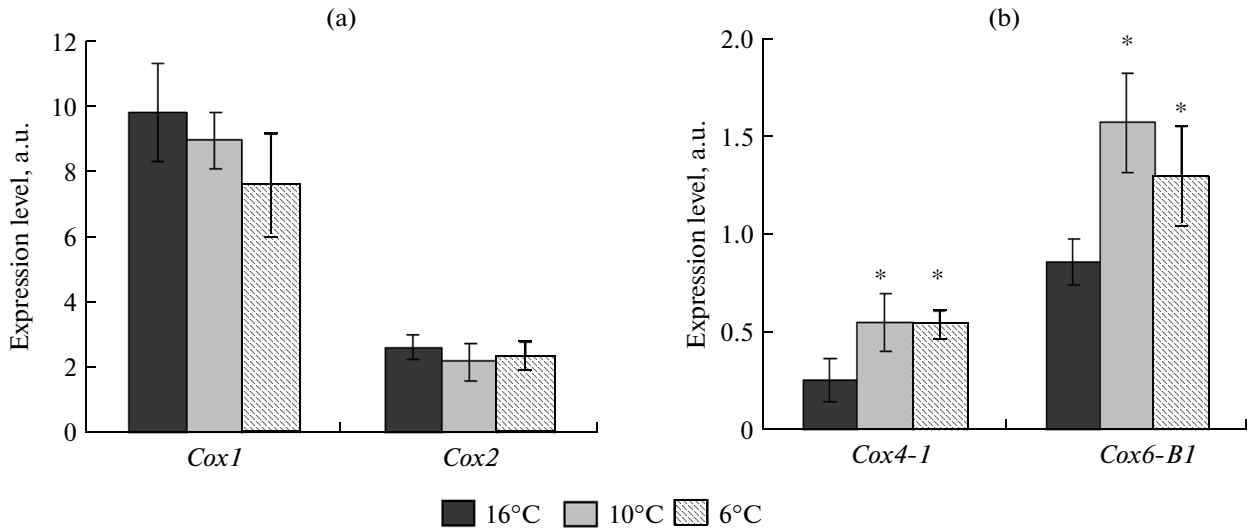

Fig. 2. The expression level of cytochrome c oxidase subunit genes *Cox1*, *Cox2* (a) and *Cox4-1*, *Cox6-B1* (b) in white muscle of brown trout caught at water temperature 16°C (July), 10°C (October) and 6°C (November), $M \pm m$. *Differences compared with the value at 16°C are significant at $p < 0.05$.

brown trout revealed two features: firstly, excessive *Cox1* gene transcription, compared with the expression level of the *Cox2* gene and nuclear subunit genes *Cox4-1*, *Cox6-B1*; and secondly, the absence of significant correlation with COX activity under decreasing temperature for both mitochondrial subunits (Fig. 2a).

In contrast to mitochondrial subunit genes, expression level of genes of nuclear subunits 4 and 6 are correlated with an increase in the enzyme activity and the number of mitochondria. Relative *Cox4-1* gene expression in white muscles of brown trout in October and November (10 and 6°C) increased 2 times compared with that in July (16°C). The level of *Cox6-B1* gene expression in autumn was 1.8 and 1.5 times higher, respectively, than in July (Fig. 2b).

It is known that subunits encoded by the mitochondrial genome are involved in catalysis and evolutionarily conservative [8]. They form the catalytic core, or "heart" of the enzyme, which is the structural and initiating complex. Heme *a* and binuclear center heme *a*3-CuB are linked with COX1 subunit. COX2 subunit includes CuA center and is involved in cytochrome *c* binding. We assume that overexpression of the *Cox1* gene and the absence of correlation with enzyme activity during cold adaptation of brown trout suggest that the amount of COX1 catalytic subunit transcripts is not a regulating factor for increasing the enzyme activity during cold, but it can be of some importance at the posttranslational level when the catalytic core of the enzyme is assembling.

Reasons for overexpression of COX1 gene subunit, encoded by the mitochondrial genome, have not yet been completely established, but a similar phenomenon was observed in studies in fish [14], as well as in mammals [19, 20]. A high level of gene expression may be associated with the necessity to maintain a certain concentration of corresponding polypeptide. It is known that during the assembly of oligomeric complexes, initiating subunits are generally present in excessive amounts that provide the beginning of molecule assembly. The catalytic subunit COX1 is the "priming" of COX molecules in this case [21]. The increase in its amount activates heme synthase and incorporation of heme *a*, and that is a key and leading event in the beginning of the enzyme assembly. This provides correct folding of COX1, stabilizing the membrane and the further formation of catalytic center of the molecule [22]. Perhaps, that is why this subunit should be present in sufficient quantity to provide a permanently accessible pool of initial elements for oligomer assembly.

Analysis of publications concerning the structure and function of the COX4 and COX6B1 subunits shows that these two nuclear subunits are expressed in white muscle in relatively small amounts compared with other subunits, but play an important role in the structure and activity regulation of the enzyme [23]. Subunit 4 (COX4) is one of the first nuclear subunits, included in the enzyme structure, forming a subassembly consisting of three subunits (1, 4 and 5) with heme *a* and binuclear center heme *a*3-CuB. In the next step, this complex interacts with COX2 subunit [20]. It was established that in mammalian cells free COX1 and COX 5 subunits are always present in excessive amounts, whereas the concentration of COX4 and COX2 is significantly lower. Our results were comparable: COX1 subunit overexpression, a relatively low expression of COX2, COX6 and, especially, COX4-1 in brown trout muscle at all investigated temperatures may indicate that assembly of the enzyme, regardless

of temperature, is regulated at the transcriptional level by expression limitation of the second, the sixth and, especially, the fourth subunits. Moreover, the observed correlation between the enzyme activity and the level of expression of subunits 4 and 6 also indicates the involvement of these polypeptide types in temperature dependent modulation of the enzyme activity in brown trout. Similar results, that are the correlation of COX activity with the amount *Cox4* mRNA and the absence of a relationship between COX1 and *Cox1* expression under decreasing water temperatures was shown previously in studies on goldfish and dace [9, 10].

Also, increased COX4 expression is thought to be targeted adaptation to changes in partial oxygen pressure in the cells of higher organisms [24]. Decreased temperatures cause an increase of capillarization in fish muscle tissue and oxygen diffusion into the cells that along with the enzyme activation leads to an increase in mitochondrial oxidative phosphorylation. It is found that oxygen is involved in regulation of COX4 and COX 5 nuclear subunit expression and COX1 and COX2 mitochondrial genes expression via signal transduction [8].

An increase in the expression level of the COX4 subunit can affect not only the enzyme assembly, but its activity. The regulating effect on enzyme activity is associated with the formation of an allosteric center for ATP binding in this subunit. At the same time, ATP molecules act as a negative modulator. The enzyme containing COX4-1 isoform of this subunit is activated by a low ratio of ATP/ADP, which is typical for metabolism at lower temperatures [5, 23].

COX6B subunit incorporates into the structure on the one of the last stages of enzyme assembling. It provides the formation of a unique contact site for dimerization of two complexes in the inner mitochondrial membrane and also binds cytochrome *c* substrate with participation of 3, 5B and 6A subunits [5, 23, 25]. We observed an almost two-fold increase in *Cox6-B1* gene expression under decreasing temperatures in brown trout; which indicates the participation of this subunit in the regulation of enzyme activity by increasing the amount of active dimers.

3. The Activity and Kinetic Characteristics of mtLDH Isozymes

Tetraploidy, which emerged as a result of the evolutionary process, contributed to the formation of a special adaptive capacity in salmonids. Doubling of gene loci, in comparison with diploid species, led to the formation of a large set of subunit and/or isozyme variants, including mitochondrial ones differentiated in their catalytic and kinetic characteristics. Lactate dehydrogenase (LDH, EC 1.1.1.27, L-lactate: NAD^+-oxidoreductase) is an enzyme catalyzing one of the major reactions of common metabolic pathways of the cells, the interconversion of pyruvate and lactate. In fish and higher animals, LDH is presented mainly in the form of five isozymes: homo- or heterotetramers, composed of A and B subunits. Salmonids and other tetraploid fish species express two times more subunits: A', A'', B', and B'', which increase their isozymes set up to 14–16. Isozymes containing A subunits predominantly catalyze reduction of pyruvate, while isozymes containing B subunits mainly catalyze oxidation of lactate. Previously, it was thought that the enzyme is present only in cytoplasm, but now the existence of the mitochondrial lactate oxidation complex (mLOC) was proved for the cells of skeletal muscles, heart, and neurons [26, 27]. It was found that it consists of membrane-bound mtLD, cytochrome *c* oxidase, lactate transporter protein MCT-1 and its chaperone OX-47 (CD-147), which control its expression. Pyruvate produced during the oxidation of lactate incoming into mitochondria is transferred to the mitochondrial matrix via MCT-1 and oxidized there in the tricarboxylic acid cycle (TCA)

Analysis of LDH isozyme spectrum of the white muscle mitochondrial fraction in brown trout identified preferably homo- and hetero isozymes of B group: B'_4 (R_f = 0.68), B'_3B'' (0.60), $B'_2B''_2$ (0.43), $B'B''_3$ (0.34) and B''_4 (0.25). Isozymes containing the A' and A'' subunits were represented in the mitochondrial fraction in trace amounts. Investigation of B group isozyme activity at 6 and 16°C shows their dependence on temperature (Fig. 3). During the warm period (16°C) the highest activity was shown for B'_4 isozyme. Its summer activity was 1.5 times higher than in autumn. B'_3B'' and $B'_2B''_2$ hetero isozymes showed no difference in activity when two temperatures were compared. The activity of $B'B''_3$ and B''_4 isozymes significantly increased when adapting to low temperatures (6°C). Wherein, the total mtLDH activity at 6°C was just 10% higher compared with the activity at 16°C.

Determination of the apparent Michaelis–Menten constant (K_M) showed that the studied B group isozymes are differentiated by affinity towards the substrate (Fig. 3). The B'_4 isozyme possesses the highest affinity to lactate, i.e., it is saturated with small amounts of the substrate. The K_M value for B''_4 isozyme is three times higher; it means that it works and reaches a maximum rate at higher lactate concentrations.

Based on obtained K_M values and activities of the isozymes during cold adaptation in brown trout, one can say that an increase in activity of isoforms containing the B" subunits with low affinity to lactate provides conditions to increase in amount of oxidizable lactate and enhance formation of pyruvate without significantly increasing the amount of the enzyme. Selective activation of mtLDH isozymes with necessary kinetic properties in brown trout under decreasing temperature, probably, is aimed at increasing the rate of mitochondrial oxidative phosphorylation through

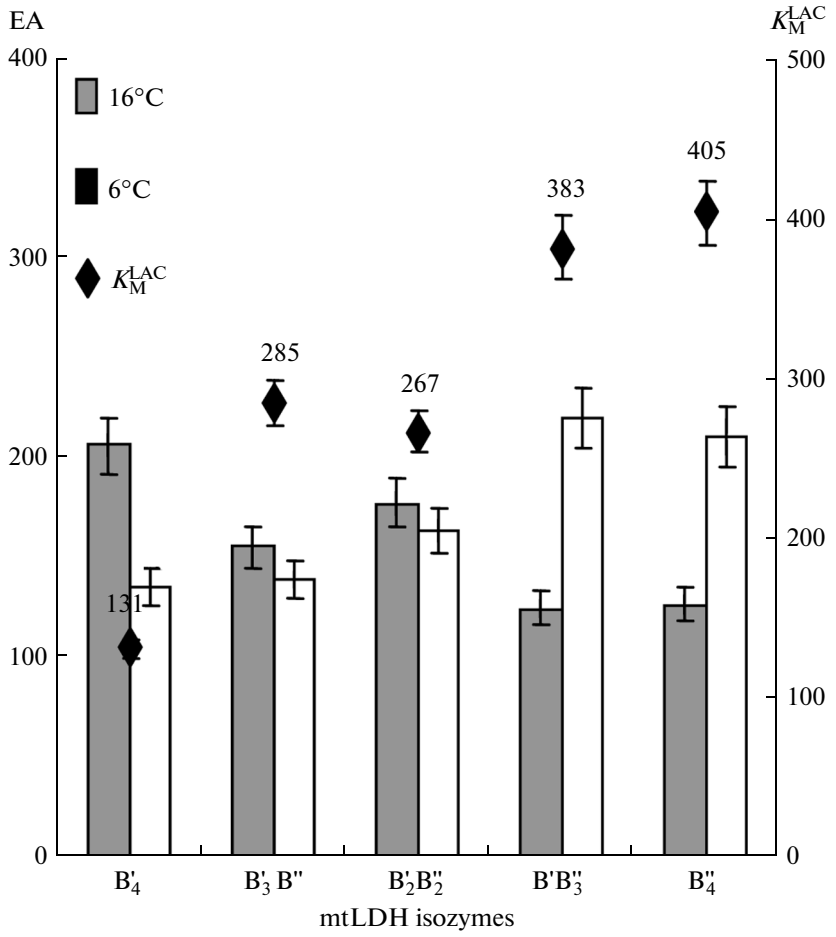

Fig. 3. On the left scale: Activity (EA) of mtLDH isozymes (group B) in white muscle of brown trout at 16°C (July) and 6°C (October) in μmol of lactate/min/g of tissue, $M \pm m$. *Differences are significant at $p < 0.05$. On the right scale: value of the apparent Michaelis–Menten constant for lactate (K_M^{LAC}) for the same isozymes measured at 20°C.

the formation of the necessary pyruvate pool, which is a substrate for the Krebs cycle. Our assumptions are confirmed by the results of this study on the increase of COX activity, as well as data on mLOC, which shows that mtLDH is located on the outer side of the inner mitochondrial membrane and associated with COX [26, 27]. This compartmentalization of the studied enzymes provides a coupling of endergonic lactate oxidation reaction and exergonic change of redox potential in the mitochondrial electron transport chain during oxidation of cytochrome *c*.

Thus, the behavior of mitochondrial parameters investigated suggests a complicated and multilevel system of mitochondrial metabolism regulation in brown trout during adaptation to cold. Increased activity of COX and features of *Cox1*, and *Cox2* catalytic subunit and *Cox4* and *Cox6* nuclear subunit gene expression indicate that required enzyme efficiency may be regulated at the stages of molecular biogenesis by a change in amounts of different subunit types, and further modulation of the enzyme activity by COX4 participation in its allosteric regulation. The established relationship between an increase in COX activity, activity of $B'B''_3$ and B''_4 isozymes and low affinity to lactate suggests regulation of mitochondrial metabolism by activation of the isozymes with the required catalytic properties. It confirms the hypothesis about the existence of a coordinated mechanism of interaction of these two enzymes under ATP deficiency, which occurs at low temperatures. The ability to maintain the energy status at appropriate level and the possibility of quick adjustment under alterations of water temperatures are essential for the adaptation and survival of juvenile salmonids during selection of the optimal habitat, and further realization of the growth and development strategy.

CONCLUSIONS

1. It was found that during seasonal adaptation to cold of juvenile brown trout (16°C → 6°C) there occurs an increase in the number of mitochondria by

28% and the activity of cytochrome *c* oxidase, which compensates for the direct effect of low temperatures on mitochondrial metabolism rate.

2. The analysis of *Cox1*, *Cox2*, *Cox4*, and *Cox6* subunit gene expression level shows that expression of *Cox1* and *Cox4* catalytic subunit genes is not dependent on temperature, wherein the amount of *Cox1* mRNA significantly exceeds the amount of other subunit gene transcripts. Unlike catalytic subunits, *Cox4* and *Cox6* nuclear subunit gene expression level correlates with an increase in the enzyme activity under decreasing temperature.

3. Low temperature adaptation of juvenile brown trout is accompanied by a significant increase in the activity of mtLDH B'B$_3^{''}$ and B$_4^{''}$ isozymes with low affinity to lactate, which indicates an increase in oxidation of lactate to pyruvate at low temperature.

EXPERIMENTAL

Reagents. Tris(hydroxymethyl) aminomethane (MP Biomedicals, France), hydrochloric acid (NevaReaktiv, Russia), EDTA (PanReac, Spain), heparin (Applichem, Germany), sucrose (NevaReaktiv, Russia) (PanReac, Spain), Triton X-100 (Acros, Germany), cytochrome *c* (Serva, Germany), sodium phosphate dibasic (NevaReaktiv, Russia) (PanReac, Spain), sodium phosphate monobasic (NevaReaktiv, Russia) (PanReac, Spain), ascorbic acid (Vekton, Russia) (PanReac, Spain), glycine (Applichem, Germany), sodium hydroxide (NevaReaktiv, Russia), D-,L-lactic acid sodium salt (Dia-M, Russia), NAD (Applichem, Germany), imidazole (PanReac, Spain), acrylamide (NevaReaktiv, Russia), *N,N'*-methylenebisacrylamide (Vekton, Russia), ammonium persulfate (Khimreactiv, Russia), riboflavin (Vekton, Russia), phenazine methosulfate (Applichem, Germany), nitrotetrazolium blue (Vekton, Russia), ExtractRNA (Evrogen, Russia), DNase (10 U/mL) (Sileks, Russia), a set of reagents M-MLV RT kit (Evrogen, Russia), qPCRmix-HS SYBR (Evrogen, Russia).

Equipment. High speed refrigerated centrifuge Rotina 35R; (Hettich, Germany), spectrophotometer SF-2000 (OKB Spectr, Russia), electrophoretic chamber (Reanal, Hungary), thermocycler Tercyc (DNA Technology, Russia), Real-Time PCR Detection System i-Cycler iQ5 (BioRad, United States)

Isolation of mitochondria. Mitochondria were isolated from the organs of freshly slaughtered fishes using the common method of differential centrifugation in 0.01 M Tris-HCl buffer (pH 7.4) supplemented with 1 mM of EDTA and 2 mM of heparin containing 0.25 M sucrose (buffer A). All the steps of mitochondria isolation were performed at a temperature of 4°C. The extracted tissue or organ (about 1 g) was homogenized. The resulting homogenate was centrifuged for 10 min at 2500 *g*. The precipitate containing cell debris, nuclei, and myofibrils was discarded and the supernatant was centrifuged for 10 min at 10000 *g*. The resulting precipitate containing mitochondria was washed with 2 mL of buffer A, centrifuged four times for 10 min at 10000 *g*, discarding the supernatant each time. Extraction of mitochondrial enzymes was performed by twofold volume (relative to the volume of mitochondria precipitate) 0.01 M Tris-HCl buffer (pH 7.5) containing 0.1% of Triton X-100. Enzymatic control of the isolated mitochondrial fraction was performed by the determination of cytochrome *c* oxidase by the Smith method [28].

The activity of cytochrome *c* oxidase (EC 1.9.3.1) was determined by measuring the increase in the amount of oxidized cytochrome *c* [28]. To obtain the reduced form of the substrate, 15 mg of cytochrome *c* and 30 mg of ascorbic acid were dissolved in 1 mL of 0.1 M phosphate buffer for 2 hours. The reduced cytochrome was purified from the excess of ascorbic acid on Sephadex G-25 column (15 mm x 400 mm) in 0.02 M phosphate buffer. For 1 mL of reduced cytochrome the volume of purified reduced cytochrome eluate was 5 mL at a rate of 5 mL/min. Enzyme activity was expressed as *k*/g of tissue (*k* is the first order constant of the reaction). Measurement of absorbance was performed at 550 nm. One unit of activity was defined as the amount of the enzyme that oxidizes 1 mol of cytochrome *c* in 1 min per 1 g tissue at 20°C.

Determination of the activity and K_M for lactate dehydrogenase isozymes. The total mitochondrial lactate dehydrogenase activity was determined by the rate of lactate → pyruvate reaction using common method [29]. The concentrations of the reagents: glycine buffer, 0.1 M; pH 10.0; lactate 0.5 M; NAD, 20 mM; pH 6.0; and enzyme 0.3 U/mL. Alterations of absorbance at 340 nm over 2–3 min on a spectrophotometer SF-2000 were measured. One unit of the activity was defined as the amount of the enzyme which catalyzes the conversion of 1 μmol of lactate substrate in 1 min per 1 g tissue at 20°C.

mtLDH isozymes were purified on 6% cylindrical PAG in imidazole/veronal buffer at pH 7.8 in a vertical preparative electrophoresis chamber according to Richards [30]. The amount of extract loaded on the gel was 25 mL. Electrophoresis scheme: 150 V for 10 min, 250 V for 10 min and 400 V for 3 h. Staining of the isozymes was performed using the tetrazolium method. The relative electrophoretic mobility of the isozymes (R_f) was calculated by measuring the movement from the anode to the cathode.

The activity of each isozyme was determined as a percentage of the total enzyme activity in the mitochondrial fraction on the densitogram using a densitometry method, allowing the evaluation of the area of the colored zone of isozyme on PAG. The activity of each isozyme was calculated as μmol of lactate/min/g of tissue based on the value of the total activity of mtLDH, determined spectrophotometrically.

Table

Gene	Nucleotide sequences (5'-3')	Annealing temperature, °C	GenBank identifier
Cox	Forward: CCCAGCCATCTCCCAATATC Reverse: TTCGGTCTGTGAGTAGCATAG	57	EF609450.1
Cox	Forward: CCAGGCCAATTCCGTCTTCT Reverse: TACTCCAGGTCGAGAGGCAA	57	HQ167688
Cox4-1	Forward: TCAATCTGTGTACGTGGGGC Reverse: CACAAACTGGACGTCTGGGA	60	BT043749
Cox6B-1	Forward: ATTGAGGAGAAGATAAAGAACTAC Reverse: GGACAGATACTCTTGTAGACC	58	BT073270.1
Ef-1	Forward: GGTGGTGTGGGTGAGTTTGAG Reverse: CAGGCGATGTGAGCAGTATG	57–60	EF406271.1

To determine the apparent Michaelis–Menten constant, each isozyme was isolated from the gel, extracted with 0.01 M Tris-HCl buffer (pH 7.5) and purified on a Sephadex G-25 column (15 mm × 500 mm) in 0.1 M glycine buffer (pH 9.5). For 1 mL of reduced cytochrome the volume of purified reduced cytochrome eluate was 6 mL at a rate of 5 mL/min, the isozyme concentration was 0.45 U/mL. Determination of lactate → pyruvate reaction rate was performed at 20°C for different concentrations of lactate from 10 μM up to 1 mM (step 30 μM) by standard method. [29] The concentrations of the reagents: glycine buffer, 0.1 M; pH 10.0; NAD, 20 mM; pH 6.0; and the isozyme, 0.3 U/mL. The value of the apparent Michaelis–Menten constant (K_M^{LAC}) was calculated by the Cornish–Bowden method [31].

Determination of gene expression level for cytochrome *c* oxidase subunits. The level of gene expression was determined by real-time PCR. Total RNA was isolated from brown trout white muscle with an ExtractRNA reagent. RNA isolation was performed according to the protocol of the manufacturer. Total RNA was treated with DNase (10 U/mL). Complementary DNA (cDNA) was synthesized from total RNA using M-MLV reverse transcriptase and random hexanucleotides. Amplification was performed using qPCRmix-HS SYBR master mix. Primers for the nucleotide sequences of cytochrome *c* oxidase subunit genes and reference elongation factor (Ef-1) gene were selected using the Beacon Designer 5.01 (Premier Biosoft, United States) (table). Due to the absence of the nucleotide sequences for *Cox4-1* and *Cox6-B1* genes of brown trout in the database, the primers for genes of salmon (*Salmo salar* L.) and trout (*Oncorhynckus mykiss* Walb.) were selected. PCR protocol: denaturation of DNA at 95°C for 5 minutes; repeated cycles (45): DNA denaturation at 95°C for 20 s, primer annealing for 30 s at the temperature indicated in the table, DNA elongation at 72°C for 30 s. After PCR, melting of DNA fragments (from 59 to 95°C, step 0.5°C) was performed. The level of studied gene transcripts was calculated relative to the transcript level of the reference gene *Ef-1*. The relative expression level of the tested gene (Ratio) is calculated by the formula [32]:

$$\text{Ratio (test/ref)} = 2^{-\Delta Ct}, \text{ where}$$
$$\Delta Ct = Ct\ (test) - Ct\ (ref),$$

where Ct (test) is the threshold cycle for the tested gene; and Ct (ref), the threshold cycle for the reference gene.

Statistical data processing. Standard methods of statistical data processing using the StatGraphics 2.5 for Windows and MS Excel software packages were used. Sample comparison was performed using the nonparametric Wilcoxon–Mann–Whitney test. The influence of the studied factors was assessed using multivariate analysis of variance MANOVA

ACKNOWLEDGMENTS

The study was supported by the Russian Science Foundation (project no. 14-24-00102).

REFERENCES

1. White, C.R., Alton, L.A., and Frappell, P.B., *Proc. Biol. Sci.*, 2012, vol. 279, pp. 1740–1747.
2. Somero, G., *Comprehensive Physiology*, Published Online: JAN 1, 2011. http://onlinelibrary.wiley.com/doi/10.1002/cphy.cp130219/
3. Boldyrev, A.A., *Soros. Obrazovat. Zh.,* 1997, no. 6, pp. 21–27.
4. Capaldi, R.A., *Annu. Rev. Biochem.*, 1990, vol. 59, pp. 569–596.
5. Kadenbach, B. Huttemann, M., et al., *Free Radic. Biol. Med.*, 2000, vol. 29, pp. 211–221.
6. Carr, H.S., *Acc. Chem. Res.*, 2003, vol. 36, pp. 309–316.
7. Richter, O.M. and Ludwig, B., *Rev. Physiol. Biochem. Pharmacol.*, 2003, vol. 147, pp. 47–74.
8. Fontanesi, F., 1st., Soto, I.C., Horn, D., and Barrientos, A., *Am. J. Physiol. Cell. Physiol.,* 2006, vol. 291, pp. 1129–1147.

9. LeMoine, C.M., Genge, C.E., and Moyes, C.D., *J. Exp. Biol.*, 2008, vol. 211, pp. 1448–1455.
10. Duggan, A.T., Kocha, K.M., Monk, C.T., Bremer, K., and Moyes, C.D., *J. Exp. Biol.*, 2011, vol. 214, pp. 1880–1887.
11. Dong, Y. and Somero, G., *J. Exp. Biol.,* 2009, vol. 212, pp. 169–177.
12. Somero, G.N., *Comp. Biochem. Physiol., B: Biochem. Mol. Biol.*, 2004, vol. 139, pp. 321–333.
13. Meshcheryakova, O.V., Churova, M.V., and Nemova, N.N., *Trudy Karel. Nauch. Tsentra RAN*, 2013, no. 3, pp. 136–142.
14. Battersby, B.J. and Moyes, C.D., *Am. J. Physiol.*, 1998, vol. 275, pp. 905–912.
15. Hardewig, P., van Dijk, M., Moyes, C.D., and Portner, H.O., *Am. J. Physiol. Regulat. Integrat. Comp. Physiol.*, 1999, vol. 277, no. 2, pp. 508–516.
16. Lucassen, M., Koschnik, N., Eckerle, L., and Portner, H., *J. Exp. Biol.*, 2006, vol. 209, pp. 2462–2471.
17. Guderley, H., *Comp. Biochem. Physiol. B: Biochem. Mol. Biol.*, 2004, vol. 139, pp. 371–382.
18. O'Brien, K.M., *J. Exp. Biol.*, 2011, vol. 214, pp. 275–285.
19. Williams, R., Salmons, S., Newsholme, E., Kaufman, R., and Mellor, G., *J. Biol. Chem.,* 1996, vol. 261, pp. 376–380.
20. Stiburek, L., Hansikova, H., Tesarova, M., Cerna, L., and Zeman, J., *Physiol. Res.*, 2006, vol. 55, Suppl. 2, pp. 27–41.
21. Fontanesi, F., Soto, I.C., and Barrientos, A., *IUBMB Life*, 2008, vol. 60, pp. 557–568.
22. Hyung, J., Khalimonchuk, O., Smith, P., and Winge, D., *Biochim. Biophys. Acta*, 2012, vol. 1823, pp. 1604–1616.
23. Little, A.G., Kocha, K.M., Lougheed, S.C., and Moyes, C.D., *Physiol. Genomics*, 2010, vol. 42, pp. 76–84.
24. Huttemann, M., *Biochim. Biophys. Acta,* 2000, vol. 1492, pp. 242–246.
25. Ugalde, C., Coenen, M.H., Farhoud, S., Gilinsky, S., Koopman, W.J., Heuvel, L.P., Smeitink, J.A., and Nijtmans, L.G., *Mitochondrion*, 2002, vol. 2, pp. 117–128.
26. Hashimoto, T. and Brooks, G.A., *Med. Sci. Sport Exerc.*, 2008, vol. 40, no. 3, pp. 486–494.
27. Hashimoto, T., Hussien, R., Cho, H.S., Kaufer, D., and Brooks, G.A., *PLoS ONE*, 2008, vol. 3, p. 2915.
28. Smith, L., *J. Methods Biochem. Analysis,* 1955, vol. 2, no. 427, pp. 427–434.
29. Kochetov, G.A., in *Prakticheskoe rukovodstvo po enzimologii* (A Practical Guide to Enzymology), Moscow: Vysshaya shkola, 1980.
30. Maurer, H.R., *Disc Electrophoresis and Related Techniques of Polyacrylamide Gel Electrophoresis*, Berlin: Walter de Gruyter, 1971.
31. Cornish-Bowden, A., *Fundamentals of Enzyme Kinetics,* London: Portland Press, 1976.
32. Livak, K.J. and Schmittgen, T.D., *Methods*, 2001, vol. 25, pp. 402–408.

Translated by D. Novikova