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Research paper

Alternative splicing generates two lactate dehydrogenase subunits differentially expressed during hypoxia via HIF-1 in the shrimp *Litopenaeus vannamei*

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ABSTRACT

Metabolic adjustment to low oxygen exposure (hypoxia) in the white shrimp *Litopenaeus vannamei* implies a shift to anaerobic metabolism. Lactate dehydrogenase (LDH) is a key enzyme of the anaerobic metabolism described in most organisms. The structure and expression of the LDH gene, as well as the LDH isoenzymes in marine crustacean are not well defined. In the present study we characterized a gene that codes for two LDH subunits, measured their expression and detected the isoenzymes in tissues from white shrimp. We also silenced the transcriptional activator hypoxia inducible factor 1 (HIF-1) to elucidate the regulation of LDH in tissues from white shrimp in response to hypoxia. The complete LDH gene coding sequence is 7571 bp (*LvanLDH*) and encodes two different LDH subunits (LDHvan-1 and LDHvan-2) generated by alternative splicing and composed of 332 amino acids with conserved domains important for the function and regulation. Phylogenetic analysis shows that LvanLDH -1 and -2 are closer to the invertebrate counterparts. The LDHvan-1 transcript increased 2.5-fold after hypoxia in gills but not in hepatopancreas, while the LDHvan-2 transcript decreased 14-fold in muscle but not in gills and hepatopancreas. Three bands with LDH activity of ~60–90 kDa were detected in hepatopancreas, while one band of ~140 kDa was detected in gills and muscle. The silencing of HIF-1 blocked the increase of LDH mRNA and activity produced by hypoxia in gills. These results demonstrate a single gene for LDH (*LvanLDH*) that by alternative splicing generates two different LDH subunits (LDHvan-1 and LDHvan-2) that are expressed in a tissue-specific manner during hypoxia via the HIF-1 pathway.

1. Introduction

Accumulation of lactate as an end product of anaerobic glycolysis in the white shrimp *Litopenaeus vannamei* during low oxygen (hypoxia) is well known [1–3]. Lactate dehydrogenase (L-Lactate: NAD oxidoreductase, E.C. 1.1.1.27; LDH) produces lactate from pyruvate in the last step of anaerobic glycolysis. LDH enzymes belong to a family of highly conserved proteins and have been used as a model to investigate molecular evolution of isoenzymes [4]. The vertebrate LDHs are tetramer of ~140 kDa formed by four subunits of 34 kDa coded by three independent genes, *LDH-A* (muscle), *LDH-B* (heart) and *LDH-C* (testis) [5]. Each LDH isoenzyme exhibits distinct tissue expression, kinetic, physicochemical and immunochemical properties [5]. The LDHs found in *Homo sapiens* are the LDH-5 (also called A or M_4) present in anaerobic tissues as skeletal muscle and liver, and LDH-1 (also called B or H_4) present mainly in aerobic tissues as cardiac muscle. A third type found mainly in testes and sperm is known as the X or C type and is formed by type C subunits. The LDH subunits can form homotetramers and heterogeneous tetramers such as MH_3 (LDH-2), M_2H_2 (LDH-3) and M_3H (LDH-4) [4].

The invertebrate LDH is not yet well characterized. D (−) or L (+) lactic acid specific LDHs have been found in invertebrate tissues [6–8] and all LDHs from crustaceans appear to be also tetrameric proteins [9–14] but interestingly, some dimeric forms are also active. For example, the LDH obtained from the tail muscle from *Homarus americanus* can be active as dimer or tetramer depending on ionic conditions [9,15]. The crustacean LDHs enzymes are usually represented by a single gene–enzyme system [10,14,16,17], but multiple forms of LDH are found in the lobster *H. americanus*, king crab *Paralithodes camtschatica*, the northern krill *Meganyctiphanes norvegica*, the Antarctic krill *Euphausia superba* and the snow crab *Chionoecetes opilio* [9,18–23].

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The transcriptional activator, hypoxia inducible factor 1 (HIF-1) is a global regulator of oxygen homeostasis that controls hundreds of target genes, including erythropoietin (EPO), vascular endothelial growth factor (VEGF) and proteins associated with glucose and energy metabolism [24]. In mammals exposed to hypoxia, HIF-1 is the main activator of the LDH gene [25]. HIF-1 is a heterodimer composed of one regulatory α-subunit (HIF-1α) and a constitutive β-subunit (HIF-1β/ARNT) [26]. During hypoxia, HIF-1α is stabilized, translocated into the nucleus, bound to HIF-1β and then, it binds to hypoxia-responsive elements (HREs) up-regulating transcription. In shrimp, we characterized the cDNA sequence of both HIF-1 subunits and showed that are differentially expressed in gills, hepatopancreas and muscle during normoxia (5 mg/L O_2) and hypoxia (2.5 and 1.5 mg/L O_2) [2]. Moreover, the silencing of α or β subunits affected the glucose and lactate concentration in hemolymph and gills [3], and the expression and activity of hexokinase in shrimp exposed to hypoxia [27]. These results suggest that the metabolic responses of shrimp to hypoxia are mediated by the HIF-1 pathway.

The accumulation of lactate in shrimp tissues after exposure to hypoxia reveals the participation and importance of LDH. Although this enzyme has been studied for decades in vertebrates, very little is known about the structure, regulation, subunits and isoenzymes in penaeid shrimp. In the present study, the complete coding sequence of the LDH gene that codes for two LDH subunits was obtained. The mRNA levels were quantified in gills, hepatopancreas and muscle from shrimp after exposure to hypoxia. Also, the two full-length LDH cDNA sequences encoded by the LDH gene were used for phylogenetic analysis. Furthermore, the LDH isoenzymes were detected in gills, hepatopancreas and muscle from shrimp. Finally, the hypoxia inducible factor 1 (HIF-1) was silenced to investigate the regulation of the LDH gene via HIF-1 quantifying by RT-qPCR the mRNA and the LDH activity in tissues from shrimp exposed to hypoxia. The white shrimp is able to withstand large changes in environmental conditions, making it an interesting model to understand the physiological mechanisms by which, marine crustaceans support hypoxia.

2. Materials and methods

2.1. LDH gene characterization and tissue-specific expression during hypoxia

The complete LDH gene sequence was obtained from genomic DNA (gDNA) using primers designed with the Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) [28] (Table 1) and based on cDNAs sequences for LDH as described below. The gDNA was isolated from 2 g of muscle using proteinase K digestion, repeated phenol–chloroform extraction and precipitation with cold ethanol [29]. The genomic DNA was used for PCR. The first PCR fragments of the LDH gene were obtained using the following conditions. For a 25 μL final volume reaction, 22 μL of Platinum PCR SuperMix (Invitrogen, Carlsbad, CA, USA), 100 ng of gDNA, 1 μL (20 μM) of each primer (for the 5′-end, LDHMinFw + LDHrtMRv and for the 3′-end, LDH2CBRTFw + LDH2CBRv) were mixed and subjected to the following conditions: 75 °C for 5 min for 1 cycle; 94 °C for 3 min for 1 cycle; 36 cycles of 94 °C for 30 s, 57–60 °C for 1 min and 68 °C for 4 min; and an overextension step of 68 °C for 7 min. PCR fragments of ~4000 bp towards the 5′-end (*LDHvan-G5*') and ~3000 bp towards the 3′-end (*LDHvan-G3*') were obtained, sequenced and identified as LDH by comparison against the cDNA sequences and GenBank data. The complete sequence of the LDH gene (named *LvanLDH*) was obtained by overlapping all PCR products. The *LvanLDH* sequence was compared to the cDNAs sequences (see below) to identify the exons and introns and this led to the identification of two different LDH subunits – LDHvan-1 and LDHvan-2. Both strands of the PCR fragments were thoroughly sequenced.

Table 1
Primers used for LDH and L8.

Primer name	Nucleotide sequences (5′-3′)
LDH2CBRTRv	AACACATCCTTGTCGATAC
LDH2CBRv	CTGGCCTTTAGGCACTC
LDH2rtRv	CATTGTTGCATCACGCGCCTTA
LDHCBRTFw	CATTGTTGCATCACGCGCCTTA
LDHGrRv	ACAAGGGACAAACGAGACTCTC
LDHBL3Fw	GAGAGTCTCGTTTGTCCCTTGT
LDHMinFw	ATGGCCTCTGTTCCTGAAATG
LDHrtMRv	GTAGACACGGCGTAAACAGTA
LDH2CBRTFw	ATGAAGGGATACACTTCAT
LDH2rtFw	CCATGGTATCGACAAGGATG
LDHrtBHFw2	GTCGCCTCTCTGGTGCAT
LDHrtMFw3	GTGCTGTTCTAACCAGGG
L8F2	TAGGCAATGTCATCCCCATT
L8R2	TCCTGAAGGAAGCTTTACACG

Ten shrimp were kept in individual fiberglass tanks (150-L) and sampled during normoxia (5.5 ± 1.2 mg/L O_2) and hypoxia by 1 h (1.5 ± 0.1 mg/L O_2) as described previously [3] to investigate tissue-specific expression of LDHvan -1 and -2. Fixed ratios of nitrogen gas and air were used to obtain the experimental hypoxia. Dissolved oxygen and temperature were monitored every 1 h during all the experiments using a YSI Model 55D dissolved oxygen meter (Geo Scientific Inc, Vancouver, CAN). Gills, hepatopancreas and muscle were collected, frozen by immersion in liquid nitrogen and stored at −80 °C until analyzed. Total RNA was isolated individually from shrimp tissues using TRIzol (Invitrogen, Carlsbad, CA, USA) following the manufacturer instructions. RNA integrity was confirmed by measuring the absorbance at 260 nm/280 nm and by 1% agarose gel electrophoresis [30]. Contamination of genomic DNA in total RNA was eliminated by digestion with DNase I (Roche, Indianapolis, IN, USA), as specified by the manufacturer. Two separate cDNAs from each tissue were synthesized from total DNA-free RNA (1 μg) using oligo-dT and the QuantiTect Reverse Transcription kit (Qiagen, Valencia, CA, USA).

Specific primers for LDHvan-1 and LDHvan-2 were designed and validated by PCR using specific clones from each LDH subunits. The expression of the ribosomal protein L8 (DQ316258) was used as an internal standard to compare LDH expression data. LDHvan-1, LDHvan-2 and L8 mRNA were measured by quantitative RT-qPCR using the LDHrtBHFw2 + LDH2rtRv, LDHrtMFw3 + LDH2rtRv and L8F2 + L8R3 primers, respectively. Two separate cDNA reactions and two PCR reactions for each individual shrimp and tissue were run in an iQ5 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) in reactions containing 10 μL of iQ SYBR Green Supermix (Bio-Rad), 6 μL of H_2O, 0.5 μL of each primer (20 μM) and 3 μL of cDNA (equivalent to 150 ng of total RNA). The amplifications were done using the following conditions: 95 °C for 5 min; 40 cycles at 95 °C for 30 s, 62 °C for 1 min and 72 °C for 40 s, with a single fluorescence measurement and a final melting curve program increasing 0.3 °C each 20 s from 60 °C to 95 °C. Positive and negative controls were included and the expression levels (ng/μL) were normalized to L8 and expressed as relative values (LDH/L8).

2.2. LDH cDNAs cloning and characterization

The complete cDNAs sequences for the LDH subunits encoded by the *LvanLDH* gene were obtained using cDNAs libraries constructed previously in the SMART cDNA Library Construction Kit (Clontech, Mountain View, CA, USA) and ZAP Express XR library