The cDNA sequence of the transferrin gene in tongue sole (Cynoglossus semilaevis) and its transcriptional changes under acute hypoxic stress

Zi-Sheng Wang,1,2 Zh-Tao Qi,1,3 Qi-Huan Zhang,3 Ming Qiu,1,3 Jin-Tian Huang,1 Wei-Hong Zhao,1 Xi-Jie Guo1

1Department of Ocean Technology, Yancheng Institute of Technology, Yancheng, China
2School of Biology and Chemical Engineering, Jiangsu University of Science and Technology, Zhenjiang, China
3Chemical and Biological Engineering College, Yancheng Institute of Technology, Yancheng, China

Abstract

In this study, the cDNA sequence of the transferrin (Tf) gene in tongue sole (Cynoglossus semilaevis) (CsTf) was identified and characterised. The full length of CsTf cDNA was 2281 bp encoding 677 amino acids (aa). The mature CsTf protein was found to be made up of 656 aa and it consisted of two lobes (N- and C-lobes). Two- and three- dimensional (2-D and 3-D) structure analysis showed that CsTf possesses a similar structure (multi-α helix)/β-sheet to human Tf. Real-time polymerase chain reaction (PCR) analysis showed that CsTf was mainly expressed in the liver, and moderately expressed in the heart, spleen and kidney. Moreover, CsTf was up-regulated in different tissues under acute hypoxia [dissolved oxygen (DO)=0.8 mg/L]. Our results suggest that CsTf plays a role in fish’s adaptation to hypoxic stress.

Introduction

Aquatic hypoxia has become a complex ecological phenomenon because of the increasing industrialisation and urbanisation of coastal zones (Wawrowski et al., 2011). Research has shown an increasing amount of genes that are down- or up-regulated under hypoxia. These genes are mainly involved in maintaining iron homeostasis, energy levels and muscle structure (Wulff et al., 2012).

Transferrin (Tf) is one of the members of a super-family of iron-binding proteins. It is expressed mainly in the liver and is also found in other tissues, including the brain, testes, ovary, spleen, mammary gland and kidney. Transferrin possesses the typical structure of the Tf family, which includes a N-terminal domain (N-lobe) and a C-terminal domain, connected by a short hinge region. Under hypoxia, mammalian Tf is up-regulated, thus demonstrating that it plays a role under hypoxic stress (Wenger et al., 1995). The exact molecular mechanism of hypoxic enhancement of Tf expression was elucidated by Rolfs et al. (1997). They found that the Tf promoter contained two hypoxia-inducible factor-1 (HIF-1) binding sites (HBSs), which could be bound by HIF-1 to confer oxygen regulation of Tf expression.

To date, most research on Tf has been carried out on several fishes, including Atlantic salmon (Salmo salar) (Kvingedal, 1994), Atlantic cod (Gadus morhua) (Denovan-Wright et al., 1996), rainbow trout (Oncorhynchus mykiss) (Tange et al., 1997), mrigal carp (Cirrhinus mrigala) (Sahoo et al., 2009) and roughskin sculpin (Trachidermus fasciatus) (Liu et al., 2012). The fish Tf gene is very similar to mammalian Tf and is mainly expressed in the liver or brain too. Fish Tf serves as a cadmium transport protein (De Smet et al., 2001) and activator of immune response of macrophages (Stafford et al., 2001; Jurecka et al., 2009). However, little is known regarding the response of Tf under hypoxia in fish.

The tongue sole (Cynoglossus semilaevis) is an important marine aquaculture species in China (Shao et al., 2010). Our previous study found that this species possesses specific adaptations to hypoxia (Wang et al., 2011a). However, there is limited information on its molecular responses under hypoxia. Therefore, in the current study, the Tf gene from tongue sole was cloned and characterised. Its structure and tissue distribution were determined, and the expression pattern under hypoxia was examined as well.

Materials and methods

Fish

The fishes used for the experiments were adult tongue sole (Cynoglossus semilaevis) with a mean length of 24.64±1.01 cm (±SD) and a mean weight of 77.44±8.21 g. Prior to the experiments, the fishes were acclimated on a 14-h light/10-h dark cycle at 21°C for 2 weeks.

Cloning the CsTf

Total RNA was extracted from the liver using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). After digesting with RNase-free DNase I (TaKaRa Bio Inc., Otsu, Japan) for 30 min at 37°C, total RNA was reverse transcribed into first-strand cDNA using SuperScript Reverse Transcriptase (Invitrogen) according to the manufacturer’s instructions.

The Japanese flounder (Paralichthys olivaceus) Tf sequence (GenBank accession no.: ACZ92269) was used to search the Expresed Sequence Tag (EST) database [National Center for Biotechnology Information (NCBI) (http://blast.ncbi.nlm.nih.gov/blast.cgi)] using the BLASTP tool which identified one tongue sole EST (GenBank accession no.: GH229616). Further analysis showed that this EST shared a high identity with the middle sequence of Japanese flounder Tf. Specific primers (Tf-F1 and Tf-R1, listed in Table 1) were designed according to the identified EST in order to clone this partial sequence from the liver tissue of tongue sole. After confirmation by
sequencing, primers used for 3‘ and 5‘ rapid amplification of cDNA ends (RACE) were designed on the basis of the sequence obtained. The polymerase chain reaction (PCR) cycling program was: 1 cycle of 94°C for 5 min; 9 cycles of 94°C for 30 s, 64°C for 30 s, and 72°C for 90 s; 25 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 90 s; 1 cycle of 72°C for 10 min. A 1225-bp 3‘-RACE product was obtained and it contained the C-terminal of the transferrin (Tf) gene in Cynoglossus semilaevis (CsTf) coding region and 3‘-prime untranslated region (UTR). A 395-bp 5‘-RACE product was also obtained. It enclosed the 5‘-UTR and N-terminal partial coding region. Primers used for the gene clone are listed in Table 1.

Sequence analysis

The deduced amino acids (aa) were predicted using the software at the ExPaSy molecular biology server (http://www.expasy.org). Multiple sequence alignments were performed by CLUSTAL 1.8 (Clewell and Arnold, 1997) and shaded with Genedoc software (Nicholas et al., 1997). The signal peptide was predicted using the SignalP programme (http://www.cbs.dtu.dk/services/SignalP/) (Bendtsen et al., 2004), while the DISULFIND programme (http://disulfind.dsi.unifi.it/) was used to predict Disulfide bonds (http://disulfind.dsi.unifi.it/). The protein secondary structure was predicted foreseen using PSIPRED (http://bioinf.cs.ucl.ac.uk/psipred/). Phylogenetic trees were created by following the neighbour-joining (N-J) method using the MEGA programme (version 4.1), and were bootstrapped for 10,000 times.

Three-dimensional model of CsTf

We searched for the best template for CsTf using GeneSilico Metaserver (Kurowski and Bujnicki, 2003), Pcons.net (Wallner and Elofsson, 2005), and BLAST analysis. After determining the template, the three dimensional (3-D) structure of CsTf was constructed in the SWISS-MODEL server (http://expasy.org/swissmod/SWISS-MODEL.html) on the basis of the comparative modelling method. The model obtained was then validated by PROCHECK (Laskowski et al., 1993). We performed protein visualisation using the DeepView software.

Acute hypoxic stress

In total, 90 tongue soles were used for acute hypoxic stress. Groups of five fish were randomly assigned to the hypoxic group or control group. The control group was maintained under normoxic conditions (DO=6.2 mg/L).

Acute hypoxic conditions (DO=0.8 mg/L) in the hypoxic stressed group were maintained by bubbled with nitrogen gas as previously described (Wang et al., 2011a, 2011b). During the experiments, the DO, pH and temperature were monitored using the YSI 556 multiprobe system (YSI Inc., Yellow Springs, OH, USA). The heart, liver, spleen, kidney, intestine, stomach, blood and gills from three fishes in each group were sampled after continuous exposure for 5, 30, 60, 90 and 120 min, and stored at -80°C until use.

Quantitative real-time polymerase chain reaction

The basal and hypoxic stressed expression patterns of CsTf were examined using the SYBR green-based real-time PCR method. RNA preparation and cDNA synthesis from different tissues were as previously described (Wang et al., 2011a, 2011b). The primers used for real-time PCR analysis were confirmed by the reverse transcription polymerase chain reaction (RT-PCR) and melting curve analysis on an ABI 7300 real-time PCR system (Applied Biosystems, Carlsbad, CA, USA). The real-time PCR was run in duplicate for each sample and carried out using a two-step method: 30 s at 95°C, followed by 40 cycles of 95°C for 5 s and 60°C for 31 s. Basal gene expression in normal tissue was measured relative to the gene expression of β-actin. The expression of CsTf under hypoxic stress was measured using the standard curve method and calculated using the following formula (Wang et al., 2011b):

fold = fold change in target gene expression (experimental group/control group) / fold change in reference gene expression (experimental group/control group).

Real-time PCR data were analysed using Origin 6.0. Results are expressed as mean±SD. The significance between the stressed and the control groups was evaluated using the Student’s t test, with P<0.05.

Results

Sequence analysis of Cs Tf

The CsTf cDNA sequence was 2281 bp, and it contained a 5‘-UTR of 28 bp, an open reading frame of 2034 bp, and a 3‘-UTR of 219 bp. There was one mRNA instability motif (AATAA) and a polyadenylation signal (AATAAA) 16 bp upstream of the poly(A) tail (Figure 1). The CsTf cDNA encoded 677 aa with a 21 aa signal peptide which was predicted using SignalP 3.0. The mature secreted CsTf was 656 aa, with a theoretical molecular mass of 74.90 kDa and an isoelectric point of 6.15. Similar to human Tf, CsTf also contained two lobes: the N-lobe comprising 23-326 residues and the C-lobe comprising 337-656 residues. The two lobes were connected by a short hinge region (327-336 residues). The secondary structure analysis showed that each lobe contained two subunits possessing a conserved /1structure. Eight iron-binding residues (Asp71/385, Tyr 101/198/420/512 and His523/576) were predicted using ScanProsite. Four conserved Tf super-family motifs were also found in CsTf at the following residues: 101-110 (YYAVAVKEK), 420-429 (YFVAVIKRD), 198-213 (YDGAFRCLEKGDVA), and 512-528

Table 1. Primers used for cloning and expression analysis of Cs Tf

| Primer     | Sequence (5’ to 3’)      | Usage                  |
|------------|--------------------------|------------------------|
| Ti-F1      | TATAATGCTGCTTGGCTGTGCA   | Gene cloning           |
| Ti-R1      | GCTAAATGACCCCACTTTA      | RACE PCR               |
| Ti-5out    | CTCCCACCCAGTGACACAG      |                        |
| Ti-5in     | GATGCACATGGTGGCTCGAG     |                        |
| Ti-3out    | TCCAGTTGGGTGTACATGAGAG  |                        |
| Ti-3in     | CCAGACCTGAGAGCTCAC       |                        |
| UPM        |                          |                        |
| Long       | CTAATACGACTCTACTAGGGCGACAGTGG-TATCAACGACAG      |                        |
| Short      | CTAAATGACCATCATAAGGCC    | Real-time PCR          |
| QTF-F1     | GGTGAAAAGGTGGAGTGGT      |                        |
| QTF-R1     | CCTTTACGACCATGAGGAGAT    |                        |
| Qactin-F1  | CGCCCATCTGTGCCATC       |                        |
| Qactin-R1  | TCCTTTAGTTCAGCAAGCAGAT  |                        |

CsTf, transferrin gene in tongue sole Cynoglossus semilaevis; Ti, transferrin; RACE, rapid amplification of cDNA ends; PCR, polymerase chain reaction; UPM, universal primer mix.
(YTGALKCLADGVGDVAF) (Anderson et al., 1989). Four conserved iron-binding sites in each lobe (Asp71, Tyr101, His195 and His376 in the N-lobe) and two anion sites in each lobe (Gly127 and Lys130 in the N-lobe; His417 and Arg449 in the C-lobe) remained conserved in CsTf (Ford, 2012). A total of 32 cysteines were found in CsTf. Analysis using DISULFIND showed that 24 cysteines might form 12 disulphide bonds, with six bonds in each lobe (Figure 2). The CsTf shared 48.1% to 63.3% sequence identity with Tf from other vertebrates; the highest was with the Japanese flounder Tf (63.3%) and the lowest was with human Tf (48.1%). Analysing the sequence identity of the N-lobe and C-lobe of CsTf showed that these lobes shared a high identity with those in different species. The N-lobe of CsTf shared 65.6% identity with the N-lobe of Japanese medaka Tf, 66.5% with the N-lobe of Japanese Flounder Tf, and 49.3% with the N-lobe of human Tf. The C-lobe of CsTf shared 58.5% identity with the C-lobe of Japanese medaka Tf, 59.4% with the C-lobe of Japanese Flounder Tf, and 49.8% with the C-lobe of human Tf. The CsTf N-lobe shared 36% identity with its C-lobe.

Phylogenetic analysis

To further analyse the evolutionary context of the CsTf gene, a phylogenetic tree was constructed with the MEG4 programme using human lactotransferrin as an outgroup. The phylogenetic tree was divided into two main clades. One clade included Tf from mammals and the other from amphibians and fish. In the second clade, the amphibian Tf formed a separate cluster, and fish Tf formed a large cluster. In the latter, the fish in the same order formed a sub-cluster, e.g., fish (grass carp, common carp and Japanese crucian carp) in cyprinids were clustered together. As expected, CsTf was included within the sub-cluster of Pleuronectiformes (Japanese flounder) with a high bootstrap value (Figure 3).

Structure of the tongue sole transferrin

The two-dimensional structure analysis showed that tongue sole Tf possessed an α-helix/β-sheet structure (Figure 4a). Further, the 3-D structure of tongue sole Tf was constructed in the SWISS-MODEL server using the comparative modelling method. The results showed that CsTf was divided into a bilobal structure with an amino-terminal lobe (N-lobe, residues 23-326) and a carboxyl-terminal lobe (C-lobe, residues 337-656). The lobes were connected by a linker peptide (residues 327-336).

Figure 1. Nucleotide and deduced amino acid (aa) sequence of CsTf. The full-length cDNA sequences of CsTf were deposited to GenBank (GenBank accession no. HQ099442). Translated amino acid sequence is shown above the nucleotide sequence. Numbers to the left of each row refer to nucleotide or amino acid position. The initiation codon (ATG) is boxed and the translational stop site (TAA) is indicated by an asterisk. The mRNA unstable signal (attta) and the polyadenilation site (aataaa) are single and double underlined, respectively.
336). Each lobe was further divided into two sub-domains containing a multi-α-helix/β-sheet structure (Figure 4b).

**Tissue distribution of CsTf**

The tissue distribution of CsTf mRNA was determined using real-time PCR. As shown in Figure 5, the expression of CsTf was relatively high in the liver. Moderate levels of CsTf expression were detected in the heart, spleen and kidney, with the lowest levels being in the stomach, blood, gills and intestine.

**CsTf expression profile under acute hypoxic stress**

The expression change in CsTf under acute hypoxic stress (DO=0.8 mg/L) was detected using real-time PCR. Under hypoxic stress, CsTf expression exerted a tissue-specific expression pattern. For example, Tf in the heart and liver was increased from 30 min to 120 min under hypoxic stress (P<0.05), while Tf in the spleen was increased from 60 min to 120 min under hypoxic stress compared with that in the control group (P<0.05). As the hypoxic time was prolonged from 90 min to 120 min, Tf in the blood, gills and stomach returned to normal expression levels, similar to those in the control group (P>0.05; Figure 6).

**Discussion**

In the present study, the tongue sole Tf gene was cloned, characterised, and its expression under hypoxic stress was examined as well. Our study confirmed that tongue sole exactly possesses Tf gene homology. In addition, tongue sole Tf was up-regulated in different tissues after hypoxic stress, indicating that this gene plays a role in fish’s response to hypoxia.

We found that the tongue sole Tf gene was 2281 bp in length, and encoded 677 aa, which shared similar aa lengths with Tf in other vertebrates (615-695 aa). Also, the full sequence of tongue sole Tf shares a high identity with Tf in other vertebrates. Some important aa, e.g., four iron-binding sites in each lobe and two sites of anions of each lobe, remained unchanged during evolution (Figure 2). The CsTf possessed the same 2-D and 3-D structure as human Tf, thus implying again that the structure of Tf remained conserved during evolution. The N-lobe and C-lobe also shared higher sequence identities with their counterparts from different species. It is speculated that Tf might have evolved from a common ancestor gene during evolution. The analysis of the sequence identity of the N-lobe and C-lobe showed that the N-lobe was more conserved than the C-lobe in different species. This suggested that the sequence coding of the primordial N-lobe might be the ancestor of Tf.

The expression of Tf has been studied in several fishes and appears to be tissue-specific in different species. The Atlantic salmon Tf expression is found at high levels in the liver, but at lower levels in the kidney and stomach. Transferrin of the Atlantic cod and roughskin...
Figure 3. Phylogenetic tree of putative transferrin (Tf) sequences. Bootstrap values were calculated from 10,000 replicates. The GenBank accession no. of the Tf sequences used for this analysis were as follows: human, AAH59367; mouse, AAL34533; cow, AA96735; western clawed frog, AAH96012; Japanese crucian carp, AAP86287; common carp, ACD99642; grass carp, AAR20997; tongue sole, HQ909442; Japanese flounder, AAF33233; rainbow trout, BAA84103; Nile tilapia, ABB70391; Japanese medaka, BAA10901; emerald rockcod, CAL92188; black rockcod, CAL92189; large yellow croaker, CAM96032; European seabass, ACN80997; red seabream, AAP94279; gilthead seabream, AEA41140; and black porgy, AAQ63949. Human lactotransferrin (LTF) accession no. was EAW64767.

Figure 4. The predicted secondary structure and homology modeling of CsTf. a) 2-D structure of CsTf. The α helix is indicated as a cylinder and β-sheet as a line with arrows. b) 3-D structure of CsTf. The α helix is shown in green, β-sheets are shown in red, and other residues are shown in blue. The iron-binding sites are shown as gray dots and expressed as FE1 and FE2.
Sculpin is highly expressed in the liver and brain, but less expressed in other tissues (Denovan-Wright et al., 1996; Liu et al., 2012). Our study found that CsTf was highly expressed in the liver and moderately expressed in the heart, spleen and kidney, which is consistent with the expression pattern of the Atlantic salmon (Kvingedal, 1994). The high expression of Tf in the liver suggests that the fish liver is the main tissue that synthesises and secretes Tf. The reason for varying expression patterns in other tissues (e.g., the brain and spleen) needs to be further investigated by focusing on the regulation pathway of Tf in closely related species.

Aquatic hypoxia affects fish directly since it jeopardises their habitat. However, research on fish’s response to hypoxia has only recently begun. Wulff et al. (2012) found that more than 40 proteins involved in maintaining iron homeostasis, energy levels and muscle structure are down- or up-regulated in rainbow trout under hypoxic stress, thus suggesting that fish’s response to hypoxia is a complex process.

Previous studies indicated that Tf might play a role under hypoxic conditions (Simpson, 1992; Rolfs et al., 1997). Still, little is known about hypoxia in fish. Therefore, we selected the marine fish tongue sole – which possesses a specific hypoxic adaptation strategy – as an animal model in order to detect the expression pattern of Tf under hypoxia. Our previous studies found that some genes (e.g., globins) might play a role at different stages of hypoxia (Wang et al., 2011a, 2011b). In the present study, we found that tongue sole Tf was up-regulated in some tissues (e.g., the liver and heart) under acute hypoxia (DO=0.8 mg/L). However, as hypoxia became prolonged, Tf returned to normal levels to save energy for maintaining the physiological function of important tissues (liver and heart).

**Conclusions**

In this paper, the Tf sequence from the marine fish tongue sole was cloned and analysed for the first time. Tongue sole Tf possesses the same structure as mammalian Tf with the N-lobe and C-lobe. Some aa sites are conserved in tongue sole Tf. Moreover, tongue sole Tf is mainly expressed in the liver and up-regulated under hypoxic stress, thus indicating that Tf also plays a role in fish’s adaptation to hypoxia.

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Figure 5. Tissue distribution of transferrin (Tf) in healthy tongue sole. β-actin was used as a control for the amount and quality of cDNA.

Figure 6. Expression patterns of transferrin (Tf) in different tissues of tongue sole under hypoxia. Asterisks indicate that the copy number is significantly higher than that of the corresponding control sample (*P<0.05).
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