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Heme metabolism enzymes are dynamically expressed during *Xenopus* embryonic development

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ABSTRACT: As the key component of many hemoproteins (heme-containing proteins), heme is involved in a broad range of biological processes. Enzymes required for heme biosynthesis and degradation pathways are evolutionarily conserved. While heme metabolism has been studied extensively, the expression of heme metabolism enzymes during development has not been described. Here, we report that all heme biosynthases and two heme oxygenases, which initiate heme degradation, are dynamically expressed during *Xenopus* embryonic development. All heme synthases, with the exception of aminolevulinic acid synthase 2, are maternally expressed. At neurula stage, heme synthases are expressed in the developing neural tissue and in migrating neural crest cells. At the swimming tadpole stage, expression of heme synthases can be detected in multiple lineages, including eyes, neural crest cells, developing central nervous system, ventral blood island, pronephron, and pronephric tubule. Similar to heme synthases, heme oxygenases are expressed maternally. Zygotic expression of heme oxygenases is mainly restricted to the developing neural and neural crest lineages. Unlike heme synthases, heme oxygenases are not expressed in the ventral blood island and are expressed at a very low level in the pronephron and pronephric tubule. This indicates that heme metabolism may play important roles during development.

Introduction

Heme is a prosthetic group with an iron in the center of a porphyrin. Many proteins contain heme and are called hemoproteins. Hemoproteins have diverse functions, ranging from electron transfer to signal transduction regulation (Hou *et al*., 2006; Mense and Zhang, 2006). A recent study has indicated that heme is involved in the maturation of microRNA as well (Faller *et al*., 2007). The heme biosynthetic pathway is evolutionarily conserved and consists of eight enzymes. (Kaplan *et al*., 1974; Sassa, 1976, 1990; Woodard and Dailey, 2000). The first reaction of heme biosynthesis, which is the rate-limiting step, occurs in the mitochondrial where succinyl-CoA and glycine are converted into δ-aminolevulinic acid (ALA). Enzymes catalyzing this reaction are aminolevulinic acid synthases (ALASs). There are two ALAS isoenzymes encoded by two different genes, ALAS-1 and ALAS-2. ALAS-2 is specifically expressed in erythroid cells, whereas ALAS-1, which is considered as the "nonspecific ALAS", is expressed ubiquitously in adult animals. Interestingly, heme suppresses ALAS1 through a number of negative feedback loops. This results in a precise regulation of ALAS1 expression and, consequently, the level of heme biosysthesis. After δ-ALA is produced, the rest seven enzymes work sequentially to catalyze a series of chemical reactions, which ultimately leads to the formation of heme. These enzymes are ALA dehydratase (ALAD),

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PBG deaminase (PBGD), uroporphyrinogen III synthase (URO3S), uroporphyrinogen decarboxylase (UROD), coproporphyrinogen oxidase (CPO), protoporphyrinogen oxidase (PPO), and ferrochelatase (FECH). Once synthesized, heme is incorporated into heme binding proteins to form hemoproteins and regulates a variety of biological processes (Ajioka *et al*., 2006). The heme biosynthetic pathway has been extensively studied. All heme synthases have been cloned and the three-dimensional crystal structures of these enzymes have been determined (Ajioka *et al*., 2006).

While heme is essential for the functions of hemoprotein, free heme is toxic. The level of intracellular heme is precisely controlled by heme degrading enzymes. Two heme oxygenases (HO1 and HO2), the rate-limiting enzymes for heme degradation, catalyze the first reaction of heme turnover, leading to the production of biliverdin, iron, and carbon monoxide. Once this happens, biliverdin reductase reduces the central methene bridge of biliverdin, producing bilirubin. (Snyder and Baranano, 2001; Unno *et al*., 2007). HOs are involved in cell cycle and cell survival regulation. Recent studies have indicated that HOs may be involved in human diseases, for example, colon cancer (Oates and West, 2006).

Heme regulates the erythroid differentiation (Igarashi and Sun, 2006; Keel *et al*., 2008). A recent work has indicated that heme metabolism may be important for *in vitro* neuronal differentiation (Shinjyo and Kita, 2006). These observations promoted us to investigate the expression pattern of heme biosynthetic enzymes during *Xenopus* embryonic development. And also, since free heme is toxic to cells, we further investigated the expression pattern of the enzymes initiating heme degradation (HO1 and HO2).

Material and Methods

Embryos

Xenopus laevis were obtained from *Nasco*. Eggs were obtained from hCG-stimulated female *X. laevis* and *in vitro* fertilized. Fertilized eggs were dejellied in 2% cystein solution according to standard protocols. Staging of embryos was according to Nieuwkoop and Faber (1967).

Xenopus heme metabolism enzymes

The IMAGE clones for *X. laevis* heme metabolism enzymes were obtained from American Type Culture Collection. These include: *ALAS1* (Image:4033270), *ALAS2* (IMAGE:7011935), *ALAD* (Image:5543150), *PBGD* (Image:7391702), *URO3S* (Image:7206195),

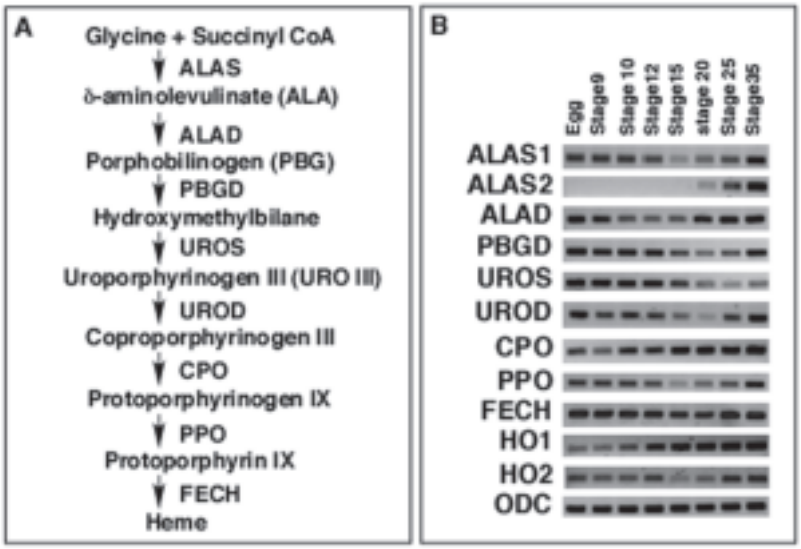

FIGURE 1. Temporal expression profile of heme metabolism enzymes during *Xenopus* embryonic development. A. Schematic presentation of the heme biosynthetic pathway. B. RT-PCR result showing the temporal expression of all heme synthases and heme oxygenases at the one-cell stage (egg), late blastula stage (stage 9), gastrula stages (stage 10 and stage 12), neurula stage (stage 15), tailbud stages (stage 20 and stage 25), and tadpole stage (stage 35).

UROD (Image: 5515775), *CPO* (Image:6638065), *PPO* (Image:5079368), *FECH* (Image:7979365), *HO1* (Image: 6859921), and *HO2* (Image: 5154723).

RT-PCR and whole-mount in situ hybridization

RNA extraction and RT-PCR methods were as described previously (Yang *et al*., 2002). RT-PCR primers used were as follows: *ALAS1*: (5'-ATT ACT CCA TCC CGA TCT CC-3', 5'-TCC AAG GCA TCC GAA ACA GC-3'); *ALAS2*: (5'-ACC CAT ACG GGT TGG AAA CG-3', 5' - AGC AGA TGG TGT GTG CAA GG - 3'); *ALAD*: (5' - CTG TGG GAT TCT ACG AGA AG - 3', 5' - CTG TAA CTC ATG ACG GAC AC - 3'); *PBGD*: (5' - GAA TGT CTC TAT GCT GTG GG - 3', 5' - ATC CAG GCT GTA TAC AGC AC - 3'); *URO3S*: (5' - AGC CCA AGA GCA GTA GAA GC - 3', 5' - ACC GTA ATG GTC TCC AGA GG - 3'); *UROD*: (5' - AAG CTC AGT GGA TGT GTC AG - 3', 5' - GTG AGT TGC TTG AGA AGC TG - 3'); *CPO*: (5' - GAT GAT CAT GGG AAC TCA GG - 3', 5' - TCA TTT GCC GTA TGG ACT CC - 3'); *PPO*: (5' - AAG CAC TGG AGG ACT TCC TG - 3', 5' - TGG CTG AAC TCA TCT GCT GC - 3'); *FECH*: (5' - GAA GTG GAG CGT CAT AGA CC - 3', 5' - CCT TGG ATT GCC AGA CCA GC - 3'); *HO1*: (5' - TGC ACT CAT CAC TCT CAT GG - 3', 5' - AAT GAG TCA GGT ACC AAG GC - 3'); *HO2*: (5' - TCT GAG CTG CTC AAA GAA GG - 3', 5' - ATC AGA GCC TCC TTC CTG TG - 3'); and *ODC*: (5'-AAT GGA TTT CAG AGA CCA-3'; 5'-CCA AGG CTA AAG TTG CAG-3').

Whole-mount in situ hybridization was carried out as described by Sive *et al*. (2000). Embryos were hybridized with either anti-sense probes or with sense probes as negative controls.

Results

Expression of heme biosynthetic enzymes

Xenopus heme biosynthetic enzymes were identified from the GenBank database based on their homologies to human heme biosynthetic enzymes. As shown in Table 1, *Xenopus* heme synthases are highly similar to heme synthases in other vertebrates. RT-PCR was performed to investigate the temporal expression profile of heme synthases during *Xenopus* embryonic development. As shown in figure 1B, most heme synthases, with the exception of ALAS2, are maternally expressed. The expression level of these enzymes remains grossly constant during early developmental stages. In contrast, ALAS2, which is known to be expressed in erythroid cells in most species, is not detectable until stage 20. This correlates well with the timing of ventral blood island development in *Xenopus*.

We next examined the spatial expression patterns of heme synthases by whole-mount *in situ* hybridization (Fig. 2). Heme synthases are ubiquitously expressed from the cleavage stage to gastrula stage (not shown).

TABLE 1.

Percent identity of amino acid sequences between *Xenopus* heme metabolism enzymes and their homologues in human, mouse, and zebrafish.

	Human	Mouse	Zebrafish
ALAS1	75	74	71
ALAS2	67	67	66
ALAD	77	74	80
PBGD	75	75	76
URO3S	57	55	50
UROD	72	71	75
CPO	53	52	55
PPO	51	51	55
FECH	86	81	73
HO1	61	58	59
HO2	70	67	60

The sequences of heme biosynthetic enzymes and HOs are retrieved from GenBank database (http://www.ncbi.nlm.nih.gov). The GenBank accession number of these proteins are: human ALAS1 (NP_000679), mouse ALAS1 (NP_065584), zebrafish ALAS1 (NP_958444), human ALAS2 (NP_000023), mouse ALAS2 (NP_033783), zebrafish ALAS2 (NP_571757), human ALAD (NP_000022), mouse ALAD (NP_032551), zebrafish ALAD (NP_001017645), human PBGD (P08397), mouse PBGD (P22907), zebrafish PBGD (NP_001019559), human URO3S (NP_000366), mouse URO3S (EDL17774), zebrafish URO3S (AAO49476), human UROD (NP_000365), mouse UROD (NP_033504), zebrafish UROD (NP_571422), human CPO (AAH23554), mouse CPO (NP_031783), zebrafish CPO (CAK04614), human PPO (NP_000300), mouse PPO (NP_032937), zebrafish PPO (AAI54349), human FECH (BAA00628), mouse FECH (NP_032024), zebrafish FECH (NP_571706), human HO1 (AAH01491), mouse HO1 (EDL10826), zebrafish HO1 (NP_955972), human HO2 (P30519), mouse HO2 (O70252), and zebrafish HO2 (AAI52490).

Asymmetric expression of heme synthases was first detected at the beginning of the neurula stage. At this stage, expression of heme synthases is slightly stronger on the dorsal side of the embryo (data not shown). The expression of heme biosynthetic enzymes is gradually diminished on the ventral side of the embryo. By late neurula stage, heme synthases become clearly localized on the dorsal side. All enzymes are accumulated in the neural tube, the eye fields, and the migrating neural crest cells. Weak and diffused expression of all eight enzymes can be detected in some epidermis cells adjacent to the neural tube. It is worth to mention that in situ hybridization with ALAS1 or ALAS2 probes resulted in identical staining patterns. Since ALAS1 and ALAS2 share 73% identities, it is likely that these probes cross-react with both ALAS isoforms. At the tadpole stage, the highest level of heme biosynthetic enzymes was observed in the ventral blood island, the site of primitive erythropoiesis. In addition, multiple tissues express heme synthases at fairly high levels. These include head, eyes, central nervous system, migrating neural crest cells, developing pronephron, and pronephric tubule (Fig. 2). Given that all 8 heme synthases are expressed in an identical pattern, it is likely that heme biosynthesis occurs in these tissues during development.

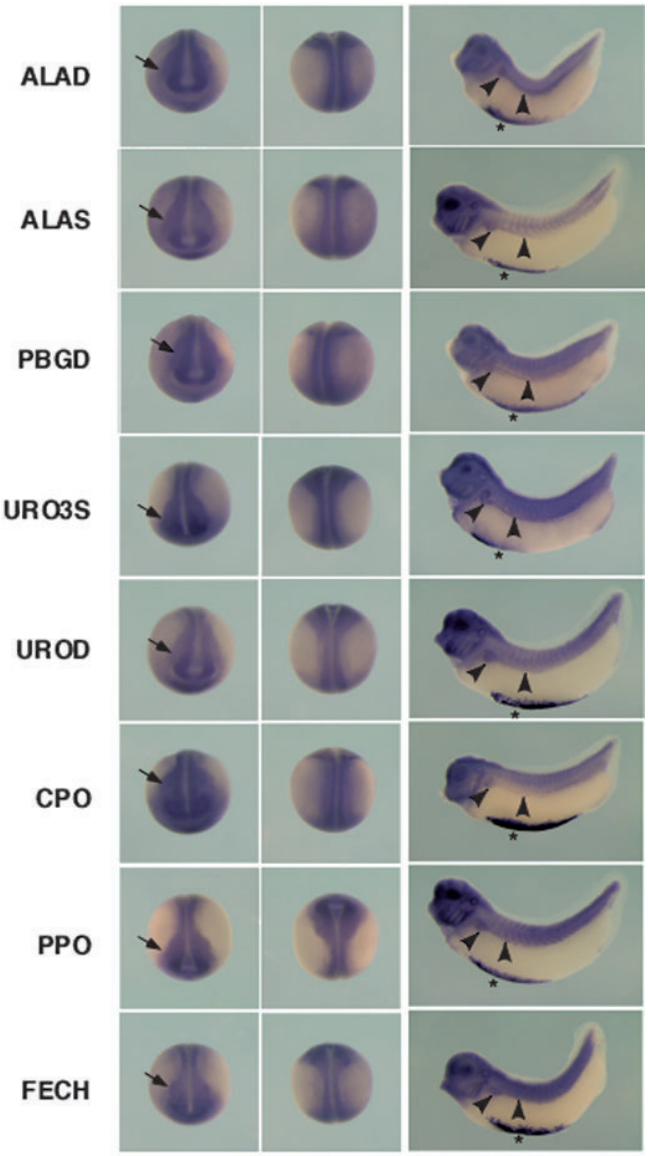

FIGURE 2. Whole mount *in situ* hybridization to show the expression of heme synthases at the late neurula stage and the tadpole stage. From left to right are: anterior views of neurula stage embryos (left column), dorsal views of neurula stage embryos (middle column), and lateral views of tadpole stage embryos (right column). Note that the expression patterns of heme synthases are identical to each other. Arrows point to the migrating neural crest cells. Arrowheads indicate the pronephron and pronephric tubule. * indicates the ventral blood island.

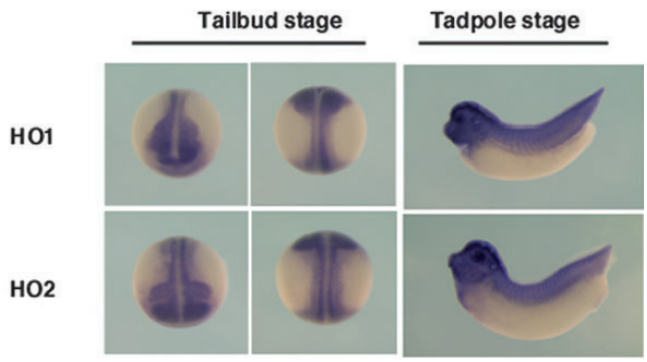

FIGURE 3. Whole mount in situ hybridization to show the expression of heme oxygenases (HO1 and HO2) at the neurula stage and the tadpole stage. From left to right are: anterior views of neurula stage embryos (left column), dorsal views of neurula stage embryos (middle column), and lateral views of tadpole stage embryos (right column).

Expression of heme oxygenases

Xenopus HO1 and HO2 were identified by blasting the GenBank database with their human homologues. *Xenopus* HO1 and HO2 share significant identities with HOs in other vertebrates (Table 1). Similar to most heme biosynthetic enzymes, both HO1 and HO2 are maternally expressed (Fig. 1B). Transcripts of HO1 and HO2 are distributed ubiquitously prior to the neurula stage (not shown). Expression of HO1 and HO2

becomes dorsally localized when embryos reach the neurula stage (Fig. 3). At this stage, the expression pattern of HO1 and HO2 is very similar, if not identical, to that of heme synthases. Strong expression of both enzymes were observed in the neural tube, developing eye field, and migrating neural crest cells. Weaker expression was detected in some epidermis cells adjacent to the neural tube. The expression of HO1 and HO2 persists in the neural and neural crest lineages by the tadpole stage. Unlike heme synthases, however, HO1 and HO2 are not expressed in the ventral blood island at this stage. In addition, they are expressed only very weakly in the developing pronephron and pronephric tubule (Fig. 3).

Discussion

Heme is an essential compound of hemoproteins and is expected to play important roles in many biological processes, including development. Previous studies have indicated that heme metabolism is critical for erythroid differentiation (Igarashi and Sun, 2006; Keel *et al*., 2008). Recently, it has been reported that the expression level of heme biosynthetic enzymes changes dynamically during *in vitro* neuronal differentiation (Shinjyo and Kita, 2006). This suggests that heme metabolism may have a broad impact on development. In agreement with this view, here we show for the first time that enzymes involved in heme metabolism are dynamically expressed during *Xenopus* embryonic development. All heme synthases share identical expression pattern and are expressed in the neural tissue, neural crest cells, developing pronephron, pronephric tubule, and ventral blood island. This raises the possibility that heme biosynthesis is indeed happening in these tissues and may be important for the development of these lineages. Heme synthases and heme oxygenases play opposite roles in heme metabolism. Interestingly, we observed that HO1 and HO2, which are responsible for heme degradation, were mainly restricted to the developing neural and neural crest lineages during early development. While further experiments are needed, above observations suggest that precise regulation of heme metabolism may be important for *Xenopus* early development.

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References

Ajioka RS, Phillips JD, Kushner JP (2006). Biosynthesis of heme in mammals. Biochimica et Biophysica Acta 1763: 723-736.

Faller M, Matsunaga M, Yin S, Loo JA, Guo F (2007). Heme is involved in microRNA processing. Nature Structural & Molecular Biology 14: 23-29.

Hou S, Reynolds MF, Horrigan FT, Heinemann SH, Hoshi T (2006). Reversible binding of heme to proteins in cellular signal transduction. Accounts of Chemical Research 39, 918-924.

Igarashi K, Sun J (2006). The heme-Bach1 pathway in the regulation of oxidative stress response and erythroid differentiation. Antioxidants & Redox Signaling 8, 107-118.

Kaplan BH, Tricoche M, Vanderhoff G (1974). Regulatory role of heme. Ann N Y Acad Sci. 241: 334-346.

Keel SB, Doty RT, Yang Z, Quigley JG, Chen J, Knoblaugh S, Kingsley PD, De Domenico I, Vaughn MB, Kaplan J, Palis J, Abkowitz JL (2008). A heme export protein is required for red blood cell differentiation and iron homeostasis. Science 319: 825-828.

Mense SM, Zhang L (2006). Heme: a versatile signaling molecule controlling the activities of diverse regulators ranging from transcription factors to MAP kinases. Cell Res. 16: 681-692.

Nieuwkoop PD, Faber J (1967). Normal Table of *Xenopus laevis* (Daudin). North Holland Publishing Company, Amsterdam.

Oates PS, West AR (2006). Heme in intestinal epithelial cell turnover, differentiation, detoxification, inflammation, carcinogenesis, absorption and motility. World J Gastroenterol. 12: 4281-4295.

Sassa S (1976). Sequential induction of heme pathway enzymes during erythroid differentiation of mouse Friend leukemia virus-infected cells. J Exp Med. 143: 305-315.

Sassa S (1990). Regulation of the genes for heme pathway enzymes in erythroid and in non-erythroid cells. Int J Cell Cloning. 8: 10-26.

Shinjyo N, Kita K (2006). Up-regulation of heme biosynthesis during differentiation of Neuro2a cells. J Biochem. 139: 373-381.

Sive H, Grainger R, Harland R (2000). Early Development of *Xenopus laevis*; A Laboratory Manual. Cold Spring Harbor Press, Cold Spring Harbor.

Snyder SH, Baranano DE (2001). Heme oxygenase: a font of multiple messengers. Neuropsychopharmacology 25: 294-298.

Unno M, Matsui T, Ikeda-Saito M (2007). Structure and catalytic mechanism of heme oxygenase. Nat Prod Rep. 24: 553-570.

Woodard SI, Dailey HA (2000). Multiple regulatory steps in erythroid heme biosynthesis. Arch Biochem Biophys. 384: 375-378.

Yang J, Tan C, Darken RS, Wilson PA, Klein PS (2002). Beta-catenin/Tcf-regulated transcription prior to the midblastula transition. Development 129: 5743-5752.