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CLONING AND ONTOGENIC EXPRESSION OF SEPIAPTERIN REDUCTASE mRNA IN ZEBRAFISH EMBRYO: POSSIBLE ROLE IN PTERIDINE BIOSYNTHESIS

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CLONING AND ONTOGENIC EXPRESSION OF SEPIAPTERIN REDUCTASE mRNA IN ZEBRAFISH EMBRYO: POSSIBLE ROLE IN PTERIDINE BIOSYNTHESIS

by

KUAH MENG KIAT

Thesis submitted in fulfilment of the requirements for the degree of Master of Science

APRIL 2008

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TABLE OF CONTENTS

		Page
ACKNOWLEDGEMENTS		ii
TABLE OF CONTENTS		iii
LIST OF TABLES		vi
LIST OF FIGURES		vii
LIST OF ABBREVIATIONS		viii
ABSTRAK		xi
ABSTRACT		xii
CHAPTER 1: INTRODUCTION		1
1.1	General	1
1.2	Objectives	3
CHAPTER 2: LITERATURE REVIEW		4
2.1	Colouration	4
2.2	Chromatophores	4
2.2.1	Melanophores	5
2.2.2	Cyanophores	5
2.2.3	Iridophores	6
2.2.4	Leucophores	7
2.2.5	Xanthophores and erythrophores	7
2.3	Pteridines	8
2.4	Zebrafish	10
2.5	Strengths of the zebrafish as a model organism	12
2.5.1	Mutagenesis screens	12
2.5.2	Reverse genetics	13
2.5.3	Genome sequencing	13
2.6	Pigmentation of zebrafish	14
2.7	The development of zebrafish pigment pattern	16
2.8	The biosynthesis pathway of pteridines in zebrafish	18
2.9	Sepiapterin reductase (SR)	20
2.9.1	Structure, chromosomal localisation and expression of SR gene	20
2.9.2	Structure and localisation of SR enzyme	20
2.9.3	Functional roles of SR in the biosynthesis of tetrahydropteridines	21

CHAPTER 3: MATERIALS AND METHODS 24
3.1 Stock solutions and reagents 24
3.2 Culture media and antibiotic 24
3.2.1 LB medium and LB-ampicillin plate 24
3.2.2 Antibiotic 24
3.3 Host strain and cloning vector 24
3.4 Animals 26
3.5 Isolation of total RNA 26
3.6 Quantification of total RNA 26
3.7 DNase treatment of total RNA 27
3.8 5' and 3' RACE 27
3.9 Cloning of PCR products 29
3.9.1 Gel extraction of PCR products 29
3.9.2 Ligation of PCR products 29
3.9.3 Preparation of competent cells 30
3.9.4 Transformation of competent cells 30
3.9.5 PCR-screen of recombinant colonies 31
3.9.6 Purification of recombinant plasmids 31
3.10 Sequencing of PCR products 32
3.11 Computational sequence analysis and phylogenetic analysis 32
3.12 Semi-quantitative RT-PCR analysis 32
3.12.1 Primer design 32
3.12.2 RT-PCR 33
3.12.3 Validation of semi-quantitative RT-PCR analysis 35
3.12.4 Data analysis 35
3.13 RNA probe synthesis 35
3.13.1 Linearisation of cDNA clone 35
3.13.2 DNA cleanup 36
3.13.3 *In vitro* transcription 36
3.13.4 Ethanol precipitation of RNA transcripts 37
3.14 Whole-mount *in situ* hybridisation 37

CHAPTER 4: RESULTS 40
4.1 Isolation and characterisation of zebrafish SR cDNA 40
4.1.1 Isolation of total RNA from adult zebrafish 40
4.1.2 5' and 3' RACE 40
4.1.3 Nucleotide sequence of the full-length zebrafish SR cDNA 43

4.1.4 Analysis of the deduced amino acid sequence of zebrafish SR 43
4.1.5 Phylogenetic analysis of zebrafish SR with other SR proteins 46
4.2 Comparative ontogenic expression of zebrafish SR, GCH and XDH 49
4.2.1 Total RNA isolation from zebrafish embryos 49
4.2.2 Validation of semi-quantitative RT-PCR analysis 49
4.2.3 Semi-quantitative RT-PCR analysis of zebrafish SR, GCH and XDH 54
4.3 Spatio-temporal expression of zebrafish SR in embryo 57

CHAPTER 5: DISCUSSION 60

CHAPTER 6: CONCLUSION 66

BIBLIOGRAPHY 67

APPENDICES

Appendix A Stock solutions and reagents used in this study 77
Appendix B Nucleotide and deduced amino acid sequences of the 5' and 3' end of zebrafish SR together with the result of BLASTP analysis showed at the bottom 78
Appendix C Theoretical molecular weight and isoelectric point predicted from zebrafish SR 80
Appendix D Motifs identified in the deduced amino acid sequence of zebrafish SR 81
Appendix E One-way ANOVA analysis and Tukey's HSD Post Hoc Test for the data from semi-quantitative RT-PCR analysis of zebrafish SR, GCH and XDH mRNA expression in embryo 82

LIST OF TABLES

		Page
Table 3.1	Genotype of *Escherichia coli* strain DH5*α*	25
Table 3.2	Nucleotide sequences of the primers used in RT-PCR analysis	34
Table 3.3	Duration of proteinase K digestion for zebrafish embryos	38
Table 4.1	Amino acid identities (%) of the deduced amino acid sequences encoding SR of zebrafish and other species	45

LIST OF FIGURES

		Page
Figure 2.1	Ring structures of pteridines and pterins (Ziegler, 2003)	9
Figure 2.2	Adult male and female zebrafish	11
Figure 2.3	Chromatophores present in the lateral trunk of an adult zebrafish (Parichy *et al*., 2000)	15
Figure 2.4	The distribution of pigment cells, in relation to the stripe pattern in zebrafish (Hirata *et al*., 2003)	17
Figure 2.5	Proposed biosynthesis pathway of pteridines in zebrafish (Ziegler, 2003)	19
Figure 2.6	A proposed BH4 salvage pathway (Blau *et al*., 2001)	22
Figure 3.1	Circle map and sequence reference points of the pGEM®-T Easy Vector from Promega, USA (Technical manual of pGEM®-T and pGEM®-T Easy Vector Systems)	25
Figure 4.1	Total RNA isolated from adult zebrafish	41
Figure 4.2	Amplification of 5' cDNA end of zebrafish SR	42
Figure 4.3	Amplification of 3' cDNA end of zebrafish SR	42
Figure 4.4	Nucleotide and deduced amino acid sequences of the full-length cDNA of zebrafish SR	44
Figure 4.5	Comparison of the deduced zebrafish SR protein with other known SR proteins	47
Figure 4.6	Phylogenetic analysis of zebrafish SR and other SR proteins	48
Figure 4.7	Total RNA isolated from different stages of zebrafish embryos during development	50
Figure 4.8	Comparison between the sequences of PCR products from RT-PCR analysis against zebrafish SR (DQ336569), GCH (AJ311846), XDH (XM_685717) and β-actin (BC045846)	51
Figure 4.9	Kinetics of PCR amplifications with the representatives of the electrophoretic images shown at the bottom	53
Figure 4.10	Ontogenic expression profiles of SR, GCH and XDH during the embryogenesis of zebrafish	55
Figure 4.11	Spatio-temporal expression of SR mRNA during the development of zebrafish	58

LIST OF ABBREVIATIONS

ANOVA	analysis of variance
BAC	bacterial artificial chromosome
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BH4	L-*erythro*-tetrahydrobiopterin
BLAST	basic local alignment search tool
BSA	bovine serum albumin
$CaCl_2$	calcium chloride
cDNA	complementary deoxyribonucleic acid
dH_2O	distilled water
DH4	D-threo-tetrahydrobiopterin
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxyribonucleoside triphosphate
EC	Enzyme Commission
EDTA	ethylenediaminetetraacetic acid
EST	expressed sequence tag
GC	guanine-cytosine
GCH	GTP cyclohydrolase I
GFP	green fluorescence protein
GTP	guanosine triphosphate
HSD	honestly significantly different
IPTG	isopropyl β-D-thiogalactopyranoside
KCl	potassium chloride
KH_2PO_4	potassium dihydrogen phosphate
LB	Luria-Bertani
LiCl	lithium chloride
mCR2	mouse carbonyl reductase 2

$MgCl_2$	magnesium chloride
M-MLV	Moloney murine leukemia virus
MOPS	4-morpholinepropanesulfonic acid
mRNA	messenger RNA
$Na_2HPO_4.2H2O$	di-sodium hydrogen phosphate dihydrate
NaCl	sodium chloride
$NADP^+$	nicotinamide adenine dinucleotide phosphate, oxidised form
NADPH	nicotinamide adenine dinucleotide phosphate, reduced form
NaOH	sodium hydroxide
NBT	nitro blue tetrazolium
NCBI	National Centre for Biotechnology Information
NO	nitric oxide
ORF	open reading frame
PAC	P1-derived artificial chromosome
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PKU	phenylketonuria
PTPS	6-pyruvoyl tetrahydropterin synthase
PTU	1-phenyl-2-thiourea
RACE	rapid amplification of cDNA ends
RE	restriction enzyme
RNA	ribonucleic acid
RNAi	RNA interference
RNase	ribonuclease
rRNA	ribosomal RNA
RT-PCR	reverse transcription-polymerase chain reaction
SDR	short-chain dehydrogenase/reductase
SEM	standard error of the mean
SR	sepiapterin reductase

SSC	saline-sodium citrate
TBE	Tris-Borate-EDTA
TILLING	targeting induced local lesions in genomes
Tris	tris(hydroxymethyl)aminomethane
TTS	transcription start site
Tween® 20	polyoxyethylenesorbitan monolaurate
UTP	uridine 5'-triphosphate
UTR	untranslated region
XDH	xanthine dehydrogenase
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

PENGKLONAN DAN PENGEKSPRESAN ONTOGENIK mRNA SEPIAPTERIN REDUCTASE DI DALAM EMBRIO ZEBRAFISH: PERANAN YANG MUNGKIN DI DALAM BIOSINTESIS PTERIDIN

ABSTRAK

Sepiapterin reductase (SR, EC1.1.1.153) memangkinkan langkah terakhir di dalam laluan biosintesis *de novo* bagi tetrahidrobiopterin (BH4), yang mana merupakan kofaktor sangat penting untuk penghasilan pteridin di dalam kromatofor teleost. Walaupun demikian, pemahaman pada peringkat transkripsi gen SR, khususnya ketika perkembangan embrio adalah terhad. Dalam penyelidikan ini, jujukan penuh cDNA zebrafish yang mengekodkan SR telah dipencilkan dan dicirikan. Protein sepanjang 261 asid amino tersebut mempunyai homologi sebanyak 29 hingga 51% dengan jujukan asid amino protein SR lain yang telah diketahui. Analisis jujukan menunjukkan residu-residu tapak fungsi yang tipikal bagi SR adalah terpelihara di dalam zebrafish. Analisis RT-PCR separa-kuantitatif telah dilakukan untuk menentukan corak pengekspresan ontogenik bagi gen ini di dalam embrio yang sedang berkembang. Keputusan menunjukkan tahap pengekspresan SR adalah meningkat secara beransuran, bersama-sama dengan penampilan melanofor dan xantofor yang mengandungi pteridin. Tambahan lagi, “whole-mount *in situ* hybridisation” telah dilakukan untuk menentukan pengekpresan SR zebrafish secara lebih mendalam. Pengkespresan SR yang sederhana telah dikesan kebanyakannya di bahagian kepala, badan dan ekor pada 36 hpf yang terawal, berkolerasi dengan corak perkembangan melanofor. Dari 48 hpf seterusnya, pengekspresan SR telah meningkat terutamanya pada bahagian dorso-anterior kepala, dan di seluruh kawasan lateral badan, selari dengan corak perkembangan xantofor. Kesimpulannya, penyelidikan ini telah menunjukkan corak pengekspresan SR zebrafish semasa perkembangan embrio dan konsistensinya dengan penampilan melanofor dan xantofor yang berasal daripada puncak saraf.

CLONING AND ONTOGENIC EXPRESSION OF SEPIAPTERIN REDUCTASE mRNA IN ZEBRAFISH EMBRYO: POSSIBLE ROLE IN PTERIDINE BIOSYNTHESIS

ABSTRACT

Sepiapterin reductase (SR, EC 1.1.1.153) catalyses the final step in the *de novo* biosynthesis pathway of tetrahydrobiopterin (BH4), which is the essential cofactor for the pteridine production in teleost chromatophores. However, the understanding on the gene transcriptional level of SR, especially during the embryonic development is limited. In this present study, full-length zebrafish cDNA encoding SR was isolated and characterised. The deduced 261 amino acid protein shares 29 to 51% homology with amino acid sequences of other known SR proteins. Sequence analysis revealed that typical functional site residues of SR are well conserved in zebrafish. Semi-quantitative RT-PCR analysis was performed to determine the ontogenic expression pattern of this gene in the developing embryo. The result demonstrated that the expression levels of SR increased gradually, in tandem with the appearance of melanophores and pteridine-contained xanthophores. In addition, whole-mount *in situ* hybridisation of embryo was also carried out to further determine the spatio-temporal expression of zebrafish SR. Moderate expression of SR was mostly detected at the head, trunk and tail regions during the first 36 hpf, correlating with the development pattern of melanophores. From 48 hpf onwards, the expression of SR intensified especially at the dorso-anterior part of head, and throughout the lateral trunk region, parallel to the development pattern of xanthophores. In summary, this study revealed the expression pattern of zebrafish SR during the development of embryo and its consistency with the appearance of the neural crest-derived melanophores and xanthophores.

CHAPTER 1

INTRODUCTION

1.1 General

In vertebrates, chromatophores are pigmented cells derived from neural crest during embryonic development at the neurulation stage. Based on differences in interaction, distribution and density, chromatophores associated within the integuments give rise to the attractive body hues and striking patterns in brightly coloured vertebrates such as fish and amphibians. Pigments within chromatophores play an integral role in colouration as they adsorb, scatter or reflect lights from different wavelengths to form the skin colouration. In parallel, chromatophores are classified into six distinct colour types based on the presence of different pigments.

Pteridines, the major pigments contained in bright-coloured chromatophores, are enclosed in the pterinosomes, which are presumably derived from the smooth endoplasmic reticulum. Pteridines are chemical compounds comprising fused pyrimidine and pyrazine rings. In fact, the capacity to generate pteridines from guanosine triphosphate (GTP) is a common feature to most of the chromatophores. Among pteridines, tetrahydrobiopterin (BH4) is well known for its functions in many physiological pathways.

Danio rerio, commonly known as zebrafish, is a popular ornamental fish originated from the Ganges region in Northern India as well as Pakistan, Bangladesh, Nepal and Myanmar. However, this humble tiny fish has gained popularity among scientists during the past decade, being one of the model organisms especially in vertebrate developmental and gene functional studies. It is easy to maintain in laboratory environment, has a short and rapid generation time, breeds readily, and produces large numbers of transparent embryos, which are accessible for all the embryonic stages due to the *ex utero* development. In addition, the availability of various types of mutants, sequence information of an almost completed genome

project and the growing armamentarium of techniques have strengthened the usefulness of zebrafish as a model organism in the modern biological research.

In zebrafish, some of the pigments of chromatophores are believed to be pteridines which are synthesised *de novo* in the fish. The biosynthesis of pteridines begins with GTP, through the formation of BH4 as intermediate, and finally to the production of pteridines in the chromatophores. During the BH4 synthesis process, enzymes involved are GTP cyclohydrolase I (GCH), pyruvoyl tetrahydropterin synthase (PTPS) and sepiapterin reductase (SR). However, the origin and regulation of these enzymes especially at the molecular level is still not well studied. Furthermore, the exact pathway leading to the formation of pteridines from BH4 is still getting to be fully understood.

Melanophores, iridophores and xanthophores are the three types of chromatophores found in zebrafish. They contain melanins, purines and pteridines as pigments or reflective platelets, respectively in the pigment organelles. In some way, the synthesis of melanin from tyrosine in the melanophores depends on the availability of BH4, which its biosynthesis process involves SR as one of the key enzymes. On the other hand, the coloured pteridines found within the pterinisomes of xanthophores were demonstrated to be sepiapterin, which is also believed to be synthesised by SR from BH4.

These facts indicate that SR plays a crucial role in the biosynthesis of pteridines within chromatophores. However, the understanding on the transcriptional level of this gene during the development of pteridines biosynthesis pathway remains unknown. By using zebrafish as a model, the ontogenic expression levels of SR were analysed by semi-quantitative RT-PCR assay. Concurrently, the spatio-temporal expression patterns of SR were determined by whole-mount *in situ* hybridisation in the developing embryos.

1.2 Objectives

In order to unlock the mechanism of pteridine biosynthesis within different types of chromatophores in teleosts during development, the gene regulation and functional role of the key enzymes involved in this biosynthesis pathway must be fully understood. As the pre-requisite of this, the ontogenic expression pattern of one of these key enzymes, sepiapterin reductase (SR) was determined in this study using the embryo of zebrafish. The objectives of this study were listed as below:

1. To clone and characterise the full-length cDNA of SR from zebrafish.
2. To determine the expression changes of zebrafish SR during its embryonic development, in comparison to other pteridine-synthesised enzymes.
3. To determine the spatio-temporal expression pattern of zebrafish SR in the embryo.

CHAPTER 2

LITERATURE REVIEW

2.1 Colouration

Among vertebrates, fish probably possess the most diversified and fascinating body hues and patterns due to the differentiation, proliferation and migration of different pigmented cells known as chromatophores within the integument (Quigley & Parichy, 2002; Kelsh, 2004). Under neuronal and hormonal influences, chromatophores are able to undergo changes in colour patterns almost instantaneously via cellular motile activities and changes in morphology or density of chromatophores (Burton, 1981; Fujii, 2000; Sugimoto, 2002). Fish body colouration and background adaptation are critically important and essential for protection and predation. Further importance of colouration is seen in social interactions through signal aggression or submission (Höglund *et al*., 2002), signal of sexual interest by development of nuptial colouration (Kodric-Brown, 1998; Amundsen & Forsgren, 2001; Bourne *et al*., 2003), and explosive speciation under sexual selection (Seehausen *et al*., 1999; Allender *et al*., 2003). These extraordinary sophisticated colouration systems that we observed now are certainly developed during the long run of evolution in fish.

2.2 Chromatophores

Like other lower vertebrates, fish form their bodily patterns through the interaction, density and distribution of different types of chromatophores (Hirata *et al*., 2003; Kelsh, 2004). Generally, chromatophores are characterised based on the content of the pigmentary substances, which are enclosed in the organelles termed chromatosomes. These pigment granules absorb, scatter or reflect lights from different wavelengths of the visual light spectrum to the colours of chromatophores. Hitherto, melanophores, xanthophores, erythrophores and cyanophores have been identified as the light-absorbing pigment cells, while iridophores and leucophores are light-reflecting

chromatophores. In total, six types of chromatophores are now known in poikilothermal vertebrates (Fujii, 2000).

2.2.1 Melanophores

Melanophores, a group of dendritic cells that contain eumelanins as the black or dark brown pigments enclosed in specific organelles termed eumelanosomes, are one of the most common chromatophores that present in poikilothermal vertebrates. In teleosts, melanophores are usually present in the dermis with relatively limited distribution in the epidermis. In contrast, the black pigment cells in homeothermal vertebrates, melanocytes are mostly abundant in epidermis (Fujii, 2000).

Teleost melanophores usually display high motile activities and play principal roles in the physiological colour changes. Under the neuronal and hormonal control, numerous melanosomes associated with the cytoskeletal filaments such as microtubules and actins are aggregated rapidly or dispersed throughout the cytoplasm slowly in response to various stimuli (Murphy & Tilney, 1974; Schliwa *et al*., 1979; Rodionov *et al*., 1998; Rogers & Gelfand, 1998; Kimler & Taylor, 2002). Due to the flat and discoidal shape of cell, and the size, colour and rapid rate of pigment granule transportation, melanophores have been considered as one of the most useful models in cellular motility studies (McNiven & Porter, 1984; Stearns, 1984).

2.2.2 Cyanophores

Cyanophores are the latest discovered chromatophore and hence, not much is known for this type of chromatophore. They were found in two species of callionymid fish, the mandarin fish (*Synchiropus splendidis*) and psychedelic fish (*S*. *picturatus*) by Goda and Fujii (1995). Like other chromatophores, the blue pigments are enclosed in the pigmentary organelles designated cyanosomes within the dendritic chromatophores. These granules are approximately 500 nm in diameter and are composed of fibrous material enclosed by a limiting membrane. Cyanophores respond

to various stimuli by the aggregation and dispersion of cyanosomes (Bagnara *et al*., 2007). However, further investigations are still needed on this chromatophore to reveal its importance in fish colouration.

2.2.3 Iridophores

Unlike other chromatophores aforementioned, iridophores (also known as guanophores) are light-reflecting chromatophores which are located in the dermis of fish. They reflect light through the reflective platelets. In guppy, these platelets vary from 0.2 to 0.7 µm in length with the diameter approximately 35 µm (Takeuchi, 1976). They are flat-appearing organelles that contain stacks of empty spaces occupied by purine crystals especially guanine, although hypoxanthine, adenine or uric acid may be employed in small quantity. These purines do not serve as true pigment but they are involved in imparting structural colours. The iridescent colours arise from the interference of light scattered from precisely ordered crystalline purines in the cytoplasm of iridophores (Ziegler, 2003; Lynn Lamoreux *et al*., 2005). Usually, iridophores are immotile and inactive physiologically, meaning the distance between the adjacent platelets in a stack does not change.

However, teleost iridophores can change colours by varying the distance between adjacent platelets in response to stimuli. This phenomenon was first observed in the blue spots of the male killifish, *Fundulus heteroclitus* by Foster (1933). The motile iridophores were able to change their colour from blue to red within a few seconds and took less than a minute to regain the original colour. Furthermore, similar colour changes of iridophores were also observed in Neon tetra (Clothier & Lythgoe, 1987; Nagashi & Oshima, 1989) and blue damselfish (Kasukawa *et al*., 1986). In addition, aggregation and dispersion of reflecting platelets were demonstrated by dendritic iridophores of freshwater goby (Iga & Matsuno, 1986; Matsuno & Iga, 1989).

2.2.4 Leucophores

Similar to iridophores, leucophores are white or cream light-reflecting chromatophores that found only in certain groups within the Osteichthyes among vertebrates. Fishes that possess leucophores are killifish, guppy and medaka (Fries, 1942; Takeuchi, 1976; Lynn Lamoreux *et al*., 2005). In guppy, *Lebistes reticulatus*, leucophores are highly dentritic chromatophores that contain spherical or ellipsoidal pigment granules measuring 500 to 800 nm in diameter. Furthermore, these pigment granules showed degenerative changes during melamine treatment which are similar to xanthophores in some mutants of the goldfish (Takeuchi, 1976). On the other hand, pteridine derivatives were found within the drosopterinosomes, membranous pigmentary organelles of leucophores in medaka (Lynn Lamoreux *et al*., 2005).

2.2.5 Xanthophores and erythrophores

Xanthophores and erythrophores are bright coloured-chromatophores present mainly in dermis although some xanthophores also known to be present in epidermis (Obika & Meyer-Rochow, 1990; Ferrer *et al*., 1999). They contain yellow and red pigments within their pigment granules termed pterinosomes. Generally, pterinosomes are spherical or ellipsoidal, about 500 to 700 nm in diameter and contain internal series of concentric lamellae (Matsumoto, 1965; Matsumoto & Obika, 1968). Similar to melanosomes, pterinosomes have been demonstrated to involve in the pigment translocation activities in chromatophores (Kotz & McNiven, 1994; Kimler & Taylor, 2002; Sato *et al*., 2004). In tandem, this feature has made xanthophores and erythrophores play a critical role in physiological colour changes in teleosts (Fujii, 2000). In cyprinid fishes, sepiapterin has been identified as one of the major pteridines in their pterinosomes (Matsumoto & Obika, 1968; Le Guyader & Jesuthasan, 2002). On the other hand, pterinosomes for poeciliid fishes have been proven to contain drosopterin as major pteridines (Matsumoto, 1965; Grether *et al*., 2001). Furthermore,

sepiapterin and three drosopterins were also abundant in bright coloured-chromatophores from amphibians (Obika & Bagnara, 1964).

Pteridines are present within the chromatophores, either alone or together with another distinctive pigmentary compound, carotenoids (Goodrich *et al*., 1941). However, animals have lost the ability to *de novo* biosynthesise carotenoids due to the lack of enzymes involved in the biosynthesis pathway, but they obtain carotenoids exclusively from their food sources (Olson & Owens, 1998). Carotenoids are present in xanthophores and erythrophores as lipid droplets within vesicles (Takeuchi & Kajishima, 1972; Ferrer *et al*., 1999).

2.3 Pteridines

Pteridines, including pterins, are derivatives from purines with addition of one more carbon. They are chemical compounds consisting of a pyrimidine ring and a pyrazine ring. Pteridines are heterocyclic compounds containing different substitutions on their ring structure as shown in Figure 2.1. The first pterin compound was discovered in the pigments of Brimstone yellow butterfly by Hopkins (Smith, 1962).

In contrast to mammals, teleosts are one of the vertebrates that *de novo* synthesise and deposit various pteridines into the pterinosomes for colouration purposes. Among chromatophores, sepiapterin and drosopterin are the major coloured pteridines abundant in xanthophores and erythrophores (Grether *et al*., 2001; Le Guyader & Jesuthasan, 2002). Besides, other colourless pteridines including 7-oxobiopterin, biopterin, isoxanthopterin, xanthopterin, 2,4,7-trioxopteridine and tetrahydrobiopterin (BH4) are also present in chromatophores (Matsumoto, 1965; Ziegler *et al*., 2000).

Among these pteridines, BH4 is well studied due to its various functions in many other cell-types. BH4 serves as the cofactor for aromatic amino acid hydroxylation, synthesis of all three forms of nitric oxide (NO), and glyceryl-ether hydroxylation (Thöny *et al*., 2000). BH4 is also involved in proliferation of murine erythroid cells (Tanaka *et*

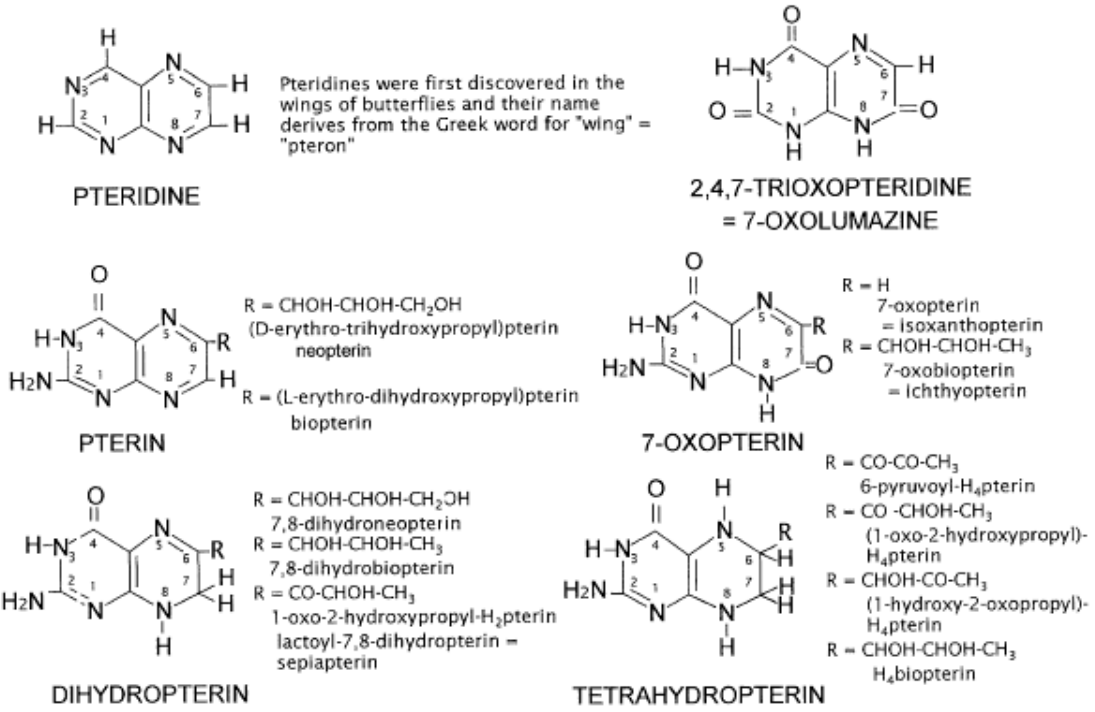

Figure 2.1 Ring structures of pteridines and pterins (Ziegler, 2003).

al., 1989), promotion of neurotransmitter release in brain (Mataga *et al*., 1991) and regulation of melanogenesis in human epidermis (Schallreuter *et al*., 1994). In a rare variant of hyperphenylalaninaemia (atypical phenylketonuria, PKU), BH4 deficiency can result in the deficit of neurotransmitter dopamine and serotonin. Reduced levels of BH4 in the brain and cerebrospinal fluid have also been documented to associate with several neurological diseases presenting phenotypically without hyperphenylalaninaemia, such as Parkinson's disease, Alzheimer's disease, depression and autism (Thöny *et al*., 2000).

2.4 Zebrafish

Zebrafish (*Danio rerio*), belongs to the minnow family (Cyprinidae), is a common ornamental fish throughout the world (Figure 2.2). It was first described by the British surgeon Francis Hamilton in 1822. This small freshwater fish, with full-grown body length approximately 4 to 5 cm is native to streams, canals, ditches, ponds and even rice fields in the Ganges region in Northern India, as well as Pakistan, Nepal and Myanmar. Zebrafish is named for its bodily pattern comprising of longitudinal blue-black and silvery-yellow stripes on the side of body. After years in the aquarium trade, two other forms, 'long finned' and 'leopard' were developed through selective breeding from the typical 'short finned' form. Zebrafish prefers to live in water with a temperature range of 18 to 24°C, pH range of 6.0 to 8.0 and hardness of 5.0 to 19.0 dGH. Normally, zebrafish mates and spawns at dawn (Axelrod, 1995). It is considered to be one of the easiest aquarium fish to breed. A female fish is able to produce up to 300 eggs in a single spawning. The larvae take about two days to hatch and grow rapidly. Subsequently, zebrafish grows through larval and juvenile stages, and continue to the adult stages in 90 days. However, zebrafish is able to achieve reproductive maturity in just six weeks (Quigley & Parichy, 2002).

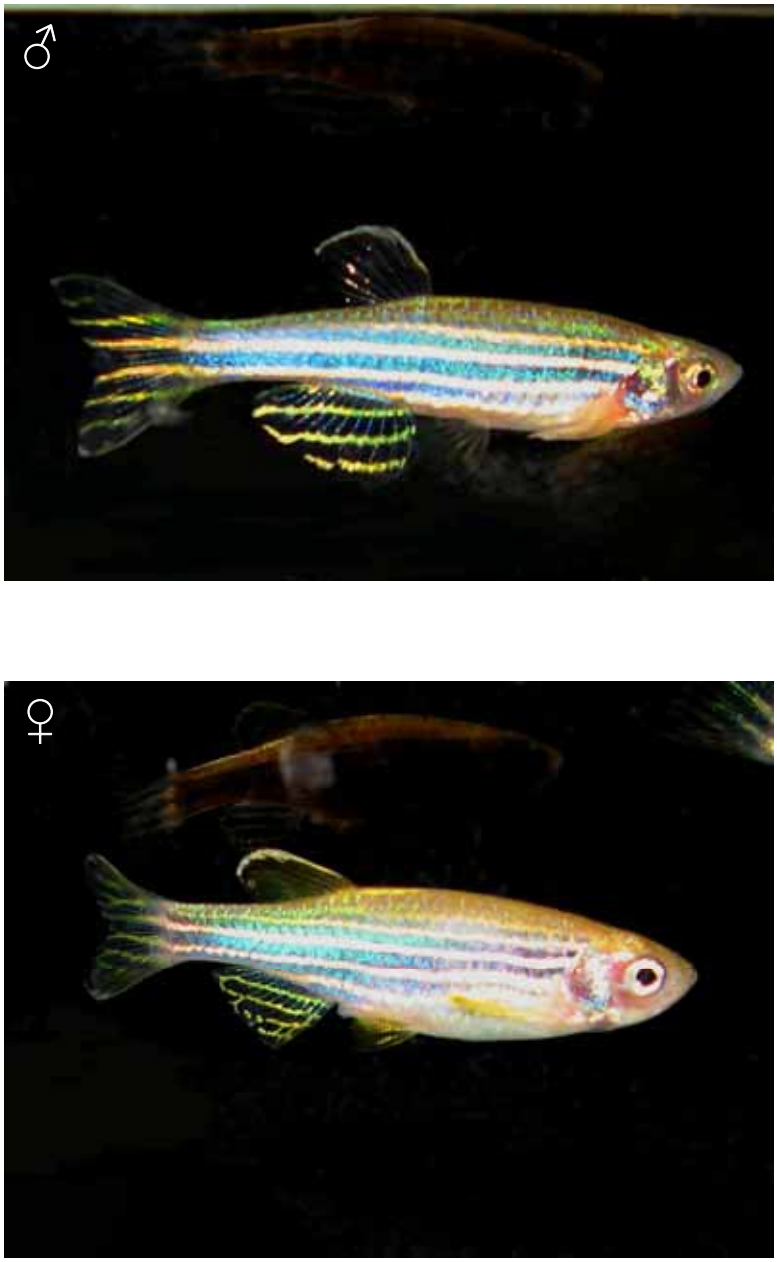

Figure 2.2 Adult male and female zebrafish.

2.5 Strengths of the zebrafish as a model organism

Zebrafish has emerged as one of the most versatile model organism in life sciences since the pioneering work of the late George Streisinger and colleagues (Streisinger *et al*., 1981). A particular field that has recognised the potential of zebrafish as a model organism is developmental biology. Several factors contributing to this are:

i) Zebrafish is easy to maintain and breed under laboratory conditions in large scale (Brand *et al*., 2002).
ii) The production of relatively large and transparent embryos. In addition, the *ex utero* development of embryos facilitates visual observation of their rapid developing anatomical structures within 48 h (Nüsslein-Volhard *et al*., 2002).
iii) The existence of several techniques such as *in situ* hybridisation, green fluorescence protein (GFP) labelling coupled with advances in microscopy imaging has enabled the rapid and precise detection of gene expressions in developing embryos (Gilmour *et al*., 2002; Schulte-Merker, 2002).

Nevertheless, the importance of zebrafish as a model organism is also being recognised in other fields, including gerontology (Gerhard & Cheng, 2002), toxicology (Spitsbergen & Kent, 2003), cancer (Stern & Zon, 2003), behaviour (Gerlai, 2003), pigmentation (Pickart *et al*., 2004), aquaculture (Dahm & Geisler, 2006) and cell death research (Pyati *et al*., 2007).

2.5.1 Mutagenesis screens

While the visual accessibility of developing embryos is an essential feature, the existence of zebrafish mutants also provides researchers with valuable insights in the determination of gene functions. Over the past decade, large numbers of mutants with various developmental defects, ranging from neural degeneration to organ

development and pigmentation patterns were identified either from large-scale chemical mutagenesis screens (Driever *et al*., 1996; Haffter *et al*., 1996) or through insertional mutagenesis screens (Amsterdam *et al*., 1999, 2004; Golling *et al*., 2002). Both of these methods randomly induce mutation without prior knowledge of targeted gene and thus, allowing the identification of novel genes. However, they can only identify genes with unique or partially irredundant functions based on morphological defects. Genes with redundant functions will go undetected.

2.5.2 Reverse genetics

In order to determine the function of a particular gene during development, it is necessary to generate organisms carrying loss-of-function mutations. This can be accomplished in zebrafish through reverse genetic approaches, either by specific knockout or knockdown of genes of interest. These methods include RNAi-mediated knockdown (Wargelius *et al*., 1999; Li *et al*., 2000), morpholino-mediated knockdown (Nasevicius & Ekker, 2000) and TILLING (Wienholds *et al*., 2003). Compared to the other two methods, TILLING seems to be more efficient and easier to be performed for target-selected mutagenesis in zebrafish. However, morpholino-mediated knockdown method is still widely used in zebrafish studies due to its various advantages despite its major drawback. The effect of gene knockdown by morpholino is transient and only stable for 3 to 4 days (Dahm & Geisler, 2006).

2.5.3 Genome sequencing

The current existence of an array of molecular tools needs to be backed by availability of genome sequence information. The existing zebrafish genome sequencing project was started in February, 2001. The genome sequencing strategies comprised of mapping and sequencing clones from BAC and PAC libraries. Secondly, whole genome shotgun sequencing is carried out to fill in the gaps between clone contigs. The assemblies are then automatically annotated and accessible in Ensembl

(http://www.ensembl.org/Danio_rerio/index.html; Vogel, 2000). At the time of writing of this thesis, the latest assembly is version 6 (Zv6), which was released on 30 March 2006, meaning an approximate 1.2 Gb of the zebrafish genome sequence is released to the public from the estimated whole genome of 1.7 Gb.

2.6 Pigmentation of zebrafish

Zebrafish is famously characterised by its horizontal-stripe bodily pattern comprising different distribution of three types of pigment cells: melanophores, xanthophores and iridophores (Figure 2.2). Hirata *et al*. (2003) reported that these three types of chromatophores in zebrafish are generally similar to the respective chromatophores in other teleosts under the transmission electron microscopic study. Furthermore, they classified two types of iridophores from zebrafish based on the size of the reflecting platelets contained therein. Type L iridophores contain relatively large (~10 µm) but small number (<20) of platelets. On the other hand, type S iridophores contain uniform-sized (2 µm) and large number (>50) of platelets.

The adult pigmentation pattern of zebrafish consists of five alternating dark and light stripes (Schilling, 2002). In general, the dark stripes comprising principally melanophores and iridophores, although occasionally xanthophores can be found within this region. The light interstripes lack of melanophores but consist of xanthophores and iridophores only (Parichy *et al*., 2000; Parichy & Turner, 2003; Figure 2.3). However, a study by Hirata *et al*. (2003) using transmission electron microscopy had showed the details of the interaction and distribution of these three chromatophores in the stripe and interstripe regions of zebrafish. In the interstripe region, xanthophores were found lying between the dermis and type S iridophore layer, which is just above the muscular layer. On the other hand, xanthophores were located immediately below the dermis at the stripe region, but the density of xanthophores in stripe region is lower compared to the interstripe region. A layer of type S iridophores was found below the xanthophores. Just below these two layers of xanthophores and

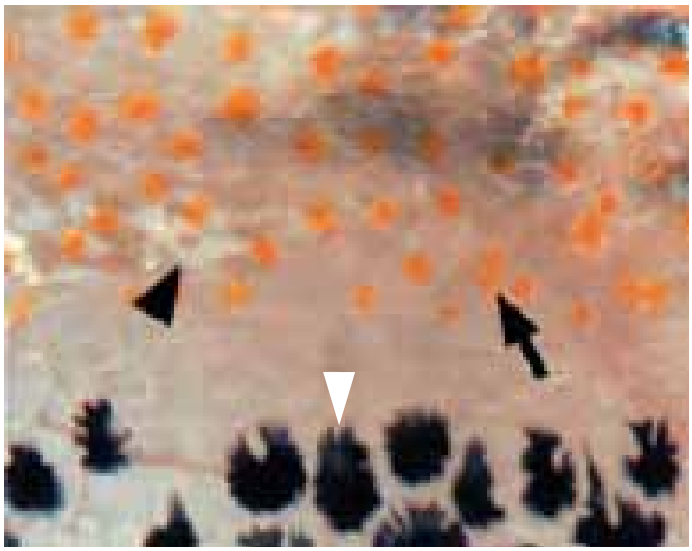

Figure 2.3 Chromatophores present in the lateral trunk of an adult zebrafish (Parichy *et al*., 2000). Xanthophores (black arrow) are distributed within the light interstripe whereas melanophores (white arrowhead) are found within the dark stripe. Iridophores (black arrowhead) are widely distributed within both the regions.

iridophores, there is a layer of melanophores and they showed ubiquitous distribution throughout the whole stripe region. Additionally, there is another layer of type L iridophores present just above the muscular layer. No type L iridophores have been observed in contact with xanthophores (Figure 2.4).

2.7 The development of zebrafish pigment pattern

In zebrafish, pigment cells are derived from the neural crest cells during the embryogenesis and the chromatoblasts (melanoblasts, xanthoblasts and iridoblasts) are derived from two migration pathways, lateral and medial pathway. The former pathway is below the developing epidermis and the latter is in between the somites and neural tube. The melanophores of zebrafish migrate on both the lateral and medial pathways while xanthophores and iridophores are restricted to the lateral pathway. These chromatoblasts are later proliferated and differentiated into specific chromatophores forming the early larval pattern (Kelsh, 2004).

The early larval pattern is clearly observed at 5 days post-fertilisation (dpf), comprises of four longitudinal stripes of melanophores, which include dorsal, lateral, ventral and yolk-sac stripes. In addition, the melanophore stripes are accompanied by iridophores formed in overlapping sequence essentially in order of their distance from the neural tube. In contrast, xanthophores are distributed homogeneously over the lateral face of the underlying myotome, in a slightly graded fashion with the density is higher at dorsal part of body (Schilling, 2002; Kelsh, 2004). Melanophores begin to be visible around 24 hours post-fertilisation (hpf) in the eye and then extended from the hindbrain to posterior and laterally. The first appearance of xanthophores is around 42 hpf as a very pale tinge at the dorsal of the head. Iridophores only started to scatter in the eye around 43 hpf and formed lateral patches at the swim bladder at 48 hpf (Lister, 2002; Ziegler, 2003).

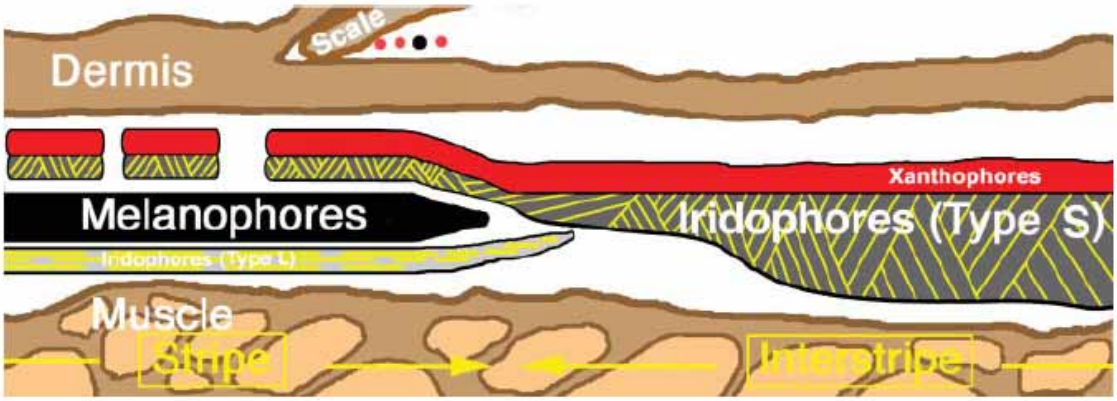

Figure 2.4 The distribution of pigment cells, in relation to the stripe pattern in zebrafish (Hirata *et al*., 2003).

2.8 The biosynthesis pathway of pteridines in zebrafish

Like other teleosts, zebrafish is able to synthesise pteridines as pigments within their chromatophores, especially in xanthophores. The *de novo* biosynthesis of pteridines starts from GTP, via the formation of dihydroneopterin triphosphate and 6-pyruvoyl tetrahydropterin to the formation of BH4 (Thöny *et al*., 2000; Ziegler *et al*., 2000). Three key enzymes involved in this initial biosynthesis pathway, including GTP cyclohydrolase I (GCH, EC 3.5.4.16), 6-pyruvoyl tetrahydropterin synthase (PTPS, EC 4.6.1.10) and sepiapterin reductase (SR, EC 1.1.1.153). GCH is the first rate-limiting enzyme that catalyses the formation of dihydroneopterin triphosphate from GTP through elimination of C(8) from the purine ring. The second rate-limiting enzyme, PTPS eliminates the phosphate groups from dihydroneopterin triphosphate and catalyses an internal redox transfer to yield 6-pyruvoyl tetrahydropterin. Both of the keto groups from this unstable intermediate are rapidly reduced by SR using NADPH as an electron donor, via the formation of 6-(1'-hydroxy-2'-oxopropyl)-tetrahydropterin, isomerisation to 6-lactoyl-tetrahydropterin, and final reduction to BH4 (Figure 2.5).

Interestingly, a second route yielding other forms of pteridines such as 7-oxopteridines has been proposed to be started from the sepiapterin by Ziegler *et al*. (2000). It is proposed that sepiapterin is reduced to dihydrobiopterin by SR and later oxidised to biopterin by dihydrobiopterin oxidase. The pathway is later en route to the formations of different 7-oxo derivatives, where xanthine oxidoreductase/dehydrogenase and pterin deaminase are believed to be involved. The precise origin of sepiapterin remains unknown although it has been proposed to be arisen by air oxidation of 6-lactoyl-tetrahydropterin in *Drosophila* (Switchenko & Brown, 1985). It has also been suggested to originate from 6-pyruvoyl-tetrahydropterin or 6-lactoyl-tetrahydropterin under certain enzymatic control although this is still hypothetical (Ziegler *et al*., 2000).

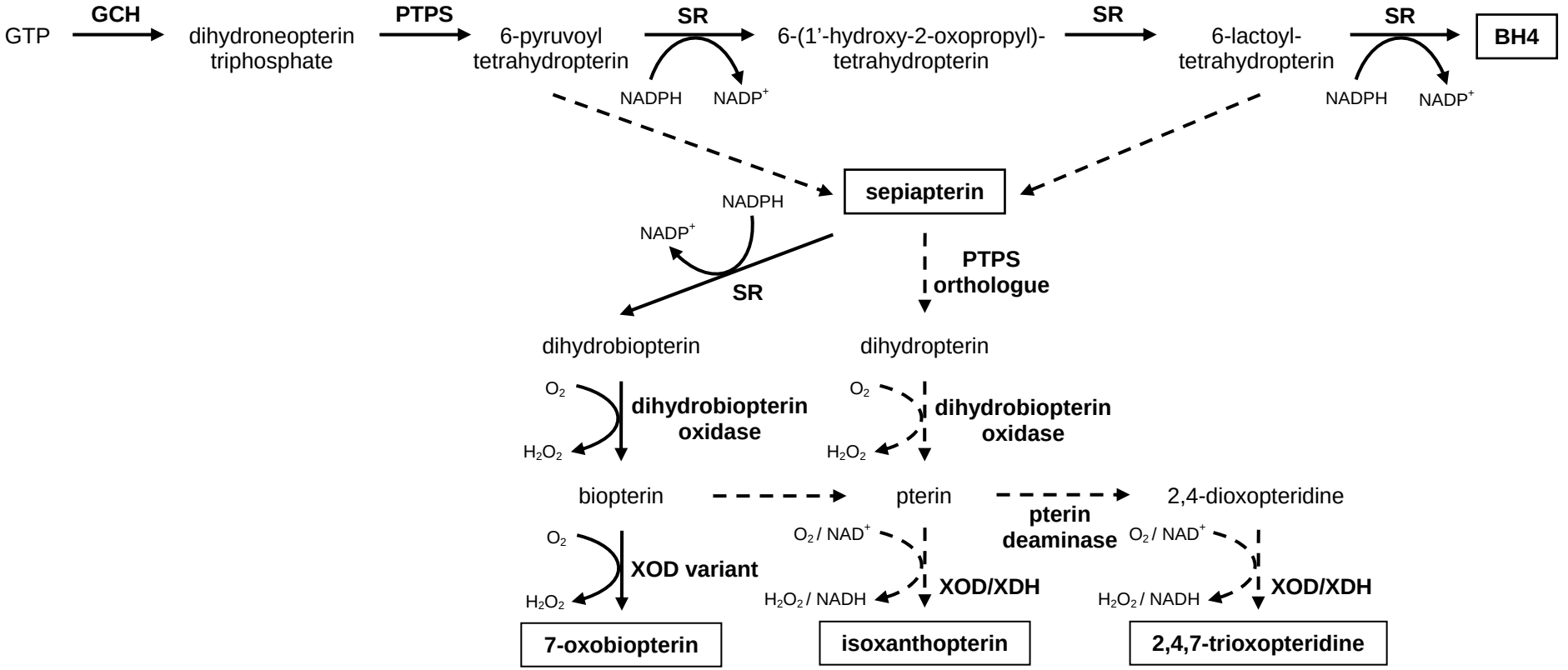

Figure 2.5 Proposed biosynthesis pathway of pteridines in zebrafish (Ziegler, 2003). GTP, guanosine triphosphate; BH4, tetrahydrobiopterin; GCH, GTP cyclohydrolase I; PTPS, 6-pyruvoyl tetrahydropterin synthase; SR, sepiapterin reductase; XOD, xanthine oxidase; XDH, xanthine dehydrogenase; – –► hypothetical pathway. Pteridines identified in zebrafish was boxed.

2.9 Sepiapterin reductase (SR)

2.9.1 Structure, chromosomal localisation and expression of SR gene

In mammals, the genomic organisation of SR genes is highly conserved between human and mouse (Ohye *et al*., 1998; Lee *et al*., 1999). They both consist of three exons, span a region of 4 to 5 kb and no splice variants have been observed so far. However, a highly homologue pseudogene (Sprp) was isolated and characterised from the mouse genome. It is composed of exon 1 and exon 2 region of the authentic SR gene with overall homology of 82%. Precisely, human SR gene was mapped to the p13 region of chromosome 2, while mouse SR gene was located in the central region of chromosome 6, which is also the known syntenic region of human chromosome 2 (Kim *et al*., 1997). In contrast to mammalian SR genes, *Drosophila* SR gene does not have any intron and produce a single transcript of 1.4 kb (Seong *et al*., 2000). It was mapped to 15A on the X chromosome.

The transcription start sites (TSS) of mammalian and *Drosophila* SR genes have been determined, which are located within 100 bp upstream from the ATG codon. The promoter region studies revealed that there is devoid of distinctive TATA- and CAAT-box motifs in all three SR genes. Furthermore, sequence between -83 and -51 at the promoter region was shown to be essential for the mouse SR gene expression (Lee *et al*., 1999). Previous works done by Maier *et al*. (1993) have shown that rat SR mRNA was expressed in different types of tissue including liver, kidney, spleen and brain. Moreover, SR mRNA was also detected throughout different developmental stages, and in both heads and bodies of *Drosophila* (Seong *et al*., 2000).

2.9.2 Structure and localisation of SR enzyme

The 1.25 Å crystal structure of mouse SR in its ternary complex with oxaloacetate and $NADP^+$ has been demonstrated by Auerbach *et al*. (1997). SR is a homodimer comprising 2 monomers with 261 amino acids each. The monomers form a single-domain *α*/*β*-fold with a central four-helix bundle connecting two seven-stranded

parallel *β*-sheets, each sandwiched between two arrays of three *α*-helices. However, the two parallel *β*-sheets of the dimer are in an anti-parallel orientation enclosing an angle of 90°. At the C-terminal end of the *β*-sheets contains the active site of SR, a 15 Å-deep pocket that is suitable to receive pterin and small carbonyl substrates. The substrate is later anchored by the guanidine moiety of a specific Asp residue (Asp-258 in mouse SR).

Recently, Ikemoto *et al*. (2002) has demonstrated that the wide distribution patterns of SR enzyme in different parts of human brain, such as substantia nigra (SN), caudate nucleus (CN), grey and white matters of the cerebral cortex (CTX), and dorsal and ventral parts of the medulla oblongata (MO). On the other hand, researchers from Japan revealed that the distributions of SR enzyme are mainly in the xanthophores but very little in the melanophores of medaka fish (*Oryzias latipes*), although SR is very likely to be included in melanophores due to the biosynthesis of BH4 within the cells (Negishi *et al*., 2003).

2.9.3 Functional roles of SR in the biosynthesis of tetrahydropteridines

Commonly, SR is well known to be the enzyme involved in the terminal step of the *de novo* biosynthesis of L-*erythro*-tetrahydrobiopterin (BH4), which is a well-known cofactor important for various physiological processes in higher mammals. Under the presence of NADPH, SR first reduces the keto group at the side-chain C-1 and forms 6-(1'-hydroxy-2'-oxopropyl)-tetrahydropterin. Following this, the internal rearrangement of the keto group takes place via side-chain isomerisation, and leads to the formation of 6-lactoyl-tetrahydropterin. Immediately, the intermediate is then converted into BH4 in a second NADPH-dependent reduction step (Thöny *et al*., 2000).

Beside this, Blau *et al*. (2001) suggested that SR may be involved in a BH4 salvage pathway by catalysing the conversion of sepiapterin into dihydrobiopterin (Figure 2.6). However, Sawada *et al.* (2005) showed that the proposed pathway in which the non-enzymatic conversion of 6-lactoyl-tetrahydropterin to sepiapterin is

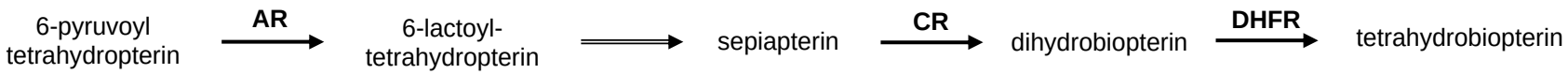

Figure 2.6 A proposed BH4 salvage pathway (Blau *et al*., 2001). AR, aldose reductase; CR, carbonyl reductase; DHFR, dihydrofolate reductase; ⟹ non-enzymatic conversion.

difficult or unlikely to proceed in human. Recently in *Dictyostelium discoideum*, SR has also being demonstrated that its novel capability to convert 1'-oxo-2'-D-hydroxypropyl-tetrahydropterin into D-threo-tetrahydrobiopterin (DH4). Moreover, *Dictyostelium* SR prefers 1'-oxo-2'-D-hydroxypropyl-tetrahydropterin as substrate than 6-pyruvoyl tetrahydopterin to maintain the dominant production of DH4 over BH4 in this species (Choi *et al*., 2006).

CHAPTER 3

MATERIALS AND METHODS

3.1 Stock solutions and reagents

All the stock solutions and reagents used in this study were prepared according to Appendix A.

3.2 Culture media and antibiotic

3.2.1 LB medium and LB-ampicillin plate

LB medium was prepared according to Maniatis *et al*. (1982). A total of 10 g of tryptone, 5 g of yeast extract and 10 g of NaCl were dissolved in a final volume of 1000 ml dH_2O. The pH of medium was adjusted to 7.5 with NaOH, and sterilised by autoclaving at 121°C for 20 min.

To prepare LB-ampicillin plate, 15 g of agar was added to 1000 ml of LB medium. The medium was sterilised by autoclaving and allowed to cool to 50°C, before adding ampicillin to a final concentration of 100 µg/ml. Approximately 15 to 20 ml of the medium was poured into an 85 mm petri dish and allowed to solidify. LB-ampicillin plate was then stored at 4°C for up to 1 month. For blue-white colony selection, 100 µl of 100 mM IPTG and 20 µl of 50 mg/ml X-Gal were spread over the surface of an LB-ampicillin plate and allowed to absorb for 30 min at 37°C prior to use.

3.2.2 Antibiotic

Sodium salt of ampicillin was dissolved in dH_2O to a final concentration of 100 mg/ml, followed by filter-sterilisation and stored in aliquots at -20°C.

3.3 Host strain and cloning vector

The genotype of the bacteria host strain used in this study is presented in Table 3.1 and the map of pGEM®-T Easy Vector (Promega, USA) is shown in Figure 3.1.