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Critical Review

Biosynthesis of Drosopterins, the Red Eye Pigments of *Drosophila melanogaster*

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Abstract

Drosophila melanogaster has red eyes. Scientists have been curious about the biosynthesis of the red eye pigments and have completed a number of investigations on these compounds. Scientific contributions made over the past 50 years have improved our understanding of the red eye pigments. Researchers have elucidated the chemical structures of some pigments and have successfully purified and identified the enzymes that participate in the biosynthesis of the red eye pigments. In this article, we will review the characteristics of the *Drosophila* red eye pigments and of the enzymes and genes involved in its biosynthetic pathway. © 2013 IUBMB Life, 00(0):000–000, 2013

Keywords: *Drosophila;* drosopterins*;* GTPCH I*;* PTP synthase*;* PDA synthase*;* dihydropterin deaminase

INTRODUCTION

Since the discovery of many eye color mutants, the eye color pigments of *Drosophila melanogaster* have been the subject of numerous investigations. Two classes of pigments, the brown "ommochromes" and the red "drosopterins", contribute to the typical eye color phenotype of *Drosophila* and serve as light-screening pigments (1). The biosynthetic pathways of these two pigments are distinct and do not share enzymes; ommochromes are synthesized from tryptophan, whereas drosopterins are synthesized from guanosine-5′-triphosphate (GTP). In this review, we will describe the drosopterins and the pathways leading to the synthesis of three of these red pigments.

Abbreviations *GTP, guanosine-5′-triphosphate; TLC, thin layer chromatography; ^{1}H NMR, proton nuclear magnetic resonance; GTPCH I, GTP cyclohydrolase I; H_2-NTP, 7,8-dihydroneopterin triphosphate; 6-PTP, 6-pyruvoyltetrahydropterin; PTPS, PTP synthase; PDA, pyrimidodiazepine; BH_4, tetrahydrobiopterin; GFRP, GTPCH I feedback regulatory protein; GSH, reduced glutathione; GST, glutathione S-transferase; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight*

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The family of drosopterins is composed of at least five compounds, which have been referred to as drosopterin, isodrosopterin, aurodrosopterin, neodrosopterin, and fraction e. To avoid confusion in this review, the terms "drosopterins" and "drosopterin" will be used to refer to the class of red eye pigments and to the specific member of this pigment class, respectively (2). Drosopterins were first isolated by Lederer (3) in 1940, and the five components have been separated by cellulose thin layer chromatography (TLC). Of these red eye pigments, drosopterin and isodrosopterin are generally considered the major pigments, whereas aurodrosopterin and neodrosopterin are the minor components (4). Aurodrosopterin constitutes <10% of the total drosopterins in wild-type flies. Little is known about neodrosopterin and fraction e.

THE CHEMICAL STRUCTURES OF DROSOPTERINS

Drosopterins are composed of a pteridine moiety and a pyrimidodiazepine (PDA; 6-acetyl-2-amino-3,7,8,9-tetrahydro-4*H*-pyrimido[4,5-*b*][1,4]diazepin-4-one) moiety in a pentacyclic ring system (4,5). The chemical structure of drosopterin was elucidated by Theobald and Pfleiderer (5) in 1978. In 1972, Schlobach and Pfleiderer (6) first proposed a dipteridyl structure of drosopterin, which later turned out to be incorrect. Based on new spectral data obtained from proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy and circular

dichroism spectroscopy experiments, Pfleiderer's group (5) proposed a new, correct, structure for drosopterin, consisting of a pentacyclic ring system containing a 5,6,7,8-tetrahydropterin (2-amino-5,6,7,8-tetrahydropteridin-4(1*H*)-one) moiety, a PDA moiety, and a pyrrole ring (Fig. 1). They also showed that drosopterin and isodrosopterin are enantiomers of each other.

The chemical structure of aurodrosopterin was determined by Yim et al. (4) in 1993. ^{1}H-NMR and ultraviolet (UV)/visible (VIS) spectral analyses revealed that this compound has one fewer amino group in the pteridine portion relative to drosopterin (Fig. 1). Yim et al. (4) synthesized aurodrosopterin by the nonenzymatic condensation of 7,8-dihydrolumazine with PDA and showed that the spectra of the synthetic aurodrosopterin were identical to those of natural aurodrosopterin. They also reported the presence of isoaurodrosopterin in *Drosophila* based on the thin layer chromatographic analyses of *Drosophila* head extracts using various solvent systems (4). Aurodrosopterin and isoaurodrosopterin appear to be enantiomers.

Unlike the pigments discussed above, natural neodrosopterin seems to be optically inactive. Yim et al. (4) tried to resolve the enantiomeric forms of neodrosopterin on cellulose TLC with many different solvent systems but failed. They synthesized a reddish pigment, which was named aminodrosopterin, by the nonenzymatic condensation of 2,4-diamino-7,8-dihydropterin with PDA and compared the UV/VIS spectra of this synthetic compound and natural neodrosopterin. The spectral properties of synthetic aminodrosopterin were different from those of natural neodrosopterin.

BIOSYNTHETIC PATHWAY OF DROSOPTERINS

In contrast to the biosynthetic pathway of ommochromes, which consists of four steps and has been well characterized both biochemically and genetically, the biosynthetic pathway of drosopterins has not yet been firmly established. The biosynthesis of drosopterins is accomplished by a series of enzymatic and nonenzymatic reactions (Fig. 1) (7). The first step in the biosynthesis of drosopterins is the conversion of GTP into 7,8-dihydroneopterin triphosphate (H_2-NTP) with the release of formic acid by GTP cyclohydrolase I (GTPCH I; EC 3.5.4.16) (8). H_2-NTP is then converted into 6-pyruvoyltetrahydropterin (6-PTP) by 6-PTP synthase (PTPS; EC 4.6.1.10) (9,10). Next, 6-PTP is converted into PDA by PDA synthase (EC 1.5.4.1) (2,11).

Drosopterin and isodrosopterin are produced nonenzymatically by the one-to-one condensation of 7,8-dihydropterin with PDA under acidic conditions (12). The origin of 7,8-dihydropterin is not well established. Yim et al. (13) have reported the presence of enzymatic activity in *Drosophila* that releases the three-carbon side chain from H_2-NTP. Owing to the impurity of the enzyme system, the true substrate of the enzyme that produces 7,8-dihydropterin was not identified. However, the biochemical properties of the enzyme strongly suggest that 6-PTP is the substrate of the side chain releasing enzyme. Aurodrosopterin and isoaurodrosopterin are also produced in a similar fashion by the nonenzymatic one-to-one condensation of 7,8-dihydrolumazine with PDA under acidic conditions (4). 7,8-Dihydrolumazine is produced from 7,8-dihydropterin by deamination that is catalyzed by dihydropterin deaminase (EC 3.5.4.11) (7,14).

The first two steps in the biosynthesis of drosopterins are shared in the *de novo* biosynthesis of tetrahydrobiopterin (BH_4), which is accomplished by three consecutive enzymatic reactions catalyzed by GTPCH I, PTPS, and sepiapterin reductase (EC 1.1.1.153) (15). BH_4 is an essential cofactor required for the synthesis of important biogenic amines such as catecholamines and serotonin.

ENZYMES

GTP Cyclohydrolase I (EC 3.5.4.16)

The first step of the biosynthesis of drosopterins is catalyzed by GTPCH I, which converts GTP into H_2-NTP (8). GTPCH I was first partially purified from the extracts of late pupae and young adults of the species *Drosophila melanogaster* and characterized by Fan and Brown (16) in 1976. The enzyme was later completely purified from young adult flies and further characterized by Weisberg and O'Donnell (17) in 1986. The crystal structure of GTPCH I from *Escherichia coli* was solved by Nar et al. in 1995 and it showed a homodecameric enzyme composed of a dimer of pentamers (18).

GTPCH I is encoded by the *punch* gene (*CG9441*), which is located at 57C7-57C8 on the second chromosome. The *punch* locus is genetically complex (19) and produces at least four different transcripts as a result of alternative promoters and alternative splicing (20). Three of these transcripts, transcripts A (1.70 kb), B (1.75 kb), and C (1.80 kb), have been well characterized (20–22). Transcript A and transcripts B and C are produced from two distinct promoters, P1 and P2, respectively, and are expressed in different tissues and at different stages of development. The P1 promoter is active only from the late pupal stage to the adult stage, whereas the P2 promoter is active throughout the development. Both the P1 and P2 promoters are more active in the adult head than the adult body. Transcripts B and C are produced by the differential use of 3′-splice sites in the first exon. All three isoforms have identical catalytic core domains but different N-terminal domains (20). The N-terminal domain of isoform A is completely different in sequence from those of isoforms B and C. Isoforms B and C are identical except for a 16-amino acid addition to the N-terminal domain of isoform C. The variation in the N-terminal domains of the *Drosophila* GTPCH I isoforms confers differential enzymatic activities and regulatory properties to these enzymes (20).

Drosophila GTPCH I is regulated at the posttranslational level by a variety of mechanisms. First, all the three isoforms are subjected to end product feedback inhibition by BH_4 (20).

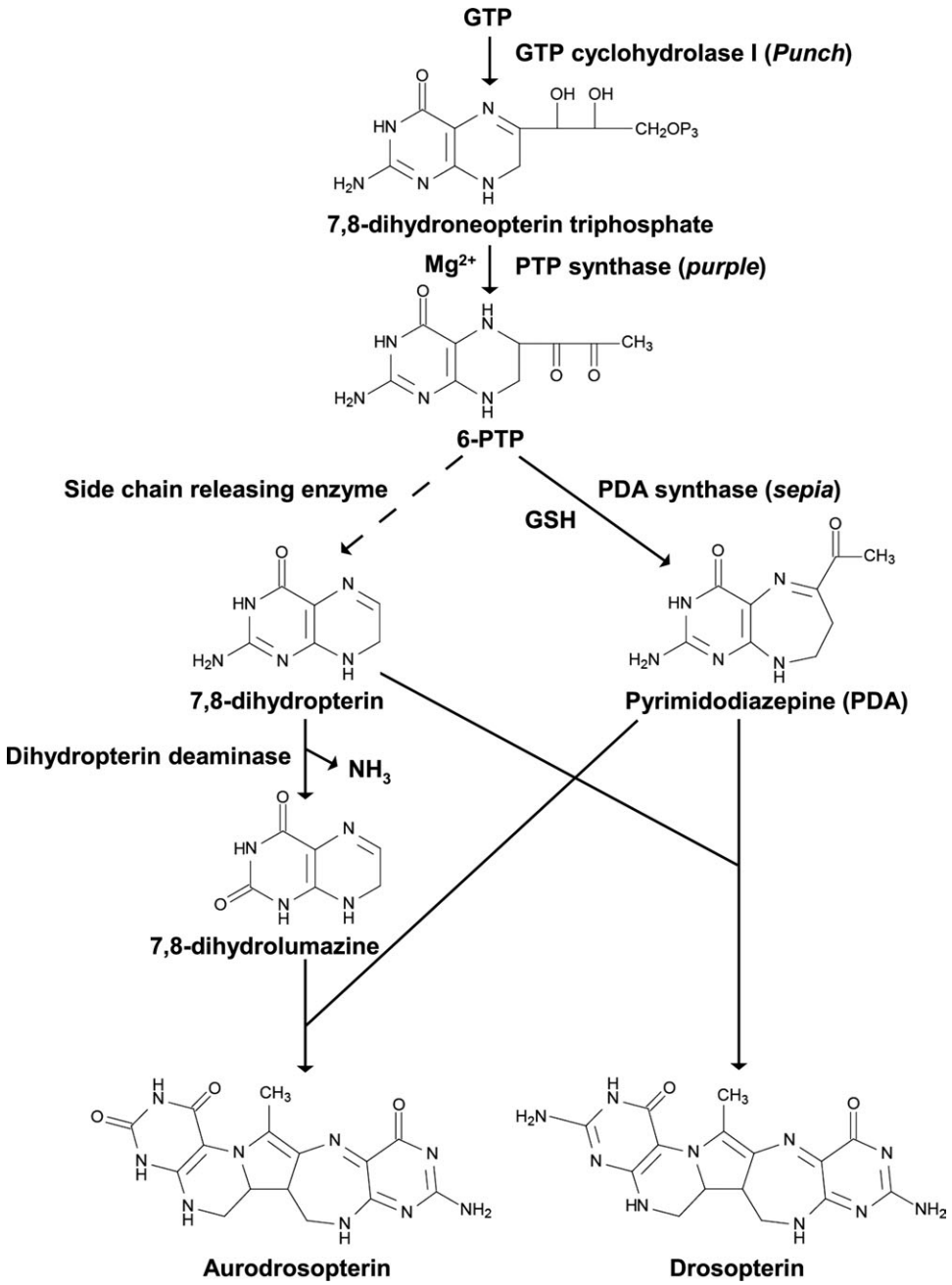

FIG 1 *Pathway for the biosynthesis of drosopterins in Drosophila melanogaster (7).*

Unlike the mammalian GTPCH I, which requires GTPCH I feedback regulatory protein (GFRP) for end product feedback inhibition by BH_4 (23,24), *Drosophila* GTPCH I does not need GFRP. GFRP is not even present in the *Drosophila* genome (20). The N-terminal domains of *Drosophila* GTPCH I serve as functional homologs of the mammalian GFRP. Second, the N-terminal domains of isoforms B and C have a negative regulatory role (20). Despite the fact that all three isoforms have identical catalytic domains, the negative regulatory functions of the N-terminal domains of isoforms B and C result in reduced activity relative to that of isoform A. Third, *Drosophila* GTPCH I is also positively regulated by phosphorylation (20). The N-terminal domains of isoforms B and C serve as substrates for protein kinases A and C. The phosphorylation of the N-terminal domains of isoforms B and C results in a significant elevation in activity. It seems that phosphorylation of the negative regulatory domain alleviates the negative regulation of the enzyme by the negative regulatory domain.

Consistent with the expression profile of GTPCH I, *Drosophila* exhibits a characteristic activity profile of GTPCH I

during the life cycle (25,26). Two peaks of GTPCH I activity occur during development. The first peak of activity occurs at the time of pupation, and the second peak of activity occurs at the time of eclosion. The activity of the second peak is restricted almost entirely to the head and decreases rapidly to 20% of its maximum activity within 48 H after eclosion, supporting the hypothesis that this enzyme is responsible for the synthesis of drosopterins (8).

PTP Synthase (EC 4.6.1.10)

PTPS catalyzes the conversion of H_2-NTP into 6-PTP in the presence of a divalent cation, Mg^{2+} (9,10). PTPS is encoded by the *purple* gene, which is located at 38B3 on the second chromosome. In the early studies, *purple* was originally described as a sepiapterin synthase (27) because a *purple* mutant exhibited reduced sepiapterin synthase activity, which was presumed to be responsible for the synthesis of sepiapterin from dihydroneopterin triphosphate. The sepiapterin synthase enzyme system actually consists of two enzymes, termed sepiapterin synthase A and sepiapterin synthase B (9), which were later identified as PTPS and PTP reductase, respectively (28). Sepiapterin synthase A was first purified from the heads of *Drosophila melanogaster* by Switchenko and Brown (29). In their study, they identified the reaction product of this enzyme as 6-pyruvoyl-5,6,7,8-tetrahydropterin, and they therefore named the enzyme 6-pyruvoyltetrahydropterin synthase. The biochemical properties of *Drosophila* PTPS were confirmed and further characterized by Park et al. (10) in 1990. The crystal structure of rat PTPS was determined by Burgisser et al. in 1995 and it showed a homohexameric enzyme composed of a dimer of trimers (30).

PTPS complementary DNA clones were first isolated by Kim et al. (31) in 1996. *Purple*, the structural gene for PTPS, produces two transcripts from two different promoters (31). The small 1.1 kb transcript is head specific and is highly expressed during the late pupal and young adult stages, when eye pigment synthesis is highest. The 1.3 kb large transcript is expressed constitutively and presumably supports the synthesis of BH_4. These two transcripts use different promoters. The large transcript uses the distal promoter, whereas the small transcript uses the proximal promoter for transcription initiation.

PDA Synthase (EC 1.5.4.1)

The key step in the biosynthesis of drosopterins is catalyzed by PDA synthase. During the reaction, the reduced pyrazine ring of the substrate PTP is opened and enlarged to a seven-membered diazepine ring (2,11). The chemical structure of PDA was determined by Jacobson et al. (32) in 1982. Pyrimidodiazepine, sometimes called 6-acetylhomopterin, is an interesting compound. Unlike other pteridines, it quenches UV light at room temperature but fluoresces an intense green color at very low temperatures (33).

The PDA synthase activity was first discovered by Brown and coworkers while analyzing the heads of *Drosophila* (2). In that study, they demonstrated that the presence of reduced glutathione (GSH) is required for the conversion of 6-PTP into PDA by the purified enzyme. Because the reaction product PDA is reduced by two electrons relative to the precursor 6-PTP, it seems that the reducing power required for the reaction is supplied by GSH. The molecular weight of the enzyme is 48 kDa under native conditions and it is composed of two identical polypeptide chains (34). Brown and coworkers also found that the enzyme activity is decreased in the fly mutants *sepia* and *clot* (2). *Clot* is a leaky mutant, which encodes a protein related to the glutaredoxin family of the thioredoxin-like enzyme superfamily (35). The *sepia* mutant is characterized by the accumulation of the yellow pigment sepiapterin and by the lack of drosopterin pigments in the eye. The *sepia* mutant has almost no PDA synthase activity, suggesting that *sepia* might be the structural gene for PDA synthase.

To search the structural gene of the PDA synthase in *Drosophila*, Kim et al. (11) identified five candidate genes from the proposed *sepia* locus (66D5 on the third chromosome) by examining the molecular masses of the gene products and the abilities of these gene products to interact with GSH *in silico*. They found that among the five candidates, only *CG6781* exhibited PDA synthase activity *in vitro* and that all the candidates, including *CG6781*, had high glutathione-dependent thiol transferase and dehydroascorbate reductase enzymatic activities *in vitro*. These activities are the characteristic of Omega class glutathione S-transferases (GSTs) (36), and therefore, *CG6781* is classified as a member of the Omega class GSTs together with the other four candidates.

Kim et al. (11) also demonstrated the function of PDA synthase *in vivo*. They showed that the naturally occurring mutant se^1 has a frameshift mutation from AAGAA to GTG in *CG6781* that causes a premature stop codon. The se^1 allele causes a dark brown eye color phenotype. The expression of *CG6781* in se^1 mutants using the eye-specific *GMR-gal4* system rescued the eye color phenotype (Fig. 2), as well as the PDA synthase activity and the PDA content, in a *UAS-CG6781* dose-dependent manner (11).

The *sepia* gene *CG6781* is expressed only during the late pupal stage to the young adult stage. Moreover, the expression of *CG6781* is restricted to the adult head. The developmental expression profile and head-specific expression of *CG6781* are consistent with the characteristics of other enzymes participating in the biosynthesis of drosopterins, such as GTPCH I and PTPS (11). This expression profile also corresponds with the fact that the formation of drosopterins begins during the late pupal stage and continues for several days after eclosion.

Dihydropterin Deaminase (EC 3.5.4.11)

Dihydropterin deaminase deaminates 7,8-dihydropterin, yielding 7,8-dihydrolumazine (7,14). The product of the reaction, 7,8-dihydrolumazine, produces aurodrosopterin via nonenzymatic one-to-one condensation with PDA (4).

Takikawa et al. (14) partially purified dihydropterin deaminase from *Drosophila* for the first time. In 2009, Kim et al. (7)

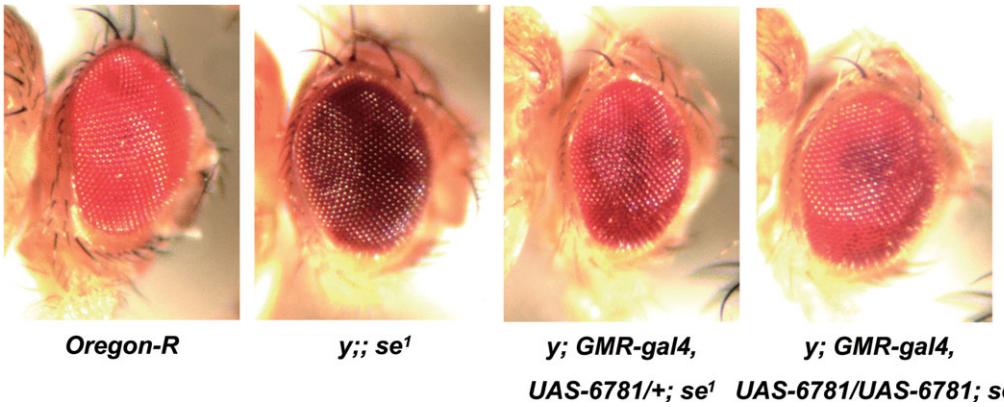

FIG 2 *The defective eye color phenotype due to the se^1 allele is rescued by the eye-specific expression of CG6781 in a dose-dependent manner. Wild-type flies (Oregon-R), sepia mutants (y;; se^1), flies with one copy each of GMR-gal4 and UAS-CG6781 in the se^1 mutant background (y; GMR-gal4,UAS-CG6781/+; se^1), and flies with one copy of GMR-gal4 and two copies of UAS-CG6781 in the se^1 mutant background (y; GMR-gal4,UAS-CG6781/UAS-CG6781; se^1) were used. From Kim, J., Suh, H., Kim, S., Kim, K., Ahn, C., et al. Biochem. J. 2006, 398, 451 – 460, ©The Biochemical Society.*

purified dihydropterin deaminase to homogeneity from the heads of *Drosophila* using conventional purification procedures. The enzyme is a monomeric protein with a molecular weight of 48 kDa. The purified enzyme showed high deaminase activity toward guanine in addition to 7,8-dihydropterin. The K_M values for 7,8-dihydropterin and guanine are 1.6 mM and 76 μM, respectively, and the *k*cat values are 16 and 650 molecules/Sec, respectively. *k*cat/K_M, a measure of catalytic efficiency, is 860-fold higher for guanine than 7,8-dihydropterin. The enzyme exhibited optimal catalytic activities at pH 7.5 and 40 °C with both substrates (7). Similar to the activities of other enzymes involved in eye pigment synthesis, the deaminase activity increased sharply immediately before and after eclosion and remained relatively high in young adults (7).

The structural gene of the dihydropterin deaminase was identified as *CG18143* by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry analysis (7). *CG18143* is located at 82A1 on the third chromosome and produces one transcript. The theoretically predicted molecular mass and pI value of the *CG18143* gene product (48,884 and 5.97) are in close agreement with the values determined for the purified deaminase. It appears that this enzyme can deaminate guanine as well as 7,8-dihydropterin because the chemical structures of the two compounds are similar.

The *in vivo* function of the 7,8-dihydropterin deaminase was analyzed biochemically and genetically (7). Mutant flies were constructed using a deficient line lacking the *CG18143* gene and a line that carries a *P*-element inserted immediately upstream of the 5′-end of the transcription initiation site of the gene. The transheterozygous mutant showed reduced levels of *CG18143* messenger RNA and 7,8-dihydropterin deaminase activity along with a defective eye color phenotype (Fig. 3A). The analysis of the red pigments revealed that the deaminase mutant had a dramatically decreased amount of aurodrosopterin, whereas the levels of the other drosopterins were normal (Fig. 3B), suggesting that this enzyme is indeed involved in the biosynthesis of the minor red pigment aurodrosopterin.

COMMENTS AND CONCLUDING REMARKS

Pteridines are widely distributed as pigments in nature (1,37). Some are blue, some are yellow, and certain pteridines have a beautiful red color. In the 1950s and 1960s, the coenzyme functions of pteridines were discovered (38–41). The most important and most well-known pteridine coenzymes are pteroylglutamate (folic acid) (42,43), BH_4 (38–41), and riboflavin (42,44).

Owing to the biological significance of pteridines, the biosynthesis of various pteridine compounds has been the subject of numerous investigations. Many studies of pteridine biosynthesis have been conducted in *Drosophila melanogaster* because the use of *Drosophila* has several advantages (45). First, *Drosophila melanogaster* contains numerous pteridines, most of which are present as eye pigments. Second, the enzymes responsible for the biosynthesis and interconversion of pteridines are abundant in the heads of *Drosophila.* Third, a wealth of eye color mutants are associated with the synthesis of pteridine pigments.

Drosopterins are the major pteridine pigments found in the eyes of *Drosophila*. For the last 50 years, considerable progress has been made in the study of the biosynthesis of drosopterins. The chemical structures of some drosopterins have been elucidated, and the enzymes and genes associated with the biosynthesis of drosopterins have been successfully characterized and identified. Drosopterins, like all other pteridine compounds, are derived from GTP. GTP is converted into H_2-NTP by the enzyme GTPCH I (8), which catalyzes the rate-limiting step in the pathway. H_2-NTP is then converted into 6-PTP by PTPS (9,10). 6-PTP is the immediate precursor of BH_4,

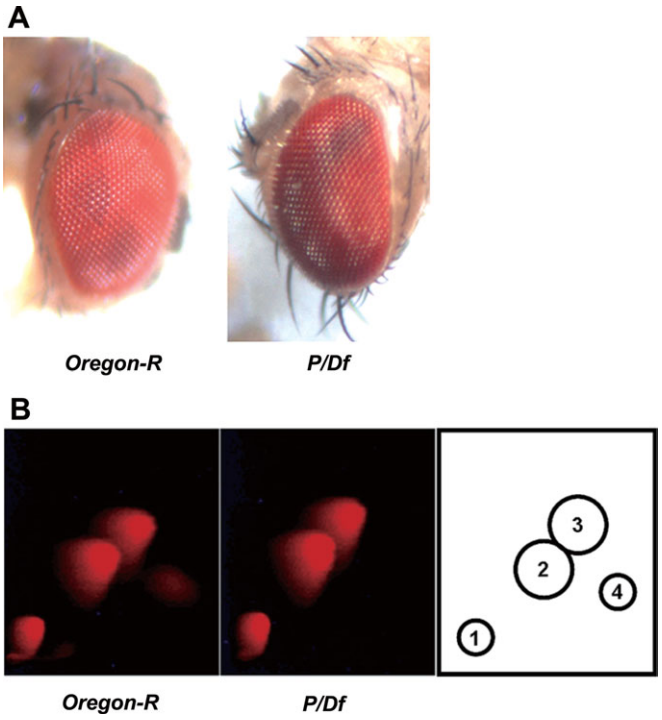

FIG 3

(A) Eye color phenotype of a 2-day-old wild-type (OR) and CG18143 transheterozygous mutant (P/Df) flies. (B) Analysis of the red pigments in the heads of OR and P/Df flies by two-dimensional TLC. In P/Df transheterozygotes, the level of aurodrosopterin was specifically decreased when compared with the level in wild-type flies. The identity of each spot is as follows: 1, neodrosopterin; 2, drosopterin; 3, isodrosopterin; and 4, aurodrosopterin. From Kim, J., Park, S. I., Ahn, C., Kim, H., and Yim, J. J. Biol. Chem. 2009, 284(35), 23426 – 23435, ©The American Society for Biochemistry and Molecular Biology.

the hydroxylation cofactor for aromatic amino acids, as well as PDA, a key compound in the biosynthesis of drosopterins. The conversion of 6-PTP into PDA is catalyzed by the enzyme PDA synthase (2,11), the product of the *sepia* gene. Finally, drosopterin and its enantiomer, isodrosopterin, are produced nonenzymatically by the one-to-one condensation of PDA and 7,8-dihydropterin (12).

Although much is known, the biosynthetic pathways of drosopterins have not yet been fully elucidated. For example, the origin of 7,8-dihydropterin, one of the precursors of drosopterin, is unclear. Moreover, little is known about the chemical nature of neodrosopterin and fraction e. A continued effort should be made to achieve a better understanding of the chemical structures and biosynthesis of drosopterins, the red eye pigments in *Drosophila*.

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