Biochemical characterization of the tetrahydrobiopterin synthesis pathway in the oleaginous fungus *Mortierella alpina*

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We characterized the *de novo* biosynthetic pathway of tetrahydrobiopterin (BH_4) in the lipid-producing fungus *Mortierella alpina*. The BH_4 cofactor is essential for various cell processes, and is probably present in every cell or tissue of higher organisms. Genes encoding two copies of GTP cyclohydrolase I (GTPCH-1 and GTPCH-2) for the conversion of GTP to dihydroneopterin triphosphate (H_2-NTP), 6-pyruvoyltetrahydropterin synthase (PTPS) for the conversion of H_2-NTP to 6-pyruvoyltetrahydropterin (PPH_4), and sepiapterin reductase (SR) for the conversion of PPH_4 to BH_4, were expressed heterologously in *Escherichia coli*. The recombinant enzymes were produced as His-tagged fusion proteins and were purified to homogeneity to investigate their enzymic activities. Enzyme products were analysed by HPLC and electrospray ionization-MS. Kinetic parameters and other properties of GTPCH, PTPS and SR were investigated. Physiological roles of BH_4 in *M. alpina* are discussed, and comparative analyses between GTPCH, PTPS and SR proteins and other homologous proteins were performed. The presence of two functional GTPCH enzymes has, as far as we are aware, not been reported previously, reflecting the unique ability of this fungus to synthesize both BH_4 and folate, using the GTPCH product as a common substrate. To our knowledge, this study is the first to report the comprehensive characterization of a BH_4 biosynthesis pathway in a fungus.

INTRODUCTION

Tetrahydrobiopterin (BH_4) is a ubiquitous, multifaceted molecule found widely in nature. It has a well-known biochemical function in higher animals as an essential cofactor for various enzymes, including phenylalanine hydroxylase (PAH), tyrosine hydroxylase, tryptophan hydroxylase, nitric oxide synthase and alkylglycerol monooxygenase (Werner-Felmayer *et al.*, 2002). The first three of these enzymes are required for the degradation of phenylalanine and for the synthesis of neurotransmitters, such as serotonin and melatonin (Nagatsu & Ichinose, 1999). Requirements for BH_4 in nitric oxide synthesis and/or phenylalanine hydroxylation have also been reported in

bacteria (He & Rosazza, 2003) and some fungi of specific taxa (Maier & Ninnemann, 1995).

BH_4 biosynthesis has been extensively characterized in higher organisms (Fig. 1). The *de novo* synthesis of BH_4 starts with the conversion of GTP to dihydroneopterin triphosphate (H_2-NTP) by GTP cyclohydrolase I (GTPCH; EC 3.5.4.16). Then, H_2-NTP is converted to 6-pyruvoyltetrahydropterin (PPH_4) by 6-pyruvoyltetrahydropterin synthase (PTPS; EC 4.2.3.12), before ultimately being converted to BH_4 by sepiapterin reductase (SR; EC 1.1.1.153) (Thöny *et al.*, 2000). The genes responsible for the synthesis of BH_4 have been identified (Cha *et al.*, 1991; Citron *et al.*, 1990; Ohye *et al.*, 1998; Shen *et al.*, 1989; Thöny *et al.*, 1992), and structural information for all pathway enzymes has been elucidated (Auerbach *et al.*, 1997; Maita *et al.*, 2004; Ploom *et al.*, 1999).

Unlike higher animals, most bacteria, fungi and plants do not synthesize BH_4, but convert the first intermediate (i.e. H_2-NTP) into folate (Stanger, 2002), a vitamin that serves as a coenzyme for a variety of one-carbon transfer reactions
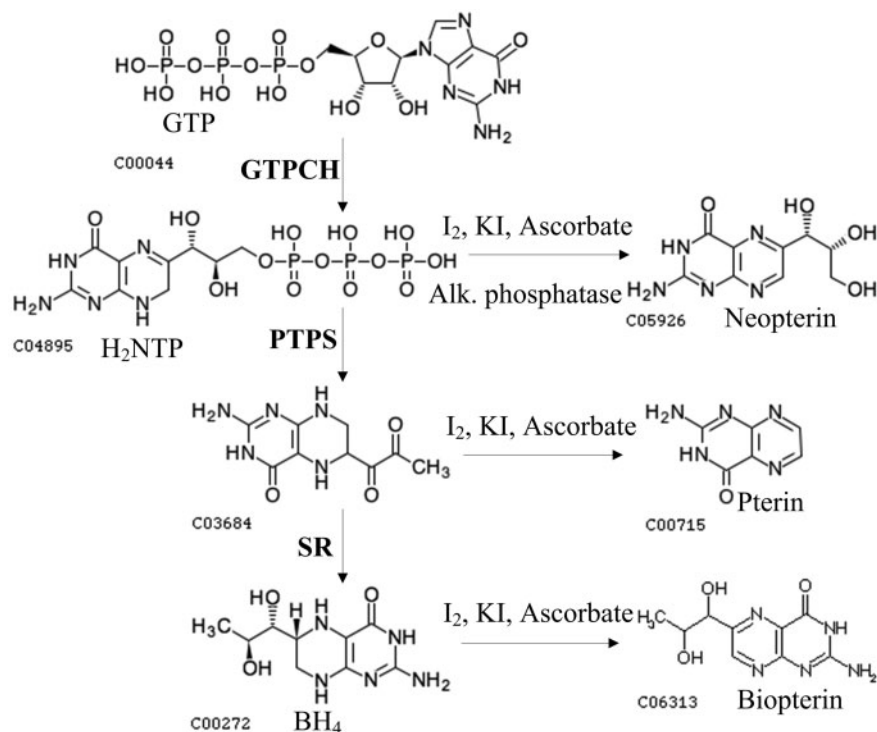
Fig. 1. Pathways for BH$_4$ biosynthesis and the activity assays for GTPCH, PTPS and SR.

(Maden, 2000). The presence of a BH$_4$ *de novo* synthesis pathway has been reported in a few bacteria based on the identification of related genes (Choi *et al.*, 2005; Kang *et al.*, 1998). In fungi, the presence of a BH4 *de novo* synthesis pathway has been reported for the zygomycete *Phycomyces blakesleeanus* and the ascomycete *Neurospora crassa*, and this was based on the isolation of related enzymes (Maier & Ninnemann, 1995). However, no putative PTPS gene involved in the *de novo* synthesis of BH$_4$ has been explored in any fungi, and the molecular basis for the synthesis of BH$_4$ in fungi has not yet been reported.

Mortierella alpina is a well-known polyunsaturated fatty acids (PUFAs)-producing oleaginous fungus commonly found in soil (Sakuradani *et al.*, 2009). It is one of the most important industrial species for the production of PUFAs (Sakuradani *et al.*, 2009). PUFAs play important roles as structural components of membrane phospholipids and as precursors of the eicosanoids of signalling molecules, including prostaglandins, thromboxanes and leukotrienes (Qi *et al.*, 2004; Sakuradani *et al.*, 2009). Recently, we have sequenced the whole genome of *M. alpina* (ATCC 32222) (results not shown). Analysis of this sequence has suggested the presence of a *de novo* pathway for the synthesis of BH$_4$ in this fungus.

In this present study, we characterized *in vitro* the functions of the genes encoding GTPCH, PTPS and SR in the BH$_4$ *de novo* pathway in *M. alpina*. Two functional GTPCH genes (GTPCH-1 and GTPCH-2) were found for

the first time to our knowledge. Kinetic parameters and other properties of GTPCH, PTPS and SR were investigated. Comparative analysis between GTPCH, PTPS and SR proteins and other homologous proteins was performed, and the physiological roles of BH$_4$ in *M. alpina* are discussed.

METHODS

Strains and growth conditions. *M. alpina* (ATCC 32222) was cultured on potato dextrose agar at 25 °C for 5–7 days. Fungal cultures were initially cultivated in 200 ml Kendrick medium (Kendrick & Ratledge, 1992) and incubated at 25 °C for 36 h. These cultures were used to provide a 10 % (v/v) seed to inoculate a 2 l (working volume) fermenter containing modified Kendrick medium, supplemented with ammonium tartrate (2 g l^{-1}) and glucose (30 g l^{-1}) (Wynn *et al.*, 1999). The fermenter contents were incubated at 25 °C and stirred at 500 r.p.m. with aeration at 0.5 vol./ vol. min^{-1}. Then, the mycelia were collected by filtration through sterile cheesecloth, and frozen immediately in liquid nitrogen for RNA extraction. The ammonium concentration in the culture filtrate was determined using the indophenol test (Chaney & Marbach, 1962). Cell lipid was extracted using the method of Bligh & Dyer (1959). BH$_4$ in *M. alpina* was prepared as described by Maier & Ninnemann (1995) and analysed by HPLC.

Gene searching. Predicted proteins in the *M. alpina* (ATCC 32222) genome (GenBank accession no. ADAG00000000) were annotated by BLAST (White *et al.*, 2005) searches with an E-value of 1E-5 against the following protein databases: NR (http://www.ncbi.nlm.nih.gov), KOGs and COGs (Tatusov *et al.*, 2003), KEGG (Kanehisa *et al.*, 2004),

Swiss-Prot and UniRef100 (Wu *et al.*, 2006), BRENDA (Chang *et al.*, 2009); and by InterProScan (Quevillon *et al.*, 2005) against protein domain databases with default parameter settings. Pathway mapping was conducted by associating EC assignment and KO assignment with KEGG metabolic pathways based on BLAST search results. Predicted GTPCH-1, GTPCH-2, PTPS and SR proteins in the *M. alpina* genome were searched against predicted proteins of sequenced fungal genomes by BLAST with an E-value of 1E-5.

Cloning and plasmid construction. Total RNA extractions were performed using TRIzol Reagent (Invitrogen) according to the manufacturer's instructions. RNA was subjected to RNase-free DNase digestion and then purified using an RNeasy Mini kit (Qiagen). The quantity and quality of total RNA were evaluated using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). Total RNA was reverse-transcribed with a PrimeScript RT reagent kit (Takara Bio) following the manufacturer's instructions, before PCR amplification of GTPCH-1, GTPCH-2, PTPS and SR genes using the primer pairs shown in Table 1. The PCR conditions used were as follows: denaturation at 95 °C for 30 s, annealing at 50 °C for 45 s and extension at 72 °C for 1 min (for 25 cycles in total); the final volume in each well was 50 µl. The amplified product was cloned into pET28a$^+$ to construct pwl39688 (containing GTPCH-1), pwl39690 (containing GTPCH-2), pwl39692 (containing PTPS) or pwl39694 (containing SR). The presence of the inserts in the plasmids was confirmed by sequencing using an ABI 3730 sequencer.

Protein expression and purification. *Escherichia coli* BL21 carrying pwl39688, pwl39690, pwl39692 or pwl39694 was grown overnight at 37 °C with shaking in Luria–Bertani (LB) medium containing 50 µg kanamycin ml^{-1}. The overnight culture (10 ml) was inoculated into 500 ml fresh medium and grown until the OD_{600} reached 0.6. Expression of both GTPCHs was induced by the addition of 0.1 mM IPTG at 15 °C for 16 h, expression of PTPS was induced by 0.5 mM IPTG at 37 °C for 16 h, and expression of SR was induced by 0.5 mM IPTG at 37 °C for 16 h. After the IPTG induction, cells were harvested by centrifugation, washed with binding buffer (50 mM Tris/HCl, 300 mM NaCl, 10 mM imidazole, pH 8.0), resuspended in the same buffer (supplemented with 1 mM PMSF and 1 mg lysozyme ml^{-1}) and then sonicated. Cell debris was removed by centrifugation, while the supernatant (containing the soluble proteins) was collected. The His-tagged fusion proteins were purified by nickel ion affinity chromatography using a Chelating Sepharose Fast Flow column (GE Healthcare) according to the manufacturer's instructions. Unbound proteins were washed through with 100 ml wash buffer (50 mM Tris/HCl, 300 mM NaCl, 25 mM imidazole, pH 8.0). Fusion proteins

were eluted with 3 ml elution buffer (50 mM Tris/HCl, 300 mM NaCl, 250 mM imidazole, pH 8.0) and dialysed overnight at 4 °C against 50 mM Tris/HCl buffer containing 20 % (v/v) glycerol (pH 7.4). Protein concentration was determined by the Bradford method. Purified proteins were stored at −80 °C.

Enzyme activity assays. Enzyme activities were assayed using the method of Maier & Ninnemann (1995) with minor modifications. The reaction mixture for GTPCH contained 100 mM Tris/HCl (pH 7.5), 5 mM EDTA, 50 mM KCl, 2 mM DTT, 0.2 mM GTP (Sigma-Aldrich) and enzyme solution in a total volume of 25 µl and was incubated at 37 °C for 1 h. The PTPS assay was performed in 100 mM Tris/HCl (pH 7.5), 10 mM MgCl$_2$, 30 µM H$_2$-NTP, 2 mM DTT and the enzyme solution in a total volume of 25 µl at 25 °C for 1 h. The SR assay was performed in 100 mM Tris/HCl (pH 6.0), 0.4 mM NADPH (Sigma-Aldrich), 5 mM sepiapterin (Sigma-Aldrich) and enzyme solution in a total volume of 25 µl at 37 °C for 1 h. After each reaction, 10 µl PTPS or SR reaction mixture was terminated and oxidized by adding 20 µl acidic iodine solution (2 % KI and 1 % I$_2$ in 1 M HCl) for 1 h in the dark. Excess iodine in the supernatant was reduced by adding 10 µl 5 % ascorbic acid. Samples were prepared for HPLC after centrifugation. The 10 µl GTPCH reaction mixtures were oxidized by adding 20 µl acidic iodine solution and then partially neutralized with 1 M NaOH. Neopterin, cleaved from the neopterin phosphates by alkaline phosphatase, was used as a comparison in quantification (Viveros *et al.*, 1981). H$_2$-NTP was freshly prepared from GTP using the recombinant *M. alpina* GTPCH purified in this study. The reaction mixture contained 50 mM Tris/HCl (pH 7.5), 50 mM KCl, 5 mM EDTA, 2 mM DTT, 1 mM GTP and enzyme solution, and this was incubated at 42 °C for 16 h, before being used directly in the enzyme assay in aliquots according to the concentration of the product. SR activity was also analysed using the *in vivo* substrate PPH$_4$, which was provided by a mixed assay with PTPS. One unit of GTPCH was defined as the amount of enzyme that catalyses the formation of one nanomole of H$_2$-NTP per hour at 50 °C. One unit of PTPS was defined as the amount of enzyme that catalyses the formation of one nanomole of PPH$_4$ per hour at 25 °C. One unit of SR was defined as the amount of enzyme that catalyses the formation of 15 nanomoles of 7,8-dihydrobiopterin per hour at 25 °C.

HPLC analysis. HPLC was performed on a Shimadzu model LC-20AT instrument equipped with a Rheodyne loop of 20 µl, an Inertsil ODS-3 C18 column (5 µm, 150 × 4.6 mm, GL Sciences), and a Shimadzu model RF-20A fluorescence detector (using excitation and emission wavelengths of 350 and 450 nm, respectively). Aliquots of

Table 1. Primers used in this study

Gene	Primer sequence used for cloning (5′–3′)*	Primer sequence used for qPCR (5′–3′)
GTPCH-1	CGGAATTCATGGCAAGCAGCATCGAGAAC	GACGGCCTTAGCTTCCCAAG
GTPCH-1	CCGCTCGAGCTAAATCCCACTGCGTCTGATAAG	CGAACTGCCTCTGCAATCCT
GTPCH-2	CGGAATTCATGTCCCACACTCCCACC	TCACGAAGCAAGTCGCAATG
GTPCH-2	CCGCTCGAGCTACACGCCACGTCGTCTG	TGATCAAGGACAAGAACTCCTCC
PTPS	CGGAATTCATGACATCCTCAACACCCGTC	TGACATCCTCAACACCCGTCAG
PTPS	CCGCTCGAGTTATTCTCCTCGGTAAACAACG	GCATTTGCCAAAGAGCTTCAC
SR	CTAGCTAGCATGTCGTCCAAGGAGCATC	CTTGGCAGGAGGCTAGAAGTTAC
SR	CCCAAGCTTCTACTCATCATAAAAGTCGATGTGT	ACGGCGAGCAGTGAGGATAT
18S rDNA	–	CTATTGGCGGAGGTCTATTCGT
18S rDNA	–	GCACGCATTCGGATAATTGGT

*Restriction sites are underlined.

reaction mixtures were injected into the column in 10 mM Na_2HPO_4 (pH 6.0) at a flow rate of 1.2 ml min^{-1} (Maier & Ninnemann, 1995). Quantities were calculated by comparison with external pteridine standards (Sigma-Aldrich). In order to isolate pteridine compounds, HPLC was performed under the same conditions as described above, except that 10 % methanol was used as the mobile phase and the flow rate was set at 1 ml min^{-1} (Son & Rosazza, 2000).

Electrospray ionization-MS (ESI-MS). The samples were injected into a Finnigan LCQ Advantage MAX ion trap mass spectrometer (Thermo Electron) in negative mode (4.5 kV, 250 °C) for ESI-MS analysis. For the SR product only, the apparatus was used in positive mode.

Measurement of kinetic parameters. To measure the K_m and V_{max} values of both GTPCHs for using GTP, reactions were carried out using various concentrations of GTP (0.4–1.5 mM), in conjunction with a fixed concentration of GTPCH (0.67 mM). To measure K_m and V_{max} values for PTPS acting on H_2-NTP, reactions were carried out using various concentrations of H_2-NTP (28–50 μM), but a fixed concentration of PTPS (4.48 mM). To measure K_m and V_{max} values for SR interacting with sepiapterin, reactions were carried out using various concentrations of sepiapterin (0.4–1.5 mM), and fixed concentrations of NADPH (1 mM) and SR (0.59 mM). To measure K_m and V_{max} values for SR using NAPDH, reactions were carried out using various concentrations of NAPDH (0.4–1.5 mM), with fixed concentrations of sepiapterin (5 mM) and SR (0.59 mM). The GTPCH reaction was performed at 45 °C for 5 min, the PTPS reaction was performed at 20 °C for 5 min, and the SR reaction was performed at 25 °C for 5 min. All reactions had final volumes of 25 μl. Conversions of GTP to H_2-NTP, H_2-NTP to PPH4, and sepiapterin to 7,8-dihydrobiopterin were monitored by HPLC. Kinetic parameters were obtained by fitting initial rates of reactions measured at varying concentrations of one substrate, at a fixed concentration of the other, to the Michaelis–Menten equation using non-linear regression.

Determination of temperature and pH optima, cation requirements, and the effects of a protein-reducing reagent. To determine the temperature optima for GTPCH, PTPS and SR, reactions were carried out at 5, 15, 25, 37, 50, 65 and 80 °C for 30 min at pH 8.0. For the determination of the optimum pH, Tris/HCl was used over a range of pH 2.0 to 12.0 for 15 min at 37 °C. To test the effects of various cations and a protein-reducing reagent on enzymic activity, reactions were carried out for 15 min at 37 °C in the presence of 5 mM of the following: KCl, $MgCl_2$, $MnCl_2$, $FeCl_2$, $CuCl_2$, $CoCl_2$ or DTT.

Real-time quantitative PCR (qPCR). qPCR was conducted with 2 μl reverse-transcribed cDNA using a 7300 Real-Time PCR system (Applied Biosystems) and Power SYBR Green PCR Master Mix (Applied Biosystems) according to the manufacturer's guidelines. The primer pairs used for qPCR are shown in Table 1. The PCR cycling conditions were: 10 s at 95 °C, followed by 30 s at 60 °C for a total of 40 cycles. The 18S rDNA gene was used as the reference gene. Fold changes in gene expression were calculated using the formula $2^{-\Delta\Delta C_t}$, where $\Delta\Delta C_t$ is ΔC_t (treatment)$-\Delta C_t$ (control), ΔC_t is C_t (target gene)$-C_t$ (18S), and C_t is the cycle value at which the fluorescence of the gene of interest crosses the threshold level (User's Manual for ABI 7300 Real-Time PCR system).

RESULTS

Gene searching

Putative GTPCH-1, GTPCH-2, PTPS and SR genes were found in *M. alpina* genomes (GenBank accession nos

JF746874, JF746875, JF746876 and JF746877), and the presence of BH_4 in *M. alpina* is shown in Fig. 2. These results revealed the presence of a *de novo* pathway for the synthesis of BH_4 in this fungus. The search results for GTPCH, PTPS and SR genes in all sequenced fungal genomes are shown in Supplementary Table S1.

Expression and purification of GTPCHs, PTPS and SR

GTPCH-1, GTPCH-2, PTPS and SR were expressed as His-tagged fusion proteins in *E. coli* BL21 by IPTG induction, and purified to near homogeneity by nickel ion affinity chromatography (Supplementary Fig. S1). The molecular masses estimated from SDS-PAGE analysis were 34 kDa for GTPCH-1, 34 kDa for GTPCH-2, 21 kDa for PTPS and 33 kDa for SR, corresponding well to the calculated masses (excluding the His-tag, these were 30, 30, 16 and 30 kDa, respectively).

HPLC analysis of GTPCHs, PTPS and SR products

The enzymic products of the recombinant proteins were identified by HPLC as shown in Fig. 2. GTP was converted to a peak eluted at 4 min, corresponding to the position of the neopterin standard in the reaction catalysed by GTPCH-1 (Fig. 2, IV) or GTPCH-2 (Fig. 2, V). In the reaction catalysed by PTPS, the 4 min peak (the GTPCH oxidized product) was converted to a new peak eluted at 12 min, corresponding to the position of the pterin standard (Fig. 2, VI). When sepiapterin was used as a substrate for PTPS, sepiapterin side chain-releasing (SSCR) activity was not obtained (data not shown), contrary to the human, *Drosophila melanogaster*, *Synechocystis* sp. and *E. coli* cases (Woo *et al.*, 2002). A new fluorescent peak at 13.5 min, which corresponded to the position of biopterin, was seen when sepiapterin was incubated with SR and NADPH and subsequently oxidized by iodine (not shown). The same result was obtained when the *in vivo* substrate of SR, PPH_4 (provided by a mixed assay with GTPCH and PTPS), was used (Fig. 2, VII). None of these peaks was observed in the sample incubated in the absence of the enzymes, while the other minor peaks remained unchanged (data not shown). It was therefore confirmed that the recombinant proteins possess GTPCH, PTPS and SR activity, respectively.

Analysis of GTPCHs, PTPS and SR products by ESI-MS

To further confirm the identities of the GTPCH, PTPS and SR reaction products, the oxidized products from the GTPCHs (the 4 min peak), PTPS (the 12 min peak) and SR (the 13.5 min peak) were purified by HPLC and analysed by ESI-MS. In negative mode, ion peaks were obtained at *m/z* 252.62 and *m/z* 252.87 for the products of GTPCH-1 and GTPCH-2, respectively. These peaks corresponded well to the mass of neopterin (*m/z* 253). Ion peaks were obtained at
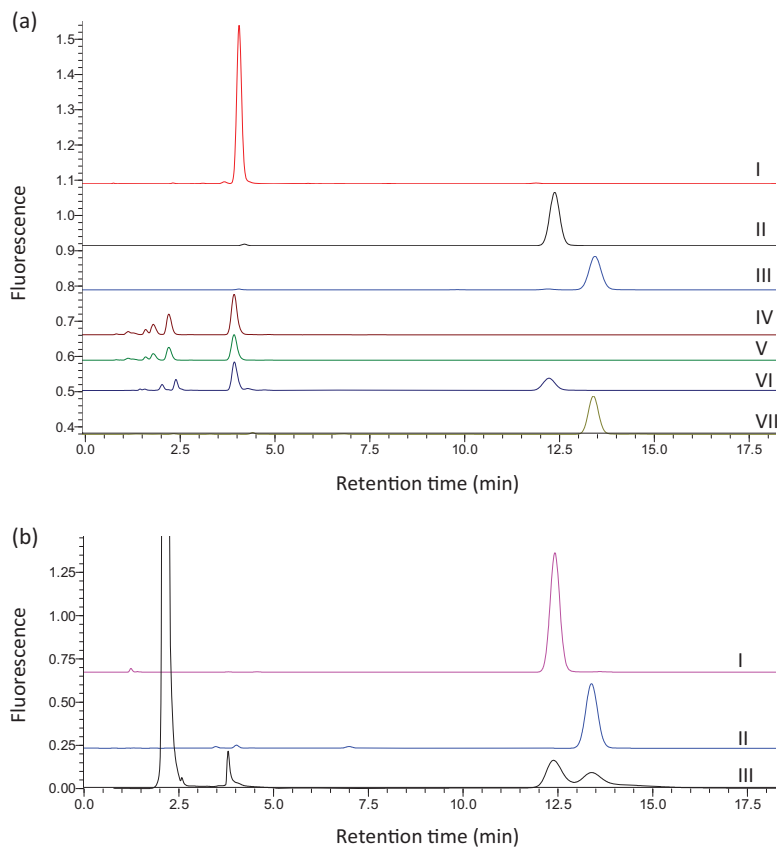
Fig. 2. (a) HPLC chromatographs of reaction products. Shown are chromatographs of a neopterin standard (I), a pterin standard (II), a biopterin standard (III), the GTPCH-1 reaction (IV), the GTPCH-2 reaction (V), a mixed assay of GTPCH and PTPS (VI), and a mixed assay of GTPCH, PTPS and SR (VII). (b) HPLC chromatographs of a pterin standard (I), a biopterin standard (II) and the pteridines in *M. alpina* (III).

m/z 162.88 (negative mode) for the PTPS product and m/z 238.87 (positive mode) for the SR product, and these corresponded well to the masses of pterin (m/z 163) and biopterin (m/z 237), respectively (Supplementary Fig. S2).

Kinetic parameters of the GTPCHs, PTPS and SR

Kinetic parameters of GTPCH (for GTP), PTPS (for H_2-NTP) and SR (for sepiapterin and NADPH) were measured. The kinetics of the reactions catalysed by these enzymes fit well with the Michaelis–Menten model, and non-linear regression of the experimental data to the Michaelis–Menten equation yielded the kinetic parameters listed in Table 2.

Effects of temperature, pH, cations and a protein-reducing reagent on the activities of the GTPCHs, PTPS and SR

GTPCH-1, GTPCH-2, PTPS and SR were active at each of the temperatures tested (15–65 °C), with the greatest activities detected at 55, 45, 20 and 25 °C, respectively (Fig. 3a). The 100 % activities were defined as the GTPCH, PTPS and SR reaction at 50, 37 and 25 °C, respectively, under the above conditions. For GTPCH-1 and GTPCH-2, high activity (90 %) was observed at temperatures up to 65 °C (Fig. 3a), which is in accordance with the data previously obtained for *D. melanogaster* (Weisberg &

O'Donnell, 1986), *Dictyostelium discoideum* (Witter *et al.*, 1996) and *Nocardia* sp. (He & Rosazza, 2003). GTPCH-1 and GTPCH-2 from *M. alpina* were heat-stable, although this organism is thermosensitive and cannot withstand temperatures above 37 °C. This may reflect the ancient origin of GTPCH from a putative thermophilic ancestor, and this property of the enzyme may have been maintained during evolution. PTPS remained active across a broad temperature range, and high activity (70 %) could even be sustained at temperatures as low as 5 °C, which is not known to have been reported in any other species before. SR had greatest activity at 25 °C, but this decreased quickly at higher temperatures (Fig. 3a), which contrasts to data obtained for SR activity in *Chlorobium tepidum* (Cho *et al.*, 1999).

Table 2. Kinetic parameters

The data shown are the mean of three independent experiments.

Enzyme	Substrate	K_m (mM)	V_{max} (μM min^{-1})
GTPCH-1	GTP	0.88 ± 0.04	0.145 ± 0.006
GTPCH-2	GTP	0.37 ± 0.02	0.408 ± 0.004
PTPS	H_2-NTP	0.164 ± 0.005	4.61 ± 0.21
SR	Sepiapterin	2.14 ± 0.12	15.93 ± 0.75
SR	NADPH	0.303 ± 0.004	13.05 ± 0.05
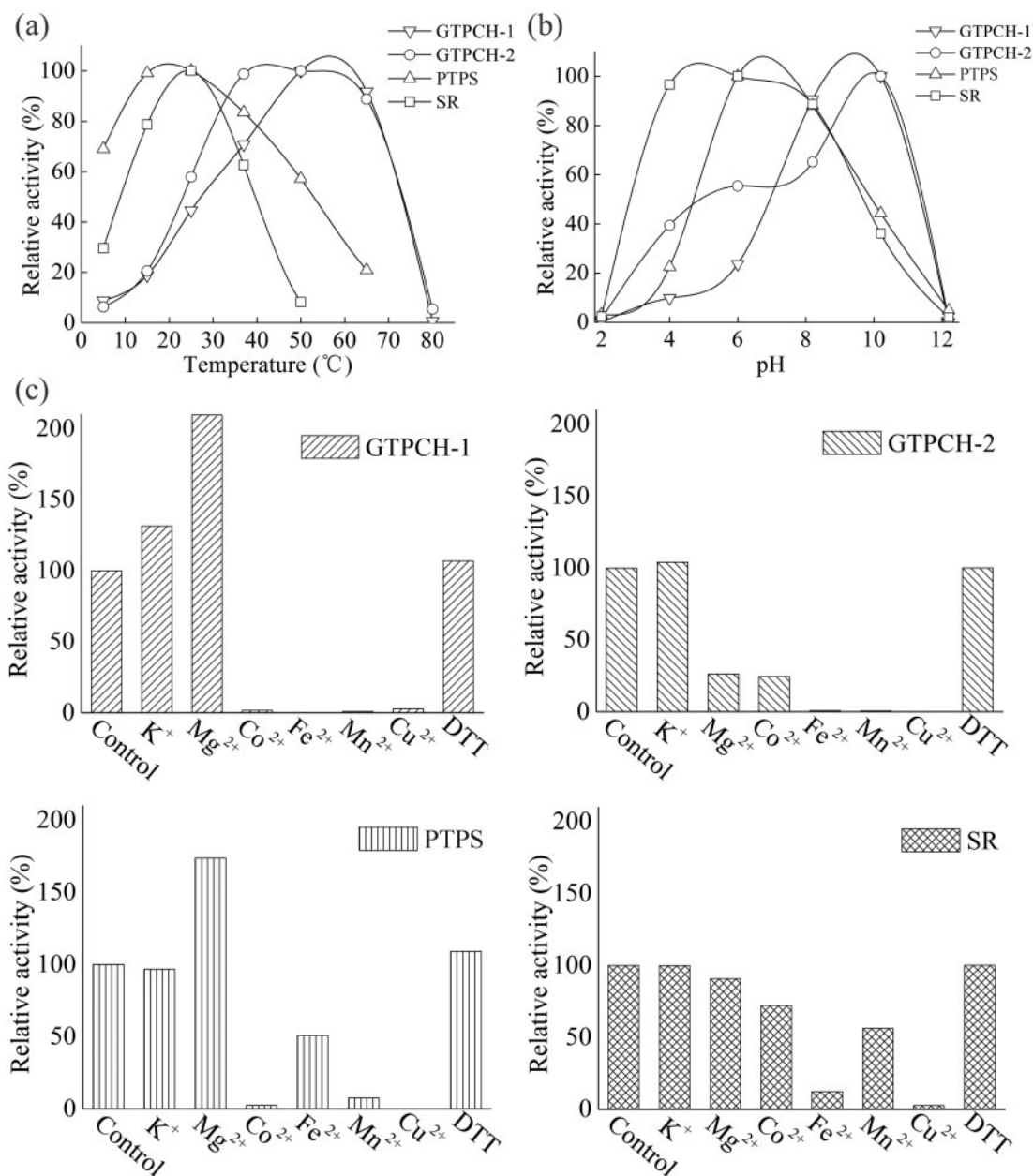
Fig. 3. Effects of temperature (a), pH (b), and cations and DTT (c) on the activity of GTPCH-1 ($\triangledown$), GTPCH-2 ($\bigcirc$) PTPS ($\triangle$) and SR ($\square$). Each point represents the mean of three independent measurements.

GTPCH-1, GTPCH-2, PTPS and SR showed optimal activities at pH 9.0, 9.5, 7.0 and 5.0, respectively (Fig. 3b). The 100 % activities were defined as the GTPCH, PTPS and SR reactions at pH 10.2, 10.2 and 6.2, respectively, under the above conditions. The GTPCHs, PTPS and SR were all active over a broad range of pH values (4–10). GTPCH-1 and GTPCH-2 were most active in more alkaline conditions than the optimal pH ranges for human, *D. melanogaster* and *Nocardia* sp. GTPCHs (He & Rosazza, 2003; Shen *et al.*, 1989; Weisberg & O'Donnell, 1986). PTPS had optimal

activity at pH 7.0, which is similar to that for recombinant rat PTPS (optimal pH 6.5–7.0) (Inoue *et al.*, 1991). SR had optimal activity at pH 5.0, which is consistent with the acidic pH optima observed for human and rat SRs (Sueoka & Katoh, 1982), but is lower than the optimal pH for SR derived from *C. tepidum* (Cho *et al.*, 1999).

GTPCH-1 activity was enhanced significantly by Mg^{2+} (100 % increase), and completely inhibited by Co^{2+}, Fe^{2+}, Mn^{2+} and Cu^{2+}. The 100 % activities were defined as the

GTPCH, PTPS and SR reactions without cations under the above conditions. GTPCH-2 activity was completely inhibited by Fe^{2+}, Mn^{2+} and Cu^{2+}, and inhibited to different degrees by Mg^{2+} and Co^{2+} (Fig. 3c). The inhibitory effect of Mg^{2+} has also been observed for the GTPCHs of *D. melanogaster* and *Bacillus subtilis* (De Saizieu *et al.*, 1995; Weisberg & O'Donnell, 1986). Neither K^+ nor DTT was found to be necessary for the activity of the two *M. alpina* GTPCHs, in contrast to the reported increase in activity with other homologous enzymes in the presence of K^+ and DTT (Milstien *et al.*, 1996; Yim & Brown, 1976). PTPS activity was enhanced (75 %) by Mg^{2+}, completely inhibited by Co^{2+}, Mn^{2+} and Cu^{2+}, slightly inhibited by Fe^{2+}, but unaffected by DTT. A positive effect of Mg^{2+} on PTPS activity has been reported before (Lee *et al.*, 1999; Park *et al.*, 1990). SR activity was completely inhibited by Cu^{2+}, inhibited to different degrees by Co^{2+}, Fe^{2+} and Mn^{2+}, but unaffected by K^+, Mg^{2+} and DTT (Fig. 3c). *M. alpina* SR activity was not enhanced by those cations and that protein-reducing reagent, in accordance with the fact that cations and DTT are not involved in the SR reaction (Cho *et al.*, 1999; Kim *et al.*, 2000; Seong *et al.*, 1998).

qPCR analysis

To investigate whether BH_4 synthesis is related to lipid accumulation in *M. alpina*, qPCR was performed to analyse the changes in the expression levels of genes involved in BH_4 biosynthesis during the course of the lipid accumulation which is induced by the exhaustion of nitrogen (Wynn *et al.*, 1999). Total RNA samples were purified from mycelia collected from cultures in different growth phases. RNA samples were collected before lipid accumulation when nitrogen availability was sufficient (8.5 h), during the initial stage of lipid accumulation just before nitrogen was used up completely (18.5, 20 and 22 h), and after the lipid had accumulated for a long and sustained period of time (33 and 69 h). The relative expression levels of these genes were measured in triplicate by qPCR (Fig. 4). C_t values obtained after 8 h of incubation were chosen as the control values in order to calculate the fold change in gene expression over time for each treatment. GTPCH-1, GTPCH-2, PTPS and SR genes were transcribed in all growth phases. In comparison with the sample collected at 8 h, when nitrogen was sufficient, the expression of GTPCH-1, GTPCH-2, PTPS and SR was downregulated 0.4-, 0.6-, 0.5- and 0.8-fold, respectively, in cells collected at 18.5 h when nitrogen started to decrease; 0.4-, 0.6-, 0.3- and 0.7-fold, respectively, in cells collected at 20 h when nitrogen was almost used up; and 0.3-, 0.2-, 0.3- and 0.7-fold, respectively, in cells collected at 22 h when nitrogen was completely exhausted and lipids started to accumulate (Fig. 4). Compared with the 22 h cells, the expression levels of the GTPCH-1, GTPCH-2 and PTPS genes in cells collected from other time points (33 and 69 h), when lipids were rapidly accumulating, were not changed significantly (Fig. 4). To evaluate the GTPCH-1 transcript/GTPCH-2 transcript ratio, C_t values of GTPCH-2 obtained at each phase were chosen as the control values.

The GTPCH-1/GTPCH-2 transcript ratios were 3.9, 2.9, 2.4, 7.0, 5.5 and 3.7 at the respective sampling times (Fig. 4f), which suggests that GTPCH-1 is more active than GTPCH-2 in all phases of growth.

DISCUSSION

GTPCH-1 and GTPCH-2 share 28–48 % identity with other characterized GTPCH proteins and are most similar to those of *Gallus gallus*. Multiple sequence alignment of the *M. alpina* GTPCHs and other characterized GTPCH proteins reveals the presence of certain conserved motifs and residues (Supplementary Fig. S3), including five conserved motifs (Lys-Thr-Pro-Xaa-Arg-Xaa-Ala, Ser-Xaa-Cys-Glu-His-His, Gly-Leu-Ser-Lys-Leu-Ala-Arg, Gln-Val-Gln-Glu-Arg-Leu-Thr and Cys-Met-Val-Met-Arg-Gly-Val) that form a GTP-binding pocket (Du *et al.*, 2009; He *et al.*, 2004; Maita *et al.*, 2004); a Lys-Thr-Arg-Glu motif at the C terminus involved in 7,8-dihydrobiopterin binding, resulting in inhibition of GTPCH activity (Du *et al.*, 2009; Maita *et al.*, 2004); four residues (Thr108, Ser153, Ser191 and Thr255) involved in multiple phosphorylation regulation, both positively and negatively (Du *et al.*, 2009); and six residues (Ala205, Ala207, Pro214, Gly247, Arg250 and Lys254) involved in stimulatory or inhibitory effects on the GTPCH feedback regulatory protein (GFRP) (Maita *et al.*, 2004). Compared with the highly conserved central and C-terminal amino acids of rat GTPCH, which are responsible for substrate binding and catalysis, the N-terminal parts of GTPCH differ depending on the species examined. The N terminus exerts auto-inhibitory control over rat GTPCH and is required for GFRP binding on its own (Higgins & Gross, 2011). This indicates that the N termini of *M. alpina* GTPCHs may exert allosteric control over the enzymic activities on their own, or via interactions with GFRP, or even with other regulatory factors. All of these conserved motifs and residues in *M. alpina* GTPCHs are also present in other fungi, and these fungal GTPCHs cluster in the same branch of the phylogenetic tree (Supplementary Fig. S4). However, two GTPCH genes were found and functionally confirmed in *M. alpina*, and such a case has never been reported before to our knowledge. In most fungi, the GTPCH product is converted to folate and not BH_4. In *M. alpina*, there also exists a folate synthesis pathway (as suggested by genome analysis), indicating that both folate and BH_4 are synthesized by this fungus. As the reaction catalysed by GTPCH is the first and rate-limiting step in the *de novo* biosynthesis of BH_4 (Higgins & Gross, 2011), the presence of two distinct GTPCH genes likely reflects the importance of BH_4 synthesis in *M. alpina*. Thus, the presence of two GTPCH genes is advantageous for the fungus to produce sufficient H_2-NTP to supply substrates for both the BH_4 pathway and the folate pathway. The enzymic properties of GTPCH-1 and GTPCH-2 are generally the same, but the contrasting effects of Mg^{2+} on GTPCH-1 and GTPCH-2 may reflect different origins for the two genes. GTPCH-1 is more active than GTPCH-2 in
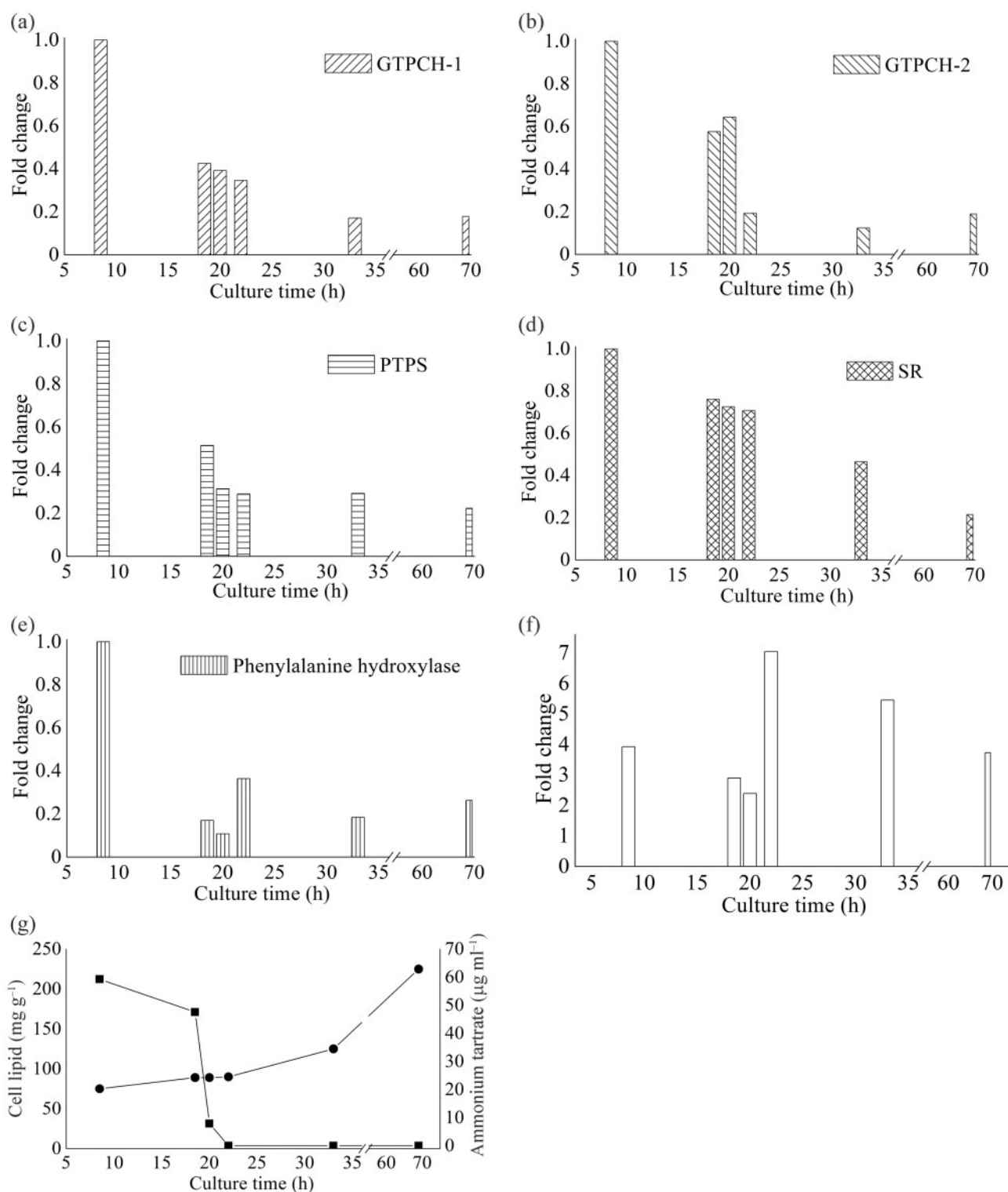
Fig. 4. Relative expression levels of GTPCH-1 (a), GTPCH-2 (b), PTPS (c), SR (d) and PAH (e) genes during the accumulation of lipids in *M. alpina*; (f) the ratio of the transcripts of GTPCH-1/GTPCH-2; (g) exhaustion of ammonium tartrate (■) and accumulation of cell lipid (●) in *M. alpina*. Each point represents the mean of three independent measurements.

all phases of growth, indicating that GTPCH-1 makes the major contribution to BH_4 synthesis. GTPCH-1 and GTPCH-2 display different substrate binding and catalytic activity, contributing to the increased K_m of GTPCH-1 compared with GTPCH-2. The physiological roles of the two GTPCH genes in the synthesis of BH_4 and folate in *M. alpina* require further investigation.

PTPS shares 28–48 % identity with other characterized PTPS proteins and shares most homology with the human protein. Multiple sequence alignment of the PTPS proteins reveals the presence of various conserved residues characteristic of PTPSs (Supplementary Fig. S5), including four catalytic residues (Cys41, Asp87, His88 and Gly145) and two residues (Thr104 and Thr105) responsible for substrate binding (Ben *et al.*, 2003; Woo *et al.*, 2002). The first 20 amino acid residues at the N terminus are highly variable, but the other portions of the protein are well conserved between vertebrates and *M. alpina*. Putative PPTS genes were found in the genomes of the fungi *Allomyces macrogynus*, *Batrachochytrium dendrobatidis*, *Rhizopus oryzae*, *Spizellomyces punctatus* and *P. blakesleeanus*. The conserved residues characteristic of PTPS in *M. alpina* are also shown by these fungal PTPSs. When displayed on a phylogenetic tree, the fungal PTPSs were more related to animal than to microbial enzymes (Supplementary Fig. S6).

M. alpina SR shares 15–36 % identity with other characterized SR proteins, and again is most similar to the human enzyme. Multiple sequence alignment of the SR proteins reveals the presence of conserved motifs and residues described before for SRs (Supplementary Fig. S7), including a motif (Gly-Xaa-Xaa-Xaa-Gly-Xaa-Gly) that acts as an NADP-binding site at the N terminus (Cho *et al.*, 1999); a motif (Tyr-Xaa-Xaa-Xaa-Lys) known to be a catalytic site (Cho *et al.*, 1999); three residues (Ser176, Tyr189 and Met226) involved in the active site cavity in the NADP–sepiapterin complex (Supangat *et al.*, 2006); a specific anchor (Asp278) for pterin substrates (Auerbach *et al.*, 1997); and a residue (Arg42) conserved in all known members of the SR family that prefer NADPH to NADH (Auerbach *et al.*, 1997). However, the Tyr165 of mouse SR is not conserved in the *M. alpina* enzyme, since this residue is proposed to interact with indoleamines via π electrons (Auerbach *et al.*, 1997). Moreover, the Phe99 and Trp196 residues of the *C. tepidum* SR, which are known to play a key role in stereospecific enzyme catalysis by holding the substrate in the active site (Supangat *et al.*, 2008), are also not conserved in *M. alpina*. The significantly increased K_m of *M. alpina* SR compared with *C. tepidum* SR could be due to the loss of one of these residues (i.e. Phe99 or Trp196), which might consequently impair substrate binding and decrease catalytic activity. Compared with SRs from fungi which cannot synthesize BH_4, *M. alpina* SR contains a specific anchor (Asp278) for pterin substrates that is essential in determining the substrate-binding specificity and anchors pterin derivatives for reduction at the monoketo or diketo side chains (Auerbach *et al.*, 1997). This is why *M. alpina* SR maintains the ability to bind PPH_4, which is not synthesized in most fungi due to the absence of PTPS. Based on the search results of fungal genomes (Supplementary Table S1), *M. alpina*, *Batrachochytrium dendrobatidis*, *R. oryzae*, *S. punctatus* and *P. blakesleeanus* are the fungi which contain all of the GTPCH, PTPS and SR enzymes involved in the BH_4 *de novo* pathway. The existence of these fungi and other members of the fungal SR cluster as a distinct group in the phylogenetic tree could also explain why *M. alpina* SR has the ability to synthesize BH_4 (Supplementary Fig. S8). This tree illustrates the clear distinction between *M. alpina* SR and other fungi that cannot synthesize BH_4, and also shows the close relationship between *M. alpina* and mammalian SRs (Supplementary Fig. S8). Based on the sequence comparisons and enzymic properties, SRs from *M. alpina* and mammals are thought to have evolved from a common ancestor.

The functions of BH_4 in fungi are not well understood, but it can act as a cofactor in nitric oxide synthesis (Maier *et al.*, 2001). To investigate the functions of BH_4 in *M. alpina*, the genome was searched for the presence of potential BH_4-dependent genes, including PAH, tyrosine hydroxylase, tryptophan hydroxylase, nitric oxide synthase and alkylglycerol monooxygenase, but only a putative PAH (EC 1.14.16.1) gene was found. The expression of this enzyme was confirmed by qPCR analysis (Fig. 4e). BH_4 functions as a biological cofactor for three aromatic amino acid hydroxylases, including PAH, which is required for the catabolism of phenylalanine and the synthesis of neurotransmitters in animals (Nagatsu & Ichinose, 1999). Although the same function has never been demonstrated in fungi, it is highly likely that BH_4 plays the similar role in *M. alpina*, based on the identification of the PAH homologue. Still, this needs to be confirmed experimentally in future studies. We did not find the other expected genes based on homology searching, and it is possible that the fungus uses non-homologous genes instead.

M. alpina is noteworthy for its production of various PUFAs, especially arachidonic acid, and the accumulation of lipids, such as glycerolipids, phospholipids, sphingolipids and sterols. It has been suggested that BH_4 might be essential for the desaturation or omega oxidation of long-chain fatty acids, the conversion of dihydrosphingosine into sphingosine, or in sterol metabolism, but the functional significance of BH_4 and closely related compounds remains to be fully elucidated (Forrest & Van Baalen, 1970; Kaufman, 1967, 1993). The effect of BH_4 on lipid metabolism has been investigated *in vitro* (Rudzite *et al.*, 1998). BH_4 increases the incorporation of unsaturated fatty acids into phospholipids (particularly arachidonic acid) at the expense of saturated varieties, and it also decreases the cholesterol content, which means that BH_4 may act to increase membrane fluidity, stimulate the cell cycle and prevent cholesterol precipitation. In phenylketonuria patients treated with BH_4, n-3 PUFA composition increases significantly (Vilaseca *et al.*, 2010), indicating that BH_4 is important for PUFA status in humans. These

findings suggest that BH_4 biosynthesis may exert control over lipid accumulation on its own or via interactions with other as-yet-unrecognized regulatory factors in *M. alpina*. In our gene regulation study, the downregulation of BH_4 synthesis may simply be due to N limitation and have no close relationship with lipid accumulation (Fig. 4). In order to determine the exact relationships between them, gene knockout experiments could be performed in further work.

Apart from GTPCH, PTPS and SR genes for the *de novo* pathway, a putative dihydrofolate reductase (EC 1.5.1.3) gene responsible for the sepiapterin-dependent salvage pathway (Hasegawa *et al.*, 2005) and genes encoding pterin-4α-carbinolamine dehydratase (EC 4.2.1.96) and dihydropteridine reductase (EC 1.6.99.7), which are required for the regeneration of BH_4, which is essential in phenylalanine metabolism (Thöny *et al.*, 2000), were found in the *M. alpina* genome (data not shown). This provides further evidence for the importance of BH_4 in this fungus. According to KEGG (Kanehisa, 1997), the salvage pathway is rarely found in micro-organisms, and no other fungal species is known to contain the BH_4 *de novo* synthesis and salvage pathways simultaneously with the BH_4 regeneration pathway.

To the best of our knowledge, this is the first time that a BH_4 synthesis pathway has been characterized at the molecular level in a fungus. Genes encoding GTPCH, PTPS and SR were functionally characterized *in vitro*. The roles of BH_4 in *M. alpina* PAH interaction and lipid accumulation are deserving of further investigation. BH_4 is commercially produced by pharmaceutical companies for the treatment of hyperphenylalaninaemia and endothelial dysfunction (Werner-Felmayer *et al.*, 2002). As the method currently used for the organic synthesis of BH_4 is expensive, *M. alpina* could be used as an alternative source of this molecule.

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