Pterin and Folate Salvage. Plants and *Escherichia coli* Lack Capacity to Reduce Oxidized Pterins[1][OA]

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Dihydropterins are intermediates of folate synthesis and products of folate breakdown that are readily oxidized to their aromatic forms. In trypanosomatid parasites, reduction of such oxidized pterins is crucial for pterin and folate salvage. We therefore sought evidence for this reaction in plants. Three lines of evidence indicated its absence. First, when pterin-6-aldehyde or 6-hydroxymethylpterin was supplied to Arabidopsis (*Arabidopsis thaliana*), pea (*Pisum sativum*), or tomato (*Lycopersicon esculentum*) tissues, no reduction of the pterin ring was seen after 15 h, although reduction and oxidation of the side chain of pterin-6-aldehyde were readily detected. Second, no label was incorporated into folates when 6-[^{3}H]hydroxymethylpterin was fed to cultured Arabidopsis plantlets for 7 d, whereas [^{3}H]folate synthesis from *p*-[^{3}H]aminobenzoate was extensive. Third, no NAD(P)H-dependent pterin ring reduction was found in tissue extracts. Genetic evidence showed a similar situation in *Escherichia coli*: a GTP cyclohydrolase I (*folE*) mutant, deficient in pterin synthesis, was rescued by dihydropterins but not by the corresponding oxidized forms. Expression of a trypanosomatid pterin reductase (PTR1) enabled rescue of the mutant by oxidized pterins, establishing that *E. coli* can take up oxidized pterins but cannot reduce them. Similarly, a GTP cyclohydrolase I (*fol2*) mutant of yeast (*Saccharomyces cerevisiae*) was rescued by dihydropterins but not by most oxidized pterins, 6-hydroxymethylpterin being an exception. These results show that the capacity to reduce oxidized pterins is not ubiquitous in folate-synthesizing organisms. If it is lacking, folate precursors or breakdown products that become oxidized will permanently exit the metabolically active pterin pool.

Pterins have a wide range of metabolic roles, including as essential intermediates in folate biosynthesis, as products of folate breakdown (Scott et al., 2000; Suh et al., 2001), and as cofactors for aromatic hydroxylases (Zhao et al., 1994; Thony et al., 2000; Yamamoto et al., 2001). The oxidation state of the pterin ring (Fig. 1A) is crucial to biochemical function because only reduced (dihydro or tetrahydro) forms are metabolically active. The intermediates of folate synthesis are dihydro forms, as is the major pterin formed by folate breakdown (Fig. 1B). Pterin cofactors are tetrahydro forms.

Reduced pterins readily autoxidize to their fully oxidized (aromatic) state (Fig. 1B, mauve arrows; Pfleiderer, 1985). A capacity to reduce oxidized pterins to their bioactive forms would thus seem a likely auxiliary to folate and pterin pathways. So far, however, only Leishmania and other trypanosomatid parasites are definitively known to have this capacity. These organisms, which are pterin and folate auxotrophs, contain an NADPH-dependent pterin reductase (PTR1, EC 1.5.1.33) that mediates the two-stage reduction of pterins to their 7,8-dihydro and tetrahydro states (Bello et al., 1994; Dawson et al., 2006). PTR1 is a member of the short chain dehydrogenase/reductase (SDR) family with a distinctive variant of the usual SDR motif (TGX_3GXG, where X is any amino acid) in which R replaces the second G (Gourley et al., 2001). PTR1 is distinct from 6,7-dihydropterin reductase (EC 1.5.1.34, also termed quinonoid dihydropteridine reductase), which acts on 6,7-dihydropterins. These pterins are intermediates in the recycling of tetrahydropterin cofactors (Thony et al., 2000).

It is not clear whether folate-synthesizing organisms (plants, bacteria, and fungi) have a reductase that acts on oxidized pterins. Some evidence suggests not. Thus, in tomato (*Lycopersicon esculentum*) fruit with upregulated pterin synthesis, most of the accumulated pterins became oxidized as ripening proceeded (Díaz de la Garza et al., 2004). In *Lactobacillus plantarum*, 6-hydroxymethylpterin ($PtCH_2OH$) and pterin-6-aldehyde (PtCHO) were far poorer folate synthesis precursors in cell extracts than their reduced forms (Shiota, 1959). Similarly, in *Escherichia coli*, neither $PtCH_2OH$ (Shiota and Disraely, 1961) nor pteroic acid (Griffin and Brown, 1964) supported in vitro folate synthesis, although their dihydro forms did. Furthermore, the only PTR1-like enzyme in *E. coli*, FolM, acts on dihydrobiopterin but not on its oxidized form (Giladi et al., 2003).

While it is unclear whether bacteria or plants reduce oxidized pterins to the dihydro level, it is certain that bacteria reduce dihydropterins to the tetrahydro level,

[1] This work was supported by the National Research Initiative of the U.S. Department of Agriculture Cooperative State Research, Education, and Extension Service (grant no. 2005–35318–15228) and by an endowment from the C.V. Griffin Sr. Foundation.

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www.plantphysiol.org/cgi/doi/10.1104/pp.106.093633

Compound	Abbrev.	R
Neopterin	NPt	*(1′S,2′R)*CH(OH)CH(OH)CH_2OH
Monapterin	MPt	*(1′R,2′R)*CH(OH)CH(OH)CH_2OH
6-Hydroxymethylpterin	$PtCH_2OH$	CH_2OH
Pterin-6-aldehyde	PtCHO	CHO
Pterin-6-carboxylate	PtCOOH	COOH
Pteroate	Pte	CH_2-*p*-aminobenzoate
Folate	PteGlu	CH_2-*p*-aminobenzoylglutamate

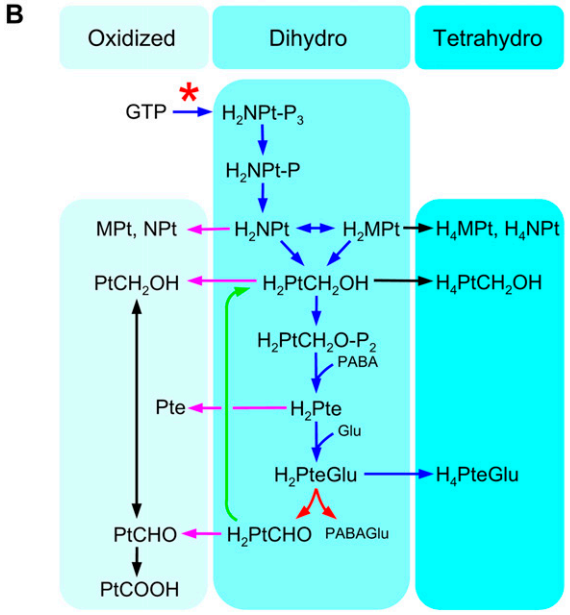

Figure 1. Pterin structures and metabolism. A, Structures of the pterin ring in its fully oxidized, 7,8-dihydro, and 5,6,7,8-tetrahydro forms, and of the substituent (R) at the 6 position for the pterins in this study (chiral designations in parentheses). B, The participation of pterins in folate synthesis, breakdown, and salvage reactions. The red asterisk marks the first step of pterin synthesis, mediated by GTP cyclohydrolase I. The oxidation state of the pterin ring is indicated by three shades of blue background color. Mauve arrows show autoxidation of the pterin ring. Blue arrows show folate synthesis reactions. Red arrows show the oxidative breakdown of dihydrofolate to H_2PtCHO and *p*-aminobenzoyl-glutamate; tetrahydrofolate undergoes similar cleavage to yield tetrahydropterin-6-aldehyde, which can autoxidize to the dihydro form. The green arrow shows the only route by which plants and *E. coli* appear to be able to salvage H_2PtCHO (see "Discussion"). -P, Phosphate, $-P_2$, diphosphate; $-P_3$, triphosphate; PABAGlu, *p*-aminobenzoyl-glutamate; H_2, dihydro; H_4, tetrahydro.

and likely that plants do. Thus, tetrahydromonapterin and tetrahydrohydroxymethylpterin (the latter as a glycoside) occur in bacteria, both being reduction products of folate synthesis intermediates (Fig. 1B; Guroff and Rhoads, 1969; Lee et al., 1999; Ikemoto et al., 2002). Furthermore, the tetrahydropterin-dependent enzyme Phe hydroxylase occurs naturally in Pseudomonas and some other bacteria, and functions if introduced into *E. coli* (Zhao et al., 1994). Lastly, plant genomes encode homologs of mammalian pterin 4a-carbinolamine dehydratase, an enzyme needed to recycle tetrahydropterin cofactors (Thony et al., 2000) that may be considered diagnostic for their presence.

The reduction of oxidized pterins could impact the availability of folate synthesis intermediates and the salvage of folate breakdown products. It is also relevant to biofortification projects in which pterin synthesis is up-regulated to enhance folate accumulation (Sybesma et al., 2003; Díaz de la Garza et al., 2004; Hossain et al., 2004) since overproduced pterins, which can autoxidize, are at best useless and may even inhibit folate synthesis (Brown et al., 1961). We therefore investigated pterin reduction in plants, focusing particularly on the possible reduction of $PtCH_2OH$, the oxidized form of the folate synthesis intermediate 6-hydroxymethyldihydropterin (H_2PtCH_2OH), and PtCHO, a folate breakdown product (Fig. 1B) that is convertible to $PtCH_2OH$ by side chain reduction (Kobayashi, 1982; Orsomando et al., 2006). Having found no reductive capacity in plants, we checked the generality of this surprising result in *E. coli* and yeast (*Saccharomyces cerevisiae*).

RESULTS

Absence of PTR1-Like Plant Proteins

Because trypanosomatids acquired many genes from photosynthetic organisms (Hannaert et al., 2003), we searched plant genomes for SDR sequences resembling Leishmania PTR1 in having a TGX_3RXG motif. Among 86 SDRs in the Arabidopsis (*Arabidopsis thaliana*) genome and similar numbers in rice (*Oryza sativa*) and poplar (*Populus* spp.) genomes, none had this motif. Nor did any of them have higher overall sequence identity to PTR1 than the 15% to 30% typical for SDR family members (Jörnvall et al., 1999). Plants thus do not have PTR1 orthologs, suggesting possible inability to reduce oxidized pterins.

Absence of in Vivo Pterin Reduction in Plants

When Arabidopsis, pea (*Pisum sativum*), and tomato tissues were incubated with $PtCH_2OH$ it was readily absorbed and in most cases underwent some side chain oxidation to pterin-6-carboxylate (PtCOOH; Fig. 2, A and B). Neither the remaining $PtCH_2OH$ nor the PtCOOH showed measurable ring reduction (Fig. 2C). The absence of detectable reduced $PtCH_2OH$ (di- or tetrahydro, henceforth for simplicity H_2PtCH_2OH), cannot be ascribed to in vivo decomposition or metabolism since it accumulated substantially when dihydropterin-6-aldehyde (H_2PtCHO) replaced $PtCH_2OH$ (Fig. 2C); H_2PtCH_2OH in this case comes from side chain reduction (Orsomando et al., 2006). These data imply that plants lack $PtCH_2OH$ and PtCOOH ring reductase activity.

To test for ring reductase activity against PtCHO, we reasoned that the product, H_2PtCHO, would undergo

rapid side chain reduction to give H_2PtCH_2OH, as occurs with exogenously supplied H_2PtCHO (Fig. 2C). As expected (Orsomando et al., 2006), Arabidopsis, pea, and tomato tissues all reduced the PtCHO side chain to give $PtCH_2OH$, but none of this was in reduced form (Fig. 3). Consistent with the observed conversion of $PtCH_2OH$ to PtCOOH (Fig. 2A), all tissues also metabolized PtCHO to PtCOOH, none of which was reduced. These results indicate that PtCHO ring reductase activity is lacking, and corroborate the results of Figure 2C for $PtCH_2OH$ and PtCOOH.

Absence of [^{3}H]Folate Synthesis from 6-[7-^{3}H]Hydroxymethylpterin by Arabidopsis

The above results were obtained with quite short incubation times (15 h) and with pterin doses (approximately 20 nmol g^{-1} fresh weight) that exceeded endogenous pterin contents (e.g. Fig. 2B). We therefore carried out longer experiments with a more physiological pterin dose, exploiting the fact that pterins need reduction to the dihydro level before incorporation into folates (Fig. 1B). Axenically cultured Arabidopsis plantlets (initial fresh weight 0.25 g) were given 166 pmol (1.6 μCi) of [7-^{3}H]$PtCH_2OH$ for 7 d, then analyzed for ^{3}H incorporation into folates. Control plantlets received a similar dose of *p*-[^{3}H]aminobenzoate ([^{3}H]PABA; 65 pmol, 1.7 μCi), PABA being readily incorporated into folates by plants (Imeson et al., 1990). At 7 d, similar amounts of label from [^{3}H]$PtCH_2OH$ and [^{3}H]PABA were present in tissue extracts (0.74 and 1.15 μCi, respectively), demonstrating comparable uptake. However, while folates were heavily labeled in [^{3}H]PABA-fed samples they contained no detectable label in [^{3}H]$PtCH_2OH$-fed samples (Fig. 4). In the latter, 98% of the ^{3}H in the extract was in the form of PtCOOH, consistent with the extensive $PtCH_2OH \rightarrow$ PtCOOH conversion in Figure 2A, and 2% remained in $PtCH_2OH$.

This lack of conversion of [^{3}H]$PtCH_2OH$ to folates implies that it was not reduced to its bioactive dihydro form. It is unlikely that the lack of folate labeling from [^{3}H]$PtCH_2OH$ was due to loss of ^{3}H by exchange with

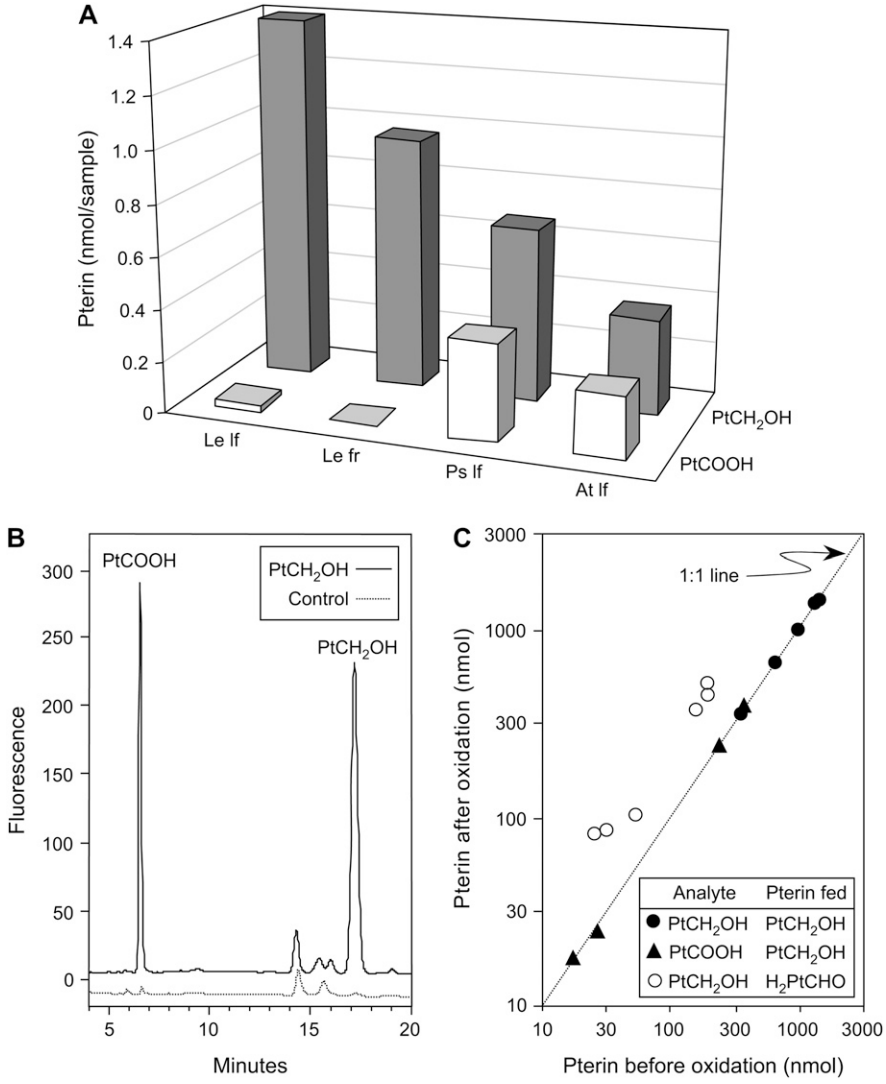

Figure 2. Metabolism of $PtCH_2OH$ by plant tissues. A, Conversion of $PtCH_2OH$ to PtCOOH by Arabidopsis (At), pea (Ps), and tomato (Le) leaf (lf) and tomato fruit pericarp (fr) tissues. Tissues (0.11–0.21 g fresh weight) were incubated with $PtCH_2OH$ (1.9 nmol) for 15 h in darkness and washed before analysis. $PtCH_2OH$ and PtCOOH values are after oxidation, corrected for the low endogenous levels of these pterins in tissues incubated without $PtCH_2OH$. B, Representative HPLC separation (for pea leaf tissue) showing the accumulation of $PtCH_2OH$ and PtCOOH in the $PtCH_2OH$ treatment and their near absence in controls not given $PtCH_2OH$. C, Oxidation state of pterins in $PtCH_2OH$-fed tissues. $PtCH_2OH$ and PtCOOH values (black symbols) before and after oxidation are plotted against each other. The data points fall on the 1:1 line, showing that neither pterin is reduced. White symbols are values for $PtCH_2OH$ extracted from Arabidopsis leaf or tomato fruit tissues given H_2PtCHO (3.4 nmol) instead of $PtCH_2OH$. These data points are well above the line, showing that much of the $PtCH_2OH$ is reduced and thereby confirming the in vivo stability of reduced pterins.

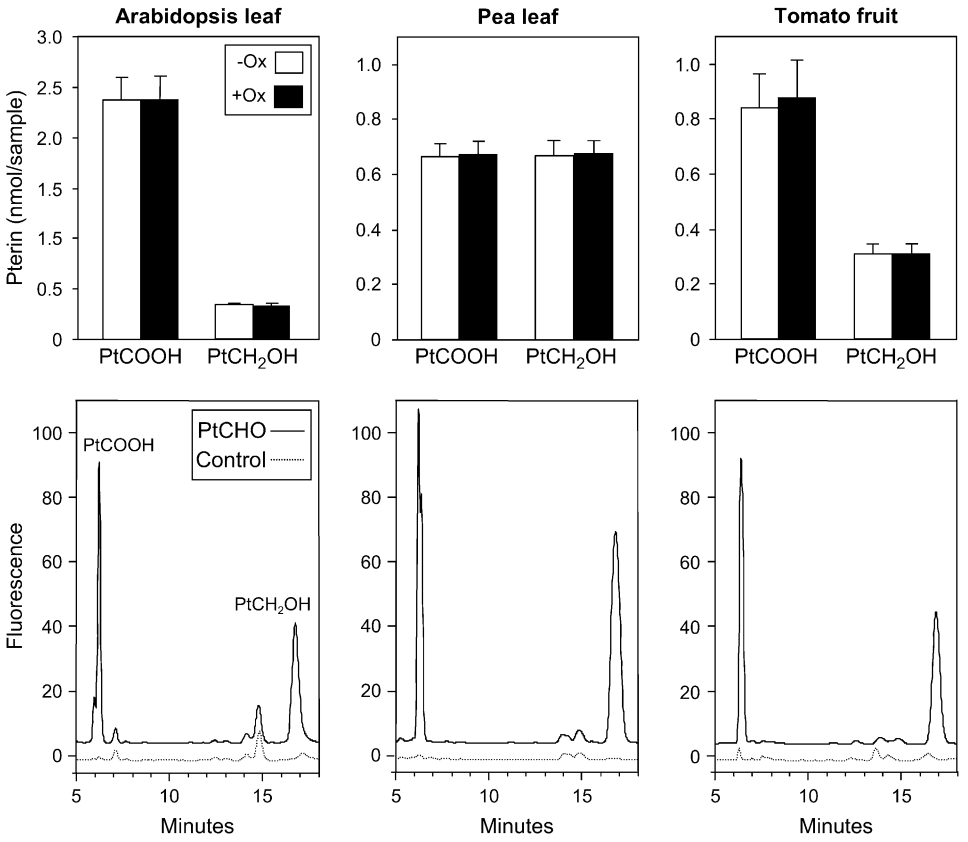

Figure 3. Metabolism of PtCHO by plant tissues. Arabidopsis leaf, pea leaf, and tomato pericarp tissues (0.15–0.23 g fresh weight) were incubated with PtCHO (4 nmol) for 15 h in darkness and washed. Pterins were analyzed before (−Ox) and after (+Ox) oxidation. Top frames show formation of $PtCH_2OH$ and PtCOOH (means of three replicates and SE). Values were corrected for the low endogenous levels of these pterins in controls incubated without PtCHO, shown for representative nonoxidized samples in the bottom frames.

water either before or after incorporation into folates. Tritium or deuterium at position 7 of the pterin ring is subject to very little if any spontaneous exchange, or to exchange during enzymatic reduction and tetra- to dihydro oxidation (Kaufman, 1964; Zakrzewski, 1966; Scott, 1984; Charlton et al., 1985). Experimental evidence against exchange as an explanation for lack of folate labeling is that only 3% of the [^{3}H]$PtCH_2OH$ label supplied was recovered from the medium as ^3H_2O at the end of the experiment. Nor can the absence of [^{3}H]folate synthesis from [^{3}H]$PtCH_2OH$ be explained by massive isotopic dilution by the endogenous $PtCH_2OH$ pool, because this pool is far smaller in Arabidopsis than the total PABA pool (<0.2 versus approximately 5 nmol g^{-1} fresh weight; Fig. 3; Orsomando et al., 2006).

Absence of Pterin Reduction by Plant Extracts

We tested for PtCHO or $PtCH_2OH$ ring reductase activity simultaneously using a coupled assay that exploits the high NADPH-linked pterin aldehyde reductase activity of plant extracts (Orsomando et al., 2006). PtCHO was used as substrate; the predicted end product was H_2PtCH_2OH, the pathway to it being PtCHO → H_2PtCHO → H_2PtCH_2OH for PtCHO ring reductase, and PtCHO → $PtCH_2OH$ → H_2PtCH_2OH for $PtCH_2OH$ ring reductase. In the presence of NADPH and an NAD(P)H regenerating system, Arabidopsis, pea, and tomato extracts converted PtCHO to $PtCH_2OH$, but none of this was reduced (Fig. 5). Tests with $PtCH_2OH$ as substrate likewise gave no NADPH- or NADH-dependent H_2PtCH_2OH formation (data not shown).

In view of the oxidation of PtCHO to PtCOOH seen in vivo (Fig. 3), we also tested for pyridine

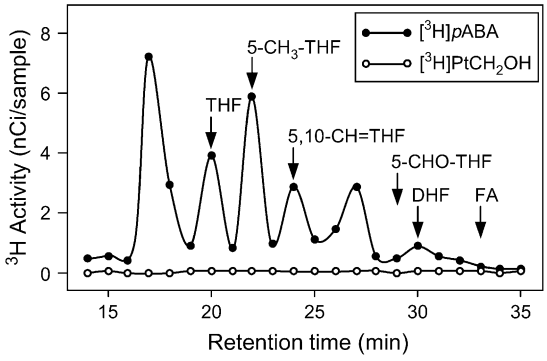

Figure 4. Metabolism of 6-[^{3}H]hydroxymethylpterin and [^{3}H]PABA by Arabidopsis plantlets. Plantlets (initial fresh weight 0.25 g) in 100 mL of medium were supplied with 1.6 μCi (166 pmol) of [^{3}H]$PtCH_2OH$ or 1.7 μCi (65 pmol) of [^{3}H]PABA and harvested after 7 d. Tissue fresh weight at harvest was 4.9 g. The folate fraction was deglutamylated, isolated by affinity chromatography, and separated by HPLC. Running positions of authentic folates cochromatographed with the samples are indicated. The experiment was repeated, with similar results. THF, Tetrahydrofolate; 5-CH_3-THF, 5-methyltetrahydrofolate; 5,10-CH=THF, 5,10-methenyltetrahydrofolate; 5-CHO-THF, 5-formyltetrahydrofolate; DHF, dihydrofolate; FA, folic acid. The radioactive peaks at approximately 17 and 27 min are unidentified metabolites.

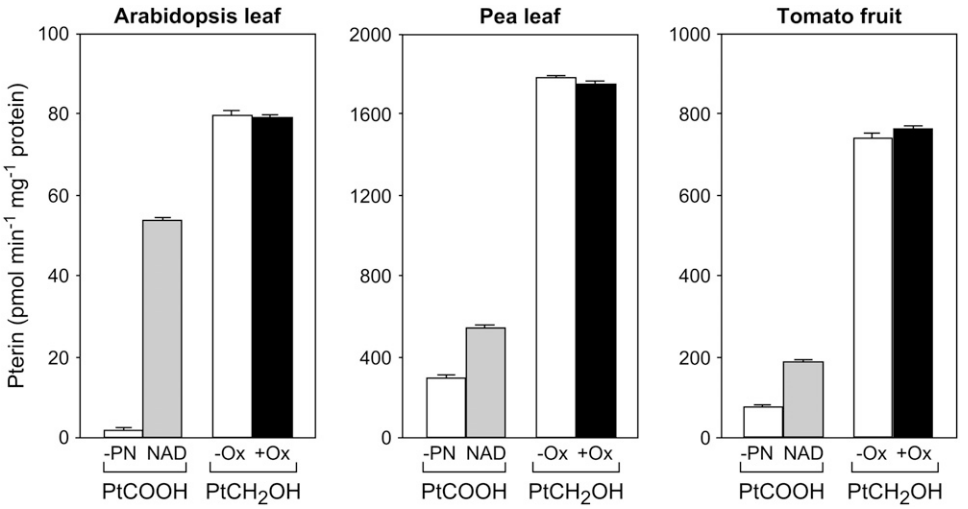

Figure 5. Fates of PtCHO in plant tissue extracts. Desalted extracts of Arabidopsis leaves, pea leaves, and tomato pericarp were incubated with 20 μM PtCHO. To measure side chain and ring reduction, 200 μM NADPH plus an NAD(P)H regenerating system were included, and the reaction product $PtCH_2OH$ was analyzed before (−Ox) and after (+Ox) oxidation. To measure side chain oxidation, 200 μM NAD was included and PtCOOH was analyzed. Controls contained no added pyridine nucleotide (PN), with or without the NAD(P)H regeneration system (which in the absence of pyridine nucleotides had no significant effect on PtCHO conversions). Data are means of three or four replicates and SE.

nucleotide-dependent and -independent PtCHO oxidation (Fig. 5). Arabidopsis, pea, and tomato extracts had substantial NAD-linked activity, and pea and tomato also had considerable activity without added NAD or NADP. The latter activity was not due to traces of endogenous NAD(P) left after desalting as it persisted when these were removed by an NAD(P)H regenerating system.

Folate Synthesis from Dihydropterins But Not Oxidized Pterins in *E. coli*

The above evidence for plants prompted us to investigate whether other folate-synthesizing organisms, *E. coli* and yeast, also lack pterin-reducing capacity. To find whether *E. coli* has pterin reductase activity, we used a *folE* deletant (Klaus et al., 2005) that lacks GTP cyclohydrolase I (the first enzyme of pterin synthesis) and therefore cannot make pterins or folates (Fig. 1B). This deletant can be maintained on Luria-Bertani (LB) medium plus thymidine, but its growth is slow (Fig. 6A, top row). Various oxidized pterins and dihydropterins were tested for the ability to support growth (Fig. 6A); they were related either to folate synthesis ($PtCH_2OH$, neopterin, monapterin, pteroic acid, and their dihydro forms) or to folate breakdown (PtCHO and H_2PtCHO; Fig. 1B). All the dihydropterins allowed rapid growth of the deletant, comparable to that of wild-type cells (Fig. 6A, first column). That H_2PtCHO was as effective as H_2PtCH_2OH confirms the in vitro evidence (Mitsuda and Suzuki, 1971) that *E. coli* can reduce the H_2PtCHO side chain. Unlike the dihydro forms, oxidized pterins did not support growth of the deletant (Fig. 6A, second column). This was not to due to toxicity of oxidized pterins, since these did not affect the growth of wild-type cells (Fig. 6A, third column). Nor was it due to failure to take up oxidized pterins, since deletant cells expressing Leishmania PTR1 were able to utilize $PtCH_2OH$ or PtCHO (Fig. 6B), both of which are PTR1 substrates (Nare et al., 1997). The failure of the *folE* deletant to grow on oxidized pterins thus argues strongly that, like plants, *E. coli* lacks capacity to reduce them.

Folate Synthesis from Dihydropterins or $PtCH_2OH$ in Yeast

The ability of yeast to reduce oxidized pterins was tested by the strategy used for *E. coli*. A GTP cyclohydrolase I (*fol2*) mutant, which cannot make pterins or folates and is auxotrophic for folate (Nardese et al., 1996), was supplied with oxidized pterins or dihydropterins (Fig. 7). After two passages on yeast peptone dextrose (YPD) medium alone, the mutant ceased growing, but continued growth when dihydropterins were given. As in *E. coli*, H_2PtCHO was as effective as H_2PtCH_2OH, demonstrating the capacity for H_2PtCHO side chain reduction. Among the oxidized pterins, only $PtCH_2OH$ consistently supported growth of the mutant, indicating that yeast can reduce this pterin at least. PtCHO occasionally gave some growth of the mutant (data not shown); presumably it was less potent than $PtCH_2OH$ due to the need for two reduction steps (side chain and ring) instead of one before entering the folate pathway.

DISCUSSION

We report here several lines of evidence that plants and *E. coli* have no detectable capacity to reduce the

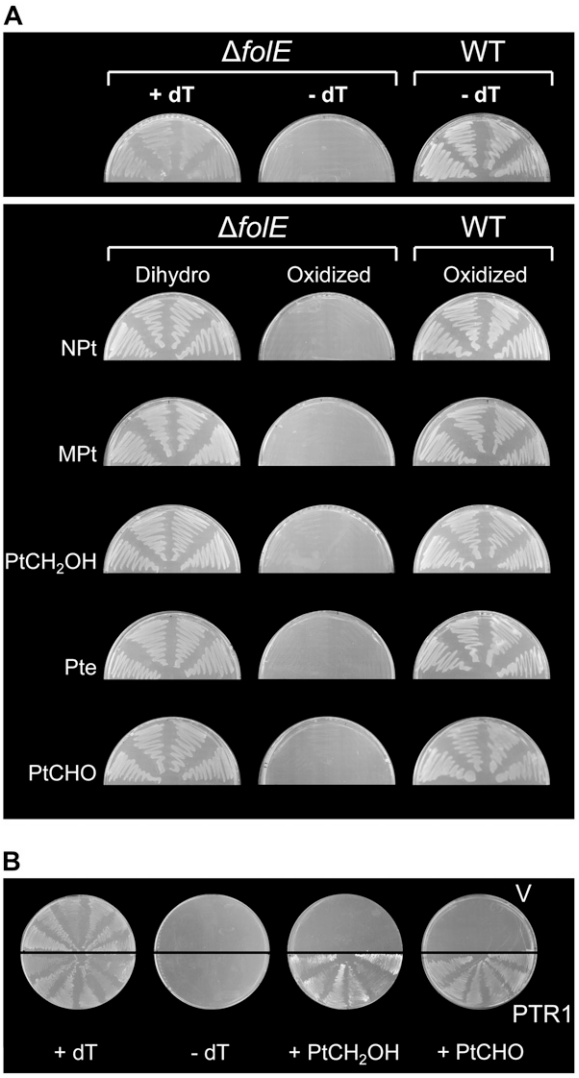

Figure 6. Pterin utilization by an *E. coli* pterin synthesis mutant. A, Cells of four independent clones of the *folE* deletant (Δ*folE*) were streaked on LB medium containing 0.1% Na ascorbate, 1 mM dithiothreitol, and kanamycin (50 μg mL^{-1}), without or with 300 μM thymidine (dT) or 11 μM pterin (dihydro or oxidized). The inoculum was from a thymidine-containing plate. Incubation was at 37°C under N_2. Conditions were the same for the K12 wild-type (WT) strain except that kanamycin was omitted and the inoculum was from an LB plate. Plates were photographed after 1 d. B, *folE* Deletant cells were transformed with pBluescript alone (V) or harboring *Leishmania major* PTR1 (PTR1). Six independent clones of each construct were streaked on LB medium containing 0.5 mM isopropyl-β-D-thiogalactopyranoside, kanamycin (30 μg mL^{-1}), and ampicillin (60 μg mL^{-1}) without or with 300 μM thymidine or 11 μM pterin. Plates were incubated at 37°C under N_2 and photographed after 5 d. NPt, Neopterin; MPt, monapterin; Pte, pteroate. The experiment was repeated, with similar results.

ring of oxidized pterins, although oxidation or reduction of the side chain is easily measurable. For plants, the metabolic, radiotracer, and biochemical lines of evidence are individually criticizable, on the grounds of abnormal compartmentation of pterins fed in vivo, for example. Collectively, however, these strands of evidence are quite persuasive, and made more so by the unequivocal genetic evidence for a similar situation in *E. coli*. We therefore infer that, unlike trypanosomatids, plants and *E. coli* have very little if any potential to salvage oxidized pterins, and that if this potential exists at all, it is physiologically insignificant compared to the capacity to modify the side chain.

This seems surprising, given the instability to oxidation of di- and tetrahydropterins, for it implies (1) that the dihydropterin intermediates of folate synthesis (H_2PtCH_2OH, dihydroneopterin, and dihydromonapterin) cannot be reclaimed if they become oxidized, and (2) that the folate breakdown product H_2PtCHO can only be recycled to folate synthesis if its side chain is reduced before its ring gets oxidized. Mechanisms can, however, be envisioned that would obviate the need to reduce oxidized pterins. The dihydropterin intermediates of folate synthesis could well be largely protein bound, and in this state resist oxidation. This is the case for tetrahydrofolates in mammals (Suh et al., 2001; Jones and Nixon, 2002). For the breakdown product H_2PtCHO, a sufficiently high side chain reductase activity could intervene to reduce it to H_2PtCH_2OH almost as it formed, preempting the chance of ring

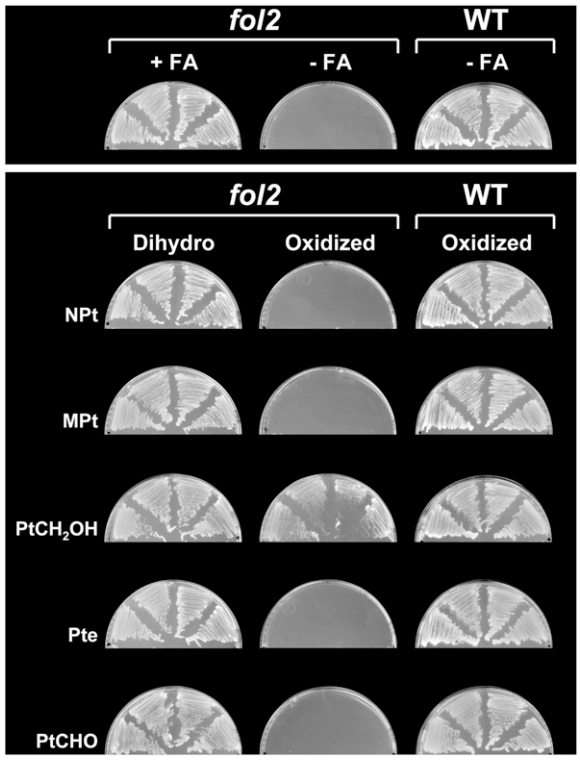

Figure 7. Pterin utilization by a yeast pterin synthesis mutant. Cells of four independent clones of the *fol2* mutant and the corresponding wild type (WT) were streaked twice in succession on YPD medium containing 0.2% Na ascorbate and 1 mM dithiothreitol, without or with 100 μM folinic acid (*FA*) or 20 μM pterin (dihydro or oxidized). Plates were incubated at 30°C under N_2 and photographed after 2 d. Pterin abbreviations as in Figure 6. The experiment was repeated, with similar results.

oxidation to give PtCHO (Fig. 1B, green arrow). Supporting this possibility, high in vivo and in vitro H_2PtCHO-reducing activities occur in plants (Figs. 3 and 4; Orsomando et al., 2006) and *E. coli* (Fig. 6; Mitsuda and Suzuki, 1971). Such mechanisms for dihydropterin preservation might, however, be overwhelmed under unusual circumstances. Metabolic engineering of pterin overproduction may be an instance of this, since >80% of the pterins that accumulate in engineered tomato fruits (up to 140-fold the wild-type level) become oxidized (Díaz de la Garza et al., 2004).

The discovery that yeast has the capacity to reduce $PtCH_2OH$ (although not other pterins) shows that reductases acting on oxidized pterins are not confined to trypanosomatids, the only organisms in which such enzymes were known hitherto. Since the yeast genome encodes no SDR with the characteristic PTR1-type TGX_3RXG motif, this implies the existence of an unknown class of pterin reductase. By the same token, the absence of a PTR1-like sequence from a genome can no longer be taken to signal lack of oxidized pterin reductase (or dihydropterin reductase) activity.

Lastly, we found in this study that plants readily oxidize the side chains of PtCHO and $PtCH_2OH$ to give PtCOOH, and that PtCHO oxidation is due to two types of activity, one NAD dependent, the other not. The former activity has also been found in *E. coli* extracts (Suzuki and Mitsuda, 1971); the latter may be due to aldehyde oxidase, which has a broad substrate range that includes aromatic aldehydes (Schwartz and Mendel, 2006). Whatever the case, the high potential for side chain oxidations indicates that H_2PtCH_2OH or H_2PtCHO molecules that undergo ring oxidation will be promptly converted to PtCOOH, a stable end product that is not salvaged by bacteria (Shiota, 1959) or Leishmania (Nare et al., 1997), nor probably by plants (Stakhov et al., 2002). This may explain why PtCOOH is often a major pterin in dry seeds (Kohashi, 1980; Kohashi et al., 1980).

MATERIALS AND METHODS

Chemicals

Pteridines were from Schircks Laboratories; near-saturated solutions were freshly prepared in N_2-gassed K phosphate 2 mM, pH 8.5, and quantified spectrophotometrically using published extinction coefficients (Blakley, 1969; Pfleiderer, 1985; Orsomando et al., 2006). Pterin solutions were protected from light. *p*-[3,5-^{3}H]Aminobenzoic acid (26.2 Ci mmol^{-1}) and [3′,5′,7,9-^{3}H]folic acid (47.9 Ci mmol^{-1}; diammonium salt in aqueous ethanol) were from Moravek Biochemicals.

Synthesis of 6-[7-^{3}H]Hydroxymethylpterin

[^{3}H]Folic acid (48 μCi, 1 nmol) was dried in vacuo, redissolved in 100 μL of 50 mM K phosphate, pH 7.5, and irradiated for 230 s in a microcuvette in the UV beam of a Beckman DU 7400 diode array spectrophotometer. The [7-^{3}H]PtCHO formed was enzymatically reduced to [7-^{3}H]$PtCH_2OH$ as follows. The volume was brought to 200 μL, and 10 mM glutathione, 100 μM NADPH, and 10% (v/v) glycerol (final concentrations) were added along with 20 μg purified recombinant aldehyde reductase (the Arabidopsis [*Arabidopsis thaliana*] *At1g10310* gene product; A Noiriel and A.D. Hanson, unpublished data). After incubation for 1 h at 30°C, 6 μL of 10 N HCl were added to stop the reaction and to destroy excess NADPH, and after a further 1 h at 4°C denatured enzyme was removed by centrifugation (10,000*g*, 10 min). The [7-^{3}H]$PtCH_2OH$ product was isolated using the HPLC conditions given below. Overall radiochemical yield was approximately 30% and radiochemical purity was ≥94%. The specific activities of product batches were 9 to 11 Ci mmol^{-1}.

Plant Materials

Arabidopsis L. Heynh. (ecotype Columbia) leaves were from rosettes of plants grown in a chamber for 4 to 6 weeks at 23°C to 28°C in 12-h d (photosynthetic photon flux density 80 μE m^{-2} s^{-1}) in potting soil. Pea (*Pisum sativum* L. cv Laxton's Progress 9) leaves were from 9- to 14-d-old plants grown as described (Orsomando et al., 2006). Tomato (*Lycopersicon esculentum* Mill.) pericarp was from mature green fruits of cv Micro-Tom, grown as described (Díaz de la Garza et al., 2004); tomato leaves were fully expanded, from a derivative of cv M82 growing in soil in a naturally lit greenhouse in September, 2005.

Escherichia coli and Yeast Strains

Escherichia coli K12 *folE* deletant P1-7B (Klaus et al., 2005) and strain P1-6E obtained by the same procedure were grown at 37°C on LB medium containing 300 μM thymidine and 30 μg mL^{-1} kanamycin. Both strains were used in experiments. Yeast (*Saccharomyces cerevisiae*) strains 971/6c (*Mata ade2-1 his3-11,15 leu2-3,112 ura3-1 can1*) and 971/6a (Matα *ade2-1 his3-11,15 leu2-3,112 ura3-1 can1 fol2:HIS3*) were obtained from M. L. Agostoni Carbone (Università di Milano). Yeast was cultured at 30°C in YPD medium (1% Difco yeast extract, 1% Difco bacto-peptone, and 2% Glc). The *fol2* mutant strain 971/6a was maintained on YPD containing folinic acid (Ca^{2+} salt; Sigma) at a final concentration of 50 μg mL^{-1}.

Pterin Metabolism Experiments

Arabidopsis leaf sections and tomato pericarp discs were prepared as described (Orsomando et al., 2006). Pea and tomato leaf discs (1 cm diameter) or pea leaflets were stripped of the midvein on the abaxial surface. Pterin doses (19–32 μL of solution) were equally divided among sets of five or six leaf sections, leaflets, or discs, or two pericarp discs, applying droplets to the cut surfaces. Tissues were then incubated for 14.5 h in darkness on moist filter paper, shaken (75 rpm) for 0.5 h in 5 mL of water to remove nonabsorbed pterins, and frozen and ground in liquid N_2. Ground samples were extracted in 2 mL of MeOH:$CHCl_3$:water (12:5:1, v/v/v) containing 0.1% (w/v) Na ascorbate and 10 mM β-mercaptoethanol, warmed to 50°C for 5 min, and centrifuged to clear. The supernatant was mixed with 0.5 mL of $CHCl_3$ and 0.75 mL of water and centrifuged to separate the aqueous phase, which was concentrated in vacuo to 200 μL. Pairs of 50-μL samples were taken for pterin analysis: one received 80 μL of 1 N HCl containing 1% I_2 plus 2% KI (w/v; to oxidize pterins), the other 80 μL of 1 N HCl alone. After incubation for 1 h in darkness, both samples received 10 μL of 5% (w/v) Na ascorbate (to destroy excess I_2) and 7 μL of 1 N NaOH (to partly neutralize the HCl). Aliquots (50 μL) were analyzed by HPLC. Recoveries from tissue samples of 200-pmol pterin spikes (used to correct experimental data) were: H_2PtCH_2OH, 21% to 50%; $PtCH_2OH$, 54% to 83%; and PtCOOH, 8% and 78%. Recoveries from Arabidopsis were consistently lower than from pea or tomato. $PtCH_2OH$ and PtCOOH recoveries from oxidized and nonoxidized samples were not significantly different.

^{3}H-Labeling Experiments

Arabidopsis seeds were surface sterilized and germinated on Murashige and Skoog agar. Plantlets 2 weeks old were then cultured axenically in 250-mL flasks (seven plantlets/flask) containing 100 mL of 0.33× liquid Murashige and Skoog medium plus 10 g L^{-1} Suc. Flasks were shaken at 80 rpm; temperature and lighting were as given above for Arabidopsis. Filter-sterilized [7-^{3}H]$PtCH_2OH$ or [3,5-^{3}H]PABA was added to 11-d-old cultures, which were darkened for the first 24 h, then returned to the normal light regime. At 7 d, plantlets were washed twice for a total of 4 h with 100 mL of culture medium containing 1 μM unlabeled $PtCH_2OH$ or PABA (to remove nonabsorbed label), then taken for analysis. Folates were extracted, deglutamylated, purified by affinity chromatography, and separated by HPLC as described (Díaz de la Garza et al., 2004).

In Vitro Pterin Reduction and Oxidation Assays

Extracts of Arabidopsis leaves, pea leaves, and tomato pericarp were prepared by grinding in liquid N_2, thawing in two volumes of 100 mM K

phosphate, pH 7.5, containing 5 mM dithiothreitol and 3% (w/v) polyvinylpolypyrrolidone, centrifuging to clear (10,000*g*, 10 min), and desalting on PD-10 columns equilibrated in 100 mM K phosphate, pH 7.5, containing 5 mM dithiothreitol and 10% (v/v) glycerol. The desalted extracts were frozen in liquid N_2 and stored at −80°C. Enzyme assays (50 μL final volume) contained 4 to 29 μg of protein, 100 mM K phosphate, pH 7.5, 10 mM glutathione, and 20 μM PtCHO. Reduction assays contained 200 μM NADPH and an NAD(P)H regenerating system comprising 1 mM Glc-6-P and 0.15 units (1 unit = 1 μmol min^{-1}, measured in the above assay buffer) of *Leuconostoc mesenteroides* Glc-6-P dehydrogenase. Oxidation assays contained 200 μM NAD or NADP. Assays were incubated for 30 min at 30°C and stopped by freezing or acidification. A pair of 20-μL samples was used for pterin analysis: one received 10 μL of HCl-I_2/KI solution, the other 10 μL of 1 N HCl; both were incubated for 1 h in darkness and then received 10 μL of 5% (w/v) Na ascorbate and 60 μL of 10 mM Na phosphate, pH 6.0, containing 10 mM β-mercaptoethanol and 1% (w/v) Na ascorbate.

HPLC Analysis of Pterins

Pterins (50-μL injections) were separated on a 4-μm, 250- × 4.6-mm Synergi Fusion-RP 80 column (Phenomenex) eluted isocratically with 10 mM Na phosphate (pH 6.0) at 1.5 mL min^{-1}. Peaks were detected by fluorescence (350 nm excitation, 450 nm emission) and quantified relative to standards. Because pterins are highly fluorescent when oxidized but not when reduced, the difference in peak area between oxidized and nonoxidized samples is a measure of reduced (di- and tetrahydro) forms (Fukushima and Nixon, 1980).

Pterin Reduction Tests with *E. coli* and Yeast

E. coli cells were streaked on LB medium containing 0.1% (w/v) Na ascorbate, 1 mM dithiothreitol, 30 or 50 μg mL^{-1} kanamycin, and (for cells harboring pBluescript plasmids) 60 μg mL^{-1} ampicillin and 0.5 mM isopropyl-β-D-thiogalactopyranoside. Pterin concentration was 11 μM. Yeast cells were streaked on YPD medium containing 0.2% Na ascorbate and 1 mM dithiothreitol. Pterin concentration was 20 μM. *E. coli* and yeast plates were incubated at 37°C and 30°C, respectively, in a 10-L vessel flushed with N_2 at 10 mL min^{-1}.

PTR1 Expression Construct

A *Leishmania major* PTR1 amplicon preceded by a Shine-Dalgarno sequence and a stop codon in frame with Lac*Zα* was cloned between the *Bam*HI and *Kpn*I sites of pBluescript SK-, which contains the Amp^r marker. The PCR template was pSNAR-HR1A, which contains a 15-kb *L. major* strain CC-1H genomic fragment including the *ptr1* gene (the gift of S.M. Beverley, Washington University); primers were 5′-GTCA*GGATCC*CTGAAGGAAACAGCTATG-ACTGCTCCGACCGTGCCG-3′ and 5′-AGTGTA*GGTACC*TCAGGCCCGGG-TAAGGCTGTA-3′; amplification was with KOD HiFi polymerase (Novagen). The amplicon was first cloned into pGEM-T Easy (Promega), sequenced, excised with *Bam*HI and *Kpn*I, then cloned into pBluescript. The deduced amino acid sequence differed from that published in having Val not Phe at position 162. The constructs were made in *E. coli* strain DH5*α*, then introduced into *E. coli folE* deletant strains. Transformants were grown on LB plates containing 300 μM thymidine, 60 μg mL^{-1} ampicillin, and 30 μg mL^{-1} kanamycin.

ACKNOWLEDGMENTS

We thank M.J. Ziemak for technical help, and Drs. S.M. Beverley, V. de Crecy-Lagard, and S.W. Bailey for advice.

Received November 23, 2006; accepted December 20, 2006; published January 12, 2007.

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