

Folate synthesis in higher-plant mitochondria: coupling between the dihydropterin pyrophosphokinase and the dihydropteroate synthase activities

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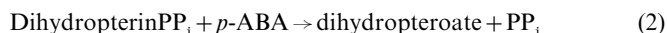
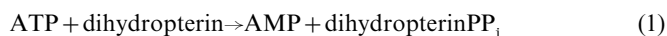
The plant enzyme 6-hydroxymethyl-7,8-dihydropterin pyrophosphokinase/7,8-dihydropteroate synthase (HPPK/DHPS) is a mitochondrial bifunctional protein involved in tetrahydrofolate synthesis. The first domain (HPPK) catalyses the pyrophosphorylation of 6-hydroxymethyl-7,8-dihydropterin (dihydropterin) by ATP, leading to 6-hydroxymethyl-7,8-dihydropterin pyrophosphate (dihydropterinPP_i) and AMP. The second domain (DHPS) catalyses the next step, i.e. the condensation of *p*-aminobenzoic acid (*p*-ABA) with dihydropterinPP_i to give 7,8-dihydropteroate (dihydropteroate) and PP_i. In the present article we studied the coupling between these two reactions. Kinetic data obtained for the HPPK domain are consistent with an ordered Bi Bi mechanism where ATP binds first and dihydropterinPP_i is released last, as proposed previously for the monofunctional *Escherichia coli* enzyme. In the absence of *p*-ABA, AMP and dihydropterinPP_i accumulate and negatively regulate the reaction. In the

presence of *p*-ABA, the rates of AMP and dihydropteroate synthesis are similar, indicating a good coupling between the two reactions. DihydropterinPP_i, an intermediate of the two reactions, never accumulates in this situation. The high specific activity of DHPS relative to HPPK, rather than a preferential channelling of dihydropterinPP_i between the two catalytic sites, could explain these kinetic data. The maximal velocity of the DHPS domain is limited by the availability of dihydropterinPP_i. It is strongly feedback-inhibited by dihydropteroate and also dihydrofolate and tetrahydrofolate monoglutamate, two intermediates synthesized downstream in the folate biosynthetic pathway. Thus the HPPK domain of this bifunctional protein is the limiting factor of the overall reaction, but the DHPS domain is a potential key regulatory point of the whole folate biosynthetic pathway.

Key word: C1 metabolism.

INTRODUCTION

Plants and micro-organisms, in contrast to animals, are able to synthesize tetrahydrofolate *de novo*. From 6-hydroxymethyl-7,8-dihydropterin (hereon referred to as dihydropterin), this biosynthetic pathway requires the sequential operation of five enzymes (for a review, see [1]). The enzymes catalysing the first three steps are absent in animals. These enzymes are therefore potential targets for antimicrobial or herbicide drugs. The 6-hydroxymethyl-7,8-dihydropterin pyrophosphokinase (HPPK; reaction 1) and the 7,8-dihydropteroate synthase (DHPS; reaction 2) catalyse the first two steps of this pathway, leading to the formation of dihydropteroate.



where dihydropterinPP_i is 6-hydroxymethyl-7,8-dihydropterin pyrophosphate and *p*-ABA is *p*-aminobenzoic acid. The DHPS activity (reaction 2) is the target of sulphonamides, a family of molecules that are potent antibiotics. These chemicals are *p*-ABA analogues that are recognized by the enzyme as alternative substrates [2,3] and their effects in bacteria and protozoa have been studied intensively [4–8]. DHPS from prokaryotes is a dimeric protein with identical subunits of about 30–35 kDa [9,10]. The three-dimensional structures of the *Escherichia coli* and *Staphylococcus aureus* DHPS proteins have been solved, giving new insights into how substrates and inhibitors bind and react in the catalytic site of the protein [11,12]. The protein belongs to the triose phosphate isomerase (TIM) barrel group

and kinetic and crystallographic studies suggest a random binding of the two substrates and a half-site reactivity (the substrates bind to only one monomer) [12].

On the other hand, there are few data concerning the kinetic behaviour of the HPPK-catalysed reaction. This reaction was generally measured in association with DHPS activity [13,14], and the regulation of this pyrophosphoryl-transferring enzyme remains to be determined. In prokaryotes, the HPPK protein is a monomer of 18 kDa [13]. The three-dimensional structures of the *E. coli* and *Haemophyllum influenzae* enzymes have been determined [15–17], showing that the monomer consists of a single globular α/β domain. The positions of the substrates in the catalytic site are compatible with a mechanism involving a nucleophilic attack from the hydroxyl group of the pterin molecule to the β -phosphate of ATP, followed by a direct transfer of the PP_i group from ATP to the 6-hydroxymethyl side chain of dihydropterin. Two Mg²⁺ ions, co-ordinated with the carboxyl groups of two conserved aspartate residues, are involved in this transfer and stabilize the transition state.

In contrast with the situation found in prokaryotes, HPPK and DHPS activities are associated in all the eukaryotes studied so far. Indeed, in the protozoa *Plasmodium falciparum* and *Toxoplasma gondii* a bifunctional protein [18,19] supports both activities. The situation is even more complex in the sporozoan *Pneumocystis carinii*, where the enzyme is a trifunctional polypeptide supporting dihydroneopterin aldolase, HPPK and DHPS activities [20]. Dihydroneopterin aldolase catalyses the conversion of dihydroneopterin into dihydropterin, the substrate of HPPK activity. In plants, the DHPS and HPPK activities are

Abbreviations used: *p*-ABA, *p*-aminobenzoic acid; HPPK, 6-hydroxymethyl-7,8-dihydropterin pyrophosphokinase; DHPS, 7,8-dihydropteroate synthase; dihydropterin, 6-hydroxymethyl-7,8-dihydropterin; dihydropterinPP_i, 6-hydroxymethyl-7,8-dihydropterin pyrophosphate.

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also supported by a bifunctional enzyme located in the matrix space of mitochondria [21,22]. In fact, all the five steps required for tetrahydrofolate polyglutamate synthesis in plants were localized in mitochondria [21], which strongly suggests that these organelles are a major site for folate synthesis in these organisms.

In previous studies [22] we have purified and determined the primary sequence of the bifunctional HPPK/DHPS from pea leaves. The molecular data (the presence of a mitochondrial transit peptide, a single-copy gene) confirmed the biochemical evidence for this protein being localized in the mitochondria. Preliminary kinetic studies of the DHPS activity suggested a random bireactant system strongly retro-inhibited by dihydropteroate, a product of the reaction (K_i , 8–10 μM [22]). However, the catalytic properties of HPPK and its coupling with DHPS have not been studied yet. The biochemical characterization of the native HPPK/DHPS from higher-plant mitochondria is hampered by the difficulty in obtaining sufficient amounts of protein. Indeed, it represents only 0.04–0.06 % of the soluble proteins of the mitochondrial matrix [22]. An alternative approach is to produce a recombinant enzyme in a prokaryotic host, such as *E. coli*. In this report, we describe for the first time the catalytic properties and coupling of the HPPK and DHPS activities of the plant recombinant bifunctional enzyme.

MATERIALS AND METHODS

Materials

Pterate and pterin were obtained from Sigma. PterinPP_i was obtained from Schircks Laboratory (Jona, Switzerland). These products were reduced as dihydro- compounds as described by Scrimgeour [23]. They were quantified by their typical absorption spectra [24,25] and MS analysis, then stored at -80°C in flasks saturated with argon.

Expression of the recombinant HPPK/DHPS in *E. coli*

The cDNA fragment encoding the mature pea leaf HPPK/DHPS [22] was ligated into the appropriate sites of a modified version of the plasmid pET-3a (Stratagene), provided kindly by Professor D. Macherel (University of Angers, Angers, France). This vector contained the nucleotide sequence of *argU*, the gene encoding a tRNA^{ARG} that is only rarely found in bacteria. Indeed, the HPPK/DHPS protein contains 19 arginine residues, a situation that could strongly limit its prokaryotic expression. The resulting pET-HPPK/DHPS vector was used to transform the BL21 (DE3) *E. coli* strain (Stratagene). The bacteria were then grown at 37°C in 2 litres of Luria–Bertani medium supplemented with 100 $\mu\text{g}/\text{ml}$ carbenicillin. The expression of recombinant HPPK/DHPS protein was induced at 18°C by the addition of 4 μM isopropyl β -D-thiogalactoside. Cells were harvested 48 h later by centrifugation. Most of the recombinant protein was produced as inclusion bodies. However, a small part remained soluble. The presence of the recombinant HPPK/DHPS was monitored by SDS/PAGE analysis [26] and by Western blotting using rabbit polyclonal antibodies raised against the recombinant enzyme purified from inclusion bodies (Elevage Scientifique des Dombes, Chatillon-Chalaronne, France).

Purification of the recombinant HPPK/DHPS

Cell pellets from 1 litre cultures were incubated at 37°C for 15 min in 100 ml of a medium containing 2 mM EDTA, 10 $\mu\text{g}/\text{ml}$ lysozyme and 50 mM Tris/HCl (pH 8.0). The cells were disrupted by three consecutive freeze/thaw cycles followed by a 10 min ultrasonic period at 4°C . All subsequent steps were performed at 4°C . The suspension was centrifuged for 15 min at 15000 *g* to

pellet membrane fragments and inclusion bodies. Lipids and small vesicles were removed by ultra-centrifugation for 1 h at 30000 *g*. The resulting supernatant containing soluble recombinant HPPK/DHPS was then concentrated 10-fold by ultrafiltration through a 100 kDa-cutoff membrane (Amicon). This procedure removed the two bacterial HPPK (18 kDa) and DHPS (70 kDa) proteins present in vanishing amounts. The remaining protein extract was thereafter loaded on to a $0.5\text{ cm} \times 5\text{ cm}$ folate-agarose affinity column (Sigma) that had been equilibrated previously with buffer I [0.1 M KH_2PO_4 , pH 7.5/10 % (v/v) glycerol]. The flow rate was 0.3 ml/min. The column was washed for 45 min with buffer I, then eluted with a linear gradient (0–100 %) of buffer II [0.1 M KH_2PO_4 , pH 8.5/10 % (v/v) glycerol/1 mM folate]. Fractions containing the purified recombinant HPPK/DHPS were dialysed against buffer I and concentrated to a final volume of 800 μl . The N-terminal part of the protein was analysed as described previously [22]. The obtained sequence (MFHTAPNSSI) matched the one determined with the native enzyme [22], thus confirming its identity. The protein was stored at -80°C in 10 % (v/v) glycerol until use.

Determination of HPPK and DHPS activities

These activities were estimated at 30°C . All the solutions were maintained under a stream of argon to minimize the oxidation of the various dihydropterin substrates. The HPPK + DHPS activity (total activity, i.e. reaction 1 + reaction 2) was estimated according to [21]. The standard reaction medium (medium A) contained, in a total volume of 120 μl : 40 mM Tris (pH 8.0), 20 mM 2-mercaptoethanol, 10 mM MgCl_2 , 200 μM ATP and various amounts of the purified protein. [¹⁴C]p-ABA (2 μl , 2 mM, 1.85 GBq \cdot mmol⁻¹; ICN Biomedicals) was added to the assay medium, and then the reaction was started by the addition of 100 μM dihydropterin. After various incubation periods, 100 μl of the assay medium were injected into a reversed-phase HPLC system (Merck 655A-11 Liquid Chromatograph, equipped with a Shandon, Zorbax ODS Z225 5 μm column) coupled with a Berthold (LB 506D) scintillation counter, as described earlier [22]. The HPLC conditions were: solvent A, 0.1 M sodium acetate, pH 6; solvent B, acetonitrile; solvent B increased linearly by 0.8 %/min at a flow rate of 1 ml \cdot min⁻¹. Within these experimental conditions, excess [¹⁴C]p-ABA was not retained in the column, whereas [¹⁴C]dihydropteroate, the final product of the reaction, was eluted after 19 min of chromatography.

The DHPS activity (reaction 2) was measured in medium A (final volume, 120 μl) devoid of ATP [22]. For a standard assay, 2 μl of 2 mM [¹⁴C]p-ABA (1.85 GBq \cdot mmol⁻¹) were added in the medium, and the reaction was started by the addition of 100 μM dihydropterinPP_i. After various times of incubation the [¹⁴C] dihydropteroate formed was estimated as described above.

The HPPK activity (reaction 1) was estimated through the formation of either [2,8-³H]AMP in the presence of [2,8-³H]ATP (Amersham Bioscience) or β -[³²P]dihydropterinPP_i in the presence of [γ -³²P]ATP (Amersham Bioscience). The standard reaction medium contained, in a total volume of 120 μl : 40 mM Tris (pH 8.0), 20 mM 2-mercaptoethanol, 10 mM MgCl_2 and 200 μM [2,8-³H]ATP (1.85 GBq \cdot mmol⁻¹) or 200 μM [γ -³²P]ATP (1.85 GBq \cdot mmol⁻¹) and various amounts of the purified recombinant enzyme. Then the reaction was started by the addition of 100 μM dihydropterin. After various periods of incubation, the products of the reaction were analysed by the HPLC system described above. The HPLC conditions were: solvent A, 50 mM KH_2PO_4 , pH 6.0/5 mM tetrabutylammonium phosphate/4 % (v/v) acetonitrile; solvent B, acetonitrile; solvent B increased linearly by 0.8 %/min at a flow rate of 1 ml \cdot min⁻¹. Within

these experimental conditions, [2,8-³H]AMP and [2,8-³H]ATP (or [γ -³²P]ATP) were eluted after 14 and 32 min of chromatography, respectively, whereas β -[³²P]dihydropterinPP_i was eluted after 15 min. We verified in separate control experiments that non-enzymic formation of [2,8-³H]AMP was negligible during the course of the experiment.

RESULTS

Expression and purification of the recombinant HPPK/DHPS

The recombinant HPPK/DHPS was expressed almost exclusively as inclusion bodies in *E. coli*. However, with the culture conditions described in the Materials and methods section (i.e. low temperature, low isopropyl β -D-thiogalactoside concentration) DHPS and HPPK activities were higher in the transformed cells than in the control, indicating that some of the recombinant protein was soluble and active. Although the bifunctional HPPK/DHPS was not clearly visible on SDS/PAGE analysis of soluble proteins (Figure 1A, lane 2), the antibodies raised against

HPPK/DHPS recognized a band of approx. 53000 Da, corresponding to the molecular mass of the plant enzyme (Figure 1B, lane 1). This band was not present in non-transformed cells (result not shown). Unfortunately, all our attempts to improve the yield of recombinant enzyme production (expression as a fusion protein with thioredoxin, co-expression with GroES and GroEL, and expression in the yeast *Pichia pastoris*) were unsuccessful. Nevertheless, the production of the recombinant HPPK/DHPS in *E. coli* was a good alternative compared with the time-consuming purification of the enzyme from isolated plant mitochondria [22]. Indeed, the recombinant HPPK/DHPS enzyme was purified in a single step by affinity chromatography on a folate-agarose column, leading to a final enzyme preparation apparently devoid of contaminants (Figure 1A, lane 3). Sequencing of the N-terminal part of the protein (see the Materials and methods section) further confirmed the identity of the enzyme. According to our protocol, about 300 μ g of purified HPPK/DHPS could be obtained from 1 litre of *E. coli* cell culture.

In preliminary experiments, we compared the catalytic properties of the native HPPK/DHPS with those of the recombinant enzyme. The various substrates used for K_m determinations were highly susceptible to degradation and were therefore freshly prepared (see the Materials and methods section). As shown in Table 1, the kinetic parameters of the two types of enzyme were similar, justifying the use of the recombinant protein in this study. However, it must be pointed out that the specific activities reported in Table 1 were measured immediately after the final step of purification. These values decreased with the length of the storage period, suggesting some instability in the purified native and recombinant proteins. This loss of activity was reduced when the enzyme was kept at -80°C in presence of 10% (v/v) glycerol.

Identification of the dihydropterinPP_i formed by HPPK activity

HPPK activity can be determined in the absence of *p*-ABA either by the amount of [2,8-³H]AMP formed in the presence of [2,8-³H]ATP or the amount of [³²P]dihydropterinPP_i formed in the presence of [γ -³²P]ATP (see reaction 1). To study the coupling between HPPK and DHPS activities it is necessary to estimate the level of dihydropterinPP_i, the product of the first reaction and the substrate for the second. HPLC identification of trace amounts of dihydropterinPP_i is not simple because there is no commercially available labelled standard. Using [γ -³²P]ATP or [β , γ -³²P]ATP, we identified within our HPLC conditions a peak (retention time, approx. 15 min) that increased with the time course of the reaction. This peak was identified as dihydropterinPP_i for the following reasons. First, it was not present when

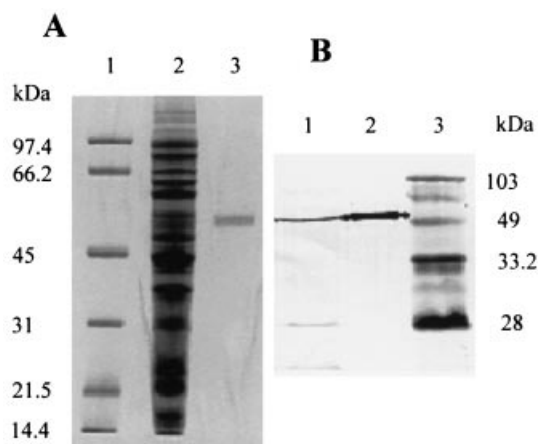


Figure 1 Purification and immunodetection of the recombinant HPPK/DHPS expressed in *E. coli*

(A) Coomassie Brilliant Blue-stained SDS/PAGE of the *E. coli* proteins during the course of purification. Lane 1, molecular-mass standards; lane 2, soluble proteins (50 μ g) collected after concentration on an XM-100 ultrafiltration membrane; lane 3, purified HPPK/DHPS (2 μ g) recovered from the folate-agarose column. (B) Immunodetection of the recombinant HPPK/DHPS. Lane 1, soluble proteins (50 μ g) collected after concentration on a XM-100 ultrafiltration membrane; lane 2, purified HPPK/DHPS (2 μ g); lane 3, molecular-mass standards.

Table 1 Kinetic parameters of the HPPK/DHPS protein purified from pea leaf mitochondria and the recombinant HPPK/DHPS protein produced in *E. coli*

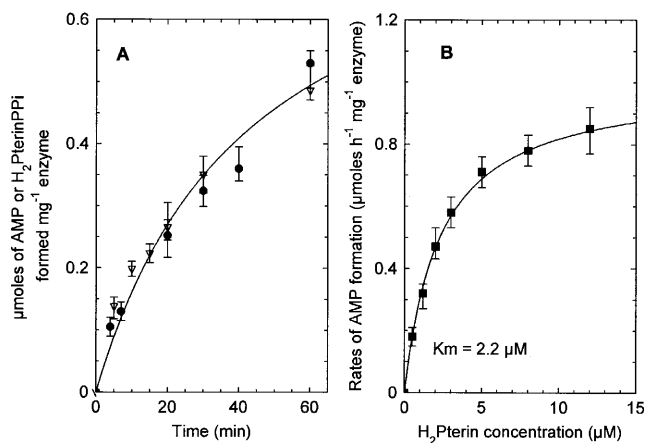
The results are means \pm S.D. from three to five separate determinations. The specific activities were measured immediately after purification of the enzymes and the K_m values were estimated by direct fitting to Michaelis-Menten curves using non-linear regression and EasyPlot software (Spiral Software).

Kinetic parameter	Pea HPPK/DHPS		Recombinant HPPK/DHPS	
	HPPK + DHPS activity	DHPS activity	HPPK + DHPS activity	DHPS activity
K_m (μM)				
Dihydropterin	1 \pm 0.5		2 \pm 1	
ATP	70 \pm 15		70 \pm 15	
DihydropterinPP _i		8 \pm 4		10 \pm 5
<i>p</i> -ABA		1.0 \pm 0.5		2.5 \pm 1.0
Specific activity ($\mu\text{mol} \cdot \text{mg}^{-1} \cdot \text{h}^{-1}$)	2.5 \pm 0.5	20 \pm 4	1.8 \pm 0.5	16 \pm 4

Table 2 Identification of AMP and dihydropterinPP_i by HPLC

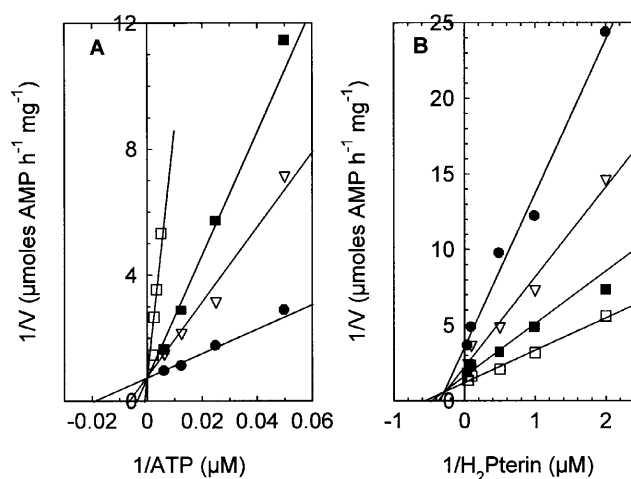
The reaction medium (120 μ l) contained 1 μ g of the recombinant HPPK/DHPS protein, 100 μ M dihydropterin and 200 μ M of various labelled ATPs ([2,8-³H]ATP, [α -³²P]ATP, [β , γ -³²P]ATP and [γ -³²P]ATP). After 60 min of incubation, 100 μ l of the reaction medium were injected into the HPLC system and the amounts of radioactivity present in the AMP (14 min) and dihydropterinPP_i (15 min) peaks were determined as described in the Material and methods section. The controls (without dihydropterin or without enzyme) were determined with all the various labelled ATP species. The small amount of AMP observed in these controls was present in the initial ATP solution. It was subtracted from all the following data. The values are means \pm S.D. from two determinations for [α -³²P]ATP and [β , γ -³²P]ATP and at least three determinations for the other conditions; ND, not detectable.

	Specific activity (μ mol \cdot mg of protein ⁻¹)	
	AMP	DihydropterinPP _i
No dihydropterin	0.038 \pm 0.008	ND
No enzyme	0.035 \pm 0.008	ND
[³ H]ATP	0.53 \pm 0.04	ND
[α - ³² P]ATP	0.55 \pm 0.03	ND
[β , γ - ³² P]ATP	ND	0.46 \pm 0.055
[γ - ³² P]ATP	ND	0.49 \pm 0.04

**Figure 2 Kinetic study of the HPPK domain**

(A) Time-course formation of AMP (∇) and dihydropterinPP_i (H₂PterinPP_i; \bullet). HPPK activity is expressed as μ mol of products (AMP or dihydropterinPP_i) formed/mg of protein. The reaction medium (120 μ l) contained 1 μ g of recombinant HPPK/DHPS protein, 100 μ M dihydropterin and 200 μ M ATP. Each point is the mean of two to five measurements. (B) Rate of AMP formation as a function of dihydropterin (H₂Pterin) concentration. The reaction medium (120 μ l) contained 1 μ g of recombinant HPPK/DHPS protein, 200 μ M ATP and various concentrations of dihydropterin. The K_m value (2.2 μ M) was estimated by direct fitting to the Michaelis–Menten curve using non-linear regression and EasyPlot software (Spiral Software). Each point is the mean from three determinations.

either dihydropterin or the enzyme (Table 2) was omitted. Secondly, the peak could not be observed in presence of [2,8-³H]ATP or [α -³²P]ATP, a situation where AMP accumulated, indicating that it was not related to the purine ring and did not contain the α -Pi group of ATP. Thirdly, the peak, but not AMP, was observable in the presence of [β , γ -³²P]ATP and [γ -³²P]ATP (Table 2). The amount of product calculated from the specific activities of the initial ATP substrates was similar in both cases, indicating the presence of two P_i groups. Indeed, if only one P_i was transferred from [β , γ -³²P]ATP, the specific activity of the final product would have been half as much. Furthermore, this amount was similar to the amount of AMP formed in the presence

**Figure 3 Inhibition of the HPPK activity by dihydropterinPP_i**

(A) Competitive inhibition by dihydropterinPP_i of ATP in the presence of a saturating concentration of dihydropterin (100 μ M). DihydropterinPP_i concentrations were: \square , 75 μ M; \blacksquare , 30 μ M; ∇ , 7.5 μ M; \bullet , 0 μ M. The K_i value was 5 μ M. (B) Mixed-type (non-competitive) inhibition by dihydropterinPP_i of dihydropterin (H₂Pterin) in the presence of saturating ATP (200 μ M). DihydropterinPP_i concentrations were: \bullet , 30 μ M; ∇ , 15 μ M; \blacksquare , 7.5 μ M; \square , 0 μ M. The K_i values were 10 and 15 μ M. For both panels each point is the mean from three separate estimations.

Table 3 Inhibition of the HPPK activity by the end products of the reaction

The K_i values for AMP or dihydropterinPP_i versus ATP or dihydropterin were calculated from the initial velocities of the reaction measured in the presence of one particular product (AMP or dihydropterinPP_i) and variable concentrations of one particular substrate (ATP or dihydropterin), as described by Segel [28]. The results are means \pm S.D. from three determinations.

Substrate	DihydropterinPP _i		AMP	
	K_i (μ M)	Type of inhibition	K_i (μ M)	Type of inhibition
ATP	5 \pm 3	Competitive	700 \pm 200	Mixed
Dihydropterin	13 \pm 5	Mixed	400 \pm 100	Mixed

of [2,8-³H]ATP or [α -³²P]ATP, confirming that the P_i groups were β - and γ -P_i from ATP.

Catalytic properties of the HPPK reaction

In the absence of *p*-ABA and in the presence of saturating concentrations of dihydropterin (100 μ M) and ATP (200 μ M), the amount of [2,8-³H]AMP and [³²P]dihydropterinPP_i increased with time in a stoichiometric manner (Figure 2A). The K_m values for dihydropterin (2.2 \pm 0.7 μ M, Figure 2B) and ATP (50 \pm 15 μ M, results not shown) were similar to the values reported in Table 1 for the overall reaction (that is, in the presence of *p*-ABA). As shown in Figure 3 and Table 3, dihydropterinPP_i appeared as a competitive inhibitor of ATP (approx. K_i , 5 μ M) and a mixed-type (non-competitive) inhibitor of dihydropterin (approx. K_i , 10–15 μ M). AMP was a poor inhibitor of the reaction (summarized in Table 3), in agreement with the relatively

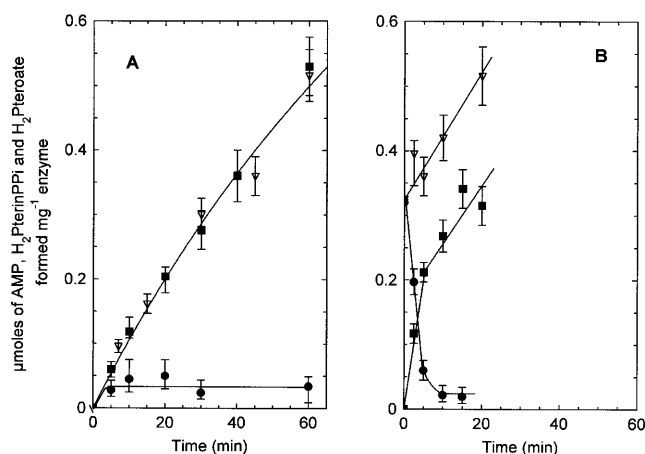


Figure 4 Kinetic study of the coupled HPPK/DHPS reactions

(A) Time-course formation of AMP (∇), dihydropteroate (\blacksquare) and dihydropterinPP_i (\bullet). The reaction medium (120 μ l) contained 1 μ g of recombinant HPPK/DHPS, 100 μ M dihydropterin, 200 μ M ATP and 100 μ M *p*-ABA. (B) Time-course evolution of AMP, dihydropteroate and dihydropterinPP_i in the presence of *p*-ABA, added 30 min after the addition of the other substrates. The reaction medium (120 μ l) contained 1 μ g of recombinant HPPK/DHPS, and the initial concentrations of dihydropterin and ATP were 100 and 200 μ M, respectively. After 30 min of incubation, 100 μ M *p*-ABA were added and the evolution of the various substrates was monitored as described in the Materials and methods section. Each point is the mean from two to four determinations.

low affinity reported for the monofunctional *E. coli* enzyme [27], and appeared as a mixed-type inhibitor against both ATP and dihydropterin. The crystallographic data obtained with the *E. coli* monofunctional enzyme clearly indicate that both substrates have to be complexed with the enzyme for the one-step reaction of pyrophosphoryl transfer [16], an observation that is indicative of a sequential mechanism [28]. Other studies using analogues of ATP [29] suggest an ordered reaction where ATP is bound first. The product-inhibition pattern is also representative of the type of sequential mechanism (ordered or random) that is involved. The pattern presented in Table 3 is compatible with an ordered sequential Bi Bi mechanism [28] where ATP is bound first and dihydropterinPP_i released last (in a random sequential mechanism in rapid equilibrium, dihydropterinPP_i and AMP would have been competitive inhibitors of both substrates). Thus the plant enzyme mechanism is apparently similar to the one described recently for *E. coli*.

Coupling between HPPK and DHPS activities

The coupling of these two activities was achieved by adding *p*-ABA (100 μ M) together with ATP and dihydropterin. As shown in Figure 4(A), when *p*-ABA was added at the beginning of the experiment, the rates of AMP and dihydropteroate formation were identical, indicating a good coupling between the two reactions. The velocity of the overall reaction was roughly similar to the one recorded for the HPPK reaction alone (see Figure 2A). In this situation however, dihydropterinPP_i was barely detectable, suggesting either a very rapid transformation through the DHPS activity or limited diffusion in the bulk medium. This last hypothesis is not supported by the accumulation of dihydropterinPP_i observed in Figure 2(A) when the reaction was carried out in the absence of *p*-ABA. However, a possible conformational change favouring a 'direct' connection between the two catalytic sites (channelling situation) could take place when the two reactions were coupled; that is, when DHPS

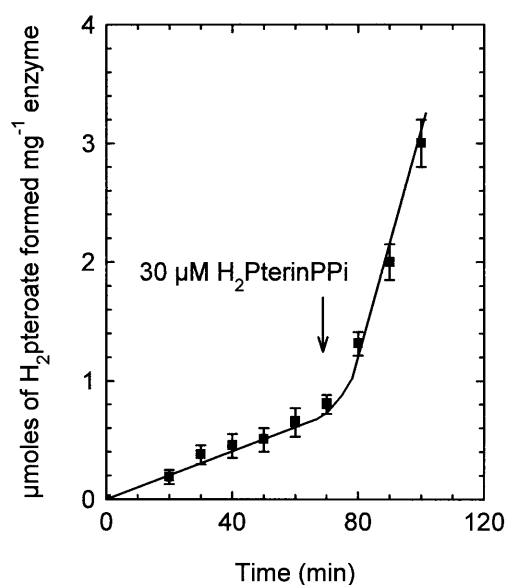


Figure 5 Effect of the addition of dihydropterinPP_i on the coupled activities of HPPK and DHPS

HPPK/DHPS activity is expressed as μ mol of dihydropteroate ($\text{H}_2\text{Pteroate}$) formed/mg of protein. The reaction medium contained 1 μ g of protein, 100 μ M dihydropterin, 100 μ M *p*-ABA and 200 μ M ATP. Each point is the mean from two determinations. $\text{H}_2\text{PterinPP}_i$, dihydropterinPP_i.

was working. In order to test this hypothesis, *p*-ABA was added 30 min after the beginning of the experiment, i.e. when dihydropterinPP_i accumulated in the medium. Indeed, one might expect that a channelling situation would limit the exchange of dihydropterinPP_i between the DHPS catalytic site and the bulk medium. This was apparently not the case since we observed (Figure 4B) a rapid decrease in the dihydropterinPP_i initially present in the external medium, down to the level recorded in Figure 4(A). This drop was concomitant with a symmetrical increase in dihydropteroate. Thus rapid exchanges of dihydropterinPP_i between the DHPS binding site and the external medium were still possible when the two reactions were coupled. The initial rate of dihydropteroate formation shown in Figure 4(B) was about three times higher than the steady-state rate measured in Figure 4(A), suggesting that the DHPS activity could be limited by the availability of dihydropterinPP_i. Indeed, as shown in Figure 5, the rate of dihydropteroate synthesis from dihydropterin increased about 9–10-fold when 30 μ M dihydropterinPP_i was added to the external medium. Thus the HPPK activity was the rate-limiting step of the overall reaction and the production of dihydropteroate kept pace with that of AMP.

Regulation of the HPPK/DHPS by folate derivatives

In a previous paper [22] we studied some of the kinetic parameters associated with the DHPS activity. This enzyme catalysed a random bireactant reaction and was feedback-inhibited by dihydropteroate, a competitive inhibitor of both dihydropterinPP_i and *p*-ABA. Interestingly, the monoglutamate forms of dihydrofolate and tetrahydrofolate were also potent inhibitors of the DHPS reaction (Table 4). Like dihydropteroate, these derivatives were competitive with dihydropterinPP_i and *p*-ABA with similar K_i values for the two substrates. However, the pentaglutamate forms of dihydrofolate and tetrahydrofolate had less effect, and 5-formyl-, 5-methyl- and 5,10-methylene-tetrahydrofolate had no

Table 4 Effect of various folate derivatives on HPPK and DHPS activities

H_2FGlu_1 and H_4FGlu_1 are dihydro- and tetrahydro-folate monoglutamates; H_2FGlu_5 and H_4FGlu_5 are dihydro- and tetrahydro-folate pentaglutamates. These derivatives and dihydropteroyl were competitive inhibitors of *p*-ABA and dihydropterinPP₁, with similar K_i values for the two substrates. The results are means \pm S.D. from three different determinations; NSE, no significant effect for concentrations up to 500 μ M.

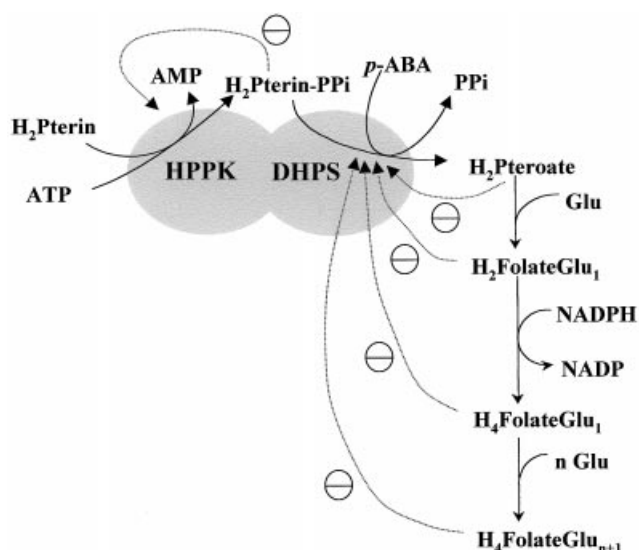
	K_i (μ M)	
	HPPK	DHPS
Dihydropteroyl	NSE	8 \pm 3
H_2FGlu_1	NSE	12 \pm 3
H_4FGlu_1	NSE	12 \pm 3
H_2FGlu_5	NSE	40 \pm 5
H_4FGlu_5	NSE	45 \pm 5
5-Formyl/ H_4FGlu_1	NSE	NSE
5-Methyl/ H_4FGlu_1	NSE	NSE
5,10-Methylene/ H_4FGlu_1	NSE	NSE

detectable effect on the rate of the DHPS reaction. On the other hand, we were not able to detect any significant change in the velocity of the HPPK reaction in the presence of these various compounds, at least for concentrations up to 500 μ M.

DISCUSSION

The plant bifunctional HPPK/DHPS is an important enzyme of the folate biosynthetic pathway. This study represents the first attempt to obtain kinetic information concerning the coupling of the two sequential reactions catalysed by the plant enzyme. The HPPK domain of the protein catalyses the Mg^{2+} -dependent pyrophosphorylation of dihydropterin and our results suggest a sequential Bi Bi ordered system where ATP binds first to the catalytic site and dihydropterinPP₁ is released last. These results are in good agreement with the enzyme mechanism described recently for the monofunctional HPPK from *E. coli* [16,29]. Our data also indicate that the level of dihydropterinPP₁ remains very low when the two reactions are coupled. Several folate-dependent enzymes involved in sequential reactions were shown to increase their efficiency by channelling intermediates between the catalytic sites. This has been observed with formiminotransferase-cyclo-deaminase, an enzyme that presents a channelling specificity that is optimal for the pentaglutamate form of tetrahydrofolate [30]. Another example of channelling of a folate derivative is shown with the bifunctional dihydrofolate reductase/thymidylate synthase. Indeed, the X-ray structure of this enzyme indicates that transfer of dihydrofolate between the two active sites does not occur by a diffusional pathway but rather by electrostatic channelling at the surface of the protein [31]. The term channelling implies that intermediates do not equilibrate with the external medium. In the case of the HPPK/DHPS our results indicate, on the contrary, that exchange of dihydropterinPP₁ between the external medium and the protein always remains possible. Although we cannot totally exclude the possibility of a limited diffusion pathway due to the close vicinity of the two catalytic sites, these observations are not in favour of a channelling hypothesis. In our experimental conditions it is likely that the low level of dihydropterinPP₁ recorded when the two domains are operating results from the high specific activity of the DHPS versus the HPPK.

In conclusion, it appears from our results that the HPPK activity in plants is the limiting factor of the overall reaction and is highly dependent on the DHPS activity. Indeed, dihydropterinPP₁ exerts strong feedback inhibition upon HPPK activity

**Scheme 1** Schematic representation of the tetrahydrofolate biosynthetic pathway

The scheme emphasizes the regulation of bifunctional HPPK/DHPS by the different intermediates of the pathway. H_2 Pterin, dihydropterin; H_2 PterinPP₁, dihydropterinPP₁; H_2 Pteroyl-Glu, dihydropteroyl-Glu; H_2 FolateGlu₁, dihydro- ($n = 2$) or tetra- ($n = 4$) hydrofolate glutamate.

and its rate of formation is thus dependent on its utilization by DHPS. As summarized in Scheme 1, the DHPS domain is in turn regulated tightly. Indeed, it is feedback-inhibited by dihydropteroyl, the product of the reaction [22], and it is also inhibited strongly by the monoglutamate forms of dihydrofolate and tetrahydrofolate, two intermediates synthesized downstream in the folate biosynthetic pathway. From all these data it is tempting to postulate that the DHPS domain of the HPPK/DHPS protein is a key regulatory point of the folate biosynthetic pathway in plants. If this holds true, this mitochondrial enzyme could be a good target for folate enhancement in plants of nutritional interest. Indeed, serious diseases are correlated with folate deficiency and one possible answer to these health problems could be to increase the folate content of plant food (for a review, see [32]). We are currently investigating the effects of varying the level of expression of the bifunctional HPPK/DHPS on the folate status in plants.

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