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ARABIDOPSIS DIHYDROPTEROATE SYNTHASE: GENERAL PROPERTIES AND INHIBITION BY REACTION PRODUCT AND SULFONAMIDES

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Key Word Index—*Arabidopsis*; Cruciferae; folate metabolism; dihydropteroate synthase; *p*-aminobenzoic acid; properties; product inhibition; sulfonamide inhibition.

Abstract—Dihydropteroate synthase (DHPS) (EC 2.5.1.15) was extracted from leaves of *Arabidopsis thaliana* and purified 21-fold by ion-exchange chromatography. This enzyme preparation was then characterized for several of its properties. Michaelis–Menten constants for the substrates, *p*-aminobenzoic acid and dihydropteridine diphosphate were estimated to be 2.5 and 91 μM, respectively. In an optimized assay, the reaction product, dihydropteroic acid, competitively inhibited the enzyme activity with a K_i of 81 μM. However, neither dihydrofolate nor tetrahydrofolate, products further downstream in the biosynthetic pathway inhibited enzymatic activity. This appears to be the first report of product inhibition of DHPS from a higher plant. The relative inhibitory properties of several sulfonamides, analogues of *p*-aminobenzoic acid, were also examined. The substitutions on the amide nitrogen of the sulfonamides influenced the degree of inhibition; thus I_{50} values for the inhibition of the DHPS activity by sulfanilamide, sulfacetamide and sulfadiazine were estimated to be 18.6, 9.6 and 4.2 μM, respectively. The competitive pattern of inhibition was shown in experiments with sulfadiazine.

INTRODUCTION

Tetrahydrofolate co-enzymes are required in a wide variety of cellular processes which include formation of formylmethionyl-*t*RNA, the biosynthesis of purine nucleotides and thymidylate, and metabolism of the amino acids serine, glycine, histidine and methionine [1, 2]. The folate-derived methyl group of methionine is utilized for a spectrum of methylation reactions via *S*-adenosylmethionine. The regular availability of folates is critical for the maintenance of cellular activities in all organisms.

Thus, folate deficiency results in direct and indirect retardation of metabolism, and in humans manifests itself in the form of potentially fatal disorders [3]. Animals lack the enzymes for folate biosynthesis and obtain their folates from ingestion of plant tissues. However, information on the biosynthesis of tetrahydrofolates in plants is fragmented and superficial. We have begun to investigate $H_4PteGlu_n$† biosynthesis and $H_4PteGlu_n$-dependent metabolism in *Arabidopsis* and cultured cells of *Datura* at both the genetic and biochemical levels [4–7].

One objective in our studies was to identify potential control points in the folate biosynthetic pathway of higher plants and describe the possible mechanisms of control at each locus. Dihydropteroate synthase (DHPS) (EC 2.5.1.15) catalyses the condensation of dihydropteridine diphosphate and PABA to yield H_2Pte (Fig. 1). The substrates are derived from two independent cellular sources; the pteridine substrate is derived from GTP [8], while PABA is derived from shikimic acid [9]. This step could be a locus of regulation in the flow of folate precursors. Indeed, the DHPS from *Escherichia coli* was shown to be competitively inhibited by 7,8-dihydropteroic acid (H_2Pte) [10, 11]. Among higher plants, only the DHPS from pea has been studied for its properties [12–16]; however no information on any regulatory properties were reported.

A second objective of our studies was to examine folate-dependent single-carbon metabolism in whole plants through the use of ^{13}C NMR spectroscopy [7]. In such studies we wish to alter endogenous levels of $H_4PteGlu_n$ by using inhibitors of $H_4PteGlu_n$-synthesizing enzymes. For this purpose, the *in vitro* effectiveness of the inhibitors against the target enzymes must be confirmed. The sulfonamide drugs are analogues of PABA (Fig. 2) and are competitive inhibitors

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†5,6,7,8-Tetrahydrofolate-polyglutamate.

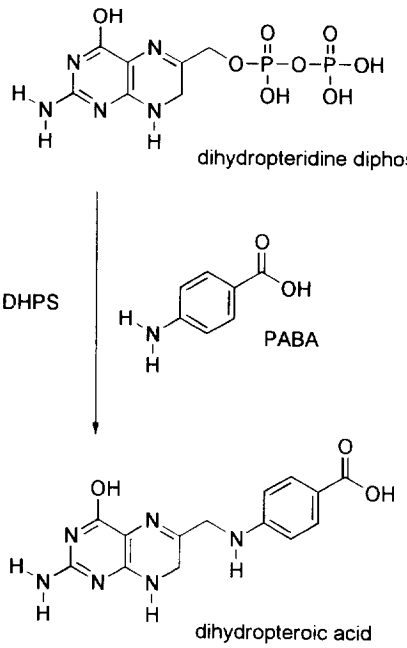

Fig. 1. Biochemical reaction catalysed by DHPS.

of DHPS [17]. The inhibitory properties of the sulfonamides have been intensively examined in microorganisms due to their applications in drug therapy. Considerable variability in the inhibition of the various DHPS enzymes by individual sulfonamides has been found [18, 19]. Among the higher plants, only the DHPS from pea seedlings has been examined in this respect [15]. The relative effectiveness of the individual sulfonamides against particular plant enzymes is unknown.

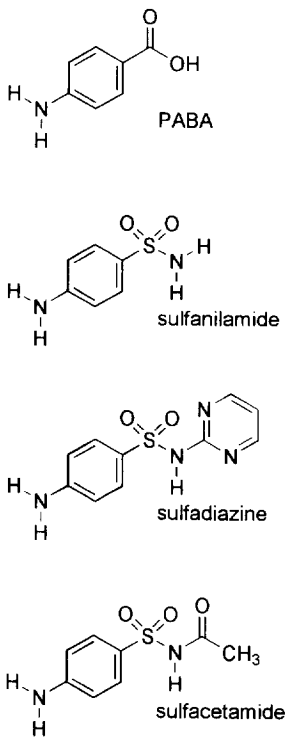

Fig. 2. Structures of three sulfonamide analogs in comparison to that of PABA.

In view of these two objectives, the DHPS from *Arabidopsis* was partially purified and its catalytic properties were determined. The experiments revealed that the product of the biosynthetic reaction, H_2Pte, competitively inhibited the enzyme activity. The substituted groups on the amide nitrogen of the sulfonamides influenced their degree of inhibition of the *Arabidopsis* DHPS.

RESULTS AND DISCUSSION

General properties of the enzyme preparation

A strong single peak of enzyme activity was eluted from the DEAE column between KCl concentrations of 85 and 130 mM. [This allowed its convenient separation from the bulk protein, which was eluted in a subsequent broad peak.] The fractions making up this activity peak were pooled and concentrated using ammonium sulfate. This partially purified preparation was divided into aliquots and stored at −80°C. The desalted enzyme preparation was unstable at 4°, but could be stored with only minimal losses in ammonium sulfate, overnight at −20°, or for 4–6 weeks at −80 .

One unit of enzyme activity is defined as one pkat and specific activity is defined as pkat/mg protein. The pooled fractions from the DEAE column had a specific activity of 1 ± 0.1 pkat/mg protein when assayed with saturating concentrations of both substrates and thus showed a 21-fold purification. Calculation of the purification of this preparation was made based upon the specific activity of the initial 20–45% ammonium sulfate saturation fraction, rather than the crude extract, as the enzyme activity could not be reliably assayed in the crude. All enzyme assays were performed with this single preparation and each experiment was repeated three or more times, using a new aliquot of enzyme desalted by gel filtration each time.

The dissociation constants for dihydropteridine diphosphate and PABA were estimated by varying the concentration of the substrate of interest while maintaining the other at saturation. The range of concentrations were determined from preliminary experiments with a previous preparation of the enzyme. In experiments in which the PABA concentration was varied, the activity of *Arabidopsis* DHPS appeared as a hyperbolic saturation curve in a Michaelis–Menten plot (not shown) and the K_m was estimated to be 2.5 μM from a Lineweaver–Burk plot (not shown). This estimate is similar to the values obtained for enzymes from several other organisms [10, 19, 20], including pea [15]. When the concentration of dihydropteridine diphosphate was varied, the enzyme activity once again followed Michaelis–Menten kinetics, and the K_m for dihydropteridine diphosphate was estimated to be 91 μM from a Lineweaver–Burk plot (not shown).

Thus the *Arabidopsis* enzyme appeared to have a relatively lower affinity for the pteridine when compared to the enzymes from many organisms for which the K_m ranged from 1–10 μM [15, 19, 20].

For further studies of inhibition of the enzyme activity by several compounds, an assay was prepared using the information from the above kinetic experiments to allow non-limiting amounts of both substrates. To confirm this, experiments were conducted in which the amount of enzyme protein was varied. In such experiments, the amount of product increased linearly when the amount of protein was increased between 10 and 50 μg (not shown). This also illustrated that the amount of product formed in the regular enzyme assay did not cause detectable inhibition.

Inhibition of enzyme activity by dihydropteroic acid

To determine if H_2Pte could inhibit the enzymatic reaction, this compound was added at a concentration of 50 μM to a series of reactions in which the concentration of PABA was varied. The double-reciprocal plot (Fig. 3) showed that H_2Pte was indeed a competitive inhibitor of the enzymatic reaction and from this the K_i was estimated to be 81 μM. A similar competitive inhibition pattern was observed when the pteridine substrate was varied (not shown). When H_2PteGlu or H_4PteGlu, products further downstream in the biosynthetic pathway, were tested (100 μM) the enzyme activity did not differ from the control (not shown). This appears to be the first demonstration of product inhibition of DHPS from a higher plant.

Several H_4PteGlu$_n$ biosynthetic enzymes have been shown to be inhibited by one or more products of the pathway including the DHPS from *Escherichia coli* [11]. DHPS may be a general control point in the pathway of H_4PteGlu$_n$ biosynthesis. It could, therefore, be that the cellular accumulation of H_2Pte, from one of several processes, could inhibit the activity of DHPS and regulate the flow of H_4PteGlu$_n$ precursors. However, *in vivo* confirmation of such a mechanism is lacking and, therefore, at this point remains as speculation. It is of interest to us to pursue this aspect using ^{13}C NMR spectroscopy with whole plants in which H_2Pte has accumulated either through feeding of this compound or by inhibition of a downstream enzyme such as dihydrofolate reductase.

Inhibition by sulfonamides

We examined three sulfonamide analogues (Fig. 2) for their inhibitory properties against the *Arabidopsis* DHPS. The three analogues, sulfanilamide, sulfacetamide and sulfadiazine showed considerable variation in their inhibition of the *Arabidopsis* enzyme (Fig. 4) with I_{50} values of 18.6, 9.5 and 4.2 μM, respectively. These analogues were competitive inhibitors of the *Arabidopsis* enzyme as demonstrated with sulfadiazine (Fig. 5). From this double-reciprocal plot the K_i for sulfadiazine was estimated to be 0.5 μM.

Other studies have suggested that the inhibitory property of the sulfonamides are related to the electron density in their -SO_2- region as influenced by substitutions on the amide nitrogen with corresponding changes in the pK_a of the compound [17]. Thus the antibacterial property of sulfonamides against *E. coli* was found to be maximized in ana-

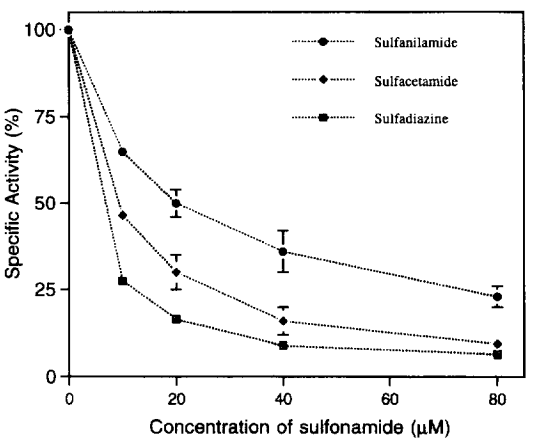

Fig. 4. Inhibitory effect of individual sulfonamides on DHPS activity.

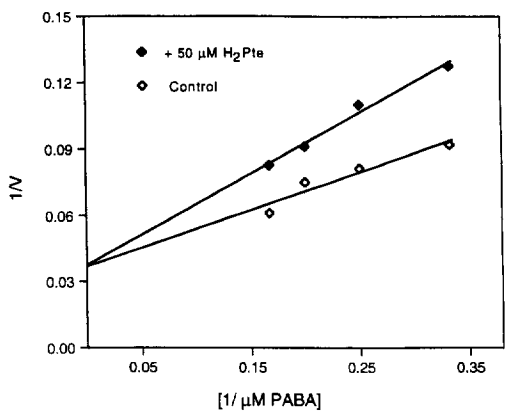

Fig. 3. Double-reciprocal plot showing the inhibition of the DHPS enzyme activity by 50 μM H_2Pte when compared to controls to which it was not added.

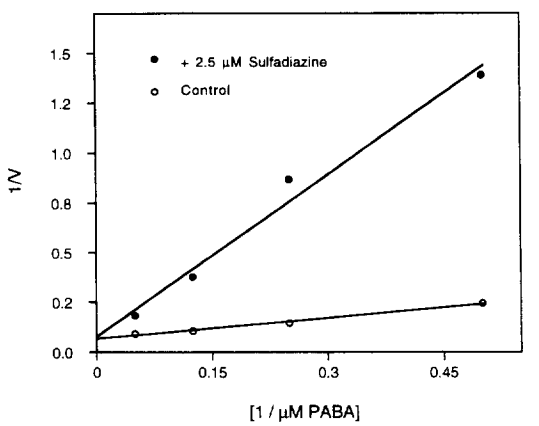

Fig. 5. Double-reciprocal plot showing the competitive pattern of inhibition of DHPS by sulfadiazine.

logues with a pK_a between 6 and 7 in a medium at pH 7.0 [17]. Sulfadiazine with a pK_a of 6.5 was the most effective inhibitor of the *Arabidopsis* enzyme, while sulfanilamide, with a pK_a of 10.4, and sulfacetamide, with a pK_a of 5.4, were less effective.

Separate from the relationship with pK_a, the inhibitory effect of a given sulfonamide against different enzymes is highly variable. For example, I_{50} values for sulfadiazine inhibition of the DHPS varied from 0.42 μM for the *Pneumocystis carinii* enzyme [19] to 500 μM for the enzyme from *Plasmodium falciparum* [18]. However, the sulfadiazine I_{50} value for the *Plasmodium berghei* enzyme was 180 μM [20] suggesting that variation within groups of relatively closely related organisms may also be high. A 34-fold variation was found in the potency of sulfadiazine when comparing its effect on the *Arabidopsis* enzyme (K_i 0.5 μM) to the pea enzyme (K_i 17 μM) [15]. Since point mutations resulting in single amino acid changes have been shown to alter the susceptibility of DHPS to specific sulfonamides [21] it is possible that variations in susceptibility to a single drug reflect inherent variation in the amino acid sequences of the individual DHPS proteins.

As DHPS has proved to be an effective target for drug control of many microorganisms it may also serve as a herbicide target in higher plants. The inherent variability in the antagonism of particular analogues to different enzymes might also allow the design of drugs to be more selective towards certain plants. Our current efforts are aimed at examining the *in vivo* effectiveness of some sulfonamide analogues in ^{13}C NMR spectroscopic studies of folate-dependent metabolism.

EXPERIMENTAL

Chemicals. Pyrophosphoric acid was purchased from Aldrich, DEAE–Sephacel from Pharmacia and [^{14}Carboxyl]-PABA (58 mCi-mmol^{-1}) from Moravek. All other chemicals were from Sigma.

Plant material. Arabidopsis thaliana (L.) Heyn. Columbia wild type was grown in flats containing commercial potting material using an 8 hr photoperiod and light/dark temperature of 25 . The lighting was supplied by a 1 : 1 mixt. of fluorescent Sylvania Gro-Lite and incandescent Sylvania 60 W lamps with a total photosynthetic photon flux density of 900 μmol m^{-2} s^{-1}. The leaves were collected from plants that were 4–5 weeks old and stored if necessary at −80 .

Enzyme preparation. The procedures were carried out at 4 . About 1000 g of leaf tissue were ground using a mortar and pestle in a 100 mM Tris–HCl buffer (pH 8.5) containing 1 mM Na_2EDTA, 10% glycerol, 20 mM β-mercaptoethanol, and 1 mM PMSF. Small amounts of acid washed sand were added when necessary during the grinding. The resulting homogenate was squeezed through two layers of miracloth, and then centrifuged at 10 000 *g* to remove cellular debris. Protein that precipitated between 20 and 45% $(NH4)_2SO_4$ saturation was dialysed for 10 hr against a pH 8.5 buffer containing 50 mM Tris, 20 mM β-mercaptoethanol and 1 mM PMSF. The protein was then applied to a 27 × 400 mm DEAE–Sephacel column in a 100 mM Tris–HCl buffer (pH 8.5) containing 10 mM β-mercaptoethanol and 25 mM $(NH_4)_2SO_4$. The column was washed with this buffer until no protein could be detected in the eluate. The column was then developed using a linear gradient of 50 mM KCl to 350 mM KCl in the washing buffer.

Preparation of other substrates and inhibitors. The phosphorylated form of dihydropteridine was prepared, purified by ion exchange chromatography and reduced as described by Shiota *et al.* [22]. For the preparation of H_2Pte we attempted to reduce Pte by the often cited method for PteGlu [23]. However, the ^1H NMR spectrum revealed that the majority of the Pte was not reduced after the reaction. We therefore reduced Pte by a modification as described below. The purity of the reduced compound was confirmed by ^1H NMR spectroscopy (at 500 MHz using a Bruker AMX instrument). The assignments of proton chemical shifts for Pte and the 7,8-dihydro- compound in 0.1 N NaOH were similar to those for the pteridine moiety of PteGlu and its derivatives [24].

Pte was reduced to 7,8-H_2Pte as follows: 8 mg were dissolved in 5 ml 0.1 N NaOH and then 300 mg $Na_2S_2O_4$ was added and the reaction mixt. stirred at 25 for 30 min. The pH was then reduced to 2.8 by adding 2 N HCl containing 5 mM β-mercaptoethanol and the resulting ppt. was separated by centrifugation. This pellet was then redissolved in 5 ml of 0.1 N NaOH, a further 300 mg of $Na_2S_2O_4$ added and the mixture stirred as above. After precipitating with HCl, again as above, the pellet was washed ×4 in 0.005 N HCl. This pellet was freeze-dried and could be stored for several weeks at −20 C and redissolved in 0.1 N NaOH when required. The purity of the H_2Pte was routinely monitored by ^1H NMR spectroscopy just prior to use in the assay.

Enzyme assay. The method for the enzyme assay was adopted from ref. [25]; protein was estimated from ref. [26]. The standard reaction was conducted in microfuge tubes in a final volume of 100 μl containing 100 mM Tris–HCl (pH 9.0), 1mM $MgCl_2$, 100 mM KCl, 20 μM PABA (116 nCi), 500 μM dihydropteridine diphosphate and 25–50 μg of protein from the above enzyme preparation. For some experiments individual inhibitors at various concentrations were included in this final volume. The reaction was carried out at 37 for 60 min and stopped by adding 100 μl of 95% EtOH. The stopped reaction was kept at −20 for 30 min; the protein was pelleted by centrifugation and 40 μl of the reaction mixt. then spotted on Whatman 1 chromatography paper. The product was separated by ascending PC in 100 mM K-Pi (pH 7.0) as described in ref. [22] and the radioactivity measured by liquid scintillation counting (Packard 2000 CA Tricarb).

All experiments were repeated three or more times

and the s. e. associated with the mean is provided in the results. Where the s. e. is not apparent for individual means it was too small to be displayed on the scale of the graph.

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