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Research Article

Toxoplasma gondii is a common protozoan disease that often causes life-threatening disease, particularly among patients with the acquired immunodeficiency syndrome. This study demonstrates that the dihydropteroate synthase in *T. gondii* is kinetically distinct from the enzyme characterized from other sources and can be highly purified with a high yield using sequential dye-affinity chromatography. Conditions have been identified that allow for stabilization of the purified enzyme, and its physical characteristics have been elucidated. The molecular weight of the native protein was 125,000 and the protein appeared to contain both dihydropteroate synthase and 6-hydroxymethyl-dihydropterin pyrophosphokinase activities. The sulfonamide class of compounds vary in inhibitory potency by more than three orders of magnitude. Sulfathiazole, sulfamethoxazole, and sulfamethazine, with 50% inhibitory concentrations (IC50's) of 1.7, 2.7, and 5.7 microM, respectively, represent the most potent of this class of inhibitors. Several sulfone analogues, including dapsone, were identified as highly potent inhibitors with IC50's less than 1 microM. The results of these cell-free experiments were corroborated by investigating the metabolic inhibition produced by the various inhibitors in intact organisms. The qualitative and quantitative relations among the inhibitors were preserved in both the cell-free and intact cell assay systems. These studies suggest that the sulfones may be important therapeutic agents for the treatment of toxoplasmosis.

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Interaction of Sulfonamide and Sulfone Compounds with *Toxoplasma gondii* Dihydropteroate Synthase

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Abstract

Toxoplasma gondii is a common protozoan disease that often causes life-threatening disease, particularly among patients with the acquired immunodeficiency syndrome. This study demonstrates that the dihydropteroate synthase in *T. gondii* is kinetically distinct from the enzyme characterized from other sources and can be highly purified with a high yield using sequential dye-affinity chromatography. Conditions have been identified that allow for stabilization of the purified enzyme, and its physical characteristics have been elucidated. The molecular weight of the native protein was 125,000 and the protein appeared to contain both dihydropteroate synthase and 6-hydroxymethyl-dihydropterin pyrophosphokinase activities. The sulfonamide class of compounds vary in inhibitory potency by more than three orders of magnitude. Sulfathiazole, sulfamethoxazole, and sulfamethazine, with 50% inhibitory concentrations (IC_{50} 's) of 1.7, 2.7, and 5.7 μ M, respectively, represent the most potent of this class of inhibitors. Several sulfone analogues, including dapsone, were identified as highly potent inhibitors with IC_{50} 's < 1 μ M. The results of these cell-free experiments were corroborated by investigating the metabolic inhibition produced by the various inhibitors in intact organisms. The qualitative and quantitative relations among the inhibitors were preserved in both the cell-free and intact cell assay systems. These studies suggest that the sulfones may be important therapeutic agents for the treatment of toxoplasmosis. (J. Clin. Invest. 1990. 85:371-379.) antimetabolites • enzymology • protozoan • sulfonamides

Introduction

Toxoplasma gondii infects up to 40% of individuals in North America (1). In immunocompetent patients, *T. gondii* exposure can result in a febrile lymphadenopathic syndrome, although most acutely infected adults are asymptomatic (1, 2). Fetal infection with *T. gondii* can occur in women who become infected during pregnancy, and may be accompanied by serious sequelae including neurologic abnormalities (1). *T. gondii* infection is prevalent in immunocompromised patients, particularly those with AIDS. Clinical disease occurs in 5–10% of all patients with AIDS and in ~ 30% of toxoplasma

antibody-positive AIDS patients in the United States (1). If the adult who has *T. gondii* cysts has diminished function of T lymphocytes, especially due to AIDS, fatal disseminated disease or cerebral disease can occur.

Folate compounds are essential cofactors for a number of critical metabolic steps including the formation of purines and thymidine nucleotides. Dihydrofolate reductase (DHFR)¹ is the enzyme responsible for the maintenance of folate pools in their physiologic reduced states. Dihydropteroate synthase (DHPS) is one of the enzymes responsible for the de novo synthesis of the folate molecule. Unlike mammalian cells, which possess a carrier-mediated active transport system responsible for the uptake of performed folates, *T. gondii* lack this system and must synthesize their required folates de novo (3). Several of the enzymes of the de novo folate pathway, including DHPS, are unique to nonmammalian cells, and as such provide targets for drug therapy with a potentially high therapeutic index. Therapy of *T. gondii* disease has traditionally depended on a combination of pyrimethamine, a DHFR inhibitor, and sulfadiazine, an inhibitor of DHPS. In general, DHFR and DHPS inhibitors are most effective when used in combination (4). While combination therapy is effective, the incidence of adverse side-effects in the AIDS population is substantial (30–70%) (5, 6). Due to the severity of side-effects, this therapy must be discontinued in a large percentage of patients. Clearly, alternative therapy is urgently needed.

A limited number of sulfonamide and sulfone compounds have been tested for antitoxoplasma activity in the murine animal model. Sabin and Warren initially demonstrated the utility of these compounds for the treatment of murine toxoplasmosis (7). These studies were subsequently confirmed by several independent laboratories (8–13). The classic works of Eyles and Coleman assessed the relative in vivo potency of various sulfonamide inhibitors against *T. gondii* and found sulfapyrazine, sulfamethazine, sulfamerazine, and sulfadiazine to be the most potent of the sulfonamides tested, and approximately equivalent in their ability to prolong the survival of infected mice (9–12). Notably, sulfisoxazole and sulfanilamide were found to be ~ 20 times less capable of prolonging survival. Subsequent studies from these same laboratories demonstrated the relative activity of several sulfone analogues, including 4,4'-diaminodiphenyl sulfone (dapsone) (13). They found dapsone to be equivalent in activity to sulfadiazine and the most potent of the tested sulfones.

Based on the effectiveness of antifolate therapy and the presumption that many of the side-effects are dose- and inhibi-

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tor specific, we initiated an investigation of alternate DHPS inhibitors. Previous studies from our laboratory have indicated that the interaction of inhibitors with folate-dependent enzymes isolated from *T. gondii*, such as DHFR, may vary by several orders of magnitude when compared with the interactions of comparable enzymes isolated from other nonmammalian sources (4). Thus, we felt it critical to investigate the interaction of DHPS inhibitors with the target enzyme specifically isolated from the *T. gondii* organisms to guide the development of new therapies for this infection. To this end, we have developed techniques to isolate, purify, and characterize the DHPS of *T. gondii*. Further, we investigated the interaction of various DHPS inhibitors with the cell-free target enzyme and with intact *T. gondii* organisms using the metabolic activity of the organisms as a measure of drug activity.

Methods

Materials

6-Hydroxymethyl-7,8-dihydropterin pyrophosphate (H_2PtCH_2OPP) was obtained from the Drug Synthesis and Chemistry Branch, National Cancer Institute, National Institutes of Health (Bethesda, MD). The compound was synthesized according to the method of Shiota et al. (14) and the structure was confirmed by mass spectroscopy and nuclear magnetic resonance spectroscopy. The purity of the product was determined to be 82% by HPLC and was used without further purification. The HPLC method consisted of the development of a C-8 μ bondapak column with a concave gradient (No. 8) of methanol from 0 to 40% with Pic A, pH 5.5, constituting the balance of the mobile phase. The gradient was run over 40 min at a flow rate of 2 ml/min. Under these conditions, the H_2PtCH_2OPP substrate had a retention time of 9 min and was easily separable from the monophosphate (retention time = 4 min). The compound was stored as a powder under nitrogen at $-80^{\circ}C$ and found to be stable for at least one year. The starting compounds for the synthesis of H_2PtCH_2OPP , pyrophosphoric acid and 6-hydroxymethyl-pterin, were supplied by Fluka Biochemicals (Buchs, Switzerland) and Schiricks Laboratories (Jona, Switzerland), respectively. $3',5'[^3H]Paroaminobenzoate$ (pABA) (sp act 50 Ci/mmol) was purchased from Moravek Biochemicals (Brea, CA) and was found to be $> 98\%$ pure by HPLC (see below). ATP, DTT, BSA, fraction V, 2-mercaptoethanol (2-ME), and various sulfonamides including sulfabenzamide, sulfacetamide, sulfadiazine, sulfamerazine, sulfamethazine, sulfapyridine, and sulfathiazole were obtained from Sigma Chemical Co. (St. Louis, MO). 3MM paper was purchased from Whatman Laboratory Products Inc. (Clifton, NJ). Matrix gel blue A and matrix gel green A were obtained from Amicon Corp. (Lexington, MA). A protein assay kit was obtained from Bio-Rad Laboratories (Richmond, CA). Other sulfonamide compounds were obtained from Hoffman-La Roche Inc. (Nutley, NJ), including sulfamethoxazole, sulfisoxazole, and sulfadoxine. Sulfanilamide, sulfapyrazone, and sulfamethizole were obtained from Lilly Research laboratories, Ciba-Geigy (Suffern, NY) and Ayerst Laboratories (New York, NY), respectively. 4,4'-Diaminodiphenyl sulfone (Dapsone) was obtained from Jacobus Pharmaceutical Co. (Princeton, NJ). All other sulfone analogues were obtained from Warner-Lambert/Parke-Davis Inc. (Ann Arbor, MI) or from the Drug Synthesis and Chemistry Branch, National Cancer Institute, National Institutes of Health. Partially purified *Escherichia coli* DHPS was a generous gift from Dr. Robert Ferone (Burroughs-Wellcome Co., Research Triangle Park, NC).

Enzyme source and purification

T. gondii organisms (RH strain) were passaged every 3–4 d in the peritoneal cavities of Balb/c mice. Tachyzoites were isolated from the peritoneal exudates of 30–40 infected mice and separated from inflammatory cells by differential centrifugation as previously described (4).

The sedimented toxoplasma organisms were then resuspended in 1 ml of PBS and either used immediately or frozen at $-80^{\circ}C$.

Extraction

Preparations of *T. gondii* trophozoites were disrupted by a 60-s burst from a Branson sonifier 350 (Branson Sonic Power Co., Danbury, CT) equipped with a microtip, and the supernatant was clarified by centrifugation ($4^{\circ}C$) at 2,000 g for 15 min.

Affinity chromatography

DHPS was purified by the sequential use of two dye affinity resins, blue and green Sepharose. The enzyme-containing cytosolic supernatant was first applied at room temperature to a 0.7×4 -cm glass column (Bio-Rad Laboratories) containing blue Sepharose that had been pre-equilibrated with 100 mM potassium chloride (KCl). After loading, the column was washed with 3 ml of 100 mM KCl followed by 2 ml of 200 mM KCl. DHPS activity was then eluted from the column with a linear gradient of KCl from 0.2 to 1 M. 1.2-ml fractions were collected in glass tubes (12×75 mm) containing 10 mM Tris/HCl, pH 8.3, 5 mM DTT, and 5 mM 2-ME in 120 μ l. These fractions were collected on ice and then assayed for DHPS activity. The fractions containing DHPS activity were pooled and concentrated by ultrafiltration using an ultrafiltration cell (Amicon Corp.) equipped with a 30,000-mol wt cut-off membrane (PM-30; Amicon Corp.). The pooled fractions were concentrated 10-fold at $4^{\circ}C$ then diluted with 40 mM Tris/HCl, pH 8.3, to yield a final salt concentration < 200 mM. The concentrated preparation was then loaded onto a second affinity column containing green Sepharose prepared in an identical fashion as the blue dye column. Elution of the DHPS activity was carried out as described above. The active fractions were again pooled and concentrated 10-fold by ultrafiltration. The purified enzyme was stored at $-80^{\circ}C$.

Molecular weight determination

Column chromatography. Purified DHPS preparations were applied to a protein sizing column (TSK 2000 SW; Beckman Instruments, Inc., Palo Alto, CA) having a usable molecular weight range of 12,000–300,000. Protein standards including glutamate dehydrogenase (290,000), lactate dehydrogenase (142,000), bovine albumin (67,000), and adenylate kinase (32,000) were used to calibrate the column (United States Biochemical Corp., Cleveland, OH). Column elution was accomplished using an HPLC (LKB-Bromma, Uppsala, Sweden) composed of a model 2150 pump, 2152 system controller, and model 2211 fraction collector. The column was developed using a flow rate of 0.3 ml/min of PBS (pH 7.0) under isocratic conditions. 0.3-ml fractions were collected at $4^{\circ}C$ in glass tubes containing 10 mM Tris/HCl, pH 8.3, 5 mM DTT, and 5 mM 2-ME. The molecular weight was determined by comparison of the retention time with standards.

Gel electrophoresis. NaDODSO₄ gel electrophoresis was carried out on 10% gels according to the methods of Weber et al. (15) using myosin (200,000), phosphorylase b (92,500), BSA (68,000), ovalbumin (43,000), and α -chymotrypsinogen (25,700) as molecular weight standards. Protein bands were detected using a silver staining kit (Bio-Rad Laboratories).

Isoelectric focusing

Ampholine pagplates, pH 3.5–9.5 (LKB-Bromma), were used with a Multiphor apparatus (LKB-Bromma) to determine the isoelectric focusing point (Pi) of the purified DHPS. The focusing procedure was carried out at $4^{\circ}C$ and according to the manufacturer's instructions included with the apparatus. The gel was developed over 60 min at a current of 40 mA. Human hemoglobin (Pi 6.8) served as a control for each experiment. Proteins were considered to have reached their isoelectric points when the movement of hemoglobin, which was loaded on separate lanes at either end of the electrophoretic plate, stabilized at parallel positions. After development, the gel was cut into 0.5-cm strips. One-half of each strip was placed into 0.5 ml of deionized water for pH determination after equilibration at room temperature for 1 h. The remaining portion of the strips were equilibrated over a 3-h period at $4^{\circ}C$ in a buffered solution containing 50 mM Tris/HCl, pH 8.3, and

5 mM DTT. DHPS activity was then measured using the eluted enzyme according to the described methods (see below) with the exception that the reaction time was increased to 18 h and performed at 4°C. The DHPS Pi was determined by comparison of the enzyme activity with a plot of gel pH versus distance from the anode.

DHPS catalytic assay

DHPS was assayed using a modification of an assay previously published (16). Assay tubes contained 5 mM MgCl₂, 5 mM DTT, 10 μM H₂PiCH₂OPP, 1 μM [³H]pABA (final sp act, 2 Ci/mmol), and 40 mM Tris/HCl, pH 8.3, in a total reaction volume of 100 μl. Various concentrations of inhibitors were added when applicable. Reactions were initiated by the addition of 0.5 mU of enzyme (1 U = 1 nmol of product formed/min). After a 30-min incubation at 37°C the reaction tubes were placed on ice to terminate the reactions, and then spotted onto 3 × 30-cm strips of 3 MM chromatography paper (Whatman Laboratory Products Inc.). The strips were developed in a descending chromatography tank using an elution buffer of 0.1 M KH₂PO₄, pH 7.0. The origins containing the labeled products of the reaction were cut from the strips, placed in scintillation vials, and counted in a liquid scintillation counter (Packard Tri-Carb; Packard Instrument Co. Inc., Downers Grove, IL) 24 h after the addition of 9.5 ml of counting cocktail (3A70b; Research Products International Corp., Mt. Prospect, IL).

6-Hydroxymethyl-7,8-dihydropterin pyrophosphokinase assay

Kinase activity was measured as a coupled reaction that included kinase and DHPS activity. The kinase assay was performed in an identical fashion as that for DHPS except: 50 mM ATP was added to the reaction tubes; the substrate was changed to 50 μM 6-hydroxymethyl-7,8-dihydropterin; and the final pABA concentration was increased to 5 μM. These reactions were allowed to proceed for 1 h at 37°C to allow adequate conversion of the substrate to the dihydropteroate product.

Protein analysis

Proteins were quantitated according to the methods of Bradford using BSA as the protein standard (17).

pABA uptake studies

The potency of the various inhibitors was assessed in intact organisms according to published methods (18). These methods quantitated the ability of drugs to inhibit the metabolism of [³H]pABA to intracellular folates. Briefly, *T. gondii* trophozoites (10⁵ organisms) were placed into tissue culture flasks containing a monolayer of Wi-38 human lung fibroblast cells and 10 ml of pABA and folate-free RPMI media supplemented with 10% fetal bovine serum. Plated organisms were treated with various concentrations of the drug under study for 2 h, followed by an 18-h exposure to 0.6 μM [³H]pABA (sp act 50 Ci/mmol). At the end of the exposure the cells and organisms were harvested with the aid of a rubber policeman and washed three times with ice-cold PBS. Labeled intraorganism folates were then extracted by a 1-min immersion into boiling water after the addition of 2 ml of boiling 2% ascorbate, pH 6.0, and 2% 2-ME. Cellular debris and denatured protein were then removed by centrifugation for 5 min at 2,000 g, followed by hydrolase treatment with 1 ml of partially purified hog kidney hydrolase (12–15 mg protein/ml) for 30 min at 37°C. Porcine proteins were denatured in boiling water, followed by clarification at 2,000 g for 5 min. The treated folates were concentrated using a C-18 Sep-Pak (Waters Associates, Milford, MA) and separated by HPLC according to previously described methods (19). The separated folates were quantitated by an in-line liquid scintillation counter (Flow-one Beta, model CR; Radiomatic Instruments & Chemical Co., Inc., Tampa, FL).

Data analysis

All kinetic parameters were computed using a nonlinear least squares kinetics program designed by Dr. Rodbard and Dr. Lutz (20) and

converted for use on a Macintosh computer. IC₅₀ values for both the intact cell and cell-free experiments were computed using the ALLFIT program on the National Institutes of Health DEC 10 computer system (21), except for the values determined for the sulfone analogues presented in Table V. These IC₅₀'s were determined by weighted least squares fitting of the pure competitive inhibition equation. Beginning with the usual expression for this type of inhibition,

$$v = \frac{V}{1 + \frac{K}{S} \left(1 + \frac{I}{K_i} \right)},$$

where *v* is the enzyme reaction rate, *V* is the maximal rate (this *V* also includes second substrate terms as long as this substrate has constant concentration), *K* is the Michaelis constant of the inhibited substrate (*s*), *K_i* is the inhibitor constant, and *I* is the inhibitor concentration. An expression, *A*, for fractional remaining activity in the presence of an inhibitor may be derived from the definition *A* = *v*(*I*)/*v*(0). Expressed as an inverse,

$$A^{-1} = 1 + \frac{K}{K_i(K + S)} I = 1 + \beta I. \quad (1)$$

For a given sulfone, *β* is a constant since *K_i* is inhibitor specific, and *S* (i.e., pABA) is not varied during the course of inhibition measurements. *β*⁻¹ is equivalent to the IC₅₀. Because Eq. 1 is linear, a simple regression to this yielded an IC₅₀ for each of the inhibitors investigated.

In performing these regressions, we used a local variance weighting factor proportional to A², a relationship holding under the assumption that the enzyme activities measured both in the presence and absence of inhibitor are characterized by a constant coefficient of variation.

Results

DHPS preparation

Purification and stability. Affinity columns of blue and green Sepharose bound the DHPS enzyme with adequate affinity to allow a high degree of purification with high yield when the affinity columns were used sequentially. Table I illustrates a representative purification and Fig. 1 is typical of the elution profile of the enzyme from the green Sepharose column. Elution from the blue and green columns occurred with KCl concentrations of 0.6 and 0.8 M, respectively. Generally, purification resulted in the recovery of 5–10-fold more enzyme activi-

Table I. Affinity Purification of *T. gondii* DHPS

	Protein	Specific activity	Fold purification		Yield
			mg/ml	pmol/min per mg	
Crude	24	1.2	1	100	
Blue Sepharose					
affinity column	0.34	297.5	250	1,396	
Green Sepharose					
affinity column	<0.001	>42,500	>35,750	974	

Purification scheme for the isolation of DHPS enzyme from a cytosolic preparation of *T. gondii*. The cytosolic fraction from the *T. gondii* organisms was applied to the blue Sepharose column which was developed using a KCl gradient. The active fractions from the blue affinity column were pooled and concentrated before loading onto the green affinity column, which was also developed using a KCl gradient. The active fractions collected from the green column with the highest specific activity had protein concentrations < 1 μg/ml.

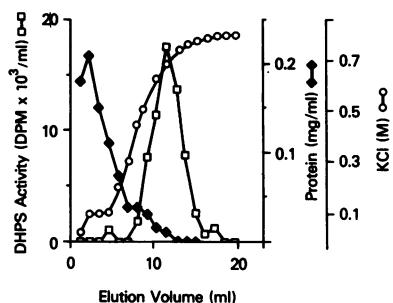


Figure 1. Purification of DHPS by green Sepharose chromatography. The DHPS protein was purified using a green Sepharose dye affinity column. After elution from the prior affinity step, the active fractions were pooled, concentrated by ultrafiltration, and diluted to a final

salt concentration of < 200 mM. This preparation was applied to the green Sepharose column preequilibrated with 100 mM KCl buffer. After washing with 5 column volumes of the loading buffer, the column was developed at room temperature using a linear KCl gradient. Protein elution (●), DHPS activity (□), and KCl gradient (○).

ity than was present in the crude preparations, suggesting the elimination of an inhibitor or enzymatic activity capable of degrading the substrate. In addition, 6-hydroxymethyl-7,8-dihydropterin pyrophosphokinase activity was also assayed and found to copurify with DHPS such that its fold-purification after the final step (green Sepharose) was identical to that of DHPS. This activity was also found in identical fractions as DHPS using an HPLC gel filtration column to measure the molecular weight of the green Sepharose purified enzyme (see below).

While the crude *T. gondii* cytosolic preparations were relatively stable with repeated freeze-thawing, and when stored at -80°C, the purified DHPS activity was extremely unstable, having a half-life of only 1-2 d. Because of the limited availability of *T. gondii* DHPS, we conducted preliminary stability experiments with DHPS purified from *E. coli* using the exact methods outlined above. As shown in Table II, we found little effect on DHPS stability with the addition of sucrose or protease inhibitors, or with storage at either 4 or -80°C despite the presence of 2-ME and Tris/HCl buffer. However, while the addition of either BSA or DTT alone had little effect, the combination of the two agents resulted in stabilization of enzyme activity. Table II represents the effect of combining DTT, BSA, and 2-ME in a buffered solution on the stability of *T. gondii* DHPS. The combination of these agents resulted in a marked stabilization of the enzyme for at least 14 d. We found that concentrations of BSA of 0.01 or 1% resulted in identical stabilization. Other potential stabilizers led to no additional improvement when tested in samples stored at -80°C as illustrated in Table II.

Characterization. The kinetic constants of both substrates for the crude and purified DHPS enzyme are shown in Table III. Under the assay conditions used we found the DHPS reaction to be linear for up to 60 min and up to an enzyme concentration of 20 mU. The Michaelis-Menten constant for pABA was found to be highly dependent on the purity of the enzyme with an apparent affinity 10-fold greater for the purified enzyme (0.36 μM) when compared with the crude enzyme (3.6 μM). The molecular weight of the enzyme in its native state was determined by molecular sizing chromatography using an HPLC system equipped with a protein sizing column capable of separating proteins with molecular weights in the range of 12,000-300,000. The sizing chromatography revealed the enzymatic activity to elute at a molecular weight of

Table II. Stability of Purified DHPS from *E. coli* and *T. gondii*

Addition*	Activity remaining (% initial)				
	1	2	3	5	9
<i>E. coli</i> DHPS					
None (4°C)	23	18	11		
None (-80°C)	38	5	18		
10% sucrose	23	0	2		
Protease inhibitors [‡]	9	3	6		
BSA [§]	35	14			
DTT (5 mM)	42	18			
BSA/DTT (5 mM)	99	58	66		
<i>T. gondii</i> DHPS					
None (0.5 M KCl)	56	28			
None (1 M KCl)	96	32			
BSA/DTT (5 mM)	100	96	93	77	
BSA/DTT (5 mM) plus					
DMSO (30%)	96	60	70		
Ethylene glycol (12%)	85	44	53		
Triton X-100 (0.02%)	4	0			
EDTA (8 mM)	72	49	49		
Protease inhibitors	85	79	71		
H ₂ PtCH ₂ OPP (10 μM)	79	76	76		

* All preparations contained Tris/HCl (pH 8.3), 100 mM/2-ME 50 mM.

† Soybean trypsin inhibitor (50 μg/ml), chymostatin (50 μg/ml), leupeptin (20 μg/ml), benzamidine (1 mg/ml), aprotinin (50 μg/ml), and phenanthroline (2 mg/ml).

§ Concentrations used were 0.01 and 1%.

125,000 (Fig. 2 A). 6-Hydroxymethyl-7,8-dihydropterin pyrophosphokinase activity was present in the identical fractions as the DHPS activity. Samples of protein from each of the purification steps, including that from the peak activity fractions of the sizing column studies, were applied to 10% SDS polyacrylamide gels. We noted the consistent appearance of a protein band of 120,000 mol wt that was not apparent in the crude preparations but that became more intense with the purification process. In addition to this protein band, green column

Table III. Kinetic Parameters of *T. gondii* DHPS

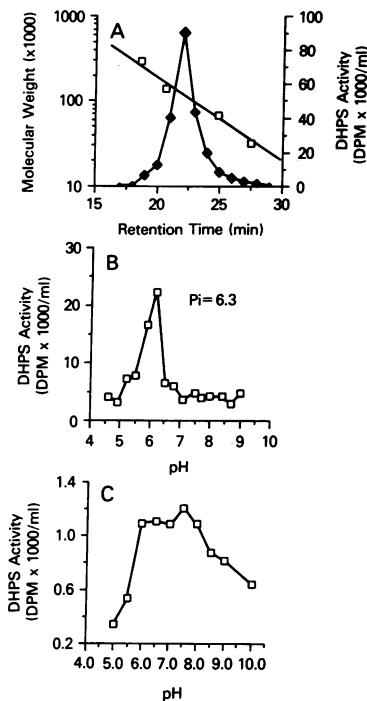
Substrate	K _m	
	Crude	Purified
μM		
pABA	3.6±0.29	0.36±0.13
H ₂ PtCH ₂ OPP	5.5±1	5.2±1.7

The Michaelis-Menten constants for the substrates were determined for both crude and purified enzyme preparations of *T. gondii* DHPS. The K_m value for the pABA substrate was determined over a range of 0.1-2 μM for the purified enzyme and 0.5-10 μM for the crude preparation. The pterin substrate was determined over a range of 1-40 μM. The second substrate was maintained at saturating concentrations. The kinetic values represent the mean and SE of three to six experiments.

purified protein also contained a consistent protein band at 74,000 and, in three of the six tested preparations, a band of 31,000 mol wt. After the molecular sizing chromatography, only the 120,000 mol wt protein remained apparent in the fractions containing DHPS activity. The isoelectric focusing point of the enzyme was found to be 6.3 (Fig. 2 B). As shown in Fig. 2 C, the enzyme retained activity over a broad pH range of 6.0–8.0.

Interaction of DHPS with paraaminobenzoate analogues

Various sulfonamides, as shown in Table IV, were tested for their ability to inhibit the DHPS enzyme. The sulfonamides varied by over a three log range in IC_{50} 's with sulfathiazole, sulfamethoxazole, and sulfamethazine being the most potent of the sulfonamides tested. These studies were performed with both crude and purified DHPS. The purified enzyme proved to be more susceptible to inhibition when compared with the crude form by variable degrees depending on the potency of the sulfonamide. This difference was most apparent for the least potent agents, wherein the difference exceeded 40-fold, and least apparent for the more potent inhibitors (< 4-fold). K_i 's were generated for selected inhibitors (Table IV). In all cases tested, the inhibition was found to follow a pure competitive inhibition pattern with respect to pABA. Of interest was the finding that the sulfone, dapsone (4,4'diamino-diphenyl sulfone), was more potent than any of the sulfonamide analogues tested with an IC_{50} value of 1.2 and 0.25 μM for inhibition of the crude and purified enzyme, respectively. Fig. 3 illustrates the competitive nature of inhibition by dapsone and sulfamethoxazole with respect to the pABA substrate.



represents the average of three separate determinations (range, 6.1–6.4). The effect of pH on synthetic function (C) was determined by performing the assay at each of the denoted pH intervals using 100 mM Tris/HCl buffer. Assay tubes were tested for pH accuracy after the inclusion of all reactants, including enzyme.

Table IV. Inhibition of *T. gondii* DHPS by pABA Analogues

Drug	IC_{50}		
	Crude	Pure	K_i
	μM		
Sulfathiazole	5.9±1*	1.7±0.5	1.9±1
Sulfamethoxazole	14±3	2.7±0.7	21±6
Sulfamethazine	7.7±3	5.7±2	
Sulfadiazine	19±5	11±2	19±5
Sulfabenzamide	16±12	15±5	
Sulfamethizole	94±40	7.9±3	
Sulfisoxazole	165±50	17±6	21±2
Sulfapyridine	280±132	4.6±2	
Sulfamerazine	324±40	7.2±2	
Sulfacetamide	495±151	42±16	
Sulfanilamide	1,365±652	24±7	68±26
Sulfadoxine	1,940±1,090	110±8	
Sulfapyrazone	>10,000	1,000±1,300	
Dapsone	1.2±0.03	0.25±0.08	0.81±0.4

* Mean±SEM represent two to six separate experiments.

Interaction of DHPS with sulfone analogues

Because of the strength of dapsone as an inhibitor of DHPS, we investigated the ability of various sulfone analogues to inhibit the DHPS enzyme relative to dapsone. Using the DHPS assay described above with purified enzyme, enzyme activities were determined in the presence of 1- and 10- μM concentrations of various sulfone inhibitors. For the 25 sulfone analogues summarized in Table V, all compounds except AA had at least one of the residual activities lying between 2 and 80%, and nearly half had both activities within this range. The IC_{50} 's obtained by the data analysis method described previously closely reproduce the experimental activities for all inhibitors examined. Experimental activities were generally within 6% of theoretical. The IC_{50} for dapsone was obtained from a separate

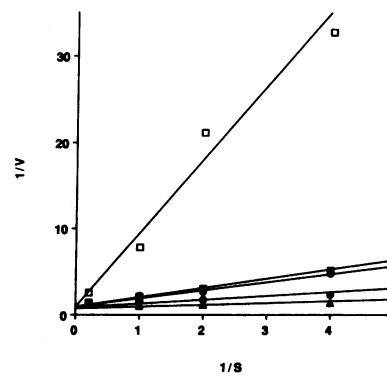


Figure 3. Representative double-reciprocal plots of DHPS inhibition by dapsone and sulfamethoxazole. A representative double-reciprocal plot of the interactions of dapsone and sulfamethoxazole is illustrated. For these experiments, the concentration of the pterin substrate was held constant (10 μM) and variable amounts of the pABA (0.1–10 μM) were tested against the concentrations of each inhibitor as noted. ▲, No inhibitor; ○, 50 μM sulfamethoxazole; ●, 10 μM sulfamethoxazole; ■, 1 μM dapsone; □, 10 μM dapsone. All reactions were initiated with 0.5 mU of the purified DHPS enzyme and allowed to proceed for 30 min before processing as outlined in Methods. All points represent the mean of duplicate determinations from a representative experiment. S, Concentration of pABA $\times 10^6$; V, moles of product formed per minute $\times 10^{10}$.

Table V. Inhibition of *T. gondii* by Dapsone Analogs

Analogue	2'	3'	4'	5'	6'	Potency relative to dapsone
						$\left(\log \frac{IC_{50} \text{ dapsone}}{IC_{50} \text{ sulfone}} \right)^*$
A	CH ₃	H	NH ₂	H	H	-0.910
B	H	H	CH ₃ CH ₂ CH ₂	H	H	-1.419
D	CH ₃	H	H	H	H	-1.452
F	H	H	AcNH	H	H	-1.319
H	H	Cl	H	H	H	-1.385
I	H	H	Cl	H	H	-1.265
J	H	H	CH ₃	H	H	-1.747
L	SO ₂ NHAc	H	H	H	H	-1.343
M	OH	H	H	OH	H	-0.948
O	H	SO ₂ NH ₂	NH ₂	H	H	-0.982
P	H	H	NHCH ₂ CH ₂ OH	H	H	-0.531
S	H	Cl	NH ₂	H	H	-0.157
T	H	NH ₂	NH ₂	H	H	-1.866
U	H	H	Br	H	H	-1.898
W	H	NH ₂	CH ₃ CH ₂ OCH ₂ CH ₂ NH	H	H	-2.017
X	H	H	CH ₃ O(CH ₂) ₃ NH	H	H	-0.841
AA	H	CH ₃	H	H	H	-2.314
EE	NH ₂	H	SO ₂ NH ₂	H	H	-1.553
GG	H	H	NH(CH ₂) ₃ NH ₂	H	H	-1.146
HH	OH	H	NH ₂	H	H	-2.027
II	NH ₂ SO ₂	H	NH ₂ SO ₂	H	H	-1.710
JJ	H	CH ₃	NH ₂	H	H	-1.199
KK	H	NH ₂	H	H	H	-1.613
MM	H	SO ₃ H	NH ₂	H	H	-1.411
NN	H	SO ₂ NHAc	NH ₂	H	H	-0.949
Dapsone	H	H	NH ₂	H	H	0.000

* $-\log IC_{50} \text{ dapsone} = 0.6021$ (IC₅₀ in micromolar units).

set of experiments using five inhibitor concentrations and was found to be 0.25 μM .

Potencies of the various sulfone inhibitors relative to dapsone were defined as

$$\log \left(\frac{IC_{50} \text{ dapsone}}{IC_{50} \text{ sulfone}} \right)$$

and are listed in Table V. By this definition, the stronger sulfone inhibitors are characterized by more positive potencies. Thus, the strongest inhibitors found among the 25 tested were the 3'-Cl derivative (compound S) and the 4'-aminoethanol derivative (compound P). Compound S has a potency similar to dapsone, while compound P is slightly less inhibiting. No compounds tested exhibited greater mean inhibitory potential than dapsone when tested against purified enzyme.

To ascertain whether high potency sulfones exist bearing combinations of substituents other than those investigated experimentally, we also performed a quantitative structure-activity analysis of the data using the Fujita-Ban model (22-24). This technique yields the contribution of each substituent group to sulfone potency and, once such group contributions are in hand, the positive ones may then be arbitrarily summed

to identify new compounds that are likely to exhibit high overall potency.

This analysis showed that all best-estimate group contributions to potency were negative quantities, i.e., these substituents, either alone or in arbitrary combination with others, are unlikely to yield analogues that are substantially better inhibitors of purified DHPS than is dapsone itself. (OH substituents are excluded from this finding since the pH used in the DHPS assay alters the charge state of these groups from that encountered intracellularly [25, 26].)

Metabolic studies using [³H]pABA

In addition to direct enzymatic inhibition in cell-free preparations, we felt it critical to assess the potency of the inhibitors in intact organisms. A variety of factors may alter the effectiveness of a given compound in intact cells including compound stability under physiologic conditions, membrane transport, and intracellular metabolism. We used the ability of the intact organisms to incorporate radiolabeled pABA into their folate pools as a measure of the antifolate's effect on cellular metabolism according to methods previously described in detail (18).

The results of these studies are illustrated in Fig. 4 for selected DHPS inhibitors. Also shown for comparative purposes are the results using the DHFR inhibitors trimetrexate and pyrimethamine. In general, the inhibitory potency of the DHPS inhibitors in the intact cell assay closely paralleled the findings in the cell-free experiments. The DHFR inhibitor trimetrexate was found to be a potent inhibitor of toxoplasma metabolism with an IC_{50} of 3.5 nM. Pyrimethamine, the inhibitor currently used to treat *T. gondii* infections, was > 200-fold less potent than trimetrexate. Methotrexate, which requires an active transmembrane transport system not found in toxoplasma organisms, had no metabolic effects at concentrations > 10 μ M (data not shown). These results are in concert with those previously reported using the DHFR inhibitors in cell-free enzymatic studies. The inhibitory potency of three sulfonamides and dapsone in the intact cell assay are also illustrated in Fig. 4. Unlike the cell-free experiments, sulfadiazine and sulfamethoxazole appeared to be two- to threefold better inhibitors than dapsone. Finally, since there was a great dependence of substrate and inhibitor affinity on the purity of the enzyme, we compared the inhibitory results of selected sulfonamide and sulfone inhibitors when their potency was assayed using each of the three assay systems described in this report (Table VI). When the inhibitory potency of these inhibitors was compared, we noted that the cell-free results using the crude enzyme provided a more accurate quantitative indication of inhibitory potency when either cell-free result was compared with the intact cell system. From a qualitative perspective, either set of cell-free data accurately predicted the relative inhibitory potency of the inhibitors in intact cells. The sulfone analogues, S and P, demonstrated similar potency in the intact and cell-free assay systems. In particular, analogue S was fivefold more potent than dapsone and twofold more potent than even the most potent sulfonamides in the intact cell assay system.

Discussion

We report here a novel, high-yield, and simple purification scheme capable of purifications of *T. gondii* DHPS > 30,000-fold. The initial characterization of the *T. gondii* enzyme revealed it to be a 125,000 mol wt protein (native state), which is intermediate between that reported for *E. coli* (50,000) and plasmodia (190,000–250,000) (27–29). Given the copurification

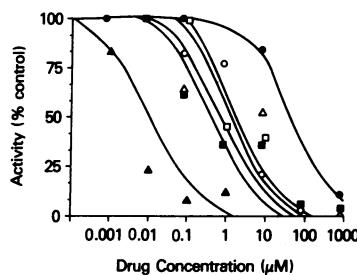


Figure 4. Inhibition of intact *T. gondii* organisms by antifolates. Various antifolates were tested as metabolic inhibitors of intact *T. gondii* organisms. ▲, Trimetrexate; ■, sulfamethoxazole; □, pyrimethamine; ○, dapsone; ●, sulfisoxazole; △, sulfadiazine. In each case, the organisms were

exposed to various concentrations of the inhibitors for 2 h before the addition of radiolabeled pABA. After an additional 18 h the organisms were harvested and their intracellular folates quantitated. These values served as a measure of the inhibitory potency of the inhibitors when total intracellular folates were compared with those in control organisms (100%). Each point represents the mean of three to six independent experiments.

Table VI. Comparison of Selected Inhibitors in the Three Study Systems

Inhibitor	IC_{50}		
	Crude DHPS	Pure DHPS	Intact cells
μ M			
Sulfonamides			
Sulfathiazole	5.9±1*	1.7±0.5	0.88±0.4
Sulfamethoxazole	14±3	2.7±1	0.88±0.1
Sulfadiazine	19±5	11±2	1.5±0.3
Sulfisoxazole	164±50	18±6	57±9
Sulfanilamide	1,365±652	24±7	>1,000
Sulfones			
Dapsone	1.2±0.03	0.25±0.08	2.4±0.2
S	1.1±0.5	0.35±0.08	0.47±0.2
P	1.2±0.5	0.81±0.2	2.1±0.9

* Mean±SEM represent four to eight separate experiments using five inhibitor concentrations for each of the three study systems.

tion of 6-hydroxymethyl-7,8-dihydropterin pyrophosphokinase activity, which also appeared in identical fractions throughout the purification scheme as the DHPS activity, it is conceivable either that both activities reside on the same protein or that the protein is comprised of separate subunits with distinct functions. Richey and Brown were able to resolve the DHPS and 6-hydroxymethyl-7,8-dihydropterin pyrophosphokinase activities in *E. coli* into proteins with M_r 's of 50,000 (DHPS) and 15,000 (dihydropterin pyrophosphokinase) using a Sephadex G-100 column (27). While DHPS activity was also separable from 6-hydroxymethyl-7,8-dihydropterin pyrophosphokinase activity by DEAE-Sephadex chromatography in *Plasmodium berghei*, these activities were not separable in *Plasmodium chabaudi*, nor were they separable in pea seedlings (16, 30, 31). DHPS protein purified through the green Sepharose affinity column did not appear to be homogeneous, as at least one additional protein (74,000 mol wt) was consistently noted to copurify. DHPS further purified by sizing chromatography contained both synthase and pyrophosphokinase activity and appeared as a single protein band on a silver-stained denaturing polyacrylamide gel.

As reported by others, we also noted increased enzymatic yield (up to 10-fold) during purification. This apparent increase in activity may be explained by the presence of enzymatic activity in the crude preparations capable of degrading the substrate. Upon examination of the crude preparations, we identified enzymatic activity capable of degrading a diphosphate (ADP) that was not present after the first purification step (data not shown). Whether this activity is capable of degrading the pyrophosphate substrate remains to be elucidated. Purification of DHPS from potential inhibitors present in the crude preparations represents an additional possibility that has not been excluded. Instability of the DHPS enzymatic activity isolated from other organisms has not previously been noted and may be due to differences in the purity or source of the preparations used in other studies (16, 27–30). The DHPS activity in crude cytosolic preparations of *T. gondii* was stable and became unstable only with purification > 100–200-fold. The purified enzyme appears to be highly susceptible to oxidation and adherence to inert surfaces given the stability of

ferred by an inclusion of DTT and BSA. The DHPS enzyme was found to have an acidic Pi of 6.3 and was capable of maintaining catalytic activity over a broad pH range. Previous reports have noted that the optimum pH for plastic enzyme is ~ 8.5 (29, 30), and this is consistent with that noted for *T. gondii*.

In investigating the Michaelis-Menten constants for the DHPS enzyme with respect to both substrates, we found the affinity constant for pABA to be similar to those reported for enzymes isolated from both *P. berghei* (0.28 μ M) and *E. coli* (0.21 μ M) by Ferone and co-workers (16, 28), but somewhat tighter than that reported by others (1.1–2.8 μ M) (29, 30). These differences may relate to the dependence of affinity on the purity of the enzyme preparation. We found that the K_m for pABA differed by a factor of 10 between crude and pure enzyme, while the K_m for the pterine substrate showed little dependence on the purity of the enzyme. The affinity of *T. gondii* DHPS for the pterin pyrophosphate substrate is slightly weaker (~ 5 μ M) than that reported for either *P. berghei* or *E. coli* (0.5–1.4 μ M) (16, 28).

These kinetic studies allow only speculation as to why the substrate and inhibitor affinities are dependent on enzyme purity. It is plausible that the conformation of the enzyme changes with purification to allow greater access to the active site and thereby enhance the apparent affinity of the substrates for the enzyme. This postulate is additionally supported by the finding that the potency of various inhibitors depended on the enzyme purity. This difference was most apparent for the weaker inhibitors but is, in general, apparent even for the most potent compounds including the sulfones. This postulate would account for the crude enzyme being a better predictor of inhibitory activity in intact cells, as the enzyme in the crude preparations may more closely reflect the native conformation in intact cells.

The inhibition of DHPS isolated from bacterial and plasmoidal sources by sulfonamides and sulfones and their utility as therapeutic agents has previously been reported (16, 28, 29). Investigations of the interactions of sulfonamides and sulfones with the DHPS isolated from *T. gondii* demonstrated that the sulfonamides exhibited a broad range of inhibitory potency, with sulfadiazine, the DHPS inhibitor of choice for the treatment of toxoplasma encephalitis, being one of the most potent of the sulfonamides tested. Of some note was the finding that sulfadoxine, commonly used in prophylaxis regimens for *Pneumocystis carinii*, was a particularly poor toxoplasma DHPS inhibitor. These results are in conformity with those reported from in vivo experiments using the acute murine toxoplasma model. In the murine model, sulfadiazine, sulfamethazine, sulfamerazine, and sulfathiazole were noted to be of high potency, while sulfisoxazole, sulfapyridine, and sulfanilamide were found to be therapeutically poor compounds (9–12). As others have previously reported using DHPS from *E. coli* and Plasmodia, we found both classes of inhibitors to be purely competitive with respect to the pABA substrate (16). A finding that was in contrast with previously reported bacterial studies but consistent with results from the murine model, was the potency of the sulfone, dapsone, relative to the sulfonamides. Generally, the sulfones have been found to be relatively poor inhibitors of bacterial DHPS. These studies suggest that the *T. gondii* enzyme is substantially different from that of bacteria in its interaction with these agents and thus probably distinct in its overall protein structure.

Several investigators have noted that the potency of the

sulfonamides may be directly related to their ability to ionize at physiologic pH (32–34). Since these drugs penetrate cellular membranes with difficulty, the best inhibitors of intact organisms were those that were half-ionized at physiologic pH, i.e., their pK_a was between 6 and 7.4. The nature of the sulfone structure does not allow these compounds to be fit into a similar relationship; however, the activity of these compounds as inhibitors of bacterial growth has been related to the molecular charge distribution of the inhibitor with respect to the natural substrate (pABA). De Benedetti and co-workers investigated such structure-activity relationships for the sulfones and identified several analogues with activity exceeding that of dapsone (25). Given the potency of dapsone as an inhibitor of *T. gondii* DHPS, we studied additional sulfone analogues for inhibitory potency. Direct determination of the activity of the sulfones in Table V against purified DHPS identified compounds S and P as those most similar to dapsone. Quantitative structure-activity analyses then identified the contributions to inhibitory potency from individual substituent groups in the analogues investigated. In the mean, none of these groups were found to contribute positively to potency, and thus no strong candidates for new analogues could be suggested.

Examination of the error factors associated with the various group contributions did reveal the possibility that the 3'-Cl, 4'-NH(CH₂)₂OH, 2'-NH₂, and SO₂NH₂/SO₂NHAc groups could be associated with increased potency. Indeed, detailed examination of compound S in the cell-free system led to a statistically insignificant difference in activity from dapsone, leaving open the possibility of its 3'-Cl group being inhibition enhancing. In contrast, the 4'-NH(CH₂)₂OH group, characteristic of compound P and quite similar to the 4' group of compound W, failed to significantly enhance the activity of dapsone. The 2'-NH₂ group, when it is the only additional substituent on dapsone, has not been investigated in our assay. However, De Benedetti et al. (25), in presenting the activity of sulfones against the DHPS from *E. coli*, reported the 2'-NH₂ derivative of dapsone to be twice as active as the parent compound. It is consistent with our structure-activity analysis that this level of increased activity may also exist against *T. gondii* DHPS, and further testing, perhaps involving the 2',4',6'-(NH₂)₃ compound as well, is warranted.

To further enhance our understanding of the interactions of pABA analogues with *T. gondii* DHPS, we investigated their effects on intact organisms using the metabolism of radiolabeled pABA as a measure of inhibitor potency. Unlike the cell-free experiments, this system accounts for events in addition to enzyme inhibition, including inhibitor stability, membrane transport, and intracellular metabolism. We found an excellent quantitative and qualitative relation between the results obtained in the cell-free system and those from the pABA experiments, suggesting that enzyme inhibitor interactions are the critical rate-limiting step for these compounds. Interestingly, the cell-free results using crude enzyme correlated most closely with those obtained from the intact cell experiments when compared with those with purified DHPS, suggesting once again that purification may alter the native state of the enzyme. While compound S had the same potency as dapsone in the cell-free systems, it was fivefold more potent in the intact cell experiments, suggesting an advantage perhaps due to improved membrane transport. The results obtained using DHFR inhibitors, particularly trimetrexate, illustrate the extraordinary potency of these compounds compared with DHPS inhibitors in this assay system. These results document

the presence of the DHPS enzyme in *T. gondii* organisms and show that it is kinetically distinct from the DHPS of other sources. While we have been able to purify and characterize the enzyme, it appears that kinetic experiments with crude enzyme preparations are quantitatively more representative of the potency that may be expected from pABA analogues in intact organisms. Of importance is the demonstration that sulfonamides vary in potency by over three orders of magnitude, supporting the utility of direct enzyme studies in guiding the selection of sulfonamides for clinical testing. Further, the finding that dapsone and several analogues are highly potent inhibitors of *T. gondii* DHPS supports the consideration of therapeutic trials using dapsone for the treatment of *T. gondii* infections and serves as a focal point for the identification and design of new pABA analogues that may potentially have improved physical, pharmacokinetic, and therapeutic properties.

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