Identification, partial purification and inhibition by guanine analogues of a novel enzymic activity which phosphorylates guanosine to GMP in the protozoan parasite *Eimeria tenella*

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From oocysts of the protozoan parasite *Eimeria tenella*, responsible for avian coccidiosis, we have partially purified and characterized a novel enzymic activity which specifically phosphorylates guanosine to GMP. The enzyme is able to use several phosphate donors, in the order: acetyl phosphate (Ac-P) > ATP > UTP > CTP > phosphoribosyl pyrophosphate (PRPP) > dUTP ≥ dATP. The low specificity of this enzyme for the phosphate donor suggested that it be named guanosine phosphotransferase (GPTase). This enzyme is biochemically distinct from the previously described adenosine kinase (AK) and hypoxanthine/xanthine/guanine phosphorib-

osyltransferase (HXGPRTase), and may enable the parasite to synthesize guanine nucleotides under conditions of imbalance between adenine and guanine nucleotides. Because of its possible role in the purine salvage pathways, we have studied the effect of several guanine and guanosine analogues, recently synthesized in our laboratory, on the activity of GPTase *in vitro*. GPTase is specifically inhibited in the micromolar range by several substituted *N*²-phenylguanine bases. These results indicate that, as previously found for AK and HXGPRTase, GPTase could be a potential target for antiparasitic chemotherapy.

INTRODUCTION

Diseases caused by protozoa are often difficult to prevent or cure. At present, control relies mainly on chemotherapy, because effective vaccines are still not available for any of the major diseases. It has been observed that protozoa sometimes possess unique enzymic activities not found in the host cell. Moreover, the mere evolutionary distance between host and parasite usually provides ample differences in substrate specificity for those enzymes shared by both the protozoa and the host cell. These facts suggested the possibility to synthesize inhibitors specific for protozoan enzymes, which, when present, are not recognized by the cellular counterparts [1].

It has been shown that the functioning of various purine salvage pathways in the protozoa is essential for their survival and growth, because they are deficient in the synthesis of purine nucleotides *de novo* [2–6]. Thus it appears that protozoa are able to utilize cellular nucleosides, but not, or to an insufficient extent, the cellular nucleotides. This is most probably due to the inability of the charged nucleotides readily to traverse the protozoan membrane. The lack of synthesis of purine nucleotides *de novo* has prompted investigators to exploit protozoan enzymes of the salvage pathway as potential targets for antiparasitic chemotherapy. The block of the salvage pathway of purine, in fact, could be lethal for protozoan parasites, but not for the host cells, even if the inhibitors were not able to discriminate efficiently between protozoan and host enzymes, because host cells could bypass the block through synthesis *de novo*.

Among protozoan parasites, the members of the coccidia family cause a diseased condition of the digestive tract in domestic

birds, known as coccidiosis. One of them, *Eimeria tenella*, develops inside the caecal epithelial cells of infected chickens. This parasite has been found to be unable to synthesize purine nucleotides *de novo* and to depend for their synthesis on the salvage pathway [7].

Thus AMP is provided by adenosine kinase (AK), which can phosphorylate exogenous adenosine to AMP [8], whereas IMP and GMP are provided by a purine phosphoribosyltransferase. This enzyme, in contrast with the chicken liver enzyme, uses not only hypoxanthine and guanine but also xanthine as substrates, hence its name hypoxanthine/xanthine/guanine phosphoribosyltransferase (HXGPRTase) [9]. Protozoan AK and HXGPRTase were found to phosphorylate nucleoside or base analogues as well, which accumulate in the nucleotide pool and, after conversion into their triphosphate forms, are finally incorporated into RNA, where they exert their toxic effect. Thus these enzymes, because of their low substrate specificity, are possible targets for anticoccidial chemotherapy. AK has been shown to phosphorylate the anticoccidial drugs ethylthio- and cinnamylthio-pyrazolo[3,4-*d*]pyrimidines [8], which have anticoccidial activity against *E. tenella*. Also, the hypoxanthine analogue allopurinol, a competitive inhibitor of protozoan HXGPRTase [9], has some antiproliferative activity against *E. tenella* in cell cultures [10]. However, allopurinol should more drastically decrease the proliferation of *E. tenella*, because the absence of AMP deaminase [9] should not allow the parasite to convert AMP into GMP. These results lead us to hypothesize that this parasite, like *Trichomonas vaginalis* [11], could possess an enzyme activity able to phosphorylate guanosine directly to GMP under conditions of imbalance between AMP and GMP.

Abbreviations used: GPTase, guanosine phosphotransferase; Ac-P, acetyl phosphate; PRPP, phosphoribosyl pyrophosphate; AK, adenosine kinase; HXGPRTase, hypoxanthine/xanthine/guanine phosphoribosyltransferase; DTT, dithiothreitol; PMSF, phenylmethanesulphonyl fluoride; PEI, poly(ethyleneimine).

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METHODS

Cells and chemicals

Sporulated oocysts of *E. tenella* were obtained from the Institute for Animal Health, Houghton Laboratory, Houghton, Huntingdon, U.K. [^{14}C]Guanosine (528 mCi/mmol) was from New England Nuclear, and [^3H]adenosine (26 Ci/mmol) and Hyperfilm-MP were purchased from Amersham. Unlabelled nucleosides, cofactors, dithiothreitol (DTT), allopurinol, allopurinol riboside, formycin A, formycin B, formycin A 5'-phosphate and the protease inhibitor phenylmethanesulphonyl fluoride (PMSF) were from Sigma. DE-81 filters were from Whatman. T.l.c. plastic sheets of poly(ethyleneimine) (PEI)-cellulose F (layer thickness 0.1 mm) were from Merck. Enzyme chromatography was performed by Pharmacia f.p.l.c. using Mono-Q and AMP-Sepharose columns. All other chemicals and reagents (analytical grade) were from BDH.

Synthesis of guanine and guanosine analogues

This was done as previously described [12,13].

Guanosine phosphotransferase (GPTase) assay

The enzyme was assayed at 37 °C for 30 min in 25 μl of a mixture containing 30 mM Hepes/ K^+ (pH 7.5), 1 mM Ac-P, 1 mM DTT, 0.2 mg/ml BSA and 10 μM [^{14}C]guanosine (1100 c.p.m./pmol). The reaction was terminated by spotting 20 μl of the incubation mixture on a 25 mm DE-81 paper disc. The disc was washed twice in an excess of 2 mM ammonium formate, pH 5.6, to remove unconverted nucleoside, and then once in distilled water and finally in ethanol. Radioactive GMP was estimated by scintillation counting in Omnifluor (NEN). One unit of enzyme is defined as the amount of enzyme which converts, under the above assay conditions, 1 nmol of guanosine into GMP in 30 min.

AK assay

The enzyme was assayed at 30 °C for 30 min in 25 μl of a mixture containing 50 mM Tris/HCl (pH 7.5), 1.5 mM MgCl_2 , 1 mM ATP, 0.5 mM DTT, 0.5 mM NaF, 50 mM KCl, 0.1 mg/ml BSA and 70 μM [^3H]adenosine (170 c.p.m./pmol). The reaction was terminated by spotting 20 μl of the incubation mixture on a 25 mm DE-81 paper disc. The disc was washed twice in an excess of 2 mM ammonium formate, pH 5.6, to remove unconverted nucleoside, then once in distilled water, and finally in ethanol. Radioactive AMP was estimated by scintillation counting in Omnifluor (NEN). One unit of enzyme is defined as the amount of enzyme which converts, under the above assay conditions, 1 nmol of adenosine into AMP in 30 min.

Purification of GPTase and AK from oocysts of *E. tenella*

Sporulated oocysts of *E. tenella* (0.25 g) were thawed in 0.7 ml of 10 mM Hepes/ K^+ (pH 7.5)/1 mM KCl/0.5 mM DTT/1.5% DMSO/1 mM PMSF, and then kept on ice for 10 min. The oocysts were then frozen in liquid nitrogen, immediately melted, and homogenized in a Dounce homogenizer (pestle B) to favour the breakage of the oocysts' and sporocysts' walls. This step was repeated until complete breakdown of the walls. The cell extract was then centrifuged at 8000 g for 20 min at 4 °C. The supernatant (crude extract fraction) was immediately ultracentrifuged at 100000 g for 1 h at 4 °C. The supernatant (S-100 fraction) was loaded at a flow rate of 0.5 ml/min on a f.p.l.c. Mono-Q column

(volume 1 ml) previously equilibrated with 10 column vol. of buffer A (20 mM Tris/HCl, pH 7.5, 1 mM DTT, 1 mM PMSF and 10% glycerol). Enzymic activities were eluted with a linear gradient between 0 and 0.6 M KCl in buffer A. AK and GPTase were recovered as single peaks at 0.2 M and 0.3 M KCl respectively. The fractions containing the GPTase activity were pooled and stored in batches in liquid nitrogen until use.

The fractions containing AK activity were pooled, dialysed against buffer B (100 mM phosphate buffer, pH 7.5, 1 mM DTT, 1 mM PMSF and 5% glycerol) and loaded at a flow rate of 0.25 ml/min on an AMP-Sepharose column (0.5 ml) previously equilibrated with 10 vol. of buffer B. After a wash with buffer B (2 column vol.), non-specifically bound proteins were eluted with 2 column vol. of buffer B containing 1 M KCl. The column was then washed with 2 column vol. of buffer C (100 mM Tris/HCl, pH 9.5, 20% glycerol, 1 mM DTT and 1 mM PMSF), and the enzyme was finally eluted with 1 ml of buffer C containing 5 mM adenosine. The enzyme was dialysed against buffer C and stored in batches in liquid N_2 until use.

RESULTS

Detection and partial purification of GPTase from oocysts of *E. tenella*

The presence of an enzymic activity which phosphorylates guanosine to GMP in other parasites [11], and the modest inhibitory effect of allopurinol on *E. tenella* proliferation [10], despite the inhibition by this drug of HXGPRTase, led us to reinvestigate other possible pathways of GMP formation of *E. tenella* oocysts. In an early study [9], no GPTase activity was detected in extracts of *E. tenella* oocysts assayed in the presence of 20 mM phosphate donor (ATP, Ac-P etc.) or 100 mM *p*-nitrophenyl phosphate (p-NPHP). However, we found evidence for the direct phosphorylation of guanosine to GMP in the presence of 10–100 times lower concentrations of phosphate donors, or even in their absence. This activity has now been purified from *E. tenella* as described in the Methods section, both for biochemical characterization and for study as a target of several guanine and guanosine analogues recently synthesized in our laboratories. For comparative studies of these and other inhibitors, we have simultaneously purified *E. tenella* AK, the

Figure 1 Resolution of GPTase (●) and AK (○) of *E. tenella* by f.p.l.c. on Mono-Q

GPTase and AK activities were separated on a Mono-Q column and measured as described in the Methods section. Fraction volume 0.15 ml.

Table 1 Purification of GPTase and AK activities from *E. tenella* oocysts

Enzyme	Step	Protein (mg)	Activity (units)	Sp. activity (units/mg)	Recovery (%)	Purification (fold)
GPTase	Crude extract	2.500	5.020	2.01	100	—
	S-100	1.450	4.833	3.30	96	1.6
	Mono-Q	0.016	4.617	276.85	92	137.0
AK	Crude extract	2.500	1.820	0.72	100	—
	S-100	1.450	1.589	1.10	87	1.5
	Mono-Q	0.050	0.487	9.75	26	13.5
	AMP-Sepharose	0.004	0.340	90.66	18	125.0

other purine-phosphorylating activity previously described by Miller et al. [8].

GPTase and AK activities from sporulated oocysts of *E. tenella* were completely separated on a FPLC Mono-Q column by use of a salt gradient. AK was eluted first at 0.2 M KCl, followed by GPTase at 0.3 M KCl. AK co-eluted with the bulk of proteins, as shown in Figure 1. After this step, GPTase was purified approx. 150-fold to a specific activity of 277 units/mg (Table 1). AK activity was further purified to 90 units/mg on an AMP-Sepharose affinity column [14]. Both preparations were free from contaminating 5'-nucleotidase activity, assayed as described previously [15].

Characterization of *E. tenella* GPTase

As mentioned above, in crude extracts as well as in S-100 fractions the GPTase was active even in the absence of the phosphate donors. Surprisingly, this behaviour was observed also when the fractions were gel-filtered through a Sephadex G-50 column. Only after the Mono-Q column did the GPTase activity become strictly dependent on a phosphate donor. As shown in Table 2, the enzyme is capable of transferring phosphate from a variety of compounds to guanosine, in the order: Ac-P > ATP > UTP > CTP > phosphoribosyl pyrophosphate (PRPP) > dUTP ≥ dATP. With Ac-P or ATP, maximal activity was observed at 1 mM and 2 mM respectively (Figure 2). Neither

Mg²⁺ nor Mn²⁺ is essential for the enzymic activity; both bivalent cations inhibited the GPTase activity in the presence of 1 mM Ac-P as phosphate donor. On the contrary, when 2 mM ATP was used, both Mg²⁺ and Mn²⁺ slightly stimulated the enzymic activity, with a maximum at an ATP/Mg²⁺ or Mn²⁺ ratio of 1:1. No measurable GPTase activity was observed at 10 mM Ac-P or ATP in either the absence or the presence of Mg²⁺ or Mn²⁺ in molar ratios (phosphate donor/bivalent cation) ranging between 10:1 and 1:10. This indicates that the inhibition of GPTase activity by a high concentration of phosphate donor is unaffected by the presence of bivalent cations.

In contrast with its low specificity for the phosphate donor, the enzyme from *E. tenella* is highly specific for guanosine. In fact, no other unlabelled ribonucleoside or deoxyribonucleoside was found to compete for the phosphorylation of [¹⁴C]guanosine to [¹⁴C]GMP. The only product of the reaction catalysed by our enzyme preparation is GMP, as demonstrated by PEI t.l.c. analysis (Figure 3a). Figure 3(b) shows the kinetics of phosphorylation of [¹⁴C]guanosine to [¹⁴C]GMP. The kinetic parameters for guanosine (*K_m* and *V_{max}*) are 9.1 (±0.5) μM and 18.4 (±0.4) pmol/min per unit respectively. The Hill coefficient, calculated from a Hill plot, is 0.9, indicating a single substrate-binding site on the enzyme. The enzyme displayed a bell-shaped pH/activity curve, in which the optimal pH is 7.5. Total inactivation was seen at pH ≥ 9.5, and 50% inactivation occurred at pH 6.7 and pH 8.7. Because the enzyme is specific for guanosine, but does not show an absolute preference for ATP or

Table 2 Effect of different phosphate donors on GPTase activity from *E. tenella*

Abbreviations: PHP, phenyl phosphate; PEP, phosphoenolpyruvate; pNPHP, *p*-nitrophenyl phosphate.

Phosphate donor (1 mM)	GPTase activity (%)
None	< 0.01
ATP	100
CTP	35
UTP	63
dATP	20
dCTP	< 0.1
dUTP	21
AMP	< 0.1
Ac-P	195
PRPP	32
PHP	1
PEP	< 0.1
pNPHP	< 0.1

Figure 2 Effect of different concentrations of Ac-P (●) and ATP (○) on GPTase activity

Apart from the concentration of the phosphate donor, the enzyme activity was assayed as described in the Methods section.

Figure 3 (a) Autoradiogram of the PEI-cellulose t.l.c. of the product of the GPTase activity; (b) kinetics of GMP synthesis

(a) Assay was performed as described in the Methods section. A 1 μ l of portion of the reaction mixture was loaded at the indicated times on a PEI-cellulose F t.l.c. plastic sheet. Unlabelled guanosine and GMP (50 nmol) were added as optical markers in lane 1. Chromatography was performed in 0.1 M LiCl. The sheet was then dried and exposed for 10 h. (b) Quantitative determination of GMP formation was performed by cutting the radioactive spots from the dried PEI-cellulose sheet, counting the radioactivity in a β scintillation counter and subtracting the radioactivity (c.p.m.) of the sample corresponding to 0 min, left for 40 min on ice in the presence of the enzyme.

other nucleoside triphosphate as phosphate donor, we termed the new enzyme purified from *E. tenella* GPTase rather than guanosine kinase.

Search for inhibitors of GPTase activity

The inability to synthesize purine nucleosides *de novo* and the low substrate specificity of the enzymes involved in the salvage pathways make protozoan enzymes good targets for anticoccidial chemotherapy [1]. For this reason we have tested several novel N^2 -substituted guanine and 2'-deoxyguanosine derivatives [12,13] for their possible inhibitory activity on the *E. tenella* GPTase described here. Surprisingly, the results presented in Table 3 show that only the base analogues are inhibitors of the enzyme. Compared with the unsubstituted N^2 -phenylguanine (compound 1), halogen substituents in positions *m* (compounds 4 and 7) and *p* (compounds 8 and 5) have significant inhibitory activity. Disubstituted derivatives are generally weaker than those with *m* or *p* substituents alone. In fact, compounds carrying halogen groups in both *m* and *p* positions (compounds 6, 9 and 13) are almost completely inactive. However, the addition of a methyl group in the *p* position to compound 4, producing the di-substituted compound 14, only slightly decreased the potency of the analogue. We conclude that an electron-attracting substituent such as halogen in the *p* position probably prevents the efficient binding of the *m*-substituted compound to the enzyme. Further-

Table 3 Effect of N^2 -substituted guanine and 2'-deoxyguanosine derivatives on GPTase and AK activities from *E. tenella*

Abbreviations: n.d., not done; dR, 2'-deoxyribose; dRMP, 2'-deoxyribose 5'-phosphate; Pur, purine; β -napt, β -naphthyl; inact., inactive.

Compound	X	Y	Z	R	IC ₅₀ (μ M)	
					GPTase	AK
1 PhG	H	H	OH	H	inact.	n.d.
2 PhdG	H	H	OH	dR	inact.	n.d.
3 PhdGMP	H	H	OH	dRMP	inact.	n.d.
4 <i>m</i> -ClPhG	Cl	H	OH	H	2.9	272
5 <i>p</i> -ClPhG	H	Cl	OH	H	17.4	n.d.
6 di-ClPhG	Cl	Cl	OH	H	inact.	inact.
7 <i>m</i> -BrPhG	Br	H	OH	H	8.7	n.d.
8 <i>p</i> -BrPhG	H	Br	OH	H	8.6	inact.
9 di-BrPhG	Br	Br	OH	H	84.0	n.d.
10 <i>m</i> -CF ₃ PhG	CF ₃	H	OH	H	inact.	inact.
11 <i>m</i> -CF ₃ PhdG	CF ₃	H	OH	dR	inact.	n.d.
12 <i>p</i> -CF ₃ PhG	H	CF ₃	OH	H	58.9	n.d.
13 di-FPhG	F	F	OH	H	inact.	n.d.
14 ClCH ₃ PhG	Cl	CH ₃	OH	H	11.5	inact.
15 β -naptG	-CH=CH-CH=CH-		OH	H	12.7	n.d.
16 Anilino-6ClPur	H	H	Cl	H	n.d.	250
17 Anilino-6NH ₂ Pur	H	H	NH ₂	H	n.d.	inact.

Table 4 Effect of allopurinol and its derivatives on GPTase and AK activities from *E. tenella*

Abbreviations: R, ribose; RMP, ribose 5'-phosphate.

Compound	X	Y	Z	W	IC ₅₀ (μ M)	
					GPTase	AK
Allopurinol	OH	N	CH	H	inact.	inact.
Allopurinol-R	OH	N	CH	R	inact.	inact.
Formycin-A	NH ₂	CH	N	R	62	189
Formycin-AMP	NH ₂	CH	N	RMP	inact.	inact.
Formycin-B	OH	CH	N	R	75	288

more, the fact that compound 1, which has no substituent in either *m* or *p* position, is not active indicates that the activity of the compound is dependent on the presence of a halogen or other related substituent in one of these positions. Table 3 also shows

that the guanine and guanosine analogues, active against GPTase, are inactive against the other protozoan purine kinase, AK, indicating that these compounds could be considered prototypes for the development of specific anti-GPTase drugs.

Effect of known antiprotozoal drugs on *E. tenella* GPTase and AK

In Table 4 we report the effect of allopurinol and some related compounds, with recognized antiprotozoal activity, on *E. tenella* GPTase and AK. Our data indicate that formycin A and formycin B are active against both enzymes. Interestingly, these compounds are structurally related to the only specific inhibitors of AK so far described, i.e. ethylthio- and cinnamylthio-pyrazolo[3,4-*d*]pyrimidines [8,16,17]. On the other hand, allopurinol, a known inhibitor of coccidial HXGPRTase [9,10], has no effect on either enzyme.

DISCUSSION

In this work we have reported identification, partial purification and characterization of a novel enzymic activity, purified from sporulated oocysts of *E. tenella*, which specifically phosphorylates guanosine to GMP. This activity differs in some respects from the guanosine kinase previously purified only 20-fold from *Trichomonas vaginalis* [11]. In fact, guanosine kinase from *T. vaginalis* is more specific for the phosphate donor, being able to utilize ATP and a few other nucleoside triphosphates, but it has a low substrate specificity, since it phosphorylates not only guanosine but also inosine, adenosine, uridine and 2'-deoxyguanosine. On the contrary, the guanosine-phosphorylating activity in oocysts of *E. tenella* described here uses several phosphate donors in the order $Ac-P > ATP > UTP > CTP > PRPP > dUTP \geq dATP$, but it is highly specific for the substrate, being able to phosphorylate only guanosine. For these reasons we propose for this activity the name of guanosine phosphotransferase (GPTase). Our results conflict with the findings of Wang and Simashkevich [9], who did not detect, in *E. tenella* crude extracts, any enzymic activity which could phosphorylate exogenous guanosine to GMP. Therefore, they suggested that the only pathway of GMP synthesis in *E. tenella* was the

utilization of exogenous guanine through the HXGPRTase activity [1,9]. However, in their assay conditions these authors used as phosphate donor either 20 mM ATP, a concentration which completely inhibited the GPTase characterized in this work (see Figure 2), or 100 mM *p*-nitrophenyl phosphate, which is not a phosphate donor for *E. tenella* GPTase at 1 mM (see Table 2) or at 100 mM (results not shown).

The enzymic activity described in the present work provides a new pathway of GMP synthesis in *E. tenella* through the direct phosphorylation of guanosine (Figure 4), and could thus explain the modest effect of allopurinol on *E. tenella* growth in cell culture [10]. In fact, the inhibition of HXGPRTase by allopurinol, which limits the supply of GMP necessary for the proliferation of *E. tenella*, can in part be by-passed by the GPTase activity described here. This enzyme can thus represent a novel target for anticoccidial chemotherapy.

In the attempt to search for inhibitors of GPTase activity, we screened several *N*²-substituted guanines and related derivatives [12,13] that we have found active against the herpes simplex type 1 and type 2 thymidine kinases [12,13,18–22], enzymes known to phosphorylate not only thymidine but also a variety of nucleoside analogues, including guanosine analogues. Among several active *N*²-(halophenyl)guanine bases, we have found one compound, namely *N*²-(*m*-chlorophenyl)guanine, which strongly inhibits protozoan GPTase with an IC_{50} of 2.9 μ M. In comparative experiments we found that none of the anti-GPTase compounds is active against *E. tenella* AK. On the other hand, formycin A and formycin B, chemically related to the specific anti-AK compounds ethylthio- and cinnamylthio-pyrazolo[3,4-*d*]pyrimidines [8,16,17] are active against both AK and GPTase. The availability of specific anti-GPTase and anti-HXGPRTase drugs could represent a way to block completely GMP synthesis and, therefore, proliferation of the protozoan.

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Figure 4 Proposed role for GPTase (dashed arrow) in the adenosine and guanosine salvage pathways in *E. tenella*

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