Characterization of prenylated protein methyltransferase in *Leishmania*

Marie-Pierre HASNE[1] and Françoise LAWRENCE[2]

Centre National de la Recherche Scientifique, Institut de Chimie des Substances Naturelles, Avenue de la Terrasse, 91 198 Gif-sur-Yvette Cedex, France

Prenylated protein methyltransferase, an enzyme involved in the post-translational modification of many signalling proteins, has been characterized in a parasitic flagellated protozoan, *Leishmania donovani*. The activity of this enzyme was monitored by the methylation of an artificial substrate, an S-prenylated cysteine analogue, with *S*-adenosyl-L-[*methyl*-^{3}H]methionine as methyl donor. More than 85% of the methyltransferase activity was associated with membranes. The enzyme methylates *N*-acetyl-*S*-*trans*,*trans*-farnesyl-L-cysteine and *N*-acetyl-*S*-all-*trans*-geranylgeranyl-L-cysteine, but *N*-acetyl-*S*-*trans*,*trans*-geranyl-L-cysteine only very weakly. In contrast with the enzyme from mammals, the leishmanial enzyme had a greater affinity for the farnesylated substrate than for the geranylgeranylated one. Activity *in vitro* was not modulated by cAMP, protein kinase C activator or guanosine 5′-[γ-thio]triphosphate. An analysis of the endogenous substrates showed that the carboxymethylated proteins were also isoprenylated. The main carboxymethylated proteins have molecular masses of 95, 68, 55, 46, 34–23, 18 and less than 14 kDa. Treatment of cells with *N*-acetyl-*S*-*trans*,*trans*-farnesyl-L-cysteine decreased the carboxymethylation level, whereas treatment with guanosine 5′-[γ-thio]triphosphate increased the carboxymethylation of various proteins, particularly those of molecular masses 30–20 kDa.

Key words: Kinetoplastidae, *Leishmania donovani*, promastigote, protozoans, sinefungin.

INTRODUCTION

Some major diseases are caused by intracellular parasites of the reticulo-endothelial cells. Among these are the leishmaniases, generated by different species of *Leishmania*, haemoflagellate protozoans transmitted by haematophagous biting insects, the sandflies. The reservoir hosts are humans and domestic or wild animals. These diseases affect millions of people in tropical, subtropical and some temperate regions of the world. *Leishmania donovani* is the agent of visceral leishmaniasis, which produces considerable morbidity and mortality worldwide. Classical treatments are toxic and resistance to them increases each year. Research for new anti-leishmanial drugs remains a priority. To achieve this goal, the search for a new potential therapeutic target is necessary. Because inhibitors of methyltransferases, such as sinefungin (SF), are highly effective against *Leishmania*, we focused our interest on prenylated protein methyltransferase [(protein-*S*-isoprenylcysteine *O*-methyltransferase, EC 2.1.1.100) (PPMTase)].

PPMTase has been identified in various cells [1–9]. This enzyme catalyses the methylesterification of the α-carboxy group of prenylcysteine in native proteins as well as in synthetic S-farnesylated and geranylgeranylated cysteine peptides and isoprenylated analogues of cysteine [1,7,10]. This post-translational modification is the last step in the processing of a group of proteins that are synthesized with a Cys-Xaa-Xaa-Xaa, Cys-Xaa-Cys or Xaa-Cys-Cys C-terminal sequence [11,12].

It has been reported that carboxymethylation is involved in the activation of platelets, neutrophils and macrophages [1,7,11] as well as that of normal pancreatic rat islets [13,14] and a pancreatic β-cell line [8]. It is also implicated in the regulation of intracellular pH homeostasis [15]. The inhibition of protein carboxymethylation alters chemotactic responses [1,7] and insulin secretion [8,13]. Both heterotrimeric and small G-proteins have been implicated in these events [1,7].

The aim of the present study was to characterize the prenylated protein methyltransferase activity in *Leishmania* and to compare its properties with that of the known mammalian enzyme, because differences between the leishmanial and mammalian enzymes could have therapeutic implications.

EXPERIMENTAL

Materials

N-Acetyl-*S*-*trans*,*trans*-farnesyl-L-cysteine (AFC), *N*-acetyl-*S*-*trans*,*trans*-geranyl-L-cysteine (AGC), *N*-acetyl-*S*-all-*trans*-geranylgeranyl-L-cysteine (AGGC), *S*-*trans*,*trans*-farnesylthiosalicylic acid, cAMP, PMA, guanosine 5′-[γ-thio]triphosphate (GTP[S]), *S*-adenosyl-L-homocysteine, methylthioadenosine (MTA) and mevastatin (compactin) were purchased from Sigma-Aldrich (Saint Quentin Fallavier, France).

Rainbow molecular mass markers and *S*-adenosyl-L-[*methyl*-^{3}H]methionine ([*methyl*-^{3}H]AdoMet) were from Amersham (83 Ci/mmol; 1 mCi/ml) and (*R*,*S*)-[5-^{3}H]mevalonolactone (30 Ci/mmol; 1 mCi/ml) was a product from DuPont-NEN. SF was a gift from Rhône-Poulenc Santé (Vitry, France). Protein assay was from Bio-Rad (Ivry-sur-Seine, France). Culture medium components were from Gibco and serum was from Flobio.

Strains and culture conditions

L. donovani (strain LRC L52) originated from the strain collection of the World Health Organisation's International Reference Center for leishmaniasis (WHO-LRC), and was provided by Dr L. F. Schnur (Kuvin Center for the Study of Infectious and

Abbreviations used: AdoMet, *S*-adenosyl-L-methionine; AGGC, *N*-acetyl-*S*-all-*trans*-geranylgeranyl-L-cysteine; AFC, *N*-acetyl-*S*-*trans*,*trans*-farnesyl-L-cysteine; AGC, *N*-acetyl-*S*-*trans*,*trans*-geranyl-L-cysteine; GTP[S], guanosine 5′-[γ-thio]triphosphate; MTA, methylthioadenosine; PPMTase, prenylated protein methyltransferase; SF, sinefungin.

[1] Present address: University of Glasgow, Joseph Black Building, Institute of Biomedical and Life Science, Division of Infection and Immunity, North Laboratory, Glasgow G12 8QQ, Scotland, U.K.

[2] To whom correspondence should be addressed (e-mail Lawrence@icsn.cnrs-gif.fr).

Tropical Diseases, Jerusalem, Israel). An SF-resistant clone was obtained from the wild-type cells by increasing drug pressure [16].

Promastigotes were grown as described earlier in a semi-defined RPMI 1640 medium containing 2 mM glutamine, 25 mM Hepes, pH 7.4, 10 % (v/v) heat-inactivated fetal calf serum, 5 μg/ml streptomycin and 5 i.u./ml penicillin [17]. Experiments were performed with cells in exponential-phase [(20–35) $\times 10^6$ cells/ml] or stationary-phase [(40–45) $\times 10^6$ cells/ml] growth, as mentioned in the text.

Parasite lysis and fractionation

All procedures were conducted at 4 °C. Promastigotes were harvested by centrifugation at 5000 ***g*** for 5 min, then washed twice with PBS and resuspended in 100 mM Tris/HCl, pH 7.5, containing 1 mM EDTA and 250 mM sucrose. To ascertain whether the enzyme was particulate or soluble, parasites were lysed by three cycles of freezing (−170 °C) and thawing (25 °C) and centrifuged (12000 $\boldsymbol{g}_{av}$ for 10 min) to yield particulate 'P12' and soluble fractions [18]. Parasite breakage was assessed by phase-contrast microscopy.

In further experiments, the lysate was fractionated by differential centrifugations: 2100 ***g*** for 10 min, 15800 ***g*** for 10 min and 100000 ***g*** for 1 h. The pellets were resuspended in Tris/HCl supplemented with 250 mM sucrose to give fractions P1, P2 and P3 respectively and the final supernatant S3. Membrane-bound and soluble acid phosphatases were measured at 37 °C with 5 mM *p*-nitrophenyl phosphate in 50 mM acetate buffer, pH 5, in the presence or absence of 200 mM tartrate respectively [19,20]. Cytochrome *c* oxidase was assayed spectrophotometrically by measuring the decrease in A_{550} of a solution containing 35 μM reduced cytochrome *c* in 30 mM sodium phosphate, pH 7, containing various amounts of the enzyme fraction [21].

Protein determination

Protein concentration was measured by the dye-binding method, with BSA as a standard [22].

Prenylated protein methyltransferase assay

The activity of PPMTase was measured, within 1 h of cell breakage, by using AFC or AGGC as substrate. The enzyme forms a hydrophobic, base-labile methyl ester in the presence of AdoMet. Assays were performed in 100 mM Tris/HCl, pH 7.5, containing 1 mM EDTA, approx. 1 μCi of [*methyl*-^{3}H]AdoMet, 100 μM AFC and 20–40 μg of cellular extract, in a final volume of 50 μl. Samples were incubated at 37 °C for 30 min; the reaction was stopped by the addition of 50 μl of 20 % (w/v) trichloroacetic acid and vortex-mixed for 10 s. The AFC methyl ester was extracted by the addition of 200 μl heptane followed by vigorous vortex-mixing for 15 s and processing as described by Pillinger et al. [5]. Assays were performed in triplicate.

Labelling of the promastigotes

Promastigotes in exponential growth (1 ml of culture, containing 2×10^7 cells) in Nunclon wells were incubated 3 h at 25 °C in the presence of either 75 μCi of [^{3}H]mevalonolactone and 24 μg of compactin, as described by Field et al. [23], or 25 μCi of [*methyl*-^{3}H]AdoMet. Cells were collected by centrifugation and washed with PBS.

Identification of carboxymethylated endogeneous proteins

The methylated or isoprenylated labelled cells were lysed and proteins were separated by SDS/PAGE [24]. Samples were boiled for 3 min and applied to 12.5 % (w/v) polyacrylamide gels. Gels were blotted to a nitrocellulose membrane [25]; the membrane was then either cut into slices or used for immunodetection. Cutting of the blots gave more rapid and reproducible results than direct cutting of the dried gel. The incorporation of radioactivity into protein methyl esters was measured with a vapour diffusion assay [26]. In brief, blots were sliced into 1.5 mm sections; slices were placed in 1.5 ml polypropylene tubes containing 200 μl of 1 M NaOH. The tubes were immediately placed in vials containing 5 ml of scintillation fluid and incubated for 48 h at 20 °C, after which the radioactivity was measured in a Wallac liquid-scintillation counter. Results are expressed as c.p.m./mg of protein applied to each well or c.p.m./10^9 cells.

RESULTS

Localization of PPMTase

The distribution of PPMTase was first examined in the two subcellular fractions obtained after centrifugation at 12000 ***g***. The pellet contained 80 ± 8 % ($n = 3$) of the activity recovered. (All results are given ± S.D.) Similar results were observed with cells in the exponential or stationary phase of growth. By using differential centrifugation, most of the activity was found in the 2100 ***g*** pellet along with the tartrate-resistant acid phosphatase activity used as a plasma membrane marker (Table 1). A more detailed localization using time-consuming steps could not be performed owing to the instability of the activity.

Optimum pH

The maximum activity of PPMTase occurred at pH 6.5–8. The sharp decrease observed at pH 8 was due partly to the methyl ester hydrolysis known to occur at alkaline pH (results not shown).

Substrate specificity of leishmanial PPMTase

To determine the specific activity and prenyl chain specificity of the leishmanial enzyme we incubated the 'P12' fraction with [*methyl*-^{3}H]AdoMet in the presence or absence of various concentrations of prenylcysteine analogues and measured methylesterification. Both AFC and AGGC were efficiently methylated by the leishmanial enzyme, whereas AGC was poorly methylated (Figure 1). The apparent K_m of PPMTase for AFC was estimated as 60 ± 9 μM ($n = 4$) and the apparent V_{max} as 0.230 ± 0.017 pmol/min per mg of protein ($n = 4$). The enzyme could also use AGGC as a substrate with a K_m of 166 ± 27 μM ($n = 4$) and a V_{max} of 0.097 ± 0.007 pmol/min per mg of protein ($n = 4$). The apparent K_m of PPMTase for AGC was estimated as 657 ± 200 μM ($n = 3$) and the apparent V_{max} as 0.011 ± 0.001 pmol/min per mg of protein ($n = 3$). The affinity for AFC was not affected by increasing the concentration of AdoMet by up to 5-fold. *S-trans,trans*-Farnesylthiosalicylic acid was not a substrate for the enzyme but was inhibitory (IC_{50} 50 μM).

Activity as a function of AdoMet concentration

A typical experiment shows that PPMTase exhibited typical Michaelis–Menten kinetics for AdoMet (Figure 2). The apparent K_m value for the enzyme from the wild-type promastigotes was estimated as 0.13 ± 0.01 μM ($n = 3$) and the apparent V_{max} as 0.487 ± 0.020 pmol/min per mg of protein ($n = 3$). Compared

Table 1 Subcellular distribution of PPMT activity in *Leishmania* promastigotes

Cells were homogenized and separated into P1, P2, P3 and cytosol (S3) as described in the Materials and methods section. Acid phosphatase (AP) was used as both a plasma membrane (tartrate-resistant) and a cytosolic (tartrate-sensitive) marker; cytochrome *c* oxidase activity was used as a marker for mitochondria. Results show the activity recovered in each fraction as a percentage of the activity present in the homogenate and are means ± S.D. Assays were performed in duplicate.

		Enzyme activity (%)				
Enzyme	Fraction ...	Homogenate	P1	P2	P3	S3
PPMT		100	80.3 ± 7.1	5.4 ± 2.1	3.4 ± 1.7	2.4 ± 1.2
Tartrate-resistant AP		100	64.8 ± 7.4	11.0 ± 5.5	8.9 ± 3.6	5.1 ± 2.1
Cytochrome *c* oxidase		100	2.7 ± 1.1	92.2 ± 4.3	0.4 ± 0.3	0 ± 0.1
Tartrate-sensitive AP		100	0 ± 0.1	0.1 ± 0.1	9.3 ± 1.9	54.1 ± 5.3

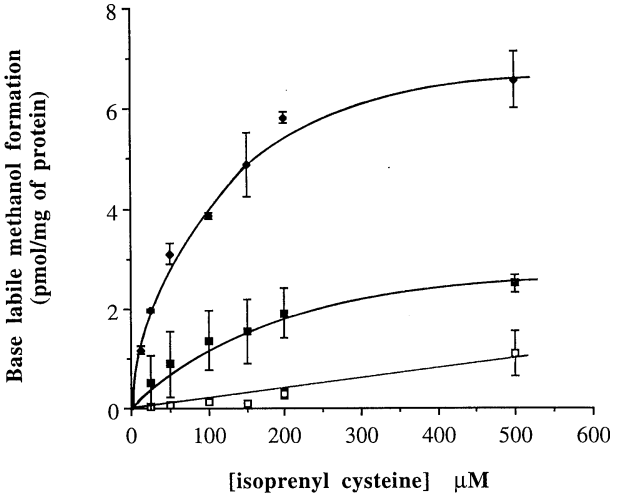

Figure 1 Substrate specificity of leishmanial PPMTase for prenylated substrates

The particulate ('P12') fraction from wild-type promastigotes (20–40 μg of protein) was incubated in 50 mM Tris/HCl containing 0.14 μM [*methyl*-^{3}H]AdoMet and various concentrations of prenylcysteine (◆, AFC; ■, AGGC; □, AGC). The assays were processed as described in the Materials and methods section. Results are means ± S.D. for four independent determinations. Error bars not shown are within symbols.

with the wild type, the enzyme from SF-resistant promastigotes had an apparent affinity for AdoMet of approx. 29% (K_m 0.45 ± 0.09 μM); its maximal velocity was decreased to approx. 5% (V_{max} 0.024 ± 0.007 pmol/min per mg of protein).

Inhibition of PPMTase activity by analogues of AdoMet

Agents that inhibit PPMTase might prove pharmacologically useful. We examined the ability of three general methylation inhibitors, which are AdoMet analogues, to inhibit the carboxymethylation of AFC: SF, a natural antibiotic containing the structural elements of 5′-deoxyadenosine and ornithine linked by a carbon–carbon bond [27]; *S*-adenosylhomocysteine, the reaction product of the demethylation of AdoMet [28]; and MTA, a ubiquitous nucleoside biosynthesized from AdoMet [29]. Figure 3 shows that prenylcysteine carboxymethyltransferase was inhibited by SF, *S*-adenosylhomocysteine and MTA with IC_{50} values of 20 nM, 25 μM and approx. 900 μM respectively.

SF inhibited leishmanial PPMTase competitively with respect to AdoMet (Figure 2). The enzyme from the wild-type cells had a greater affinity for SF than that from the SF-resistant cells (K_i values 9 and 60 nM respectively). This antibiotic behaves as a non-competitive inhibitor with regard to AFC.

Regulation *in vitro*

Carboxymethylation is implicated in cell activation [1,7–9,11,13,30,31] and GTP[S], the non-hydrolysable analogue of guanosine triphosphate, stimulates the methylation of some small G-proteins [1,3,13]. Therefore the possibility that this enzyme was regulated by GTP or by a signalling pathway such as Ca^{2+}, cAMP or a protein-kinase-C-mediated process was examined. With AFC as substrate, the activity of leishmanial PPMTase was not increased in the presence of 100 μM GTP[S], suggesting that GTP[S] does not act directly on the enzyme. Similarly, we did not observe any significant increase in PPMTase activity *in vitro* in the presence of 100 μM cAMP (an activator of protein kinase A) or 100 nM PMA (a potent activator of protein kinase C). However, a slight stimulation (1.4-fold) was observed in the presence of 250 μM Ca^{2+} (results not shown).

Endogenous substrates

Known endogenous substrates for PPMTase are prenylated proteins [11,12]. Prenylation of proteins by farnesyl and geranylgeranyl isoprenoids occurs in a variety of eukaryotic cells, including protozoans [16,23,32–34]. We thus examined the spectrum of polypeptides that were both carboxymethylated and isoprenylated.

Taking advantage of the permeability of *L. donovani* to the biological methyl donor AdoMet [17], we incubated the promastigotes with [*methyl*-^{3}H]AdoMet and analysed the extracts by SDS/PAGE. The analysis of the profile of methyl esters by a vapour diffusion assay allowed the detection of several (seven to ten) peaks, with molecular masses of 140, 95, 68, 53, 46, 34, 23, 18 and 14 kDa (Figure 4).

Culturing promastigotes in the presence of [^{3}H]mevalonolactone (which is hydrolysed in cells to give mevalonic acid, the precursor of prenyl groups) and an inhibitor of mevalonic acid biosynthesis leads to the radiolabelling of a specific set of proteins when analysed by SDS/PAGE (Figure 4). Their molecular masses have been assessed as 95, 68, 48, 34–23 and 14 kDa and low-molecular-mass species migrating below 14 kDa. Thus there is a relatively good correlation between both profiles.

Regulation *in vivo*

In contrast with the results obtained *in vitro*, GTP[S] stimulates the carboxymethylation of endogenous substrates in cultured

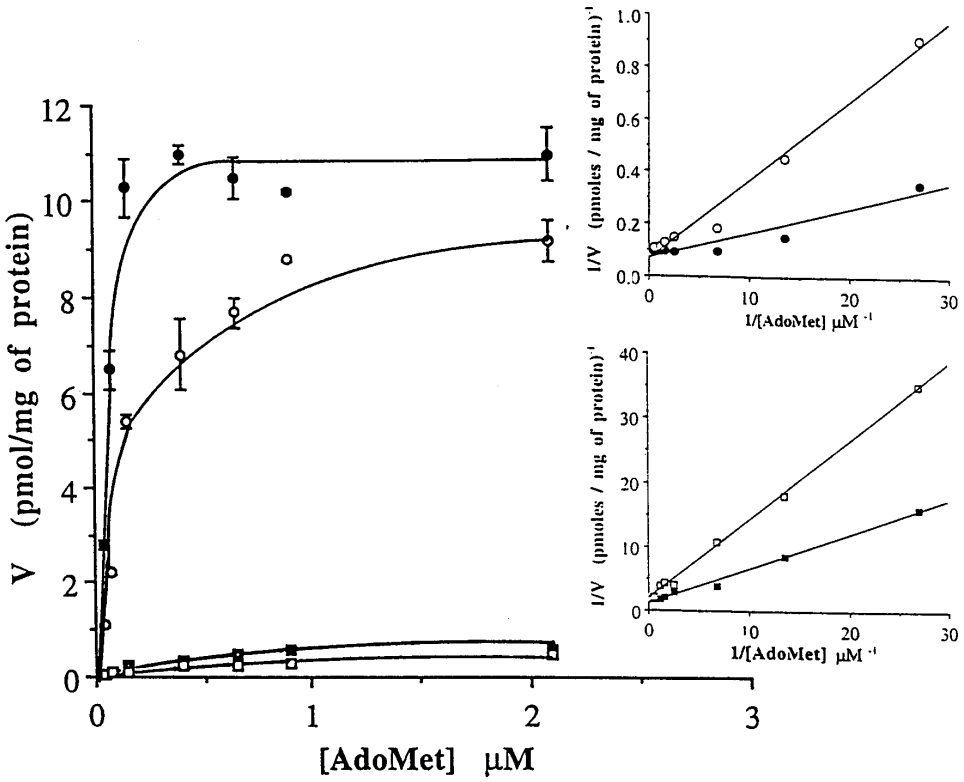

Figure 2 Kinetics of PPMTase in the particulate fraction from *L. donovani* promastigotes

Particulate ('P12') fractions from wild-type (○,●) or SF-resistant (□,■) promastigotes (15 or 20 μg of protein respectively) were incubated in 50 mM Tris/HCl containing 100 μM AFC (because the concentration of AFC was non-saturating, results yield only an apparent K_m) and various concentrations of labelled AdoMet, with (○,□) or without (●,■) 26 nM SF. The assays were processed as described in the Materials and methods section. Results are means ± S.D. for three determinations. Error bars not shown are within symbols. The inset shows the same results expressed as double-reciprocal plot; top, wild-type; bottom, SF-resistant.

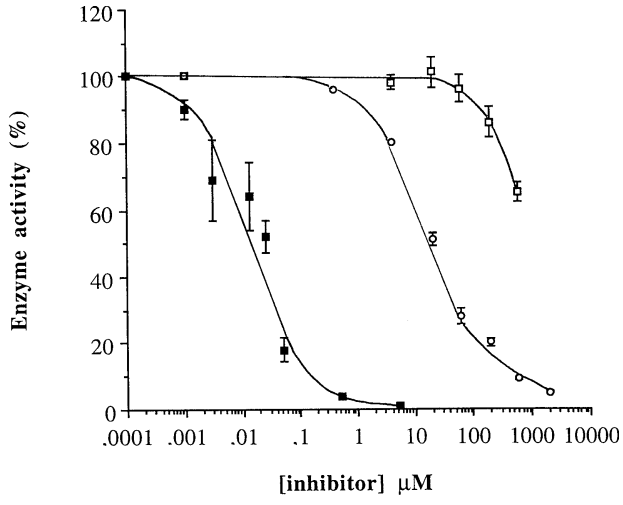

Figure 3 Inhibition of PPMTase activity by AdoMet analogues

The assays were performed in the presence of 100 μM AFC, 0.14 μM AdoMet, 25 μg of the particulate fraction ('P12') from wild-type promastigotes and various concentrations of inhibitors. Results are means ± SD ($n = 3$). Symbols: ■, SF; ○, *S*-adenosylhomocysteine; □, MTA.

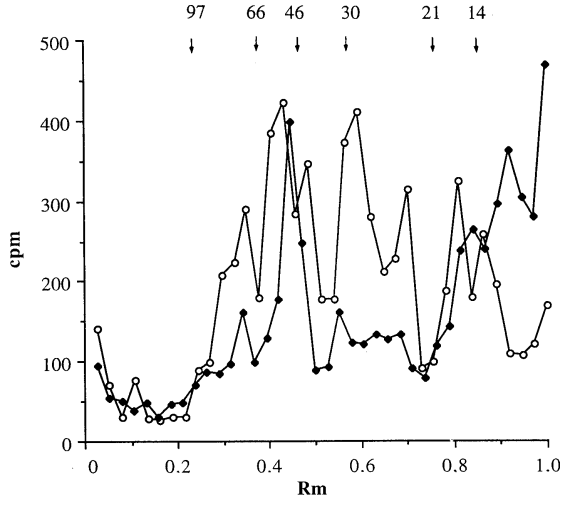

Figure 4 Vapour diffusion assay from SDS/PAGE profiles of carboxymethylated and isoprenylated proteins from intact promastigotes

Cells were incubated for 4 h in the presence of [*methyl*-^{3}H]AdoMet (○) or [^{3}H]mevalonolactone plus compactin (◆), as described in the Materials and methods section. Proteins were separated by SDS/PAGE and processed as described in the Materials and methods section. The positions of molecular mass markers in parallel lanes are indicated (in kDa) at the top. R_m, reference migration, i.e. reference front.

Table 2 Effect of GTP[S], AFC and AGGC on protein carboxylmethylation in intact cells

Promastigotes were labelled for 4 h at 25 °C with 25 μCi of [*methyl*-^{3}H]AdoMet in the presence or the absence of GTP[S] (50 μM), AFC (100 μM) or AGGC (100 μM). Results are expressed as the amount of methanol obtained from the various molecular mass peaks, in c.p.m./mg of protein loaded; numbers in parentheses are percentages of the activities of the controls.

	Activity (c.p.m./mg of protein)			
Molecular mass (kDa)	Control	+ GTP[S]	+ AFC	+ AGGC
140	7426 (100)	9320 (125)	2500 (33)	4606 (62)
85	33911 (100)	35777 (105)	15205 (45)	23937 (71)
70	14026 (100)	24369 (173)	10573 (75)	11496 (82)
46	13614 (100)	20048 (147)	8524 (63)	13779 (101)
36	13696 (100)	16699 (122)	5369 (39)	8779 (64)
26	8085 (100)	17864 (220)	5984 (74)	8308 (102)
19	8251 (100)	24514 (297)	5779 (70)	9803 (119)
< 14	32343 (100)	31650 (98)	13279 (41)	16890 (53)
Total	131352 (100)	180241 (137)	67213 (51)	97598 (74)

promastigotes. The quantity of carboxymethyl esters formed in cells incubated with 50 μM GTP[S] for 4 h was 137 % that of control cells. Analysis by SDS/PAGE indicated that four zones were increased, the highest stimulation being observed in the 26–19 kDa region (Table 2).

Incubation of the promastigotes with [*methyl*-^{3}H]AdoMet in the presence of AFC (100 μM) decreased the labelling of most peaks (Table 2), suggesting that these molecular species are substrates of PPMTase. When AGGC was used instead of AFC, the inhibition was weaker. This might reflect the lower affinity of AGGC than AFC for the PPMTase, as shown above.

DISCUSSION

Our results reveal that most of the PPMTase activity in *Leishmania* promastigotes is associated with the particulate fraction. Similar observations have been described in other cell systems [1–3,7]. The precise subcellular distribution of PPMTase is controversial. The enzyme was found mainly in the plasma membrane in neutrophils [5], mainly associated with microsomes in rat hepatocytes [1] in both plasma membrane and ER-enriched fractions of kidney cortex [3] and in the endoplasmic reticulum in mammals and in *Saccharomyces cerevisiae* [35,36].

As with enzymes from other sources [1,11], AFC and AGGC are substrates of the leishmanial enzyme. In murine tissues and human neutrophils the K_m values of the *N*-acetyl-*S*-prenylcysteine compounds AGC, AFC and AGGC decrease strongly with increasing hydrophobicity, probably owing to increased partitioning in the membrane [1,5]. Except for the enzyme from bovine retinal rod outer segment, which has equal affinities for AFC and AGGC [1], mammalian PPMTases possess a greater affinity for AGGC (3–8-fold) than for AFC [1,5,9]. In contrast, the leishmanial enzyme has a greater affinity for AFC than AGGC and its apparent V_{max} is greater with the former substrate, suggesting that it might be different from that of mammals.

SF, a carba-analogue of AdoMet of fungal origin, inhibits various methyltransferases from different origins *in vitro* [5,17]. In *Leishmania*, which are very sensitive to SF both *in vitro* and *in vivo* [37], this antibiotic is a strong inhibitor of protein methyltransferases *in vitro* [38]; however, in promastigotes in culture it exhibits a relative specificity, inhibiting mainly the protein carboxymethylations [17]. Our present results indicate that PPMTase has a strong affinity for SF: it inhibits leishmanial prenyl-directed carboxymethylation *in vitro* with an apparent K_i more than an order of magnitude lower than the K_m for AdoMet. In SF-resistant cells, the enzyme has a lowered affinity for the drug and a smaller V_{max}. Therefore it seems that PPMTase is a good target for this antibiotic.

Published results have been variable with regard to the capacity of MTA to inhibit PPMTase activity. In intact Chinese hamster ovary cells, MTA inhibits the methylation of nuclear lamin B (36–60 % at 3 mM) [39] as well as the methylation of an unidentified membrane protein in a mouse pre-B-cell line (more than 50 % inhibition at 2 mM) [40]. In canine microsomal preparation, 3 mM MTA completely inhibits the methylation of a Ras protein *in vitro* [41], whereas it is inactive (up to 6.5 mM) towards the rat liver microsomal enzyme in the methylation of farnesylpeptides [21]. MTA is a weak competitive inhibitor (K_i 3.95 mM) of AFC carboxymethylation by the human neutrophil enzyme [5]. We observed a modest inhibition of AFC carboxymethylation by the leishmanial PPMTase in the presence of MTA, the IC_{50} (approx. 900 μM) being at least 36-fold that for *S*-adenosylhomocysteine.

The presence of GTP[S] in assay *in vitro* did not increase the methylation of AFC by leishmanial PPMTase. Similar observations have been reported in other systems [3,5,9], although GTP[S] promotes the methylation of Ras-related proteins in broken cell preparations [1,3,42,43] and in intact cells [1,5]. These findings suggest that the stimulation of carboxymethylation by GTPγ[S] might be due to its effect on conformation: the binding of GTP could regulate the methylation of G-proteins *in vivo* by increasing their affinity for PPMT [5,42].

In mammals, three classes of protein bearing isoprene moieties have been observed: a 69 kDa protein corresponding to the isoprenylated nuclear lamins [32], an unassigned 46 kDa protein and a 20–30 kDa group including Ras superfamily members and low-molecular-mass subunits of heterotrimeric GTPases [32]. Similarly, in *Trypanosoma*, 14 isoprenylated proteins have been detected and classified in four main size groups: 67 kDa, 45 kDa, a complex between 20–30 kDa and low-molecular-mass species migrating at or below 14 kDa [23]. In the 20–30 kDa region at least eight different species were labelled [23]. In *Giardia lamblia* the same high-molecular-mass and low-molecular-mass bands were observed [33]. We found a similar pattern of isoprenylated proteins in promastigotes of *Leishmania*, indicating that isoprenylation is a conserved feature in eukaryotes.

A comparison of methylesterified proteins from different murine tissues show many similar features on SDS/PAGE

profiles: 70 kDa (lamin B) except in brain, 36 kDa, 33 kDa and several peaks in the 20–30 kDa region [43]. In *S. cerevisiae* four types of STE14-dependent methyl-accepting polypeptide of molecular masses 38, 33, 31 and 26 kDa, localized in the membranes, have been detected [44]. (STE14 is the gene encoding the isoprenylcysteinyl C-terminal methyltransferase.) The carboxymethylation profile of leishmanial proteins shows several peaks with molecular mass estimates of 140, 95, 68, 53, 46, 34, 23, 18 and 14 kDa. The profile is very close to that of the isoprenylated proteins.

The treatment of promastigotes with GTP[S] increases the activity of leishmanial membrane-associated PPMTase toward endogenous substrates, the highest stimulation being observed in the 26–19 kDa region. Similar results have been obtained with the human neutrophil enzyme [5]. It is noteworthy that guanine nucleotide-dependent stimulation has previously been observed only for substrates with molecular masses in the range 21–23 kDa [1,3] and some of these have been identified as the small G-proteins Rap1 and G25K [1]. The similarity of the molecular masses of proteins for which carboxymethylation is stimulated by GTP[S] (26–19 kDa) and of small G-proteins (which are reported to be carboxymethylated) suggests that in promastigotes some of the carboxymethylated proteins might be small G-proteins. Indeed, the presence of such proteins has been reported in Kinetoplastidae [45–49]. However, in *Leishmania*, the stimulation of carboxymethylation is observed not only in the region of small G-proteins but also for proteins with apparent molecular masses of approx. 70 and 46 kDa. It is tempting to speculate that these proteins represent lamin B, a 70 kDa carboxymethylated nuclear envelope protein, and its principal methylated degradation product of molecular mass 45 kDa [43]. However, additional results will be required before this assertion can be made with confidence.

In conclusion, our results revealed that the enzyme is bound to the cell membranes; it is not modulated by conventional protein kinase effectors. The enzyme methylates AFC and AGGC. However, in contrast with the enzyme from mammals, the leishmanial enzyme has a greater affinity for the farnesylated substrate than for the geranylgeranylated one; it is also inhibited by MTA. Exploitation of the underlying physical properties offers the possibility of developing specific inhibitors of the parasite enzyme that will be potential lead compounds for novel chemotherapeutic reagents.

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