Protein methylases in *Trypanosoma brucei brucei*: activities and response to DL-α-difluoromethylornithine

NIGEL YARLETT,* AARON QUAMINA and CYRUS J. BACCHI

Introduction

*S-Adenosyl-L-methionine* (S-AdoMet) is the methyl donor for many methylation reactions resulting in the formation of *S*-adenosyl-L-homocysteine (S-AdoHcy: Fig. 1). The ratio of substrate to product (methylation index) plays a central role in the control of methylation reactions (Ueland, 1982). Methylation is a reversible pathway for post-translational protein modification, resembling the process of protein phosphorylation (Kim, 1977; Paik & Kim, 1980). The multi-faceted roles of protein methylation include chemotactic sensitization, hormone storage and secretion, repair of aged proteins, modification of calmodulin sensitivity to calcium, and control of DNA–histone association (van Waarde, 1987; Paik, 1980). The multi-faceted roles of protein methylation include chemotactic sensitization, hormone storage and secretion, repair of aged proteins, modification of calmodulin sensitivity to calcium, and control of DNA–histone association (van Waarde, 1987; Paik & Kim, 1969). The rapidity with which methyltransferases are activated makes these enzymes important in organisms undergoing major changes in metabolism and life cycles. Three distinct protein methylases have been identified and classified according to the type of amino substituent methylated. Protein methylase I (EC 2.1.1.23) is a cytosolic enzyme which methylates the guanidino group of arginine residues in proteins resulting in the formation of α-N-methylarginine (Paik & Kim, 1968). Protein methylase II (EC 2.1.1.24), also located in the cytosol, methylates protein carboxyl groups (Kim & Paik, 1970). Protein methylase III (EC 2.1.1.43), located in the nucleus, methylates the ε-amino groups of lysine (Paik & Kim, 1970).

Protein methylation reactions in kinetoplastids have been relatively unexplored. Because of the rapid morphological and biochemical changes in their life cycles, these organisms must be subject to rapid changes in activation of their genomes.

In this study, we examined the types of protein methylation reactions in bloodstream forms of African trypanosomes and determined their subcellular distribution. We examined the effects of the polyamine antagonist DL-α-difluoromethylornithine (DFMO; Ornidy) on protein methylation activities since this agent blocks growth of the parasite in vivo and causes numerous morphological and biochemical effects (Bacchi & McCann, 1987). These include a dramatic (48-fold) increase in S-AdoMet which results in a 17-fold increase in the methylation potential of bloodstream parasites (Yarlett & Bacchi, 1988). The naturally occurring nucleoside antibiotic sinefungin, which is structurally similar to S-AdoHcy and S-AdoMet, has strong antimicrobial activity towards many protozoa, including the kinetoplastids (Bachrach *et al*., 1980; Nadler *et al*., 1982; Dube *et al*., 1983; Bacchi *et al*., 1987) and was a potent inhibitor of protein methylase II in *Leishmania* spp. (Avila & Avila, 1987). Since sinefungin was active...
against *Trypanosoma brucei brucei* in vivo (Bacchi et al., 1987) we examined the effect of this antibiotic upon trypanosome protein methylase II.

**Methods**

*Parasites.* Bloodstream trypomastigotes of *T. b. brucei* Lab 110 EATRO were obtained from infected rats (10⁶ ml⁻¹) and isolated by DEAE-cellulose chromatography after cardiac puncture (Lanham & Godfrey, 1970). Rats with a 60 h infection were treated with 4% (w/v) DFMO for 12 h or 36 h as described by Bacchi et al. (1988). Cells were washed in 70 mM-potassium phosphate buffer containing 8.4 nmol S-AdoMethyl-[^14]C\[Met\] (7.3 x 10⁵ c.p.m.) in 0.2 M-potassium phosphate buffer pH 7.2, 15 mg histone II-A and 200–300 μg trypanosome protein in a total volume of 0.5 ml (Paik & Kim, 1968). Reactions were stopped after 30 min by addition of 0.5 ml 30% (w/v) trichloroacetic acid. Protein was collected onto filter discs (Whatman GF/C, 2 cm), resuspended in 10% trichloroacetic acid and heated to 90°C for 20 min to remove nucleic acids. The precipitated protein was washed with hot 95% (v/v) ethanol, followed by diethyl ether/ethanol/chloroform (2:2:1, by vol.) and finally diethyl ether alone. The air-dried protein was dissolved in 0.5 M-potassium phosphate buffer (pH 8.0) and left at room temperature for 2 h to hydrolyse the ester bond of carboxymethylated amino acids due to protein methylase II. The precipitated protein was resuspended in aceton, absorbed onto filter paper and radioactivity determined by scintillation counting.

Protein methylase I (EC 2.1.1.23) was determined in incubations containing 8.4 nmol S-AdoMethyl-[^14]C\[Met\] (7.3 x 10⁵ c.p.m.) in 0.2 M-potassium phosphate buffer pH 7.2, 15 mg histone II-A and 200–300 μg trypanosome protein in a total volume of 0.5 ml (Paik & Kim, 1968). Reactions were stopped after 30 min by addition of 0.5 ml 30% (w/v) trichloroacetic acid and heated to 90°C for 20 min to remove nucleic acids. The precipitated protein was washed with hot 95% (v/v) ethanol, followed by diethyl ether/ethanol/chloroform (2:2:1, by vol.) and finally diethyl ether alone. The air-dried protein was dissolved in 0.5 M-potassium phosphate buffer (pH 8.0) and left at room temperature for 2 h to hydrolyse the ester bond of carboxymethylated amino acids due to protein methylase II. The precipitated protein was resuspended in aceton, absorbed onto filter paper and radioactivity determined by scintillation counting.

**Enzyme analysis.** Trypanosome fractions from control and DFMO-treated rats were dialysed in 70 mM-potassium phosphate buffer (pH 7.0), as previously described (4 h at 4°C; Yarlett & Bacchi, 1988) to reduce levels of S-AdoMet and S-AdoHcy to those found in control cells. Activities of protein methylases were determined at 30°C by measuring the incorporation of [[^14]C]methyl groups from S-AdoMethyl-[^14]C\[Met\] (46 mCi mmol⁻¹; 1702 MBq mmol⁻¹) into various protein acceptors.

**Fig. 1.** Metabolism of S-adenosylmethionine. Abbreviations: ODC, ornithine decarboxylase; DFMO, DL-α-difluoromethylornithine; S-AdoMet, S-adenosylmethionine; S-AdoMet DC, S-AdoMet decarboxylase; DC S-AdoMet, decarboxylated S-AdoMet [(5-deoxy-5-adenosyl)(3-aminopropyl)methylsulphonium salt]; S-AdoHcy S-adenosylhomocysteine; Hcy, homocysteine.

Protein methylase I (EC 2.1.1.23) was determined in incubations containing 8.4 nmol S-AdoMethyl-[^14]C\[Met\] (7.3 x 10⁵ c.p.m.) in 0.2 M-potassium phosphate buffer pH 7.2, 15 mg histone II-A and 200–300 μg trypanosome protein in a total volume of 0.5 ml (Paik & Kim, 1968). Reactions were stopped after 30 min by addition of 0.5 ml 30% (w/v) trichloroacetic acid. Protein was collected onto filter discs (Whatman GF/C, 2 cm), resuspended in 10% trichloroacetic acid and heated to 90°C for 20 min to remove nucleic acids. The precipitated protein was washed with hot 95% (v/v) ethanol, followed by diethyl ether/ethanol/chloroform (2:2:1, by vol.) and finally diethyl ether alone. The air-dried protein was dissolved in 0.5 M-potassium phosphate buffer (pH 8.0) and left at room temperature for 2 h to hydrolyse the ester bond of carboxymethylated amino acids due to protein methylase II. The precipitated protein was resuspended in aceton, absorbed onto filter paper and radioactivity determined by scintillation counting.

Protein methylase II (EC 2.1.1.24) was measured in incubations containing 8.4 nmol S-AdoMethyl-[^14]C\[Met\] (7.3 x 10⁵ c.p.m.) in 0.1 M-disodium phosphate and 0.03 M-citric acid, pH 6.0, containing 6 mol 2-mercaptoethanol, 15 mg acceptor substrate protein and 200–300 μg trypanosome protein in a total volume of 0.5 ml (Kim & Paik, 1970). The reaction was terminated after 30 min with 30% trichloroacetic acid. Nucleic acids and phospholipids were removed as for protein methylase I, and the remaining protein was absorbed onto filters and counted. The identity of the methylated carboxyl group was checked by measuring the amount of [methyl-[^14]C]methyl formed after treatment of the reaction mixture with 1 ml 0.5 M-sodium borate buffer (pH 11.0) and 6 ml isoamyl alcohol/toluene (4:1, v/v) (Diliberto & Axelrod, 1974). The radioactivity in the organic phase was determined before and after overnight evaporation at room temperature (van Waarde & van Hoof, 1985). The difference in[^14]CH₃ recovered in the two samples represents the volatile fraction, which is assumed to be methanol.

Protein methylase III (EC 2.1.1.43) was determined in incubations containing 0.1 M-Tris/HCl (pH 9.0), 8.4 nmol S-AdoMethyl-[^14]C\[Met\] (7.3 x 10⁵ c.p.m.), 15 mg histone V-S and 200–300 μg trypanosome protein in a final volume of 0.5 ml (Paik & Kim, 1970). The reaction was stopped after 30 min with 30% trichloroacetic acid and treated as described for protein methylase I to recover volatile acids and...
phospholipids. The precipitated protein was resuspended in 0·2 M-NaOH at 100 °C for 2 h to remove methylarginine and carboxylmethylated amino acids, neutralized with 0·5 M-HCl, absorbed onto filter paper and the radioactivity measured.

Protein methylase activities are expressed as pmol $^{14}$CH$_3$- incorporated min$^{-1}$ (mg trypanosome protein)$^{-1}$. S-AdoMet synthase (methionine adenosyltransferase: EC 2.5.1.6) and S-AdoHcy hydrolase (EC 3.3.1.1) were determined as described previously (Yarlett & Bacchi, 1988).

Partial purification of protein methylase II. Approximately 900 mg of T. b. brucei crude homogenate was obtained from a freeze-thawed cell pellet after centrifugation at 2000 g for 70 min at 4 °C. The homogenate was treated with 25% saturation (NH$_4$)$_2$SO$_4$ at 4 °C for 30 min. The supernatant obtained by centrifugation was brought up to 50% saturation (NH$_4$)$_2$SO$_4$ by gradual addition with gentle stirring. After centrifugation, the pellet was suspended in 3 ml 70 mM-potassium phosphate buffer containing 4·3 mM-NaCl and 300 mM-glucose. An equal volume of calcium phosphate gel was added to the above suspension, the gel was washed with 40 ml of water and the protein eluted with 2 × 6 ml portions of 0·2 M-potassium phosphate buffer, pH 7·2. The resulting eluate was concentrated to 2 ml by sucrose dialysis. Protein was estimated by the Lowry method.

Chemicals. S-Ado[methyl-$^{14}$C]Met was obtained from E. I. Dupont (New England Nuclear Products). Sinefungin, histone II-A (undefined from calf thymus), III-S (lysine-rich), V-S (lysine-rich subgroup f), VIII-S (arginine-rich subgroup f3) were detected in homogenates of T. b. brucei (Table 1). Protein methylase I appeared to have the highest activity, while protein methylases II and III had similar but lower activities in fractionated homogenates. Subcellular fractionation of homogenates revealed that protein methylases I and II were predominantly cytosolic, whereas protein methylase III was confined to the nuclear fraction. As detailed below, protein methylase II activity increased significantly during DFMO treatment of trypanosomes and for this reason it was studied in detail.

In terms of substrate specificity, protein methylase II had highest activity with histone type VIII-S (arginine-rich subgroup f3) as exogenous substrate (Table 2), and had a broad pH optimum which peaked at 6·0 in citrate/phosphate buffer (range 4–14). Under the assay conditions described, the incorporation of $^{14}$CH$_3$- into substrate protein by protein methylase II was linear up to 30 min (not shown). The identity of the methyl group was confirmed by alkali treatment of the acidified pellet. The counts present in the pellet were taken to be 100%, and this was compared to counts present after overnight incubation of the pellet with pH 11·0 borate (aqueous phase), and extraction with an organic phase of 4:1 (v/v) isoamyl alcohol/toluene (Table 3). The organic and aqueous fractions were dried at 30 °C under N$_2$, and the differences in counts recovered were assumed to be the result of the evaporation of methanol formed from the alkaline esterification of the carboxyl group (Diliberto & Axelrod, 1974). The temperature used to evaporate the methanol formed by borate treatment is critical to the correct identification of the volatilized product since high temperature causes degradation of methylmethionine (van Waarde & van Hoof, 1985). This treatment revealed that 73% of the incorporated label was present as the alkali-labile carboxyl form when the assay was

### Results

#### Enzyme studies

Protein methylases I (arginine), II (aspartate and glutamate), and III (lysine) were detected in homogenates of T. b. brucei (Table 1). Protein methylase I

Table 1. Subcellular distribution of protein methylases

<table>
<thead>
<tr>
<th>Protein methylase</th>
<th>Homogenate</th>
<th>Nuclear</th>
<th>Large particle</th>
<th>Cytosolic</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>7·2 ± 0·7</td>
<td>0·10 ± 0·01 (0·6)</td>
<td>5·9 ± 0·90 (12·5)</td>
<td>20·3 ± 1·2 (108)</td>
</tr>
<tr>
<td>II</td>
<td>4·1 ± 1·0</td>
<td>0·07 ± 0·04 (0·7)</td>
<td>0·3 ± 0·18 (1·1)</td>
<td>8·3 ± 1·2 (79)</td>
</tr>
<tr>
<td>III</td>
<td>3·5 ± 0·4</td>
<td>7·70 ± 0·80 (89)</td>
<td>1·0 ± 0·20 (4·4)</td>
<td>0·4 ± 0·2 (4·4)</td>
</tr>
</tbody>
</table>
Table 2. Substrate specificities for protein methylase II

Histone II-A consists of a mixed histone fraction, histone III-S is a lysine-rich fraction, Histone V-S has a lysine-rich subgroup f1, histone VIII-S has an arginine-rich subgroup f3 (Sigma designations). $K_m$ and $V$ (saturation velocity) values were determined from Lineweaver-Burk plots using a substrate range of 2-30 mg ml$^{-1}$. $V$ is expressed as pmol incorporated methyl group min$^{-1}$ (mg protein)$^{-1}$. Percentage of control is the activity of the enzyme in the presence of the substrate compared to that in controls lacking exogenous protein acceptor substrate. The enzyme assays contained 200-300 µg trypanosome protein. The results are presented as the arithmetic mean ± population standard deviation of the number of experiments shown in parenthesis.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$V$</th>
<th>$K_m$ (mg ml$^{-1}$)</th>
<th>Percentage of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histone II-A</td>
<td>2.75 ± 0.85</td>
<td>3.2 ± 1.40</td>
<td>1101 ± 208 (3)</td>
</tr>
<tr>
<td>Histone III-S</td>
<td>0.93 ± 0.31</td>
<td>2.0 ± 0.44</td>
<td>661 ± 37 (3)</td>
</tr>
<tr>
<td>Histone V-S</td>
<td>0.26 ± 0.08</td>
<td>0.8 ± 1.20</td>
<td>288 ± 31 (3)</td>
</tr>
<tr>
<td>Histone VIII-S</td>
<td>3.6 ± 1.20</td>
<td>26.2 ± 2.00</td>
<td>2772 ± 209 (5)</td>
</tr>
<tr>
<td>Gelatin</td>
<td>0.36 ± 0.00</td>
<td>1.4 ± 0.22</td>
<td>366 ± 19 (2)</td>
</tr>
<tr>
<td>γ-Globulin</td>
<td>0.70 ± 0.01</td>
<td>2.6 ± 0.40</td>
<td>479 ± 90 (2)</td>
</tr>
<tr>
<td>Bovine serum albumin</td>
<td>0.45 ± 0.17</td>
<td>4.0 ± 1.60</td>
<td>1108 ± 304 (2)</td>
</tr>
</tbody>
</table>

Table 3. Effect of alkali treatment, organic extraction and evaporation at 23°C on the incorporated $^{14}$CH$_3$-

<table>
<thead>
<tr>
<th>Percentage of starting material</th>
<th>Assay incubated at:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH 6.0</td>
</tr>
<tr>
<td>Pellet prior to extraction</td>
<td>100</td>
</tr>
<tr>
<td>Pellet after borate incubation</td>
<td>24</td>
</tr>
<tr>
<td>Organic phase</td>
<td>64</td>
</tr>
<tr>
<td>Organic phase evaporated</td>
<td>3</td>
</tr>
<tr>
<td>Aqueous phase</td>
<td>0</td>
</tr>
<tr>
<td>Aqueous phase evaporated</td>
<td>0</td>
</tr>
<tr>
<td>Volatile fraction</td>
<td>73</td>
</tr>
</tbody>
</table>

incubated at pH 6.0, as compared to only 26% when the assay was performed at pH 9.0. The methylated product was stable at acid pH (15% TCA) for up to 1 h.

The activities of the three transmethylases, and of S-AdoMet synthase and S-AdoHcy hydrolase were determined in DFMO-treated and control cells since in previous studies we had shown a 48-fold increase in S-AdoMet levels in DFMO-treated trypanosomes and a rise in the cell methylation index from a normal of 6.5 to 114 (Yarlett & Bacchi, 1988). S-AdoMet synthase and S-AdoHcy hydrolase activities increased to 227% and 366%, respectively, that of control cells after 36 h of DFMO treatment (Table 4). Protein methylase activities were determined in the same extracts which had been dialysed to remove excess S-AdoMet and S-AdoHcy. The activity of protein methylase I under these conditions was about 60% lower than in dialysed controls. Protein methylase II activity increased dramatically in extracts of DFMO-treated trypanosomes, approaching a six-fold greater activity after 36 h compared to dialysed controls (Table 4). Protein methylase III appeared initially to decrease to about 40% of control activity after 12 h of DFMO exposure; however the activity then increased to about the normal control level after 36 h (Table 4).
from *T. b. brucei* revealed that the enzyme had an apparent affinity ($K_m$) for histone VIII-S of 28 mg ml$^{-1}$, which is similar to the value determined for the unpurified enzyme (Table 2). The $K_m$ for histone VIII-S corresponds to 11.4 mm-aspartyl and 18.4 mm-glutamyl residues, assuming that all the carboxyl amino acids in histone VIII-S are methylated (Fig. 2a). An apparent $K_m$ for S-AdoMet of 8.4 μM was determined for the unpurified enzyme (Fig. 2b).

**Inhibitor studies**

The nucleoside antibiotic sinefungin was an effective inhibitor of protein methylase II from *T. b. brucei*, with an apparent $K_i$ (concentration causing 50% inhibition of activity) for the crude enzyme of $1.6 \pm 0.6$ μM (range 0.5–10 μM) for triplicate determinations (not shown). The kinetics of protein methylase II inhibition by sinefungin were investigated by measuring the enzyme activity with varying concentrations of histone in the absence and presence of 1 μM-sinefungin. Lineweaver-Burk analysis indicated non-competitive inhibition of histone methylation (Fig. 2a). Repeating the experiment with varying S-AdoMet concentrations indicated competitive inhibition of protein methylase II activity as determined by Lineweaver-Burk analysis (Fig. 2b). S-AdoHcy was less effective as an inhibitor of the enzyme from *T. b. brucei*, with an apparent $K_i$ of 12.9 ± 0.05 μM (range 0.5–25 μM) for triplicate determinations (not shown).

**Discussion**

S-AdoMet is formed from methionine and ATP by S-AdoMet synthase and has a pivotal role in polyamine biosynthesis as amino propyl group donor to putrescine and spermidine, and in methylation reactions as methyl donor to proteins, lipids and nucleic acids (Fig. 1). It was previously demonstrated that treatment of *T. b. brucei* with the polyamine antagonist DFMO elevates levels of both decarboxylated S-AdoMet and S-AdoMet (Fairlamb *et al.*, 1987; Yarlett & Bacchi, 1988), resulting in a 17.5-fold increase in the methylation index of the cell and with it the likelihood of aberrations in activities of methyltransferase enzymes (Yarlett & Bacchi, 1988). In the present study both S-AdoMet synthase and S-AdoHcy hydrolase activities increased in DFMO-treated bloodstream trypanosomes. After 36 h of treatment the synthase was elevated more than twofold and the hydrolase had increased more than threefold (Table 4). Both of these enzymes are critical to cellular methylation: the synthase for the provision of methyl substrate groups and the hydrolase in the removal of S-AdoHcy, a potent inhibitor of most methyltransferase reactions.
Three distinct protein methylases, designated I, II and III, according to the definition of Paik et al. (1972) were detected in extracts of T. b. brucei. Protein methylase II acts on the carboxyl groups of aspartate and glutamate, resulting in the formation of a carboxyl methyl ester. The preferred substrate for the parasite protein methylase II was histone VIII-S, which possesses an arginine-rich subgroup f3, and the nature of the methylated product was confirmed by alkaline hydrolysis and evaporation (Diliberto & Axelrod, 1974; van Waarde & van Hoof, 1985). The enzyme was initially found to have a broad pH optimum, but based upon alkal lability of the carboxyl methyl ester, we determined that the parasite enzyme has a narrow pH range of 5.5-6.0, which is similar to that determined for the mammalian enzyme (Kim & Paik, 1970; Diliberto & Axelrod, 1974; Paik et al., 1988), but different from the plant enzyme, which has a pH optimum of 7-0 (Trivedi et al., 1982).

The reduction in activities of protein methylases I and III after 12 h exposure to DFMO was consistently observed. This may be due to negative control by S-AdoMet, or possibly to the rapid increase in S-AdoHay (Yarlett & Bacchi, 1988), a product of the methylase reaction. That protein methylase III recovers in cells treated with DFMO for 36 h, even though S-AdoMet continues to increase, suggests that the observed reduction in the activity of the cells treated for 12 h is not due to elevated S-AdoMet, but may be due to initial perturbations of S-AdoHay prior to increase in hydrolyase activity. In contrast, the activity of protein methylase II consistently increased in trypansomes exposed to DFMO. The differential activities of these enzymes when trypansomes are exposed to DFMO suggests that these enzymes are under tight control by either the substrate, or the product, or the substrate/product ratio, a significant property for any control mechanism which might participate in the rapid changes of transformation which accompany DFMO administration (Giffin et al., 1986; Giffin & McCann, 1989).

The increased activity of protein methylase II may be indicative of hypermethylation of protein (histone, cytoskeletal, calcium-binding, etc.) in T. b. brucei, which may have profound effects. Duschak & Cazzulo (1990) have shown that total histones from the trypansome Crithidia fasciculata are similar in content of acidic amino acids (aspartate and glutamate) to calf thymus histone. In addition these authors show that band 5 of the H1 histone fraction is unusually rich in acidic amino acids (27.1 mol% compared to 9.5 mol% from calf thymus H1 histone fraction: Dushak & Cazzulo, 1990). This unusual feature may play a functional role in control of histone by carboxyl methylation. Several studies suggest that histone methylation may be involved in the condensation of euchromatin to heterochromatin prior to mitosis (Tidwell et al., 1968; Shepherd et al., 1971; Paik et al., 1972). Methyl substitution on the amino group influences the pK value of histone (Fieser & Fieser, 1963), and since histone exists in situ in conjunction with DNA or RNA, modification of histone molecules by methylation would be expected to affect gene expression. The parasite protein methylase II was found to be a cytosolic enzyme, and since histone is synthesized in the cytoplasm in mammalian cells (Robbins & Borun, 1967), methylation may play an important role in controlling the rate of transport of this protein through the nuclear membrane (Paik et al., 1972). The natural substrate for the enzyme in T. b. brucei is not known. Histone VIII-S proved to be the best substrate of those tested but it is possible that the natural substrate is not a histone protein. It is also likely that several proteins are methylated by the enzyme and that perturbations resulting from hypermethylation result in uncontrolled methylation at more than one site. It is clear from the literature that post translational control of proteins by carboxyl methylation does play an important role in the modification of proteins involved in secretion, chemotaxis and cytoskeletal structure during differentiation, and the activity of protein methylase II increases in rapidly dividing and differentiating cells (Zukerman et al., 1982; Kloog et al., 1983; Paik et al., 1988; Haklai & Kloog, 1990).

The production of hypermethylated end-products due to an elevated methylation index and increased activity of protein methylase II is one possible mechanism for the major morphological and biochemical changes observed in T. b. brucei cells undergoing DFMO treatment (Bacchi et al., 1983; de Gee et al., 1984; Giffin et al., 1986; Giffin & McCann, 1989). These events feature the development of stumpy forms from slender blood forms and activation of the quiescent mitochondrial genome to produce cytochromes and other respiratory proteins (Giffin & McCann, 1989; Bienen et al., 1983; Feagin et al., 1986). The involvement of methylation in these events is further bolstered by the recent studies of Penketh et al. (1990), who studied the effects of known methylating agents on trypansome infections. These agents were therapeutically active in extending the lifespan and/or curing model infections and at low doses were able to synchronously transform slender blood forms to stumpy forms.

Sinefungin, a naturally produced antibiotic and structural analogue of S-AdoMet and S-AdoHay, is a potent growth inhibitor of Leishmania spp. and Trypanosoma spp. (Bachrach et al., 1980; Dube et al., 1983; Paolantonacci et al., 1985; Avila & Avila, 1987; Bacchi et al. 1987; Nolan, 1987). In Leishmania, growth inhibition in vitro was completely reversible by concurrent addition of exogenous S-AdoMet (Paolantonacci et
et al., 1987; Nolan, 1987). Although sinefungin, due to its structural similarity to S-AdoHcy, has been shown to be a strong inhibitor of methyltransferase activity in mammalian cells (Fuller & Nagarajan, 1978), its target in kinetoplastids has been elusive. Sinefungin inhibits DNA synthesis in intact Leishmania (Blanchard et al., 1986), by a mechanism that is not related to uptake or phosphorylation of thymidine (Paolantonacci et al., 1987). The compound is weakly inhibitory to protein methylases I and III from Leishmania (Paolantonacci et al., 1986), but highly inhibitory to protein methylase II (Ki, 2 µM: Avila & Avila, 1987). T. b. brucei protein methylase II was found to be sensitive to sinefungin inhibition with an apparent Ki of 1-6 µM. There appear to be multiple binding sites on the enzyme, for which sinefungin is a competitive inhibitor of the S-AdoMet binding site and a non-competitive inhibitor of the histone binding site. The strain of T. b. brucei used in the present study was susceptible to sinefungin in vivo in mouse infections (Bacchi et al., 1987). We intend to examine protein methylase II in strains of parasites refractory to sinefungin in vitro to determine whether, as in Leishmania, the enzyme from these sources also has diminished sensitivity to the antibiotic.

The T. b. brucei protein methylase II was inhibited by S-AdoHcy with an apparent Ki of 12.9 µM, which is higher than that reported for the enzymes of Leishmania mexicana or L. braziliensis strains (Avila & Avila, 1987), which have apparent Ki values in the range 0.9-1.6 µM. In contrast to other organisms which have been examined (Avila & Avila, 1987; Haklai & Kloog, 1987; Paik et al., 1988) the trypanosome protein methylase is sixfold more sensitive to sinefungin than the natural regulator S-AdoHcy.

The aim of this study was to characterize protein methylases in T. b. brucei. It is likely that nucleic acid methylases and lipid methylases are also present and would be affected by the altered methylation index in DFMO-treated cells. The presence of methylated DNA has been described in Trypanosoma cruzi (Rojas & Galanti, 1990), although no methylcytosine residues were detected in African trypanosome DNA (Bernards et al., 1984; Pays et al., 1984). However, the African trypanosomes do methylate RNA: in particular the common leader sequence of the mRNA has a 7-methylguanosine (m7G) cap (Perry et al., 1987). The role of DNA and RNA methylations in T. b. brucei and their modulation by DFMO is presently being explored in our laboratory.

In summary, we have demonstrated the presence of three distinct protein-methylating activities in T. b. brucei, and found that in DFMO-treated cells, the levels of protein methylase II increased significantly. Elevation of the methylating potential of the trypanosome upon DFMO treatment and resultant post-translational modifications of protein may be one effect in a cascade of events which result in the observed morphological changes and trypanocidal efficacy of DFMO.

Note added in proof: Since this paper went to press, Byers, T. L., Bush, T. L., McCann, P. P. & Bitonti, A. J. (1991) Biochemical Journal (in the Press) have demonstrated that a greater correlation exists between the efficacy of several polyamine antagonists and the increase of intracellular AdoMet pools than depletion of polyamine pools.

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