The *Entamoeba histolytica* Dnmt2 Homolog (Ehmeth) Confers Resistance to Nitrosative Stress

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Nitric oxide (NO) has antimicrobial properties against many pathogens due to its reactivity as an S-nitrosylating agent. It inhibits many of the key enzymes that are involved in the metabolism and virulence of the parasite *Entamoeba histolytica* through S-nitrosylation of essential cysteine residues. Very little information is available on the mechanism of resistance to NO by pathogens in general and by this parasite in particular. Here, we report that exposure of the parasites to S-nitrosoglutathione (GSNO), an NO donor molecule, strongly reduces their viability and protein synthesis. However, the deleterious effects of NO were significantly reduced in trophozoites overexpressing Ehmeth, the cytosine-5 methyltransferase of the Dnmt2 family. Since these trophozoites also exhibited high levels of tRNA Asp methylation, the high levels suggested that Ehmeth-mediated tRNA Asp methyltransferase activity is part of the resistance mechanism to NO. We previously reported that enolase, another glycolytic enzyme, binds to Ehmeth and inhibits its activity. We observed that the amount of Ehmeth-enolase complex was significantly reduced in GSNO-treated *E. histolytica*, which explains the aforementioned increase in tRNA methylation. Specifically, we demonstrated via site-directed mutagenesis that cysteine residues 228 and 229 of Ehmeth are susceptible to S-nitrosylation and are crucial for Ehmeth binding to enolase and for Ehmeth-mediated resistance to NO. These results indicate that Ehmeth has a central role in the response of the parasite to NO, and they contribute to the growing evidence that NO is a regulator of epigenetic mechanisms.

Amebiasis is a parasitic infection of the human intestine and is caused by the single-celled protozoa *Entamoeba histolytica*. The disease has a worldwide distribution with substantial morbidity and mortality, and it is one of the three most common causes of death from parasitic disease (1). The clinical spectrum of amebiasis ranges from asymptomatic infection to colitis, dysentery, or liver abscess. The parasite has two stages in its life cycle: the infective cyst and the invasive trophozoite. In the host, the parasite is exposed to various environmental challenges and is capable of adapting to the demands of its surrounding environment, such as extreme changes in the glucose concentration and the oxidative and nitrosative attacks of the host immune system (2–5).

*E. histolytica* belongs to the so-called family of “Dnmt2-only” organisms, in that it does not contain any of the canonical DNA methyltransferases (Dnmt1 and Dnmt3). *E. histolytica* Dnmt2 (Ehmeth) is a weak, but genuine, DNA methyltransferase (6–8), and its ability to catalyze tRNA Asp methylation has been recently demonstrated (9). This dual specificity of Ehmeth for DNA and tRNA has also been proposed for the Dnmt2 homolog in *Drosophila melanogaster* (10). Although control of gene expression by Ehmeth has been reported (6), this function is apparently not its most important function (11). Since Ehmeth expression fluctuates significantly (2- to 3-fold) between laboratory strains where its expression is barely detectable and strains isolated from patients, these fluctuations suggest that Ehmeth is associated with the parasite’s adaptation to its host (reference 8 and unpublished observations).

While the overall biological functions of Dnmt2/Ehmeth are not yet completely understood, recent work has enabled us to view their expressions in terms of the parasite’s survival, longevity, and adaptability to metabolic and oxidative stresses. We have recently reported that glucose starvation, with the help of the glycolytic enzyme enolase, regulates the parasite’s methylation status (9). Enolase interacts with the catalytic site of Ehmeth and inhibits its methyltransferase activity. Dnmt2 expression has been implicated as a necessary component for maintaining the normal life span in *D. melanogaster*, and its overexpression induces longevity in fruit flies (12). It has been proposed that the underlying mechanism of extended longevity is an increased resistance to oxidative damage, which has a well-established association with both degenerative diseases and aging (13). Dnmt2 overexpression induces the expression of small heat shock protein (HSP) in *D. melanogaster* (12) and promotes resistance to H2O2 exposure in *E. histolytica* (14).

Nitric oxide (NO) is the major cytotoxic molecule that is released by activated macrophages, natural killer cells, and other phagocytic cells for killing *E. histolytica* trophozoites (15). We have previously reported that NO controls the activity of some of the parasite’s virulence factors (16, 17). It has also been recently reported that NO triggers stress responses in *E. histolytica* and that NO directly inhibits glycolysis and stimulates cysteine synthase activity (18). Evidence is emerging that NO is also a regulator of epigenetic events, because it can modify components of the chromatin remodelling machinery (19, 20). While knowledge on NO as an epigenetic regulator is increasing (20, 21), little is known about the effects of NO on Dnmt activity in general and on Dnmt2 in particular. It is also not known whether the protective effects of Dnmt2 against oxidative stress or heat shock (22) apply to nitrosative stress (14).
In this report, we describe the results of our investigation and describe the underlying molecular mechanisms of increased tolerance to nitrosative stress in *E. histolytica* trophozoites that overexpress Dnmt2. The findings in this report provide the first evidence of NO-mediated regulation of a Dnmt2 protein.

**MATERIALS AND METHODS**

**Microorganisms.** *E. histolytica* trophozoites strain HM-1:IMSS were grown under axenic conditions in Diamond’s TYI-S-33 medium at 37°C, and trophozoites in the exponential phase of growth were used in all experiments. *Escherichia coli* strain BL21(DE3) [F−ompT gal dcm lon hsd Sgal(r−m−) λ (DE3 [lacIqUV5−T7 gene 1 ind1 sam7 min5]), a derivative of the *E. coli* B strain, was used for transformation and protein expression.

**DNA constructs.** The pJST4 expression vector and the pJST4-Klp5 vector were kindly provided by A. Lohia, Department of Biochemistry, Bose Institute, India. The pJST4 expression vector enables the expression of the CHH (calmodulin binding domain, hemagglutin [HA], and histidine [His])-tagged protein in *E. histolytica*; expression of this protein is driven by an actin promoter. The pJST4-Klp5 vector expresses Klp5, a 99-kDa protein that belongs to the kinesin 5 family. This plasmid was used as a control in our previous study in order to exclude the possibility that the CHH tag regulates Ehmeth activity, and we used this plasmid for the same purpose in this study. For more details about the construction of pJST4-Ehmeth, see reference 9. The transfec- tion of *E. histolytica* trophozoites was performed using a previously described protocol. Details about the construction of the glutathione S-transferase (GST)–Ehmeth plasmid were previously described in references 8 and 9.

**Site-directed mutagenesis.** The expression of the mutagenic plasmids used for recombinant proteins in *E. coli* BL21(DE3), namely, Ehmeth C228S-GST, Ehmeth C229S-GST, and Ehmeth C228S-C229S-GST variants, were created by site-directed mutagenesis. Briefly, pairs of complementary mutagenic primers (Ehmeth C228S 5’ and 3’, Ehmeth C229S 5’ and 3’, and Ehmeth C228S-C229S 5’ and 3’ [Table 1]) were used to amplify the entire GST-Ehmeth plasmid with a high-fidelity non-strand-displacing DNA polymerase (Pfu DNA polymerase; Promega). The template DNA was eliminated by enzymatic digestion with DpnI, which is

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**FIG 1** Overexpression of Ehmeth protects *E. histolytica* against nitrosative stress. (A) Northern blot analysis was performed using total RNA that was extracted from pJST4-Ehmeth and pcontrol *E. histolytica* trophozoites. rDNA whose expression was not changed in pJST4-Ehmeth and pcontrol trophozoites were used as controls for RNA loading. The figure displays a representative result from three independent experiments. (B) Western blot analysis was performed on nuclear protein fractions that were prepared from pJST4-Ehmeth and pcontrol *E. histolytica* trophozoites. The proteins were separated on 12% SDS-PAGE gels and analyzed by Western blotting with an anti-HA (α HA) antibody or an anti-actin antibody. The figure displays a representative result from three independent experiments. (C) The viabilities of wild-type *E. histolytica* trophozoites from strain HM-1:MSS, *E. histolytica* trophozoites from a strain that was transfected with a control vector (pcontrol), *E. histolytica* trophozoites that overexpressed Ehmeth (pJST4-Ehmeth), and *E. histolytica* trophozoites that overexpressed pJST4-Ehmeth C228S-C229S exposed to 350 μM GSNO for 30, 60, 90, and 120 min were measured. The number of trophozoites at the beginning of each experiment was set at 100%. Bars represent the standard deviations of the means. The means of the different groups for three independent experiments were compared using Student’s *t* test, and statistical significance was set at 5%. The viabilities of the wild-type *E. histolytica* trophozoites of strain HM-1:MSS, the pcontrol *E. histolytica* trophozoites, and the pJST4-Ehmeth C228S-C229S *E. histolytica* trophozoites were not significantly different from each other at any time point. In contrast, the viability of the pJST4-Ehmeth *E. histolytica* trophozoites was significantly different (*P < 0.05*) from that of the wild-type *E. histolytica* trophozoites of strain HM-1:MSS, the pcontrol *E. histolytica* trophozoites, and the pJST4-Ehmeth C228S-C229S *E. histolytica* trophozoites after a 60- or 120-min exposure to GSNO.
specific for methylated DNA, while the mutated plasmid that was generated in vitro was unmethylated and was left undigested. All created mutants were sequenced to ensure the presence of desired mutations and the absence of undesired mutations.

For the expression of CHH-tagged Ehmeth C228S-C229S (pST4 Ehmeth C228S-C229S) in E. histolytica, Ehmeth C228S-C229S was amplified from the plasmid C228S-C229S-GST using the primers Ehmeth-kpn and EhmethBgl (Table 1) and then cloned in the pST4 expression vector.

Induction of protein S-nitrosylation. Trophozoites that were grown in Diamond’s TYI-S-33 medium were incubated with freshly prepared -nitrosoglutathione (GSNO) solution (350 μM; Sigma) for 1 h at 37°C. The trophozoites were then pelleted by centrifugation at 300 g for 3 min at 4°C, and either total protein extract or total RNA extract was prepared for further analysis.

Northern blotting. For Northern blotting, total RNA was extracted using the RNA isolation kit TRI reagent (Sigma). RNA (10 μg) was separated on a 1% agarose, 0.3% formaldehyde gel in morpholinepropanesulfonic acid (MOPS) buffer (0.2 M MOPS, 50 mM sodium acetate, 5 mM EDTA; pH 7.2) and then blotted to Genescreen membranes (NEN Bioproducts, Boston, MA). The RNA was cross-linked to the membrane by UV irradiation (1,200 J/cm²) in a UV Stratalinker apparatus (Stratagene) followed by drying at 80°C for 2 h. The membrane was washed in hybridization buffer (0.5 M NaP, 7% SDS, 1 mM EDTA) and then blocked with 100 μg/ml salmon sperm DNA for 1.5 h. Probes were randomly labeled with [α-32P]dCTP by using a random primer DNA labeling mix (Biological Industries, Kibbutz Beit Haemek, Israel) and cleaned on a G-50 column (GE Healthcare). Hybridization with the probes was performed at 60°C overnight. The membrane was then washed several times at 60°C with washing buffer 1 (5% SDS, 40 mM NaP, 1 mM EDTA; pH 7.2), and then with washing buffer 2 (1% SDS, 40 mM NaP, 1 mM EDTA; pH 7.2). The membrane was then exposed to X-ray film (Fujiﬁlm), and the ﬁlm was developed for detection of the signal.

Western blotting. For Western blotting, nuclear fractions of pST4-Ehmeth- or pcontrol-transformed E. histolytica trophozoites were prepared using a previously described protocol (24). Proteins were resolved on 12% SDS-polyacrylamide gel electrophoresis. The proteins in each gel slice were reduced with 2.8 mM dithiothreitol and digested overnight in 10% acetonitrile and 10 mM ammonium bicarbonate with modified trypsin (Promega) at 37°C. The resulting tryptic peptides were resolved by reverse-phase chromatography on 0.075- by 0.25-μm silica capillaries (J&W) packed with Reprosil reverse-phase material (Dr. Maisch GmbH, Germany). The peptides were eluted with linear gradients of 7 to 40% acetonitrile with 0.1% formic acid in water at a flow rate of 0.25 μl/min over 94 min and 95% acetonitrile with 0.1% formic acid in water at a flow rate of 0.25 μl/min over 12 min. Mass spectrometry (MS) was performed by using an ion trap mass spectrometer (Orbitrap; Thermo) in positive mode and repetitively full MS scan followed by collision-induced dissociation (CID) of the seven most dominant ions selected from the first MS scan.

The mass spectrometry data were analyzed using the MaxQuant 1.4.1.2 software (26), which searched the E. histolytica section of the NCBI-nr database with a 1% false-discovery rate, and quantiﬁed by label-free analysis using the same software.

Immunoprecipitation assays. Aliquots of nuclear protein fractions (100 μg) were diluted in 20 mM HEPES (pH 7.5), 150 mM NaCl, 0.1% Triton, 10% glycerol (HNTG buffer; 300 μl ﬁnal volume) and then incubated with protein G-Sepharose beads (10 μl; Sigma) for 30 min at 4°C. Nonspeciﬁc interacting proteins were excluded by centrifugation (3,000 rpm at 4°C for 5 min). The supernatant was incubated with either 1:200 HA antibody or enolase antibody for 2 h at 4°C. Following incubation, protein G-Sepharose beads (20 μl) were added to the samples, which were then incubated for 16 h at 4°C. Immunoprecipitated proteins were collected by centrifugation, washed three times with HNTG buffer, and then resolved by 12% SDS-polyacrylamide gel electrophoresis. The proteins were then transferred to nitrocellulose membranes for Western blot analysis and detected with either a mouse monoclonal enolase antibody (sc-7183; Santa Cruz Biotechnology) or a rabbit six-histidine antibody (sc-805; Santa Cruz Biotechnology). Expression of recombinant Ehmeth and Ehmeth mutant proteins in E. coli BL21. E. coli BL21(DE3) cells that were transfected with the respective vectors (GST-Ehmeth, Ehmeth C228S-GST, Ehmeth C229S-
GST, and Ehmeth C228S-C229S-GST) were grown overnight at 37°C in Luria broth (LB) medium that contained 100 μg/ml ampicillin. These precultures were inoculated with a 500 ml of a 1:100 dilution of fresh LB medium that contained 100 μg/ml ampicillin and were further grown for 3 h until the optical density at 600 nm of the medium reached 0.8. These bacteria were induced with 0.5 mM isopropyl-beta-D-thiogalactopyranoside (IPTG) for 16 h at 25°C. At the end of incubation, the induced cells were harvested and lysed in lysis buffer (100 mM KCl, 1 mM DTT, 1 mM phenylmethylsulfonylfluoride, 100 μg/ml lysozyme, and 100 μg/ml leupeptin in PBS). The lysed cells were then sonicated for 5 min with 30-s pulses, with 30 s between each pulsing session. The lysis was completed by adding 1:100 BugBuster protein extraction reagent (Novagen). The lysate was then centrifuged at 2,000 × g for 20 min in order to recover the soluble proteins in the supernatant. GST fusion proteins were purified by affinity purification on glutathione-Sepharose beads. The recombinant proteins were then eluted with glutathione elution buffer (50 mM Tris-HCl [pH 8.0], 10 mM glutathione [Sigma]), and their concentrations were measured by using Bradford's method (27).

**Exposure of Ehmeth and mutants proteins to GSNO.** Aliquots (0.04 nmol) of recombinant protein were treated with 5 μM GSNO for 1 h at 37°C in 20 μl of methylation buffer without DTT (100 mM Tris-HCl [pH 7.5], 5% glycerol, 5 mM MgCl2, and 100 mM NaCl). DTT (20 mM) was added to certain protein samples following their exposure to GSNO to revert their S-nitrosylation. Recombinant proteins were resolved by 12% polyacrylamide gel electrophoresis under native conditions and then transferred to nitrocellulose membranes. Membranes were blocked (3% skim milk powder) and then probed with 1:800 rabbit polyclonal S-nitrosocysteine (S-NO-Cys) antibody (N5411; Sigma) for 1 h at room temperature. The membrane was then incubated with 1:5,000 secondary antibody to rabbit IgG (Jackson ImmunoResearch) for 1 h at room temperature and developed by using enhanced chemiluminescence. Ponceau S staining of the blots prior to their blocking was used to control the loading and transfer of the proteins to the membranes.

**Molecular docking.** The coordinates for the E. histolytica enolase structure (GenBank accession number XP_649161.1; PDB ID 3QTP) and the E. histolytica methyltransferase EhMeth/Dnmt2 (GenBank accession number XP_655267.2; PDB ID 3QV2) were obtained from the Protein Data Bank (http://www.rcsb.org/pdb/home/home.do). All nonprotein residues were removed prior to the docking procedure. Subunit B of enolase was considered the receptor, because it was larger (438 residues) than Ehmeth (320 residues), which served as the ligand during docking. The protein-protein docking was done using the Hex 6.3 platform (28) and the shape correlation. Similar clusters of docked proteins were obtained using SwamDock (29) and ClusPro (30). Docked complexes were visualized, and the images were produced using PyMol (http://www.pymol.org/).

**RESULTS**

**Overexpression of Ehmeth protects E. histolytica against nitrosative stress.** In order to test the hypothesis that Ehmeth is involved in the protection of the parasite against nitrosative stress, we determined the viability of three strains of E. histolytica trophozoites, namely, wild-type strain HM-1: MSS, E. histolytica trophozoites transfected with pcontrol, and E. histolytica trophozoites transfected with pJST4-Ehmeth and that overexpressed Ehmeth, after their exposure to 350 μM GSNO for 120 min. GSNO was selected as the NO donor molecule because it is the main nonprotein S-nitrosothiol (SNO) in human cells and extracellular fluids (31).

The overexpression of Ehmeth as a CHH-tagged protein was confirmed by both Northern and Western blotting (Fig. 1A and B). Figure 1C displays the time-dependent changes in viability of the three types of E. histolytica trophozoites. After 60 min, the viabilities of the wild-type and pcontrol trophozoites were less than that of the pJST4-Ehmeth trophozoites. These differences in viability were exacerbated after 120 min: only 25% of the wild-type E. histolytica trophozoites and pcontrol trophozoites were viable, whereas 50% of the pJST4-Ehmeth trophozoites were viable. These results indicate that Ehmeth contributes toward protecting E. histolytica trophozoites against nitrosative stress.

**tRNA<sup>Asp</sup> methylation and protein synthesis in NO-treated trophozoites.** Eukaryotic protein synthesis is regulated by a variety of tRNA modifications (32). In addition, stress-specific reprogramming of modified ribonucleosides in tRNA is involved in the selective translation of survival proteins (33). We decided to investigate whether Ehmeth is involved in the mechanism of NO resistance in the parasite. For this purpose, we determined the levels of tRNA<sup>Asp</sup> methylation and protein synthesis in pcontrol trophozoites and pJST4-Ehmeth trophozoites before and after GSNO treatment.

For determining the level of tRNA<sup>Asp</sup> methylation, we used a recently developed method that is based on bisulfite sequencing of tRNA and enables direct detection of cytosine methylation in tRNA by accurately localizing the methylated cytosines within the sequence (25). The cytosine 38 residue in tRNA<sup>Asp</sup> is a well-known substrate of Dnmt2 enzymes (25, 34, 35). Amplicons (PCR products) of tRNA<sup>Asp</sup> were generated from bisulfite-treated total RNA samples that were extracted from E. histolytica strains, and the sequences of several independent amplicons were determined. We observed that cytosine 38 methylation in the pJST4-Ehmeth trophozoites was 5 times greater than that in the pcontrol trophozoites (Fig. 2A). This increase of cysteine 38 methylation in the pJST4-Ehmeth trophozoites that were exposed to 350 μM GSNO for 1 h was even higher (6.7 times) than that in the pcontrol trophozoites (Fig. 2A). There were no differences in the levels of cysteine 38 methylation in the untreated and GSNO-treated pcontrol trophozoites. In contrast, the levels of cysteine 38 methylation in the GSNO-treated pJST4-Ehmeth trophozoites were substantially greater than those in the pJST4-Ehmeth trophozoites that were not exposed to GSNO. We observed 100% methylation of the cysteine 49 residue in the three types of trophozoites, irrespective of whether or not they were exposed to GSNO. These findings suggested that the methylation of the cysteine 49 residue is not catalyzed by Ehmeth (Fig. 2A). We also found complete demethylation of other cysteine residues (32, 33, 37, 48, and 56), and these findings indicated that the bisulfite treatment was efficient (Fig. 2A). Collectively, these results confirm our previous observations about the ability of Ehmeth to methylate tRNA<sup>Asp</sup> (9) and indicate that Ehmeth is similar to other Dnmt2 proteins in that it methylates the cysteine 38 residue of tRNA<sup>Asp</sup>. Additionally, our results showed an unexpected positive effect of NO on tRNA<sup>Asp</sup> methylation in the pJST4-Ehmeth trophozoites.

Next, we hypothesized that the hypermethylation of tRNA<sup>Asp</sup> that we detected in the pJST4-Ehmeth trophozoites promotes protein synthesis. In order to test this hypothesis, we used the surface sensing of translation (SunSET) technique (36), which uses the antibiotic puromycin (a structural analog of tyrosyl-tRNA) and puromycin antibodies to detect the amount of puromycin that was incorporated into nascent peptide chains.

We observed that pcontrol and pEhmeth trophozoites had comparable rates of protein synthesis under control conditions (Fig. 2B). Treating pcontrol trophozoites with either 35 μM or 175 μM GSNO for 15 min inhibited protein synthesis by 30% and 90%, respectively (Fig. 2B). These inhibitory effects of 35 μM and 175 μM GSNO were less pronounced (10% and 40% inhibition, respectively) compared to the pJST4-Ehmeth trophozoites that were treated with 350 μM GSNO, which were virtually nonviable (Fig. 2B). These results indicate that Ehmeth contributes toward protecting E. histolytica trophozoites against nitrosative stress.
respectively) in the pJST4-Ehmeth trophozoites (Fig. 2B). Cycloheximide is an inhibitor of protein biosynthesis (37). We also found that the extent of inhibition of protein synthesis by cycloheximide was the same (90%) in the untreated pcontrol and the pJST4-Ehmeth trophozoites. The observation that the inhibitory effect of GSNO in the pJST4-Ehmeth trophozoites was less pronounced than that in the pcontrol trophozoites was confirmed independently by measuring the rate of protein synthesis in these trophozoites by using [35S]methionine (see Fig. S1 in the supplemental material).

We next determined whether Ehmeth overexpression selectively influences the synthesis of proteins that are involved in the resistance to nitrosative stress. For this purpose, we performed quantitative proteomic analysis of pcontrol and pJST4-Ehmeth trophozoites that were exposed or not exposed to 350 μM GSNO for 1 h (see Table S1 in the supplemental material for results of the complete analysis). For the purpose of this study, we decided to focus on proteins that were upregulated in the GSNO-treated pJST4-Ehmeth trophozoites and compare them to those from GSNO-treated pcontrol trophozoites (Fig. 2C). Among these proteins, we identified proteins that are involved in protein translation, such as the 60S and 40S ribosomal proteins and glycyl-tRNA synthetase, protein transport, such as the coatomer beta-subunit and vacuolar sorting protein, and signaling, such as the Rab family...
GTPases. Interestingly, two proteins that were significantly upregulated were alcohol dehydrogenase 2 (ADH2; 3-fold increase) and the antioxidant peroxiredoxin (4-fold increase). ADH2 is essential for energy metabolism of oxidatively stressed parasites (38), and peroxiredoxin has been reported to be associated with resistance to nitrosative stress in *Leishmania* spp. (39).

Collectively, these results strongly suggest that Ehmeth-mediated tRNA methylation has a positive effect on protein synthesis in general and on stress response-related proteins in particular when the parasite is nitrosatively stressed.

**The amount of the Ehmeth-enolase inhibitory complex is reduced in NO-treated trophozoites.** Although NO usually inhibits enzymatic activity, it can also activate enzymatic activity, as reported for the *E. coli* transcription factors OxyR and SoxR (21). Hence, we decided to investigate whether NO also modulates Ehmeth activity. Ehmeth is devoid of any tRNA methyltransferase activity in the absence of DTT (data not shown). On the other hand, the effect of NO on Ehmeth activity could not be determined in the presence of DTT, because DTT reverses the S-nitrosylation of cysteine.

We previously reported that enolase binds to Ehmeth and inhibits its activity (9). We hypothesized that the hypermethylation of tRNA<sup>A<sup> spoof</sup></sup> in NO-treated pJST4-Ehmeth trophozoites (Fig. 2A) is due to reduced formation of the enolase-Ehmeth complex. In order to test this hypothesis, we used trophozoites that were transfected with pJST4-Ehmeth plasmids. In the absence of an efficient Ehmeth antibody, we used an HA antibody to immunoprecipitate Ehmeth and visualized it by Western blotting. Using this approach, which had been previously validated (9), we showed that the amount of Ehmeth-enolase complex in the pJST4-Ehmeth trophozoites that were exposed to 350 μM GSNO for 1 h was significantly lower than that found in the untreated pJST4-Ehmeth trophozoites (Fig. 3A). This result can be explained by either a direct effect of NO on the amount of the Ehmeth-enolase complex or by NO limiting the availability of either one or both of the constituents of the complex. According to the results of Western blot analysis, the amount of Ehmeth did not change in the GSNO-treated parasites (Fig. 3B). Collectively, these results indicate that NO can directly influence the amount of the Ehmeth-enolase complex and the methylation status of tRNA<sup>A<sup> spoof</sup></sup>.

Although the crystal structure sizes of Ehmeth and enolase have been determined to be 2.15Å and 1.9Å, respectively (40, 41), the molecular details of the Ehmeth-enolase interaction remains uncharacterized.

In order to predict which cysteine residues in Ehmeth are involved in the formation of the Ehmeth-Enolase complex and might be potential targets for S-nitrosylation, we performed an *in silico* docking analysis. Prior to screening for possible complex interfaces, the structures were stripped of nonprotein constituents. The program Hex 6.3 explores the possible energies of protein-protein interactions by using both shape complementarity and electrostatic effects. The top-rated structure, which is displayed in Fig. 4A, showed strong surface complementarity with the loop between residues 250 and 260 of enolase that includes the glutamic acid 253 (Glu253) residue and juts into the aperture that is formed between two globular lobes of the Ehmeth protein. This strong surface complementarity brings the Glu253 residue of enolase into close proximity with the Cys229 residue of Ehmeth. In addition, both the Cys229 residue and its neighbor, the Cys228 residue, of Ehmeth are on a rather unstructured loop, with their SH groups pointing outwards. Hence, the two Cys residues are potentially accessible to NO and to an interacting protein. Figure 4B details the putative interaction between the Glu253 residue of enolase and the Cys229 residue of Ehmeth; the Cys229 residue of Ehmeth was computationally modified by adding NO to the terminal sulfur atom in Fig. 4C.

In Fig. 4D, the complex components have been separated and each protein turned 90° in order to reveal the interaction interface. These interfaces have been overlaid with the surface electrostatic potential, which was calculated using the algorithm in PyMol, a molecular visualization system. This analysis clearly showed that a strong negative potential surrounds the Glu253 residue of enolase, while the area that surrounds the Cys229 residue of Ehmeth is strongly positive.
Collectively, the output of the in silico computer-based modeling of the Ehmeth-enolase interaction suggested that the Cys228 and Cys229 residues are accessible to NO and are important sites for the binding of Ehmeth to enolase.

In order to test the accuracy of this model, we created three Ehmeth mutant proteins: Ehmeth228, Ehmeth229, and Ehmeth228-229, in which the Cys228 residue, the Cys229 residue, and the two cysteine residues were, respectively, replaced with a serine. Following the exposure of the wild-type and mutated proteins to 5 μM GSNO for 1 h, their levels of S-nitrosylation were compared by Western blot analysis by using an S-NO-Cys antibody (Fig. 5A). The presence of S-nitrosylated cysteine(s) was detected in the wild-type Ehmeth protein, but the signal was significantly attenuated in the single mutants and even more so in the double mutants. The specificity of the S-NO-Cys antibody was confirmed by the loss of signal in Ehmeth proteins that were treated with DTT immediately after their S-nitrosylation by GSNO (Fig. 5A). Collectively, these results indicated that Cys228 and Cys229 are efficiently S-nitrosylated.

We next determined the involvement of these cysteines in the formation of the Ehmeth-enolase complex. For this purpose, Ehmeth228-229 was expressed as a CHH-tagged protein in the parasite. According to the results of our Western blot analysis (Fig. 5B), the amounts of Ehmeth and Ehmeth228-229 proteins in the pJST4-Ehmeth and the pJST4-Ehmeth228-229 trophozoites were the same. The amounts of enolase-Ehmeth complex in the pJST4-Ehmeth and the pJST4-Ehmeth228-229 trophozoites were then determined by immunoprecipitation analysis (Fig. 5C). We found that the amount of the Ehmeth-enolase complex in the pJST4-Ehmeth and the pJST4-Ehmeth228-229 trophozoites was substantially smaller than that found in the pJST4-Ehmeth trophozoites (Fig. 5C). These results indicated that the Cys228 and Cys229 residues in Ehmeth are involved in the binding of Ehmeth to enolase and strongly suggest that the S-nitrosylation inhibits the formation of the Ehmeth-enolase complex. We next determined the involvement of the Cys228 and Cys229 residues in Ehmeth activity. We found that the levels of tRNAASp methylation in pcontrol and pJST4-Ehmeth228-229 trophozoites were the same, and this result strongly suggested
that these mutations impair the catalytic activity of Ehmeth (Fig. 2A). We also found that untreated and GSNO-treated pcontrol and pJST4-Ehmeth228-229 trophozoites had comparable rates of protein synthesis (Fig. 2B), and this result strongly suggested that the level of protein synthesis correlates with the level of tRNA Asp methylation.

Finally, we compared the protective effect of Ehmeth of nitrosatively stressed pJST4 Ehmeth228-229 E. histolytica trophozoites to that of nitrosatively stressed wild-type, pJST4 pcontrol, and pJST4Ehmeth E. histolytica trophozoites. We found that the protective effect of Ehmeth in nitrosatively stressed trophozoites was lost when the Ehmeth228-229 protein was overexpressed (Fig. 1C).

DISCUSSION

Mammalian defense strategies against pathogens include the production of a chemical arsenal, such as reactive oxygen and nitrogen species. NO plays a major role in this defense process, and NO-induced inhibition of protein synthesis is part of its cytostatic action in mammalian cells. Different mechanisms, such as NO-mediated cleavage of 28S and 18S rRNA (42) and NO-induced phosphorylation of eukaryotic initiation factor 2α (eIF-2α) (43) have been proposed to explain this inhibitory activity. Therefore, one might surmise that any action of the parasite to counteract the deleterious effect of NO on its protein synthesis is an effective means of resistance against nitrosative stress. Our data indicate the existence of a strong correlation between Ehmeth-mediated tRNA methylation and the control of protein synthesis in nitrosatively stressed E. histolytica trophozoites. Most of the recent efforts to explain the role of Dnmt2 in the protection of different organisms from environmental stresses have focused on the organisms’ abilities to methylate tRNA (22). Recently, it was proposed that cysteine-5 tRNA methylation in mice promotes tRNA stability and protein synthesis and prevents stress-induced RNase cleavage by angiogenin (44). According to the results of our bioinformatics analysis, the absence of an angiogenin homolog in E. histolytica (data not shown) suggests that this protective mechanism does not exist in E. histolytica, or that a still-undiscovered RNase in E. histolytica has a similar function to that of angiogenin. In contrast, the upregulation of ribosomal proteins 40S and 60S subunits in the GSNO-treated pJST4-Ehmeth and pJST4-Ehmeth E. histolytica trophozoites determined by using a histidine antibody.

The preservation of protein synthesis as a mechanism of resistance against nitrosative stress is somewhat counterintuitive. The general stratagem in most oxidatively stressed and heat-shocked species, including E. histolytica, typically includes downregulation of protein synthesis (46, 47) in order to stop energy waste and the toxic buildup of damaged or misfolded proteins. In this investigation, we found paradoxical evidence on the
maintenance of protein synthesis in GSNO-exposed pJST4-Ehmeth trophozoites. We surmise that the additional protein synthesis is a mechanism of titration/competition against the intracellular accumulation of S-nitrosoylated proteins. Essentially, an intracellular protein reservoir is created in order to buffer the effects of nitrosative damage. This notion is well illustrated by the fact that the synthesis and turnover of Fe-S cluster-containing proteins in *E. histolytica* that were exposed to the NO donor sodium nitroprusside increased in order to overcome the deleterious effects of NO treatment (18).

Our data indicate that expression of specific stress-related proteins is upregulated in GSNO-treated pJST4-Ehmeth trophozoites. This finding is in agreement with those of Santi-Roca and others (18), who reported that the amount of peroxiredoxin transcripts was increased by NO in *E. histolytica*. It has been reported that ADH2 (48, 49) and peroxiredoxin (50) are associated with the resistance of other organisms to oxidative stress and nitrosative stress (51–53). Accordingly, we posit that these enzymes contribute to the resistance to nitrosative stress of pJST4 Ehmeth trophozoites.

Growing evidence indicates that NO can regulate key epigenetic events, including chromatin remodelling (for a recent review, see reference 21). Our data indicate that S-nitrosylation regulates Dnmt2 activity by inhibiting the formation of an Ehmeth–enolase complex. Since enolase binds to Ehmeth and inhibits its activity (9), this result can be used to explain the significant increase in tRNA<sup>Asp</sup> methylation that we observed in the GSNO-treated trophozoites. We previously showed that the deletion of the catalytic site in Ehmeth (motif IV) partially suppresses the formation of the Ehmeth–enolase complex, and this finding suggests that other components of Ehmeth are involved in its binding to enolase (9). The results of this investigation shed new light on the formation of this complex and emphasize the importance of the Cys228 and Cys229 residues in Ehmeth in this process. In the future, it will be interesting to challenge and confirm this information for the role of the Glu253 residue of enolase in the formation of the Ehmeth–enolase complex that was obtained in our molecular docking analysis.

In summary, the results of this investigation show that Ehmeth-mediated tRNA<sup>Asp</sup> methylation is crucial in the protection of *E. histolytica* against nitrosative stress by maintaining active protein synthesis. Another important finding of this study is that NO influences the amount of Ehmeth–enolase complex and consequently regulates Ehmeth activity. The results of this analysis open the door to many important questions about the regulation of Dnmt activity and the role of NO in Dnmt–protein interactions in other organisms. Finally, the results of this investigation indicate that Ehmeth-mediated tRNA<sup>Asp</sup> methylation is a potential target for the development of drugs to treat amoebiasis.

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