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Short communication

Kinetic analyses and inhibition studies reveal novel features in peptide deformylase 1 from *Trypanosoma cruzi*

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ABSTRACT

In eubacteria and eukaryotic organelles N-terminal methionine excision requires the sequential action of two activities, a peptide deformylase (PDF), which systematically removes the N-formyl group present on all nascent polypeptides and methionine aminopeptidase (MAP), which exscinds methionine specifically and depends on the previous removal of the N-formyl group. In *Trypanosoma cruzi* two genes encoding bacterial PDF homologues have been identified and referred to as *Tc*PDF-1 and *Tc*PDF-2. Here we report the biochemical characterization of a truncated soluble version of *Tc*PDF-1 lacking the hydrophobic N-terminal domain that is active with the bacterial PDF substrate formyl-methionyl-alanyl-serine but, in contrast to other PDFs, is not inhibited by actinonin. The enzyme is strongly activated by Cu^{2+} and inhibited by Ni^{2+}. Our results show that *T. cruzi* PDF exhibits unique features thus providing a new avenue for the design of potential inhibitors for use in the treatment of diseases caused by trypanosomatid parasites.

N-terminal methionine excision (NME) is an essential co-translational process conserved from bacteria to eukaryotes that determines the cellular fate of proteins [1]. First, the initiator methionine is modified previously to translation initiation by the incorporation of a formyl group in a reaction catalyzed by a specialized enzyme, methionyl-$tRNA^{fMet}$ transformylase (MTF). The formyl group is necessary for optimal ribosomal translation [2]. Once the nascent protein emerges from the ribosomal tunnel, the formyl group must be removed for further recognition by methionine aminopeptidases. This enzymatic reaction plays a crucial role in the posttranslational maturation of proteins and is catalyzed by peptide deformylase (PDF), the enzyme responsible for the hydrolytic excision of the formyl group (Fig. S1). PDF constitutes a growing family of hydrolytic enzymes that contain, in addition to the HEXXH signature sequence strictly conserved in the zinc metalloprotease family, two short stretches of conserved amino acids, EGCLS and GXGXAAXQ, that altogether build up the substrate and metallic cofactor binding site [1]. PDF was previously believed to be a unique feature to eubacteria, where the enzyme is essential for viability. More recently, several studies have demonstrated that PDF orthologues are present and functional in eukaryotes [3] including several parasites of biomedical importance such as *Plasmodium* spp. [4] and species of the *Trypanosomatidae* family [5] but they have not been found in *Saccharomyces cerevisiae* or *Caenorhabditis elegans*. The enzyme is essential in procyclic forms of *Trypanosoma brucei* [5].

Trypanosoma cruzi is a kinetoplastid parasite that causes American trypanosomiasis or Chagas' disease which is one of the most prevalent neglected parasitic diseases in the American continent. In this organism, mitochondrial protein synthesis relies on imported $tRNA^{Met\text{-}e}$ that becomes formylated by MTF after import and is optimally used in translation initiation, while non-formylated $tRNA^{Met\text{-}e}$ is used in elongation [6]. The formyl group is the main determinant associated with the recognition of initiation factor 2 [7]. Recently it has been shown that mitochondrial translation is essential in the clinically relevant bloodstream form of *T. brucei* [8], which is an indication that formylation and probably deformylation might be functional in this stage of the parasite. In trypanosomatids, the mitochondrial DNA or kinetoplast encodes several proteins involved in oxidative phosphorylation and the respiratory chain [9]. All these proteins are putative substrates of PDF which emphasizes the potential importance of formylation-deformylation cycles in the mitochondrion of these parasites.

A comparison of the amino acid sequence of *T. cruzi* PDF-1 with other prokaryotic and eukaryotic PDFs reveals an overall low degree of similarity (Fig. S1). The three typical motifs of this family

Abbreviations: PDF, peptide deformylase; EDTA, ethylenediaminetetraacetic acid; f-MAS, formyl-methionyl-alanyl-serine.

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of metalloproteases involved in catalysis are conserved in the *T. cruzi* protein: motif 1 ^{114}GVGLAAPQ^{121} in *Arabidopsis thaliana* and ^{44}GIGLAATQ^{51} in *Escherichia coli*, motif 2 ^{176}EGCLS^{180} in *A. thaliana* and ^{89}EGCLS^{93} in *E. coli* and motif 3, HEXXH which is strictly conserved in all PDFs studied to the date. The Gly of motif 1 (Gly^{114} in *A. thaliana*, Gly^{44} in *E. coli*) and Leu of motif 2 (Leu^{179} in *A. thaliana*, Leu^{92} in *E. coli*) are crucial for catalysis and are substituted in *H. sapiens* for Cys^{108} and Glu^{173} in motif 1 (^{108}CVGLSAPQ^{115}) and motif 2 (^{170}EGCES^{174}) respectively. *T. cruzi* PDF-1 displays a similar substitution in motif 1, Cys^{83} (^{83}CISFSAPK^{90}) but maintains a hydrophobic Ile in motif 2 (^{134}ENCIS^{138}).

All eukaryotic PDF orthologues have an extended N-terminal domain which makes expression in bacterial systems difficult. This extension plays a role in organellar targeting of the protein to plastids and mitochondria in *A. thaliana* [1], to the apicoplast in apicomplexan parasites [4] and to the mitochondria in mammals [3]. In this study, we explored the properties of a truncated version of PDF-1 from *T. cruzi* lacking 37 amino acids from the amino terminus using the formylated tripeptide formyl-methionyl-alanyl-serine (f-MAS).

Initial attempts to produce a recombinant full-length soluble *T. cruzi* enzyme were unsuccessful. Considering previous reports on PDF from *H. sapiens* [3], and *Plasmodium falciparum* [4], we generated two deletion mutants that lacked the N-terminal 33 and 37 amino acids. The full-length *PDF-1* gene was amplified by PCR from *T. cruzi* CL Brener genomic DNA with primers 5′-GCC ATA TGC TGA GCC GTC TGT CAC GGA-3′ and 5′-GGA TCC CAT TGG TTC AGA CGA GCA TCC-3′, which were specifically designed according to the information obtained from the genome sequence database (GenBank accession number TcCLB.506871.100). Additional primers, 5′-GCC ATA TGG CGG AGG CGC AGG TGA AGT 3′ and 5′-GCC ATA TGG TGA AGT CCC GAG TGG CCT-3′, were designed to generate the truncated proteins where 33 (ΔN33) and 37 (ΔN37) amino acids were eliminated from the N-terminus respectively. The primers contain *Nde*I and *BamH*I restriction sites (underlined) for cloning into the pET-22b vector. The cloning procedure resulted in the addition of a six-histidine tag to the C-terminus of PDF. Of the two constructs tested, major amounts of soluble enzyme were obtained with *Tc*PDF-1ΔN37, a PDF lacking 37 amino acids from the amino terminus.

The purification and biochemical characterization of bacterial PDFs have largely remained a major challenge because the enzyme is extraordinarily labile due to oxidation of its catalytic Fe^{2+}. It has been shown that substitution of the ferrous ion for Co^{2+} or Ni^{2+} during the purification procedure of bacterial [10,11], *P. falciparum* [4] and human PDFs [12] gives highly active and stable enzyme preparations. Here *Tc*PDF-1ΔN37 protein was overexpressed in *E. coli* cells that were grown in medium enriched with Co^{2+} (100 μM). Under these conditions, *Tc*PDF-1ΔN37 was purified to over 99% purity by metal-affinity chromatography and exhibits an estimated molecular mass of ∼28–30 kDa (Fig. S2).

Truncation of the first 37 N-terminal amino acids gave a protein that proved to be catalytically active and exhibited kinetic parameters similar to other eukaryotic PDFs. A similar strategy was employed by Schneiders and coworkers who obtained an active version of *T. brucei* PDF-1 by eliminating the first 30 amino acids, thus suggesting the presence of a structural core responsible for deformylase activity [5].

The PDF activity assay was based on the quantification of formate using formate dehydrogenase [13]. The reaction mixture contained 50 mM KH_2PO_4, pH 7.5, 12 mM NAD^+, 1.2 U/ml FDH, and *Tc*PDF-1ΔN37 enzyme as indicated. The reaction was performed at 37 °C for 300 s and the resulting increase in absorbance of NADH at 345 nm was measured with a UV-VIS spectrophotometer.

The catalytic activity of *Tc*PDF-1ΔN37 was assessed with f-MAS as substrate. f-MAS is a bacterial PDF specific substrate used in the characterization of *E. coli*, human and *Plasmodium* peptide-deformylase activity. As shown in Fig. 1A, while the activity increased with substrate concentration up to 35 mM, it drastically dropped thereafter, showing that *Tc*PDF-1ΔN37 is inhibited by an excess of substrate. A similar substrate inhibition has been described for bacterial [14] and human PDFs [12]. Consequently, 20 mM f-MAS was used in the enzymatic assay henceforth. K_m and V_{max} values were obtained by hyperbolic regression fit of the data to the Hill equation using Sigma Plot® software. The K_m and V_{max} for f-MAS were 22.37 ± 0.137 mM and 200 ± 2.3 nmol min^{-1} mg^{-1}, respectively. The calculated k_{cat} value was 0.093 ± 0.065 s^{-1}. While we cannot fully discard that the His-tag may be detrimental to enzyme activity, these values are of similar order of magnitude to those described for other truncated recombinant eukaryotic enzymes when f-MAS was used as substrate although the V_{max} was significantly lower in comparison to that of bacterial PDFs [4,5,12].

It is evident that the peptide f-MAS used in this study is not the optimal substrate for *Tc*PDF-1. It was designed to characterize bacterial PDFs and is based on the fact that the complete bacterial proteome is associated to formylation. In contrast, formylation in trypanosomatids is limited to only 13 mitochondrial DNA-encoded proteins [9]. Recent studies on human PDF have shown that the enzyme is more efficient in deformylating substrates based on the organelle-encoded proteome than other nonspecific substrates including f-MAS [15]. We therefore designed a tripeptide based on the N-terminal sequence of subunit 7 of the NADH dehydrogenase complex (formyl-methionyl-leucyl-phenylalanine, f-MLP) coded by mitochondrial DNA but its high hydrophobicity made it inadequate for assay in aqueous media.

The influence of the ionic strength on deformylase activity was assessed by supplementation of the reaction buffer with increasing concentrations of KCl. An increase in ionic strength had a significant impact on *Tc*PDF-1ΔN37, giving rise to a stimulation of the activity which reached a maximum at 0.5 M KCl (Fig. 1B).

Next, we studied the effect of the nature and concentration of the metallic cofactor on *Tc*PDF-1 deformylation capacity. To this end, several oxidation insensitive divalent cations were added to the reaction buffer at different concentrations ranging from 10 to 100 μM. The enzyme was preincubated with the metallic cofactor for 10 min before initiating the reaction. While Co^{2+}, Mg^{2+}, Mn^{2+} did not affect significantly the activity, Ni^{2+} had a pronounced inhibitory effect on peptide deformylation at all concentrations tested. On the other hand, the addition of Cu^{2+} induced a 50% increase in the measured activity at 50 μM (Fig. 1C). To our knowledge, our study is the first to report the activation of a PDF enzyme by this metal ion. Both *P. falciparum and E. coli* PDFs differ from *Tc*PDF-1 and are highly active with both Co^{2+} and Ni^{2+}, retaining the same catalytic activity as the iron-native enzyme with the latter [4,10,11]. In contrast, human PDF is only active with Co^{2+} [12] while *A. thaliana PDF* is highly active when associated to Zn^{2+}.

The effect of the chelating agents EDTA and 1,10-phenantroline on deformylase activity was studied at concentrations ranging from 1 to 20 mM, and 1 to 5 mM respectively. *E. coli* PDF was included as a control assay. For this purpose, a segment of the *E. coli def* gene was amplified by PCR using forward primer 5′-CGC ATA TGT CAG TTT TGC AAG TGT TAC-3′ and reverse primer 5′-GGA TCC TTC AGC GGT GAC AGA TAA TCC AT-3′ and cloned in pET22b. The resulting expression construct codes for a bacterial PDF (*Ec*PDFΔC18) that lacks 18 C-terminal residues and contains a C-terminal His-tag. The conditions for expression and purification were similar to those used for *Tc*PDF-1ΔN37 (see supplementary information). Purified *Ec*PDFΔC18 was active and more stable than the full-length enzyme [16]. Before measuring the activity, the enzyme was preincubated with the chelating agent for 10 min. Strikingly, both EDTA and 1,10-phenantroline strongly activated deformylase activity while 1,10-phenantroline was a potent inhibitor of

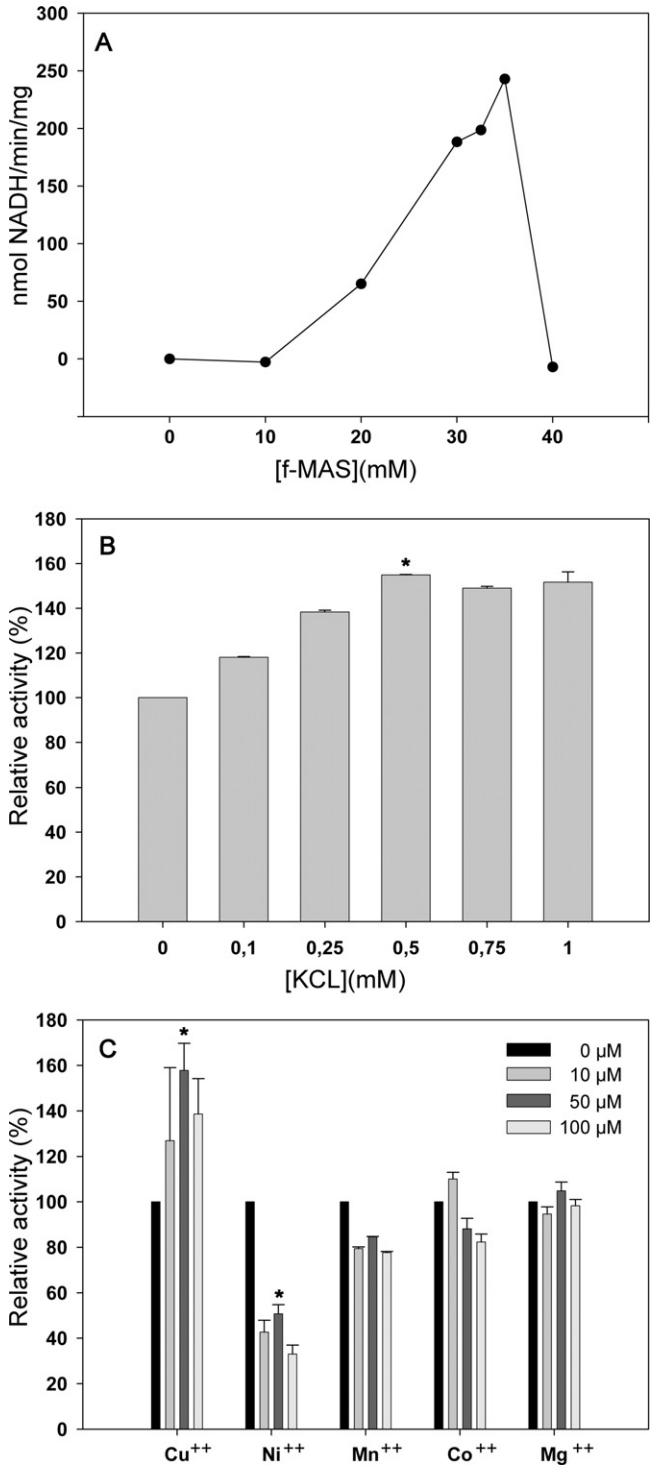

Fig. 1. Substrate inhibition, divalent ion requirements and influence of ionic strength on Δ37*Tc*PDF-1. (A) Substrate inhibition. The concentration of f-MAS ranged between 10 and 40 mM. The activity was measured as a function of substrate f-MAS using the formate dehydrogenase-coupled PDF assay. The experiments are representative of at least 2 independent determinations. (B) Influence of ionic strength on the relative rate of deformylation of Δ37*Tc*PDF-1. Relative activity of 100% was assigned to the rate value obtained under standard assay conditions. (C) Graphical representation of the effect of several divalent metallic cations on deformylase activity using standard assay conditions (50 mM KH_2PO_4, 12 mM NAD^+, 0.1 mg/ml Δ37*Tc*PDF-1 and 20 mM f-MAS). The error bars correspond to standard deviations. The asterisk indicates significant differences with control data ($p < 0.05$; Student's *t* test).

the *E. coli* enzyme. In the case of EDTA, *Tc*PDF-1ΔN37 activity increased up to 70% at 10 mM (Fig. 2A) while a stunning 6-fold increase was observed in the presence of 1 mM 1,10-phenantroline (Fig. 2B). This unusual observation suggests that an unknown divalent cation metal with inhibitory properties is strongly associated to the enzyme in our preparation. We can hypothesize that other active divalent cations present in the bacterial expression system during cell growth or in the chemicals used for enzyme purification such as inhibitory Ni^{2+} ions could have been incorporated to the enzyme active site during the purification process.

The occurrence of the HEXXH motif involved in metal-binding characteristic of zinc metalloproteases and present in all PDFs, prompted us to explore whether the parasite enzyme would be inhibited by a series of previously characterized metalloprotease inhibitors. Bestatin is a compound originally isolated from *Streptomyces olivoreticulli* and was one of the first potent inhibitors of metallo-aminopeptidases with broad spectrum of action and has been extensively investigated in biological systems both in *vitro* and *in vivo*. Other compounds tested included CP-101537, a matrix metalloprotease (MMP) inhibitor and CL-82198 which is a selective inhibitor of MMP-13. However, none of the compounds had significant effect on PDF-1 activity at concentrations up to 100 μM.

Actinonin is a naturally occurring inhibitor of bacterial peptide deformylase with an IC_{50} value of 0.8–90 nM, depending on the metal cation of the bacterial enzyme [17]. The inhibitor has been shown to be also active on eukaryotic PDFs such as human, *Plasmodium* and plants both *in vitro* and *in vivo* [4,12,18]. Wiesner *et al.* have reported that actinonin inhibits the growth of both wild-type *P. falciparum* (IC_{50} = 3.0 μM) and a multidrug-resistant strain (IC_{50} = 3.6 μM) [18]. In addition, actinonin inhibits human deformylase activity with an IC_{50} of 43 nM [12].

Here, we examined the inhibitory effect of actinonin at concentrations up to 100 μM which completely inhibit the activity of *E. coli* PDF, yet this compound had no impact on the *T. cruzi* deformylase activity (Fig. 2C). The antiproliferative effects of actinonin were also evaluated on the epimastigote form of *T. cruzi* in culture (Fig. 2D). The drug induced a dose-dependent inhibition of growth with an IC_{50} of 500 μM. These observations suggest that the structural elements that determine actinonin binding in other PDF enzymes may be absent in this parasite. Several crystallographic studies available describe the binding of actinonin to representative members of type I and type II PDFs [19]. In *E. coli PDF*, the actinonin binding site consists in a deep invagination that involves 18 residues. Four of these amino acids are strictly conserved in eukaryotic PDFs, including *A. thaliana*, *Plasmodium* spp. and human enzymes; three of them, Gly^{46}, Leu^{47} and Gln^{51} are involved in the interaction with the hydroxamate moiety of actinonin and the fourth, Gly^{90}, contacts its valine group. In *T. cruzi*, the actinonin-binding residues described for *E. coli* and *H. sapiens* are substituted by Ser^{85}, Phe^{86}, Lys^{90} and Asn^{135} respectively, thus providing a reasonable explanation for the apparent insensitivity of the parasite PDF to actinonin (Fig. S1). To elucidate whether the absence of one or more of these residues, and in particular of Gly^{90}, is responsible for lack of inhibition of *Tc*PDF-1, a systematic mutation analysis is currently underway. These studies together with the structural characterization of *Tc*-PDF1 would help to identify the residues that shape the inhibitor binding site in PDFs. This information would be especially relevant in the design of compounds with improved activity and in devising strategies to overcome potential drug resistance resulting from point mutations.

In summary, we have purified and characterized biochemically one of the PDF orthologues identified in the *T. cruzi* genome and demonstrated that the enzyme possesses deformylating activity *in vitro*. The enzyme is strongly modulated by the nature of the metallic cofactor and the ionic strength suggesting that the activity might be physiologically relevant under the adequate reaction conditions. The unique features regarding metal ion requirements

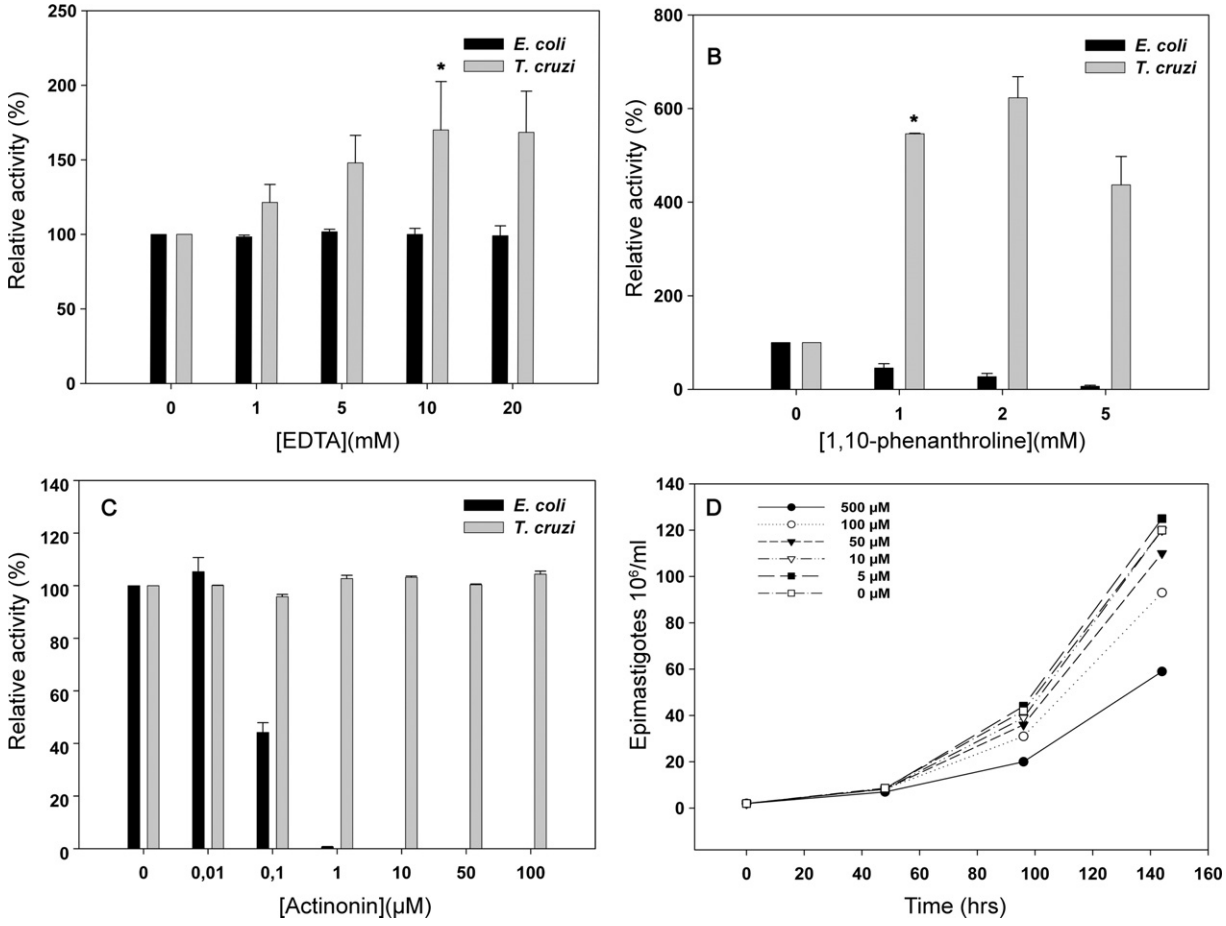

Fig. 2. Effect of chelating agents and inhibitors on the deformylase activity of Δ37*Tc*PDF-1 and *Ec*PDFΔC18 and on the growth of *T. cruzi* epimastigotes. (A) EDTA. (B) 1,10-phenantroline. The enzyme was incubated for 5 min at 37 °C with the indicated concentration of the chelating agent. The asterisk indicates significant differences with control data ($p < 0.05$; Student's *t* test). (C) Effect of actinonin on the deformylase activity of Δ37*Tc*PDF-1 and *Ec*PDFΔC18. The enzyme was incubated for 5 min at 37 °C in the presence of increasing concentrations of compound. Relative activity of 100% was assigned to the rate value obtained in the standard assay (50 mM KH_2PO_4, 12 mM NAD^+, 0.1 mg/ml Δ37*Tc*PDF-1 and 20 mM f-MAS). Assay conditions for *Ec*PDFΔC18 were 50 mM KH_2PO_4, 12 mM NAD^+, 0.005 mg/ml enzyme and 2 mM f-MAS. The error bars correspond to standard deviations. (D) Effect of actinonin on the proliferation of *T. cruzi* epimastigotes. The (Y) strain of the epimastigote form of *T. cruzi* was cultured in liver infusion tryptose (LIT) medium, supplemented with 10% newborn calf serum (Invitrogen) at 28 °C with agitation. For determination of inhibitor sensitivity, the cultures were initiated at 2×10^6 epimastigotes/ml and cell densities were measured with an electronic particle counter (model Z1 Beckmam Coulter™). Time zero corresponds to the moment of addition of the drug.

and inhibition profile exhibited by *T. cruzi* PDF and the observation that PDF-1 is essential in procyclic forms of *T. brucei* [5], support the study of trypanosomal peptide deformylase as a biochemical target for antiprotozoan drug design.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molbiopara.2011.12.003.

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