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Phenothiazine Radicals Inactivate *Trypanosoma cruzi* Dihydrolipoamide Dehydrogenase: Enzyme Protection by Radical Scavengers

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Phenothiazine cation radicals ($PTZ^{+\bullet}$) irreversibly inactivated *Trypanosoma cruzi* dihydrolipoamide dehydrogenase (LADH). These radicals were obtained by phenothiazine (PTZ) peroxidation with myeloperoxidase (MPO) or horseradish peroxidase (HRP/H_2O_2) systems. LADH inactivation depended on PTZ structure and incubation time. After 10 min incubation of LADH with the MPO-dependent systems, promazine, trimeprazine and thioridazine were the most effective; after 30 min incubation, chlorpromazine, prochlorperazine and promethazine were similarly effective. HRP-dependent systems were equally or more effective than the corresponding MPO-dependent ones. Chloro, trifluoro, propionyl and nitrile groups at position 2 of the PTZ ring significantly decreased molecular activity, specially with the MPO/H_2O_2 systems. Comparison of inactivation values for LADH and *T. cruzi* trypanothione reductase demonstrated a greater sensitivity of LADH to chlorpromazine and perphenazine and a 10-fold lower sensitivity to promazine, thioridazine and trimeprazine. Alkylamino, alkyl-piperidinyl or alkyl-piperazinyl groups at position 10 modulated PTZ activity to a limited degree. Production of $PTZ^{+\bullet}$ radicals was demonstrated by optical and ESR spectroscopy methods. $PTZ^{+\bullet}$ radicals stability depended on their structure as demonstrated by promazine and thioridazine radicals. Thiol compounds such as GSH and *N*-acetylcysteine, L-tyrosine, L-tryptophan, the corresponding peptides, ascorbate and Trolox, prevented LADH inactivation by the MPO/H_2O_2/thioridazine system, in close agreement with their action as $PTZ^{+\bullet}$ scavengers. NADH (not NAD^+) produced transient protection of LADH against thioridazine and promazine radicals, the protection kinetics being affected by the relatively fast rate of NADH oxidation by these radicals. The role of the observed effects of PTZ radicals for PTZ cytotoxicity is discussed.

Keywords: Dihydrolipoamide dehydrogenase; *Trypanosoma cruzi*; Phenothiazines; Cation radical; Myeloperoxidase; Horseradish peroxidase

Abbreviations: *T. cruzi* LADH dihydrolipoamide dehydrogenase (E.C. 1.6.4.3); TR trypanothione reductase; MPO myeloperoxidase; HRP horseradish peroxidase; PTZ phenothiazine; CPZ chlorpromazine; FFZ fluphenazine; PCP prochlorperazine; PCYZ propericyazine; PFZ perphenazine; PMTZ promethazine; PPZ propionylpromazine; PZ promazine; TFP trifluoperazine; TFPZ trifluopromazine; TMPZ trimeprazine; TRDZ thioridazine; NAC *N*-acetylcysteine; CPT captopril [1-(3-mercapto-2-methyl-1-oxopropyl-1-proline)]; PAM penicillamine (3-mercapto-D-valine); DMPO 5,5-dimethyl-1-pyrroline-*N*-oxide; Trolox 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid

Structures: PTZ, dibenzothiazine; PZ, 10-(3-dimethylaminopropyl)-PTZ; TMPZ, 10-(2-methyl-3-dimethyl aminopropyl)-PTZ; PMTZ, 10-(2-dimethylaminopropyl)-PTZ; CPZ, 2-chloro-10-(3-dimethylaminopropyl)-PTZ; PCP, 2-chloro-10-[3-(1-methyl-4-piperazinyl)-propyl]-PTZ; PFZ, 2-chloro-10-[3-[1-(2-hydroxyethyl)-4-piperazinyl]propyl]-PTZ; TRDZ, 2-methylmercapto-10-[2-(1-methyl-2-piperidinyl)-ethyl]-PTZ; TFPZ, 2-trifluoromethyl-10-[3-(dimethylamino)propyl]-PTZ; TFP, 2-trifluoro-methyl-10-[3-(1-methyl-4-piperazinyl)propyl]-PTZ; FFZ, 2-trifluoromethyl-10-[3-[1-(2-hydroxyethyl)-4-piperazinyl]propyl]-PTZ; PPZ, 2-propionyl-10-(3-dimethylaminopropyl)-PTZ; PCYZ, 2-cyano-10-[3-(4-hydroxypiperidinyl) propyl]-PTZ

INTRODUCTION

Phenothiazines (PTZs) are potential chemotherapeutic agents against *Trypanosoma cruzi*, the causative

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agent of American trypanosomiasis (Chagas' disease).[1–9] CPZ,[5,6] PMTZ,[7] TFPZ,[5,6] TFP[3,4] and TRDZ[8,9] exert various activities in *T. cruzi*, namely, disruption of cell membranes, inhibition of cyclic nucleotides phosphodiesterase, inhibition of epimastigote proliferation, prevention of *T. cruzi* infection in mice and inhibition of trypanothione reductase (TR).[10,11] In a previous study,[12] we demonstrated that PTZ cationic radicals ($PTZ^{+\bullet}$) inhibit *T. cruzi* TR at a relatively low concentration, as compared with TR inhibition by the neutral molecule.[11] Moreover, TR inhibition was irreversible,[12] a circumstance enhancing the relevance of cation radicals cytotoxicity in *T. cruzi*. Thiol compounds, such as GSH, prevented TR inactivation.[12] Preliminary observations demonstrated that chlorpromazine (CPZ) cation radicals exerted a similar action on *T. cruzi* LADH,[13] an enzyme that, like TR, has been postulated as a possible target for trypanocidal drugs.[14,15] LADH reversibly catalyses NADH dependent reduction of lipoamide (Reaction (1): LA, lipoamide and $LA.H_2$, dihidrolipoamide) (Reaction (1)). The enzyme is

$$NADH + H^+ + LA \rightarrow NAD^+ + LA.H_2 \qquad (1)$$

a component of alpha-oxo acid dehydrogenase complexes, such as pyruvate dehydrogenase, alpha-oxoglutarate and branched-chain oxoacid dehydrogenase complexes, all of them playing a major role in cell metabolism. Accordingly, we have now extended our observation on *T. cruzi* LADH inactivation by PTZ cation radicals, using a series of structurally different PTZs and a highly purified LADH preparation.[16]

In order to produce the $PTZ^{+\bullet}$ radical cations for LADH-inactivation, we used the MPO/H_2O_2 systems. MPO plays a major role in drug peroxidation in neutrophils, monocytes and macrophages,[17–21] a set of cells accessible to PTZs *in vivo*. The following topics deserved special attention: (a) PTZ structure/activity relationships; (b) peroxidase influence on PTZ/H_2O_2 systems activity and; (c) the action of cation radical scavengers which, under physiological conditions, may counteract PTZs toxicity. These scavengers include, thiol compounds, amino acids and LADH substrate NADH.

MATERIALS AND METHODS

Enzyme Preparations

Recombinant LADH from *T. cruzi* was obtained by expression in *Escherichia coli* JRG 1342 cells as described.[16] The enzyme was stored in 100 mM K-phosphate, 100 mM KCl, 1.0 mM EDTA, pH 7.0, containing 50% (v/v) glycerol, at −20°C and remained stable for at least one year. Specific activity was about 400 U/mg protein. LADH stock solution was diluted 25% (v/v) with 50 mM K-phosphate, pH 7.4 and kept at 4°C for experiments. Porcine heart lipoamide dehydrogenase, human leukocyte MPO and HRP were obtained from Sigma Chemical Co, St. Louis, MO, USA. MPO specific activity ranged from 50 to 100 U/mg as determined by the standard guaiacol/H_2O_2 assay.[22] Other experimental conditions were as described previously.[12,13]

Reagents

PTZ were obtained from Sigma Chemical Company. $PTZ^{+\bullet}$ radicals were prepared by oxidation with peroxidase/H_2O_2 systems in 50 mM K-phosphate, pH as described under Results. Cation radical concentrations were determined spectrophotometrically in an Aminco DW UV/VIS (SLM) spectrophotometer in 50 mM K-phosphate, pH 6.5 using the following wavelengths (in nm) and ϵ values (in $M^{-1}.cm^{-1}$, in parenthesis): PZ, 518 (7714); TRDZ, 633 (7682).[23–25] NADH, NAD^+, Tyr, Trp, Gly, His, Met and ascorbic acid were obtained from Sigma Chemical Co, St. Louis, MO, USA. Trolox was from Aldrich Chemical Company Inc., Milwaukee, WI, USA. Peptides TyrHis, TyrGly, TrpGly and $(TyrCys)_2$ were from Bachem Biosciences Inc., King of Prussia, PA, USA. Other reagents were as described previously.[12,13]

LADH Inactivation

The LADH inactivation mixture contained LADH, peroxidase, H_2O_2, K-phosphate and additions, in a final volume of 0.1 ml, as stated in the "Results" Section. The mixture also contained 1.25 mM KCl, 12.5 μM EDTA, and 82 mM glycerol, resulting from dilution of the original LADH suspension. Control experiments (omitted) showed that glycerol failed to affect LADH or cation radical activity. Samples were incubated at 30°C for the respective time; 10-μl aliquots were added to the assay mixture and residual LADH activity was measured as described in the following section. Control samples without peroxidase or other components of the inactivation mixture were incubated simultaneously.

LADH Assay

LADH activity was measured at 30°C by the rate of NADH oxidation using lipoamide as electron acceptor.[12,13] The standard assay mixture contained 10 μl inactivation mixture, 0.2 mM NADH, 1.0 mM lipoamide, and 50 mM K-phosphate, pH 7.4; total volume, 3.0 ml.

ESR

Measurements were performed in a Bruker ER 106 ESR spectrometer (Bruker Analytische Messtechnik, Karlsruhe, Germany) at room temperature. General instrumental conditions were: microwave power, 20 mW; modulation frequency, 50.00 kHz; microwave frequency, 9.75 GHz; modulation amplitude, 0.948G; time constant, 1310 ms; scan rate, 167.7 s; and gain, 1×10^5. The reaction mixture composition was: 1.5 U/ml MPO, 0.5 mM H_2O_2, 100 μM TMPZ (or PMTZ), 50 mM DMPO and 50 mM K-phosphate, pH 7.4; total volume, 0.2 ml. Control samples without PTZ or MPO were also examined.

Radical Scavengers Assay

Scavenger activity was measured by their action on $PTZ^{+\bullet}$ radical production by the peroxidase/H_2O_2/PTZ system. Unless stated otherwise, the reaction mixture contained 0.5 U/ml HRP or MPO, 0.2 mM H_2O_2, 100 μM PTZ and 50 mM K-phosphate, pH 6.5; final volume, 3.0 ml. In most of these experiments, HRP was used in order to obtain the greatest radical production. Scavengers were added either at the beginning of incubation or when $PTZ^{+\bullet}$ radical concentration reached its maximum value, using a fast-mixer device. Radical concentration was measured spectrophotometrically, as described above.

Expression of Results

Taking into account the variation in LADH specific activity throughout the period of experimental work, relative activity values are presented as a percentage value of control sample activity (100%). LADH inactivation (I%) by the pro-oxidant systems was calculated from the equation $I(\%) = 100(A_c - A_{in}/A_c)$, where A_c and A_{in} are the activities of the control sample and the inactivated sample, respectively. Protection (P) by $PTZ^{+\bullet}$ presumptive scavengers against the peroxidase/H_2O_2/PTZ systems was calculated from the equation $P(\%) = 100(I(\%) - ip(\%)/I(\%))$, where P, I and ip are relative radical scavenger activity (LADH protection), inhibition of LADH activity by the peroxidase/H_2O_2/PTZ system, and inhibition of LADH activity by the latter system plus scavenger (P), respectively. Values presented are means ± SD. Statistical analysis was performed with the INSTAT programme using Student's t-test for one or two samples.

RESULTS

LADH Inactivation by $PTZ^{+\bullet}$ Radicals

LADH was inactivated by the MPO/H_2O_2/PTZ systems, as a function of PTZ concentration and incubation time. Figure 1 shows the time-dependent loss of LADH activity with the MPO/H_2O_2/PZ system which is representative of other systems studied. With 100 μM PZ, LADH inactivation approached a maximum and after 10 min incubation, 95% inactivation was observed. Lower concentrations of PZ produced less LADH inactivation within the same time. At 10 μM PZ, about 50% of LADH activity was inhibited within 30 min. Omission of PZ from the reaction mixture prevented LADH inactivation, ruling out a direct action of the MPO/H_2O_2 system on LADH. Table I summarizes the effect of different PTZ systems on *T. cruzi* LADH activity. PTZs were assayed at a fixed 100-μM concentration which allowed immediate comparison of effects. After 10 min incubation with the MPO/H_2O_2/PTZ systems, inactivation values for TMPZ, PZ, TRDZ, PMTZ and PCP ranged from 50 to 90%. The most effective PTZs were those with H (PZ, TMPZ, PMTZ) and a mercaptomethyl group (TRDZ) in position 2, followed by those with chloro in the same position (PCP, CPZ, PFZ). Molecules with propionyl, fluoromethyl or cyano groups at position 2 (PPZ, TFP, TFPZ, FFZ and PCYZ) were much less or not active. After 30 min incubation, LADH inactivation values for chloro substituted PTZs (PCP, CPZ and PFZ) approached levels obtained with the more active PTZs. Comparison of PCP, CPZ and PFZ, all having chloro at position 2 but diverse substituents at position 10 failed to show major differences in their action (Table I). Comparative

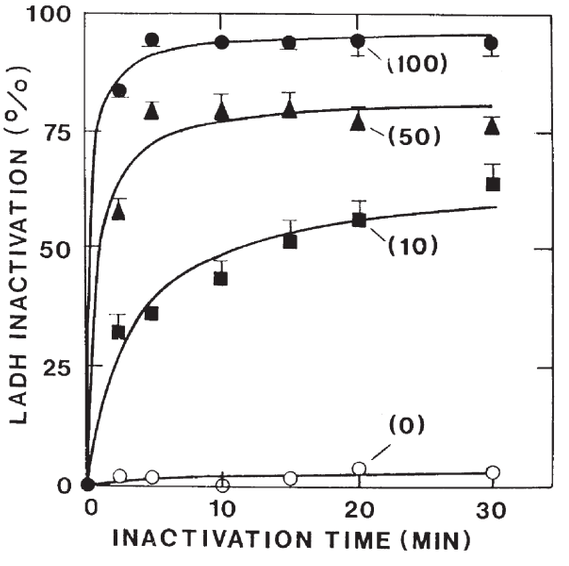

FIGURE 1 Effect of PZ concentration on LADH inactivation by the MPO/H_2O_2/PZ system. In a total volume of 0.1 ml, the inactivation mixture contained 0.8 μM LADH, 0.5 U/ml MPO, 0.1 mM H_2O_2, 50 mM K-phosphate, pH 7.4 and PZ at the concentration (μM) indicated in the figure; incubation time as indicated on the abscissa. Other conditions as described under "Materials and Methods" Section. Values represent means ± S.D. ($n = 3$–5) for all single values, $P < 0.05$.

TABLE I Inactivation of *T. cruzi* LADH by peroxidase/H_2O_2/PTZ system*

	LADH inactivation (%)		
	MPO/H_2O_2/PTZ system		HRP/H_2O_2/PZ system
Phenothiazine	Incubation: 10 min	Incubation: 30 min	Incubation: 10 min
PZ	87 ± 3	89 ± 1	94 ± 1
TMPZ	90 ± 1	90 ± 1	94 ± 1
TRDZ	77 ± 4	82 ± 3	85 ± 1
PMTZ	60 ± 3	79 ± 1	72 ± 2
PCP	54 ± 2	80 ± 1	90 ± 2
CPZ	45 ± 1	75 ± 1	89 ± 4
PFZ	42 ± 1	69 ± 1	79 ± 3
PPZ	11 ± 1†	32 ± 3	83 ± 7
TFP	5 ± 1†	16 ± 1†	67 ± 2
FFZ	1 ± 1†	8 ± 1†	61 ± 3
TFPZ	2 ± 1†	2 ± 1†	16 ± 1
PCYZ	0†	0†	0†

* The inactivation mixture contained: 0.8 μM *T. cruzi* LADH; 0.5 U/ml MPO or HRP; 0.1 mM H_2O_2 (with MPO); 0.20 mM H_2O_2 (with HRP); 100 μM PTZ and 50 mM K-phosphate, pH 7.4. Other conditions as in Fig. 1 legend and "Materials and Methods" Section. Incubation time as indicated above. Values represent means ± S.D. Statistical one-sample *t*-test analysis was performed using 0 ± 5 (theoretical mean ± S.D. (%) as control value. † $P < 0.001$; except values that were not significant.

assays with the MPO/H_2O_2/PTZ systems, using porcine heart lipoamide dehydrogenase, yielded the following inactivation values (%; 30 min incubated samples): PZ, 63 ± 2; TMPZ, 45 ± 1.0; PMTZ, 41 ± 1.0; TRDZ, 51 ± 2.0 and CPZ, 44 ± 1.5, which underline the greater sensitivity of *T. cruzi* LADH to the /PTZ systems. The HRP/H_2O_2/PTZ systems proved to be more effective than their MPO/H_2O_2 counterparts (Table I). In fact, after 10 min incubation, LADH inactivation values were above those observed with MPO/systems. The difference was noteworthy with most of the assayed PTZs. Direct inhibition of LADH by PTZs was investigated by adding 100 μM PTZ to an assay mixture containing 3.3 nM LADH, 0.2 mM NADH, 1.0 mM lipoamide and 50 mM K-phosphate, pH 7.4. Percentage inhibition values obtained (in parenthesis, the PTZ used) were 3.2 ± 1 (PZ), 0 (CPZ); −1.0 ± 0.9 (TMPZ), 0.5 ± 0.5 (PMTZ), 5.0 ± 1.5 (PCP) and 3.0 ± 1.0 (TRDZ). These results ruled out a direct effect of PTZs on LADH.

$PTZ^{+\bullet}$ Radical Production by the Peroxidase/H_2O_2/PTZ Systems

Production of $PTZ^{+\bullet}$ radicals was demonstrated spectroscopically using the HRP/H_2O_2/PZ or /TRDZ systems. Such radicals presented characteristic transient absorption peaks at 518 (PZ) and 633 (TRDZ) nm (Fig. 2). The time-course of radical production resulted from two opposite reactions, namely, (a) PTZ peroxidation and (b) $PTZ^{+\bullet}$ radical decay by dismutation or other reactions, depending on the PTZ structure as shown by the dissimilar kinetics observed with the PZ and TRDZ systems. The influence of PTZ structure on radical production was confirmed by the different rates of $PCP^{+\bullet}$, $PFZ^{+\bullet}$, $TFP^{+\bullet}$ and $FFZ^{+\bullet}$ production catalysed by the HRP/H_2O_2/system (Fig. 2 inset). Under these experimental conditions, the Cl-substituted molecules produced the corresponding radical whereas the CF_3 substituted derivatives were much less or not effective. The MPO/H_2O_2/CPZ system produced the $CPZ^{+\bullet}$ radical detectable by its ESR signal, as previously demonstrated.[12,26] Under the experimental conditions described under "Materials and Methods" section, $TMPZ^{+\bullet}$ and $PMTZ^{+\bullet}$ radicals

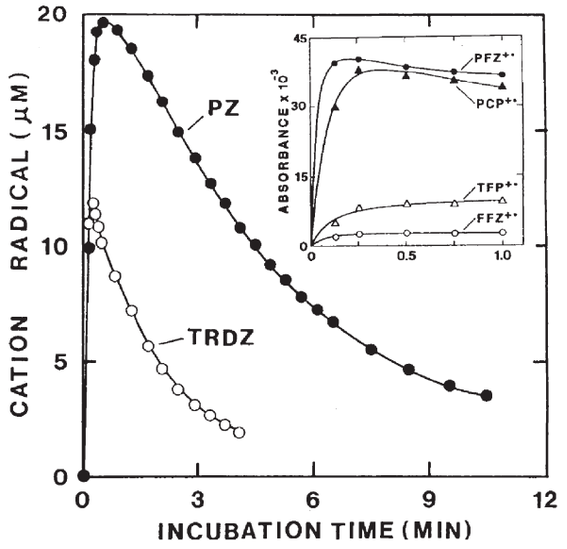

FIGURE 2 Time-dependent production and decay of $PZ^{+\bullet}$ and $TRDZ^{+\bullet}$ cation radicals. The reaction mixture contained 0.5 U/ml HRP, 0.5 mM H_2O_2, 100 μM PZ or TRDZ and 50 mM K-phosphate, pH 6.5; total volume, 3.0 ml. The reaction was started by adding H_2O_2 and absorbance was measured at 518 (PZ) or 633 (TRDZ) nm. Other experimental conditions as described in "Materials and Methods" Section. Inset: same experimental conditions, except for the PTZ (PCP, PFZ, TFP and FFZ). Incubation times as indicated on the abscissa. Absorbance was measured at 532 (PCP), 525 (PFZ), 500 (TFP and FFZ) (nm). Typical experiments.

were produced but their concentrations were relatively small, so that these radicals were detected as their DMPO radical adducts (spectra omitted).

LADH Protection by Thiols, Amino Acids and Antioxidants against $PTZ^{+\bullet}$ Radicals

Table II shows that thiol compounds prevented LADH inactivation by the $MPO/H_2O_2/TRDZ$ system. Thiols were added to the inactivation mixture before the $MPO/H_2O_2/TRDZ$ system. GSH, NAC and CPT strongly protected LADH (>88%), but PAM was somewhat less effective (71% protection). Under the same experimental conditions, GSH and NAC prevented by 97–100% the action of $MPO/H_2O_2/TMPZ$ or $HRP/H_2O_2/TRDZ$ systems (experimental data omitted). The consistent effects of thiol compounds strongly supported the anti-radical role of the thiol group. Finally, addition of 0.2 mM GSH to a LADH sample inactivated by the $MPO/H_2O_2/TRDZ$ system as described in Table II failed to restore LADH activity, thus supporting the operation of an irreversible LADH inactivating mechanism depending on $TRDZ^{+\bullet}$ radicals. MPO was used in these experiments considering the possibility of PTZ activation by MPO in MPO-containing cells.

Table II also shows the effect of amino acids and peptides on LADH inactivation by the $MPO/H_2O_2/TRDZ$ system. L-Tyrosine and its peptides produced roughly 70% protection of LADH after 10 and 30 min incubation. L-Tryptophan and its peptide also showed protective action, though less than L-tyrosine and its peptides. Interestingly enough, under similar conditions, glycine, L-histidine and L-methionine were ineffective (experimental data omitted), underlining the specificity of L-tyrosine and L-tryptophan effects. Finally, two general antioxidants, ascorbate and Trolox were found to protect LADH effectively against the $MPO/H_2O_2/TRDZ$ system (Table II). Ascorbate is known to be a CPZ radical scavenger[27] and Trolox is also a well known effective antioxidant. Figure 3 shows the time-course of LADH protection by L-tryptophan, L-tyrosine and GSH against the $MPO/H_2O_2/PZ$ system. With GSH and L-tyrosine, protection agreed generally with that observed with TRDZ (Table II) and remained constant throughout incubation time. However, with $MPO/H_2O_2/PZ$, L-tryptophan was less effective than with the /TRDZ system. Significant LADH protection was observed only at 2.5 and 5.0 min of incubation, the corresponding values being 21 ± 2.0 and 10 ± 1.0%, respectively (mean ± S.D.; in both cases $P < 0.001$).

Protection of LADH by thiols, aminoacids and antioxidants involved scavenging of $PTZ^{+\bullet}$ radical. Figure 4 show GSH effect on $PZ^{+\bullet}$ decay after its production by the $HRP/H_2O_2/PZ$ system. PZ was chosen for this experiment because of the relatively slow rate of $PZ^{+\bullet}$ radical decay. It is to be seen that addition of GSH to the reaction mixture after 30 s incubation produced a fast decay of $PZ^{+\bullet}$ concentration (Fig. 4; plot A). GSH might, however, affect $PZ^{+\bullet}$ production by inhibiting the HRP/H_2O_2 system activity. In order to test that possibility, catalase was added to the reaction mixture after 14 s incubation (Fig. 4, plot B), in order to decompose

TABLE II Protection of LADH by thiol compounds, aminoacids and other scavengers against the $MPO/H_2O_2/TRDZ$ system*

$TRDZ^{+\bullet}$ scavenger	LADH inactivation (%)
Thiol compounds	
None	84 ± 3
GSH	10 ± 1 (88)
NAC	2 ± 1 (98)
CPT	9 ± 2 (89)
PAM	24 ± 1 (71)
Amino acids and peptides	
None	84 ± 3
Tyr	24 ± 1 (71)
TyrGly	18 ± 1 (80)
TyrHis	27 ± 2 (68)
(TyrCys)2	24 ± 1 (71)
Trp	54 ± 3 (36)
TrpGly	33 ± 1 (61)
Other scavengers	
Ascorbate	1.0 ± 0 (99)
Trolox	2.0 ± 1.0 (98)

*The inactivation mixture contained 0.8 μM LADH, 0.5 U/ml MPO, 0.1 mM H_2O_2, 100 μM TRDZ, 50 mM K-phosphate, pH 7.4 and 0.2 mM scavenger as indicated above. Incubation time, 30 min. Other conditions are described in "Materials and Methods" Section. Values in parenthesis indicate LADH protection (%). Statistical analysis was performed using the two-sample *t*-test method. $P < 0.005$ for all protection values.

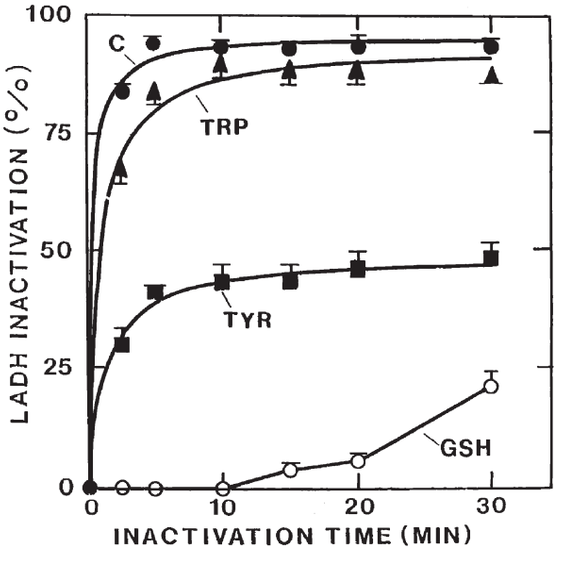

FIGURE 3 Effect of L-tryptophan, L-tyrosine and GSH on the time-course of LADH inactivation by the $MPO/H_2O_2/PZ$ system. The inactivation mixture contained 0.8 μM LADH, 0.5 U/ml MPO, 0.1 mM H_2O_2, 100 μM PZ and 50 mM K-phosphate pH 7.4. 0.2 mM L-tryptophan (TRP), L-tyrosine (TYR) or GSH were added as indicated in the figure. Other experimental conditions as in Fig. 1 legend. C, control sample, without scavenger.

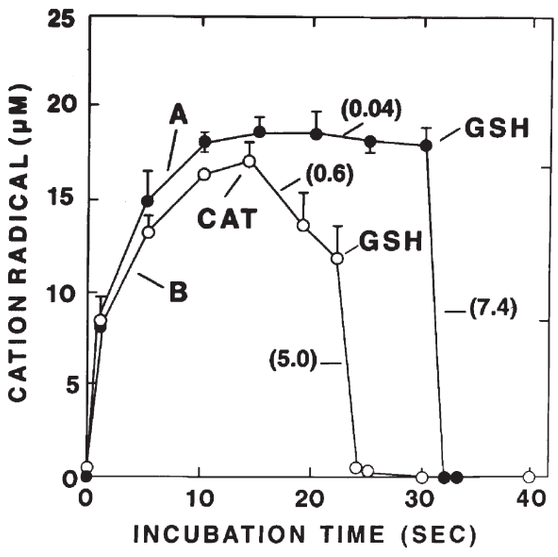

FIGURE 4 Effect of GSH and catalase on $PZ^{+\bullet}$ production and decay. The reaction mixture contained 0.5 U/ml HRP, 0.2 mM H_2O_2, 100 μM PZ and 50 mM K-phosphate, pH 6.5; total volume, 3.0 ml. The reaction was started by adding H_2O_2, and absorbance was measured at 518 nm. 0.2 mM GSH and 250 U/ml catalase (CAT) were added as indicated in the figure. Other conditions as described under "Materials and Methods" section. The figures in parenthesis indicate production of $PZ^{+\bullet}$ (μM/s). A and B as explained in the text.

the H_2O_2 present in the reaction mixture as HRP substrate. Catalase enhanced to a limited extent $PZ^{+\bullet}$ decay as indicated by the corresponding rate values (Fig. 4, plot B), thus evidencing the production of $PZ^{+\bullet}$ at a rate that in the absence of catalase (plot A) balanced the dismutation rate of $PZ^{+\bullet}$. Addition then of GSH to the catalase-supplemented sample, increased $PZ^{+\bullet}$ decay rate about 10-fold (Fig. 4, plot B), that approached the value observed after GSH addition to the catalase-free control sample (Fig. 4, plot A). These observations support the notion that GSH action was mainly due to $PZ^{+\bullet}$ scavenging. The scavenging action of other thiol compounds and amino acids was confirmed using the same experimental model and the results obtained are summarized in Table III. All the assayed compounds decreased $PZ^{+\bullet}$ concentration, the greater effects being produced by NAC and PAM. L-Tyrosine and L-tryptophan also decreased $PZ^{+\bullet}$ concentration but less than the thiol compounds, as occurred with their action as LADH protectors (Table II).

Table IV summarizes the action of LADH protectors on $TRDZ^{+\bullet}$ production by the HRP/H_2O_2/TRDZ system when added to the reaction mixture before the TRDZ system. GSH, NAC, CPT, PAM and the other anti oxidants behaved similarly keeping $TRDZ^{+\bullet}$ concentration at a level about 15% or less of control value. L-Tyrosine, TyrHis and L-tryptophan were less effective. In order to investigate the role of further HRP inhibition in $TRDZ^{+\bullet}$ production and consequently, in LADH protection, LADH protectors were assayed on MPO and HRP activity, measured with guaiacol as substrate. The results obtained indicated that L-tyrosine and L-tryptophan failed to inhibit the peroxidase reaction but GSH scavenged guaiacol peroxidation products, thus interfering with peroxidase activity measurement (data omitted).

Further evidence for PTZ radical/scavenger interaction was obtained by using the TRDZ-dependent system, under experimental conditions

TABLE III Effect of thiol compounds and aminoacids on $PZ^{+\bullet}$ radical decay*

$PZ^{+\bullet}$ scavenger (μM)	$PZ^{+\bullet}$ concentration after incubation (μM)
Thiol compounds	
None	22 ± 0.6
GSH (100)	11 ± 0.1 (50)
GSH (200)	3.0 ± 0.8 (86)
NAC (100)	5.0 ± 1.3 (77)
PAM (100)	8.0 ± 0.9 (63)
Amino acids and peptides	
None	26 ± 1.3
Tyr (100)	16 ± 1.5 (38)
Tyr Gly (100)	11 ± 0.5 (58)
Trp (200)	15 ± 1.0 (42)
Trp Gly (200)	15 ± 1.0 (42)

*The reaction mixture contained 0.5 U/ml HRP, 0.2 mM H_2O_2, 100 μM PZ and 50 mM K-phosphate, pH 6.5. The reaction was started with H_2O_2 and $PZ^{+\bullet}$ production was measured by absorbance increase at 518 nm. When it reached its maximum value (35 μM $PZ^{+\bullet}$), the scavenger was added and, after 1 min incubation, $PZ^{+\bullet}$ concentration was measured. Other conditions in "Materials and Methods" section. In parenthesis, decay (%) of $PZ^{+\bullet}$ concentration, as compared with 0-time value. $P < 0.001$ for all LADH protection values.

TABLE IV Effect of thiol compounds, aminoacids, and anti-oxidants on $TRDZ^{+\bullet}$ radical production by the HRP/H_2O_2/TRDZ system*

	$TRDZ^{+\bullet}$ production (μM)	
LADH protector (0.2 mM)	Incubation: 0–5 s	Incubation: 80 s
Thiol compounds		
None	14.3 ± 2.0	4.5 ± 1.0
GSH	0.4 ± 0.1 (97)	0.5 ± 0.1 (89)
NAC	0.3 ± 0.1 (98)	0.8 ± 0.2 (82)
CPT	0.4 ± 0.1 (98)	0.4 ± 0.1 (91)
PAM	0.1 ± 0.0 (99)	0.2 ± 0.1 (95)
Amino acids and peptides		
None	14.8 ± 0.9	4.5 ± 0.7
Tyr	7.2 ± 1.4 (51)	1.9 ± 0.6 (58)†
TyrHis	7.2 ± 0.2 (51)	1.2 ± 0.2 (73)
Trp	9.8 ± 0.7 (33)	2.1 ± 0.5 (53)†
Other antioxidants		
Ascorbate	0.3 ± 00 (98)	0.7 ± 0.1 (85)
Trolox	0.0 (100)	0.0 (100)

*The reaction mixture contained 0.5 U/ml HRP, 0.2 mM H_2O_2, 100 μM TRDZ, 50 mM K-phosphate, pH 6.5 and 0.2 mM scavenger. The reaction was started by adding H_2O_2 and $TRDZ^{+\bullet}$ production was measured by absorbance increase at 633 nm. Other conditions are described in "Materials and Methods" section. In parenthesis, inhibition (%) of cation radical production. † $P < 0.005$ except < 0.05.

consistent with those in Tables III and IV. In the absence of scavenger, addition of H_2O_2 (0.2 mM) to the HRP (0.5 U/ml), TRDZ (100 μM) system at pH 6.5 produced a broad absorption band peaking at 633 nm, due to the $TRDZ^{+\bullet}$ radical. The band reached its maximum absorbance 10 s after H_2O_2 addition, decreasing subsequently. GSH (0.2 mM) and NAC (0.2 mM) prevented $TRDZ^{+\bullet}$ radical production after 60 s incubation. With L-tyrosine (0.2 mM), the scavenging effect was time-dependent and after 80 s incubation, $TRDZ^{+\bullet}$ absorbance was suppressed. Similar results were obtained with L-tryptophan (0.2 mM) (spectra omitted).

Effect of NADH on LADH Inactivation by PTZ Radicals

NADH and NAD^+ are specific substrates of *T. cruzi* LADH[28] and, therefore, they could (a) protect LADH against cation radical or (b) enhance (only NADH) LADH sensitivity towards these radicals by reducing LADH disulfide linkage to more reactive SH-groups. Accordingly, NAD(H) effects were investigated with the MPO/H_2O_2/TRDZ or /PZ systems. Table V shows that NADH prevented LADH inactivation by the /TRDZ system, the degree of protection depending on incubation time and NADH concentration. Thus, after 2.5 or 5.0 min incubation with the /TRDZ system, protection values for NADH were 59 and 14% ($P < 0.001$), respectively (experiment with 100 μM NADH) or 61 and 64%, respectively ($P < 0.001$) (experiment with 200 μM NADH). Protection values, however, decreased as a function of incubation time and after 10 min incubation, protection was observed only with TRDZ and 200 μM NADH. After 30 min incubation, no protection was observed (data omitted). In these experiments, NADH was added to the reaction mixture immediately before the MPO/TRDZ system, LADH inactivation being started with H_2O_2. With the MPO/H_2O_2/PZ system, addition of 20 μM NADH 10 min before the MPO/PZ system failed to modify significantly the inactivating system effect. With 200 μM NADH, added 10 min before the MPO/PZ system, protection values were 57 and 11% ($P < 0.001$), after 2.5 and 5.0 min incubation, respectively. With the same NADH concentration, added immediately before the MPO/H_2O_2/PZ system, protection values were 66 and 26% ($P < 0.001$), after 2.5 and 5.0 min incubation, respectively, but no protection was observed after 10 min incubation. With 200 μM NADH-preincubated samples (P samples), protection values were somewhat lower, as described above. It should be noted that LADH preincubated with 200 μM NADH was more sensitive to the /PZ system than the non-preincubated enzyme since protection values (%) were 57/66 or 11/26 (preincubated/non-preincubated samples ratio) after 2.5 or 5.0 min incubation, respectively. At variance with the above described observations, 20 μM NADH and 200 μM, NAD^+ failed to protect LADH.

Reduced pyridine nucleotide coenzymes may be direct antioxidants.[29] In close agreement with this hypothesis, NADH addition to the HRP/H_2O_2/PZ system initially prevented the appearance of the $PZ^{+\bullet}$ signal (Fig. 5A). However, this was a temporary effect since after 60 s incubation, an attenuated $PZ^{+\bullet}$ signal was observed, which appeared after extensive NADH oxidation (Fig. 6, plot A).

Moreover, addition of NADH to the HRP/H_2O_2/PZ system when radical concentration reached its maximum value (Fig. 5B), produced a fast decay of the $PZ^{+\bullet}$ signal. In the absence of NADH, $PZ^{+\bullet}$ concentration followed typical kinetics, that is a fast initial increase followed by a slow decay (Fig. 5B, plot C). NAD^+ failed to produce any significant variation in cation radical kinetics (Fig. 5B inset), in close agreement with NAD^+ inactivity as LADH protector (Table V).

Measurement of NADH oxidation rate yielded results compatible with $PZ^{+\bullet}$ kinetics. Thus, addition of PZ to the reaction mixture containing

TABLE V Effect of pyridine dinucleotides on LADH inactivation by the MPO/H_2O_2/PTZ systems*

		LADH inactivation (%)		
PTZ (100 μM)	NAD(H) (μM)	Incubation: 2.5 min	Incubation: 5 min	Incubation: 10 min
TRDZ	None	66 ± 3.0	74 ± 3.5	76 ± 4.0
	100	27 ± 0.8 (59)†	64 ± 0.8 (14)†	85 ± 1.0 (-12)
	200	26 ± 1.6 (61)†	27 ± 2.5 (64)†	58 ± 3.0 (24)‡
PZ	None	89 ± 0.5	91 ± 0.5	92 ± 1.0
	200	30 ± 0.7 (66)†	67 ± 2.0 (26)†	94 ± 0.6 (-2)
	20P	89 ± 1.3 (0)	93 ± 0.7 (-2)	96 ± 0.8 (-4)
	200P	38 ± 0.5 (57)†	81 ± 1.0 (11)†	94 ± 0.3 (-2)
	200 (NAD^+)	89 ± 0.2 (0)	93 ± 0.5 (-2)	93 ± 0.5 (-1)

* The inactivation mixture contained 0.8 μM LADH, 0.5 U/ml MPO, 0.1 mM H_2O_2 and 50 mM K-phosphate, pH 7.4. PTZ and NADH or NAD^+ were added as indicated above. P indicates that NADH was added to the inactivation mixture 10 min before the MPO/H_2O_2/PZ system (preincubated samples). In the other assays, NADH and NAD^+ were added immediately before MPO and PTZ. In all assays, the reaction was initiated by adding H_2O_2. Other conditions as described in "Materials and Methods" section. † $P < 0.005$; ‡ $P < 0.003$; unmarked values were not significantly different from control values. Values in parenthesis indicate LADH protection (%).

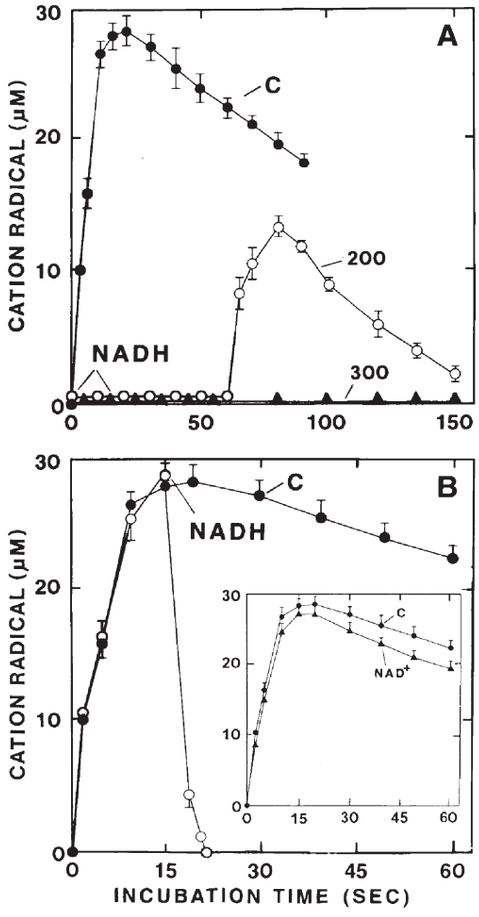

FIGURE 5 (A) Effect of NADH on time-dependent production and decay of $PZ^{+\bullet}$. The reaction mixture contained 0.5 U/ml HRP, 0.2 mM H_2O_2, 100 μM PZ, 50 mM K-phosphate, pH 6.5, 200 or 300 μM. NADH added as indicated in the figure. The reaction was started by adding H_2O_2 and absorbance was measured at 518 nm. C, control sample lacking NADH. (B) Experimental conditions as in figure A except that 0.2 mM NADH was added when $PZ^{+\bullet}$ concentration reached its maximum value. Inset: experimental conditions as in figure A, except that 200 μM NAD^+ replaced NADH. Other conditions in "Materials and Methods" section.

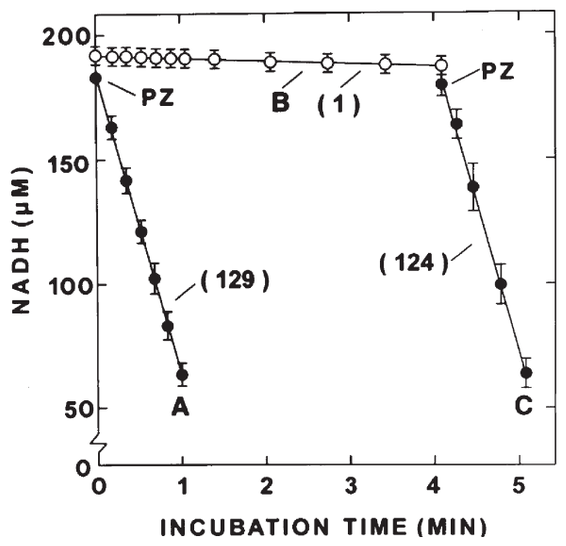

FIGURE 6 Effect of PZ on NADH oxidation by the HRP/H_2O_2 system. The reaction mixture contained 0.2 mM NADH, 0.5 U/ml HRP, 0.2 mM H_2O_2 and 50 mM K-phosphate, pH 6.5. Tracing A and C, 100 μM PZ was added after 0 and 4 min incubation, respectively. Tracings B, PZ addition omitted. NADH concentration was measured spectrophotometrically at 340 nm. Other conditions in "Materials and Methods" section. Values are the means of triplicate measurements ±S.D. The figures in parenthesis indicate the rate of NADH oxidation (μM/min).

the HRP/H_2O_2/NADH system produced an immediate, fast decay of NADH concentration (Fig. 6, plot A). In the absence of PZ (Fig. 6, plot B), NADH decrease was negligible (<1.0 μM/min), thus ruling out significant oxidation of NADH by the HRP/H_2O_2 system. Addition of PZ caused a rapid decrease in NADH concentration (plot C), similar to NADH decay when PZ was added initially (plot A). Comparison of Figs. 5A and 6 shows that the increase in $PZ^{+\bullet}$ concentration in Fig. 5A occurred when NADH concentration in Fig. 6A reached its lowest level.

DISCUSSION

Present findings demonstrate that after oxidation to their corresponding radicals structurally dissimilar PTZs can react with specific biological targets such as *T. cruzi* LADH (Table I). LADH inactivation resulted from an irreversible modification of the protein structure by $PTZ^{+\bullet}$ radicals. The inhibitory role of these radicals was supported by (a) the peroxidase requirement for PTZ activity (text); (b) optical spectroscopy (Fig. 2) and ESR data; (c) the protective action of radical scavengers including NADH (Tables II and V); and (d) the effect of LADH protectors on $PTZ^{+\bullet}$ radical production and decay (Tables III and IV). Taken together, our observations suggest that LADH cysteine, tyrosine and tryptophan residues may act as targets for $PTZ^{+\bullet}$ radicals. Comparison of Table I inhibition data with similar data for TR[12] shows that the latter enzyme was about 10-fold more sensitive to the PTZ system than LADH. On the other hand, LADH was more sensitive to /CPZ and /PFZ systems than TR. These differences points to selective effects of PTZ systems on *T. cruzi* enzyme systems, thus supporting further investigation of PTZs on potential parasite targets.

MPO/H_2O_2/PTZ systems activity depended, essentially, on PTZ structure. Substituents at position 2 of the PTZ exerted significant influence on PTZ systems activity (Table I), since molecules with trifluoromethyl, propionyl or cyano groups were significantly less active than those with H or chloro at the same position (Table I). The influence of substituents on PTZ activity may be explained by

the dissimilar rate of access to MPO active site for the phenothiazine as shown by comparing the effects of the MPO/ and HRP/H_2O_2/PTZ systems (Table I).[30]

Protection of LADH by thiol compounds (Table II) provided additional evidence for the role of the $PTZ^{+\bullet}$ radicals in LADH inactivation, given the known reactions of these radicals with thiol compounds.[31,32] Dissimilar decay rates of the $PZ^{+\bullet}$ radical, after catalase, GSH, and catalase plus GSH addition (Fig. 4), confirmed that $PZ^{+\bullet}$ scavenging, rather than the inhibition of $PZ^{+\bullet}$ production, was the major factor in LADH protection by GSH and in all probability by the other thiol compounds (Tables III and IV; Fig. 4). Electron transfer between thiols and $PTZ^{+\bullet}$ radicals occurs according to Reaction (2) in which, depending on pH, the electron is transferred from

$$GS^- + PTZ^{+\bullet} \leftrightarrow GS^\bullet + PTZ \qquad (2)$$

the thiol group (e.g. GS^-) to the cation radical ($PTZ^{+\bullet}$) to form the thiyl radical ($GS^\bullet$) and the neutral PTZ molecule. This reaction produces cytotoxic thiyl radicals as well as thiol depletion, two processes that may contribute to PTZs cytotoxicity in different cells, including mammalian cells.

L-Tyrosine and its peptides protected LADH against the MPO/H_2O_2/TRDZ, and /PZ systems (Table II, Fig. 3). L-Tyrosine action on $PTZ^{+\bullet}$ radical production (Tables III and IV) may imply a complex mechanism since both PTZ and L-tyrosine are peroxidase substrates.[33–36] Competition of PTZ and L-tyrosine for the peroxidase active site, should then be possible. However, the relative rates of HRP Compound II reduction by PTZs and L-tyrosine are different: 4.5×10^5 and $1.1 \times 10^3\,M^{-1}s^{-1}$, respectively.[35] Therefore, PTZs would be able to compete successfully with L-tyrosine for Compound II active site. When two Compound II reductants are present, one of them, termed the "primary substrate" may behave as a redox mediator of the other (the "secondary substrate").[37] The "primary substrate" oxidation by the peroxidase/H_2O_2 system would produce a diffusible radical (e.g. $PTZ^{+\bullet}$) which can carry on the oxidation of the "second substrate". With the PTZ/L-tyrosine and /L-tryptophan systems, that possibility was borne out by the difference between the corresponding redox potentials, e.g. 0.71 (PZ), 0.78 (CPZ), 0.86 (PMTZ), 0.93 (L-tyrosine) and 1.02 (L-tryptophan) V.[38–40] Accordingly, the redox potential for the couple $PTZ^{+\bullet}$/PTZ and the corresponding values for the L-tyrosine or L-tryptophan fit in well with the role of L-tyrosine and L-tryptophan as scavengers of the $PTZ^{+\bullet}$ radicals. L-Tryptophan reacts slowly with MPO Compound II (rate constant of Compound II reduction $7\,M^{-1}s^{-1}$)[39] and therefore, L-tryptophan competition with PTZ may be ruled out. Summing up, L-Tyrosine and L-tryptophan would play the "secondary substrate" role, thus leading to $PTZ^{+\bullet}$ radicals scavenging.

Protection of LADH by NADH against $TRDZ^{+\bullet}$ and $PZ^{+\bullet}$ radicals deserves special attention because NADH is an LADH substrate and, moreover, LADH plays an essential role in many metabolic reactions in cells. A characteristic feature of NADH protection was its transient kinetics (Table V and Fig. 5), which would essentially depend on NADH oxidation by $PTZ^{+\bullet}$ radicals, as shown by $PZ^{+\bullet}$ decay in Fig. 5. Reciprocally, the fast rate of NADH oxidation by HRP/H_2O_2/PZ, though not by HRP/H_2O_2 (Fig. 6, plot B), would decrease NADH concentration to an ineffective level, so that the $PZ^{+\bullet}$ radical could be expressed (Fig. 5A, 200 μM NADH), and produce LADH inactivation. Moreover, NADH oxidation product (in all probability NAD^+) was ineffective as LADH protector (Table V).

Protection of LADH by NADH-dependent $PTZ^{+\bullet}$ radical scavenging fits in well with (a) LADH protection kinetics (Table V); (b) NADH action on $PZ^{+\bullet}$ production characterized by a lag period depending on NADH concentration (Fig. 5A); and (c) the NADH-dependent $PZ^{+\bullet}$ decay, once $PZ^{+\bullet}$ concentration reached maximum value (Fig. 5B). Possible protection of LADH by NADH-binding at the LADH active site was ruled out by the inactivity of NADH at a relatively low concentration (Table V). Finally, the possibility that NADH should act as a competitive substrate for peroxidase, thus preventing PTZ peroxidation, was not supported by NADH inactivity as a substrate of the HRP/H_2O_2 system (Fig. 6, plot B). An irreversible inactivation of HRP by NADH was ruled out by the effect of PZ (Fig. 6, plot C), when added to the HRP/H_2O_2/NADH containing sample after 4 min preincubation (Fig. 6, plot B). Under these latter conditions the rate of NADH oxidation was similar to the one observed when PZ was added without preincubation (Fig. 6, plot A).

MPO/H_2O_2 induced $PTZ^{+\bullet}$ production may have biological implications for PTZ trypanocidal action since MPO is present in neutrophils, monocytes and macrophages.[17–20] These cells internalize *T. cruzi*;[5,6,41] and treatment of *T. cruzi* infected macrophages, or heart muscle cells with CPZ or TFPZ, causes damage to intracellular parasites.[5] The observations here described extend those previously reported with TR. It is therefore not unreasonable to assume that other *T. cruzi* enzymes could be affected by $PTZ^{+\bullet}$ radicals. Scavenging of $PTZ^{+\bullet}$ radicals by thiols, L-tyrosine, L-trypotphan, and NADH may prevent enzyme modification by $PTZ^{+\bullet}$ radicals but would extensively deplete cellular metabolites. Our observations allow us to

conclude that either as enzyme inhibitors or as metabolite depletors, $PTZ^{+\bullet}$ radicals may play leading role in PTZ pharmacology.

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