Free Rad. Res., Vol. 33, pp. 13–22
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Trypanosoma cruzi Dihydrolipoamide Dehydrogenase is Inactivated by Myeloperoxidase-Generated "Reactive Species"

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Accepted by Prof. V. Darley-Usmar

(Received 27 September 1999; In revised form 2 November 1999)

Dihydrolipoamide dehydrogenase (LADH) from *Trypanosoma cruzi* was inactivated by treatment with myeloperoxidase (MPO)-dependent systems. With MPO/H_2O_2/NaCl, LADH lipoamide reductase and diaphorase activities significantly decreased as a function of incubation time. Iodide, bromide, thiocyanide and chloride effectively supplemented the MPO/H_2O_2 system, KI and NaCl being the most and the least effective supplements, respectively. LADH inactivation by MPO/H_2O_2/NaCl and by NaOCl was similarly prevented by thiol compounds such as GSH, L-cysteine, N-acetylcysteine, penicillamine and N-(2-mercaptopropionyl-glycine) in agreement with the role of HOCl in LADH inactivation by MPO/H_2O_2/NaCl. LADH was also inactivated by MPO/NADH/halide, MPO/H_2O_2/$NaNO_2$ and MPO/NADH/$NaNO_2$ systems. Catalase prevented the action of the NADH-dependent systems, thus supporting H_2O_2 production by NADH-supplemented LADH. MPO inhibitors (4-aminobenzoic acid hydrazide, and isoniazid), GSH, L-cysteine, L-methionine and L-tryptophan prevented LADH inactivation by MPO/H_2O_2/$NaNO_2$. Other MPO systems inactivating LADH were (a) MPO/H_2O_2/chlorpromazine; (b) MPO/H_2O_2/monophenolic systems, including L-tyrosine, serotonin and acetaminophen and (c) MPO/H_2O_2/di- and polyphenolic systems, including norepinephrine, catechol, nordihydroguaiaretic acid, caffeic acid, quercetin and catechin. Comparison of the above effects and those previously reported with pig myocardial LADH indicates that both enzymes were similarly affected by the MPO-dependent systems, allowance being made for *T. cruzi* LADH diaphorase inactivation and the greater sensitivity of its LADH lipoamide reductase activity towards the MPO/H_2O_2/NaCl system and NaOCl.

Keywords: Dihydrolipoamide dehydrogenase, myeloperoxidase, peroxidase, halides, hypochlorous acid, nitrite, thiol compounds

Abbreviations and chemical terms: LADH, dihydrolipoamide dehydrogenase (NADH-lipoamide oxido-reductase, E.C. 1.6.4.3); MPO, myeloperoxidase; HRP, horseradish peroxidase; LPO, lactoperoxidase; HOCl, (OCl^-) hypochlorous acid (anion); DCI, dichlorophenol–indophenol; K-phosphate, KH_2PO_4–K_2HPO_4; GSH and GSSG, reduced and oxidized glutathione, respectively; Cys, L-cysteine; NAC, N-acetylcysteine; MPG, N-(2-mercaptopropionylglycine); PAM, penicillamine (3-mercapto-D-valine); CPT, Captopril [1-(3-mercapto-2-methyl-1-oxopropyl-L-proline)]; ABAH, 4-aminobenzoic acid hydrazide; I, isoniazid, 4-pyridine carboxylic acid hydrazide; caffeic acid, (3,4-dihydroxycinnamic acid; CPZ, chlorpromazine; NDGA, nordihydroguaiaretic acid

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INTRODUCTION

Trypanosoma cruzi, the agent of American trypanosomiasis (Chagas' disease) possesses LADH as a constitutive enzyme. *T. cruzi* LADH has been isolated from epimastigote parasites and the gene, cloned and overexpressed.[1,2] The enzyme has diaphorase activity (less than 5% of the lipoamide reductase activity) and is inhibited by arsenite, cadmium and *p*-chloromercuribenzoate, in close agreement with an essential role of thiol groups in LADH.[3] *T. cruzi* LADH is a homodimeric protein with FAD as prosthetic group and the calculated molecular mass of the holoprotein is 50,066. Comparison of the deduced amino acid sequence of *T. cruzi* LADH with that of human LADH shows 50% identities. Moreover, 21 out of 30 positions of the N-terminal amino acid sequence of the *T. cruzi* enzyme are identical with those of pig heart LADH.[1,2] The parasite LADH is probably a component of the mitochondrial 2-oxoacid dehydrogenase complexes since *T. cruzi* possesses a complete citric acid cycle.[4]

In order to obtain information on *T. cruzi* LADH response to "reactive species", capable of originating a new approach to Chagas' disease therapy, we have studied the action of MPO/H_2O_2-dependent systems, including different MPO substrates, on *T. cruzi* LADH. MPO plays a major role in oxidant production by neutrophils,[5] monocytes and macrophages.[6] MPO uses H_2O_2 and chloride ions to produce hypochlorous acid (HOCl) which is a strong enzyme inhibitor.[5] Moreover, the MPO/H_2O_2 systems produce other "reactive species" including $NO_2^{\bullet}$ and organic radicals.[5] Some MPO/H_2O_2 systems (the halide- and nitrite-dependent ones) inactivate myocardial LADH,[7] an effect which allows *T. cruzi* and myocardial LADH sensitivity to free radicals and prooxidants to be compared.

MATERIALS AND METHODS

Enzyme Preparations

Recombinant LADH from *T. cruzi* was obtained as described.[2] The enzyme was stored as a suspension in 50% (v/v) glycerol–100 mM K-phosphate buffer, pH 7.0, containing 100 mM KCl, 1.0 mM EDTA and 1.0 mM dithioerythritol. The specific activity of LADH preparations was about 70 units/mg. Enzyme suspensions were diluted (1/2 v/v) with 50 mM K-phosphate, pH 7.4, and kept at 4°C for subsequent use. Human leukocyte MPO as well as HRP, LPO and catalase were obtained from Sigma Chemical Co., (St. Louis, MO, USA). MPO specific activity ranged from 50 to 100 units/mg, as determined by the standard guaiacol/H_2O_2 assay. Other conditions were as described previously.[7]

Reagents

ABAH, CPZ, serotonin, catechol, norepinephrine, NDGA, acetaminophen, caffeic acid, quercetin and catechin were obtained from Sigma. Other reagents were as described previously.[7]

LADH Inactivation

Unless otherwise stated, the LADH inactivation mixture contained LADH, MPO, H_2O_2, EDTA, K-phosphate, pH 7.4, and additions as indicated under Results; final volume, 100 µl. The inactivation mixture also contained 1.25 mM KCl, 12.5 µM EDTA, and 12.5 µM dithioerythritol as a result of dilution of the original LADH suspension. Samples were incubated at 30°C for the time indicated in each case and aliquots of the inactivation mixture (10 or 20 µl) were added to the assay mixture, residual LADH activity being measured as described below. Control samples without MPO or other additions were incubated simultaneously, as stated under Results.

LADH Assays

LADH activity was measured at 30°C by the rate of NADH oxidation using lipoamide as electron acceptor. The standard reaction mixture contained 50 mM K-phosphate, pH 7.4, 0.2 mM NADH, 1.0 mM lipoamide, 1.0 mM EDTA and LADH resulting from the inactivation mixture

addition; total volume, 3.0 ml. Diaphorase activity was measured at 30°C by the rate of DCI reduction using NADH as electron donor. The reaction mixture contained 50 mM K-phosphate, pH 7.4, 0.2 mM NADH, 20 nM LADH and 40 μM DCI, total volume, 1.0 ml. Other experimental conditions were as previously described.[7]

Expression of Results

LADH activity values are presented as a percentage of control sample activity (100%). LADH inactivation (*I*%) and protection (*P*%) were calculated as described previously.[7] Unless otherwise stated, values represent a mean of duplicate measurements, the experimental values differing by less than 5% from the mean value. When more than two measurements were performed, the values presented are mean ± SD. Statistical analysis was performed using Student's *t*-test for paired values.

RESULTS

Effect of the MPO/H_2O_2/Halide Systems

Figure 1 shows the effect of the MPO/H_2O_2/NaCl system on LADH lipoamide reductase and LADH diaphorase activities. It is to be seen that the LADH lipoamide reductase activity dropped rapidly during the first 10 min incubation reaching its minimum value (100% inactivation) after 60 min incubation. Diaphorase inactivation followed a different kinetics: it increased slightly during the first 20 min incubation, an effect that recalls the increase of myocardial LADH diaphorase activity under similar experimental conditions.[7] Subsequently, activity continuously decreased reaching a minimum value (30% of the initial one) after 60 min incubation. Omission of MPO prevented the loss of LADH activity (Figure 1). LADH inactivation depended on the halide nature and correlation of inactivation with halide concentration values showed that the MPO/H_2O_2/KI system was the most effective; followed by the NaBr, KSCN and NaCl systems (Table I). LADH diaphorase activity was also inhibited by the MPO/H_2O_2/halide systems assayed although the differences between control and halide supplemented samples were smaller than with LADH reductase activity (Table I).

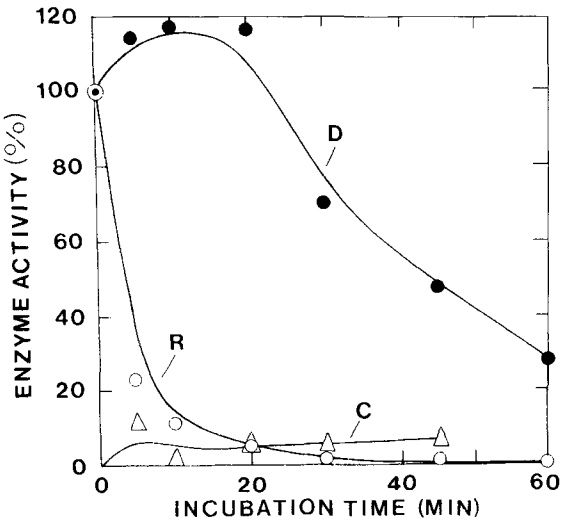

FIGURE 1 Inactivation of LADH lipoamide reductase (R) and diaphorase (D) activities by the MPO/H_2O_2/NaCl system. Inactivation mixtures contained, in a total volume of 100 μl, 100 mM NaCl and LADH, MPO, H_2O_2, EDTA, and K-phosphate, pH 7.4 as described under Materials and Methods. Incubation time was as indicated on the abscissa. Other conditions were as described under Materials and Methods. Values represent mean of duplicate measurements. C, control sample including H_2O_2 and NaCl, but not MPO.

TABLE I Effect of MPO/H_2O_2/halide systems on LADH diaphorase and lipoamide reductase activities

Pro-oxidant system	LADH activity	
	Lipoamide reductase (%)	Diaphorase (μmol DCI/min) mg LADH
None	100 ± 3.7	3.52 ± 0.10 (100)
MPO/H_2O_2/KI	1 ± 0.5[c]	1.40 ± 0.10 (40)[c]
MPO/H_2O_2/NaBr	3 ± 0.4[c]	2.79 ± 0.28 (79)[a]
MPO/H_2O_2/NaCl	2 ± 1.9[c]	2.49 ± 0.037 (71)[b]
MPO/H_2O_2/KSCN	53 ± 0.8[c]	3.53 ± 0.14 (100)
MPO/H_2O_2	96 ± 1.7	—

The inactivation mixtures contained 0.8 μM LADH, 0.5 U/ml MPO, 0.1 mM H_2O_2, 1.0 mM EDTA and 50 mM K-phosphate, pH 7.4. KI (25 μM), NaBr (1.0 mM), and NaCl (100 mM) were added as required by the assayed MPO/H_2O_2/halide system. Incubation time 30 min. Activity values represent mean ± SD ($n = 3$); in parenthesis relative activity. Other conditions were as described under Materials and Methods. [a]$P < 0.02$; [b]$P < 0.01$; [c]$P < 0.001$.

Hypochlorous acid is the product of NaCl oxidation by MPO/H_2O_2,[5] and accordingly, NaOCl was assayed on LADH. The results obtained are presented in Figure 2. It is to be seen that NaOCl inactivated LADH in a concentration-dependent manner, significant effects being obtained with 10 μM NaOCl whereas 100 μM NaOCl produced 98% inactivation. The inactivation kinetics showed an initial fast phase lasting for 2.5–5.0 min, followed by a relatively steady state phase, lasting to the end of incubation. Comparison of these results with those obtained with the myocardial LADH[7] showed that the effect of NaOCl on *T. cruzi* LADH was greater and faster than on the myocardial enzyme. Interestingly enough, L-tyrosine effectively prevented the action of NaOCl (Figure 2), thus suggesting a reaction of HOCl with LADH Tyr residues, which could be prevented by the added L-tyrosine. LADH inactivation by NaOCl did not depend on traces of transition metals such as (Fe(II) and Cu(II)) in NaOCl since the original LADH samples contained 1.0 mM EDTA. LADH inactivation by NaOCl and by the MPO/H_2O_2/NaCl system was prevented by thiol compounds such as GSH, Cys, NAC, PAM and MPG (Table II) whereas these were no significant differences between thiol effects on the MPO-system and NaOCl activities.

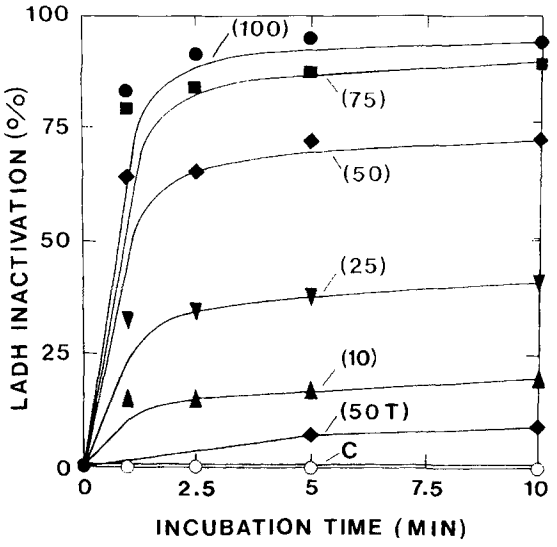

FIGURE 2 Time-course of LADH inactivation by NaOCl. The inactivation mixture contained 0.8 μM LADH, 1.0 mM EDTA, in 50 mM K-phosphate, pH 7.4. NaOCl concentration (μM) was as given in parenthesis; sample 50 T contained 200 μM L-tyrosine. Incubation time was as indicated on the abscissa. Other conditions were as described in the legend of Figure 1. C, control sample lacking NaOCl.

LADH was also inactivated by MPO halide systems in which NADH replaced H_2O_2. With these latter systems, the results obtained (not shown) closely resembled those reported with myocardial LADH.[7] Catalase prevented LADH inactivation by the MPO/NADH/halide systems as illustrated with NaCl and KI supplemented systems (Table III). The effect of catalase confirmed the role of H_2O_2 for LADH inactivation by the MPO/NADH/halide systems.

Effect of the MPO/H_2O_2/$NaNO_2$ System

T. cruzi LADH was inactivated by the MPO/H_2O_2/$NaNO_2$ system, the reaction depending on incubation time and pH in the 6.0–7.4 range, the greatest effect being obtained at pH 6.0 (experimental data omitted). The MPO/H_2O_2/$NaNO_2$

TABLE II Effect of thiol compounds on LADH inactivation by the MPO/H_2O_2/NaCl system and by NaOCl

Thiol compound	LADH inactivation (%)	
	Pro-oxidant: MPO/H_2O_2/NaCl (Expt. A)	Pro-oxidant: NaOCl (Expt. B)
None	86.8 ± 2.9	75.9 ± 3.0
GSH	19.2 ± 1.8 (78)	11.5 ± 1.6 (85)
Cys	3.2 ± 0.5 (96)	6.3 ± 1.2 (92)
NAC	4.7 ± 2.2 (94)	11.6 ± 0.5 (85)
PAM	3.8 ± 0.6 (96)	4.0 ± 1.3 (94)
MPG	6.2 ± 2.5 (93)	5.9 ± 1.8 (92)

Expt. A: The inactivation mixture contained 0.8 μM LADH, 0.5 U/ml MPO, 0.1 mM H_2O_2, 1.0 mM EDTA, 100 mM NaCl and 50 mM K-phosphate, pH 7.4. Expt. B: The inactivation mixture contained 0.8 μM LADH, 50 μM NaOCl, 1.0 mM EDTA and 50 mM K-phosphate, pH 7.4. Thiol compounds (200 μM in A or 100 μM in B) were added as stated above. Incubation time, 10 min. Other conditions were as described under Materials and Methods. Values represent mean ± SD ($n = 3$ or 4); in parenthesis, LADH protection by the thiol compound. In all cases $P < 0.05$.

TABLE III Effect of catalase on LADH inactivation by the MPO/NADH/halide system

Halide (mM)	Catalase (μg/ml)	LADH inactivation (%)	
		Incubation: 10 min	Incubation: 30 min
NaCl (100)	None	73 ± 3.5	94 ± 0.5
	5.0 (NC)	40 ± 1.0	46 ± 1.0
	5.0 (DC)	75 ± 3.0	94 ± 1.9
KI (0.1)	None	99 ± 0.3	100 ± 0.0
	5.0 (NC)	1 ± 0.2	13 ± 3.0
	5.0 (DC)	98 ± 0.0	99 ± 0.0

The inactivation mixture contained 0.8 μM LADH, 0.5 U/ml MPO, 1.0 mM EDTA and 50 mM K-phosphate pH 7.4. NaCl, KI and catalase were added as indicated above. NC; native catalase (150 U/ml); DC, denatured catalase (inactive). Other conditions were as described under Materials and Methods. LADH inactivation by the NADH/NaCl control system (MPO omitted) yielded 1% and 3% inactivation, after incubation for 10 and 30 min, respectively. Same, by the NADH/KI system, 4% and 5%, respectively.

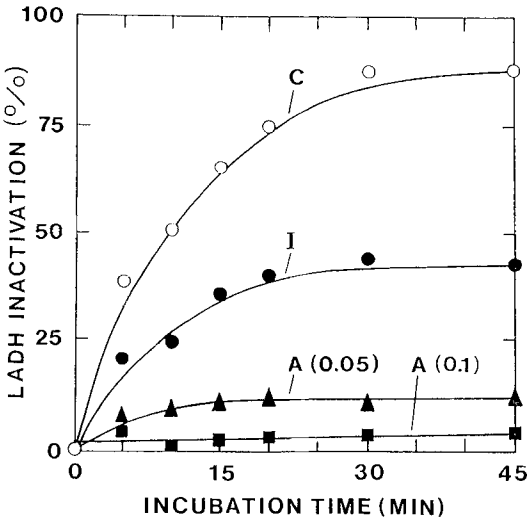

FIGURE 3 Effect of MPO inhibitors on LADH inactivation by the MPO/H_2O_2/$NaNO_2$ system. The reaction mixtures contained 0.8 μM LADH, 0.5 U/ml MPO, 0.25 mM H_2O_2, 0.25 mM $NaNO_2$, 1.0 mM EDTA in 50 mM K-phosphate, pH 6.0. Other additions were I, 0.20 mM isoniazid; A, 0.1 and 0.05 mM 4-aminobenzoate hydrazide as indicated in the figure. Incubation time was as indicated on the abscissa. Other conditions under Materials and Methods. C, control sample lacking MPO inhibitors.

effect was prevented by MPO inhibitors, such as ABAH,[8] and isoniazid,[9] the former being the more effective (Figure 3). Significant protection was also provided by L-cysteine, GSH, L-methionine and L-tryptophan but not by L-tyrosine which rather increased LADH inactivation (Table IV). LADH was also inactivated by the MPO/NADH/$NaNO_2$ system under experimental conditions as described in the legend of Table IV, except that H_2O_2 was replaced by 0.1 mM NADH). Under these experimental conditions, LADH inactivation values were 48% and 70% after 10 and 30 min incubation, respectively. Catalase (150 U/ml) protected LADH by 79% and 85%, respectively (experimental data omitted), in close agreement with results in Table III.

TABLE IV Effect of thiol compounds and L-amino acids on LADH inactivation by the MPO/H_2O_2/$NaNO_2$ system

Addition	LADH inactivation (%)
None	61 ± 3.5
L-cysteine (0.25)	0 ± 1.0 (100)[a]
GSH (0.25)	10 ± 3.8 (82)[a]
L-methionine (0.25)	2.4 ± 1.2 (96)[a]
L-tyrosine (0.25)	74 ± 1.4 (−22)[a]
L-tryptophan (0.50)	8.0 ± 2.2 (87)[a]

The inactivation mixture contained 0.8 μM LADH, 0.5 U/ml MPO, 0.25 mM H_2O_2. 0.25 mM $NaNO_2$, 1.0 mM EDTA in 50 mM K-phosphate, pH 6.0. Amino acids were added as stated above. Incubation time 15 min. Other conditions were as described in the legend of Figure 1. Values represent mean ± SD ($n = 3$); in parenthesis, protection of LADH (%).
[a]$P < 0.01$.

Effect of the MPO/H_2O_2/Phenothiazine System

Phenothiazines are effective inhibitors of *T. cruzi* enzymes, as illustrated by trypanothione reductase, also a flavoenzyme.[10] Peroxidative bioactivation of phenothiazines to the corresponding cation radical species by peroxidases[11,12] may represent one mechanism of the biochemical toxicity of these compounds. In agreement with this hypothesis, incubation of LADH with the MPO/H_2O_2/CPZ system led to LADH inactivation and after 30 min incubation, 95% of initial LADH activity was lost (Figure 4). Thiol compounds (GSH, PAM and CPT) prevented LADH inactivation by the MPO/H_2O_2/CPZ system and with PAM and CPT, an effective (about 90%), important and

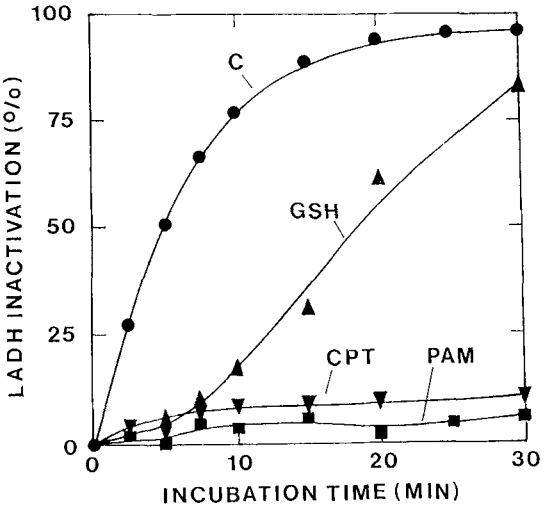

FIGURE 4 Time-course of LADH inactivation by the MPO/H_2O_2/CPZ system; effect of thiol compounds. The inactivation mixture contained 0.8 μM LADH, 0.5 U/ml MPO, 0.2 mM H_2O_2, 0.1 mM CPZ in 50 mM K-phosphate, pH 7.4. Other additions were 0.1 mM GSH, 0.1 mM PAM and 0.1 mM CPT as indicated in the figure. Incubation time was as indicated on the abscissa. C, control sample lacking thiols. Other conditions were as described under Materials and Methods.

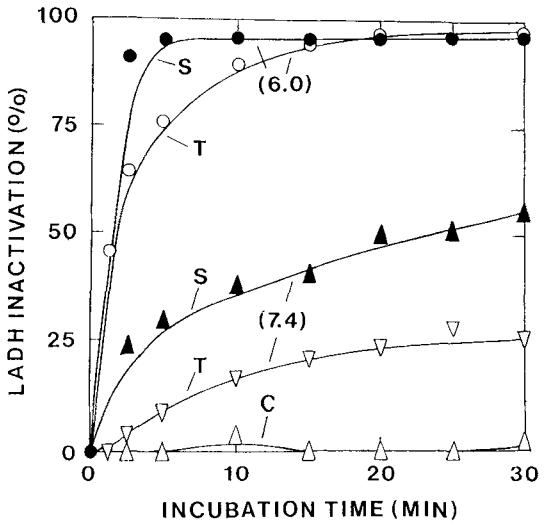

FIGURE 5 Time-course of LADH inactivation by the MPO/H_2O_2/L-tyrosine and serotonin systems at pH 6.0 and 7.4. The inactivation mixtures contained 0.8 μM LADH, 0.5 U/ml MPO, 0.2 mM H_2O_2, 1.0 mM EDTA in 50 mM K-phosphate. Other additions were 0.2 mM L-tyrosine (T), 0.1 mM serotonin (S). Other conditions were as described under Materials and Methods. C, control samples lacking MPO at 6.0; valid for all conditions.

stable protection was observed. However, with GSH, protection decreased as a function of incubation time and after 30 min, 80% of the protection effect was lost. CPZ activity depended on the peroxidase since the horseradish peroxidase and lactoperoxidase systems were more effective than the MPO/H_2O_2/CPZ system. Thus, after 2.5 min of incubation with the peroxidase/H_2O_2/CPZ systems (0.5 U/ml peroxidase, 0.2 mM H_2O_2, 100 μM CPZ), inactivation values obtained were (%; in parenthesis, the peroxidase used): 28 (MPO), 61 (LPO) and 93 (HRP).

Effect of the MPO/H_2O_2/Phenol Systems

Figure 5 shows that the MPO/H_2O_2/L-tyrosine (or serotonin) systems produced a significant time- and pH-dependent inactivation of LADH. At pH 6.0 and after 15 min incubation *circa* 95% of LADH activity was lost, irrespective of the system assayed. The effect of L-tyrosine at pH 6.0 fit in well with the results presented in Table IV. Other phenolic compounds effectively contributed to LADH inactivation. Their concentrations were selected from previously obtained concentration–inhibition curves (omitted). LADH inactivation values (%) obtained after 30 min incubation of LADH with the MPO/H_2O_2/phenol system, under standard experimental conditions, may be summarized as follows (in parenthesis, phenol concentration (μM); catechol (5), 83; norepinephrine (1.0), 59; NDGA (1.0), 78; acetaminophen (10), 80; caffeic acid (50), 93; quercetin (50), 57 and catechin (50), 46. GSH and NAC (0.2 mM) prevented LADH inactivation by the MPO/H_2O_2/NDGA or acetaminophen systems, 77% and 100% respectively (experimental data omitted).

DISCUSSION

The results presented in this paper demonstrate that adequately supplemented MPO/H_2O_2 systems inactivate *T. cruzi* LADH, in close agreement

with previously reported effects of some of these systems on myocardial LADH.[7] It is known that MPO/H_2O_2/halide systems, produce oxidizing and halogenating species[13–15] which in our study, are assumed to be the agents of LADH inactivation. The relative activity of the halide systems assayed, depended on the nature of the halide (Tables I and III). Among the species generated by halide peroxidation, HOCl is produced by the MPO/H_2O_2/NaCl system, at NaCl concentrations compatible with chloride concentration in biological media, such as mammalian tissues, in which *T. cruzi* develops. The role of HOCl in LADH inactivation was supported by (a) the negligible effect of the MPO/H_2O_2 system in the absence of NaCl (Table I); (b) LADH inactivation by NaOCl (Figure 2) and (c) the protective action of thiol compounds against MPO/H_2O_2/NaCl and by NaOCl, irrespective of thiol compound structure (Table II). The mode of action of thiol compounds against hypochlorous acid (or anion) may be explained by reactions in which one molecule of thiol inactivates three molecules of HOCl.[16–18] Peroxidizing reactions similar to those discussed above seem to occur with the MPO/NADH/halide systems as indicated by the effect of catalase (Table III). With the NADH-supplemented systems, H_2O_2 would then be produced by LADH-catalyzed NADH oxidation.[7,19]

The inhibition of LADH diaphorase activity establishes a significant difference between *T. cruzi* and myocardial LADH since this latter enzyme activity linearly increased during treatment with the MPO/H_2O_2/halide system.[7] The active site redox disulfide of *T. cruzi* LADH Cys-50 and Cys-55 would be selective targets for pro-oxidant species as indicated by the results obtained with myocardial LADH.[7] However, the pattern of enzyme modification by the pro-oxidant system (Figure 1 and Table I) suggests that in addition to the active site thiols other amino acid residues would be modified in *T. cruzi* LADH, thus preventing electron transfer from NADH to DCI acceptor.[20] The time dependence of the amino acid residues so affected would explain the biphasic kinetics of LADH diaphorase modification (Figure 1). The nature and extent of such alterations remains to be established.

Sodium nitrite was an effective supplement of the MPO/H_2O_2 system for LADH inactivation (Figure 3). The effect of MPO inhibitors (Figure 3) and catalase (referred to in text) confirmed the essential role of the peroxidase reaction for nitrite activation. Nitrite ion is a direct metabolic product of nitric oxide[21] and it is formed in macrophages.[22] Therefore, it might have access to *T. cruzi* amastigotes during phagocytosis. Reactive radicals formed by peroxidase-catalyzed oxidation of nitrite[23] can nitrate Tyr residues[24] and oxidize thiol groups in proteins.[25] Moreover, the protection of LADH by other amino acids (Table IV) agrees closely with the modifications of the corresponding residues in LADH.

CPZ was an effective inhibitor of LADH when supplementing the MPO/H_2O_2 system (Figure 4) and this inhibition might supplement other effects of CPZ in trypanosomatids.[10] CPZ effect would imply the production of the phenothiazine cation radical ($CPZ^{+\bullet}$),[11,12] which reacts with protein thiols as illustrated by bovine serum albumin[26] and similar reactions can explain LADH inactivation. Protection of LADH by GSH and other thiols against the CPZ system fits in well with that hypothesis. $CPZ^{+\bullet}$ radical reacts with thiol compounds forming the thiyl radical ($GS^{\bullet}$).[27,28] The elimination of $GS^{\bullet}$ by subsidiary reactions such as $GS^{\bullet} + GS^{-} \rightarrow GSSG^{-\bullet}$, or $GS^{\bullet} + O_2 \rightarrow GSOO^{\bullet}$ or $GS^{\bullet} + GS^{\bullet} \rightarrow GSSG$, allow an electron flow that ensures the removal of the toxic $CPZ^{+\bullet}$. The $GSSG^{-\bullet}$ radical can, however, react with O_2 to produce superoxide radicals that may lead to other "reactive oxygen species". These latter reactions probably explain the transient pattern of LADH protection by GSH, in contrast to the stable action of PAM and CPT which are less reactive towards oxygen.

It is known that the MPO/H_2O_2 system oxidizes phenols to the corresponding phenoxyl radical.[29,30] Oxidation of L-tyrosine molecules

or protein Tyr residues by MPO/H_2O_2 produce *o,o'*-dityrosine molecules or protein-linked *o,o'*-dityrosine residues[31–33] which can modify the structure of the protein molecule. In addition to these reactions, the MPO/H_2O_2/L-tyrosine system can produce L-tyrosine peroxide[34] that might also contribute to LADH inactivation. Taken together, these reactions can explain the pro-oxidant action of L-tyrosine and serotonin on LADH (Table IV and Figure 5). Tyr residues in the neighborhood of *T. cruzi* LADH active site seem to be suitable targets for MPO-dependent systems, as indicated by the inactivating effects of MPO/H_2O_2/NaCl (Figure 1), MPO/H_2O_2/KI (Tables I and III), MPO/H_2O_2/$NaNO_2$ (Figure 3 and Table IV) and MPO/H_2O_2/L-tyrosine (or serotonin) (Figure 5). The diverse "reactive species" generated by these systems can affect LADH Tyr residues by different reaction mechanisms, including chlorination, iodination, nitration and adduct production.

Polyphenolic compounds, including the flavonoids mentioned under Results, are antioxidants, which is attributed to their free radical scavenging properties.[35–37] In contrast to these effects, the results reported here show that the assayed polyphenols are pro-oxidants. These compounds, including quercetin and catechin, can react with MPO/H_2O_2, producing phenoxyl or semiquinone radicals which can be oxidized by dioxygen producing O_2^-. This latter radical can react with quercetin quinol producing the semiquinone and H_2O_2.[38,42] These reactions may be valid for other flavonoids, and the resulting *o*-quinones could covalently bind thiol groups in LADH,[39,40] thus explaining, on the one hand, enzyme inactivation and, on the other, LADH protection by thiol compounds, such as GSH. Some polyphenolic compounds are also capable of autoxidation, a reaction which is associated to production of superoxide, hydrogen peroxide and hydroxyl radical.[41,42]

LADH inactivation by MPO-dependent systems, provides an interesting example of host defensive metabolic reactions possibly involved in killing and destruction of *T. cruzi* amastigotes in host infected cells. Absence of catalase in the parasite[43,44] as well as the presence of MPO in macrophages and polynuclear leukocytes,[45,46] would effectively contribute to the production of cytotoxic species in the parasite cells. Moreover, the discharge of HOCl, nitrite related radicals and other diffusable "reactive species" would also contribute to the trypanocidal action.

Acknowledgments

This work was aided by grants from the University of Buenos Aires and the Roemmers Foundation. We are indebted to the excellent technical assistance by Edith Röckel (Heidelberg), M.G. Gutiérrez and M.A.E. Veron (Buenos Aires). Dr. R.L. Krauth-Siegel was supported by the Deutsche Forschungsgemeinschaft (R.L.K-S, DFG K 1242/1-3).

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