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CATALYSIS OF NITROFURAN REDOX-CYCLING AND SUPEROXIDE ANION PRODUCTION BY HEART LIPOAMIDE DEHYDROGENASE*

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Abstract—Heart lipoamide dehydrogenase (LADH) catalyzed redox-cycling and $O_2^{\cdot}$ production by (5-nitro-2-furfurylidene)amino derivatives using NADH as electron donor. NADH was a much more effective electron donor than NADPH for the nitroreductase activity. $O_2^{\cdot}$ production was demonstrated by cytochrome *c* reduction, adrenochrome formation and the effect of superoxide dismutase. Under optimum conditions, nitroreductase activity was about 1% of LADH activity. One electron oxygen reduction and NADH oxidation correlated in 2:1 stoichiometry. The nitroreductase kinetics was in accordance with an ordered bi–bi mechanism. Nitrofuran derivatives bearing unsaturated five- or six-membered nitrogen heterocycles were more effective substrates than those bearing other groups, namely nifurtimox, nitrofurazone, nitrofurantoin and 5-nitro-2-furoic acid. Other nitro compounds (chloramphenicol, benznidazole, 2-nitroimidazole and 5-nitroindole) were ineffective. With the triazole, traizine and imidazole nitrofuran derivatives, the nitroreductase pH curve showed a maximum at pH 8.8, different from the pH optimum for the lipoamide reductase and diaphorase activities. Spectroscopic observations demonstrated pH-dependent structural changes in the triazole(I) and triazine derivatives which would affect their behavior as nitroreductase substrates. The nitroreductase activity was inhibited by *p*-chloromercuribenzoate and enhanced by cadmium and arsenite, whereas the NADH-induced LADH inactivation failed to affect the nitroreductase activity. In the absence of oxygen, LADH catalyzed nitrofuran reduction to products more reduced than the nitroanion, which were not reoxidized by oxygen. The anaerobic nitrofuran reduction was inhibited by cadmium and arsenite. The assayed nitrofuran compounds did not inhibit LADH lipoamide reductase activity, at variance with their action on glutathione reductase (Grinblat *et al.*, *Biochem Pharmacol* **38**: 767–772, 1989).

Lipoamide dehydrogenase (NADH–lipoamide oxireductase, EC 1.6.4.3; LADH‖) is a multifunctional enzyme which catalyzes NADH-linked lipoamide reductase, nicotinamide transhydrogenase, inorganic electron transferase and quinone diaphorase reactions [1–3]. The enzyme can also reduce aromatic nitro compounds as demonstrated with several nitrofuran derivatives [4], nitropyridine and nitropyridine-*N*-oxide [5]. LADH nitroreductase activity is, however, a scarcely investigated subject, as compared with the reaction catalyzed by other flavoenzymes such as NADPH–cytochrome P450 reductase [6], xanthine oxidase [6], the respiratory redox chain NADH-dehydrogenase [7] and the mitochondrial outer membrane cytochrome b_5 reductase [8].

A characteristic feature of many nitroheterocyclic compounds ($R{\cdot}NO_2$) is their capability for redox-cycling [6, 9–12] as described by Reactions 1 and 2, where

$$NAD(P)H + 2R{\cdot}NO_2 \rightarrow NAD(P)^+ + 2R{\cdot}NO_2^{\cdot} + H^+ \quad (1)$$

$$2R{\cdot}NO_2^{\cdot} + 2O_2 \rightarrow 2R{\cdot}NO_2 + 2O_2^{\cdot}. \quad (2)$$

$R{\cdot}NO_2^{\cdot}$ is the nitroanion and $O_2^{\cdot}$ the superoxide anion radical. In the present study, we demonstrate that porcine heart LADH catalyzed redox-cycling and $O_2^{\cdot}$ production by several nitrofuran derivatives of medical interest, namely, nitrofurantoin, nitrofurazone, nifurtimox and related compounds [13] (Fig. 1). Nifurtimox is the nitrofuran used for the treatment of American trypanosomiasis (Chagas' disease). Our results establish a significant difference between LADH and glutathione reductase (NAD(P)H:glutathione disulfide oxidoreductase, EC 1.6.4.2), another FAD-containing dehydrogenase, which is inhibited by 5-nitrofuran derivatives but fails to catalyze the latter redox-cycling [14–19].

* This paper is dedicated to Professor G. B. Marini Bettolo on the occasion of his 75th birthday.

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‖ Abbreviations and chemical terms: LADH, lipoamide dehydrogenase, NF, (5-nitro-2-furfurylidene)amino; nifurtimox, 3-methyl-4-[NF]-tetrahydro-4*H*-thiazine-1,1′-dioxide; NF-triazole, 4-[NF]-1,2,4-triazole; NF-pyrazole, 1-[NF]-pyrazole; NF-benzimidazole, 1-[NF]-benzimidazole; NF-imidazole, 1-[NF]-imidazole; NF-triazole(I), 3,5-bis(methylthio)-4-[NF]-1,2,4-triazole; NF-triazine, 3-thioxo-4-[NF]-6-methyl-1,2,4-triazin-5-one; nitrofurantoin, 1[[(5-nitro-2-furanyl)methylene]-amino]-2,4-imidazolidine-dione; nitrofurazone, 5-nitro-2-furaldehyde semicarbazone; benznidazole, *N*-benzyl-2-nitro-1-imidazole-acetamide; DMFA, dimethylformamide; DCI, dichlorophenol-indophenol; CMB, *p*-chloromercuribenzoate; and SOD, superoxide dismutase.

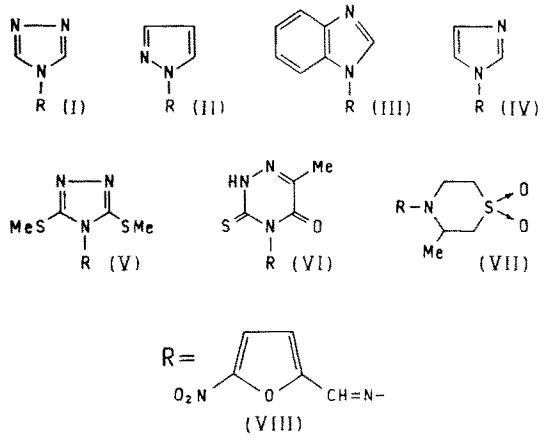

Fig. 1. Structures of LADH nitrofuran electron acceptors. Each compound consists of moieties A (structures I–VII) and B (structure VIII). (I) 4*R*-1,2,4-triazole; (II) 1*R*-pyrazole; (III) 1*R*-benzimidazole; (IV) 1*R*-imidazole; (V) 3,5-bis(methylthio)-4*R*-1,2,4-triazole; (VI) 3-thioxo-4*R*-6-methyl-1,2,4-triazin-5-one; (VII) 3-methyl-4*R*-tetrahydro-4*H*-thiazine-1,1′-dioxide; and (VIII) (5-nitro-2-furfuryl-idene)amino (R or NF in the text).

MATERIALS AND METHODS

Materials. Porcine heart LADH (Type III), DL-6,8-thioctic acid amide (lipoamide), NAD^+, NADH, dichlorophenol-indophenol (DCI), cytochrome *c* Type VI, epinephrine, catalase (from bovine liver), glucose oxidase (Type X), glucose, microperoxidase MP-11, *p*-chloromercuribenzoate (CMB), $NaBH_3$, EDTA, nitrofurazone, nitrofurantoin, superoxide dismutase (SOD, from bovine erythrocytes) and dimethylformamide (DMFA) were purchased from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). The specific activity of the original LADH preparations ranged from 115 to 150 units/mg protein (NADH-lipoamide assay), but storage of diluted enzyme solutions at 5° produced somewhat lower activities (down to 80 units/mg protein). Nifurtimox was obtained from Bayer (Leverkusen, F.R.G.); NF-triazole, NF-pyrazole, NF-benzimidazole, NF-imidazole, NF-triazole(I) and NF-triazine were synthesized by Mester *et al.* [13] and supplied by Dr. R. Claramunt, University of Madrid, Spain. Benznidazole was obtained from Hoffmann-LaRoche, Basle, Switzerland. $NaAsO_2$ and $CdCl_2$ were purchased from Mallinckrodt Chemical Works (New York, U.S.A.) and May & Baker (Dagenham, U.K.), respectively. Dihydrolipoamide was prepared from DL-lipoamide by $NaBH_3$ reduction, as described [20]. Its purity was determined spectrophotometrically at 340 nm, using 0.3 μg LADH/mL and 1.0 mM NAD^+, as described below. Other reagents were as described in Ref. 19.

Enzyme assays. Nitroreductase activity was measured at 30°, by the rate of (a) NADH-oxidation (Reaction 1) or (b) $O_2^{\cdot-}$ production (Reaction 2). The rate of NADH-oxidation was measured spectrophotomerically at 340 nm, using an Aminco 2DW™ UV/VIS spectrophotometer. Unless stated otherwise, the reaction mixture contained 1.0 μg LADH/mL, the nitrofuran compound, 0.1 mM NADH and 50 mM KH_2PO_4–K_2HPO_4, pH 7.4; total volume, 3.0 mL. The reaction was started by adding the enzyme. The reference cuvette contained the corresponding concentration of nitrofuran compound in potassium-phosphate buffer. The nitrofuran compound was added in 10–30 μL DMFA, at the concentrations stated in Results. $O_2^{\cdot-}$ production was measured by either the cytochrome *c* [21] or the adrenochrome method [22, 23]. Unless stated otherwise, the reaction mixture contained 0.16 to 1.0 μg LADH/mL, 0.1 mM NADH, 50 mM KH_2PO_4–K_2HPO_4, pH 7.4, and either 16 μM cytochrome *c* (cytochrome *c* method) or 1.0 mM epinephrine (adrenochrome method); total volume, 3.0 mL. The reaction was started by adding the nitrofuran compound. Other experimental conditions were as described above. Cytochrome *c* reduction was measured at 550–540 nm ($\varepsilon = 19\ mM^{-1}\ cm^{-1}$) and adrenochrome formation at 485–575 nm ($\varepsilon = 2.97\ mM^{-1}\ cm^{-1}$). The spectrophotometric measurements were performed with the Aminco–Chance spectrophotometer, at 30°. $O_2^{\cdot-}$ production values were subtracted from the experimental values before the nitrofuran addition. When the effect of $CdCl_2$ on $O_2^{\cdot-}$ production was measured, 80 mM Tris–HCl, pH 7.6, 0.1 mM EDTA replaced the potassium-phosphate buffer. H_2O_2 generation was measured by the microperoxidase method [24], at 419–407 nm ($\varepsilon = 78\ mM^{-1}\ cm^{-1}$). The reaction mixture contained 0.1 mM NADH, 50 mM KH_2PO_4–K_2HPO_4, pH 7.4, 3.3 μM microperoxidase and 3.6 to 22 μg LADH/mL.

LADH activity was measured by (a) the rate of NADH-oxidation using lipoamide as electron acceptor, or (b) the rate of NAD^+ reduction using dihydrolipoamide as electron donor. The reaction mixtures contained 50 mM KH_2PO_4–K_2HPO_4, pH 7.4, 0.1 to 0.4 μg LADH/mL and either 0.2 mM NADH and 1.0 mM lipoamide or 1.0 mM NAD^+ and 1.0 mM dihydrolipoamide, as substrates; total volume, 3.0 mL. NADH concentration in the reaction mixture was measured at 340 nm using the Perkin–Elmer 550S UV/VIS spectrophotometer, at 30°. When the effect of $CdCl_2$ on NADH oxidation was measured, 80 mM Tris–HCl, pH 7.6, and 0.1 mM EDTA replaced the potassium-phosphate buffer.

Diaphorase activity was measured by the rate of DCI reduction, using NADH as electron donor. The reaction mixture contained 0.2 mM NADH, 0.4 μg LADH/mL, 40 μM DCI, 50 mM potassium-phosphate buffer, at pH 7.4 or at the pH values indicated in Results. DCI reduction was measured spectrophotometrically at 600 nm ($\varepsilon = 19\ mM^{-1}\ cm^{-1}$) and 30°, using a Perkin–Elmer 550S UV/VIS spectrophotometer. When the SH-reagents were assayed, the reaction mixture contained 50 mM KH_2PO_4–K_2HPO_4, pH 7.4, 1.0 mM EDTA (in CMB experiment), or 50 mM KH_2PO_4–K_2HPO_4, pH 7.4, 0.1 mM EDTA (in $NaAsO_2$ experiment), or 80 mM Tris–HCl buffer, pH 7.6, 0.1 mM EDTA (in $CdCl_2$ experiment). In all assays with SH-reagents, samples were preincubated for 7 min (experiments with CMB) or for 2 min (experiments with $NaAsO_2$ and $CdCl_2$), before starting the reaction with NADH; total volume of the reaction mixture, 3.0 mL. CMB,

$NaAsO_2$ and $CdCl_2$ were added at the concentrations stated in Results.

Absorption spectra. These were obtained with the Aminco 2DW™ UV/VIS spectrophotometer.

Permeabilized mitochondria. Rat liver mitochondria were prepared by standard methods [25] using 0.25 M sucrose, 5.0 mM Tris–HCl, pH 7.4, 1.0 mM EDTA as homogenization medium. After centrifugation at 6000 *g*, the pellet was suspended in 20 mM KH_2PO_4–K_2HPO_4, pH 7.4, Triton X-100 (1%, v/v) and frozen–thawed three times. Protein was determined by the biuret method [26].

Expression of results. The results presented are averages of duplicate measurements. Experimental values deviated from the mean value by less than 5%.

RESULTS

As shown in Table 1, LADH catalyzed NF-benzimidazol, NF-pyrazole and NF-imidazole redox-cycling, in accordance with Reactions 1 and 2. $O_2^{\cdot}$ production was demonstrated by cytochrome *c* reduction, adrenochrome formation and superoxide dismutase effect. Measurements of H_2O_2 production under similar experimental conditions yielded negative results (experimental data omitted). Figure 2 shows the effect of increasing concentrations of LADH on the rate of NADH oxidation, using NF-triazole(I) as the electron acceptor. The nitroreductase activity was linearly related to the enzyme concentration, the molecular activity (200 mol NADH/min) being about 1.4% of the lipoamide reductase activity (1.4×10^4 mol NADH/min). In the absence of lipoamide and nitrofuran, NADH oxidation rate was about 3% of the nitrofuran-induced rate, the unstimulated molecular activity being 6 mol NADH/min. Therefore, NF-triazole(I) produced a 33-fold increase of the latter activity.

Figure 3 shows the effect of increasing NF-triazole concentrations on the rate of NADH-oxidation, at a fixed concentration of NADH, whereas the figure

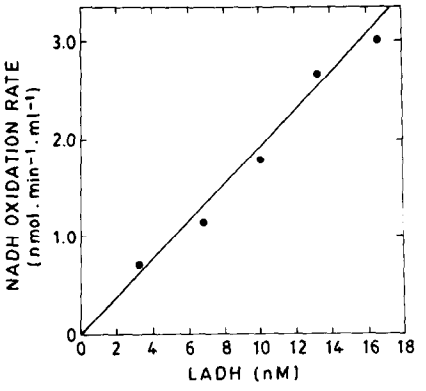

Fig. 2. Effect of LADH concentration on NADH oxidation at fixed NADH and NF-triazole(I) concentrations. The reaction mixture contained 0.1 mM NADH, 100 μM NF-triazole(I), 50 mM KH_2PO_4–K_2HPO_4, pH 7.4, and LADH as indicated on the abscissa. The reaction velocity was measured by the rate of NADH oxidation. Other experimental conditions were as indicated in Materials and Methods. Values include the unstimulated NADH-oxidation rate: 4.0 mol NADH/min/mol LADH.

inset shows the effect of increasing concentrations of NADH on $O_2^{\cdot}$ production, at a fixed concentration of NF-triazole(I). Hyperbolic curves were obtained in both cases, in accordance with Michaelian kinetics.

Table 2 data show the influence of the nitrofuran group counterpart on the activity of the whole molecule. Compounds were assayed for (a) $O_2^{\cdot}$ generation, (b) NADH oxidation, and (c) dihydrolipoamide oxidation by NAD^+. The latter assay was performed using either the purified enzyme or permeabilized mitochondria. The assayed nitrofurans enhanced the rate of $O_2^{\cdot}$ production and NADH oxidation, but they did not affect the rate of dihydrolipoamide oxidation by NAD^+, irrespective of the enzyme preparation used. The calculated values for the ($O_2^{\cdot}$ production)/(NADH consumption) molar ratio were about 2.0, as was to be expected from the combined operation of Reactions 1 and 2.

Table 1. Effects of NF-benzimidazole, NF-pyrazole and NF-imidazole on LADH-catalyzed $O_2^{\cdot}$ production

Nitrofuran	Concn (μM)	$O_2^{\cdot}$ assay	SOD (units/mL)	$O_2^{\cdot}$ production ($\frac{\mu mol/min}{mg\ LADH}$)
NF-benzimidazole	50	Cytochrome *c*	0	2.22
			0.43	1.23 (45)*
			0.86	0.70 (68)
NF-pyrazole	50	Adrenochrome	0	1.40
			0.43	0 (100)
NF-imidazole	100	Adrenochrome	0	2.09
			0.09	0.73 (65)
			0.18	0.43 (79)
			0.30	0 (100)

Experimental conditions were as described in Materials and Methods. The $O_2^{\cdot}$ production values were subtracted from the experimental values before the nitrofuran addition. Control (unstimulated) $O_2^{\cdot}$ production rate was 0.09 ± 0.005 μmol/min/(mg LADH) (mean ± SE; 14 measurements; cytochrome *c* method).

* Values in parentheses are the percent inhibition of $O_2^{\cdot}$ production

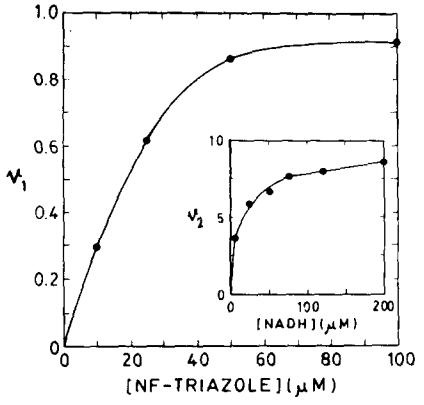

Fig. 3. Effect of NF-triazole concentration on the rate of NADH oxidation at fixed NADH and LADH concentrations. The reaction mixture contained 0.1 mM NADH, 1.0 μg LADH/mL, 50 mM KH_2PO_4–K_2HPO_4, pH 7.4, and NF-triazole as indicated on the abscissa. The reaction velocity was measured by the rate of NADH oxidation (v_1, μmol/min/mg LADH). Inset: Effect of NADH concentration on the rate of $O_2^{\cdot}$ production at fixed concentrations of NF-triazole(I) (50 μM) and LADH (0.67 μg/mL); 50 mM potassium-phosphate buffer, pH 8.1; NADH concentration as indicated on the abscissa. $O_2^{\cdot}$ production was measured by the adrenochrome method (v_2, μmol/min/mg LADH). Other experimental conditions were as described in Materials and Methods. Control (unstimulated) NADH oxidation and $O_2^{\cdot}$ production rates were as given in the legends of Fig. 2 and Table 1 respectively.

Data in Table 2 indicate that the nitrofuran activity was affected by the substituent group, since linkage to an aromatic heterocycle [e.g. triazole(I) or benzimidazole] produced more effective compounds than linkage to 4*H*-thiazine (in nifurtimox), hydrazine carboxamide (in nitrofurazone) or imidazolidinedione (in nitrofurantoin). Activity differences between compounds were apparent at all concentrations in the 25–200 μM range (results now shown). Under the same experimental conditions,

Table 3. Nitroreductase pyridine-nucleotide specificity

Nitrofuran (100 μM)	$O_2^{\cdot}$ production (μmol/min/mg LADH)	
	NADH	NADPH
NF-triazole(I)	7.15	0.18
NF-pyrazole	3.21	0.12
Nitrofurantoin	1.08	0.20
Nifurtimox	0.96	0

Samples contained 0.1 mM NAD(P)H; $O_2^{\cdot}$ production was measured by the cytochrome *c* method. Other experimental conditions were as described in Materials and Methods. Control (unstimulated) $O_2^{\cdot}$ production rate (substrate: NADH) was as given in the legend of Table 1.

other nitro compounds such as chloramphenicol (nitrophenyl derivative), benznidazole, 2-nitroimidazole and 5-nitroindole were ineffective (experimental data omitted). As regards the nitroreductase specificity for pyridine-nucleotides, NADH was a much more effective electron donor than NADPH (Table 3), in good agreement with the lipoamide reductase activity [1, 2].

Figure 4 shows the effect of pH on LADH nitroreductase activity. This latter was determined by $O_2^{\cdot}$ production, using NF-triazole(I), NF-triazine, NF-imidazole and nifurtimox as electron acceptor. The pH curves for NF-triazole(I), NF-triazine and NF-imidazole showed maxima at about pH 8.8, at variance with the optimal pH (pH 6.5) for lipoamide reductase activity [2]. The pH variation, however, scarcely affected the nifurtimox effect (Fig. 4). These results disagree with the effect of pH on diaphorase activity which, as previously reported [27], exhibited a great increase in activity at acidic pH. Since for most aromatic nitrocompounds the one-electron reduction potential is unchanged between pH 7 and 9 [28], no significant modifications in the reaction of the nitro radical with O_2 may be expected to occur in that pH range. The pH effects observed with NF-

Table 2. Effect of nitrofuran structure on LADH-catalyzed reactions

Nitrofuran (50 μM)	$O_2^{\cdot}$ generation*		NADH oxidation*	Dihydrolipoamide oxidation	
	Adrenochrome method	Cytochrome *c* method		LADH*	Mitochondria†
NF-triazole(I)	2.63	2.63	1.36	215	0.65
NF-benzimidazole		1.95			0.68
NF-triazole	1.36	1.69	0.87	209	0.58
NF-pyrazole	1.40	1.38	0.66	212	0.64
Nitrofurazone	0.53	0.50			0.66
Nifurtimox		0.35	0.22	214	0.64
Nitrofurantoin		0.26		218	0.65
5-Nitro-2-furoic acid	0	0			
None				208	0.67

Experimental conditions were as described in Materials and Methods. Control (unstimulated) NADH oxidation rate was 0.04 μmol/min/mg LADH (duplicate measurements). Control $O_2^{\cdot}$ production rate was as given in the legend of Table 1.

* Values are given in μmol/min/mg LADH.

† Values are given in μmol/min/mg protein.

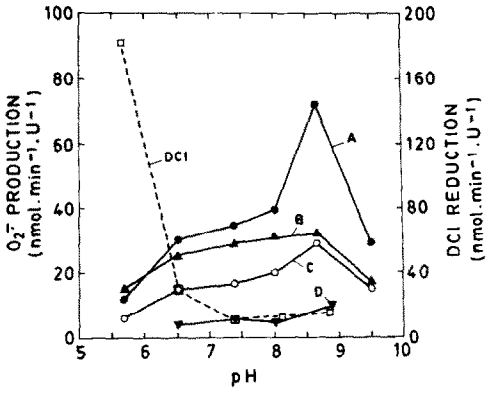

Fig. 4. Effect of pH on LADH-catalyzed nitrofuran redox-cycling. The reaction mixture contained 0.33 μg LADH/mL, 0.1 mM NADH, 50 μM nitrofuran and 50 mM potassium-phosphate buffer; pH was as indicated on the abscissa. $O_2^{\cdot}$ production was measured by the cytochrome *c* method. The reaction was started by adding the nitrofuran. Key: (A) NF-triazole(I); (B) NF-triazine; (C) NF-imidazole; (D) nifurtimox; and DCI, diaphorase activity. Other experimental conditions were as described in Materials and Methods. $O_2^{\cdot}$ production is referred to enzyme units, at pH 7.4, since the enzyme preparations used had different lipoamide reductase specific activities.

triazole(I), NF-triazine and NF-imidazole, therefore, may involve other mechanisms, such as variation of the LADH–nitrofuran interaction, depending on the nitrofuran protonation–deprotonation state. This assumption fits in well with the effect of pH on NF-triazole(I) and NF-triazine spectra (Fig. 5), in contrast to the nifurtimox spectrum (not shown), which was not affected by pH variation.

Kinetic parameters for the nitroreductase activity were measured by using NF-triazine as electron acceptor and according to the following equation:

$$V_0 = \frac{V_{max}[A][B]}{K_{ia}K_b + K_a[B] + K_b[A] + [A][B]}$$

where V_0 is the initial velocity at given concentrations of *A* (NADH) and *B* (nitrofuran), V_{max} is the maximal velocity at infinite concentrations of NADH and nitrofuran, K_{ia} is the dissociation constant for NADH, and K_a and K_b are the Michaelis constants for NADH and nitrofuran respectively [29]. The values for kinetic constants were calculated by a computation method [30]. The kinetic data set included 24 points, NADH concentration varying in the 5–100 μM range and NF-triazine concentration in the 25–200 μM range. The double-reciprocal plots of initial velocity were a family of intersecting lines and the corresponding data were fitted to the equation given above. The kinetic parameter values obtained were as follows (mean ± SE): K_a, 13.4 ± 3.4 μM; K_b, 126 ± 20 μM; K_{ia}, 22.9 ± 4.5 μM; V_{max}, 5.3 ± 0.4 μmol/min/mg LADH; σ, 0.063 and σ^2, 0.0039. These results fit in well with the ordered bi–bi reaction mechanism [29, 30].

LADH is inhibited by di- and monothiol reagents, such as arsenite [1, 31–33], cadmium [33, 34] and mercurial compounds [34–36]. To substantiate further the enzyme catalysis of nitrofuran redox-cycling, chemical modification of the enzyme thiols was performed. Table 4 shows that cadmium and arsenite greatly increased nitroreductase activity, whereas CMB produced 85% inhibition. Figure 6 shows the effect of increasing concentrations of cadmium and arsenite on (a) nitroreductase; (b) diaphorase and (c) lipoamide reductase activities. The dithiol reagents enhanced (a) and (b) activities, as revealed by $O_2^{\cdot}$ production and DCI reduction, but lipoamide reductase was strongly inhibited. Figure 7 shows the results of a similar experiment using CMB. Interestingly enough, CMB inhibited the nitro-

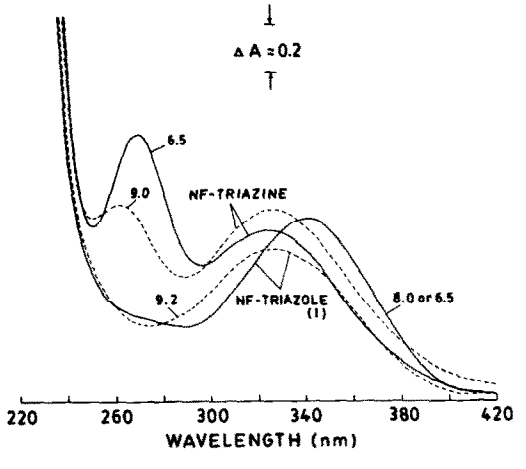

Fig. 5. Effect of pH on NF-triazole(I) and NF-triazine absorption spectra. The reaction mixture contained 50 μM nitrofuran in 50 mM KH_2PO_4–K_2HPO_4; pH was as indicated by the figures near each spectrum. Other experimental conditions were as described in Materials and Methods.

Table 4. Variation of nitroreductase activity after modification of LADH by SH-reagents

Nitrofuran (50 μM)	$O_2^{\cdot}$ production (μmol/min/mg LADH)			
	Control sample	SH-reagent-treated sample		
		$CdCl_2$	$NaAsO_2$	CMB
NF-triazole(I)	3.71	6.30	5.18	0.99
NF-pyrazole	2.94	8.00	4.13	0.45
NF-benzimidazole	1.68	2.96	2.27	0.60
Nitrofurantoin	0.75	1.24	0.96	0.33
Nifurtimox	0.65	1.17	0.74	0.23

$O_2^{\cdot}$ was measured by the cytochrome *c* method. $CdCl_2$, (300 μM), $NaAsO_2$ (300 μM) or CMB (20 μM) was added to the reaction mixture containing LADH, the nitrofuran compound and either 80 mM Tris–HCl, pH 7.6, 0.1 mM EDTA (with $CdCl_2$) or 50 mM KH_2PO_4–K_2HPO_4, pH 7.4 (with $NaAsO_2$ or CMB). The reaction was started by adding NADH. Other experimental conditions were as described in Materials and Methods. Control (unstimulated) $O_2^{\cdot}$ production rate was as given in the legend of Table 1.

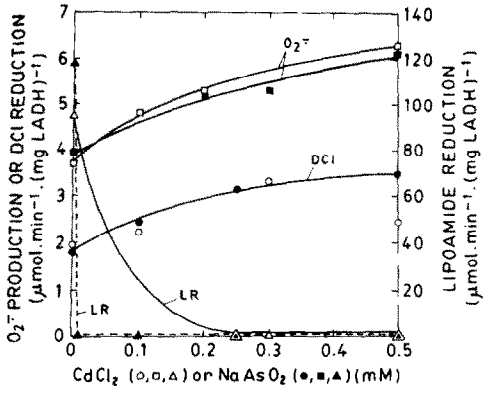

Fig. 6. Cadmium and arsenite enhancement of nitroreductase activity. SH-reagent concentration was as stated on the abscissa. Nitroreductase activity ($O_2^{\cdot-}$; □, ■) was measured by $O_2^{\cdot-}$ production (cytochrome *c* method) using 50 μM NF-triazole(I) and 0.16 μg LADH/mL. Diaphorase (DCI; ○, ●) and lipoamide reductase (LR; △, ▲) activities were measured using 0.33 and 0.16 μg LADH/mL respectively. Other experimental conditions were as described in Materials and Methods. Control (unstimulated) $O_2^{\cdot-}$ production rate was as given in the legend of Table 1.

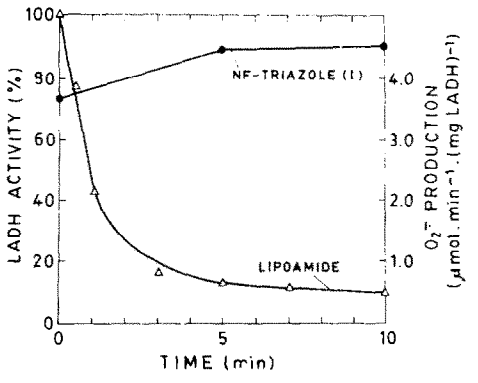

Fig. 8. Catalysis of nitrofuran redox-cycling by NADH-inactivated LADH. LADH (0.7 μg/mL; specific activity, 114 units/mg) was incubated with 0.2 mM NADH in 50 mM KH_2PO_4-K_2HPO_4, pH 7.4, for the time indicated on the abscissa. The enzyme activities were then measured by adding 1.0 mM lipoamide or 100 μM NF-triazole(I), as indicated above. Lipoamide reductase activity and NF-triazole(I) redox-cycling were measured by the rate of NADH oxidation or $O_2^{\cdot-}$ production (cytochrome *c* method) respectively. The former activity is expressed as the percentage value of the 0-time sample activity. Other experimental conditions were as described in Materials and Methods. Control (unstimulated) $O_2^{\cdot-}$ production rate was as given in the legend of Table 1.

reductase activity, unlike cadmium and arsenite, but, like the other SH-reagents, it stimulated diaphorase and inhibited lipoamide reductase activity.

LADH preincubation with NADH determined lipoamide reductase inactivation ([1] and Fig. 8), which should imply intramolecular thiol oxidation, as indicated by the enzyme protection by reduced glutathione and β-mercaptoethanol (experimental data omitted). In close agreement with the effect of dithiol reagents, the enzyme capability for nitrofuran redox-cycling was not decreased by NADH and a slight activation was observed after 5–10 min of incubation (Fig. 8).

Measurement of nitroreductase activity under strictly anaerobic conditions rendered the results presented in Fig. 9. Nifurtimox was selected for this experiment because NADH absorbance did not overlap the nitrofuran maximum at 406 nm. The difference spectra show the decrease in nifurtimox absorbance at 406 nm due to the nitro group reduction to products more reduced than the nitroanion (Expt. A). Addition of NADH to the reaction mixture determined the appearance of a small peak at 406 nm (in all probability the nitroanion radical), which disappeared at later stages of incubation. Nifurtimox reduction was irreversible since admission of oxygen to the reaction mixture failed to re-establish the original nitrofuran spectrum. Absorbance also diminished at 340 nm due to NADH-oxidation, but this effect was partly masked by nifurtimox absorption. At variance with these results, incubation of a similar sample under aerobic conditions produced difference spectra corresponding solely to NADH oxidation (Expt. B), as expected from Reaction 2. The rate of NADH aerobic oxidation was faster than that of nifurtimox anaerobic reduction (Fig. 9, inset). Cadmium and arsenite inhibited nifurtimox anaerobic reduction (Table 5), at variance with their action on the nitrofuran redox-cycling in Table 4 and Fig. 6.

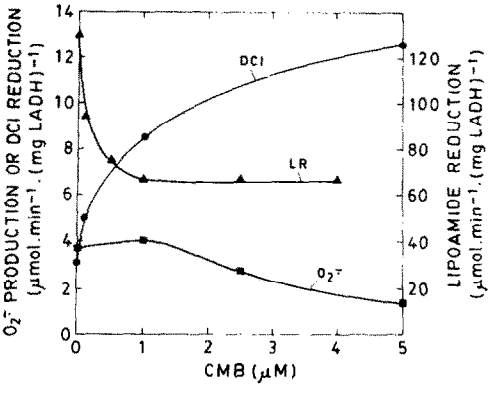

Fig. 7. CMB inhibition of nitroreductase activity. CMB concentration was as indicated on the abscissa. Nitroreductase activity ($O_2^{\cdot-}$; ■) was measured by $O_2^{\cdot-}$ production (adrenochrome method) using 50 μM NF-triazole(I) and 0.66 μg LADH/mL. Diaphorase (DCI; ●) and lipoamide reductase (LR; ▲) activities were measured using 0.40 and 0.16 μg LADH/mL respectively. Other conditions were as described in the legend of Fig. 6.

DISCUSSION

Our observations support the hypothesis that LADH catalyzes nitrofuran redox-cycling in accordance with Reactions 1 and 2. The rate of $O_2^{\cdot-}$ production via nitroreductase activity was, however, relatively slow and, accordingly, in the presence of lipoamide, electron withdrawal by nitrofuran redox-

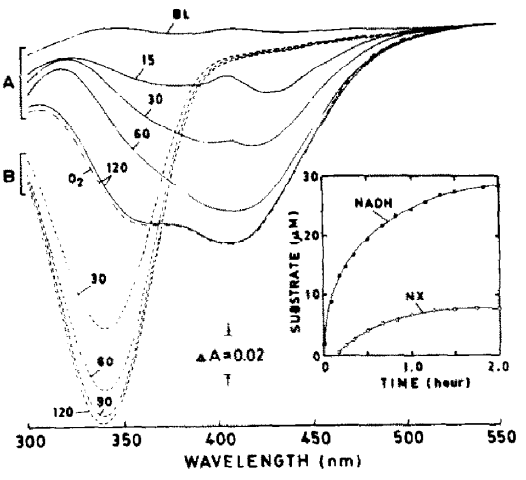

Fig. 9. Difference spectra of NADH-reduced against oxidized nifurtimox. Expt. A: the anaerobic assay contained 3.3 μg LADH/mL, 50 μM nifurtimox, 50 mM KH_2PO_4–K_2HPO_4, pH 7.4, 1.0 μg glucose oxidase/mL, 50 mM glucose, and 992 units/mL catalase. After a 5-min gasssing of the reaction mixture with argon in an air-tight cuvette, the latter was placed in the spectrophotometer cell compartment (sample light-beam) at 30°. The reference sample contained nifurtimox and potassium-phosphate buffer as above, under air. After recording the base line (BL), NADH (10 μL of a 30 mM solution, pregassed with argon; 100 μM final concentration) was added to both samples, and absorbance was recorded at the times (min) indicated by the figures near the spectra. After a 2-hr incubation, the anaerobic cuvette was opened, the reaction mixture was aerated, and the aerobic spectrum (O_2) was recorded. Expt. B: similar conditions except that (a) both samples were under air, and (b) glucose oxidase, glucose and catalase additions were omitted. Inset: Representation of NADH oxidation (NADH) and nifurtimox (NX) reduction as a function of incubation time. Values were calculated from the absorbance variation. Nifurtimox $\varepsilon_{406\,nm}$ = 14.8 $mM^{-1}\,cm^{-1}$. Other experimental conditions were as described in Materials and Methods.

cycling did not produce significant inhibition of the latter reduction (Table 2).

LADH and glutathione reductase bear striking similarity to one another, both structural and mechanistically [3]. The observations described here, taken together with previous ones by Grinblat *et al.* [19], demonstrate, however, an important difference between these two flavoproteins. Thus, LADH displays nitroreductase activity and catalyzes nitrofuran redox-cycling whereas glutathione reductase is inhibited by nitrofuran compounds, which are incapable of electron withdrawal from the reduced enzyme. The binding mode of the nitrofuran compound in the two flavoproteins would depend on the structure of the respective FAD sites, whose homology is less than 50% [3]. Further differences between FAD microenvironments have been detected by fluorescence measurements of bound FAD [37]. Moreover, these enzyme pockets binding the disulfide substrate are also different [38].

Table 5. Effect of thiol reagents on LADH-catalyzed, anaerobic reduction of nifurtimox

Thiol reagent	Concn (μM)	Nifurtimox reduction (μM)
None		7.43
$NaAsO_2$	60	5.27 (29)*
$NaAsO_2$	300	3.51 (53)
$CdCl_2$	300	0.17 (98)

Experimental conditions were as described in the legend of Fig. 9 (Expt. A; anaerobic sample). Incubation time, 90 min.

* Values in parentheses are the percentage inhibition of nifurtimox reduction.

Thiols in native LADH are remarkably unreactive, except with mercurials [27, 36] and Cu^{2+} [2]. The results in Table 4 and Fig. 7 indicate that the CMB-reactive thiols were essential for the nitroreductase, but not for the diaphorase, thus establishing a significant difference between those activities. Cadmium and arsenite effects (Table 4 and Fig. 6) suggest that Reaction 1 did not involve the active disulfide groups. On the contrary, the nitroreductase activity was enhanced in the modified LADH, in close agreement with the diaphorase variation. It is known [3] that the diaphorase activity is favored in LADH monomeric forms and, accordingly, it is enhanced when the active disulfides are blocked by cadmium and arsenite. As regards the NADH-induced inactivation (Fig. 8), which spared the nitroreductase activity, it should also imply intramolecular thiol oxidation, as indicated by LADH protection by GSH and β-mercaptoethanol.

The nitroreductase kinetics implies an ordered bi–bi mechanism, ternary complex formation, and product inhibition [30]. The initial rate of $R{\cdot}NO_2^{\cdot}$ production may be faster and the rate of $O_2^{\cdot}$ production slower the more positive the value of $E_7^1(R{\cdot}NO_2/R{\cdot}NO_2^{\cdot})$, which depends on the substituent group at the nitrofuran C-2 [28]. The nitrofuran C-2 substituent can influence electron transfer at the nitroanion level as illustrated by the LADH-catalyzed conversion of *cis*-3-(5-nitro-2-furyl)-acrylamide to its *trans* isomer [4]. Moreover, the nitrogen heterocycle structure may affect the molecular capability for binding at the LADH active site, as suggested by results in Figs. 4 and 5. Taken together, these effects may be relevant for explaining the influence of unsaturated nitrogen heterocycles on $O_2^{\cdot}$ production, as described in Tables 2–4. In this regard, the latter groups would be more effective than their counterparts in nifurtimox, nitrofurantoin, nitrofurazone and 5-nitro-2-furoic acid.

The reduction of nitroheterocyclic compounds by LADH is mechanistically interesting, because the nitro group can accept various numbers of electrons. Thus, the major product from the enzyme-catalyzed reduction of 4-nitropyridine is presumably formed in a four-electron redox reaction [5]. It should be noted that 4-nitropyridine and its *N*-oxide facilitated NADH oxidation without stimulating O_2 consumption [5], thus ruling out electron transfer to oxygen and $O_2^{\cdot}$ formation. The reduction pathway for the nitro group of nitroheterocyclic compounds is strongly influenced by the nature of the solvent [39]. In aprotic medium, a reversible one-electron

reduction takes place to form a stable nitroanion radical, whereas in aqueous medium, a single irreversible 4-electron reduction occurs to give the hydroxylamine. In LADH, the FAD flavin is bound in a hydrophobic milieu [2] and, then, the nitroanion radical would easily react with oxygen, bypassing the active center di-thiol (Fig. 6 and Table 4). Nevertheless, anaerobically, LADH catalyzed the reduction of the nitrofuran nitro group to products more reduced than the nitroanion (Fig. 9). An important feature of this reaction was its irreversiblility by oxygen, in close agreement with the production of nitroso and hydroxylamine derivatives. The four-electron reduction reaction should involve LADH thiols, as shown by the inhibitory effect of cadmium and arsenite in Table 5.

Several mammalian enzymes are known to have nitroreductase activity. In the liver cell, the major microsomal nitroreductase is NADPH–cytochrome P450 reductase, whereas in the cytosol, xanthine oxidase, DT-diaphorase and aldehyde oxidase have also nitroreductase activity [40]. LADH is located in the mitochondrial matrix and, therefore, is less accessible to nitrofuran compounds than the microsomal and cytosolic enzymes. Besides, mitochondria contain catalase, superoxide dismutase and glutathione peroxidase [41], which detoxify the peroxides generated by LADH-catalyzed nitrofuran redox-cycling. Hence, the toxicological impact of peroxide production by LADH-dependent reactions should be minor, as compared with microsomal and cytosolic production. This reasoning fits in well with the relatively small contribution of the mitochondrial system to total peroxide production by intact cells [41]. As regards the LADH-catalyzed, anaerobic, multi-electron nitrofuran reduction, that leads to toxic nitrose and hydroxylamine derivatives [6], this metabolic pathway is borne out by the results in Fig. 9. Furthermore, oxygen gradients in the liver, particularly in the intercapillary space of the Krogh tissue cylinder, may be so steep [41] that there should be insufficient oxygen for both nitroanion and NADH oxidation. In these circumstances, LADH can catalyze nitrofuran reduction to products whose toxicity should prove greater than that of superoxide related radicals. These events may well occur in the human liver, known to contain LADH [42], after administration of nitrofuran drugs.

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