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# INACTIVATION OF LIPOAMIDE DEHYDROGENASE BY COBALT(II) AND IRON(II) FENTON SYSTEMS: EFFECT OF METAL CHELATORS, THIOL COMPOUNDS AND ADENINE NUCLEOTIDES

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Fe(II)-and Co(II)-Fenton systems (FS) inactivated the lipoamide reductase activity but not the diaphorase activity of pig-heart lipoamide dehydrogenase (LADH). The Co(II) system was the more effective as LADH inhibitor. Phosphate ions enhanced the Fe(II)-FS activity. EDTA, DETAPAC, DL-histidine, DL-cysteine, glutathione, DL-dithiothreitol, DL-lipoamide, DL-thioctic acid, bathophenthroline, trypanothione and ATP, but not ADP or AMP, prevented LADH inactivation. Reduced disulfide compounds were more effective protectors than the parent compounds. Mg ions counteracted ATP protective action. Glutathione and DL-dithiothreitol partially restored the lipoamide dehydrogenase activity of the Fe(II)-FS-inhibited LADH. DL-histidine exerted a similar action on the Co(II)-FS-inhibited enzyme. Ethanol, mannitol and benzoate did not prevent LADH inactivation by the assayed Fenton systems and, accordingly, it is postulated that site-specific generated $HO^{\cdot}$ radicals were responsible for LADH inactivation. With the Co(II)-FS, oxygen reactive species other than $HO^{\cdot}$, might contribute to LADH inactivation.

KEY WORDS: Lipoamide dehydrogenase; cobalt(II); iron(II); Fenton systems; hydrogen peroxide; enzyme inactivation; nucleoside phosphates; metal chelators; lipoamide; thiol compounds; trypanothione.

Abbreviations and chemical terms used: LADH, lipoamide dehydrogenase (NADH-lipoamide oxidoreductase, EC 1.6.4.3); ROS, reactive oxygen species; Fe(II)-FS, iron(II) Fenton system; Co(II)-FS, cobalt(II) Fenton-like system; lipoamide, DL-6,8-thioctic acid amide; thioctic acid, DL-6,8-thioctic (lipoic) acid; DETAPAC, diethylenetriaminepentaacetic acid; DCI, dichlorophenolindophenol; SOD, superoxide dismutase; GSH and GSSG, reduced and oxidized glutathione, respectively; DTT, DL-dithiothreitol; TBA, thiobarbituric acid.

## INTRODUCTION

Mammalian lipoamide dehydrogenase, a flavoprotein disulfide oxidoreductase, is a common component of α-oxoacid dehydrogenase complexes, such as the pyruvate dehydrogenase complex, the α-oxoglutarate dehydrogenase complex and the branched chain α-oxoacid dehydrogenase complex[1-3]. Two Cys and one His residue seem to be essential constituents of the catalytic site[4]. In most eukaryotic organisms, LADH is a matrix-located mitochondrial enzyme and, therefore, it may

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be reasonable to assume that LADH should be accessible to ROS generated at the inner mitochondrial membrane[5,6], matrix LADH[7] or both. ROS include HO˙ radicals, hydrogen peroxide, and hypervalent iron-peroxy complexes, which are capable of modifying proteins and, accordingly, of inactivating enzymes[8-12]. Mitochondria lack catalase[13], a powerful anti-oxidant, and mitochondrial LADH may then be a candidate for oxidative damage. In order to test LADH sensitivity to ROS, we assayed the Fe(II)-FS (Fe(II) + $H_2O_2$) and a Fenton-like system (Co(II) + $H_2O_2$) on LADH. The FSs used were selected on the basis of (a) their capability of generating ROS, in particular HO˙ radicals[8-10, 14-18]; (b) the possible presence of Fe(II) in mitochondria, as a "low-molecular-mass" pool[9] and (c) the role of Co(II) as an essential trace element for mammalian nutrition, and also as a carcinogenic agent[19]. The results obtained prove that ROS can inactivate LADH but two mitochondrial metabolites, namely, ATP and glutathione can protect LADH from oxidative damage.

## MATERIALS AND METHODS

*Materials.* Porcine heart LADH (Type III), lipoamide, thioctic acid, $NAD^+$, $KH_2PO_4$, $K_2HPO_4$, ATP, ADP, AMP, EDTA, DETAPAC, bathophenanthroline, GSH, GSSG, DTT, DCI and SOD were purchased from Sigma Chemical Co., (St. Louis, MO, USA). Trypanothione was purchased from Bachem Bioscience Inc. (Philadelphia, PA, USA). The specific activity of the original LADH preparation ranged from 115 to 150 units/mg protein. Standard electrophoretic analysis on polyacrylamide dodecyl sulfate gels yielded a single component of 53000 D (experimental data omitted). $FeSO_4.7H_2O$ was purchased from Analar, BDH Ltd. (Poole, England) and $CoCl_2.6H_2O$ was purchased from Mallinckrodt Chemical Works (New York, USA). Dihydrolipoamide was prepared from lipoamide by $NaBH_4$ reduction, as described[20]. Its purity was determined spectrophotometrically at 340 nm, using 0.3 $\mu$g LADH/ml and 1.0 mM $NAD^+$. Other reagents were as stated[21].

*Enzyme assays.* LADH activity was measured by the rate of NADH oxidation using lipoamide as electron acceptor[21]. The standard reaction mixtures contained 50 mM $KH_2PO_4$-$K_2HPO_4$, pH 7.4, 4 nM LADH, 0.2 mM NADH and 1.0 mM lipoamide; total volume, 3.0 ml. NADH concentration in the reaction mixture was measured spectrophotometrically, at 340 nm. Diaphorase activity was measured by the rate of DCI reduction using NADH as electron donor[21]. The reaction mixture contained 0.2 mM NADH, 8 nM LADH, 40 $\mu$M DCI, 50 mM K-phosphate buffer, pH 7.4. Total volume, 3.0 ml. DCI reduction was measured spectrophotometrically, at 600 nm ($\epsilon = 19.mM^{-1}.cm^{-1}$). Spectrophotometric measurements were performed using a Perkin-Elmer 550S UV/VIS spectrophotometer, at 30°.

*Assay of FS activity.* Unless stated otherwise, samples containing 4.0 nM LADH in 50 mM K-phosphate buffer, pH 7.4 were incubated 90 s with the FS (25 $\mu$M Fe(II) or Co(II) and 3.0 mM $H_2O_2$). NADH and lipoamide were then added and LADH activity was measured from the initial slope of the recorded tracing. Fresh $FeSO_4$ solutions were prepared immediately before use. Similar samples of LADH less FS were incubated simultaneously and the enzyme residual activity was measured (control samples). A similar experimental procedure was utilized for measuring LADH

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diaphorase activity, except for the final composition of the reaction mixture which was as indicated above.

*Assay of TBA-reactive products.* Deoxyribose oxidation was monitored by heating solutions (0.5 ml) for 10 min at 100°C with 0.5 ml TBA (1 g/100 ml 0.05 M NaOH) and 0.5 ml trichloroacetic acid (2.8 g/100 ml). Absorbance at 532 nm was read against a blank containing both reagents[22]. Other experimental conditions were as described in Ref. 22 and Table II.

*Expression of Results.* LADH activity is expressed in $\mu$mol NADH oxidized/min per mg protein. Taking into account the limited variation of the activity of LADH preparations, in most cases relative activity values are presented (100% for the control sample). Protection (P) against FSs was calculated from Equation 1: where P, FS and FSP are the protector activity, the FS effect,

$$P(\%) = 100\ (FS(\%) - FSP\ (\%))/FS(\%) \qquad (1)$$

and the FS plus protector effect, respectively.

## RESULTS

Incubation of LADH with Fenton systems caused significant diminution of NADH-lipoamide reductase activity (Table I), especially with the Co(II) system, but at variance with these inhibitions, LADH diaphorase activity did not decrease, and even increased with Co(II) (Table I). Inhibition of lipoamide reductase activity largely exceeded the sum of $H_2O_2$ and the Me(II)-induced inhibitions, thus indicating the role of the Fenton reaction in LADH inactivation. $H_2O_2$ could not be replaced by *tert*-butylhydroperoxide (2.6 mM) or cumene-hydroperoxide (3.5 mM) (experimental data omitted). The effect of $H_2O_2$ itself was relatively small (16%). The results in Table I represent a typical experiment. However, with the Fe(II)-FS the extent of LADH inactivation varied somewhat (e.g. Table V) and occasionally, longer incubation periods were necessary in order to obtain similar inactivation.

Production of HO˙ by Fenton systems supplemented with LADH was investigated

TABLE I
Effect of Fenton systems on LADH

| Additions | Lipoamide reductase activity ($\mu$mol NADH/min per mg protein) | Diaphorase activity ($\mu$mol DCI/min per mg protein) |
|---|---|---|
| None | 104 ± 12 | 7.60 ± 0.10 |
| $H_2O_2$ | 87 ± 14 (14) | 7.27 ± 0.35 |
| Fe(II) | 69 ± 12 (33) | 6.88 ± 0.64 |
| Fe(II) + $H_2O_2$ | 24 ± 10 (77) | 7.30 ± 0.15 |
| Co(II) | 71 ± 9 (32) | 7.41 ± 0.11 |
| Co(II) + $H_2O_2$ | 9 ± 7 (91) | 14.1 ± 0.11 |

Experimental conditions were as described under Materials and Methods. 3.0 mM $H_2O_2$ and 25 $\mu$M Fe(II) or Co(II). Values represent the average ± S.E. of at least 3 determinations. In parenthesis, inhibition of LADH activity. Lipoamide reductase activity: $\mu$mol NADH/min per mg protein. Diaphorase activity: $\mu$mol DCI/min per mg protein. NADH oxidation in samples without LADH (all samples), 0.000–0.005 $\Delta$A/min.

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TABLE II
Effect of Fenton systems on deoxyribose oxidation. Assay for Fenton reaction products

| Additions (mM) | TBA reactive products ($10^3 \times A$) | |
|---|---|---|
| | 4 min incubation | 1 h incubation |
| $H_2O_2$(3.0) | 29 | 38 |
| Fe(II) (0.1) | 41 | 323 |
| Fe(II) + $H_2O_2$ | 255 | 449 |
| Fe(II) + $H_2O_2$ + EDTA (0.1) | – | 46 |
| Fe(II) + $H_2O_2$ + DETAPAC (0.1) | – | 46 |
| Co(II) (0.1) | 0 | 0 |
| Co(II) + $H_2O_2$ | 41 | 237 |

The reaction mixture contained 50 mM K-phosphate, pH 7.4, 3.0 mM deoxyribose, 1 μM LADH and additions as indicated above; final volume 0.5 ml. After incubation at 30°C for the time indicated above, TBA-reactive products were measured. Other experimental conditions were as described under Materials and Methods and Table I legend. Values after subtracting the blank absorbancy (0.045).

by the deoxyribose method[14,22]. The results obtained (Table II) indicate that (a) the Fe(II)-FS was at least twice as effective as the Co(II)-FS; (b) the Co(II)-FS was also an effective generator of $HO^{\cdot}$ radicals but a relatively longer incubation was necessary to demonstrate its maximal activity; (c) Fe(II) ions or $H_2O_2$ alone were weak generators of $HO^{\cdot}$ radicals and Co(II) ions alone did not catalyze $HO^{\cdot}$ production; (d) EDTA and DETAPAC inhibited $HO^{\cdot}$ production by the Fe(II)-FS. Blank values were relatively small and did not increase during incubation (experimental data omitted). These results fit in well with those obtained using Fenton[14] systems without LADH. Comparison of the results in Tables I and II shows no correlation between the capability of the assayed systems of inhibiting LADH, on the one hand, and of generating $HO^{\cdot}$ radicals, on the other. Moreover, Fe(II) and Co(II) were equally effective as LADH inhibitors despite the incapability of the latter of oxidizing deoxyribose. These data do not rule out the intervention of $HO^{\cdot}$ radicals in the Co(II)-FS reaction. Rather, they indicate the contribution of several ROS[15-18] to LADH inactivation by the Co(II)-FS.

Phosphate ions (50 mM) strongly increased LADH inactivation by the Fe(II)-FS (Table III), in close agreement with the increase of $HO^{\cdot}$ production by Fe(II)[22]. LADH inactivation was also affected by the ionic strength of the reaction mixture, as shown by adding 1.0 M phosphate (Table III) or 0.3 M KCl (experimental data omitted). Phosphate ions were not essential for the Co(II)-FS action but increasing phosphate concentration from 0.05 to 1.0 M prevented LADH inactivation about 90% (experimental data omitted).

EDTA, DETAPAC, DL-histidine, DL-cysteine, GSH, bathophenanthroline, DTT and trypanothione (a spermidine-glutathione conjugate, disulfide form)[23] completely, or almost completely prevented LADH inactivation by the Fe(II)-FS (Table IV). GSSG was less effective than GSH and, as regards trypanothione, the relatively low concentration at which it protected LADH fits in well with those protecting transforming DNA against γ-irradiation[24]. EDTA, DETAPAC, bathophenthroline and DL-histidine also prevented LADH inactivation by the Co(II)-FS but GSH, GSSG and DTT were less effective than with the Fe(II)-FS. LADH protection was always obtained when the metal-complexing agents were added before the Fenton system. When added after incubation (Table V), GSH and DTT partially reactivated the Fe(II)-treated enzyme, whereas DL-histidine reactivated the Co(II)-

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TABLE III
Effect of phosphate anion on LADH inactivation by the Fe(II) Fenton system

| Buffer system (M) | pH | NADH-lipoamide reductase activity | | LADH inactivation (%) |
|---|---|---|---|---|
| | | Control | Fe(II)-FS | |
| K-Phosphate (0.05) | 7.4 | 104 ± 12 | 24 ± 20 | 77 |
| K-Phosphate (1.0) | | 184 ± 1 | 184 ± 5 | 0 (100) |
| Na-Phosphate (0.05) | 7.4 | 92 ± 1 | 9.8 ± 0.8 | 90 |
| Na-Phosphate (1.0) | | 132 ± 4 | 130 ± 1.4 | 1 (99) |
| Tris-HCl (0.08) | 7.6 | 38 ± 1 | 37 ± 0.2 | 2 (98) |

Experimental conditions were as described under Materials and Methods, except the buffer system which is indicated above. Activity values represent the average ± S.D. of 3 or more determinations in $\mu$mol NADH/min per mg protein.

TABLE IV
Effect of chelators, thiol and disulfide compounds on LADH inactivation by Fenton systems

| Addition $\mu$M) | LADH inactivation (%) | | |
|---|---|---|---|
| | Fe(II) + $H_2O_2$ | Co(II) + $H_2O_2$ | FS omitted |
| None | 77 | 95 | |
| EDTA (25) | −32 (141) | −9 (109) | 0.4 |
| EDTA (50) | −38 (149) | −18 (119) | −0.5 |
| DETAPAC (50) | −17 (117) | 0 (100) | −4.4 |
| DL-histidine (50) | 4 (94) | 17 (82) | 6.6 |
| DL-cysteine (50) | −9 (111) | 7 (92) | 2.8 |
| Trypanothione (5) | 9 (91) | – | −1.9 |
| Trypanothione (10) | 7 (93) | – | 1.6 |
| Bathophenanthroline (0.10) | 3.5 (96) | 11 (89) | 1.2 |
| GSH (25) | 0 (100) | 48 (49) | 5.6 |
| GSH (50) | 0 (100) | 50 (43) | 7.0 |
| GSSG (25) | 22 (71) | 60 (32) | 3.5 |
| GSSG (50) | 35 (57) | 70 (20) | 5.7 |
| DTT (25) | 0 (100) | 40 (57) | −2.7 |
| DTT (250) | 4 (94) | 40 (57) | −3.2 |

Experimental conditions were as described under Materials and Methods. The order of additions of reagents to the LADH containing reaction mixture was: 1) the protector; 2) the FS; 3) after 90 s, the substrates. In parenthesis, protection (P) of LADH by additions. Negative inhibition values indicate that LADH activity measured in the presence of FS and chelator exceeded that of the control sample. Control sample relative activity, 100%. Values for NADH oxidation in samples without LADH were as follows (in $\Delta$A/min; in parenthesis, addition). With the Fe(II)-FS: 0.000 (none; 25 $\mu$M EDTA; 50 $\mu$M DL-histidine or DETAPAC); 0.003 (50 $\mu$M EDTA; 25 $\mu$M DETAPAC). With the Co(II)-FS: 0.000 (25 or 50 $\mu$M EDTA; 50 $\mu$M DETAPAC); 0.002 (none; 50$\mu$M GSH); 0.003 (25$\mu$M GSH or GSSG; 50$\mu$M DL-cysteine); 0.005 (50 $\mu$M GSSG); 0.010 (50 $\mu$M DL-histidine).

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TABLE V
Activation of Fenton-inhibited LADH by thiol compounds

| Fenton system | LADH activation (%) | | | | | |
|---|---|---|---|---|---|---|
| | DL-histidine | | GSH | | DTT | |
| | 25 μM | 50 μM | 25 μM | 50 μM | 25 μM | 250 μM |
| Fe(II) + $H_2O_2$ | −4.4 | −8.9 | 39 | 39 | 25 | 4.4 |
| Co(II) + $H_2O_2$ | 23 | 36 | −58 | 0 | 35 | −28 |

Experimental conditions were as described under Materials and Methods. The order of addition of reagents to the LADH containing reaction mixture was: 1) the FS; 2) after 90 s incubation, the activator; 3) the substrates. The values presented were calculated from the equation $a$ (%) = $(i - ip)/i$ (%) where $i$ and $ip$ are the inhibition of LADH by the FS, and by the FS plus activator, respectively. Negative values mean that the activator enhanced the effect of the FS. Control sample relative activity, 100%.

treated enzyme. EDTA was inactive on both FS-treated LADH preparations (experimental data omitted). In contrast to results in the presence of Fenton system, metal-complexing agents did not affect LipDH activity to any significant degree (Table IV).

Figure 1 shows the effect of increasing concentration of Cys and GSH on Fe(II)- and Co(II)-FS inhibitions. With the former system, a SH/Fe(II) molar ratio of about 1:1 determined complete protection of LADH. As regards the Co(II)-FS, total protection was obtained at a Cys/Co(II) molar ratio of about 4:1 whereas 100 μM GSH prevented LADH inactivation by 60%.

Figure 2 shows the effect of lipoamide and thioctic (lipoic) acid (reduced and oxidized forms) on the Fe(II)-FS activity. The dithiol compounds were always more

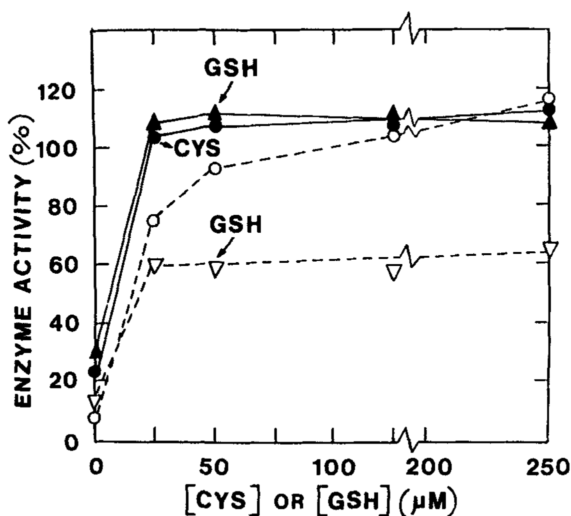


Figure 1 Effect of DL-cysteine (Cys) and reduced glutathione (GSH) on LADH inactivation by Fe(II)-FS (△, ●) and Co(II)-FS (▼, ○). Experimental conditions were as described under Materials and Methods. Samples contained the thiol compound as indicated on the abscissa and Fe(II)-FS or Co(II)-FS as indicated on the Figure. Control sample relative activity, 100%.

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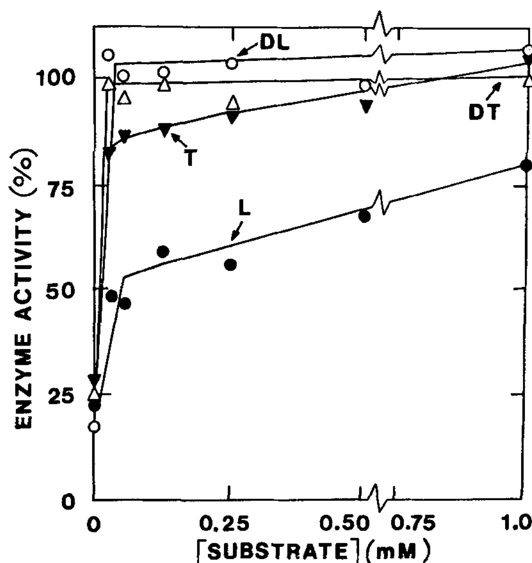


Figure 2 Effect of dihydrolipoamide (DL), lipoamide (L), dihydrothioctic (dihydrolipoic) acid (DT) and thioctic (lipoic) acid (T) on LADH inactivation by the Fe(II)-FS. Experimental conditions were as described under Materials and Methods. Concentration of additions was as indicated on the abscissa. Control sample relative activity, 100%.

TABLE VI
Effect of adenine nucleotides on LADH inactivation by Fenton systems

| Adenine nucleotide (mM) | LADH inactivation (%) | | | |
|---|---|---|---|---|
| | Fe(II) + $H_2O_2$ | Fe(II) | Co(II) + $H_2O_2$ | Co(II) |
| None | 84 | 26 | 92 | 49 |
| ATP (0.05) | 36 (57) | 13 (50) | 74 (20) | 30 (39) |
| ATP (0.25) | 20 (76) | 1.1 (95) | 44 (52) | 14 (71) |
| ADP (0.05) | 73 (13) | 28 (−8) | 92 (0) | 54 (−10) |
| ADP (0.25) | 75 (11) | 26 (0) | 92 (0) | 50 (−2) |
| AMP (0.05) | 75 (11) | 19 (13) | 91 (1) | 47 (4) |
| AMP (0.25) | 87 (−3) | 24 (8) | 92 (0) | 54 (−10) |

Experimental conditions were as described under Materials and Methods. The figures in parenthesis represent the protective action. (P%) of the adenine nucleotide. Control sample relative activity, 100%. NADH oxidation in samples without LADH (ΔA/min): (a) 0.05 mM ATP + Fe(II)-FS, 0; (b) 0.05 mM ATP + Fe(II)-FS, 0; (c) 0.05 mM ADP + 25 μM Fe (II), 0.004; (d), same as (c) plus Fe (II)-FS, 0.

effective than the parent disulfides, a remarkable difference being observed with the dihydrolipoamide/lipoamide pair.

Adenine nucleotides, especially ATP and ADP, ligate the "mobile" or "free" iron pool and as a result of complex formation, Fe(II) catalytic activity is modified[22,25-29]. Accordingly, it seemed of interest to establish the influence of adenine nucleotides on FSs action. The results presented in Table VI show that ATP protected LADH against the Fe(II)-and Co(II)-FS whereas ADP and AMP did not,

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TABLE VII
Effect of $MgCl_2$ on ATP protection of LADH against the Fe(II)-Fenton system

| $MgCl_2$ (mM) | ATP (mM) | LADH inactivation (%) | |
|---|---|---|---|
| | | Co(II)-FS | Fe(II)-FS |
| 0 | 0 | 63 | 35 |
| 2.5 | 0 | 74 (−17) | 45 |
| 0 | 0.05 | 36 (43) | 23 (35) |
| 2.5 | 0.05 | 74 (0) | 41 (10) |
| 0 | 0.125 | 36 (43) | 8.1 (77) |
| 2.5 | 0.125 | 68 (8) | 35 (17) |
| 0 | 0.250 | 16 (75) | 9.6 (73) |
| 2.5 | 0.250 | 72 (3.7) | 39 (19) |

Experimental conditions were as described under Materials and Methods and in Tables IV and V legends. In parenthesis, ATP protecive action. Values for samples containing $MgCl_2$ and ATP were calculated with (P%) respect to the value for the $MgCl_2$-containing control sample. Control sample relative activity, 100%.

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at least not to a similar extent. Omission of $H_2O_2$ from the reaction mixture did not modify the nucleotide protection pattern (Table VI). Mg(II) ions form complexes with ATP[30], thus competing with Fe(II) for the same complex[22]. As expected, Mg(II) ions prevented the effect of ATP on LADH inhibition (Table VII).

Since LADH activity was measured by the rate of NADH oxidation, it seemed necessary to establish the effect of the assayed Me(II)-complexes on the latter oxidation. The results obtained with the Fe(II)-and Co(II)-FS, using the 50 mM K-phosphate medium, were negative or negligible (foot-notes to Tables I, IV and VI), as compared with the rate of NADH oxidation by the LADH supplemented system.

Hydroxyl radical "scavengers" (ethanol, mannitol and Na benzoate) did not affect the inactivation of LADH by either the Fe(II)-FS (experimental data omitted), or the Co(II)-FS (Table VIII). SOD prevented the effect of the latter (Table VIII) but not that of the former (experimental data omitted). Thermal inactivation nullified SOD action. Bovine serum albumin also prevented LADH inactivation, as was to be expected from its metal-complexing properties.

TABLE VIII
Effect of scavengers on LADH inactivation by the Co(II) Fenton system

| Scavenger | LADH inactivation (%) |
|---|---|
| None | 88 |
| Ethanol (6.0 mM) | 89 (−1) |
| Mannitol (100 mM) | 83 (6) |
| Na Benzoate (50 mM) | 95 (−8) |
| SOD (35 μg/ml) | 78 (13) |
| SOD (70 μg/ml) | 55 (38) |
| SOD (70 μg/ml; heated[a]) | 88 (0) |
| Bovine serum albumin (70 μg/ml) | 64 (28) |

Experimental conditions were as described under Materials and Methods and Table IV legend. Scavengers were added as indicated above. Control sample relative activity, 100%.
[a]SOD inactivated by heating at 100°C, for 10 min.

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## DISCUSSION

The results here described indicate that the Fe(II)-FS inactivated LADH by a mechanism involving ROS, in all probability HO˙ radicals[31,32]. Thus, (a) Fe(II)-FS inhibition of LADH depended on both Fe(II) and $H_2O_2$ (Table I); (b) $H_2O_2$ alone was a weak inhibitor of LADH (Table I), in contrast to $H_2O_2$ effect on other enzymes[33-34]; (c) in the presence of LADH, the Fe(II)-FS oxidized deoxyribose and generated TBA-reactive products, these latter production being inhibited by EDTA and DETAPAC (Table II); (d) phosphate ions enhanced the action of the Fe(II)-FS, like the production of HO˙ by Fe(II)[22]; (e) thiol compounds (Cys and GSH; Figure 1), dihydrolipoamide and DL-dihydrothioctic acid (Figure 2), which are powerful HO˙ radical scavengers[35-37], protected LADH (mixtures of Fe(II) salts, $H_2O_2$ and GSH can produce HO˙ radicals, but this can only be demonstrated at relatively low GSH concentrations[35]); (f) Me(II)-complexing agents (EDTA, DETAPAC and DL-histidine)[38,39] prevented LADH inactivation by the Fe(II)-FS (Tables IV). The latter effect deserves special comment since Me(II) chelators increase HO˙ production by Fe(II) or the Fe(II)-FS[38,39]. Taken together with the absence of LADH protection by scavengers, the effects of the Fe(II)-complexing agents may be explained by assuming that (a), chelators diminished the concentration of Fe(II) ions available for binding to LADH and, consequently, for producing the Fe(II)-LADH complex. This complex would catalyze site-specific production of HO˙ radicals which would react with essential amino acid residues at the catalytic site, thus inactivating the enzyme. Vice versa, HO˙ radicals produced "in free solution" by chelated Fe(II) would not modify LADH because of their inaccessibility to the catalytic site. This hypothesis seems to be the most adequate for explaining LADH inactivation by the Fe(II)-FS. Nevertheless, the role of hypervalent iron complex cannot be absolutely ruled out.

As regards the nature of the oxidized amino acids residues, disulfide and thiol groups might be selective targets[8,12,35-37,40]. In close agreement with this hypothesis, (a) GSH and DTT partially restored the activity of Fe(II)-FS-treated LADH (Table V) and (b) preliminary amino acid analysis of oxidized LADH revealed significant diminution of the enzyme thiols (experimental data omitted). Nevertheless, dilute aqueous solutions of bovine serum albumin or lysozyme give positives test for peroxides after exposure to the Fe(II)-FS[41]. The ROS effective on protein peroxidation were the hydroxyl radicals and organic peroxyl. Glutamate, isoleucine, lysine, proline and valine were peroxidized[41] which means that LADH inactivation by FSs might involve several amino acid residues.

LADH inactivation by the Co(II)-FS seemed to involve a more complex mechanism and differences between the Fe(II)-and Co(I)-FS are worth noting. Thus, (a) the CO(II) system was more effective than its Fe(II) counterpart (Table I); (b) the Co(II) system was a less efficient generator of HO˙, especially after a brief incubation (Table II); (c) thiol compounds were able to counteract the effect of the Fe(II)-system completely, but partially the effect of the Co(II)-system (Table IV and Figure 1); (d) thiol compounds reverted the Fe(II)-system effect but not the Co(II) effect and the reverse occurred with DL-histidine (Table V). In this connection, it should be recalled that (a) the Co(II)-FS yields predominantly the DEMPO/˙OOH adduct whereas the Fe(II) + FS yields only the DEMPO/˙OH adduct[16,18]; (b) production of the DEMPO/˙OOH adduct is inhibited by EDTA and DETAPAC[16], in close agreement with the results in Table II; (c) the HOO˙ radical is the protonated form of the superoxide anion radical whose intervention in LADH inactivation was supported by the effect of SOD in Table VIII; (d) with the deoxyribose assay, the Co(II)-

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Fe yielded HO˙ radicals, though the kinetics differed from the Fe(II)-FS kinetics (Table II). Accordingly, combination of HO˙ and other ROS, possibly $O_2^-$ and/or singlet oxygen[15-18] would explain the greater action of the Co(II)-FS on LADH, as compared with that of the Fe(II)-FS (Table I).

The results here presented suggest that the "phosphate potential" in heart mitochondria[42] may exert a controlling action on LADH damage by ROS. In fact, the "phosphate potential" depends mainly on the ATP/ADP ratio[42]. Furthermore, a decrease of this ratio implies an increase of phosphate ion concentration. Decrease of ATP and increase of phosphate ions would enhance the probability of LADH damage by oxygen radicals and a similar effect may result from the binding of ATP by Mg(II). Thiol compounds, especially dihydrolipoamide and GSH would also contribute to protect LADH against ROS (Table III; Figures 1 and 2), since a primary function of GSH and other thiol compounds in eukaryotic cells involves protection against oxidative stress[35-37]. *Trypanosoma cruzi*, the agent of Chagas' disease, contains LADH[43], and therefore LADH inactivation might contribute towards explaining the action of oxygen radicals on the parasite[44,45]. Accordingly, trypanothione might in this organism exert a specific protective action, as suggested by the results in Table TV and Reference 24.

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