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NAD(H) enhances the Cu(II)-mediated inactivation of lactate dehydrogenase by increasing the accessibility of sulfhydryl groups

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Abstract
Copper ions are known to inactivate a variety of enzymes, and lactate dehydrogenase (LDH) is exceptionally sensitive to the presence of this metal. We now found that NADH strongly enhances the Cu(II)-mediated loss of LDH activity. Surprisingly, NADH was not oxidized in this process and also NAD^+ promoted the Cu(II)-dependent inactivation of LDH. Catalase only partly protected the enzyme, whereas hypoxia even enhanced LDH inactivation. NAD(H) accelerated sulfhydryl (SH) group oxidation of LDH by 5,5-dithio-bis(2-nitrobenzoic acid) (DTNB), and, vice versa, LDH-mediated Cu(II) reduction. LDH activity was preserved by thiol donators and pyruvate and partially preserved by lactate and oxamate. Our results suggest that reactive oxygen species (ROS) are of minor importance for the inactivation of LDH induced by Cu(II)/NADH. We propose that conformational changes of the enzymes' active sites induced by NAD(H)-binding increase the accessibility of active sites' cysteine residues to Cu(II) thereby accelerating their oxidation and, consequently, loss of catalytic activity.

Keywords: *Cu(II), lactate dehydrogenase, NAD(H), sulfhydryl groups, active sites, conformational changes*

Abbreviations: *LDH, L-lactate dehydrogenase; GSH, glutathione (reduced form); ROS, reactive oxygen species; DTNB, 5,5-dithio-bis(2-nitrobenzoic acid); neocuproine, 2,9-dimethyl-1,10-phenanthroline; SH groups, sulfhydryl groups*

Introduction

Copper, a typical redox-metal cation that alternates between Cu(I) and Cu(II) in biological systems thus serving as an one-electron shuttle, is widely distributed in nature and essential for many biochemical and physiological functions. On the other hand, copper ions are capable of catalyzing the production of harmful radicals/oxidants, such as the hydroxyl radical ($^{\cdot}OH$). This reactive intermediate is formed in a chain process[1–3] as shown in Equations 1–6.

Initiation:

$$Red + Cu(II) \rightarrow Red_{ox} + Cu(I) \quad (1)$$

$$Cu(I) + O_2 \rightarrow Cu(II) + O_2^{\cdot -} \quad (2)$$

$$2O_2^{\cdot -} + 2H^+ \rightarrow H_2O_2 + O_2 \quad (3)$$

Propagation:

$$Cu(II) + H_2O_2 \rightarrow [Cu(II) - OOH]^+ + H^+ \quad (4)$$

$$[Cu(II) - OOH]^+ \rightarrow Cu(I) + H^+ + O_2^{\cdot -} \quad (5)$$

$$Cu(I) + H_2O_2 \rightarrow Cu(II) + {}^{\cdot}OH + OH^- \quad (6)$$

The intermediate hydroxyl radical, the superoxide anion radical ($O_2^{\cdot -}$, Equation 2) as well as copper–oxygen species like copper–dioxygen complexes all are well accepted to damage biomolecules, e.g. proteins and lipids, i.e. induce oxidative stress to organisms.[2,4–7]

The harmful potential of copper ions towards enzymes, resulting in loss of enzymatic activity, is well established. For instance, Cu(II)-treated dihydrolipoamide dehydrogenase, acetylcholine esterase and gastric peroxidase lose their catalytic activity by so-called site-specifically generated $^{\cdot}OH$ at the catalytic center.[8–10]

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However, in contrast to this oxygen-dependent mechanism, Cu(II) can also directly (anaerobically) oxidize sulfhydryl (SH) groups.[11–14] In several enzymes, e.g. phosphorylase kinase, lipoyl dehydrogenase or lecithin-cholesterol acyltransferase, SH groups have been found to be the primary targets of copper ions, and their oxidation 1 to 3 result in loss of enzymatic activity.[15–17] Therefore, the oxygen-independent oxidation of protein-bound SH groups by Cu(II) has been proposed to decisively contribute to the copper-dependent inactivation of enzymes.[15–17]

It is well known that substrates and cosubstrates which can scavenge reactive species and/or shield the vulnerable active center, are highly effective in protecting the related enzymes.[18–22] The pyridine nucleotides NAD(H) and NADP(H), the most important coenzymes, are known to be almost exclusively bound to proteins (mostly dehydrogenases) within cells.[23,24] Besides playing a central role in metabolism as reducing/oxidizing equivalents,[25] these pyridine nucleotides can act as directly operating antioxidants by scavenging various harmful radicals/oxidants, e.g. $CO_3^{\cdot-}$, $NO_2^{\cdot}$, $^{\cdot}OH$, $^1O_2$ and phenoxyl radicals.[26–28] Consequently, NAD(P)H has been demonstrated to protect $NADP^+$-linked isocitrate dehydrogenase from singlet oxygen,[29] tyrosine hydroxylase from peroxynitrite[30] and to stabilize thioredoxin reductase against the attack of H_2O_2.[31] However, in marked contrast to its antioxidative properties, NAD(P)H has also been reported to be capable of reducing Cu(II) to Cu(I), thus initiating a chain reaction sequence including the Fenton-type reaction (see Equations 1–6) and, consequently, promoting DNA damaging[7,32,33] and inactivation of enzymes.[9,34] These pro- and antioxidative properties of NAD(H) as well as its association with (copper-sensitive) dehydrogenases motivated us to study the influence of NAD(H) on the Cu(II)-induced inactivation of lactate dehydrogenase (LDH) in more detail. LDH is known to be particularly sensitive to copper-induced damages, however, the mechanisms of its inactivation are still a subject of debates.[5,35–40]

Materials and methods

Materials

L-LDH from hog muscle (EC 1.1.1.28), catalase from bovine liver (EC 1.11.1.6), NAD^+ and NADH were obtained from Roche Molecular Chemicals (Mannheim, Germany). Copper dichloride (dihydrate), Chelex 100 (chelating resin), 5,5-dithio-bis(2-nitrobenzoic acid) (DTNB, Ellman's reagent), L-cystine (dihydrochloride), L-(+) lactic acid, L-ascorbic acid, glutathione (GSH), hydrogen peroxide (H_2O_2), oxamate and 2,9-dimethyl-1,10-phenanthroline (neocuproine) were purchased from Sigma (Deisenhofen, Germany). L-cysteine and the ingredients of the phosphate buffer used here, K_2HPO_4 and KH_2PO_4, were obtained from Merck (Darmstadt, Germany); the buffer was prepared with water received from a water purification system (TKA-LAB, Niederelbert, Germany, type HP 6 UV/UF). Pyruvate came from Boehringer (Mannheim, Germany). Gas mixtures were delivered by Messer Griesheim (Krefeld, Germany). All chemicals were of the highest purity commercially available.

Assessment of LDH inactivation

LDH activity was determined in phosphate buffer (50 mM, K_2HPO_4/KH_2PO_4, pH 7.5, 37°C) that had been pretreated with Chelex 100 in order to remove traces of transition metal cations.[41,42] LDH (7 nM) in 1 ml phosphate buffer was incubated in the absence or presence of Cu(II) (0.5–5 and 10 μM), NAD(H) (0.1–1 mM), catalase (100 U/ml) and H_2O_2 (100 μM). Samples (250 μl) were taken after 10, 30, 60 and 90 min of incubation, and the enzyme activity was determined photometrically according to the assay described by Bergmeyer.[43] In further experiments with LDH, oxamate (1–30 mM), pyruvate (1–30 mM)/NAD^+ (1 mM) or lactate (10–30 mM)/NADH (1 mM) were added. Oxidation of NADH (100 μM) was determined in the absence or presence of Cu(II) (100 μM) and LDH (10 μM) from the decrease in absorption at 340 nm.

Additional experiments with LDH were performed under hypoxic conditions. Within an argon-flushed glove box, phosphate buffer (6 ml, 30°C) containing Cu (II) (3, 6 or 20 μM) with or without NADH (1 mM) was transferred to a reaction tube and continuously bubbled with argon by means of a cannula. After the pO_2 of the buffer had dropped to <4.7 kPa (from 202–205 kPa at normoxia) as determined with a LICOX MCB® Oxygen Monitor (GMS, Kiel-Mielkendorf, Germany), LDH (14 nM) was added and incubated for the periods indicated above. Then 1 ml of the reaction mixture was transferred to an argon-flushed quartz cuvette using a Hamilton syringe. LDH activity was determined photometrically within the glove box. Comparative experiments at normoxia were performed under the same experimental conditions using synthetic air (20.5% O_2, rest N_2) instead of argon.

Determination of SH groups of LDH

SH groups of LDH were assessed using DTNB according to Ellman.[44] In order to avoid artifacts resulting from the presence of high amounts of glycerol used by the manufacturer for LDH stabilization, the enzyme (2 μM in phosphate buffer) was purified by ultracentrifugation (371,000*g*, 20°C, 5 h; Beckman Coulter, Krefeld, Germany; type Optima™ L-70 k, Rotor type 70 Ti). After centrifugation, the pellet was resuspended in phosphate buffer (20°C)

and the enzymatic activity determined (see above). Under these conditions, almost complete (>95%) initial catalytic activity was retained. Therefore, it was possible to calculate the concentration of the enzyme from its activity given that 1 ml of the stock solution contains 10 mg protein (as given by the manufacturer) and taking the molecular weight of LDH as 140 kDa.[43,45] The purified enzyme (5 μM) was incubated in phosphate buffer (20°C) in the presence or absence of NADH or NAD^+ (7.14 mM) prior to the addition of DTNB (1 mM). In other experiments free cysteine (1 mM) was incubated for 0–45 min with 1.5 or 3 μM Cu(II) in the presence or absence of NADH (1 mM) or free cystine (100 μM) was incubated with or without NADH (1 mM, for 5–60 min) before DTNB (30 μM) was added. Thiols were determined from the increase in absorption at 412 nm after correction for unspecific absorption. The yield of the yellow product 2 nitro-4-mercaptobenzoic acid ($\varepsilon = 13,600\,M^{-1}\,cm^{-1}$) corresponds to the SH residues oxidized by DTNB.[44]

Assessment of LDH-mediated Cu(II) reduction

Reduction of Cu(II) was determined using an assay based on the specific complexation of Cu(I) by the strong copper chelator neocuproine, leading to the orange-yellow colored $[Cu(I)\ (neocuproine)_2]^+$–complex with an absorption maximum at 454 nm.[46,47]

The reduction of Cu(II) by LDH was studied under hypoxic conditions in order to avoid the rapid autoxidation of Cu(I) in the presence of O_2. The influence of NADH on the LDH-mediated Cu(I) formation could not be studied because already NADH alone (in contrast to NAD^+) is known to reduce Cu(II) in the presence of neocuproine.[48] Cu(II) (100 μM) with or without NAD^+ (7.14 mM) were incubated in phosphate buffer (20°C) under argon atmosphere as described above. Two minutes after addition of purified LDH (5 μM), samples (1 ml) were transferred to an argon-filled quartz cuvette using a Hamilton syringe and background absorption was photometrically determined at 454 nm. Then neocuproine (200 μM) was added and the absorbance was monitored. Cu(I) formation was quantified based on calibration curves obtained from samples containing neocuproine (200 μM), NADH (200 μM) and Cu(II) standards (0.5–20 μM), after correction for unspecific absorption.

Assessment of binding of Cu(II)/Cu(I) to LDH

Cu(II) (50 μM) in the absence or presence of LDH (1, 1.5 or 2 μM) in phosphate buffer (20°C), was ultracentrifuged (see above) and the copper/LDH binding stoichiometry determined from the decrease in Cu(II) concentration in the supernantant using the Cu(I)/neocuproine assay (see above) after mixing an aliquot (1 ml) of the sample with ascorbic acid (200 μM) to reduce Cu(II). The $[Cu(I)\ (neocuproine)_2]^+$–complex was quantified from calibration curves using UV/visible spectrophotometry.

Statistical analysis

All experiments were performed in duplicate and repeated at least three times. Traces shown in the figures are representative of all the corresponding experiments performed. The data are expressed as means ± S.D. Data obtained from two groups were compared by means of Student's t test (matched values, two-tailed paired). A P value of <0.05 was considered as significant.

Results

Effect of pyridine nucleotides on the inactivation of LDH by Cu(II)

In line with previous studies,[5,35,36,49] Cu(II) (10 μM) almost completely decreased the activity of LDH (7 nM) during 90 min of incubation under normoxic conditions (Figure 1A). As expected for a Fenton-like reaction system, H_2O_2 (100 μM) strongly enhanced the effect of Cu(II) on LDH activity but did not decrease enzymatic activity in the absence of Cu(II). The Cu(II)-mediated inactivation of LDH was even more strongly accelerated in the presence of 1 mM NADH. Already after ≤10 min of incubation, LDH activity could no longer be detected. The addition of H_2O_2 to samples containing NADH and lower Cu(II) concentrations further increased the rate of LDH inactivation (data not shown). The Cu(II)-induced and NADH-enhanced inactivation of LDH strongly depended on the Cu(II) concentration, being highly effective already at 0.5 μM Cu(II) (Figure 1B; IC_{50} after 10 min of incubation in the presence of 1 mM NADH: 1.7 μM). In the absence of the coenzyme, LDH activity was comparatively insensitive to increases in [Cu(II)] during these short incubations. Given that NADH has been reported to be a reductant for Cu(II)[7,32,33,50] and the fact that the strong Cu(I) chelator neocuproine was completely protective (data not shown), the most likely explanation for the observed effect of NADH appeared to be a chain process initiated by the reduction of Cu(II) to Cu(I) (Equation 1). However, also the oxidized pyridine nucleotide NAD^+ significantly increased the inactivation of LDH by Cu(II), although less effectively than NADH (Figure 1A). Both NADH and NAD^+ diminished LDH activity in a concentration-dependent manner (Figure 1C). The IC_{50} after 10 min of incubation in the presence of 3 μM Cu(II) was about 0.16 mM for NADH and 1.8 mM for NAD^+, roughly in line with the differing Michaelis constants of LDH given for NADH (1.07×10^{-5} mol/l) and NAD^+ (2.53×10^{-4} mol/l).[51]

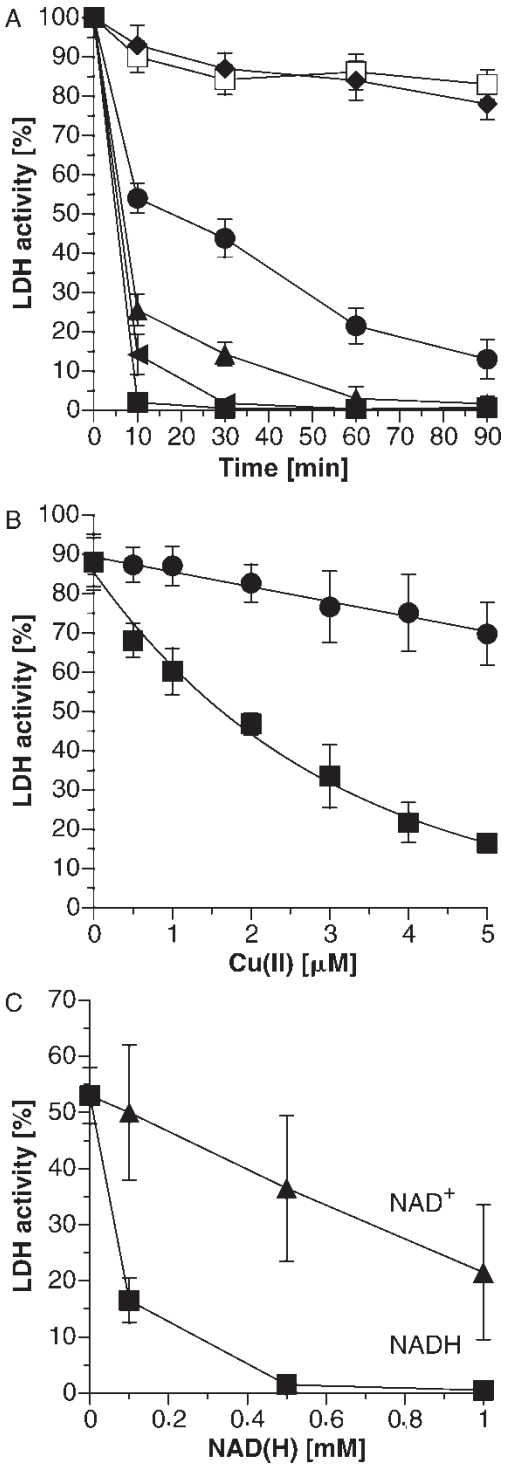

Figure 1. Effect of H_2O_2 and NAD(H) on the Cu(II)-induced inactivation of LDH. (A) LDH (7 nM) was incubated in the absence (□) and presence of (●) Cu(II) (10 μM), (■) Cu(II) (10 μM) and NADH (1 mM), (▲) Cu(II) (10 μM) and NAD^+ (1 mM), (◆) H_2O_2 (100 μM) or (◀) Cu(II) (10 μM) and H_2O_2 (100 μM). (B) LDH (7 nM) was incubated with different Cu(II) concentrations in the absence (●) and presence (■) of NADH (1 mM). LDH activity was obtained after 10 min of incubation. (C) LDH (7 nM) was incubated for 90 min with 3 μM Cu(II) and various concentrations of NADH or NAD^+. All experiments were performed in phosphate buffer (50 mM, pH 7.5, 37°C). Values shown represent means ± S.D. of at least three independent experiments performed in duplicate.

Effect of catalase and molecular oxygen on the NADH-enhanced inactivation of LDH by Cu(II)

In view of the (unexpected) effect of NAD^+, we studied to what extent Fenton-like chemistry would contribute to the Cu(II)-dependent LDH inactivation. In the absence of NADH, catalase (100 U/ml) had almost no effect on the rapid initial decrease in LDH activity induced by Cu(II) (3 μM) but significantly diminished the slower proceeding of enzyme inactivation at prolonged incubation periods, indicating a biphasic pattern of inactivation (Figure 2A). NADH (1 mM) considerably enhanced the initial (within 10 min) decrease in LDH activity but had no effect on the following slower decrease in LDH activity. Catalase hardly diminished the initial decrease in LDH activity in the presence of NADH but again significantly diminished the slower proceeding of enzyme inactivation. However, when the Cu(II) concentration was increased to 10 μM, catalase was not protective at all (data not shown). In view of the biphasic pattern of the Cu(II)-induced LDH inactivation, our results therefore suggest that H_2O_2 hardly contributed to the more rapid initial loss of enzymatic activity, which was strongly enhanced by NADH thus becoming the predominant mechanism of LDH inactivation. Reactive oxygen species (ROS) generated via the chain mechanism of Equations 1–6 only appeared to be responsible for/contribute to the slow path of inactivation but became less important with increasing Cu(II) concentrations. In line with this, LDH inactivation by Cu(II) in the absence and presence of NADH was not diminished by hypoxia ($pO_2 < 4.7$ hPa) but even increased (Figure 2B). This result suggests that the initial and predominant effect of Cu(II) on LDH activity is strongly enhanced in the absence of oxygen so that the inactivation of LDH is accelerated despite a decreased formation of reactive species. Even in the presence of 100 μM Cu(II) and 10 μM LDH, no oxidation of NADH (100 μM) to NAD^+ above autoxidation was detectable at least during the first 10 min of incubation (data not shown). This indicated that NADH did not serve as a reductant for Cu(II) and suggested that no NADH-oxidizing species had been formed. Since ROS and reduction of Cu(II) by NADH appeared to be of minor importance for the Cu(II)-induced and NADH– enhanced inactivation of LDH, we searched for alternative mechanisms.

Binding of Cu(II) to LDH and NAD(H)-dependent accessibility of SH groups to DTNB

It is well known that Cu(II) binds to LDH and oxidizes protein-bound SH groups thereby inactivating LDH.[5,35,36,38,40] Here we found by varying the [LDH]/[Cu(II)] ratio that during the prolonged incubation periods (5 h) being required for ultracentrifugation, 3–4 Cu(II) ions were maximally bound per

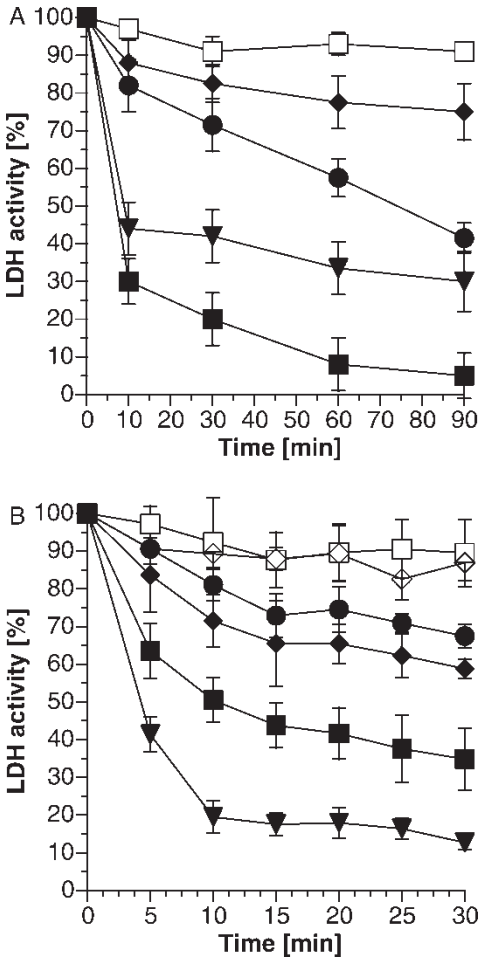

Figure 2. Effect of catalase and molecular oxygen on the Cu(II)-induced and NADH-enhanced inactivation of LDH. (A) In order to study the contribution of H_2O_2, LDH (7 nM) was incubated in the absence (□) and presence of (●) Cu(II) (3 μM), (◆) Cu(II) (3 μM) and catalase (100 U/ml), (■) Cu(II) (3 μM) and NADH (1 mM) or (▼) Cu(II) (3 μM), NADH (1 mM) and catalase (100 U/ml). (B) Experiments under hypoxic conditions were performed within a glove box that has been flushed with argon ($pO_2 < 4.7$ kPa). LDH (14 nM) was incubated in the absence (◇) and presence of (◆) Cu(II) (3 μM) or (▼) Cu(II) (3 μM) and NADH (1 mM). Comparative experiments at normoxia were performed under the same experimental conditions using synthetic air (20.5% O_2, rest N_2) instead of argon ($pO_2 = 202–205$ kPa). LDH (14 nM) was incubated in the absence (□) and presence of (●) Cu(II) (3 μM) or (■) Cu(II) (3 μM) and NADH (1 mM). All experiments were performed in phosphate buffer (50 mM, pH 7.5) at 37°C (A) or 30°C (B). Values shown represent means ± S.D. of at least three independent experiments performed in duplicate.

LDH molecule (data not shown). This finding is in line with the presence of four SH groups at the active sites of the enzyme.[20,38,40] In order to verify the hypothesis that NAD(H) may enhance the accessibility of the active centers' cysteine residues for Cu(II), we studied the effect of NAD(H) on DTNB-detectable SH groups of LDH. When 5 μM of purified LDH were incubated with DTNB (1 mM) for 1 min, approximately 4 μM of protein-bound SH groups (i.e. 0.8 ± 0.1 SH groups/LDH molecule) were detected by the sulfhydryl reagent (Figure 3). This low number of detectable thiols/LDH is partly explained by the short incubation of the enzyme with the thiol reagent and roughly in line with previous studies (1.8 DTNB-detectable thiols/LDH within 1 min in Ref. [52] and 2.81 mol of SH/mole of LDH after 24 h of incubation in Ref.[53]). In the presence of NAD^+ (7.14 mM) more than 6 μM protein-bound SH groups (i.e. 1.3 ± 0.2 SH groups/LDH molecule) reacted with DTNB, whereas almost 12 μM SH groups were detectable in samples containing NADH (7.14 mM). This effect of NAD(H), which is in line with a previous study,[53] did not result from direct reduction of DTNB by the pyridine nucleotides, as verified in controls. Similar to previous reports,[52,53] studies on the kinetics of SH detectability by DTNB (for 10 min at 10-s intervals) revealed a biphasic pattern of thiol detection/reactivity: a very rapid and predominant initial phase that was too fast (≤ 10 s) to allow temporal resolution was followed by a slow second phase of thiol oxidation (data not shown). The results strongly suggest that both NAD^+ and NADH enhance the spatial accessibility of the active centers' SH groups and, consequently, accelerate their oxidation by Cu(II). This assumption is supported by the fact that oxidation of free cysteine (1 mM) by Cu(II) (1.5 and 3 μM) was not increased in the presence of NADH (1 mM; data not shown), ruling out the possibility that NADH may influence the redox potential of Cu(II) in

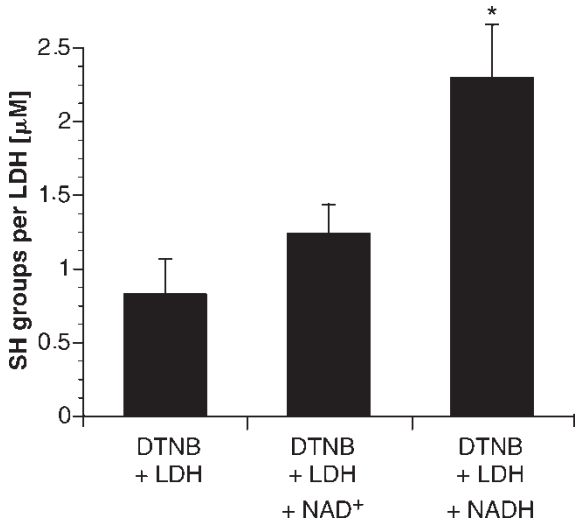

Figure 3. Effect of NAD(H) on the accessibility of LDH SH-groups to DTNB. LDH (5 μM), purified by ultracentrifugation, was incubated in phosphate buffer (50 mM, pH 7.5, 20°C) in the absence and presence of NAD^+ or NADH (7.14 mM). Then Ellman's reagent for sulfhydryl (—SH) group detection (DTNB, 1 mM) was added and absorption at 412 nm determined spectrophotometrically 1 min later. The concentration of DTNB-detectable SH groups per LDH molecule was calculated (after correction for background absorption) based on the extinction coefficient of the DTNB product 2 nitro-4-mercaptobenzoic acid ($\varepsilon = 13{,}600\ M^{-1}\ cm^{-1}$). Bars shown represent means ± S.D. of at least three independent experiments performed in duplicate. *significantly different from samples without NADH.

Table I. Effects of thiol donators on the Cu(II)-induced and NADH-enhanced inactivation of LDH and on the restoration of catalytic activity.

Additions/treatments	LDH activity [% of control]		Restoration of LDH activity [%]
LDH	100	(100)	100
+Cu(II)	85 ± 9	(88 ± 6)	88 ± 9
+Cu(II) + GSH	97 ± 3	(88 ± 5)	97 ± 9
+Cu(II) + cysteine	86 ± 11	(91 ± 3)	91 ± 8
+Cu(II) + DTT	97 ± 4	(90 ± 8)	80 ± 7
+Cu(II) + NADH	25 ± 8	(40 ± 2)	40 ± 4
+Cu(II) + NADH + GSH	97 ± 6	(36 ± 6)	42 ± 7
+Cu(II) + NADH + cysteine	85 ± 7	(36 ± 4)	45 ± 12
+Cu(II) + NADH + DTT	98 ± 2	(31 ± 6)	40 ± 5

LDH (7 nM) was incubated in phosphate buffer (50 mM, pH 7.5, 37°C) with or without glutathione (GSH, 1 mM), cysteine (1 mM) or dithiothreitol (DTT, 1 mM) in the absence and presence of NADH (1 mM). Then Cu(II) (3 μM) was added and LDH activity determined 30 min later. To study the capacity of the thiol donators to restore LDH activity after its inactivation, LDH (7 nM) was incubated for 10 min with Cu(II) (3 μM) in the absence and presence of NADH (1 mM). Then the Cu(II)-induced inactivation of LDH was stopped by adding the strong copper chelator neocuproine (100 μM). After LDH activity had been determined (in brackets), either GSH, cysteine or DTT (1 mM each) were added to the samples and LDH activity determined again 60 min later. Data are expressed in percent of LDH activity of untreated control samples (set at 100%) at the respective time points. Values shown represent means ± S.D. of at least three independent experiments performed in duplicate.

a way that a putative NADH/Cu(II) complex oxidizes LDH-bound cysteine more readily. Likewise, it is unlikely that NADH increased the amount of DTNB-detectable thiols via reduction of disulfide bridges of the enzyme, because the pyridine nucleotide (1 mM) was found to be unable to reduce —SS bridges of free cystine (100 μM; data not shown).

The Cu(II)-mediated SH oxidation has been described for LDH[5,35,36] and free cysteine[54] as well as for GSH, which has been reported to form a stable Cu(I) complex.[55] According to this property, GSH, cysteine and dithiothreitol (1 mM each) well prevented the Cu(II)-induced inactivation of LDH in the absence and presence of NADH (Table I), supporting the hypothesis that LDH was inactivated in a similar fashion. However, when the thiol donators (1 mM) were added after LDH inactivation had been stopped by adding neocuproine (100 μM), no significant restoration of LDH activity could be determined 60 min later (Table I) as well as during the following two days (data not shown).

Effect of NAD^+ on the LDH-mediated reduction of Cu(II)

Given that NAD(H) promotes Cu(II)-mediated thiol oxidation of LDH, an increased formation of Cu(I) should be detectable. When purified LDH (5 μM) and Cu(II) (100 μM) were incubated for 2 min in the absence or presence of NAD^+ (7.14 mM; NADH interferes with the Cu(I) assay; see "Materials" section) under hypoxic conditions (to prevent Cu(I) reoxidation), the enzyme alone reduced approximately 2 μM Cu(II) (i.e. 0.42 ± 0.02 Cu(II) ions/LDH molecule) but more than 3 μM Cu(I) (i.e. 0.72 ± 0.12 Cu(II) ions/LDH molecule) when NAD^+ was present (values significantly different from samples without LDH and without NAD^+, respectively). This result further supports the above assumption that NAD(H) enhances the Cu(II)-induced inactivation of LDH by increasing the oxidation of protein-bound thiols.

Effect of pyruvate, lactate and oxamate on the Cu(II)-induced and NAD(H)-enhanced inactivation of LDH

Since binding of pyruvate and lactate to the active sites of LDH is known to shift the conformation of the active center to the closed form,[56,57] the substrates should protect LDH from inhibition due to the resulting inaccessibility of the active sites to Cu(II). As expected, pyruvate strongly diminished LDH inactivation by Cu(II) and NAD^+ in a concentration-dependent manner (Figure 4). When pyruvate/NAD^+ were exchanged by lactate/NADH, LDH was also protected, but less effectively (data not shown). Oxamate (1 or 10 mM), a substrate analog of pyruvate that is not metabolized by LDH, provided sligthly better protection than lactate and abolished the effect of NADH-enhanced inactivation of LDH by Cu(II) (3 or 10 μM; data not shown). These results support the notion that the accessibility of the cysteine residues in the active sites of LDH is responsible for its inactivation by Cu(II).

Discussion

Fenton-type reaction vs. direct oxidation of sulfhydryl groups

Copper ions mediate their damaging potential to enzymes by catalyzing the formation of ROS in a chain reaction[58,59] and/or by direct oxidation of essential SH groups.[12,15,16,60] For LDH it has been reported that Cu(II) inactivates the enzyme by

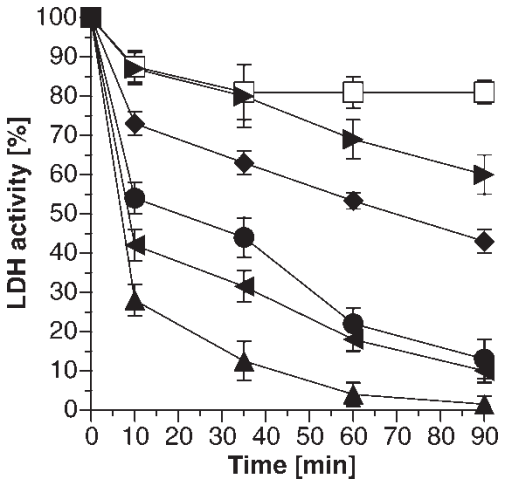

Figure 4. Effect of pyruvate on the Cu(II)-induced and NAD^+-enhanced inactivation of LDH. LDH (7 nM) was incubated in phosphate buffer (50 mM, pH 7.5, 37°C) in the absence (□) and presence of (●) Cu(II) (10 μM), (▲) Cu(II) (10 μM) and NAD^+ (1 mM) or Cu(II) (10 μM), NAD^+ (1 mM) and pyruvate at various concentrations: (◀) 1 mM, (◆) 10 mM or (►) 30 mM. Values shown represent means ± S.D. of at least three independent experiments performed in duplicate.

a direct, i.e. ROS-independent oxidation of protein-bound thiols and (subsequently) by so-called site-specifically generated $^{\cdot}OH$.[5,36,37] However, the differentiation of both mechanisms as well as the sequence of inactivation has not been clearly elucidated yet. In line with this, also our results strongly suggest that both mechanisms can be responsible for the inactivation of LDH by Cu(II). As already reported in previous studies,[35,52] Cu(II) led to a biphasic pattern of inactivation, i.e. to a rapid initial (within ≤ 10 min) decrease in catalytic activity followed by a further relatively slower course of inactivation. We here found that the initial loss of LDH activity is likely to result predominantly from a direct Cu(II)-induced oxidation of SH groups at the active center. At low Cu(II) concentrations (≤ 3 μM), the second phase of inactivation is presumably attributed to ROS, as indicated by the protective effect of catalase. At higher Cu(II) concentrations (10 μM), however, the contribution of ROS appears to be neglectable (no significant protection by catalase) and the enzyme most likely is inactivated exclusively via direct thiol oxidation.

The presence of NADH strongly enhanced the Cu(II)-dependent inactivation of LDH predominantly through an increase in initial loss of enzymatic activity so that the biphasic pattern of inactivation becomes more pronounced. The enhanced damaging potential of copper ions in the presence of NADH is commonly explained by its ability to act as an electron donor for Cu(II) thus accelerating copper redox-cycling and, consequently, copper-dependent ROS formation[50] as shown in Equations 1–8.

$$Cu(II) + NADH \rightarrow Cu(I) + NAD^{\cdot} + H^+ \quad (7)$$

$$NAD^{\cdot} + O_2 \rightarrow NAD^+ + O_2^{\cdot -} \quad (8)$$

However, in contradiction to this hypothesis, NADH was not oxidized in the presence of Cu(II)/LDH here, reflecting the complexicity of redox-processes when transition metal ion complexes are involved. Based on the standard reduction potentials ($E^{o\prime}$) of the redox-couples NAD^+, H^+/NADH (− 0.32 V,[61]) and Cu^{2+}, e^-/Cu^+ (+0.16 V; at pH 7.0 and 298 K,[62]), the reduction of Cu^{2+} by NADH yielding Cu^+ is thermodynamically allowed and appears feasible. However, transition metal ions like Cu^{2+} form complexes with a variety of organic ligands,[63–65] which thereby can strongly alter the reduction potential of the metal ion. Proteins with Fe^{3+}, e^-/Fe^{2+}- or Cu^{2+}, e^-/Cu^+-redox-centers like those of the respiratory chain and plant photosystems are only one example in this terms. Looking at the studies demonstrating or most often just proposing that NAD(P)H effectively reduces Cu^{2+} to Cu^+,[7,9,32,33,48,50,66–68] decisive differences in the experimental conditions and parameters to our study become apparent, which are likely to explain the different effects of NADH on the redox-state of Cu^{2+}. First of all, in none of these studies direct NAD(P)H oxidation by Cu^{2+} has been experimentally confirmed under physiological conditions and mostly was just supposed from the enhancing effect of the pyridine nucleotides on copper ion-dependent damages to biomolecules. In studies demonstrating a Cu^{2+}-dependent oxidation of NADH, experiments were performed at pH 5.0 or 6.0,[7,66] i.e. under conditions where NADH autoxidation and, likely, single electron transfer to Cu^{2+} is supported. NADH oxidation was also assessed in the presence of Cu^{2+} and redox-cycling drugs where it remains unclear whether Cu^{2+} or the drug itself was primarily reduced by NADH or the pyridine nucleotide was consumed by radicals that had been formed.[32,33,67,68] Reduction of Cu^{2+} by NADH has been exclusively demonstrated in the presence of the strong Cu^+ chelator/indicator neocuproine[48] which strongly shifts the reaction equilibrium due to its high affinity for Cu^+ compared to Cu^{2+} thus supporting the reducing process. Given that Cu^{2+} was actually reduced by NADH, reduction of the metal ion appears to be rather ineffective compared to other reductants for Cu^{2+} (e.g. reduced glutathione or ascorbic acid), since the contribution of NADH to the oxidative damages induced by this redox-system is comparatively weak[7] and obtained significance only in the presence of unphysiologically high concentrations of H_2O_2.[9,50] Finally, in studies dealing with Cu^{2+}-induced and NADH-enhanced DNA damages, specific patterns of DNA cleavage were found, indicating that

only DNA-bound copper ions were reduced by NADH (see above) thus leading to site-specific $^{\cdot}OH$ or Cu(I)-peroxide complex formation.[7,32]

Our findings that NADH is not oxidized by Cu^{2+} and did not enhance the inactivation of LDH by Cu^{2+} primarily via an increased formation of radicals is supported by the fact that the Cu(II)-induced inactivation of LDH was also strongly enhanced by NAD^+. In contrast to studies demonstrating protection of e.g. DNA from Cu(II)/NADH by catalase[7,32,33,67,68] and oxygen,[33] H_2O_2 contributed only weakly to the Cu(II)-derived and NAD(H)-enhanced inactivation of LDH as observed here and hypoxia (pO_2 <4.7 hPa) even increased LDH inactivation. Although conformational changes of the enzyme under hypoxia can not be excluded, the latter result may suggest that the absence of oxygen decisively supports the direct oxidation of the enzymes' thiols—probably by influencing the Cu(II)/-Cu(I) equilibrium in this reaction—so that LDH inactivation is accelerated despite a decrease of the (weak) ROS effect on enzyme activity. In line with the results obtained with LDH, oxidation of free cysteine (1 mM) in the presence of Cu(II) (1.5 or 3 μM) and NADH (1 mM) was hardly diminished by catalase (100 U/ml; Pamp, K. and Petrat, F., unpublished data) and NADH did not increase cysteine oxidation induced by Cu(II). Therefore, our results suggest that the increased inactivation of LDH in the presence of NAD(H) does not reflect an increased formation of ROS but that the coenzymes enhance the ROS-independent initial pattern of inactivation, i.e. direct thiol oxidation by Cu(II) (see Figure 2A).

Increased accessibility of sulfhydryl groups in the presence of NAD(H)

Given that ROS are of minor importance for the increased initial inactivation of LDH in the presence of Cu(II) and NAD(H), another, not so far demonstrated mechanism has to prevail. The above results strongly imply that binding of NAD(H) to LDH induces conformational changes that increase the accessibility of the (sensitive) active center sulfhydryls to Cu(II). Evidence that this mechanism plays an important role for the inactivation of LDH by Cu(II)/NADH is provided by the observations that NAD(H) accelerated the detectability of thiols by DTNB and that NAD^+ enhanced the reduction of Cu(II) by SH groups. Further, pyruvate, the binding of which shifts the active sites of LDH to the closed form,[56] completely prevented the Cu(II)-induced inactivation of LDH, although, however in line with other studies,[18,20] high concentrations were necessary. The comparable lower ability of lactate to provide protection here appears to reflect the lower binding affinity of LDH for this substrate,[43] whereas oxamate interfered with our activity assay (i.e. incomplete exchange by pyruvate) so that the protective effect of this inhibitor may be underestimated.

Oxidation of the thiols located near/at the active center of LDH has been reported to result in a complete destruction (subunit fragmentation) of the enzymes' secondary structure, a process that leads to aggregation, which is detectable as clouding.[5,35,36] Under our experimental conditions, i.e. in the presence of Cu(II), we also observed this phenomenon, which was strongly accelerated by NADH (Pamp, K. and Petrat, F., unpublished data). This supports the above findings that NADH enhances the oxidation of essential thiols and that LDH inactivation was not (predominantly) mediated by ROS, since LDH inactivation by $^{\cdot}OH/O_2^{\cdot-}$ after addition of 10 mM H_2O_2 was found to be accompanied neither by loss of secondary structure nor by aggregation.[35,36]

Our results strongly suggest the following mechanism to be responsible for the effect of NAD(H) on Cu(II)-induced LDH inactivation here: The steric structure of the active sites of LDH in the absence of the ligands hampers the binding of Cu(II) to the cysteine residues in the active center. Conformational changes of LDH following cosubstrate binding have been described in a variety of studies.[45,60,69–71] The active site of the subunit of LDH, situated in a cleft, is opened or closed by a flexible loop depending on the presence of ligands.[56,72] Binding of NADH induces a subtle change in the conformation of the active site resulting in a slight opening[45,56] thus allowing Cu(II) to enter more rapidly. Already in the 70s it was found that cysteine 165, situated in the general area of the active site and in immediate vicinity to the cosubstrate binding site,[45] is the primary target for oxidizing reagents including metal ions.[45,69,72] Cysteine 165, identified as essential for enzymatic activity, although not directly involved in the catalytic process, induces a conformational change in the enzymes active site after binding of metal ions so that an essential arginine residue can not take up its correct position in the ternary complex resulting in loss of enzymatic activity.[36,45,70,73] In this context it is most likely that NAD(H)-binding to LDH increases the accessibility of the cysteine 165 residues to Cu(II) thus presumably resulting in disulfide formation via thiol radicals and finally aggregation of the enzyme by S—S cross linking.[36,74,75] Other thiols than cysteine 165 may be oxidized in secondary processes, especially after subunit fragmentation of the enzymes' secondary structure.

In conclusion, our data demonstrate that NAD(H) strongly accelerate the Cu(II)-dependent inactivation of LDH without the intermediacy of ROS, due to the promotion of direct oxidation. This is in contrast to other enzymes, where exclusively ROS have been made responsible for their inactivation by Cu(II)/

NADH[9,34] and would explain the exceptional high sensitivity of LDH for copper ions. The mechanism of LDH inactivation by Cu(II)/NAD(H) reported here demonstrates that NAD(H) can mediate prooxidative effects without the involvement of ROS and that conformational changes of biomolecules may increase their vulnerability towards oxidants. Our findings seem to be of relevance also for the phylogenetically closely related enzyme alcohol dehydrogenase (Pamp, K. and Petrat, F., unpublished data) and may play a role for other dehydrogenases too.

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