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Enhanced oxidation of NAD(P)H by oxidants in the presence of dehydrogenases but no evidence for a superoxide-propagated chain oxidation of the bound coenzymes

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Abstract

Recently we demonstrated that lactate dehydrogenase (LDH)-bound NADH is oxidized by O_2, H_2O_2, HNO_2 and peroxynitrite predominantly via a chain radical mechanism which is propagated by superoxide. Here we studied both whether other dehydrogenases also increase their coenzymes' reactivity towards these oxidants and whether a chain radical mechanism is operating. Almost all dehydrogenases increased the oxidation of their physiological coenzymes by at least one of the oxidants. The oxidation of NADH or NADPH depended both on the binding dehydrogenase and the applied oxidant and in some cases the reactions were remarkably fast. The highest rate constant ($k = 370\,M^{-1}\,s^{-1}$) was found for the reaction of HNO_2 with NADH bound to alcohol dehydrogenase. Regardless of the applied oxidant, superoxide dismutase failed to inhibit the oxidation of protein-bound NADH and NADPH. We therefore conclude that several dehydrogenases increase the oxidation of NADH and/or NADPH by the employed set of oxidants in bimolecular reactions, but, unlike LDH, do not mediate a $O_2^{\cdot-}$-dependent chain radical mechanism.

Keywords: *Dehydrogenases, pyridine nucleotides, coenzymes, enzyme binding, oxidants, superoxide*

Abbreviations: *LDH, L-lactate dehydrogenase, ADH, alcohol dehydrogenase, AlDH, aldehyde dehydrogenase, G6-PDH, glucose-6-phosphate dehydrogenase, GlDH, glutamate dehydrogenase, GAPDH, glyceraldehyde-3-phosphate dehydrogenase, ICDH, isocitrate dehydrogenase, MDH, malate dehydrogenase, SOD, superoxide dismutase, SIN-1, 3-morpholino-sydnonimine, DTPA, diethylenetriamine-pentaacetic acid, $ONOO^-$, oxoperoxonitrate(1−)/peroxynitrite*

Introduction

In a previous study we have shown that several oxidants (O_2, H_2O_2, HNO_2 and peroxynitrite) oxidize NADH bound to lactate dehydrogenase (LDH) mainly via a superoxide-propagated chain-oxidation [1]. This chain-reaction was first discovered in the early 70s by Chan and Bielski who suggested that binding to the enzyme changes the chemical properties of the nucleotide so effectively, that its reaction rate with a non-specific oxidizing agent like $O_2^{\cdot-}$ increases many orders of magnitude [2]. Since the molecular structure responsible for the coenzyme-binding, the so-called Rossmann-fold, is remarkably similar in various dehydrogenases [3,4], it appeared likely that also dehydrogenases other than LDH increase the reaction rate between their coenzymes NADH or NADPH and the attacking oxidant. In the present study we therefore studied the dehydrogenases alcohol dehydrogenase (ADH), aldehyde dehydrogenase (AlDH), glucose-6-phosphate dehydrogenase

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(G6-PDH), glutamate dehydrogenase (GlDH), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), isocitrate dehydrogenase (ICDH) and malate dehydrogenase (MDH) regarding their capabilities to increase the reactivity of the bound coenzymes towards O_2, H_2O_2, HNO_2, authentic and *in situ*-generated peroxynitrite and to mediate a superoxide-propagated chain-oxidation of NAD(P)H.

Materials and methods

Materials

ADH (EC 1.1.1.1), AlDH (EC 1.2.1.5) and G6-PDH (EC 1.1.1.49) from yeast, GlDH (EC 1.4.1.3) and catalase (EC 1.11.1.6) from bovine liver, GAPDH (EC 1.2.1.12) from rabbit muscle, MDH (EC 1.1.1.37) from pig heart, superoxide dismutase (SOD, EC 1.15.1.1) from bovine erythrocytes, NADH and NADPH were purchased from Roche Molecular Biochemicals (Mannheim, Germany). ICDH (EC 1.1.1.42) from porcine heart, Chelex 100 (chelating resin), diethylenetriamine-pentaacetic acid (DTPA), hydrogen peroxide, manganese dioxide and isoamylnitrite were obtained from Sigma-Aldrich (Taufkirchen, Germany). The peroxynitrite generator 3-morpholinosydnonimine (SIN-1) was kindly provided by Drs K. Schönafinger and J. Pünter (Aventis, Frankfurt/Main, Germany). Oxoperoxonitrate(1−) ($ONOO^-$) was prepared by isoamylnitrite-induced nitrosation of hydrogen peroxide (0.12 mol isoamylnitrite, 100 ml H_2O_2 (1 M) containing DTPA (2 mM)), purified (i.e. solvent extraction, removal of excess H_2O_2, N_2-purging) as given by Uppu and Pryor [5] and stored at −79°C. All other chemicals were of the highest purity commercially available. Solutions were prepared using water received from a TKA-LAB purification system (Niederelbert, Germany, type HP 6 UV/UF).

Assessment of the effect of O_2, H_2O_2, and HNO_2 on the oxidation of NADH or NADPH in the absence and presence of dehydrogenases

Autoxidation of NADH and NADPH (150 μM) was determined in the absence or presence of either ADH, AlDH, GAPDH, GlDH, G6-PDH, ICDH or MDH (10 μM each) in phosphate buffer (50 mM, pH 7.0, 37°C) containing DTPA (0.1 mM) under ambient atmosphere ($pO_2 = 202–205$ kPa, i.e. $[O_2] \approx 225$ μM, determined with a LICOX MCB® Oxygen Monitor, GMS, Kiel-Mielkendorf, Germany); comparative experiments were performed either in the presence of SOD (50 U ml^{-1}) or under hypoxia (argon atmosphere, $pO_2 \leq 4.7$ kPa) within a glove box. Some experiments with AlDH were additionally carried out in the presence of catalase (130 U l^{-1}). The oxidizing effects of H_2O_2 (600 μM) and HNO_2 (as generated from 10 mM $NaNO_2$, [1]) on NAD(P)H in the absence and presence of the dehydrogenases were measured under the same experimental conditions as described for the autoxidation experiments. In some experiments SOD (50 U ml^{-1}) was added prior to the beginning of the experiments. NAD(P)H oxidation was determined photometrically (Shimadzu, Duisburg, Germany; type UV mini 1240) from the decrease in absorption at 340 nm during a period of 60 min.

Determination of the effect of peroxynitrite on the oxidation of NADH or NADPH in the absence and presence of dehydrogenases

Phosphate buffer (50 mM, pH 7.5, 25°C) with or without either ADH, AlDH, GAPDH, G6-PDH, GlDH, ICDH or MDH (10 μM each) was supplemented with NADH or NADPH (150 μM each) and transferred to reaction tubes (Eppendorf, Hamburg, Germany). Then $ONOO^-$ (130–280 μM) was added to 1 ml of the solution, using the drop-tube Vortex mixer technique as described by Kirsch et al. [6]. NAD(P)H oxidation was immediately assessed photometrically from the decrease in NAD(P)H absorption at 340 nm. In some experiments SOD (50 U ml^{-1}) was added prior to the addition of peroxynitrite.

Experiments with the peroxynitrite generator 3-morpholinosydnonimine (SIN-1) were performed under the same experimental conditions as described for authentic $ONOO^-$. SIN-1 (1 mM) was added to the buffer containing either DTPA (0.1 mM) and NAD(P)H (150 μM) or additionally one of the dehydrogenases (10 μM). Oxidation of NADH or NADPH was assessed by continuous UV/visible spectrophotometric recordings of the absorption at 340 nm (UV/Visible Lambda 40, PerkinElmer Life Sciences, Norwalk, CT, USA). Further experiments were performed in the presence of SOD (20–50 U ml^{-1}).

Controls for all experiments in this study were performed with dehydrogenases that had been dialyzed for 12 h against phosphate buffer (50 mM, pH 7.0, 4°C). Experiments with dialyzed dehydrogenases provided the same results as those described in the result section below, thus excluding artificial effects of contaminating compounds.

Kinetic simulations of oxidant-dependent consumption of NADH or NADPH

Kinetic simulations were performed with the KINTECUS V3.8 program written by Dr James C. Ianni [7]. The kinetic model outlined in Table I was used to fit the experimental data, i.e. the oxidation of NADH and NADPH under the various experimental conditions, thereby receiving the corresponding second-order rate constants. Such a procedure follows the suggestions of Chan and Bielski [8–10]. A "classical",

Table I. Background data of relevant reactions and rate constants.

Entry	Reaction	k[$M^{-1}s^{-1}$]⋆	k_{app} [$M^{-1}s^{-1}$]⋆	Remarks	References
1	$NADH + O_2 \rightarrow NAD^+ + H_2O_2$†	6.8×10^{-10}			[19]
2	$NADH + H_3O^+ \rightarrow HNADH^+ + H_2O$	7		$k_2 = 5.6 \times 10^{-5}\,s^{-1}$ at pH 5.1 and $k_2 = 5.9 \times 10^{-4}\,s^{-1}$ at pH 4.1	[20]
3	$LDH–NADH + O_2 \rightarrow LDH–NAD^{\cdot} + H^+ + O_2^{\cdot-}$		1.6×10^{-4}		[1]
4	$NADH + O_2^{\cdot-} \rightarrow NAD^{\cdot} + HOO^-$	2.7		$k_4 \ll 27\,M^{-1}s^{-1}$	[21]
5	$NADPH + O_2^{\cdot-} \rightarrow NADP^{\cdot} + HOO^-$	2.7		Estimated to be similar to entry 4	
6	$LDH–NADH + O_2^{\cdot-} \rightarrow LDH–NAD^{\cdot} + HOO^-$	3.6×10^4			[8]
7	$NADH + H_2O_2 \rightarrow NAD^+ + H_2O + HO^-$	3.5×10^{-5}		Extracted from data given in	[11]
8	$NADPH + H_2O_2 \rightarrow NADP^+ + H_2O + HO^-$	3.5×10^{-5}		Estimated to be similar to entry 7	
9	$LDH–NADH + H_2O_2 \rightarrow$ product that yields $O_2^{\cdot-}$		2.0×10^{-4}		[1]
10	$LDH–NADH + HNO_2 \rightarrow LDH–NAD^{\cdot} + NO^{\cdot} + H_2O$		520		[1]
11	$LDH + NADH \rightarrow LDH–NADH$	1.0×10^6			[1]
12	Enzyme + NAD(P)H‡ → Enzyme–NAD(P)H	1.0×10^6		Estimated to be similar to entry 11 Enzyme = MDH, G6-PDH, ADH, GAPDH, AlDH, ICDH, GlDH	
13	$LDH–NADH \rightarrow LDH + NADH$	1.0×10^1		From entry 11 and $K_m = 1.07 \times 10^{-5}$	[22]
14	Enzyme–NAD(P)H → Enzyme + NAD(P)H	1.0×10^1		Estimated to be similar to entry 13 Enzyme = MDH,G6-PDH, AlDH, ADH, ICDH, GlDH	
15	$GAPDH–NADH \rightarrow GAPDH + NADH$	3.3		From entry 12 and $K_m = 3.3 \times 10^{-6}$	[22]
16	$LDH + NAD^+ \rightarrow LDH–NAD^+$	1.0×10^6			[1]
17	Enzyme + $NAD(P)^+$ → Enzyme–$NAD(P)^+$	1.0×10^6		Estimated to be similar to entry 16 Enzyme = MDH,G6-PDH, ADH, GAPDH, AlDH, ICDH, GlDH	
18	$LDH–NAD^+ \rightarrow LDH + NAD^+$	2.5×10^2		From entry 16 and $K_m = 2.53 \times 10^{-4}$	[22]
19	$MDH–NAD^+ \rightarrow MDH + NAD^+$	2.5×10^2		Estimated to be similar to entry 18	
20	$G6\text{-}PDH–NAD^+ \rightarrow G6\text{-}PDH + NAD^+$	1.15×10^2		From entry 17 and $K_m = 1.15 \times 10^{-4}$	[22]
21	$G6\text{-}PDH–NADP^+ \rightarrow G6\text{-}PDH + NADP^+$	7.6		From entry 17 and $K_m = 7.6 \times 10^{-6}$	[22]
22	$GAPDH–NAD^+ \rightarrow GAPDH + NAD^+$	1.3×10^1		From entry 17 and $K_m = 1.3 \times 10^{-5}$	[22]
23	$AlDH–NAD^+ \rightarrow AlDH + NAD^+$	2.0×10^1		From entry 17 and $K_m = 2.0 \times 10^{-5}$	[22]
24	$AlDH–NADP^+ \rightarrow AlDH + NADP^+$	5.0×10^1		From entry 17 and $K_m = 5.0 \times 10^{-5}$	[22]
25	$ADH–NAD^+ \rightarrow ADH + NAD^+$	7.4×10^1		From entry 17 and $K_m = 7.4 \times 10^{-5}$	[23]
26	$ADH–NADP^+ \rightarrow ADH + NADP^+$	5.9×10^1		From entry 17 and $K_m = 5.9 \times 10^{-5}$	[24]
27	$NAD^{\cdot} + O_2 \rightarrow NAD^+ + O_2^{\cdot-}$	2.0×10^9			[21]
28	$LDH–NAD^{\cdot} + O_2 \rightarrow LDH–NAD^+ + O_2^{\cdot-}$	3.2×10^9			[9]
29	$2\,NAD^{\cdot} \rightarrow (NAD)_2$	5.6×10^7			[8]
30	$H_3O^+ + NO_2^- \rightarrow HNO_2 + H_2O$	5×10^{10}			[18]
31	$HNO_2 + H_2O \rightarrow H_3O^+ + NO_2^-$	7.2×10^5		From entry 30 and pK_a (HNO_2) = 3.1	[25]
32	$2\,HNO_2 \rightarrow N_2O_3 + H_2O$	13.4			[18]
33	$N_2O_3 + H_2O$ ($+HPO_4^{2-}$) → $2\,HNO_2$	$2 \times 10^3 + 8 \times 10^5 \times [HPO_4^{2-}]$			[18]

k_{app} Optimized apparent rate constants.

$k_1 = k_2 \times [H^+]$ and rearrangement of it leads to $k_2 = k_1/[H^+]$ i.e. $k_2 = (5.6 \times 10^{-5})/(7.94 \times 10^{-6}) = 7.05\,M^{-1}s^{-1}$ at pH 5.1.

⋆At ambient temperature (25°C). †The reaction outlined in entry 1 was suggested by Yokota and Yamazaki [26]. We believe that the autoxidation of NAD(P)H yields $NAD(P)^{\cdot}$ and $O_2^{\cdot-}$ but any experimental evidence for this hypothesis is presently missing. ‡The term NAD(P)H indicates that both coenzymes, i.e. NADH and NADPH, were tested for their capabilities to act as a target for the selected oxidants.

i.e. experimental determination of the second-order rate constant is very hard to achieve via a second-order plot because (i) both free NAD(P)H and protein-bound coenzymes may react, (ii) the concentration of free NAD(P)H and protein-bound one is hard to discriminate during a kinetic run, and (iii) the concentration of the attacking entities may be very low in experiments with SIN-1 and NO_2^- as donor compounds.

Statistics

All experiments were repeated at least three times. Data shown are means ± SD.

Results

The autoxidation of both NADH and NADPH proceeded rather slowly under normoxic conditions, although NADPH autoxidized several-fold faster than NADH (Figure 1 and entry 1 in Tables I and II). The enzymes MDH, G6-PDH, GAPDH and AlDH (10 μM each) enhanced the autoxidation of NADH with AlDH providing the strongest effect (Table II, entries 2–5); ADH, GlDH and ICDH did not increase the autoxidation of the coenzyme (Table II, entry 6). The autoxidation of NADPH was solely increased by AlDH (Figure 1 and Table II, entries 1, 7 and 8).

SOD (50 U ml^{-1}) and catalase (130 U l^{-1}) failed to inhibit NADH and NADPH autoxidation in each case (data not shown). Therefore, $O_2^{\cdot-}$ and contaminant H_2O_2 neither initiated nor propagated the autoxidation of the reduced coenzymes (Table II, entry 9). Hypoxia ($pO_2 \leq 4.7$ kPa), however, completely abolished NAD(P)H oxidation in all aforementioned experiments (data not shown), indicating that molecular oxygen is indeed the oxidizing species.

During 1 h of incubation with H_2O_2 (600 μM) no H_2O_2-dependent oxidation of free NADH (not shown) and NADPH was detectable (Figure 2), in line with the data of Bernofsky and Wanda ([11], Table I, entries 7 and 8). The addition of AlDH increased both the autoxidation (see above) and the H_2O_2-dependent oxidation of NADPH (Figure 2 and Table II, entries 7 and 11). Similar results were observed with NADH (Table II, entries 5 and 10). The other enzymes were without any detectable effect on NADH and NADPH oxidation by H_2O_2 (Table II, entry 12). SOD (50 U ml^{-1}) did not decrease the H_2O_2-dependent oxidation of NADH or NADPH bound to AlDH (data not shown), thus demonstrating again that no superoxide-propagated chain radical reaction occurred.

In the presence of HNO_2, as generated by addition of sodium nitrite (10 mM), free NADH or NADPH were slowly oxidized in a linear manner (Figure 3 and Table II, entries 13 and 14). ADH was the only enzyme which significantly increased the oxidation of NADH, but not of NADPH (Table II, entries 15–17). Experiments performed in the presence of SOD (50 U ml^{-1}) and additional experiments achieved under hypoxic conditions ($pO_2 \leq 4.7$ kPa; data not shown) confirmed that the HNO_2-mediated oxidation of ADH-bound NADH proceeded in a superoxide-independent manner. Consequently, no chain oxidation of NADH initiated and propagated by superoxide was observed in these experiments as well.

None of the dehydrogenases studied enhanced the oxidation of NADH or NADPH as initiated by authentic (130–280 μM) or *in situ*-generated (from 3-morpholinosydnonimine, 1 mM) peroxynitrite (data not shown). In fact, some of the enzymes even decreased the oxidation of NADH or NADPH, indicating that the protein moiety substantially reacted with peroxynitrite or radicals derived from it.

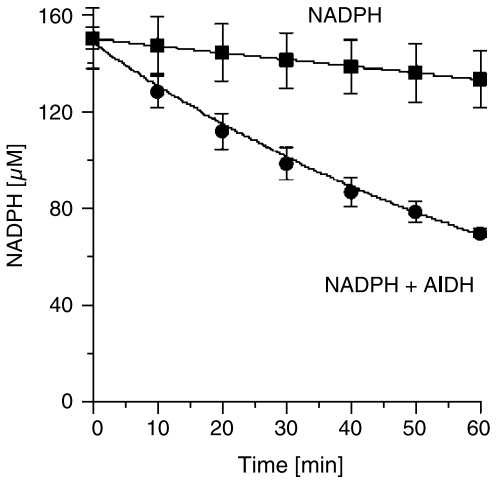

Figure 1. Effect of AlDH on the oxidation of NADPH by atmospheric O_2. NADPH (150 μM) was incubated with or without AlDH (10 μM) at normoxic conditions in phosphate buffer (50 mM, pH 7.0, 37°C). NADPH oxidation was measured by spectrophotometric recordings of the decrease in absorption at 340 nm. Data points shown are means ± SD of three independent experiments. The theoretical curves computed with the optimized rate constants $k(\text{NADPH} + O_2) = 1.5 \times 10^{-1}\,\text{M}^{-1}\,\text{s}^{-1}$ and $k((\text{AlDH–NADPH}) + O_2) = 1.5\,\text{M}^{-1}\,\text{s}^{-1}$ are shown as solid lines (Table II, entries 1 and 7).

Discussion

More than 30 years ago Bielski and Chan described a radical-chain reaction of LDH-bound NADH which was both initiated and propagated by $O_2^{\cdot-}$ [2,8,9,12,13]. Recently, we found that this LDH-dependent chain reaction can be initiated by various oxidants (O_2, H_2O_2, HNO_2, peroxynitrite and $O_2^{\cdot-}$), but that the chain is always propagated by $O_2^{\cdot-}$ ([1], Table I, entries 3, 6, 9, 10 and 28). In the present study we demonstrate that several other dehydrogenases can also increase the oxidation of NADH and/or NADPH by some of these oxidants. In contrast to

Table II. Reactions and rate constants calculated for the oxidation of NAD(P)H in the absence and presence of dehydrogenases.

Entry	Reaction	k[$M^{-1}s^{-1}$]⋆	k_{app} [$M^{-1}s^{-1}$]⋆	Remarks
1	$NADPH + O_2 \rightarrow NAPD^+ + H_2O_2$		1.5×10^{-1}	This paper
2	$MDH{-}NADH + O_2 \rightarrow MDH{-}NAD^{\cdot} + H^+ + O_2^{\cdot-}$		7.0×10^{-2}	This paper
3	$G6\text{-}PDH{-}NADH + O_2 \rightarrow G6\text{-}PDH{-}NAD^{\cdot} + H^+ + O_2^{\cdot-}$		1.0×10^{-1}	This paper
4	$GAPDH{-}NADH + O_2 \rightarrow GAPDH{-}NAD^{\cdot} + H^+ + O_2^{\cdot-}$		7.0×10^{-1}	This paper
5	$AlDH{-}NADH + O_2 \rightarrow AlDH{-}NAD^{\cdot} + H^+ + O_2^{\cdot-}$		2.0	This paper
6	$Enzyme{-}NADH + O_2 \rightarrow Enzyme{-}NAD^{\cdot} + H^+ + O_2^{\cdot-}$		0.0	This paper Enzyme = ADH, GlDH, ICDH
7	$AlDH{-}NADPH + O_2 \rightarrow AlDH{-}NAD^{\cdot} + H^+ + O_2^{\cdot-}$		1.5	This paper
8	$Enzyme{-}NADPH + O_2 \rightarrow Enzyme{-}NADP^{\cdot} + H^+ + O_2^{\cdot-}$		0.0	This paper Enzyme = MDH, G6-PDH, ADH, GlDH, ICDH, GAPDH
9	$Enzyme{-}NAD(P)H^{\dagger} + O_2^{\cdot-} \rightarrow Enzyme{-}NAD(P)^{\cdot} + HOO^-$		0.0	This paper Enzyme = MDH, G6-PDH, ICDH, GAPDH, AlDH, ADH, GlDH
10	$AlDH{-}NADH + H_2O_2 \rightarrow products$		0.33	This paper
11	$AlDH{-}NADPH + H_2O_2 \rightarrow products$		0.33	This paper
12	$Enzyme{-}NAD(P)H + H_2O_2 \rightarrow products$		0.0	This paper Enzyme = MDH,G6-PDH, GAPDH, ADH, ICDH, GlDH
13	$NADH + HNO_2 \rightarrow NAD^{\cdot} + NO^{\cdot} + H_2O$		10	This paper
14	$NADPH + HNO_2 \rightarrow NADP^{\cdot} + NO^{\cdot} + H_2O$		10	Estimated to be similar to entry 13
15	$ADH{-}NADH + HNO_2 \rightarrow ADH{-}NAD^{\cdot} + NO^{\cdot} + H_2O$		370	This paper
16	$ADH{-}NADPH + HNO_2 \rightarrow ADH{-}NADP^{\cdot} + NO^{\cdot} + H_2O$		0.0	This paper
17	$Enzyme{-}NAD(P)H + HNO_2 \rightarrow Enzyme{-}NAD(P)^{\cdot} + NO^{\cdot} + H_2O$		0.0	This paper Enzyme = MDH, G6-PDH, GAPDH, AlDH, ICDH, GlDH

k_{app} Optimized apparent rate constants in order to fit the experimental data (see Figures 1, 2 and 3).

In order to assess the second-order rate constants, experimental data were fitted with a kinetic simulation performed with the KINTECUS V3.8 program written by Dr James C. Ianni [7]. A set of reactions and rate constants as given in Refs. [1,18] and in Table I were included in the simulation of the reactions. Simulating parameters for entries 1, 7, 11, 13 and 15 see Legends to Figures 1, 2 and 3; other experimental data are given in the text or are not shown.

⋆At ambient temperature (25°C). †The term NAD(P)H indicates that both coenzymes, i.e. NADH and NADPH, were tested for their capabilities to act as a target for the selected oxidants.

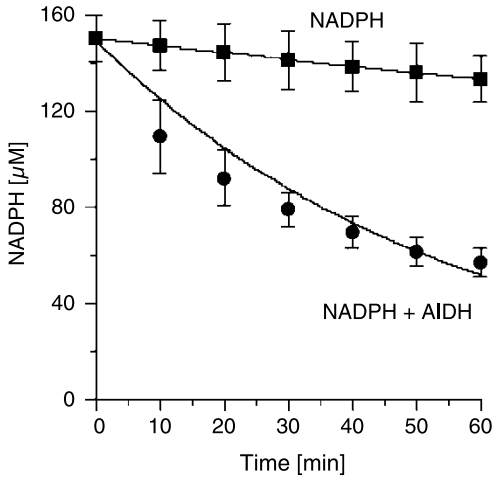

Figure 2. Effect of AlDH on the oxidation of NADPH by H_2O_2. NADPH (150 μM) was incubated with 600 μM H_2O_2 under normoxic conditions in the absence or presence of aldehyde dehydrogenase (AlDH, 10 μM) in phosphate buffer (50 mM, pH 7.0, 37°C). NAD(P)H oxidation was ascertained by spectrophotometric recordings of the decrease in absorption at 340 nm. Data points shown are means ± SD of three independent experiments. The theoretical curves computed with the optimized rate constants $k(\text{NADPH} + H_2O_2) = 3.5 \times 10^{-5}\,M^{-1}\,s^{-1}$and $k((\text{AlDH–NADPH}) + H_2O_2) = 0.33\,M^{-1}\,s^{-1}$ are shown as solid lines (Table I, entry 8 and Table II, entry 11). Note that the experimental data and the theoretical curves include the autoxidation of NADPH by O_2; compare with Figure 1.

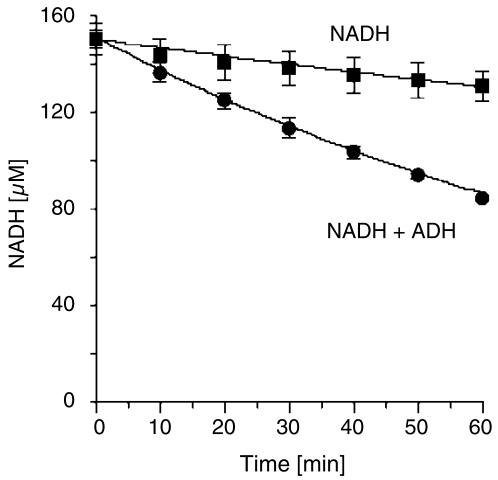

Figure 3. Effect of ADH on the oxidation of NADH by HNO_2. NADH (150 μM) was incubated with or without ADH (10 μM) under normoxic conditions in phosphate buffer (50 mM, pH 7.0, 37°C) containing $NaNO_2$ (10 mM) to generate HNO_2. NADH oxidation was assessed by spectrophotometric recordings of the decrease in absorption at 340 nm. Data points shown are means ± SD of three independent experiments. The theoretical curves computed with the optimized rate constants $k(\text{NADH} + HNO_2) = 10\,M^{-1}\,s^{-1}$ and $k((\text{ADH–NADH}) + HNO_2) = 370\,M^{-1}\,s^{-1}$ are shown as solid lines (Table II, entries 13 and 15). Note that the equilibrium concentration of HNO_2 resulting from 10 mM sodium nitrite is only 1.25 μM under these conditions [1], thus explaining the relatively low effect of this oxidant compared to the calculated rate constant. Compare with Figure 1.

LDH–NADH, however, a catalytical chain oxidation did not occur, since all the dehydrogenase–NAD(P)H complexes studied here exclusively reacted with the applied oxidants but not with the subsequently produced superoxide radicals. Our data completely align with a previous study demonstrating that the MDH–NADH, GlDH–NADH and the ICDH–NADPH complex do not react with $O_2^{\cdot-}$ as generated from the xanthine–xanthine oxidase system [10].

All of the dehydrogenases studied here, except of ICDH and GlDH, increased the oxidation of NADH and/or NADPH by at least one oxidant, and—with the exception for G6-PDH and NADH—solely the physiological coenzyme known to bind to the active center in a binary complex was oxidized. The oxidation of NADH or NADPH depended both on the binding dehydrogenase and the applied oxidant and in some cases the reactions were moderately "fast" (Table I): the binary NADH complexes of MDH, G6-PDH and GAPDH reacted even faster with molecular oxygen than LDH–NADH did ($k = 1.6 \times 10^{-4}$, [1]).

The highest rate constant ($k = 370\,M^{-1}\,s^{-1}$) was found for the reaction of HNO_2 with NADH bound to ADH (Figure 3 and Table II, entry 15) and this rate constant is similar to the one found for the LDH–NADH complex and HNO_2 ($k = 520\,M^{-1}\,s^{-1}$; Table I, entry 10). However, the reaction of LDH–NADH and the superoxide anion appears to be still the fastest one occurring under physiological conditions ($k = 3.6 \times 10^4\,M^{-1}\,s^{-1}$, [8]); the very fast reaction of GAPDH–NADH with the protonated superoxide anion, HO_2 ($k = 2.0 \times 10^7\,M^{-1}\,s^{-1}$, [10]), can be neglected at physiological pH values ($pK_a\,[HO_2] = 4.7$, [14]).

Despite the fact that the coenzyme binding regions of all the dehydrogenases studied here are known to be constructed very similar [3,4], each binary complex behaved different towards a selected oxidant. We therefore propose that the properties of the amino acid side chains in the α-helices of the coenzyme binding site, which, in contrast to the more conserved β-sheets differ to a higher degree between dehydrogenases [3,4], are responsible for the extent the bound coenzyme will be oxidized.

Our results demonstrate that binding to dehydrogenases alters the redox properties of NADH and NADPH. Since NADH and NADPH are almost exclusively bound to dehyrogenases within cells [15,16], several binary dehydrogenase–NAD(P)H complexes may be decisively involved in intracellular reactions with various oxidants. Here they should be responsible for the maintenance of the cellular redox state and possess a large antioxidative capability as well. Since oxidants reacting with NAD(P)H are generally transformed to $O_2^{\cdot-}$, which can easily be decomposed by cellular SOD [17], the accelerated reactions with oxidants as a consequence of dehydro-

genase binding should enhance the direct antioxidative capability of NAD(P)H within cells.

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