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INACTIVATION OF YEAST ALCOHOL DEHYDROGENASE BY ALKYLPEROXYL RADICALS

CHARACTERISTICS AND INFLUENCE OF NICOTINAMIDE-ADENINE DINUCLEOTIDES

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Abstract—The study of the interaction of alkylperoxyl radicals generated by the aerobic thermolysis of 2,2′-azobis(2-amidinopropane) (AAP) with yeast alcohol dehydrogenase (YADH) revealed a high reactivity of the enzyme, with an average of about 20 radicals per added YADH tetramer being needed to elicit its total inactivation. NAD^+ enhanced YADH inactivation at NAD^+/YADH molar ratios from 0.25 to 1, decreasing the rate of the process when added in excess to the enzyme concentration. At NADH/YADH molar ratios greater than 1, NADH exhibited a protective effect characterized by a poorly defined induction time and lower inactivation rates, which progressively increased during the reaction period. These changes occurred concomitantly with the oxidation of NADH into NAD^+, which might counteract the protective effect of NADH. Under similar conditions, $NADP^+$ did not modify AAP-induced YADH inactivation, while NADPH exhibited a modest protection at NADPH/YADH molar ratios greater than 1. It is concluded that YADH inactivation by alkylperoxyl radicals is strongly dependent on the redox state of the NADH-NAD^+ couple, as the rates of the process at different time intervals inversely correlate with the respective NADH/NAD^+ ratios.

Free radical species are known to interact with different types of biomolecules inducing oxidative modifications, cross-linking reactions and/or bond cleavage, thus altering their structure and function [1]. Among them, non-catalytic proteins [2–4] and enzymes [4, 5] are modified when exposed to either higher energy radiation [6] or a number of non-enzymic and enzymic free radical generating systems [5], including redox cycling agents [7]. In these conditions, enzyme inactivation has been ascribed to the presence of one or more readily oxidizable amino acid residues at the catalytic site, leading to reversible (cysteine and methionine) or irreversible (arginine, proline, lysine, histidine, tryptophan and tyrosine) oxidative alterations [1, 4, 5].

Alcohol dehydrogenase (ADH)§ is one of the metabolic enzymes studied that has been found to be susceptible to inactivation either by irradiation [8–11] or by metal-catalyzed oxidation systems, such as the NADH oxidase system (microbial diaphorase supplemented with NADH and Fe(III)) [12], NADPH cytochrome P450 reductase/cytochrome P450 plus NADPH [12] or rat liver microsomes supplemented with NADPH [7]. In these conditions, ADH inactivation seemed to involve the formation of H_2O_2 and the reduction of Fe(III) by the metal-catalyzed oxidation system, followed by oxidation of Fe(II) by H_2O_2 to produce active oxygen species which would attack the enzyme [5, 7, 12]. Since the studies were carried out using high concentrations (1 mM) of NADH or NADPH to initiate the process [7, 12], generation of significant levels of the oxidized forms of the coenzymes is likely to have occurred. Both the oxidized and reduced forms of the nicotinamide-adenine dinucleotides are known to bind ADH with different affinities [13, 14] and to interact with free radicals with different efficiencies [11, 15, 16], suggesting a possible modification of the inactivating process. This aspect was evaluated in the present work by studying the characteristics of the inactivation of yeast ADH (YADH) by free radicals, in the absence and presence of $NAD(P)^+$ and NAD(P)H. For this purpose, the thermolysis of 2,2′-azobis(2-amidinopropane) (AAP) was used as the alkylperoxyl radical generating system [17, 18]. This system generates an easily controllable output of radicals that has been found previously to be suitable for enzyme inactivation studies [19].

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§ Abbreviations: ADH, alcohol dehydrogenase; YADH, yeast alcohol dehydrogenase; AAP, 2,2′-azobis(2-amidinopropane); ROO·, AAP-derived alkylperoxyl radical; IR, inactivation rate; n_1, number of enzyme molecules inactivated/radical; n_2, number of NADH molecules oxidized/radical; and T_0, induction time.

MATERIALS AND METHODS

Enzymes and chemicals. AAP was obtained from Polysciences (Warrington, PA) and the rest of the reagents used were obtained from the Sigma Chemical Co. (St. Louis, MO). These included

crystallized and lyophilized ADH from yeast, lysozyme from hen egg white, and cells of *Micrococcus lysodeikticus* (ATCC 4698).

Enzyme inactivation system. The basic reaction system employed for enzyme inactivation studies consisted of 0.2 M potassium phosphate buffer, pH 7.4, and either YADH (monomeric concentration of 1.88 μM) or lysozyme (1.88 μM), in a final reaction volume of 2 mL. All agents studied were preincubated for 10 min with the YADH solutions, prior to AAP addition. Samples were kept on ice until the reaction was initiated by the addition of AAP to a final concentration of 3 mM, and incubations were carried out at 37° under air for periods of up to 80 min. Controls included samples without AAP and exhibited 87–100% of the initial enzyme activity at the experimental times studied.

In experiments using nicotinamide-adenine dinucleotides in the enzyme inactivation system, the initial concentration of the respective working solutions was determined by changes in the absorbance at 340 nm in either the ADH assay (for NAD^+ and NADH) [20], or in the glucose-6-phosphate/glucose-6-phosphate dehydrogenase system (for $NADP^+$ and NADPH) [21]. The rate of NADH or NADPH oxidation in the absence or presence of 3 mM AAP was determined in similar conditions by continuous measurement of the decrease in absorbance at 340 nm.

Enzyme assays. YADH activity was measured in 0.05-mL aliquots of the reaction medium taken at time zero (before AAP addition) and at different times after AAP addition. The increase in absorbance at 340 nm was determined in a reaction system containing 0.5 M Tris–HCl buffer, pH 7.2, 2.8 mM NAD^+ and 5 mM ethanol [20], in a final volume of 2 mL, at 25°. The 40-fold dilution of the aliquots of the inactivation medium and the reduction of temperature from 37 to 25° ensure a negligible production of radicals from AAP in the assay. These conditions and the use of Tris buffer, known to have a free radical scavenging activity [22], avoid further inactivation of the enzyme during the measurement of its activity. Similar experimental conditions were used for the determination of lysozyme activity, measured from the rate of turbidity loss at 436 nm when added to suspensions of *M. lysodeikticus*, prepared in 0.07 M potassium phosphate buffer, pH 6.5, containing 0.017 M NaCl [19]. The activities of the enzymes were linear with respect to time and concentration.

Data analysis. Values of enzyme activity at different incubation times were expressed as percent of the initial activity (100% at t = 0), and the

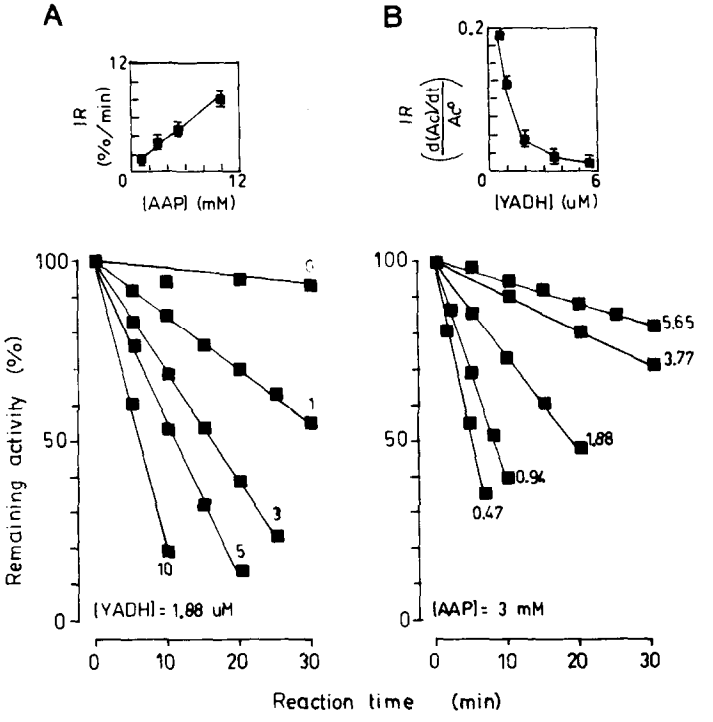

Fig. 1. Inactivation of YADH by AAP. (A) Time course of the changes in YADH activity studied in the absence and presence of millimolar concentrations of AAP, as indicated by the numbers adjacent to the traces. The monomeric concentration of YADH corresponds to 1.88 μM, and 100% activity corresponds to 1.11 ± 0.07 U/mL (N = 15). Inset: dependence of the inactivation rate (IR) of YADH on AAP concentration. Values shown are means ± SEM (N = 3) and correspond to the slopes of the remaining activity versus time plots, calculated by regression analysis. (B) Time course of the changes in YADH activity induced by 3 mM AAP, using different micromolar concentrations of the enzyme, as indicated by the numbers adjacent to the traces. Inset: dependence of the IR of YADH on enzyme concentration, expressed as $[d(Ac)/dt]/Ac^0$, where AC is the enzyme activity (Ac^0 is the initial activity). Values shown are means ± SEM (N = 3).

inactivation rates (IR) were calculated by regression analysis of the remaining activity versus time plots. Alternatively, individual inactivation rates at each time interval (IR_t) of the reaction period were also calculated. All results represent the means ± SEM of at least three separate experiments. The standard errors were omitted in the enzyme activity versus time plots, the respective values being < 10%. The statistical significance of the differences between mean values was assessed by one-way analysis of variance for unequal size groups.

RESULTS

YADH incubation in 0.2 M potassium phosphate buffer (pH 7.4) at 37° for up to 30 min did not modify the enzyme activity significantly (Fig. 1A). Addition of APP to the system led to YADH inactivation at a rate that was found to be proportional to the AAP concentration at a given enzyme concentration (Fig. 1A), and that was increased as the YADH concentration was diminished (Fig. 1B). If the remaining activity is considered to be proportional to the unmodified enzyme concentration, the data in Fig. 1B allow an estimation of the rate of enzyme inactivation, in a molar concentration basis (Table 1A). These data show that changing the monomeric concentration of YADH by a factor larger than 10 barely altered the rate of enzyme inactivation. Under the experimental conditions employed, the rate of input of AAP-derived radicals is 0.23 μM/min [23], which, when related to to the inactivation rates given in Table 1, gives an estimation of the number of YADH molecules inactivated by each radical introduced into the system (n_1). The n_1 values obtained were independent of YADH concentration and its average indicated that about 5 radicals are required to inactivate an enzyme monomer (Table 1A).

Table 1. Efficiency of YADH inactivation and NADH oxidation by AAP-derived alkylperoxyl radicals

(A) Monomeric YADH concentration (μM)	Rate of YADH inactivation* (μM/min)	n_1†
0.47	0.044	0.19
0.94	0.053	0.23
1.88	0.048	0.21
3.77	0.037	0.16
5.65	0.039	0.17
(B) Concentration of NADH (μM)	**Rate of NADH oxidation‡ (μM/min)**	**n_2§**
1.88	0.12	0.52
4.71	0.17	0.74
47.1	0.35	1.52
471.0	0.77	3.35

* Corresponds to half of the YADH concentration used divided by the time required to decrease the enzyme activity to 50% of the initial value (from Fig. 1B).

† Number of YADH molecules inactivated by each radical introduced into the system, calculated as [rate of YADH inactivation (μM/min)]/radical input (0.23 μM/min) [23].

‡ Derived from the slope of the absorbance at 340 nm versus time plots similar to that in Fig. 6A (4.71 μM NADH + 3 mM AAP), using the $E_{340} = 6.22\ mM^{-1} \cdot cm^{-1}$.

§ Number of NADH molecules oxidized per radical, calculated as [rate of NADH oxidation (μM/min)]/radical input (0.23 μM/min) [23].

Trolox is known to be an efficient scavenger of AAP-derived alkylperoxyl radicals [23], leading to a strong inhibition of lysozyme inactivation with a nearly ideal behavior characterized by an induction

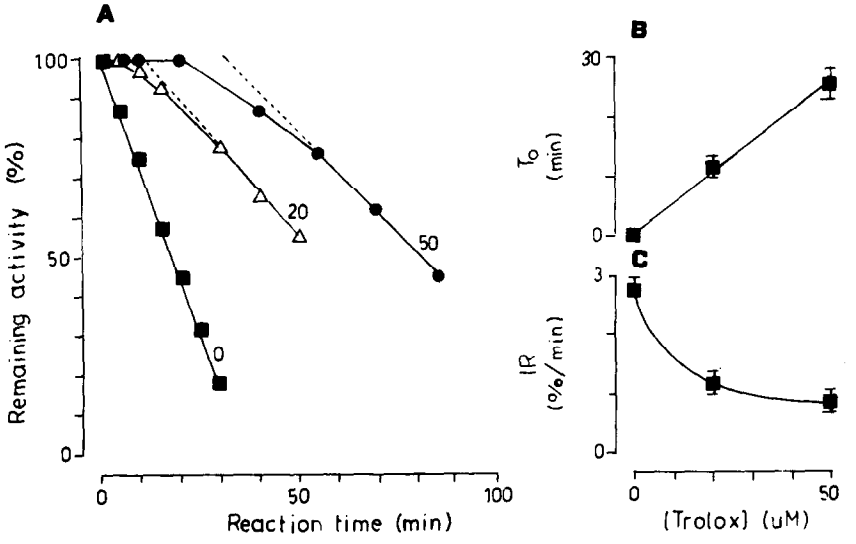

Fig. 2. (A) Effect of Trolox on the rate of YADH inactivation (1.88 μM monomeric concentration) induced by AAP (3 mM). The numbers adjacent to the traces correspond to micromolar concentrations of Trolox. One hundred percent activity corresponds to 1.07 ± 0.03 U/mL (N = 9). (B) Induction period (T_0) as a function of Trolox concentration. (C) Inactivation rate (IR) after T_0 as a function of Trolox concentration. Values shown are means ± SEM (N = 3).

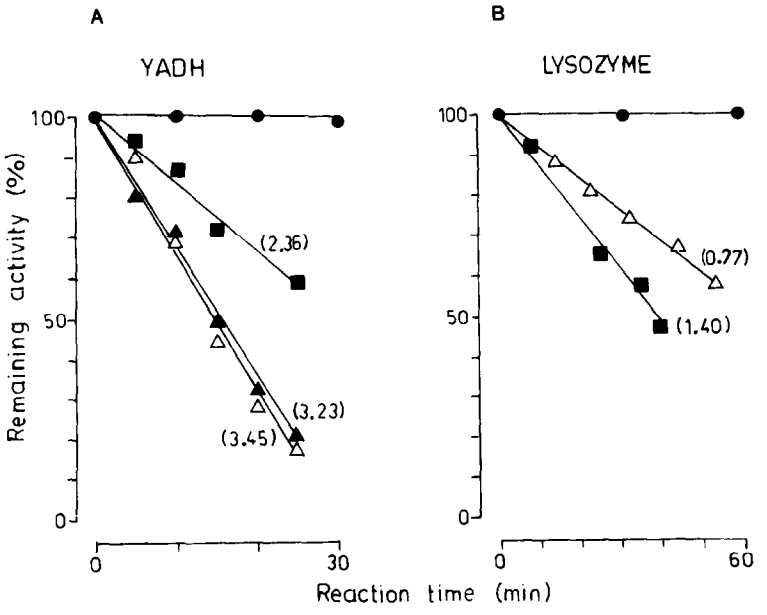

Fig. 3. Comparison of the inactivation of YADH and lysozyme by AAP (3 mM). (A) Inactivation of YADH (1.88 μM monomeric concentration) by AAP in the absence (△) and presence of either 1.88 μM lysozyme (▲) or 1.88 μM albumin (■). One hundred percent activity corresponds to 0.98 ± 0.02 U/mL (N = 12). (B) Inactivation of lysozyme (1.88 μM) by AAP in the absence (■) and presence (△) of 1.88 μM YADH. One hundred percent activity corresponds to an initial $-d(\text{Absorbance})/dt$ of 0.21 ± 0.01 min^{-1} (N = 5). The numbers in parentheses adjacent to the traces correspond to the inactivation rates, expressed as %/min. Closed circles (●) in panels A and B correspond to enzyme activity assayed in the absence of AAP.

period (T_0) after which the rate of the process is equal to that found in the absence of Trolox [24]. The presence of Trolox in the AAP-induced YADH inactivation system elicited a poorly defined T_0 after which the inactivation rate was slower than that measured in the absence of the scavenger (Fig. 2A), both parameters being dependent on Trolox concentration (Fig. 2, B and C). If it is considered that 2 radicals are trapped by each Trolox molecule [23, 24], the slope of the T_0 versus Trolox concentration plot (Fig. 2B) should be 8.33 min/μM. However, the experimentally determined value was 0.52 min/μM, suggesting that Trolox is not completely consumed after T_0 and/or that both YADH and Trolox have similar reactivities towards the alkylperoxyl radicals produced. In a molar basis, the different behavior of YADH (Fig. 2) and lysozyme [24] in the presence of Trolox could imply a significant difference in their reactivity towards the alkylperoxyl radicals. In this respect, YADH inactivation occurred at a rate of 3.45%/min (Fig. 3A) compared to 1.40%/min observed for lysozyme (Fig. 3B), measured under comparable inactivating conditions. Furthermore, while YADH addition diminished the rate of lysozyme inactivation (Fig. 3B), addition of lysozyme had no effect on the rate of YADH inactivation (Fig. 3A). At equal concentrations, albumin significantly reduced the AAP-induced YADH inactivation rate (Fig. 3A).

The addition of NAD^+ to the enzyme inactivation system to give a NAD^+/YADH molar ratio of 1 resulted in a marked increase in the rate of inactivation of YADH, either when added prior to AAP (71%; $P < 0.05$) or after 7 min of exposure of the enzyme to the free-radical generator (63%; $P < 0.05$) (Fig. 4A). YADH inactivation was found to progressively increase at NAD^+/YADH molar ratios from 0 to 1, returning to values observed in the absence of NAD^+ at a ratio of 25, with a 91% diminution ($P < 0.05$) being found at a ratio of 2500 (Fig. 4B). Induction times were not observed at all the NAD^+ concentrations used (Fig. 4B). In these conditions, the absorbance at 340 nm of a reaction medium containing 4.71 μM NAD^+ and 3 mM AAP remained constant for up to 30 min at 37°. The addition of 50 mM ethanol and 3.77 μM YADH at this time resulted in an absorbance increase corresponding to 99% of that observed for 4.71 μM NAD^+ in the absence of AAP (data not shown).

Previous work has indicated that NADH is able to trap alkylperoxyl radicals, leading to induction times, in the peroxyl-radical mediated inactivation of lysozyme [24]. However, the plot of induction times versus the initial NADH concentration presents a downwards curvature, suggesting its consumption by a short chain process, driven by $NAD^\cdot$ [24]. Data presented in Table 1B show that the estimated rate of NADH oxidation by 3 mM AAP increase by 6.4-fold by changing the NADH concentration by a factor of 250, leading to more than one coenzyme molecule oxidized per radical introduced into the system, at high NADH concentrations. These results are compatible with the occurrence of a small oxidation chain of NADH, under conditions of

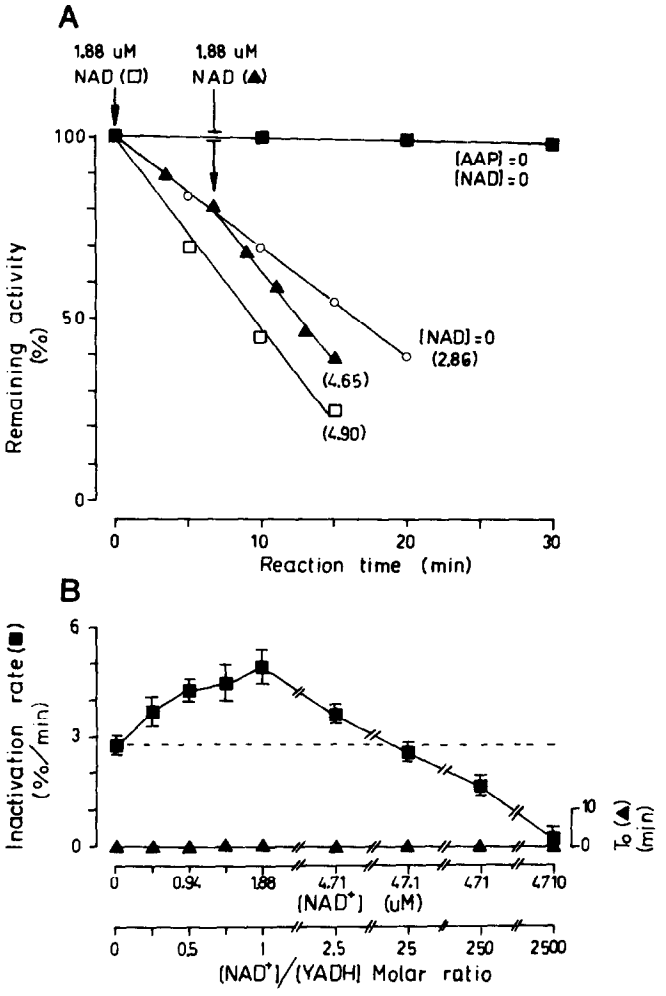

Fig. 4. Effect of NAD^+ on the rate of YADH inactivation by AAP (3 mM). (A) Time course of the AAP-induced YADH (1.88 μM monomeric concentration) inactivation in the absence or presence of 1.88 μM NAD^+ (NAD^+/YADH molar ratio of 1), added either at time zero (□) or after 7 min (▲) of exposure to AAP. The numbers in parentheses adjacent to the traces correspond to the inactivation rates expressed as %/min, calculated by regression analysis. One hundred percent activity corresponds to 1.08 ± 0.03 U/mL (N = 12). (B) YADH inactivation rates and induction times (T_0) as a function of either the initial NAD^+ concentration, in the range of 0.47 μM to 4.71 mM, or the NAD^+/YADH molar ratio. Values shown are means ± SEM (N = 3–10).

almost total radical trapping [24, 25]. Figure 5 shows the influence of NADH on the AAP-induced YADH inactivation. As can be seen, the effect of NADH was characterized by the appearance of a T_0 (Fig. 5A), which was found to increase in the concentration range of 4.71 to 471 μM NADH (Fig. 5B). At all the NADH/YADH molar ratios studied, the inactivation process took place, after the induction time, at rates comparable to those observed in the absence of NADH (Fig. 5B). In these conditions, NADH was found to disappear in the inactivation system, measured by the decrease in the absorbance at 340 nm (Fig. 6A). In this assay, the absorbance of 4.71 μM NADH alone or that of 3 mM AAP alone remained approximately constant throughout the reaction period (Fig. 6A). Furthermore, the addition of excess ethanol and YADH to the reaction medium upon NADH exhaustion resulted in a rapid recovery of the absorbance at 340 nm back to the value measured at time zero (Fig. 6a). This indicates that the alkylperoxyl radicals generated by the thermolysis of AAP led to NADH oxidation, without conversion into degraded products other than NAD^+. The procedure described above allows the calculation of the relative concentrations of NADH and NAD^+ present at different reaction times, and a significant inverse correlation between the values of the NADH/NAD^+ ratio in the system with the time of exposure to AAP is established (Fig. 6B). In this situation, the individual YADH inactivation rates (IR_t) showed a progressive enhancement during the reaction period (Fig. 6B). Although the inactivation rates (IR), in the absence of T_0 or after T_0, corresponding to the slopes of the remaining activity versus time plots did not vary as a function of the initial NADH concentration (Fig. 5B), IR_t

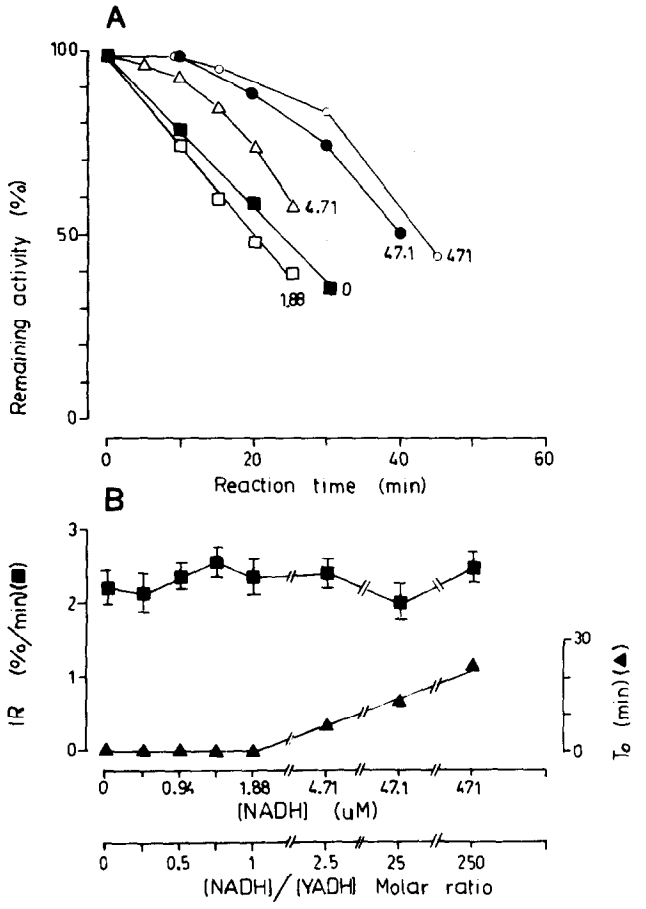

Fig. 5. Effect of NADH on the rate of YADH inactivation by AAP (3 mM). (A) Time course of the AAP-induced YADH (1.88 μM monomeric concentration) inactivation in the absence or presence of micromolar concentrations of NADH, as shown by the numbers adjacent to the traces. (B) YADH inactivation rates (IR) and induction times (T_0) as a function of either the initial NADH concentration, in the range of 0.47 to 471 μM, or the NADH/YADH molar ratio. Values shown are means ± SEM (N = 3). One hundred percent activity corresponds to 1.10 ± 0.035 U/mL (N = 24).

values in the presence of 1.88, 4.71, 47.1 or 471 μM NADH exhibited a significant inverse correlation as a function of the $NADH/NAD^+$ ratio prevailing at the different reaction times (Fig. 6C). Experiments with 4.71 and 47.1 μM NADH in the presence of 1.88 μM YADH exhibited similar results (Fig. 6C).

The influence of $NADP^+$ and NADPH on the AAP-induced inactivation of YADH is presented in Fig. 7. It can be observed that in the concentration range of 0.47 to 47.1 μM, $NADP^+$ neither modified the inactivation rate of YADH nor elicited T_0, exerting a 47% diminution ($P < 0.05$) in enzyme inactivation only at a concentration of 4.71 mM (Fig. 7A). In similar conditions, NADPH elicited a 24–45% decrease ($P < 0.05$) in the YADH inactivation rate in the concentration range of 1.88 to 471 μM, together with T_0 values of a magnitude similar to those elicited by NADH (Fig. 7B). As found for NADH (Fig. 6), NADPH oxidation occurred upon addition of AAP, as judged by the time-dependent decrease in the absorbance at 340 nm, which was reversible after the addition of excess glucose-6-phosphate and glucose-6-phosphate dehydrogenase (data not shown). However, the individual rates of YADH inactivation at each time interval (IR_t) were not significantly correlated with the respective values of the estimated $NADPH/NADP^+$ ratios (Fig. 7).

DISCUSSION

Characteristics of YADH inactivation by AAP. YADH, a homotetramer with a molecular weight of 150 kDa [13], is readily inactivated by alkylperoxyl radicals ($ROO^\cdot$) produced by the aerobic thermolysis of AAP at 37° (reaction 1), in the range of 1 to 10 mM, according to

$$AAP \xrightarrow{t°,O_2} N_2 + 2ROO^\cdot \quad (1)$$

$$YADH + ROO^\cdot \rightarrow ROOH + YADH^\cdot \text{ (inactive)} \quad (2)$$

The inactivation rates, estimated in a molar basis, were found to be independent of YADH concentration (Table 1A), suggesting that most of the AAP-derived radicals are being trapped by the

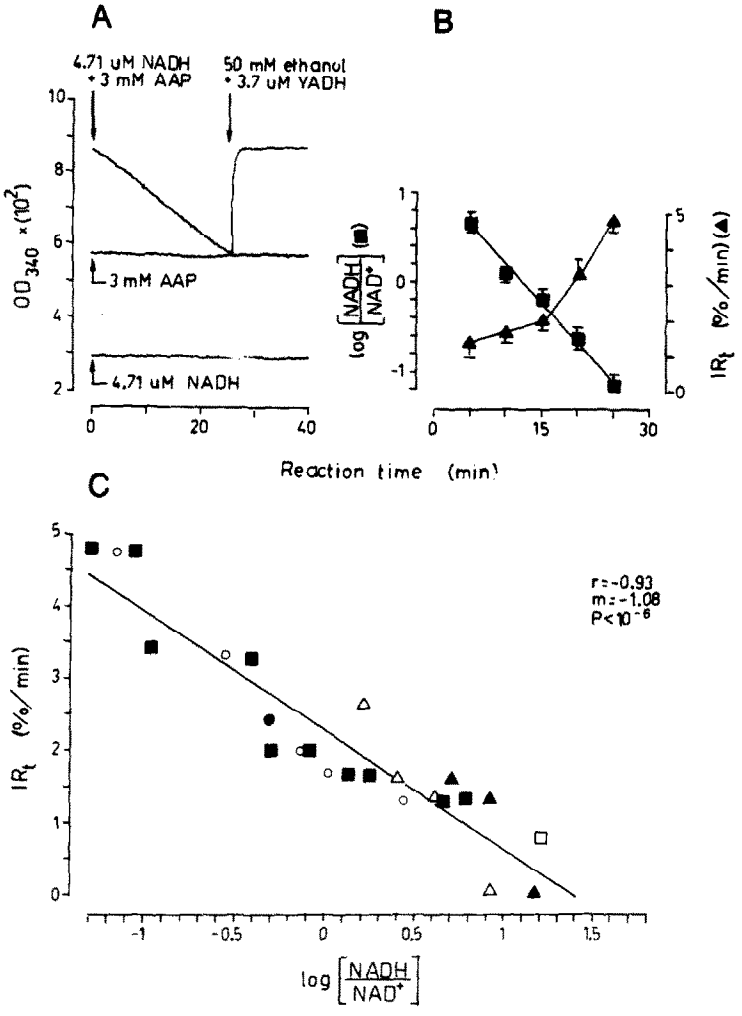

Fig. 6. Interaction of NADH with AAP. (A) Time course of the changes in absorbance at 340 nm of solutions of NADH, AAP, and NADH plus AAP at 37°, prepared in 0.2 M potassium phosphate buffer, pH 7.4. In the latter case, changes in absorbance were also measured after the addition of YADH plus ethanol. (B) Time course of the changes in either the NADH/NAD^+ ratio induced by 3 mM AAP, calculated from data presented in (A) (initial NADH concentration = 4.71 μM), or in the inactivation rate (IR_t) calculated at each time interval from data presented in Fig. 5A. Values shown are means ± SEM (N = 3). (C) Relationship between the IR_t and the NADH/NAD^+ ratio in the presence of 3 mM AAP in separate experiments using either 1.88 (●), 4.71 (■), 47.1 (▲) or 471 (□) μM NADH in the absence of YADH or 4.71 (○) and 47.1 (△) μM NADH in the presence of 1.88 μM YADH.

enzyme. In these conditions, an average of 20 radicals is needed to react with an enzyme tetramer to produce its total inactivation. The fact that the total free radical trapping capacity of YADH was observed at monomeric concentrations as low as 0.47 μM is compatible with a high reactivity of the enzyme, which appears to be similar to that of Trolox and greater than that of lysozyme.

Loss of YADH activity may be related to the presence of amino acids sensitive to reactive species [1, 5] which are structurally and functionally important. These could include two cysteinyl residues and one histidyl residue acting as zinc ligands, one arginyl residue participating in coenzyme binding, and possibly others located in the hydrophobic cores of the catalytic domain or at the active site pocket [13]. In this respect, competition studies using equimolar concentrations of proteins revealed that while YADH inactivation was not altered by lysozyme, albumin significantly diminished it (Fig. 3). Albumin contains a single -SH group per molecule and behaves as an efficient scavenger of free radicals [2, 4, 26, 27], H_2O_2 [27], and hypochlorous acid [28], the former two conditions being shown to result in thiol oxidation [27]. Thus, the high reactivity of YADH towards alkylperoxyl radicals observed could primarily be related to cysteine modification, amino acid known to be particularly susceptible to free radicals [1, 5, 27], and to chemical modifications [13, 29]. In line with this view, YADH inactivation

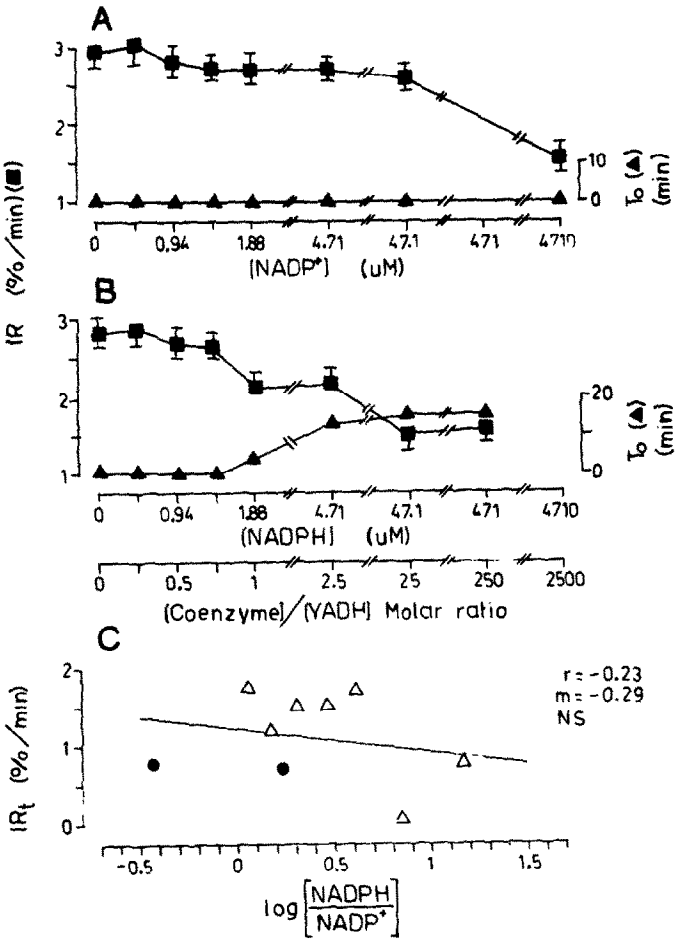

Fig. 7. Effect of $NADP^+$ and NADPH on the rate of YADH (1.88 μM monomeric concentration) inactivation by AAP (3 mM). YADH inactivation rates (IR, calculated by regression analysis of the remaining activity versus time plots) and the induction times (T_0) were measured in the absence and presence of micromolar concentrations of (A) $NADP^+$ or (B) NADPH, in the range of 0.47 μM to 4.71 mM. Values shown are means ± SEM (N = 3). (C) Relationship between the inactivation rate (IR_t) and the NADPH/$NADP^+$ ratio in the presence of 3 mM AAP, calculated at each experimental time in separate experiments using 4.71 (●) or 47.1 (△) μM NADPH in the absence of YADH. As in Fig. 6A, the time course of the decrease in the absorbance at 340 nm of the NADPH solutions induced by AAP at 37° was monitored. Upon addition of 5 mM glucose-6-phosphate and 10 U/mL of glucose-6-phosphate dehydrogenase [21], the initial absorbance was recovered (data not shown), thus allowing the estimation of the relative concentrations of NADPH and $NADP^+$ at different reaction times.

by oxygen and X-rays is accompanied by a decrement of -SH groups [29]; however, further studies are needed to verify this suggestion in the present experimental model.

Influence of nicotinamide-adenine dinucleotides on AAP-induced YADH inactivation. The addition of NAD^+ to the inactivation system resulted in a biphasic effect on YADH inactivation, depending on the coenzyme concentration. At NAD^+ concentrations leading to NAD^+/YADH molar ratios of 0.25 to 1, the enzyme inactivation was enhanced significantly, representing a fast response probably due to the relatively high rate constant describing the combination of NAD^+ with YADH ($1.1 \times 10^7 M^{-1} \cdot sec^{-1}$) [14]. The increased inactivation rate afforded by NAD^+ at low NAD^+/YADH ratios without a significant consumption of the coenzyme could be due to an interaction between the alkylperoxyl radicals and NAD^+ bound to the enzyme, to produce an enzyme bound $NAD^·$ [11] that would reactively damage essential amino acids. Alternatively, NAD^+ binding to YADH elicits changes in protein conformation [13] that may facilitate the access of $ROO^·$ to the active site of the enzyme, whose direct interaction with the target amino acids would increase YADH inactivation. At NAD^+ concentrations added in excess to that of YADH, enzyme inactivation decreased, with partial and almost total protection being observed at NAD^+/YADH molar ratios of 250 and 2500, respectively. In this situation, the molecules of NAD^+ remaining outside of the active site of the enzyme would react

with ROO·, the generated NAD· being able to either react with O_2 to produce O_2^{-}· ($k = 2 \times 10^9\,M^{-1}\cdot sec^{-1}$) [15] or to undergo dimerisation ($k = 5.6 \times 10^7\,M^{-1}\cdot sec^{-1}$) [30], thus diminishing YADH inactivation.

NADH is known to protect lysozyme from free radical-induced inactivation, possibly by mechanisms including free radical trapping and/or repair of the initial damage produced in the enzyme molecule, due to its capacity to donate a hydrogen atom or an electron [24]. Contrary to lysozyme, YADH effectively binds NADH with a rate constant ($k = 3.8 \times 10^7\,M^{-1}\cdot sec^{-1}$) which is 3.5-fold greater than that of NAD^+ [14]. However, protection of YADH against radical-induced inactivation was not observed until NADH/YADH molar ratios greater than 1 were achieved. At an NADH/YADH molar ratio of 2.5, protection was characterized by the appearance of a poorly defined T_0, with low inactivation rates being observed at early times [1.32%/min at 5 min after AAP addition (Fig. 6B) versus 2.23%/min found in the absence of NADH (Fig. 5B)], which progressively increased at later reaction times [4.80%/min at 25 min after AAP addition (Fig. 6B)]. Concomitantly, NADH was completely oxidized into NAD^+ by the alkylperoxyl radicals generated, leading to a significant time-dependent diminution in the NADH/NAD^+ ratio. Thus, the initial protective effect of NADH seems to be counteracted by the parallel and increasing production of NAD^+ in the system, which, at low concentrations, effectively enhanced YADH inactivation by AAP. Results similar to those found for an NADH/YADH molar ratio of 2.5 were observed at ratios of 25 and 250, in conditions in which high levels of NADH were still present to afford protection (33.7 and 448 μM after 30 min of exposure, for the initial concentrations of 47.1 and 471 μM NADH, respectively). However, since the number of NADH molecules oxidized per AAP-derived radical (n_2) increased with the NADH concentration (Table 1B) at a constant radical input, a chain oxidation of NADH is likely to have occurred, leading to significantly higher levels of NAD^+ in the system. This could be achieved through the production of NAD· and its further interaction with O_2 to generate O_2^{-}·and NAD^+, as this form of the coenzyme is quantitatively recovered upon addition of excess YADH and ethanol. Thus, the time-dependent enhancement in YADH inactivation by AAP at high NADH concentrations may be due to the production of a sufficient number of NAD^+ molecules, which could effectively compete with NADH for the binding site in YADH to facilitate the process.

Contrary to the marked effects of NAD^+ and NADH on AAP-induced YADH inactivation, $NADP^+$ did not influence the process (except at 4.71 mM), while NADPH exerted a modest protection at NADPH/YADH molar ratios greater than 1. The observed differences seem to be related to the high specificity of YADH towards the NADH-NAD^+ couple [29], rather than to differences in the efficiency of the coenzymes–free radical interactions. NADH binding to YADH would lead then to the protection of critical amino acid residues against radical inactivation. This suggestion is supported by the similar second-order rate constants reported for the reactions of free NADH and NADPH with hydroperoxyl radicals [16], and by the lack of correlation between YADH inactivation and the NADPH/$NADP^+$ ratio. Furthermore, although NAD^+ and $NADP^+$ are likely to trap peroxyl radicals at similar rates, protection elicited by high concentrations of NAD^+ but not by $NADP^+$ could indicate that enzyme protection is favored by NAD^+ binding to YADH. This suggestion is further supported by the finding that NAD^+ appears to be more efficient in protecting YADH than lysozyme [24], in spite of the higher reactivity of the former enzyme towards peroxyl radicals.

In conclusion, data presented in this work indicate that YADH exhibits a high reactivity towards alkylperoxyl radicals, produced by the aerobic thermolysis of AAP, whose inactivation rate is strongly dependent on the redox state of the NADH-NAD^+ couple. This is in agreement with the significant inverse correlation established between the inactivation rates at different time intervals and the respective NADH/NAD^+ ratios, with an extrapolated ratio of about 25 being needed to completely prevent YADH inactivation (Fig. 6C).

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