Sensitivity of the Essential Zinc−Thiolate Moiety of Yeast Alcohol Dehydrogenase to Hypochlorite and Peroxynitrite†

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ABSTRACT: Disruption of the zinc−thiolate center at the active site of yeast alcohol dehydrogenase results in inactivation and zinc release. Measurements of activity, zinc release, and thiol/thiolate oxidation were used to assess the effects of biologically relevant oxidants on alcohol dehydrogenase. Alcohol dehydrogenase was inactivated by 1 mM hydrogen peroxide at a rate of 1.3 M^{-1} s^{-1}. Peroxynitrite, the near diffusion-limited reaction product of nitric oxide and superoxide, inactivated alcohol dehydrogenase with an IC_{50} = 0.95 μM when catalytic concentrations of alcohol dehydrogenase subunit (0.074 μM) were present. Slow, continuous production of peroxynitrite from decomposition of SIN-1 inactivated alcohol dehydrogenase as effectively as bolus addition. The rate constants for reaction of peroxynitrite with alcohol dehydrogenase at 23 °C as determined by two different competition assays were 2.6×10^5 M^{-1} s^{-1} and 5.2×10^5 M^{-1} s^{-1}. The reaction with alcohol dehydrogenase represents one of the fastest reactions yet determined for peroxynitrite. Hypochlorite inactivated alcohol dehydrogenase at a rate of 4×10^3 M^{-1} s^{-1}. The rate constant for inactivation by taurine choramine, the reaction product of taurine and hypochlorite, was only slightly slower at 2.7×10^3 M^{-1} s^{-1}. Zinc release and thiol/thiolate oxidation were correlated with inactivation by either peroxynitrite or hypochlorite. At the concentrations of peroxynitrite or hypochlorite producing total inactivation, 0.85 zinc atom was released per subunit and 3 thiol/thiolates per subunit were oxidized. The structural similarity between the zinc−thiolate moiety of yeast alcohol dehydrogenase ($Zn_1Cys_2His_1$) and that found in zinc finger proteins ($Zn_1Cys_2His_2$) suggest that the widely distributed ubiquitous zinc finger moiety may be a major target for oxidant-induced injury.

Yeast alcohol dehydrogenase contains a zinc atom at its catalytic site, which is essential for conversion of ethanol and other primary alcohols to the corresponding aldehydes. The zinc atom exists as the tridentate ligand of two cysteine residues and one histidine ($Zn_1Cys_2His_1$). The fourth coordinate position serves as the binding site for the hydroxyl group of ethanol and is occupied by water in the absence of substrate (Vallee & Auld, 1990a). Yeast alcohol dehydrogenase is inactivated by a number of biologically relevant oxidants (Abelidis et al., 1987; Fliss & Menard, 1991). Zinc release from yeast alcohol dehydrogenase as well as from other thiolate-liganded zinc proteins (Fliss & Menard, 1991; Fliss & Menard, 1992) suggests that the zinc−thiolate center is particularly susceptible to oxidant attack.

Peroxynitrite ($ONOO^-$)[1] is a potent oxidant formed by the near diffusion-limited reaction of superoxide with nitric oxide (Huie & Padmaja, 1993). Superoxide reacts with nitric oxide 3 times faster than with superoxide dismutase, thereby allowing peroxynitrite formation to effectively compete with superoxide dismutation. Peroxynitrite has a pK_a of 6.8 (Keith & Powell, 1969; Radi et al., 1991a; Koppenol et al., 1992) and is stable in alkaline solution. It decomposes when protonated with a half-life of approximately 1 s at pH 7.4 and 37 °C (Koppenol et al., 1992). Peroxynitrous acid (ONOOH) has the reactivity of hydroxyl radical and nitrogen dioxide, although it appears to react as a vibrationally activated complex rather than physically separating into two distinct radical species (Koppenol et al., 1992; Crow et al., 1993). However, direct oxidative mechanisms, such as attack of peroxynitrite anion on sulfhydryls (Radi et al., 1991a), may be of greater toxicological significance. Peroxynitrite can nitrate and hydroxylate aromatic rings (Halfpenny & Robinson, 1952; Beckman et al., 1992), as well as oxidize lipids (Radi et al., 1991b), proteins (Moreno & Pryor, 1992), and DNA (King et al., 1992).

Hypochlorous acid (pK_a = 7.54 at 25 °C) (Morris, 1966) is produced by myeloperoxidase present in activated neutrophils. It is strongly oxidizing and readily reacts with primary amines to give the corresponding chloramine. Much of the hypochlorous acid produced *in vivo* reacts with taurine (2-aminoethanesulfonic acid) to produce *N*-chloro-2-aminoethanesulfonic acid or taurine chloramine (Thomas et al., 1983; Kalyanaraman & Sohnle, 1985).

We assessed the oxidant sensitivity of the zinc−thiolate center of yeast alcohol dehydrogenase as a model for the widely distributed zinc finger structure present in a number of proteins, most notably the DNA-binding transcription factors. The reaction with yeast alcohol dehydrogenase is among the fastest observed for peroxynitrite. Oxidation of

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[1] Abbreviations: DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); PAR, 4-(2-pyridylazo)resorcinol; SIN-1, 3-morpholinosydnonimine; $ONOO^-$, peroxynitrite; DHR, dihydrorhodamine 123.

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zinc−thiolate centers may represent a heretofore uncharacterized toxic mechanism for peroxynitrite.

MATERIALS AND METHODS

Materials. Yeast alcohol dehydrogenase, hydrogen peroxide, sodium hypochlorite, taurine, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), 4-(2-pyridylazo)resorcinol (PAR), zinc sulfate, and EDTA were purchased from Sigma. Dihydrorhodamine 123 (DHR) was purchased from Molecular Probes, Inc., Eugene OR. SIN-1 was the generous gift of Cassella AG, Frankfurt, FRG.

Peroxynitrite Synthesis. Peroxynitrite was prepared using a quenched-flow reaction apparatus as described by Reed et al. (1974). An aqueous solution of 0.6 M sodium nitrite was rapidly mixed with an equal volume of 0.7 M hydrogen peroxide containing 0.6 M HCl and immediately quenched with the same volume of 1.5 M NaOH. All reaction solutions were kept on ice. The concentration of peroxynitrite was determined spectrally in 0.3 M NaOH (ϵ_{302nm} = 1670 M^{-1} cm^{-1}) (Hughes & Nicklin, 1968). Solutions of freshly synthesized peroxynitrite ranged from 150 to 190 mM (75−95% yield). Stock solutions of peroxynitrite were stable for several weeks at −20 °C.

Treatment of Yeast Alcohol Dehydrogenase with Peroxynitrite. Alkaline conditions (0.1 M NaOH) are required for peroxynitrite stability. To minimize pH changes in reaction solutions, dilutions of peroxynitrite were made such that small volumes could be added to give the desired final concentration. The final pH of all reaction solutions was checked to ensure adequate buffering. The short half-life of peroxynitrite at pH 7.4 (1−2 s) necessitates rapid mixing of reaction solutions. Whenever possible, reaction solutions were prepared in 1.5 mL microfuge tubes which were rapidly mixed via a vortex mixer during bolus peroxynitrite additions.

Peroxynitrite solutions contain some residual nitrite and hydrogen peroxide, depending on the synthetic reaction yield. In addition, nitrate is formed from the decomposition of peroxynitrite. Possible effects of nitrite and nitrate were controlled by first adding peroxynitrite at the highest concentration used to solutions at pH 7.4, allowing it to decompose for 30 s, and then adding yeast alcohol dehydrogenase. Nitrite and nitrate had no effect. At the concentrations of peroxynitrite used, the maximum exposure of alcohol dehydrogenase to contaminating hydrogen peroxide was less than 10 μM and the duration of exposure less than 5 min. Under these conditions, hydrogen peroxide had no effect on alcohol dehydrogenase.

Peroxynitrite Generation by SIN-1. SIN-1 undergoes a base-catalyzed ring opening followed by a one-electron oxidation and subsequent release of nitric oxide. In the absence of other oxidants, molecular oxygen oxidizes SIN-1, thereby producing superoxide (Feelisch et al., 1989; Darley-Uhmar et al., 1992; Hogg et al., 1992). Peroxynitrite formation from SIN-1 results from the simultaneous production of superoxide and nitric oxide which, in turn, react at a near diffusion-limited rate.

We have previously shown that authentic peroxynitrite readily oxidizes dihydrorhodamine (DHR) whereas superoxide, hydrogen peroxide, or nitric oxide alone does not (Kooy et al., 1994). A modification of the original DHR assay was used here to make it more generally applicable for measuring peroxynitrite. DHR has no absorbance at 500 nm whereas rhodamine has a high molar extinction coefficient (ϵ_{500nm} = 74 500 M^{-1} cm^{-1}). Peroxynitrite formation from SIN-1 was assayed by continuous monitoring of rhodamine formation at 500 nm at 23 °C. The reaction solution consisted of 50 μM DHR, 0.1 mM DTPA, and 1 mM SIN-1 in 1.0 mL of 50 mM potassium phosphate, pH 7.4. Under these conditions, oxidation of DHR to rhodamine by SIN-1 serves as a specific measure of peroxynitrite formation (Crow and Beckman, unpublished observations). The rate of peroxynitrite formation from SIN-1 became linear following an initial lag period and reached a maximum of 0.55 μM peroxynitrite/min after 35 min. The efficiency of DHR oxidation was 45% of the authentic peroxynitrite added. Rates of peroxynitrite formation reported in the text have been corrected for this efficiency by multiplying the rate of rhodamine formation by 2.2.

Standardization of Hypochlorite, Preparation of Taurine Chloramine, and Inactivation of Yeast Alcohol Dehydrogenase. Solutions of sodium hypochlorite were standardized by absorbance ($\epsilon_{290\ nm}$ = 350 M^{-1} cm^{-1}) (Morris, 1966). Taurine chloramine was prepared by incubating 10 mM sodium hypochlorite with 11 mM taurine at 23 °C in 50 mM potassium phosphate, pH 7.8, for 10 min. Taurine chloramine (1 μM) was added to alcohol dehydrogenase (10 μg/mL) and allowed to react. Aliquots were removed at 5 min intervals, diluted 40-fold, and assayed for alcohol dehydrogenase activity as described below. The rate constant for the reaction of alcohol dehydrogenase with taurine chloramine was determined by activity loss over time as described for hypochlorite (Figure 7 legend).

Yeast Alcohol Dehydrogenase Activity Assay. Reaction solutions contained ethanol (0.3 M), NAD^+ (0.4 mM), and typically 0.25 μg (22.5 milliunits) of yeast alcohol dehydrogenase in 1.0 mL of 80 mM potassium phosphate, pH 7.8. Assays were carried out at either 25 or 37 °C. NADH formation was monitored continuously at 340 nm (ϵ_{340nm} = 6220 M^{-1} cm^{-1}) and rates were calculated from the initial 30 s interval.

Determination of Zinc Content of Yeast Alcohol Dehydrogenase. PAR readily complexes with zinc to form a complex which absorbs at 500 nm (Fliss & Menard, 1992). The extinction value for this complex at 500 nm was determined experimentally to be 96 200 M^{-1} cm^{-1} by titrating a 100 μM PAR solution at pH 7.8 with a known concentration of zinc sulfate. The extinction coefficient was 1.48 times greater than the that reported at pH 7.0 (Fliss & Menard, 1992). PAR (100 μM) was added to solutions containing control or oxidant-treated yeast alcohol dehydrogenase in 1.0 mL of 80 mM potassium phosphate, pH 7.8, and formation of the zinc−PAR complex was monitored at 500 nm for approximately 150−200 s until no further increase was seen. Addition of 0.5 mM EDTA, which chelates zinc more tightly than PAR, resulted in a rapid loss of absorbance, and the difference was taken as representing zinc−PAR complex. Reaction solutions containing all components except yeast alcohol dehydrogenase were subjected to zinc analysis, and appropriate subtractions were made to control for zinc contamination. Typically, zinc contamination amounted to less than 1% of that from yeast alcohol dehydrogenase.

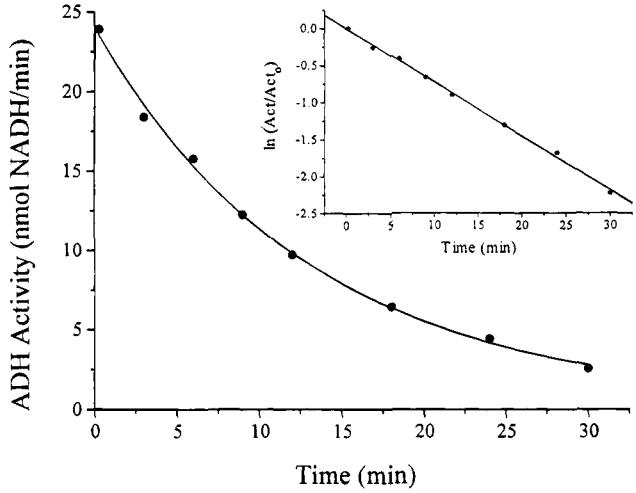


FIGURE 1: Effects of hydrogen peroxide on yeast alcohol dehydrogenase activity. Yeast alcohol dehydrogenase (20 μg) was incubated at 37 °C with H_2O_2 (1 mM) in 100 mM potassium phosphate, pH 7.4, containing 100 μM EDTA in a total volume of 1.0 mL. Aliquots were removed from incubation solutions at times shown, diluted 100-fold, and assayed for yeast alcohol dehydrogenase activity. Inset: plot for determination of pseudo-first-order rate constant.

Determination of Sulfhydryl Content of Yeast Alcohol Dehydrogenase. DTNB (Ellman's reagent) was prepared by dissolving solid to 40 mM in methanol and adding volumes of the stock solution to 1.0 or 2.0 mL reaction solutions to give a final concentration of 0.6 mM. Reaction solutions contained 0.2−0.4 mg of yeast alcohol dehydrogenase/mL of 80 mM potassium phosphate, pH 7.8. Unless otherwise specified, all solutions contained 5.7 M guanidine HCl as a denaturant. The extinction coefficient (ϵ_{412nm} = 9550 M^{-1} cm^{-1}) for 5-thio-2-nitrobenzoic acid in guanidine HCl at pH 7.8 was experimentally determined by titrating DTNB with a known amount of freshly prepared glutathione. The extinction coefficient was somewhat lower than the value (ϵ_{412nm} = 13 600 M^{-1} cm^{-1}) commonly used for Ellman's reagent (Riddles et al., 1983). Exhaustive reaction of yeast alcohol dehydrogenase with DTNB resulted in maximal release of enzyme-bound zinc, indicating that thiolate ligands binding zinc (in addition to non-ionized thiols) were reactive toward DTNB. Thus, the term "thiol/thiolate" is used to refer to all reduced sulfur present in the protein.

RESULTS

Effects of H_2O_2 on Yeast Alcohol Dehydrogenase Activity. Treatment of yeast alcohol dehydrogenase with 1 mM hydrogen peroxide resulted in time-dependent inactivation (Figure 1). The $t_{1/2}$ for hydrogen peroxide inactivation of yeast alcohol dehydrogenase was 13 min, and the second-order rate constant, as determined by activity assays over time under pseudo-first-order conditions, was 1.3 M^{-1} s^{-1} at 37 °C (Figure 1, inset).

Inactivation of Yeast Alcohol Dehydrogenase by Peroxynitrite. Bolus additions of peroxynitrite rapidly inactivated yeast alcohol dehydrogenase in a concentration-dependent manner (Figure 2). The peroxynitrite concentration required for 50% inactivation under these conditions (IC_{50}) showed a marked dependence on the initial subunit concentraton of yeast alcohol dehydrogenase. The IC_{50} for peroxynitrite was

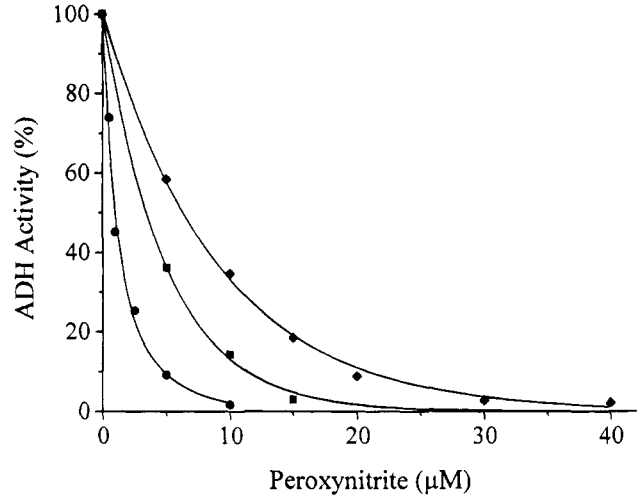
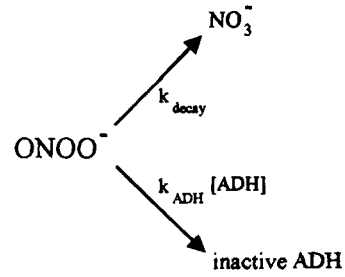


FIGURE 2: Inactivation of yeast alcohol dehydrogenase by peroxynitrite. Yeast alcohol dehydrogenase was added to 2.0 mL of 100 mM potassium phosphate, pH 7.4, containing 0.1 mM DTPA at 23 °C to give final subunit concentrations of 0.074 μM (●), 0.74 μM (■), or 5.7 μM (♦). Stock peroxynitrite was diluted in 0.3 M NaOH to give working solutions of 10 and 1 mM. Bolus additions of peroxynitrite were made to rapidly stirred yeast alcohol dehydrogenase solutions to give the final concentrations indicated using the working solution, which minimized volume change. Reaction solutions were then diluted to give a yeast alcohol dehydrogenase concentration of 0.2 μg/mL and assayed as described in Materials and Methods.

Scheme 1



6.5 μM when the concentration of yeast alcohol dehydrogenase subunit was 5.7 μM (top curve, Figure 2). An 8-fold decrease in the yeast alcohol dehydrogenase concentration decreased the peroxynitrite IC_{50} to 3.4 μM (middle curve, Figure 2). When yeast alcohol dehydrogenase subunit was decreased to 0.074 μM, well below the concentration of added peroxynitrite, the IC_{50} value decreased to 0.95 μM (bottom curve, Figure 2).

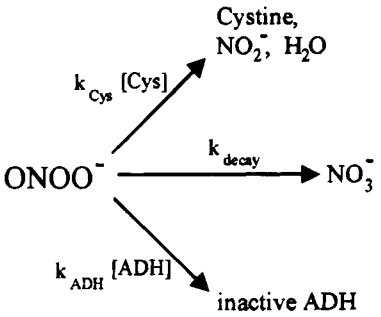
The rate of peroxynitrite inactivation of alcohol dehydrogenase may be estimated by assuming simple competition kinetics between proton-catalyzed decomposition of peroxynitrite and reaction at the active site of alcohol dehydrogenase (Scheme 1). At the lowest concentration of yeast alcohol dehydrogenase tested (0.074 μM) in Figure 2, 0.95 μM peroxynitrite resulted in 50% inactivation. Thus, a direct (1:1 molar ratio) reaction with alcohol dehydrogenase would have consumed only 4% of the total amount of peroxynitrite added. Under these conditions, the reaction of peroxynitrite with alcohol dehydrogenase is competing with spontaneous decomposition of peroxynitrite which occurs at a known rate (Beckman et al., 1990). The integrated rate equation derived in Radi et al. (1991a) was utilized to determine the second-order rate constant for yeast alcohol dehydrogenase inactivation (k_{ADH}):

$$k_{ADH} = k_{decay}(\ln 0.5)/[ONOO^-] \quad (1)$$

where k_{decay} is the rate of peroxynitrite decomposition measured by stopped-flow kinetics and $[ONOO^-]$ is the concentration of peroxynitrite producing 50% inhibition of yeast alcohol dehydrogenase. We have previously measured the rate of peroxynitrite decomposition in phosphate buffer, pH 7.4 at 23 °C, to be 0.35 s^{-1}, which yields $k_{ADH} = 2.6 \times 10^5$ M^{-1} s^{-1}. At the higher concentrations of yeast alcohol dehydrogenase used in Figure 2, the amount of peroxynitrite consumed by direct reaction with alcohol became faster than the rate of spontaneous decomposition and thereby invalidated the above equation.

A second competition assay was utilized to determine the reaction rate between peroxynitrite and yeast alcohol dehydrogenase based on the known reaction rate between peroxynitrite and cysteine (Scheme 2). The concentration of

Scheme 2



peroxynitrite required to totally inactivate 11.4 μM yeast alcohol dehydrogenase subunit was first determined (see Figure 3 legend for details). Cysteine was added at increasing concentrations to subsequent yeast alcohol dehydrogenase solutions prior to peroxynitrite addition, and the dose−response for prevention of inactivation was determined (Figure 3). The protection afforded by cysteine can be used to estimate k_{ADH} as follows. The fraction of yeast alcohol dehydrogenase activity protected by adding cysteine (F) can be described as the fraction of peroxynitrite reacting with cysteine divided by the sum of all rates of peroxynitrite decomposition:

$$F = \frac{k_{SH}[SH]}{k_{SH}[SH] + k_{ADH}[ADH] + k_{decay}} \quad (2)$$

where k_{ADH} and k_{SH} are the rates of peroxynitrite reacting with alcohol dehydrogenase and cysteine, respectively, and k_{decay} is the rate of spontaneous peroxynitrite decomposition. The fraction of inactivated yeast alcohol dehydrogenase is given by

$$1 - F = \frac{k_{ADH}[ADH]}{k_{SH}[SH] + k_{ADH}[ADH] + k_{decay}} \quad (3)$$

The ratio of these two terms is shown in the following equation:

$$\frac{F}{1-F} = \frac{k_{SH}[SH]}{k_{ADH}[ADH]} \quad (4)$$

Equation 4 can be rearranged to

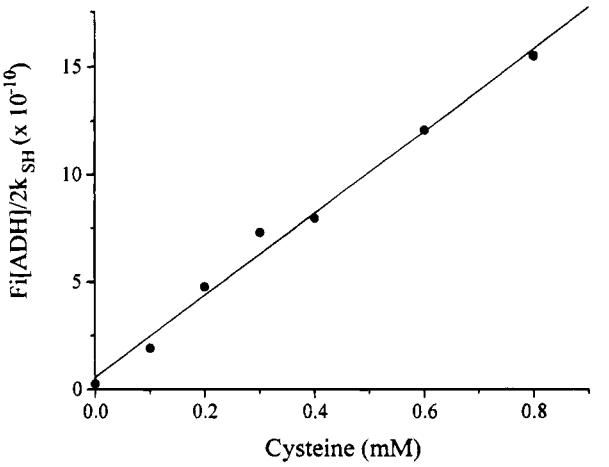


FIGURE 3: Inhibition of peroxynitrite-induced yeast alcohol dehydrogenase activity loss by cysteine. Peroxynitrite (64 μM) was added to a vigorously stirred solution of yeast alcohol dehydrogenase (80 μg) and the indicated concentration of cysteine in 0.2 mL of 100 mM potassium phosphate, pH 7.4 at 23 °C. After 60 s small aliquots were removed, diluted 1600-fold, and assayed for yeast alcohol dehydrogenase activity as described in Materials and Methods. Data were fitted by linear regression using eq 7 as described in the text, and the rate constant was determined from the slope ($1/k_{ADH}$).

$$\frac{F[ADH]}{(1-F)k_{SH}} = \frac{1}{k_{ADH}}[SH] \quad (5)$$

However, the concentration of yeast alcohol dehydrogenase changes as cysteine concentration is increased. We can approximate the loss of yeast alcohol dehydrogenase as the average between the starting and ending concentration of yeast alcohol dehydrogenase as shown in the following equation:

$$[ADH] = [ADH]_i\frac{(1-F)}{2} \quad (6)$$

$[ADH]_i$ in eq 6 is the inital amount of yeast alcohol dehydrogenase added, and F was defined previously. Substituting into eq 6 yields the following equation:

$$\frac{F[ADH]_i}{2k_{SH}} = \frac{1}{k_{ADH}}[SH] \quad (7)$$

Using the initial concentration of yeast alcohol dehydrogenase (11.4 μM) and 2.2 × 10^3 M^{-1} s^{-1} for k_{SH} at 23 °C (Radi et al., 1991a; Koppenol et al., 1992), the left-hand expresssion in eq 7 was plotted against the cysteine concentration and fitted by linear regression (Figure 3). The reciprocal of the slope yields a rate constant of 5.2 × 10^5 M^{-1} s^{-1} at 23 °C, which is close to the rate estimated by the earlier direct reaction as calculated using eq 1. Addition of cysteine or dithiothreitol 1 min after peroxynitrite did not restore enzyme activity. Thus, the assumption was made that cysteine was only acting as a competitive scavenger of peroxynitrite.

Inactivation by SIN-1. Yeast alcohol dehydrogenase was exposed to a continuous, low-level concentration of peroxynitrite using SIN-1, a compound which decomposes to give superoxide and nitric oxide. The time course for yeast alcohol dehydrogenase activity loss paralleled that for peroxynitrite production from SIN-1 as determined by its ability to oxidize dihydrorhodamine in a separate reaction (Figure 4). The half-time for inactivation is 25 min (Figure

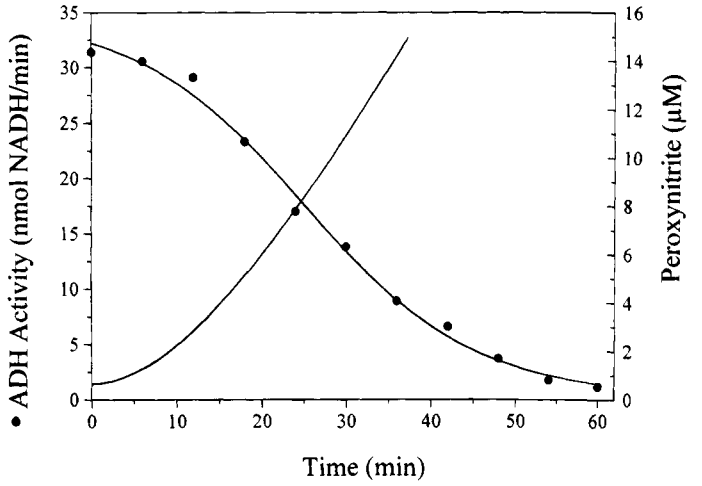


FIGURE 4: Yeast alcohol dehydrogenase inactivation time course with SIN-1. (Left axis) Yeast alcohol dehydrogenase (1.4 μM) was incubated together with 0.1 mM DTPA in 1.0 mL of 0.1 M potassium phosphate, pH 7.4 at 23 °C; SIN-1 (1 mM) was added at time zero. Aliquots were removed at intervals shown, diluted 200-fold, and assayed for yeast alcohol dehydrogenase activity as described in Materials and Methods. In a separate reaction SIN-1 was incubated under identical conditions with dihydrorhodamine (DHR) rather than yeast alcohol dehydrogenase. Absorbance was monitored continuously at 500 nm for the production of rhodamine (ϵ_{500nm} = 74 500 $M^{-1}cm^{-1}$) and converted to peroxynitrite concentration (right y-axis) based on the oxidative efficiency of authentic peroxynitrite as described in Materials and Methods.

4). The maximal rate of peroxynitrite formation during the 35 min interval where SIN-1 decomposition is linear is 0.55 μM/min. However, during the initial 25 min where 50% of the 1.4 μM yeast alcohol dehydrogenase subunit was inactivated, the total production of peroxynitrite amounted to only 7.8 μM. The stable decomposition products of SIN-1, namely, nitrate, nitrite, or SIN-1C, had no effects on yeast alcohol dehydrogenase activity. Addition of 100 μM nitric oxide to a solution of yeast alcohol dehydrogenase at ambient oxygen tension for 10 min had no effect on activity. However, addition of 1 mM nitric oxide resulted in rapid loss of 63% of initial yeast alcohol dehydrogenase activity, due to the formation of nitrogen dioxide and higher oxides (e.g., N_2O_3 or N_2O_4).

Total Thiol/Thiolate and Zinc Content versus Yeast Alcohol Dehydrogenase Activity. Loss of activity paralleled thiol/thiolate oxidation as the concentration of peroxynitrite was increased (Figure 5). The lower sensitivity of the thiol/thiolate assay required a higher concentration of yeast alcohol dehydrogenase (46 μM), which resulted in concomitantly higher concentrations of peroxynitrite to inactivate. The concentration of peroxynitrite producing essentially total inactivation under these conditions was approximately 260 μM and resulted in oxidation of 3 thiol/thiolate groups per yeast alcohol dehydrogenase subunit. A total of 4 thiol/thiolates were oxidized at 540 μM peroxynitrite (Figure 5).

Zinc release, monitored by the PAR assay, coincided with activity loss induced by increasing amounts of peroxynitrite (Figure 6). The equivalent of 0.85 zinc atom per M_r = 35 000 subunit was released at the peroxynitrite concentration (40 μM), which caused essentially complete inactivation of 5.7 μM enzyme subunit (Figure 6). Addition of peroxynitrite up to 500 μM produced no further zinc release. Addition of PAR to yeast alcohol dehydrogenase in the absence of oxidants resulted in immediate formation of the equivalent of 0.75 zinc atoms per subunit or the same amount of zinc–PAR complex seen prior to peroxynitrite addition (Figure 6). Since no activity loss was associated with this zinc, it may represent the fraction associated with the structural zinc. The addition of PAR in the absence of oxidant did not result

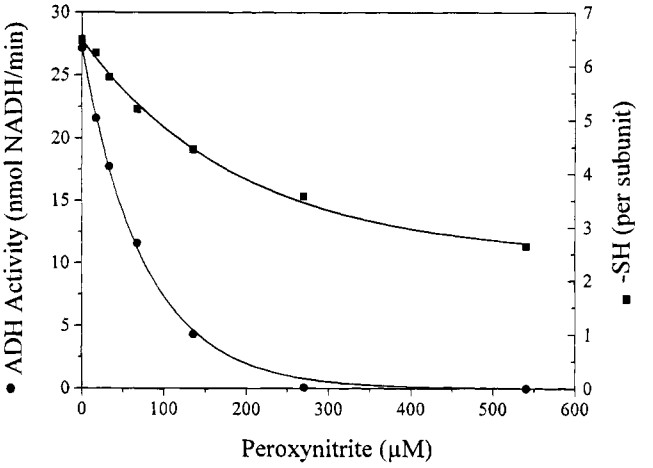


FIGURE 5: Peroxynitrite-induced -SH oxidation and activity loss. Left axis: Peroxynitrite was added to a vigorously stirred solution of yeast alcohol dehydrogenase (0.8 mg) in 0.5 mL of 100 mM potassium phosphate, pH 7.4 at 23 °C. Aliquots were removed 60 s later, diluted 6400-fold, and assayed for yeast alcohol dehydrogenase activity as described in Materials and Methods. Right axis: Simultaneous aliquots were diluted 4-fold into 80 mM potassium phosphate, pH 7.8 (2.0 mL final volume), containing 5.7 M guanidine HCl. DTNB (0.6 mM) was added, and absorbance was monitored at 412 nm.

in loss of yeast alcohol dehydrogenase activity, nor did formation of zinc–PAR complex increase over time.

Exhaustive reaction of untreated yeast alcohol dehydrogenase with DTNB under nondenaturing conditions resulted in derivatization of 5.5 thiol/thiolate groups and induced release of 0.9 zinc atom per subunit (data not shown). Under denaturing conditions (5.7 M guanidine HCl) a total of 6.5 thiol/thiolate groups were determined, but no further release of zinc was seen. A maximum of 1.6 zinc atoms per subunit was extractable either by oxidation or by thiol/thiolate derivatization. The equivalent of 0.75 mol of zinc–PAR complex/mol of enzyme subunit was formed immediately upon addition of PAR and prior to oxidant treatment. The fact that total thiol/thiolate and zinc were less than the full complement (of 8 and 2 per subunit, respectively) known to be present in yeast alcohol dehydrogenase (Eklund et al.,

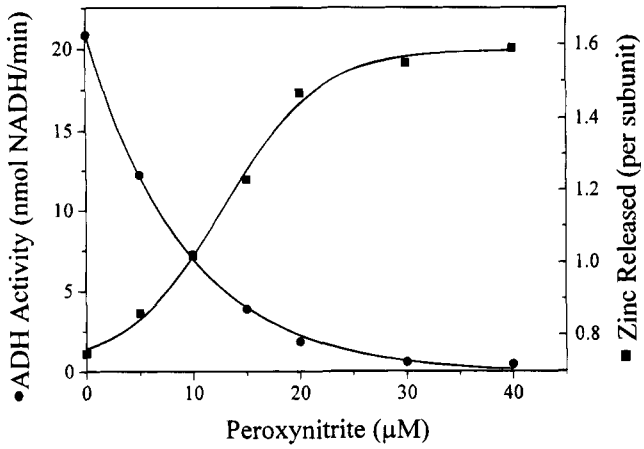


FIGURE 6: Peroxynitrite-induced zinc release and activity loss. Peroxynitrite was added to a vigorously stirred solution of yeast alcohol dehydrogenase (0.2 mg) in 1.0 mL of 80 mM potassium phosphate, pH 7.4 at 23 °C. After 60 s small aliquots were removed, diluted 800-fold, and assayed for yeast alcohol dehydrogenase activity (left axis) as described in Materials and Methods. PAR (100 μM) was added to the remaining reaction solution, and formation of zinc−PAR complex was monitored at 500 nm (right axis) until no further increase was seen (150−200 s). The change in absorbance following addition of EDTA (0.5 mM) was taken as that due to zinc−PAR complex.

Table 1: Summary of Oxidant Effects on Yeast Alcohol Dehydrogenase[a]

oxidant	total -SH's oxidized[b]	oxidant-induced Zn release[b]	rate constant[c] ($M^{-1}s^{-1}$)
H_2O_2	nd[d]	nd	1.3
hypochlorite	3.0	0.84	4×10^3
taurine chloramine	nd	nd	2.7×10^3
peroxynitrite	3.0	0.85	3.9×10^5

[a] See Materials and Methods or specific figure legends for reaction conditions. [b] Per M_r = 35 000 yeast alcohol dehydrogenase subunit at the oxidant concentration producing total inactivation. [c] Based on yeast alcohol dehydrogenase M_r = 35 000 subunit concentration. [d] nd = not determined.

1990; Vallee & Auld, 1990) likely reflects the presence of a small fraction of partially oxidized apoenzyme in the stock source.

Hypochlorite and Taurine Chloramine Inactivation of Yeast Alcohol Dehydrogenase and Determination of Rate Constants. The IC_{50} for inactivation of 5.7 μM yeast alcohol dehydrogenase subunit by hypochlorite was determined to be 14.7 μM (not shown). At concentrations producing total inactivation, 3 thiol/thiolates were oxidized and 0.84 zinc atom per enzyme subunit was released (Table 1). These results are quite similar to those seen with peroxynitrite. However, the rate of inactivation by hypochlorite was considerably slower, particularly given its much longer half-life. Incubation of 0.1 μM yeast alcohol dehydrogenase subunit with 1 μM hypochlorite resulted in the loss of more than 95% of the activity over 5 min (Figure 7). The second-order rate constant for the reaction of hypochlorite with yeast alcohol dehydrogenase at 23 °C and pH 7.4 was 4×10^3 M^{-1} s^{-1} (Figure 7, inset).

Addition of 1 μM taurine chloramine to 0.3 μM yeast alcohol dehydrogenase resulted in time-dependent inactivation (not shown) similar to that seen with hypochlorite (Figure 7). The second-order rate constant for reaction of taurine chloramine with yeast alcohol dehydrogenase was

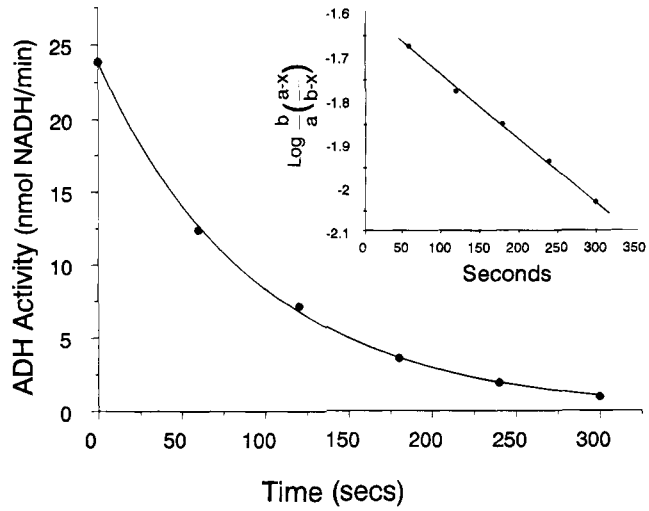


FIGURE 7: Hypochlorite-induced activity loss. Sodium hypochlorite (1 μM) was added to a solution of yeast alcohol dehydrogenase (3.5 μg) and EDTA (50 μM) in 1.0 mL of 50 mM potassium phosphate, pH 7.4 at 23 °C. Aliquots were removed at the indicated times, diluted 10-fold, and assayed for yeast alcohol dehydrogenase activity as described in Materials and Methods. Inset: Plot for determination of second-order rate constant for hypochlorite inactivation based on fractional activity and fractional hypochlorite remaining at each time point.

determined to be 2.7×10^3 M^{-1} s^{-1}. Taurine alone had no effect of yeast alcohol dehydrogenase activity.

DISCUSSION

Over three hundred zinc enzymes are known, many of which utilize thiolate functional groups of cysteine residues to coordinate the zinc atom (Vallee & Auld, 1990b; Rhodes & Klug, 1993), and the list of proteins containing "zinc fingers" ($Zn_1Cys_2His_2$), "zinc clusters" (Zn_1Cys_4), and other zinc−thiolate moieties continues to grow. Zinc fingers are a central structural feature of many mammalian transcription factors (Vallee et al., 1991) and retroviral DNA-binding proteins (Burke et al., 1992; Morellet et al., 1992). Zinc−thiolate centers are found in protein kinase C (Ono et al., 1989; Ahmed et al., 1991), the glucocorticoid receptor (Archer et al., 1990; Schwabe & Rhodes, 1991), metallothionein (Kagi et al., 1984), and many other proteins with diverse structure and function.

The zinc−thiolate ($Zn_1Cys_2His_1$-H_2O) moiety in yeast alcohol dehydrogenase is structually similar to the zinc finger ($Zn_1Cys_2His_2$). Enzymatic activity of yeast alcohol dehydrogenase is absolutely dependent on maintaining the integrity of zinc coordination at the catalytic site. Oxidant-induced activity loss was correlated with zinc release from the active site as well as with thiolate oxidation. Thus, yeast alcohol dehydrogenase is a useful and readily available model for the zinc−thiolate moiety in zinc finger proteins.

The slow rate constant for yeast alcohol dehydrogenase inactivation by hydrogen peroxide suggests that the zinc−thiolate center is not a major target of hydrogen peroxide *in vivo*. However, the rate constant for reaction of hypochlorite with the active site is 3000-fold faster, indicating that physiological concentrations of myeloperoxidase-generated hypochlorite could readily oxidize zinc−thiolates. Human neutrophil collagenase contains a "cysteine switch" (Springman et al., 1990; Van Wart & Birkedal-Hansen, 1990) at the active site which has been shown to be oxidatively

activated by neutrophil-derived hypochlorite (Saari et al., 1990; Michaelis et al., 1992). The cysteine switch contains only one zinc−thiolate bond, making it less similar to zinc fingers than is the active site of yeast alcohol dehydrogenase. However, cleavage of its sole zinc−thiolate bond by low concentrations of hypochlorite suggests an inherent oxidant lability.

Taurine is present at millimolar concentrations in many tissues, including the neutrophil, the retina, and nervous tissue (Wright et al., 1986). Its metabolic role is poorly understood, but it has been suggested to play an antioxidant role by scavenging hypochlorous acid, thereby preventing the formation of monochloramine (Grisham et al., 1984; Green et al., 1991). We report here that taurine chloramine, the product of the scavenging of hypochlorous acid by taurine, is still a potent oxidant capable of inactivating yeast alcohol dehydrogenase with a second-order rate constant of 2.7×10^3 M^{-1} s^{-1}, only slightly slower than that of hypochlorous acid itself (4×10^3 M^{-1} s^{-1}). Taurine chloramine is likewise capable of the inactivation of α_1-antiproteinase (Thomas et al., 1985).

The reaction with yeast alcohol dehydrogenase represents one of the fastest reactions yet observed for peroxynitrite. Direct measurement of the reaction between peroxynitrite and alcohol dehydrogenase was not feasible. However, the rate constants for peroxynitrite reacting with cysteine and for spontaneous decomposition of peroxynitrite had previously been directly measured by stopped-flow analysis (Radi et al., 1991a; Beckman et al., 1990). Determination of the rate constant for the peroxynitrite/alcohol dehydrogenase reaction was based on a competition assay whereby cysteine, which reacts at a known rate with peroxynitrite, was able to prevent inactivation of yeast alcohol dehydrogenase (Scheme 2). The underlying assumption is that cysteine was acting solely as a peroxynitrite scavenger without affecting yeast alcohol dehydrogenase directly. The lack of effect from cysteine or dithiothreitol added after peroxynitrite suggested that this assumption was valid. The rate constant was verified using the known rate of spontaneous peroxynitrite decomposition (to give nitrate) as an internal competition reaction (Scheme 1). The averate rate constant of 3.9×10^5 M^{-1} s^{-1} is similar to the rate at which peroxynitrite inactivates mitochondrial aconitase (Castro et al., 1994; Hausladen & Fridovich, 1994). Aconitase inactivation occurs via peroxynitrite attack on its iron−sulfur $[4Fe\text{-}4S]^{2+}$ cluster, suggesting that cationic metal−sulfur centers are particularly vulnerable to peroxynitrite (Castro et al., 1994).

Peroxynitrite reacts at least 300 000-fold faster with yeast alcohol dehydrogenase than does hydrogen peroxide and 100-fold faster than hypochlorite. The faster reaction rate allows peroxynitrite to inactivate yeast alcohol dehydrogenase at concentrations equivalent to those required for hypochlorite despite the rapid rate of spontaneous decay ($t_{1/2} \sim 1.4$ s at pH 7.4 and 25 °C relative to 6.1 h for hypochlorite). Unlike hypochlorite, which is the product of an enzyme found principally in neutrophils, peroxynitrite forms spontaneously from the near diffusion-limited reaction of superoxide and nitric oxide. Thus, peroxynitrite can be formed in any tissue that produces nitric oxide constitutively or has been stimulated to synthesize the inducible form of nitric oxide synthase.

The IC_{50} value for peroxynitrite increased with yeast alcohol dehydrogenase concentration simply because the target concentration increased and more oxidant was required to inactivate 50% of the larger targer concentration even with a 1:1 reaction stoichiometry. For example, 50% inactivation of a target present at 2 μM would require at least 1 μM of oxidant whereas 50% inactivation of a 100 μM target cannot be achieved at less than 50 μM of oxidant. The requirement for the higher oxidant concentration in the latter case does not indicate that the reaction is physiologically irrelevant, merely that the target concentration imposes an absolute minimum on the amount of oxidant required to see an effect. Thus, estimation of true oxidant potency can only be made when the target concentration is well below that of added oxidant. The dependency of the IC_{50} for peroxynitrite on yeast alcohol dehydrogenase concentration is indicative of a more generalized target concentration phenomenon and illustrates the need for caution when interpreting oxidant potency solely in terms of concentration in any *in vitro* system.

The micromolar IC_{50} value for peroxynitrite at catalytic concentrations of yeast alcohol dehydrogenase is within the concentration range around activated macrophages (Ischiropoulos et al., 1992). However, production of peroxynitrite *in vivo* from the reaction of nitric oxide and superoxide would likely result in continuous and prolonged exposure of various targets to relatively low peroxynitrite concentrations, similar to those obtained using SIN-1. Over the time course required for SIN-1 to inactivate yeast alcohol dehydrogenase by 50%, the steady-state concentration of peroxynitrite was 14.6 nM based solely on the rate of spontaneous decomposition. Using SIN-1, yeast alcohol dehydrogenase was inactivated by 50% at a peroxynitrite concentration equivalent to only a 5-fold molar excess of enzyme subunit (Figure 4). This ratio of peroxynitrite to enzyme subunit required for 50% inactivation was the same regardless of whether the exposure was from a short-lived bolus (Figure 2, middle curve) or continuous and prolonged (Figure 4) and indicates that oxidant effect is a function of total exposure (concentration $\times$ time) rather than initial concentration (Zhu et al., 1992).

Complete inactivation of yeast alcohol dehydrogenase was accompanied by oxidation of 3 thiol/thiolates per subunit. The concomitant release of all catalytically competent zinc strongly indicated that 2 of these 3 are the thiolates (Cys-46 and Cys-174) which coordinate zinc at the catalytic site. The fate of the remaining zinc coordinate (histidine-67) is not known; however, total zinc release upon derivatization of thiolates with DTNB indicates that ligation by histidine alone is insufficient to maintain zinc in association with the protein.

Zinc is not redox active in aqueous solution and is quite stable as the divalent cation. It has the highest charge-to-atomic radius ratio of any element and maintains partial cationic character even in a tetracoordinate complex like a zinc finger. Thus, zinc will attract anionic oxidants. However, when evaluating the catalytic site of yeast alcohol dehydrogenase as a model for the oxidant sensitivity of a zinc finger, small differences in the microenvironment of the zinc atom, as well as its function, must be considered. The catalytic zinc atom in yeast alcohol dehydrogenase serves as the binding site for the polar hydroxyl oxygen of alcohols and, during turnover, acts as a Lewis acid to stabilize negatively charged intermediates (Vallee & Auld, 1990b). In the absence of substrate, the fourth zinc coordinate is occupied by water. Histidine is the fourth zinc coordinate in a zinc finger. The effects of the second histidine on sensitivity to anionic oxidants like peroxynitrite and hy-

pochlorite are unknown and must await further study using a zinc finger protein or peptide.

In both yeast alcohol dehydrogenase and zinc fingers, the zinc atom links cysteine residues which are far removed from each other along the polypeptide backbone. While the mechanism of protein folding and zinc insertion is not known, the zinc atom constrains the peptide backbone, forming a loop analogous to the way a knot holds loops of ribbon into a bow. In various DNA-binding proteins these loops constitute the "fingers" which intercalate into the major groove of DNA (Rhodes & Klug, 1993). Cleavage of even one zinc−thiolate bond in such a conformationally strained loop could alter the critical tertiary structure needed to recognize and bind DNA.

Mutation of a single cysteine residue in the second zinc finger of the glucocorticoid receptor results in loss of DNA-binding properties whereas mutation of a single cysteine in the first finger actually changes the receptor from a transcription activator to a repressor (Ray et al., 1991). Thus, precedent exists for subtle changes in zinc−thiolate centers producing dramatic changes in function. The oxidant sensitivity of zinc−thiolate centers coupled with their presence in numerous proteins which play critical roles in cellular regulation suggests that they may be key targets for oxidant-mediated pathology.

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