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Ethanenitronate Is a Peroxide-dependent Suicide Substrate for Catalase*

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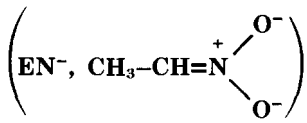
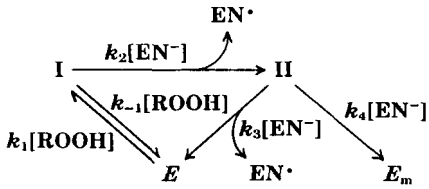
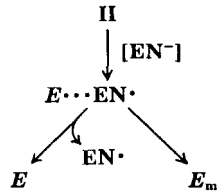
We find ethanenitronate  **to be a H_2O_2- (and peracetic acid-)dependent suicide substrate for bovine liver catalase (E) which converts E to E_m, a modified form of the enzyme. The catalytic and suicide pathways are related to E, E_m, Compound I, and Compound II according to the following scheme.**



The catalytic cycle generates free radical products (EN·) which then participate in an O_2-dependent chain reaction. Within experimental error the exclusive target for inactivation by EN$^-$ is Compound II. This partitions in the ratio (k_4 = 1.2 M^{-1} s^{-1})/(k_3 = 1.6 M^{-1} s^{-1}) to generate E_m and E, respectively. The species E_m acquires 1 eq of ^{14}C/ferriheme from [1-^{14}C]ethanenitronate which is firmly (presumably covalently) affixed to the protein moiety. According to the standard H_2O_2 assay, E_m is 7% as active catalytically as E.

We regard inactivation as resulting from that fraction of EN· in the E···EN· complex which fails to diffuse from the complex because it is trapped by reaction with a neighboring amino acid residue to generate E_m irreversibly.



This mechanism is identical to that deduced previously for suicide inactivation of horseradish peroxidase by alkane nitronates (Porter, D. J. T., and Bright, H. J. (1983) *J. Biol. Chem.* 258, 9913–9924) with the exception that EN· is trapped in that case by a methine carbon at the edge of the ferriheme rather than by the apoenzyme. The labeled residue in the catalase apoenzyme probably resides at or near the site of reduction of Compound II.

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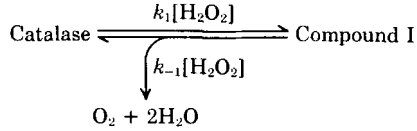
We have shown that nitroalkanes (as their nitronates) readily reduce Compounds I and II of horseradish peroxidase (EC 1.11.1.7) and, therefore, function as alternative donor substrates for this enzyme (1). We also demonstrated that one-third of the bimolecular encounters of methanenitronate with Compound II of peroxidase results in the covalent attachment of a nitromethyl moiety to a methine carbon of the ferriheme prosthetic group and that the covalently modified enzyme is about 40% as active catalytically as native peroxidase. Alkanenitronates, therefore, behave as suicide substrates for horseradish peroxidase. Because of the implications of these results for the mechanism of electron transfer in hemoprotein catalysis we extended these investigations to catalase (EC 1.11.1.6) and found, using millimolar levels of H_2O_2 and methanenitronate, that the rate of enzymatic O_2 production (from $2H_2O_2 \rightarrow O_2 + 2H_2O$) was not detectably altered by methanenitronate. These preliminary experiments suggested, therefore, that methanenitronate was neither a substrate for, nor an inactivator of, catalase.

We have re-examined our catalase findings because, upon reflection, it is clear that an alternative donor (reductive) substrate/inactivator may not be easily recognizable when it and H_2O_2 are present initially at the millimolar level. The values of k_1 and k_{-1} in the catalase mechanism (Equation 1)



(1)

exceed 10^7 M^{-1} s^{-1} (2, 3). First, therefore, an alternative donor substrate which reacts in a H_2O_2-competitive fashion with Compound I with a bimolecular rate constant as large as 10^5 M^{-1} s^{-1} will have an insignificant effect on the rate of O_2 formation (2, 3). Second, with typical concentrations of enzyme, the lifetime of Compound I during H_2O_2 turnover may be much shorter than the effective half-time for modification of the enzyme by a Compound I-specific inactivator. The detection of enzyme modification by kinetic assay under such circumstances would be difficult and would be even exacerbated if, as in the case of peroxidase (1), the inactivator were Compound II-specific (because, as is implied by the mechanism of Equation 1, Compound II is not significantly populated during H_2O_2 disproportionation).

Using protocols which generate low steady state levels of H_2O_2 (2, 3) we now demonstrate that alkanenitronates do, in fact, function as suicide substrates for catalase. As in the case of peroxidase (1), the target for inactivation is Compound II and the covalently modified enzyme, containing 1 nitroalkyl eq/ferriheme, is 7% as active as native catalase by kinetic assay. However, in contrast to peroxidase, it is the apoenzyme

of catalase, rather than the ferriheme prosthetic group, which is covalently modified.

EXPERIMENTAL PROCEDURES

Materials

Crystalline bovine liver catalase was purchased from Sigma and dissolved at room temperature over a period of 15 min after adjusting the pH of the suspension to 9.0. The dissolved enzyme was transferred into 0.01 M potassium phosphate at pH 7.0 by gel filtration through a Sephadex G-25 column equilibrated in the same buffer. Enzyme solutions were prepared fresh each day and stored at 5 °C. Nitroalkanes (Aldrich products) were purified by fractional distillation, if necessary, prior to use. The nitronates were prepared (anaerobically or aerobically on the day of the experiment, as required, and stored at 5 °C) by dissolving the nitroalkane in a molar equivalent of 1 M KOH and quantified spectrophotometrically by the absorbance of the nitronate (λ_{max} = 220–235 nm, ϵ = 9300 M^{-1} cm^{-1} (1)). The rates of reprotonation of the nitronates in 0.01 M potassium phosphate were followed spectrophotometrically at 240 nm (nitronate → nitro). In this buffer system ethanenitronate reprotonates with $t_{½}$ ≈ 40 min. Peracetic acid was a Pfaltz and Bauer product, and 30% H_2O_2 was obtained through Fisher.

[1-^{14}C]Ethanenitronate was synthesized by the displacement of iodide from [1-^{14}C]ethyl iodide by nitrite. In a typical reaction 100 μCi of [1-^{14}C]ethyl iodide (Amersham Corp., 57 mCi/mmol) was equilibrated with 250 μmol of unlabeled ethyl iodide for 2 h at room temperature and for 16 h at −10 °C. Subsequently, the reaction was initiated by addition of 1 ml of dimethyl sulfoxide containing 2 mmol of $NaNO_2$. The reaction was terminated after 1 h by addition of 25 ml of 1 mM KOH. Prior to adsorption of the product onto a column of AG 1-X8 (hydroxide form) the pH of the solution was adjusted to 11.5 with 1 M KOH. Nitrite and nitrite esters were eluted from the column with 50 ml of 5% dimethyl sulfoxide. The column was rinsed with 50 ml of deionized H_2O, after which the product was eluted with 0.1 M KOH. Fractions which contained ethanenitronate (as estimated by A_{240} measurements) were pooled, and the pH was adjusted to 5.5 with solid monobasic potassium phosphate. Nitroethane was extracted from this solution (20 ml) with two 1.5-ml aliquots of methylene chloride. The methylene chloride extracts were pooled, and the ethanenitronate was extracted into 1 ml of 0.2 M KOH. Finally, the pH of the ethanenitronate solution was adjusted to 11 with 1 M HCl. The final product was an 87 mM solution of ethanenitronate (35% yield) with a specific activity of 1.07 × 10^{12} cpm/mol and was homogeneous, as judged by ion-exchange chromatography on an AG 1-X8 column. The nitronate was eluted from this column by a gradient of 0–100 mM KOH and appeared as a single symmetrical peak with constant specific activity. [1-^{14}C]Ethanenitronate was stored at −10 °C.

Methods

Assay of Catalase—Catalase was assayed spectrophotometrically at 240 nm by following the initial velocity of H_2O_2 disappearance (initial A_{240} = 0.8 cm^{-1}). Inactivation reaction mixtures were quenched by a 100-fold dilution of an aliquot into 0.01 M potassium phosphate containing 4 mM propane-2-nitronate at pH 7.0. After 15 min any Compound II present would be reduced to resting enzyme. An aliquot from this mixture was then diluted 200-fold into the assay buffer. Relative activities are expressed as a percentage of the activity of untreated enzyme. The catalytic activity in 0.01 M potassium phosphate at pH 7.0 and 25 °C was 5.7 × 10^6 M^{-1} s^{-1} which is comparable to the value of 7.6 × 10^6 M^{-1} s^{-1} calculated from the data of Kremer (5) for purified bovine liver catalase.

Catalase, Compound I, and Compound II—Catalase concentration (as ferriheme) was quantified using $\Delta\epsilon_{403}$ = 63 mM^{-1} cm^{-1} for the difference in absorbance between Compound I (formed with excess peracetic acid) and resting enzyme (6). This measurement of active catalase eliminates interference by the absorbance at 403 nm of inactive hemin (or breakdown products) which is known to be present in bovine liver catalase preparations. Alternatively, peracetic acid, after removal of H_2O_2 with trace amounts of catalase (6), may be estimated by the absorbance change it generates at 403 nm when added to a solution containing excess catalase.

Compound I was generated by addition of peracetic acid to catalase (6). Unfortunately, our catalase preparations contain an "endogenous donor" that slowly reduces Compound I so formed back to resting enzyme. Thus, addition of 9 μM peracetic acid to 5.6 μM catalase produced 4.6 μM Compound I (by A_{403} measurements) which slowly reverted to catalase ($t_{½}$ ≈ 100 s). Concentrations of Compound I reported herein are, therefore, based upon A_{403} measurements. Furthermore, if there was less than 100% Compound I formation the residual peracetic acid concentration was considered to be zero.

Compound II was generated using the glucose oxidase/O_2/glucose system and $K_4Fe(CN)_6$ as described (7). Typically, we used 5.6 μM catalase, 25 μM $K_4Fe(CN)_6$, 2–8 μM glucose oxidase, and 4 mM glucose in air-saturated 0.01 M potassium phosphate at pH 7.0 and 25 °C. The formation of Compound II, which was followed at 434 nm, is a first order process. From the estimated end point of this reaction, we calculated $\Delta\epsilon_{434}$ = 55 M^{-1} cm^{-1} for Compound II and catalase. This value was used to calculate the amount of Compound II formed in solutions where the reaction had not proceeded to completion. Glucose and $K_4Fe(CN)_6$ were removed from the reaction mixtures by gel filtration on a Sephadex G-25 column. Compound II slowly decayed (measured by ΔA_{434}) to catalase with a $t_{½}$ ≈ 30 min. O_2 was purged from these solutions with argon.

Reaction of Peracetic Acid with Ethanenitronate—The addition of 75 μM peracetic acid to 200 μM ethanenitronate results in the disappearance of the nitronate at a rate of 3.6 μM/min (as measured by A_{240}). Assuming this to be a bimolecular process, we estimated the rate constant describing this reaction to be 220 M^{-1} s^{-1}. Thus, in the presence of 5 mM ethanenitronate, the $t_{½}$ for the disappearance of 20 μM peracetic acid would be 0.6 min. Since this half-life is less than the reaction time of ethanenitronate with catalase in our experimental protocols, meaningful turnover experiments with peracetic acid and the nitronate are not feasible.

Calculation of the Fraction of Catalase Modified from Activity Measurements—Ethanenitronate-modified catalase retains 7% of the activity of untreated catalase. Assuming that the extent of modification is proportional to the amount of activity lost, the fraction of enzyme modified (f_m) is calculated from the fraction of lost activity (a_1) from the relation $f_m = (a_1)/(0.93)$.

Spectrophotometric measurements were made with Cary 15 and Gilford spectrophotometers. Oxygen concentrations were followed polarographically on a Yellow Springs Instrument Co. oxygen electrode. Radioactivity was quantified with an Intertechnique S130 scintillation counter and ScintiVerse mixture (Fisher). All experimental solutions were maintained at 25 °C.

RESULTS

In the presence of very low steady state concentrations of H_2O_2, generated from ascorbate/O_2 or glucose/O_2/glucose oxidase (4), a variety of nitroalkanes can be demonstrated to inactivate catalase (Table I). Nitroethane, which reduces the activity of the enzyme by 93%, is the most effective inactivator tested. We note three characteristics of the inactivation phenomenon. First, both a primary nitroalkane and a H_2O_2-generating system are necessary and, furthermore, the nitroalkane must be presented to the enzyme as the anion (*i.e.* the nitronate) as shown in Table I. Second, secondary nitroalkanes fail to inactivate catalase even though they reduce Compounds I and II to resting enzyme (data for propane-2-nitronate, not shown). Third, ethanol protects catalase from inactivation by alkanenitronates and H_2O_2 (Table I). Ethanol reduces Compound I to resting enzyme without intermediate formation of Compound II (2) and, therefore, decreases the steady state concentrations of Compounds I and II when added to the enzyme during turnover with low concentrations of H_2O_2. Consequently, the direct target of ethanenitronate in the inactivation process cannot be resting enzyme.

Inactivation of catalase by ethanenitronate is attended by a burst of O_2 consumption which is superimposed upon the constant rate of O_2 consumption of the glucose/O_2/glucose oxidase system. The time courses for inactivation and for the burst of O_2 consumption are similar (Fig. 1). Since the amplitude of the O_2 burst is decreased by superoxide dismutase, free radical intermediates (such as the nitroalkyl radical and $HO_2^{\cdot}$) which participate in the chain reaction between nitroalkanes and O_2 (1) must be generated during the inactivation

TABLE I
Inactivation of catalase by alkanenitronates

Additions[a]	Activity remaining
	%
1. None	100
2. H_2O_2[b]	84
3. 1 M nitromethane-equilibrium mixture, H_2O_2	53
4. 5 mM ethanenitronate	90
5. 5 mM ethanenitronate, H_2O_2	7
6. 5 mM nitroethane, H_2O_2	104
7. 5 mM ethanenitronate, H_2O_2, 100 mM ethanol	100
8. 5 mM propane-1-nitronate, H_2O_2	33
9. 5 mM propionate-3-nitronate, H_2O_2	68
10. 10 mM propane-2-nitronate, H_2O_2	95
11. 10 mM 1-chloroethanenitronate, H_2O_2	92

[a] Catalase (4.6 μM) was equilibrated with the indicated reagents in 0.01 M potassium phosphate, pH 7.0 and 25 °C. After a 50-min reaction time, the enzyme was assayed as described under "Experimental Procedures."

[b] H_2O_2 was generated at a rate of 0.01 μM s^{-1} by 8 mM 1-ascorbate or by glucose oxidase with 4 mM glucose.

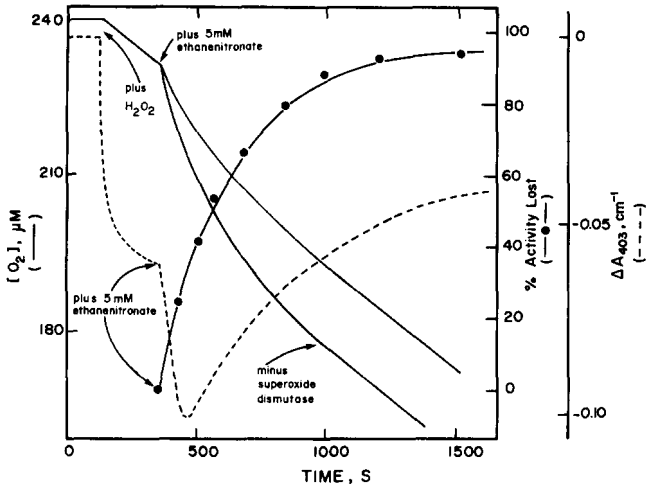
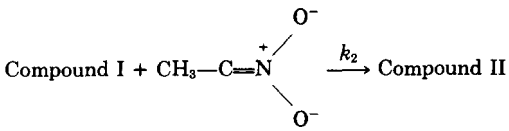
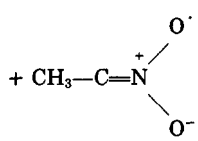


FIG. 1. **Addition of an H_2O_2-generating system and 5 mM ethanenitronate to catalase causes a loss of enzyme activity (●——●) and, with a similar time course, a burst of O_2 consumption (——) and absorbance changes in the Soret region** (– – –). Initially, reaction mixtures contained 0.01 M potassium phosphate, pH 7.0 and 25 °C, with 4.6 μM catalase ferriheme, 4 mM glucose, 12 μg/ml superoxide dismutase (unless otherwise noted), and sufficient glucose oxidase to generate H_2O_2 at a rate of 0.09 μM s^{-1} in the air-saturated solutions.

process. The presence of such species implies that ethanenitronate reduces Compound I (at least part of the time) by a one-electron process to generate Compound II (Equation 2).



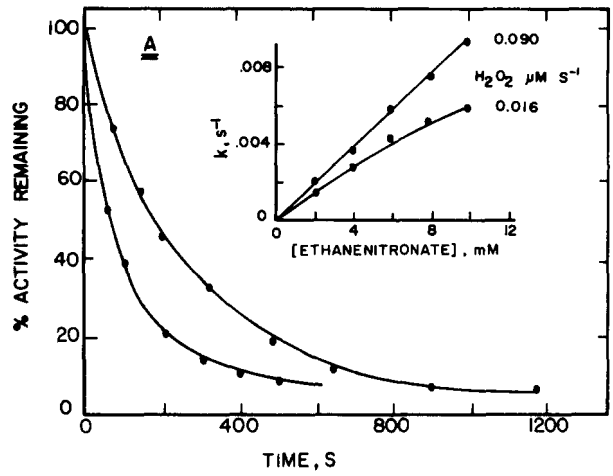
(2)



The rate of O_2 consumption doubles after addition of ethanenitronate to catalase in the presence of the H_2O_2-generating system and with a saturating concentration of superoxide dismutase (Fig. 1). Consequently, all of the H_2O_2 from the H_2O_2-generating system is reduced initially by a donor other than H_2O_2, namely ethanenitronate. The magnitude of the burst of O_2 consumption is equivalent to 4.2 molecules of H_2O_2 consumed for each subunit of catalase inactivated.

Concomitant with the burst in O_2 uptake and the inactivation of catalase by ethanenitronate there occur time-dependent spectral changes in the Soret region. A typical time course for such changes at 403 nm consists of three phases (Fig. 1). First, addition of H_2O_2 causes the concentration of Compound I to approach a steady state value (corresponding to a decrease in A_{403}). Second, addition of ethanenitronate elicits the rapid formation of Compound II (corresponding to a further decrease in A_{403}). Finally, modified catalase accumulates (corresponding to an increase in A_{403}). The modified enzyme retains about 7% of the activity of native catalase.

Inactivation data such as those shown in Fig. 2*A* yield linear first order plots with a nonzero infinity point. When H_2O_2 is generated at a rate of 0.09 μM s^{-1}, the rate of inactivation of catalase is linearly dependent upon the concentration of ethanenitronate (at values less than 10 mM). If the rate of H_2O_2 generation is decreased to 0.016 μM s^{-1}, the



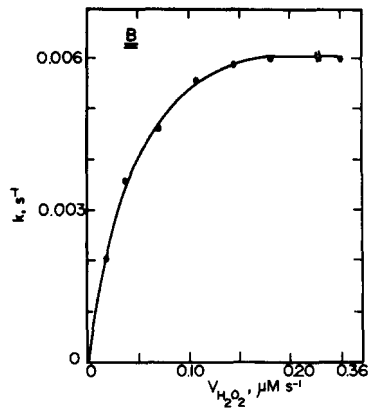
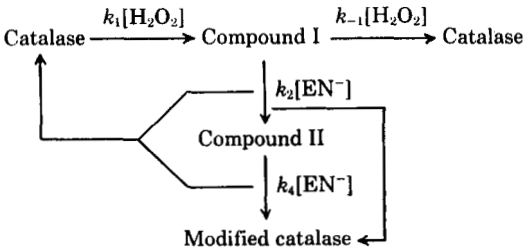


FIG. 2. **The rate at which catalase is modified by ethanenitronate depends upon the concentration of ethanenitronate and the rate of generation of H_2O_2.** Typical time courses for modification of catalase with 5 or 10 mM ethanenitronate and H_2O_2 generated at a rate of 0.09 μM s^{-1} are shown (*Panel A*). The pseudo-first order rate constants determined from data such as these are plotted against ethanenitronate concentration with two different rates of H_2O_2 generation (*inset* to *Panel A*). With a fixed concentration of ethanenitronate (5 mM) the pseudo-first order rate constants for modification (k) vary nonlinearly with the rate of H_2O_2 generation ($V_{H_2O_2}$, *Panel B*). Reaction conditions are given in the legend to Fig. 1 except for the glucose oxidase concentration which was varied to produce the reported rates of H_2O_2 generation.

inactivation rates are no longer linearly dependent upon ethanenitronate concentration (see *inset*, Fig. 2*A*). Similarly, the rate of inactivation varies nonlinearly with the rate of H_2O_2 generation ($V_{H_2O_2}$) at a fixed concentration of ethanenitronate (Fig. 2*B*). These kinetic results lead to a preliminary scheme (Equation 3) in which H_2O_2 and ethanenitronate (EN^-) compete as reductants of Compound I and/or Compound II.



(3)

Ethanenitronate-modified catalase does not recover activity after dialysis for 48 h in three different buffer systems (0.01 M phosphate at pH 7.0, 0.02 M acetate at pH 4.5, and 0.025 M Tris at pH 8.0) in the presence or absence of 0.1 M potassium fluoride. Furthermore, the electronic spectra of modified and native catalase in the Soret region are very similar; the Soret band of modified enzyme is slightly blue-shifted relative to the native enzyme while the Soret extinction coefficient of modified enzyme is slightly decreased. The modified enzyme does not exhibit the characteristic cyanide spectrum observed with the native enzyme (see Fig. 3*A*). Resolution of modified enzyme into hemin and apoenzyme components yields a hemin spectrum identical to that obtained after resolution of native catalase (see Fig. 3*B*). This suggested that only the protein portion of the enzyme is modified by ethanenitronate and was confirmed through the use of [1-^{14}C]ethanenitronate (see Table II). The modified enzyme contains 1.1 mol of radiolabel/mol of subunit of modified catalase. Resolution of the modified enzyme into its hemin and apoenzyme components with acidic 2-butanone demonstrates that all of the radiolabel is associated with the apoenzyme. The modified apoenzyme lost little radiolabel after a 24-h dialysis (either at pH 4.0 or 12.0 or in the presence of 0.05 M hydroxylamine at pH 6.0).

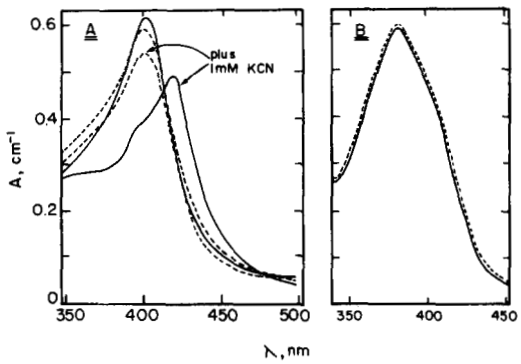


FIG. 3. *Panel A*, visible spectrum of 4 μM native catalase (——) and modified catalase (– – –) in the absence and presence of 1 mM KCN. *Panel B*, visible spectra of hemin extracted from native (——) and modified (– – –) catalase. Ethanenitronate-modified catalase (prepared as described in Table II) and native enzyme were dialyzed against 0.01 M potassium phosphate at pH 7.0 for 24 h at 0 °C. Native catalase retained over 90% of its activity while the modified enzyme retained less than 5% of the activity of the control mixture. The ferriheme from modified (– – –) and native (——) catalase from the experiments of *Panel A* (5 ml each) was extracted into 4 ml of 2-butanone, which contained 0.2 ml of concentrated HCl. The 2-butanone extracts were then diluted with an equal volume of 2-butanone.

TABLE II

[1-^{14}C]Ethanenitronate incorporation into catalase

Treatment	Total counts[a]	Stoichiometry[b]
A. Modified catalase[c]	21,480	1.12[d]
B. Resolution of enzyme[e]		
1. Protein	20,650	1.07
2. Hemin	100	0.005
C. Dialysis of protein[f]		
1. pH 4.0	16,600	0.86
2. pH 6.0, 0.05 M NH_2OH	14,780	0.77
3. pH 12.0	14,950	0.78

[a] All measurements were corrected for handling losses and may be compared with the values presented in Part A.

[b] Stoichiometry values are given as mol of ^{14}C/mol of modified catalase subunit. Modified subunit concentration was determined from the loss of relative activity as described under "Experimental Procedures."

[c] Catalase (22 nmol in 2 ml of 0.01 M potassium phosphate, pH 7.0, air saturated) was reacted with 4.7 mM [1-^{14}C]ethanenitronate for 30 min with H_2O_2 generated with 3 nM glucose oxidase and 4 mM glucose in the presence of 10 μg/ml superoxide dismutase. The 84% modified enzyme was separated from unreacted radiolabel by dialysis against pH 7.0 buffer.

[d] If H_2O_2 generation rate is zero (anaerobic solution), catalase is not inactivated and less than 0.02 ferriheme eq radiolabel are incorporated into the enzyme.

[e] See Fig. 3 for details.

[f] A fraction of the protein from Part B1 was used in each dialysis experiment. Counts are reported on the basis that the entire sample was used.

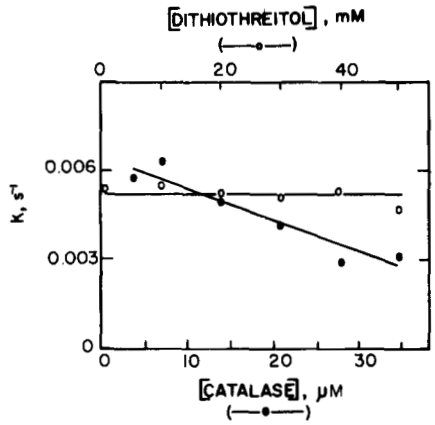


FIG. 4. **Dependence of the rate of modification of catalase by ethanenitronate on the concentrations of dithiothreitol and catalase.** When dithiothreitol concentrations were varied, the catalase concentration was fixed at 4.6 μM with H_2O_2 generated at a rate of 0.09 μM s^{-1} (○). With varying catalase concentrations and in the absence of dithiothreitol the ratio of the rate of H_2O_2 generation to ferriheme concentration was maintained at 0.013 s^{-1} by varying the glucose oxidase concentration in the presence of 4 mM glucose (●). Other reaction conditions are specified in the legend to Fig. 1.

While our preliminary scheme (Equation 3) implies "suicidal inactivation" as the basis of the effect of ethanenitronate, we have not ruled out a mechanism involving reactive free product(s). Consequently, we examined the effects of a nucleophilic scavenger and of enzyme concentration. We found that the rate of modification of catalase by ethanenitronate is not affected by dithiothreitol and is decreased only slightly when the concentration of catalase is increased 7-fold (Fig. 4). We would expect that the steady state concentration of a free product which reacts with the enzyme (and, probably, with the solvent also) would be decreased by the addition of dithiothreitol and increased when the enzyme concentration

is raised (provided the ratio of the rate of H_2O_2 generation to ferriheme concentration is fixed, as was the case). Our results are, therefore, incompatible with the hypothesis that an electrophilic free product is the modifier.

All of our results thus far support the scheme of Equation 3. However, the identity of the step or steps at which modification occurs remains ambiguous. In order to further dissect the modification mechanism we need to study Compounds I and II separately as well as assess the role of O_2 (other than as a source of slowly generated H_2O_2) and activated derivatives of O_2 in the modification process. The protocol used thus far requires O_2 for H_2O_2 formation and the consequent cyclical generation of Compounds I and II. Therefore, we changed our protocol and used peracetic acid to generate Compound I from resting enzyme (6) and glucose/O_2/glucose oxidase together with $K_4Fe(CN)_6$ to generate Compound II (7). Solutions of these species were made anaerobic with argon. We should note, too, that the reactions of Compound I with a donor such as ethanenitronate (I → II → resting catalase) are more complicated than the reaction of Compound II (II → resting catalase). Therefore, our tactic was to first study Compound II.

Anaerobic reduction of Compound II by ethanenitronate is a pseudo-first order reaction which depends linearly upon ethanenitronate concentration (Fig. 5). The reduction process corresponds to a bimolecular rate constant of 2.8 M^{-1} s^{-1} at pH 7.0 and is accompanied by a 40% loss of enzyme activity (as assessed by the H_2O_2 assay described under "Experimental Procedures") which corresponds to a 43% modification of the enzyme. Since completely modified catalase contains 1.1 mol of radiolabel/mol of ferriheme (Table II), we view partial modification during reduction of Compound II by ethanenitronate (without turnover) as occurring by partition of Compound II between enzyme with one covalently affixed ferriheme equivalent of radiolabel and resting enzyme in the ratio of 0.43 to 0.57 (Equation 4).

$$\begin{array}{l}\text{Compound II} \xrightarrow{k_4[EN^-]} \text{modified catalase} \\ \quad\downarrow k_3[EN^-] \\ \text{Catalase}\end{array} \qquad (4)$$

From $k_4/k_3 = 0.43/0.57$ and the fact that $k_3 + k_4 = 2.8$ M^{-1} s^{-1}, values of k_4 and k_3 are calculated to be 1.2 and 1.6 M^{-1} s^{-1}, respectively. Additionally, it should be noted that Compound II is modified anaerobically by ethanenitronate. Therefore, O_2 and activated derivatives of O_2 are not obligate intermediates in the modification process.

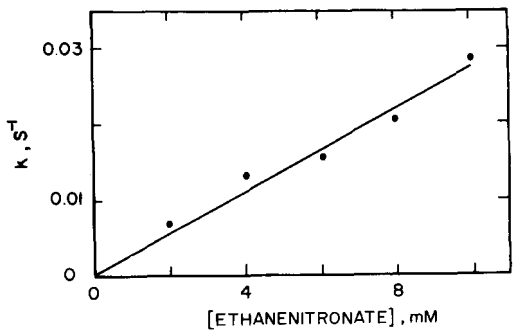


FIG. 5. **The pseudo-first order rate constants for anaerobic reduction of Compound II are linearly dependent upon ethanenitronate concentration.** Compound II was prepared using glucose/O_2/glucose oxidase, $K_4Fe(CN)_6$, and catalase as described under "Experimental Procedures." After removal of small molecules by gel filtration, the solution of Compound II was purged of O_2 by bubbling with argon. Reduction of Compound II was monitored by the decrease in absorbance at 430 nm, and pseudo-first order rate constants were derived from linear first order plots of these absorbance data. The reactions (in 0.01 M potassium phosphate, pH 7.0 and 25 °C) were initiated by the anaerobic addition of ethanenitronate.

Turning now to the behavior of Compound I, we note again that peracetic acid oxidizes resting enzyme to Compound I which, in turn, is not reduced by peracetic acid (as would be the case with H_2O_2 as substrate). Thus, peracetic acid is a convenient substrate for the selective generation of Compound I. Anaerobic addition of ethanenitronate to Compound I so generated causes the transient formation of Compound II. This reaction is conveniently followed at the isosbestic point (434 nm) for Compound I and resting catalase (see Fig. 6). To quantify the rates of reduction of Compound I to Compound II, we monitored the disappearance of Compound I at 420 nm (which is an isosbestic point between Compound II and the final products of the reaction). The pseudo-first order rate constants which describe this process are linearly dependent upon ethanenitronate concentration (*inset* of Fig. 6). From these data the bimolecular rate constant for reduction of Compound I to Compound II is calculated to be 8.1 M^{-1} s^{-1}. Since reduction of Compound II by ethanenitronate is also described by a bimolecular rate constant (see Equation 4), these results are summarized by the scheme of Equation 5.

$$\text{Compound I} \xrightarrow{k_2[EN^-]} \text{Compound II} \xrightarrow{(k_3 + k_4)[EN^-]} \text{products} \qquad (5)$$

Two additional observations affirm the correctness of Equation 5. First, the time course for Compound II formation (II(t)) in the scheme defined by Equation 5 has an analytical solution (Equation 6).

$$\frac{[II(t)]}{[I_0]} = \frac{k_2}{((k_3 + k_4)[EN^-] - k_2[EN^-])}\left(e^{-k_2[EN^-]t} - e^{-(k_3+k_4)[EN^-]t}\right) \qquad (6)$$

(This solution is a modification of the analytical solution for the system A → B → C (8).) An expression for the maximal fraction of Compound I present as Compound II ($II(t_m)/I_0$) during reduction of the former by ethanenitronate may be

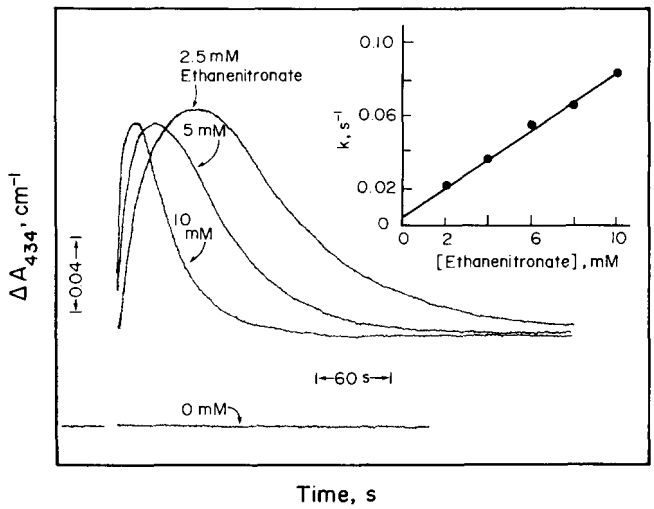


FIG. 6. **Compound II is generated transiently during the reduction of Compound I by ethanenitronate.** Compound I was obtained by the addition of 9 μM peracetic acid to 5.6 μM catalase. Because of the presence of endogenous donors in our catalase preparations, this procedure generated only 4.5 μM Compound I. Compound II formation was followed at 434 nm after the anaerobic addition of the specified concentrations of ethanenitronate. Alternatively, pseudo-first order rate constants for Compound I disappearance were obtained from the absorbance increases at 420 nm (an isosbestic point for Compound II and the products of the reaction) after anaerobic addition of the nitronate. The reaction medium was anaerobic 0.01 M potassium phosphate, pH 7.0 and 25 °C.

derived from Equation 6 to give

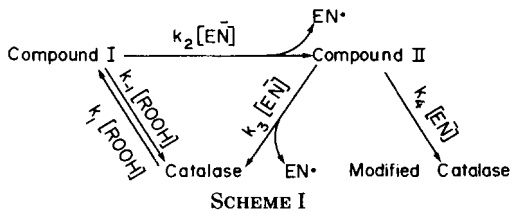
$$\frac{[II(t_m)]}{[I_0]} = K^{K/(1-K)} \quad (7)$$

where $K = (k_3 + k_4)k_2^{-1}$. This equation predicts that $II(t_m)/I_0$ will have a constant value of 0.57 when we insert our measured values of k_2 and $(k_3 + k_4)$. Experimentally, $II(t_m)/I_0$ has a value of 0.66 which is independent of ethanenitronate concentration. This ratio was estimated from the maximum absorbance change at 434 nm during reduction of Compound I (0.168 cm^{-1} in Fig. 6) and the difference extinction coefficient between Compounds I and II ($\Delta\epsilon_{434} = 55\ M^{-1}\ cm^{-1}$). Second, since we propose that Compound I is reduced by ethanenitronate to Compound II and that only Compound II is modified during this reaction with the enzyme (Equations 4 and 5), we predict that we should observe the same extent of inactivation during the reaction of either Compound I or Compound II with ethanenitronate. This prediction is verified, within experimental error, because ethanenitronate causes 43% modification when reacted with Compound II and 32% modification when reacted with Compound I. Thus, the modification reaction may be accounted for by the reaction of Compound II with ethanenitronate. However, our data are not of sufficient quality to rule out the possibility that a small fraction of Compound I is reduced directly to resting catalase without the intervention of Compound II.

The mechanism of Scheme I, which brings together the elements of Equations 3, 4, and 5, summarizes our understanding of the reaction of ethanenitronate with catalase. Since we found no evidence for complex formation between the reducing substrate and catalase Compounds I and II prior to reduction or modification, all reactions involving the nitronate are depicted as bimolecular processes. The natural substrate, H_2O_2, reacts in a similar fashion.

DISCUSSION

Ethanenitronate has proved to be a co-substrate for catalase and covalently modifies the enzyme during H_2O_2-dependent turnover. This phenomenon is characterized by the following observations. 1) Compounds I and II are reduced by ethanenitronate in one-electron steps which generate nitroethyl radicals. 2) Catalase is modified during turnover to yield an enzyme species with 7% of the enzymatic activity of native enzyme. 3) Modification requires, within experimental error, the reduction of Compound II, but not Compound I, by ethanenitronate and leads to the covalent incorporation of 1 ferriheme eq of ^{14}C from [1-^{14}C]ethanenitronate into the protein. 4) The modifying species is not an enzymatically generated electrophile released into solution because the rates of modification are unaffected by addition of a nucleophile or by changes in enzyme concentration. 5) O_2 is not required for modification of catalase. A kinetic description of the reaction of a peroxide and ethanenitronate with catalase is outlined in Scheme I. We note again that all reactions in this scheme are depicted as bimolecular processes because our kinetic results do not support saturation of Compounds I and II by ethanenitronate. Such results merely reflect the limitations of the primary kinetic data and do not disprove complex formation between Compounds I and II and the nitronate.

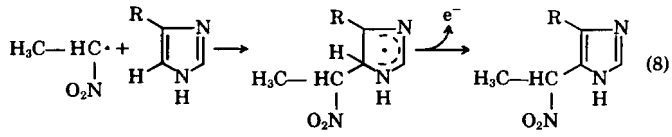


SCHEME I

As noted, modified catalase possesses 7% of the enzymatic activity of the native enzyme. We tentatively assign this residual activity as an intrinsic catalytic property of the modified enzyme. Two observations suggest, but by no means prove, that the residual activity does not arise from a small fraction of unmodified enzyme. First, catalase retains over 50% of the enzymatic activity of the native enzyme after modification by methanenitronate (Table I). This suggests that the extent of activity loss is dependent upon the nature of the nitroalkyl group. Second, catalase also retains residual activity after modification by active site-directed reagents such as cyanogen bromide (3).

The seminal finding of the present work is the observation that, within experimental error, it is the reaction of ethanenitronate with Compound II, but not Compound I, which leads to covalent modification of the protein. Since Compound II is reduced to resting enzyme by a single electron, the modification of Compound II by ethanenitronate proceeds by a radical mechanism. Thus, after reduction of Compound II by the nitronate, the nitroethyl radical so generated either diffuses from the enzyme via k_3[EN⁻] in Scheme I to yield unmodified enzyme or modifies the enzyme via k_4[EN⁻] by covalent addition to an amino acid residue which is involved in catalysis.

We have not identified the group that is modified by ethanenitronate. However, cyanogen bromide and 3-amino-1H-1,2,4-triazole (in the presence of H_2O_2) react with histidine 74 of catalase (3, 12). Such modified enzymes have properties that are similar to ethanenitronate-treated catalase. In particular, these modified catalases 1) retain some enzymatic activity, 2) do not yield the characteristic spectral shift of the Soret peak upon addition of cyanide, and 3) relative to native enzyme, display a Soret band having a slightly blue-shifted λ_{max} value and decreased intensity (3). Histidine is known to form adducts with the hydroxyl (9, 10) and semiascorbate (11) free radicals. The hydroxyl radical reacts with histidine by addition to either the 2- or 4-positions. By analogy with this reaction we propose that the nitroethyl radical, generated during the reduction of Compound II to resting enzyme, adds to histidine 74 at either position 2 or 4. Subsequently, this species is oxidized by some unspecified process to generate the stable adduct (Equation 8, with example of attack at the 4-position of histidine).



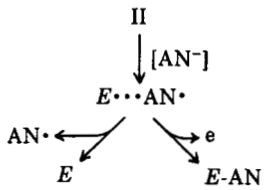
(8)

We note that the hydroxyl radical (13), the semidehydroascorbate radical (14), the 6-hydroxydopamine radical (15), and the 1,4-naphthoquinone-2-sulfonic acid radical (15) have been implicated as inactivators of catalase.

Two other peroxide-dependent inactivators of catalase are worth noting. First, unlike ethanenitronate, phenylhydrazine modifies catalase by formation of an aryl ferriheme complex in a peroxide-dependent reaction which probably proceeds by a radical mechanism (16). The enzyme species (*E*, I, II) which suffers inactivation has not been determined. Second, 3-amino-1H-1,2,4-triazole inactivates catalase through covalent modification of histidine 74. This H_2O_2-requiring reaction occurs by 2-electron oxidation of 3-amino-1H-1,2,4-triazole by Compound I (3, 12). Oxidized 3-amino-1H-1,2,4-triazole is an electrophile which is susceptible to attack by the nucleophilic histidine 74 with subsequent modification. This should be contrasted to the modification of catalase by ethanenitron-

ate in which the reactive intermediate is generated by the reaction of reductant with Compound II.

Finally, we note that the mechanism for the H_2O_2-dependent inactivation of catalase by ethanenitronate deduced in these studies (Scheme I) is identical kinetically to the mechanism we reported previously for the H_2O_2-dependent inactivation of horseradish peroxidase by alkanenitronates (1). In each case the catalytic cycle generates nitroalkyl free radicals ($AN^{\cdot}$) from the consecutive action of resting (ferri-) enzyme, Compound I, and Compound II, while the suicide pathway irreversibly generates modified enzyme containing 1 eq of covalently bound radiolabel/heme derived from ^{14}C-labeled substrate. Modified catalase, as we have shown here, retains 7% of the catalytic activity of native catalase whereas modified peroxidase is 40% as active catalytically as the native enzyme (1). The target for inactivation by the alkanenitronate (AN^-) in the suicide pathway in each case, within experimental error, is Compound II. We regard inactivation as resulting from that fraction of $AN^{\cdot}$ in the $E\cdots AN^{\cdot}$ complex derived from Compound II which fails to diffuse from the complex because it is trapped by reaction with the enzyme (Equation 9).



(9)

In the case of catalase, $AN^{\cdot}$ is trapped by reaction with an amino acid residue in the apoenzyme (Equation 8), whereas, in the peroxidase case, $AN^{\cdot}$ is trapped at a methine carbon on the ferriheme (1). While the peroxidase results support a mechanism of reduction of Compound II involving electron transfer through the edge of the heme moiety (1), the catalase data reported herein implicate an (as yet unidentified) amino acid residue which lies in close proximity to the site of Compound II reduction.

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