ISSN 0006-2979, Biochemistry (Moscow), 2008, Vol. 73, No. 1, pp. 92-96. © Pleiades Publishing, Ltd., 2008.
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Mechanism of Inhibition of Catalase by Nitro and Nitroso Compounds

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Received June 1, 2007
Revision received July 14, 2007

Abstract—Dinitrosyl iron complexes (DNIC) with thiolate ligands and S-nitrosothiols, which are NO and NO^+ donors, share the earlier demonstrated ability of nitrite for inhibition of catalase. The efficiency of inhibition sharply (by several orders in concentration of these agents) increases in the presence of chloride, bromide, and thiocyanate. The nitro compounds tested—nitroarginine, nitroglycerol, nitrophenol, and furazolidone—gained the same inhibition ability after incubation with ferrous ions and thiols. This is probably the result of their transformation into DNIC. None of these substances lost the inhibitory effect in the presence of the well known NO scavenger oxyhemoglobin. This fact suggests that NO^+ ions rather than neutral NO molecules are responsible for the enzyme inactivation due to nitrosation of its structures. The enhancement of catalase inhibition in the presence of halide ions and thiocyanate might be caused by nitrosyl halide formation. The latter protected nitrosonium ions against hydrolysis, thereby ensuring their transfer to the targets in enzyme molecules. The addition of oxyhemoglobin plus iron chelator *o*-phenanthroline destroying DNIC sharply attenuated the inhibitory effect of DNIC on catalase. *o*-Phenanthroline added alone did not influence this effect. Oxyhemoglobin is suggested to scavenge nitrosonium ions released from decomposing DNIC, thereby preventing catalase nitrosation. The mixture of oxyhemoglobin and *o*-phenanthroline did not affect the inhibitory action of nitrite or S-nitrosothiols on catalase.

DOI: 10.1134/S0006297908010148

Key words: dinitrosyl iron complex (DNIC), nitro compounds, nitroso compounds, catalase, inhibition

It was demonstrated earlier that nitrite causes a significant decrease in activity of catalase, the ferriheme-containing enzyme catalyzing decomposition of hydrogen peroxide to water and oxygen in organisms [1, 2]. An explanation of the mechanism of this phenomenon has not yet been proposed. The inhibitory effect of nitrite on catalase could be due to its known ability to incorporate into the coordination sphere of heme iron, like incorporation of azide and cyanide. In fact, formation of nitrite complexes with catalase and other ferriheme-containing enzymes is observed by spectrophotometry, but only at relatively high concentrations of nitrite (millimolar and higher) [3], which exceed by several orders of magnitude its concentrations used in experiments [1, 2].

Cohen and coworkers [1] presume that the inhibitory effect of nitrite on catalase could be determined by its ability to reduce so-called compound I of the enzyme (the complex formed by the catalase subunit with hydrogen peroxide, which is a two-equivalent oxidizer of the second peroxide molecule or some other substances): nitrite competes for compound I with the second peroxide molecule and thereby could decelerate the decomposition of the latter. However, this supposition is inconsistent with the data of our previous studies [4, 5]. They proved that nitrite in the presence of chloride, bromide, or the pseudohalide thiocyanate causes a significant decrease in catalase activity at concentrations several orders of magnitude less than the concentration of hydrogen peroxide (micromolar versus millimolar, respectively). In the absence of halides and pseudohalide, nitrite had the same effect at two orders of magnitude higher concentrations [4, 5]. Note that both in the presence and in the absence of halides the thermal effect of reaction of hydrogen peroxide decomposition into water and oxygen

Abbreviations: Cys-NO) S-nitrosocysteine; DNIC-GS) dinitrosyl iron complex with glutathione; GSH) glutathione; GS-NO) S-nitrosoglutathione; HbO_2) oxyhemoglobin; *p*-NP) *p*-nitrophenol; *o*-Ph) *o*-phenanthroline.

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remained unchanged, thus suggesting identity of reaction mechanism in both cases. The change-over of the decomposition process to either peroxidase or free-radical routes should lead to change in the value of the thermal effect of the reaction [4].

Inhibition of catalase could be due to conversion of nitrite to nitrogen monoxide (NO) that is characterized by high affinity to heme iron [6]. Testing of this hypothesis in experiments on hemolyzed erythrocytes showed that oxyhemoglobin, the effective NO scavenger, had no effect on the inhibitory effect of nitrite on catalase [4, 5]. Thus, if NO is related to catalase inactivation, it is not in free form released into solution. This could affect the enzyme in the ionized NO^+ form comprising nitrosohalogens [7-9]. Drastic increase in catalase inactivation by nitrite in the presence of halogens [4, 5] supports this supposition.

The goal of this study was to elucidate the nature of an agent that is formed from nitrite and is a direct inhibitor of catalase. To do this, we have examined other nitro and nitroso compounds besides nitrite, which can produce NO and NO^+, in particular, dinitrosyl iron complexes (DNIC) of chemical formula $[(RS^-)_2Fe^+(NO^+...^-SR)_2]^-$ and S-nitrosothiols (RS-NO).

MATERIALS AND METHODS

The following chemicals were used in experiments: sodium phosphate monobasic, sodium chloride, hydrogen peroxide, *p*-nitrophenol (*p*-NP), nitroglycerol, furazolidone, $FeSO_4 \cdot 7H_2O$, and sodium nitrate (Reakhim, Russia); *o*-phenanthroline (*o*-Ph), potassium nitrite, catalase from bovine liver, glutathione, and L-nitroarginine (Sigma, USA).

Dinitrosyl iron complex with glutathione (DNIC-GS) was synthesized in the reaction of gaseous NO with 5 mM solution of ferrous iron in distilled water followed by addition (under an atmosphere of NO) of glutathione solution in 15 mM Hepes, pH 7.4 (the ratio Fe^{2+}/glutathione was 1 : 2), according to the method described earlier [10].

S-Nitrosoglutathione (GS-NO) and S-nitrosocysteine (Cys-NO) were prepared by incubation of 0.1 M nitrite with 0.1 M glutathione/cysteine and 0.1 M HCl (1 : 1 : 2 v/v) for 15 min followed by dilution with 40 mM sodium phosphate buffer, pH 6.0, to required concentration. Nitrosothiols were determined by spectrophotometry assuming $\varepsilon_{340} = 930\ M^{-1} \cdot cm^{-1}$ [11].

Effect of oxyhemoglobin was studied using hemolysate of bovine erythrocytes prepared as described in [12]. Activity of catalase present in hemolysate served as indicator of nitrite and other tested substances in the reaction medium. Concentration of oxyhemoglobin was determined by spectrophotometry assuming $\varepsilon_{540} = 1.5 \cdot 10^4\ M^{-1} \cdot cm^{-1}$ [12].

Catalase activity was determined calorimetrically according to the method we have developed earlier. This method is based on recording of heat release kinetics upon decomposition of hydrogen peroxide by catalase, which is identical to kinetics of the peroxide decomposition [4, 5]. The enzyme activity was determined from the slope of the kinetic curve in the initial linear area, which is in direct proportion to the concentration of active enzyme [4, 5]. The initial concentration of added hydrogen peroxide was 9.0 mM.

RESULTS

The data of our study demonstrating the ability of examined substances to inhibit the decomposition of hydrogen peroxide catalyzed by catalase are summarized in the table.

Inhibition of catalase by dinitrosyl iron complex (DNIC-GS) and nitrite. It is evident from the data given in Fig. 1 that the presence of DNIC or nitrite decreased the rate of hydrogen peroxide decomposition by catalase. As this took place, the quantity of heat released until the kinetic curve flattened out was unchanged in comparison with the control. So, both substances caused a decrease in activity of the enzyme without changing the mechanism of the catalyzed reaction, because thermal effects of peroxide decomposition on free-radical (Fenton reaction type) and peroxidase pathways are several times higher [4, 5].

Efficiency of this inhibition, as follows from the data shown in the Fig. 2a, in both cases depended on medium pH and on the presence of halide ions. A decrease in cata-

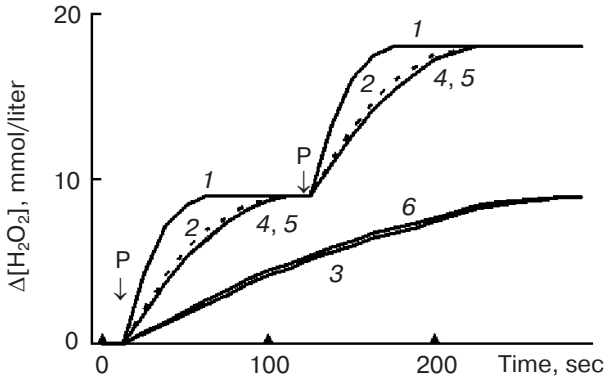

Fig. 1. Kinetic curves of hydrogen peroxide decomposition by catalase in the presence of nitrite and DNIC. *1*) Control; *2*, *3*) 0.7 and 1.4 μM nitrite, respectively; *4*, *5*) DNIC-GS, 0.7 and 1.4 μM, respectively; *6*) 0.7 μM DNIC-GS + 0.7 μM of nitrite. The reaction medium contained 40 mM sodium phosphate buffer, pH 6.5, 9.0 nM catalase, and 158 mM NaCl. The catalase reaction was initiated by addition of 9.0 mM H_2O_2 as indicated by arrows P. The ordinate represents the amount of decomposed peroxide determined by the amount of released heat, based on the thermal effect of the catalase process [4].

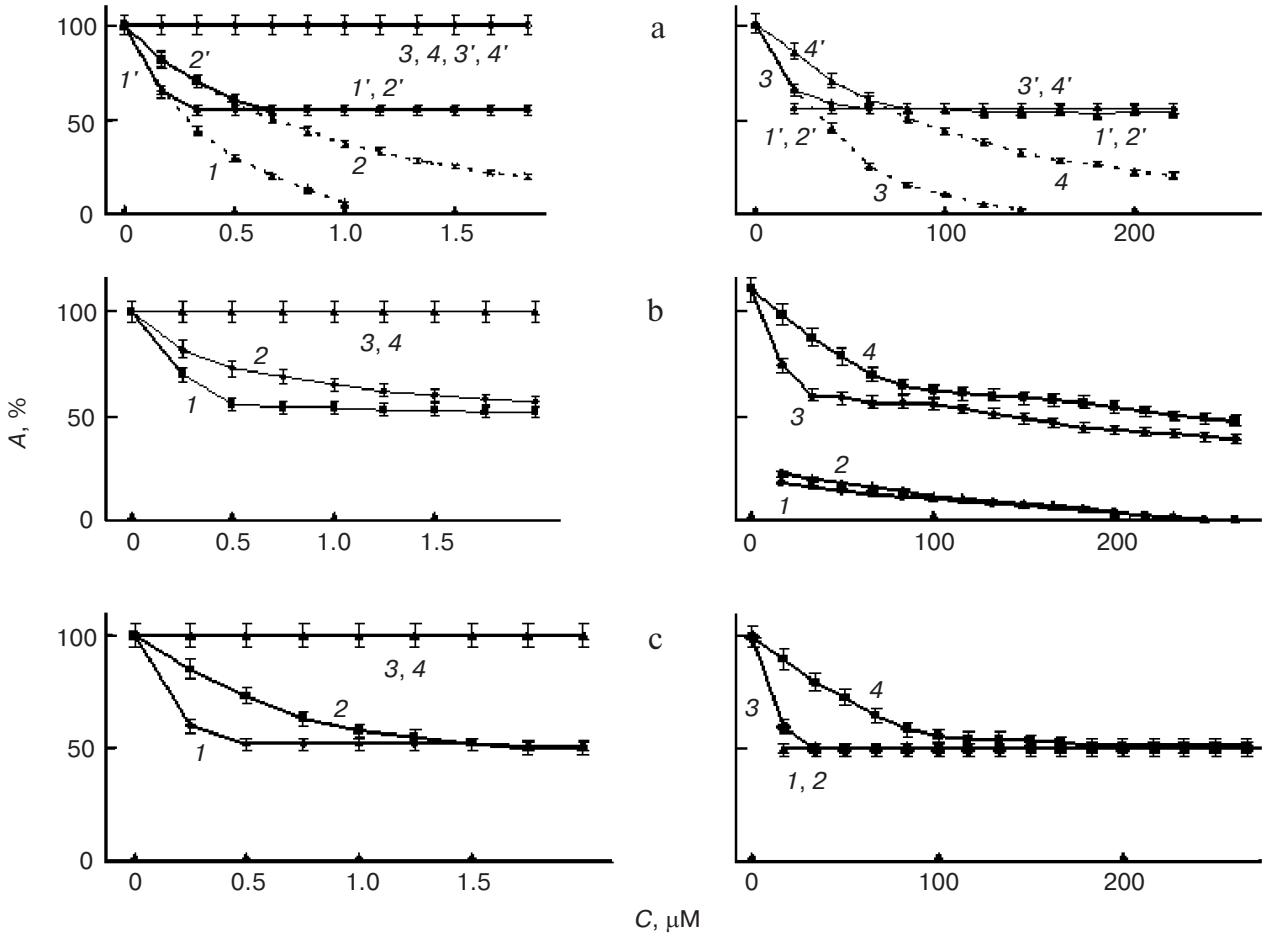

Fig. 2. Dependence of catalase activity (*A*) on concentration of: a) nitrite (*1-4*) and DNIC/GSH (*1'-4'*); b) nitrosoglutathione (GS-NO); c) *p*-nitrophenol (*p*-NP). In all cases the reaction medium contained 40 mM sodium phosphate buffer, 158 mM NaCl, 8.0 nM catalase, and, in the case of *p*-NP, 250 μM $FeSO_4$ and 500 μM glutathione. pH values: *1*) 6.0; *2*) 6.5; *3* and *4* correspond to *1* and *2* without NaCl. Activity of samples containing no tested substances was taken as 100%.

lase activity by 35% at pH 6.5 was achieved at 70-80 μM of DNIC or nitrite, whereas the same result at pH 6.0 was achieved at 25-30 μM concentration of these substances. The inhibition efficiency was elevated by two orders of magnitude and also increased with decrease in pH in the presence of 150 mM chloride or 150 mM bromide or 50 μM thiocyanate [13] (table and Fig. 2a). It is remarkable that the dependence of enzyme inhibition degree on DNIC concentration, both in the presence and absence of chloride, at both pH values achieved a plateau at 40-50% decrease in the enzyme activity. This was not observed in the case of nitrite: the enzyme activity decreased to zero with increase in the inhibitor concentration (Fig. 2a). Addition of nitrite into the medium already containing DNIC at a concentration ensuring a plateau caused further decrease in the enzyme activity (Fig. 1).

When hemolyzed erythrocytes were used, catalase from erythrocytes was inhibited by DNIC and nitrite in the same manner as the commercial enzyme. Note that oxyhemoglobin taken at concentration of 100 μM heme did not influence this process. The iron chelator *o*-phenanthroline added to hemolysate resulted in complete loss of inhibitory activity of DNIC both in the absence and in the presence of chloride. Addition of *o*-phenanthroline to DNIC solution in absence of hemolysate, as well as addition of hemolysate 1 min after *o*-phenanthroline did not lead to the loss of inhibitory effect (table).

The ability of DNIC to lose its inhibitory activity in the presence of oxyhemoglobin and *o*-phenanthroline was retained after incubation of DNIC in the hemolysate–H_2O_2 system in absence of *o*-phenanthroline. When *o*-phenanthroline was subsequently added to the reaction medium (when the kinetic curve of H_2O_2 decomposition in presence of DNIC reached a plateau), the curve observed after repeated addition of hydrogen peroxide was identical to that observed in the control (without DNIC). DNIC retained its inhibitory effect without addition of *o*-phenanthroline (Fig. 3).

Inhibitory effect of nitro- and nitroso compounds on catalase

Substance tested	Substance added							
	K	GSH	Fe	Fe + GSH	HbO_2	*o*-Ph	HbO_2 + *o*-Ph	*o*-Ph + HbO_2
Nitrite	+	+	+	+	+	+	+	+
Nitrite + Fe + GSH	+				+	+	+	+
DNIC-GS	+	+	+	+	+	+	–	+
GS-NO/Cys-NO	+	+	+	+	+	+	+	+
The same + Fe	+	+			+	+	–	+
Nitroarginine, nitroglycerol, nitrophenol, furazolidone	–	–	–	+				
The same + Fe + GSH	+	–			+	+	–	+
Nitrate	–	–	–	–	–	–	–	–

Note: +, inhibitory effect is present (decrease in activity by 40-47%); –, inhibitory effect is absent (no changes in activity of the enzyme).
The concentration of tested substances in the reaction medium was 0.5 μM; Fe ($FeSO_4$), 100 μM; GSH, 100 μM; *o*-Ph, 0.3 mM. In all experiments the reaction medium contained 40 mM sodium phosphate buffer, pH 6.0, 150 mM NaCl, 9.0 nM catalase or HbO_2–hemolysate of erythrocytes (100 μM heme). Fe, GSH, *o*-Ph, and HbO_2 in specified order at intervals of 1 min were added to 5-μM solutions of tested substances in 40 mM sodium phosphate buffer, pH 6.0. Following 5 min of incubation, the substances were placed into the reaction medium (becoming tenfold diluted), and catalase activity was determined after 1 min.
At pH 7.4, the inhibitory activity of all inhibitors presented in the table fell by two orders of magnitude compared with pH 6.0 [4, 13]. In the absence of NaCl, the inhibitory effect of all presented substances fell by two orders of magnitude. The effect of NaCl was retained on its substitution by 150 mM KBr or 50 μM KSCN.
Effects of HbO_2 and *o*-Ph were completely retained after 5 min of incubation of specified substances in the peroxide–catalase system.

o-Phenanthroline did not influence the inhibitory capability of nitrite both in the presence and in the absence of oxyhemoglobin. Addition of ferrous iron and glutathione into the solution also had no influence on this activity (table).

Inhibition of catalase by S-nitrosoglutathione and S-nitrosocysteine. GS-NO inhibited catalase in a manner analogous to that of DNIC in efficacy and dependence on pH and halides (table and Fig. 2b). However, unlike the effect of DNIC, a gradual decrease in the enzyme activity below 50% activity was observed with increasing GS-NO concentration (Fig. 2b). Unlike DNIC, GS-NO did not lose the inhibitory activity in the oxyhemoglobin + *o*-phenanthroline system. This loss only occurred when ferrous iron was added to GS-NO before addition of *o*-phenanthroline (table). Cys-NO inhibited catalase in the same manner as GS-NO (table).

Effect of *p*-nitrophenol (*p*-NP) and other nitro compounds on catalase activity. *p*-Nitrophenol itself did not inhibit catalase either in the presence or absence of halides (table). The inhibitory activity of *p*-NP solution appeared upon addition of the mixture of glutathione and ferrous iron, which presumably was due to DNIC formation with involvement of nitric oxide released from *p*-NP. In this case, the inhibitory effect of *p*-NP on catalase in all parameters was the same as the effect of DNIC: dependences on pH and halides coincided. The dependence of inhibition degree on *p*-NP concentration in the presence of iron and glutathione also formed a plateau at 40-50% decrease in the enzyme activity (Fig. 2c). As in the case of DNIC, addition of *o*-phenanthroline into the *p*-NP–Fe^{2+}–GSH–HbO_2 system led to the loss of inhibitory effect of this system. All of the nitro compounds presented in the table (except for nitrate)—nitroarginine, nitroglycerol, and furazolidone—inhibited catalase in the same manner as *p*-NP (table).

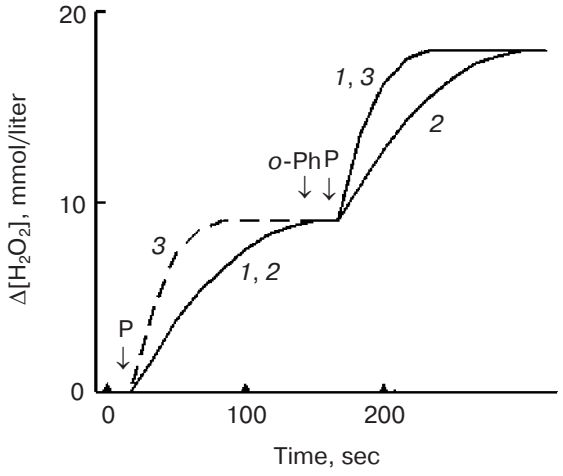

Fig. 3. Effect of hemoglobin and *o*-phenanthroline on the inhibitory activity of DNIC-GS. The DNIC-GS concentration was 1.0 μM (curves *1* and *2*). Sample *3* is a control. The reaction medium in all cases contained 40 mM sodium phosphate buffer, pH 6.0, 158 mM NaCl, and erythrocyte hemolysate with hemoglobin subunit concentration 100 μM. The catalase reaction was initiated by addition of 9 mM H_2O_2 (arrows P). Addition of 0.5 mM *o*-phenanthroline into sample *1* is denoted as *o*-Ph. Denotations for axes are the same as in Fig. 1.

DISCUSSION

As it follows from the kinetic curves of hydrogen peroxide decomposition by catalase presented in the Fig. 1, the weakening of this process in the presence of nitro- and nitroso compounds presented in the table is due to inhibition of the enzyme rather than change in the mechanism of the catalyzed reaction. This is evident from conservation of the thermal effect of the reaction in the presence of the above-mentioned substances, since peroxidase and free-radical pathways of peroxide decomposition have different thermal effects [4, 5].

The substances presented in the table can be divided into two groups differing in effect of HbO_2 + *o*-phenanthroline on their inhibitory effect on catalase. The first group includes nitrite and S-nitrosothiols, whose inhibitory effect did not depend on this combination. The second group includes DNIC with thiol-containing ligands and nitro compounds, which lose their inhibitory activity in the presence of HbO_2 and *o*-phenanthroline. Note that nitro compounds only gain the ability to inhibit catalase after treatment with the iron + glutathione mixture, which apparently leads to transformation of these substances into DNIC with glutathione as a ligand. Such transformation was earlier demonstrated for nitroglycerol [14]. S-Nitrosothiols undergo the same transformation after addition of iron, which transfers them from the first into the second group [15, 16].

Since the known NO scavenger oxyhemoglobin did not influence the inhibitory effect of all these substances on catalase, it is arguable that, as in the case of nitrite [4, 5], DNIC and S-nitrosothiols inhibit this enzyme as donors of nitrosonium NO^+ ions rather than donors of neutral NO molecules [17, 18]. The nitrosonium ions cause nitrosation of the catalase molecule, possibly at the sulfur atoms of its cysteine residues [19], which leads to its inactivation. The sharp growth of this effect in the presence of halogens is evidence for this supposition. It is known that both halides and thiocyanate increase by many times the nitrosation potential [7], which is due to their ability to form, together with nitrosonium ions, nitrosohalogens preventing hydrolysis of these ions and thus providing their transport to their targets on the enzyme molecule.

The nitrosation effect of nitrite on catalase might be due to protonation of nitrite anions, either in zones with reduced pH or within the enzyme itself with involvement of proton-donating amino acid residues. Thus produced nitrous acid can act as nitrosating agent. The spectrophotometric data suggest the appearance of nitrosonium ions in catalase molecules contacting high concentrations (10^{-2}-10^{-3} M) of nitrite [3].

Suppression of the inhibitory effect of DNIC on catalase in the presence of HbO_2 and *o*-phenanthroline might be due to competitive scavenging of nitrosonium ions, which are released from DNIC upon decomposition of these complexes under the action of *o*-phenanthroline, by HbO_2 molecules. In the absence of this competition, these ions reach their targets on the catalase molecules and thereby cause its inactivation. It is possible that due to hydrolysis some portion of the nitrosonium ions was transformed into nitrite, which had the same effect on the enzyme.

Incomplete (unlike the effect of nitrite) inhibition of catalase by DNIC and S-nitrosothiols is possibly due to steric factors, namely the greater size of DNIC and S-nitrosothiol molecules compared with nitrite. Catalase is composed of four subunits [19]. Nitrosation of two of four subunits of catalase by such large molecules as DNIC and S-nitrosothiols might hamper their contact with the other two subunits and thereby weaken the transfer of nitrosonium ions directly by these substances to their targets on catalase.

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