Vol. 105, No. 1, 1982 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
March 15, 1982 Pages 127-132

INVOLVEMENT OF PEROXIDE AND SUPEROXIDE IN THE OXIDATION OF HEMOGLOBIN BY NITRITE

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Received December 10, 1981

SUMMARY: Hydrogen peroxide exhibits significant catalytic activity for the nitrite ion oxidation of hemoglobin to methemoglobin under aerobic conditions. Peroxide is not a direct oxidant of hemoglobin, but is required for the autocatlytic transformation that is the dominant process in the nitrite oxidation. Catalase and superoxide dismutase inhibit the onset of the autocatalytic stage of hemoglobin oxidation. The composite results suggest a mechanism for hemoglobin oxidation that is initiated by the formation of monomeric nitrogen dioxide and propagated by the transfer of superoxide from oxyhemoglobin to nitrogen dioxide, oxidation of the resulting peroxynitrate ion by peroxide, and superoxide oxidation of nitrite ion to nitrogen dioxide and peroxide.

The oxidation of hemoglobin to methemoglobin by nitrite ion under aerobic conditions has been the subject of numerous investigations and is recognized to be exceptionally complex (1-5). The formation of methemoglobin occurs in two stages: a slow initial transformation that appears to be first order in nitrite (3,6) and a rapid subsequent autocatalytic oxidation (7) that is the dominant process. Hydrogen peroxide is generated, although in unspecified amounts (6,8), and the initial step in the oxidative transformation is reported to involve a single electron transfer from nitrite to the bound dioxygen of oxyhemoglobin (6). Recently, the superoxide anion has been implicated in the autocatalytic stage of the oxidation (9) and, although a composite pathway has been advanced to explain the autocatalytic behavior of nitrite oxidations, the proposed mechanism is neither autocatalytic nor consistent with the chemical behavior of the proposed intermediates.

In the course of our investigations of the biochemical effects of nitrogen oxides and nitrosyls, we have discovered that hydrogen peroxide exhibits

0006-291X/82/050127-06$01.00/0

significant catalytic activity for the nitrite ion oxidation of oxyhemoglobin. From this previously unreported observation, as well as the singular and composite inhibitory effects of catalase and superoxide dismutase, we can now report a rational mechanistic sequence of reactions that explain the complex oxidation of hemoglobin by nitrite.

MATERIALS AND METHODS

Human hemoglobin A (type IV), bovine liver catalase (EC 1.11.1.6), and human superoxide dismutase (EC 1.15.1.1) were obtained from Sigma Chemical Co. Hemoglobin was reduced with excess sodium dithionite and purified by passing the resulting aqueous solution through a G-25 Sephadex column using oxygen-saturated 0.05 M phosphate buffer at pH 7.0. Reagent sodium nitrite and stabilized stock 30% hydrogen peroxide solutions (Mallinckrodt AR) were employed. Stock solutions of all reagents were prepared in oxygen-saturated 0.05 M phosphate buffer at pH 7.0 at 25.0°C and maintained under an oxygen atmosphere.

Reactions were initiated with the injection, using a gas tight syringe, of a concentrated nitrite solution into the oxyhemoglobin solution. For reactions that employed hydrogen peroxide, nitrite and peroxide were added simultaneously. Catalase and superoxide dismutase were added from freshly prepared stock solutions to the hemoglobin reactant prior to the introduction of nitrite. Time courses for hemoglobin oxidation were determined at 25.0° C by monitoring the decrease in absorbance at 576 nm with time using a Pye Unicam SP8-200 spectrophotometer. For each set of experiments involving the introduction of peroxide, catalase, superoxide dismutase, or any combination thereof, control time courses for reactions employing only nitrite were performed at concentrations of nitrite and hemoglobin identical to those in experiments with added reagent. Methemoglobin was the only spectrophotometrically visible product from these oxidative transformations.

RESULTS

Fig. 1A describes the effect of peroxide on the time course for nitrite oxidation of hemoglobin. Without peroxide, the time course for hemoglobin oxidation is described by the characteristic pattern of a slow initial transformation followed by a rapid autocatalytic process with $t_{\frac{1}{2}}$, the time at which one-half of the reactant hemoglobin has been oxidized to methemoglobin, equal to 340 sec. The addition of peroxide in amounts as small as 0.035 molar equivalent based on heme iron causes a significant decrease in $t_{\frac{1}{2}}$, and parallel experiments with increased amounts of peroxide describe additional decreases in $t_{\frac{1}{2}}$. In the absence of nitrite, but with a concentration of peroxide equal to the highest concentration employed for these investigations, peroxide oxidation of hemoglobin is slower than even the slow stage of the nitrite ion oxidation.

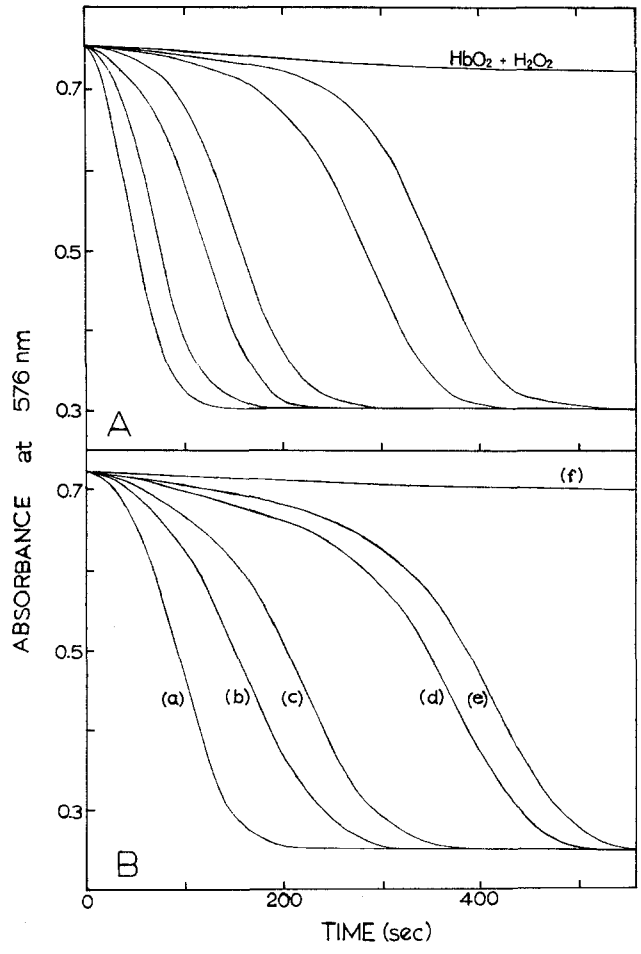

Fig. 1. Time courses for the nitrite ion oxidation of hemoglobin in oxygen-saturated 0.05 M phosphate buffer at 25.0°C. A: Effect of peroxide. $[HbO_2]$ = 49 μM, $[NaNO_2]$ = 350 μM, $[H_2O_2]/[HbO_2]$ = 1.0, 0.50, 0.20, 0.10, 0.035, and 0.00, respectively, when viewed from left to right. The upermost time course is that due to the peroxide oxidation of hemoglobin in the absence of nitrite, $[H_2O_2]/[HbO_2]$ = 1.0. B: Effect of catalase on the time course for oxidation. $[HbO_2]$ = 47 μM, $[NaNO_2]$ = 330 μM, (a)-(d) and (f) $[H_2O_2]/[HbO_2]$ = 0.50, (e) $[H_2O_2]/[HbO_2]$ = 0.00. Units of catalase: (a) 0, (b) 176, (c) 534, (d) 880, (e) 0, and (f) 1760.

Fig. 1B describes the effect of catalase on the time course for nitrite oxidation of hemoglobin when these reactions are performed in the presence of 0.50 molar equivalent of peroxide, based on heme iron. As the amount of catalase is increased so also is $t_{1/2}$ until, above 900 units of catalase, the $t_{1/2}$ for oxidation of hemoglobin is significantly greater than that for reactions performed in the absence of both peroxide and catalase.

Table 1 describes the effects of catalase and of superoxide dismutase on $t_{1/2}$ for the oxidation of hemoglobin by nitrite in the absence of added per-

Table 1. Effects of catalase and superoxide dismutase on $t_{1/2}$ for the oxidation of hemoglobin by nitrite.[a]

$[NO_2^-]/[HbO_2]$	units catalase	$t_{1/2}$, sec	$[NO_2^-]/[HbO_2]$	units superoxide dismutase	$t_{1/2}$, sec
20.0	0	166	10.0	0	240
20.0	420	194	10.0	756	312
20.0	840	312	10.0	1260	460
20.0	1200	900	10.0	1680	1040

[a] $[HbO_2]$ = 47 μM. Reactions performed at 25.0°C in oxygen-saturated 0.05 M phosphate buffer at pH 7.0.

oxide. Clearly, both catalase and superoxide dismutase inhibit the onset of the autocatalytic stage of hemoglobin oxidation. That relatively high levels of enzyme are required and their effects on $t_{1/2}$ are not linear with the amounts of catalase or superoxide dismutase employed may be a consequence of nitrite inhibition of enzyme activity, which has been suggested for catalase (8).

Although not specified in previous reports of hemoglobin oxidation by nitrite, the time courses for these transformations are markedly affected by the concentrations of nitrite and hemoglobin and are subject to sporadic behavior. As a consequence, experiments were performed at constant concentrations of hemoglobin and nitrite in oxygen-saturated solutions, and control experiments with only nitrite or with nitrite and peroxide addition were performed before and after each experiment. Uniformity in $t_{1/2}$ (± 4 sec.) was consistently observed for reactions performed in the presence of peroxide except when catalase was employed in amounts that produced $t_{1/2}$ greater than that observed in the absence of peroxide. Nitrite ion is not oxidized to nitrate by peroxide under the conditions employed for these investigations.

DISCUSSION

Results described in Fig. 1 demonstrate the significant activity of peroxide in promoting the onset of the autocatalytic stage of hemoglobin oxidation by nitrite. Peroxide is not a direct oxidant of hemoglobin under these conditions (Fig. 1A), as has been previously suggested (9), but is

required for the autocatalytic transformation. That catalase acts upon the reaction system to inhibit the onset of the autocatalytic oxidation (Fig. 1B and Table 1) lends further evidence to a catalytic role for peroxide.

The participation by superoxide in the autocatalytic oxidation has recently been documented (9) and is confirmed in this investigation (Table 1). However, the origin of superoxide in this oxidative transformation has not been adequately explained. Based on investigations by Wallace and Caughey (6), nitrite oxidation of oxyhemoglobin occurs by electron transfer from nitrite to the bound dioxygen of oxyhemoglobin to produce methemoglobin, peroxide, and nitrogen dioxide:

$$HbO_2 + NO_2^- \longrightarrow Hb^+ + O_2^{2-} + NO_2 \quad (1)$$

This process represents the slow stage of hemoglobin oxidation but, with the production of nitrogen dioxide and peroxide, initiates the following set of reactions that satisfy the autocatalytic condition:

$$HbO_2 + NO_2 \longrightarrow Hb^+ + O_2NOO^- \quad (2)$$

$$O_2NOO^- + O_2^{2-} \longrightarrow NO_2^- + 2O_2^- \quad (3)$$

$$2O_2^- + 2NO_2^- \longrightarrow 2O_2^{2-} + 2NO_2 \quad (4)$$

Thus, through reaction with oxyhemoglobin which serves as a superoxide donor (10), nitrogen dioxide forms the reactive peroxynitrate ion which, in its interaction with peroxide, produces two superoxide ions per nitrogen dioxide initiator. Superoxide oxidation of nitrite (eq. 4) completes the catalytic cycle.

The formation of peroxynitrate by superoxide transfer from oxyhemoglobin to nitrogen dioxide (eq. 2) is consistent with the parallel formation of peroxynitrite in reactions of oxyhemoproteins with nitric oxide (11). Peroxynitrate production from superoxide and nitrogen dioxide has previously been observed (12) and, although peroxide oxidation of this reactive chemical species has not been directly identified, superoxide formation constitutes an observed outcome of peroxynitrate decomposition (13). Superoxide oxidation of chemical sources for nitrite that result in the production of nitrogen dioxide is well established (12,14).

The reaction sequence that is proposed to account for the autocatalytic stage of hemoglobin oxidation by nitrite is consistent with the observed acceleration by peroxide and superoxide and with the inhibition of this oxidation by catalase and superoxide dismutase. Nitrate formation, which is a consequence of this transformation (3), can be expected to arise from nitrogen dioxide dimerization or from oxygen transfer from peroxynitrate to nitrite. Although additional details concerning oxyhemoprotein oxidations by nitrite remain to be resolved, monomeric nitrogen dioxide and peroxynitrate are clearly implicated as the functional causes for this complex transformation.

ACKNOWLEDGMENT: This research was supported by PHS Grant ES 01673 from the National Institute of Environmental Health Sciences. We are grateful to James W. Hoekstra for his preliminary investigations.

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