MECHANISMS OF HYDROGEN PEROXIDE FORMATION IN LEUKOCYTES: THE NAD(P)H OXIDASE ACTIVITY OF MYELOPEROXIDASE

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SUMMARY: The NADPH oxidase activity of polymorphonuclear leukocyte granules has not previously been attributed to myeloperoxidase because of its relative insensitivity to cyanide and its activation by aminotriazole. However it has been found that the NAD(P)H oxidase activity of myeloperoxidase or horseradish peroxidase was little affected by 2.0 mM cyanide although the peroxidase activity was nearly completely inhibited by 0.1 mM cyanide. Furthermore, the NAD(P)H oxidase activity of myeloperoxidase was considerably enhanced by aminotriazole although the peroxidase activity was inhibited.

The bactericidal activity of leukocytes has been attributed to H_2O_2 formed during the immediate increase in cyanide resistant respiration that accompanies phagocytosis, and results in increased glucose oxidation through the hexose monophosphate shunt [reviewed by Klebanoff (1) and Karnovsky (2)]. The mechanism of H_2O_2 formation is not clear but NAD(P)H oxidase activities found in subcellular fractions and which form H_2O_2 have been implicated (2, 3). The NADPH oxidase located in azurophil granules has a pH 5 optimum, is enhanced by Mn^{2+}, and can utilize NADH (3, 4). Myeloperoxidase is located in azurophil granules (4) and has an NAD(P)H oxidase activity with similar properties (5). Although Roberts and Quastel (6) suggested some time ago that myeloperoxidase was responsible for the H_2O_2 formation accompanying phagocytosis, other investigators have ruled this out because peroxidase is readily inactivated by cyanide (7) and aminotriazole (4). The NADPH oxidase activity of granules was however relatively insensitive to cyanide (3) and was enhanced by aminotriazole (4). This report shows that the NAD(P)H oxidase activities of crystalline horseradish peroxidase or a purified preparation of myeloperoxidase were also unaffected by cyanide although their peroxidase activities were inhibited. Aminotriazole considerably enhanced oxidase activity and inhibited peroxidase activity of myeloperoxidase.

MATERIALS AND METHODS

Horseradish peroxidase used was crystallized from Sigma type VI. Cetavlon[1] was the most effective agent in solubilizing myeloperoxidase from leukocyte

[1] Abbreviation: Cetavlon, cetyl trimethylammonium bromide

granules (8) and was used in the following partial purification of myeloperoxidase. Polymorphonuclear leukocytes were extracted with 30 ml of 0.9% NaCl solution from male guinea pigs (300-500 g) 15 hours after an intraperitoneal injection of 30 ml of 0.9% NaCl solution containing 1% neutralized sodium caseinate. The leukocytes were centrifuged (500 g for 5 minutes) and washed once with ice cold 0.25 M sucrose. The leukocyte content averaged 95% with a yield of 3×10^9 cells. Homogenization of the cells, suspended in 10 ml of 0.34 M sucrose at 0°C, was carried out with a teflon pestle for 4 minutes. The homogenate was centrifuged (500 g for 10 minutes) to remove unbroken cells and nuclei. The granules were sedimented by centrifuging the resulting supernatant at 13,000 g for 15 minutes. Cetavlon (final concentration of 0.05%) was added to the granule fraction in 7 ml 0.2 M NaCl and the suspension was centrifuged (105,000 for 60 minutes) to remove insoluble residue. Pharmacia CM-cellulose (CM-50) was swollen with distilled water for 2 days and washed with 25 mM acetate buffer (pH 4.7) containing 0.2 M NaCl solution. The ion-exchange resin was packed in a column (1.2 cm diameter, approximately 30 cm high), and approximately 3 cm of Pharmacia Sephadex G-100 was layered on the top of it. The granule extract (5 ml) was introduced onto the column and elution was performed at 4°C with a continuous gradient of NaCl (0.2-1.2 M) containing 25 mM acetate buffer (pH 4.7). Eluate was collected in 5 ml fractions.

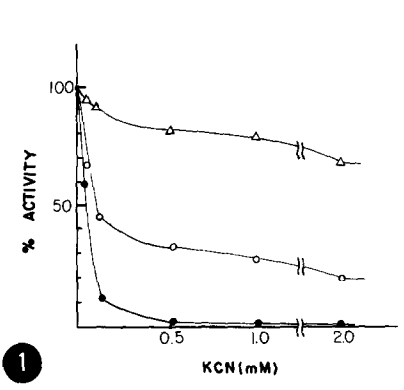
The NAD(P)H oxidase was assayed by following the oxygen consumption with a Clark oxygen electrode with a 1.5 ml vessel at 37°C. The reaction mixture contained the following: 50 mM phosphate buffer (pH 6.0), 0.5 mM $MnCl_2$ and 1.0 mM NAD(P)H. The 2,4 dichlorophenol-stimulated NAD(P)H oxidase was measured by following the decrease in light absorption at 340 nm at 37°C. The assay medium contained 50 mM phosphate buffer (pH 6.0), 0.1 mM NAD(P)H, 0.5 mM $MnCl_2$ and 1.0 mM 2,4 dichlorophenol in a 1.5 ml volume.

Peroxidase was assayed with guaiacol as substrate (9). The reaction mixture contained 10 mM guaiacol, 0.3 mM H_2O_2 and 50 mM phosphate buffer (pH 6.0).Protein was estimated by Lowry's method (10).

RESULTS

Effect of cyanide on NAD(P)H oxidase activity of horseradish peroxidase

As shown in Fig 1, the effect of cyanide on horseradish peroxidase depends upon the reaction measured. The peroxidase activity was almost completely inhibited by 0.1 mM cyanide. The NADH oxidase activity was not appreciably inhibited by even much higher concentrations of cyanide (2.0 mM). However, 2,4 dichlorophenol (1 mM) stimulated the NADH oxidase activity ten fold and the activity was now cyanide sensitive. Similar results and rates were obtained with NADPH.



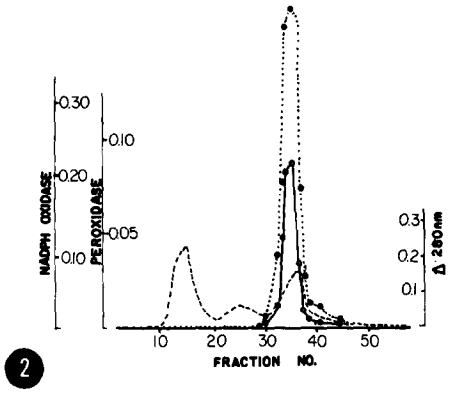


Fig. 1. Effect of cyanide on the oxidase and peroxidase activity of horseradish peroxidase. Activities were assayed as described in methods. —△— NADH oxidase activity (1.2 purpurogallin units assayed). 100% value was 25 n-moles NADH/minute/unit. —○— 2,4 dichlorophenol stimulated NADH oxidase activity (0.12 units). 100% value was 250 n-moles NADH/minute/unit. —●— Peroxidase activity (0.12 units). 100% value was 2.2 μmoles tetraguaiacol formed/minute/unit.

Fig. 2. Ion-exchange chromatography of partially purified myeloperoxidase on CM cellulose;- - - -protein (absorbance at 280 nm); —○— guaiacol peroxidase (μmoles/minute/0.1 ml);··●··NADH oxidase with dichlorophenol (μmoles/minute/ml).

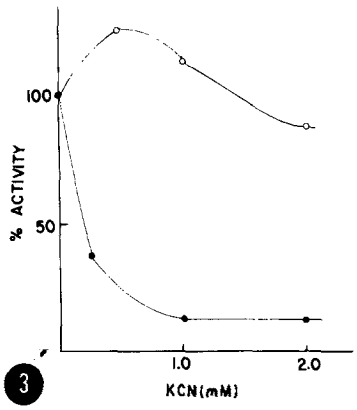
Properties of the NAD(P)H oxidase activity of myeloperoxidase

Approximately a thirty fold increase in the specific activity of myeloperoxidase was obtained by the purification procedure and as indicated in Fig 2 the peroxidase and oxidase activity were observed in the same single peak after CM cellulose chromatography.

The NADPH oxidase activity of this partially purified myeloperoxidase was enhanced four fold by a low concentration of H_2O_2 (.05 mM). Oxidase activity with NADH was similar to that with NADPH. In Fig 3 it can be seen that the oxidase activity was slightly stimulated by cyanide (2.0 mM) although the peroxidase activity was nearly completely inhibited with 0.1 mM cyanide. Dichlorophenol (1.0 mM) enhanced the activity nine fold and cyanide (1.0 mM) now inhibited 80% of the activity. In Fig 4, aminotriazole (2.0 mM) enhanced the oxidase activity five fold although the peroxidase activity was inhibited 58%. The rate of NAD(P)H oxidase activated by aminotriazole decreased gradually as a function of time within a few minutes. The results in Fig 4 are expressed in terms of initial rates.

DISCUSSION

The classical peroxidase activity is the oxidation of donors using H_2O_2 as the acceptor. However peroxidases, particularly at a pH lower than



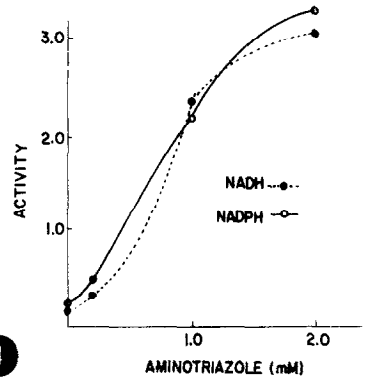


Fig. 3. Effect of cyanide on the oxidase activity of myeloperoxidase —O— NADPH oxidase activity of myeloperoxidase. 15 μg protein was assayed with 50 μM H_2O_2 present. The initial rate of oxygen uptake for the 100% value was 1.5 μmoles/minute/mg protein. —●— 2,4 dichlorophenol stimulated NADPH oxidase activity (15 μg). The 100% value was 2.8 μmoles/minute/mg protein.

Fig. 4. Effect of 3 amino-1,2,4-triazole on NADPH and NADH oxidase activity of myeloperoxidase. The activity is given in μ moles/minute/mg protein.

6.0, are also known to catalyze the oxidation of certain donors with O_2 as the acceptor. At a neutral pH Mn^{2+}, certain phenols, and H_2O_2 enhance the activity considerably (11). The oxidation reaction is autocatalytic and NAD(P)H oxidase activity is mediated by H_2O_2, O_2^-, and NAD· free radical, which are regenerated and multiplied (12). NADH is a redogenic substrate and NAD· can directly reduce molecular O_2 and peroxidase itself. A mechanism of the NAD(P)H oxidase activity of myeloperoxidase expressed in terms of the various intermediate states of peroxidase (12) is given in Fig 5. The insensitivity of the oxidase to cyanide may be because peroxidase exists as compound III during the reaction with oxygen present (13) and cyanide (10 mM) does not affect the formation of compound III from ferrous peroxidase (14). Cyanide combines with the ferric form of peroxidase to give an inactive reversible complex and can also stimulate dihydroxyfumarate oxidase activity (15, 16) possibly because the higher redox potential of the complex enhances the free radical catalyzed reduction of the ferric form (15).

Phenols enhance the oxidase activity and the reaction is now cyanide sensitive (11). Phenols are oxidogenic donors and promote the formation of the NAD· radical and consequently O_2^- radicals (12). This may shift the steady state from compound III (17) to compound II with the cyanide sensitive peroxidase reaction being rate limiting. Alternatively the super-

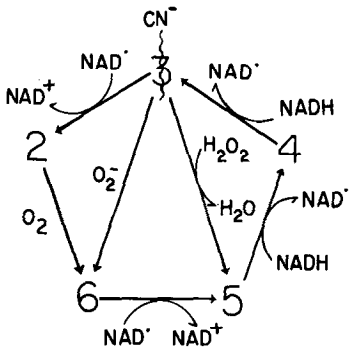


Fig. 5. Proposed scheme for the cyclic reduction and oxidation transitions of myeloperoxidase during the NAD(P)H oxidase reaction.

The formal oxidation states 2, 3, 4, 5 and 6 shown are ferrous, ferric, compound II, I, and III forms of peroxidase, respectively. NADH is oxidized by the higher oxidation states of peroxidase (4 and 5) to the one electron oxidized species, NAD·. The latter reduce states 3 and 6 and also react with oxygen to form superoxide radical anions (O_2^-) which dismutate to H_2O_2. Some O_2^- react with NADH to form H_2O_2 and NAD· and propagate a chain reaction. Dichlorophenol (DCP) enhances the oxidase activity as it is a more effective hydrogen donor than NAD(P)H for state 4 and the phenoxy radicals formed can oxidise NADH to NAD·. Cyanide (CN^-) complexes state 3 and aminotriazole acts on state 4.

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oxy radicals may short circuit the oxidase reaction by forming compound III directly from the ferric form (18).

Aminotriazole inhibits rapidly and reversibly thyroid peroxidase (19), lactoperoxidase (20) and myeloperoxidase (21) with little effect on horseradish and other peroxidases (20). Aminotriazole inhibited the guaiacol peroxidase activity and activated the NAD(P)H oxidase activity of myeloperoxidase and suggests that aminotriazole can act as an oxidogenic peroxidase donor.

Cyanide and aminotriazole cannot therefore be used to differentiate the NADPH oxidase activity of azurophil granules (3) from myeloperoxidase oxidase activity. The lack of latency of NADPH oxidase of granules in contrast to myeloperoxidase (21, 8) may also be explained if NAD(P)H, unlike guaiacol, can penetrate the granule membrane. Further research may also explain the increased NADPH oxidase activity of granules from phagocytizing leukocytes, changes of kinetic properties and increased cyanide resistance (3, 22). During phagocytosis, myeloperoxidase is released into the phagolysosomes as a result of fusion with the granules (23, 24). The latency of peroxidase in granules is less and the levels of cytosolic peroxidase are higher (25).

Myeloperoxidase may not be involved in the phagocytosis induced re-

spiratory burst as NAD(P)H oxidase activity is inhibited by catalase and superoxy dismutase (5) and aminotriazole does not enhance oxygen uptake by intact leukocytes (21). Furthermore myeloperoxidase deficient leukocytes show a normal metabolic response to phagocytosis (26) and leukocytes from patients with chronic granulomatous disease show no respiratory burst but cytochemical studies indicate that myeloperoxidase release is normal (24). A plasma membrane located NAD(P)H oxidase has been discovered which could account for the respiratory burst and peroxide formation induced by phagocytosis (27).

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