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SPECTROPHOTOMETRIC STUDIES ON NAD(P)H OXIDASE OF LEUKOCYTES

1. THE RELATIONSHIP BETWEEN GRANULE-NAD(P)H OXIDASE AND MYELOPEROXIDASE

KATSUKO KAKINUMA[a,*] and BRITTON CHANCE [b]

[a] *The Tokyo Metropolitan Institute of Medical Science, Honkomagome 3—18, Bunkyo-ku, Tokyo (Japan) and* [b] *Johnson Research Foundation, University of Pennsylvania, Philadelphia, Pa. 19104 (U.S.A.)*

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Summary

The NAD(P)H oxidase located in granules from resting leukocytes seems to be identical with myeloperoxidase on the basis of the following results. Spectral changes representing the difference between granules with and without NAD(P)H under various conditions represented the formation of compound III of myeloperoxidase, corresponding to the oxidation of NAD(P)H. The KCN difference spectrum of granules from both resting and phagocytizing leukocytes was in agreement with the KCN difference spectrum of myeloperoxidase. The affinity of KCN for myeloperoxidase was the same in both resting and phagocytizing leukocytes. The KCN-sensitive portion of NAD(P)H oxidase of granules from phagocytizing leukocytes seems to be identical with isolated myeloperoxidase and the myeloperoxidase of resting leukocytes. The KCN-insensitive oxidation of NAD(P)H by granules from phagocytizing leukocytes has not been found to be identical with myeloperoxidase.

Introduction

Polymorphonuclear leukocytes exposed to inert particles or bacteria exhibit metabolic changes such as an increase of KCN-insensitive respiration, of H_2O_2 production and of glucose oxidation through the hexose monophosphate path-

* To whom correspondence should be addressed.

way [1—3]. The explanations which have been offered for the metabolic changes are as follows. Evans and Karnovsky [4,5] have postulated that the increase of oxygen uptake is supported by an increase of oxidation of NADH. On the other hand Quastel and co-workers [2,6] have postulated that the oxidation of NADPH may be accomplished by a reaction involving myeloperoxidase. According to one hypothesis, that of Rossi and co-workers [7—9], a sharp increase in the specific activity of a granule-NADPH oxidase from phagocytizing leukocytes would be in accord with the respiratory stimulation.

In this paper a spectrophotometric study was applied to cellular and subcellular fractions of leukocytes in order to determine the nature of granule-NAD(P)H oxidase. On the basis of spectra and the oxidation of NAD(P)H, the activity of NAD(P)H oxidase in granules was discussed in relation to myeloperoxidase. Subsequently the effect of KCN on the spectra of resting cells was compared to that of phagocytizing cells in order to clarify whether the activity of myeloperoxidase is related to the KCN-insensitive respiration in granules of phagocytizing cells.

Materials and Methods

Guinea pig polymorphonuclear leukocytes were prepared according to the method of Sbarra and Karnovsky [1] with a slight modification. A guinea pig was injected intraperitoneally with 30 ml of 2% sterilized sodium caseinate in saline. After 16 h, the peritoneal exudate was collected in a plastic tube and centrifuged at 120 $\times g$ for 5 min at 4° C. The cell suspension was washed with Ca^{2+}-free Krebs-Ringer phosphate buffer (pH 7.4) and then received a brief treatment with 0.2% NaCl solution to lyse red cells. Cell number was counted by a Coulter Counter (Coulter Electronics Inc.). Granules were prepared by the method of Patriarca et al. [9] with a slight modification. The packed cells were resuspended in 0.34 M sucrose ($2 \cdot 10^8$ cells/ml) and homogenized at 0° C for 4 min (100 strokes). The homogenate was centrifuged at 480 $\times g$ for 15 min to remove cell debris and nuclei. The supernatant was then centrifuged at 13 000 $\times g$ for 20 min at 4° C. The sedimented granules were suspended in a small amount of 0.34 M sucrose. The final supernatant was used for the cytosol fraction.

For comparison of difference spectra between granules of resting and phagocytizing leukocytes, cells were incubated with polystyrene particles (100 particles/1 cell) for 5 min at 37° C and then centrifuged at 120 $\times g$ for 10 min. The packed cells were homogenized as described above. The soluble fraction of granules was prepared by the method of Patriarca et al. [10] except for the temperature and the duration of the solubilization. Granules were suspended in 0.1% cetyltrimethylammonium bromide (cetavlon) in 40 mM phosphate buffer (pH 7.0) and stirred at room temperature (25° C) for 20 min. The supernatant of the suspension was divided from granules by centrifugation at 20 000 $\times g$ for 25 min at 4° C.

Myeloperoxidase was prepared from stored granules at —20° C. Following solubilization of granules as described above, the soluble fraction was dialyzed against 25 mM acetate buffer (pH 4.7) containing 0.18 M NaCl for 24 h at

4° C and chromatographed as follows. The soluble fraction was laid on the column packed with CM-cellulose (Pharmacia CM-50) previously washed and swollen with 25 mM acetate buffer (pH 4.7) containing 0.18 M NaCl, and then washed with the same buffer. The column was eluted with a continuous gradient of NaCl (0.18—1.2 M) in 25 mM acetate buffer (pH 4.7). Fractions which exhibited the peak of peroxidase activity were collected and dialized against 0.1 M phosphate buffer (pH 7.0) for 24 h. All procedures were carried out below 5° C except for the solubilization with cetavlon. The enzyme used in this experiment had a ratio of $A_{430\ nm}/A_{280\ nm}$ of 0.6. The activity of peroxidase was measured with the guiacol test according to the method of Chance and Maehly [11]. The activity of NADH or NADPH oxidase was assayed with a Hitachi double-beam spectrophotometer model 356 as previously reported [12]. Protein was estimated according to the method of Lowry et al. [13].

The difference spectra of cellular and subcellular fractions under various conditions were measured in a single cuvette (a light path of 1.0 cm with a total volume of 1.0 ml) with a 60 Hz stirrer using a dual wavelength scanning sprectrophotometer which was connected to a computer (Chance and Sorge, in preparation) [14]. The reference wavelength was 480 nm and the measuring beam was varied from 400 to 500 nm or to 600 nm. Dynode voltages appropriated to the control spectrum of a cell fraction were fed into the memory of a Varian C-1024 time-averaging computer. In subsequent series, the control spectrum was fed back to the dynode voltage to afford a flat base line. The difference spectrum between the memorized spectrum and the subsequent spectrum was plotted. The difference spectrum of intact leukocytes was obtained as follows. $5 \cdot 10^7$ cells were incubated in the single cuvette containing Ca^{2+}-free Krebs-Ringer phosphate buffer (pH 7.4) at 37° C. Phagocytosis was carried out by adding $1.2 \cdot 10^9$ polystyrene particles after equilibration at 37° C. The difference spectrum of granules was otherwise obtained in granule suspension in 40 mM sodium-potassium phosphate buffer (pH 5.5 or 7.0) containing 0.17 M sucrose at 37° C.

NADH, NADPH and cetyltrimethylammonium bromide were purchased from Sigma Chemical Co. Polystyrene particles (average diameter 1.101 μm) were purchased from Dow Chemical Co. Other chemical reagents used here were of analytical grade.

Results

Spectral changes of granules during oxidation of NAD(P)H were studied under various conditions. Fig. 1 shows the difference spectrum between granules with and without NADPH. As indicated by the absorbance maximum at 460 nm, the NADPH difference spectrum was almost identical with that of myeloperoxidase as shown in Fig. 2 using purified myeloperoxidase. This NADPH difference spectrum was also found to be identical with the spectrum of compound III of myeloperoxidase by plotting the difference between the two absolute spectra of the enzyme in the absence and the presence of NADH which were reported by Odajima and Yamazaki [15,16]. The effects of pH,

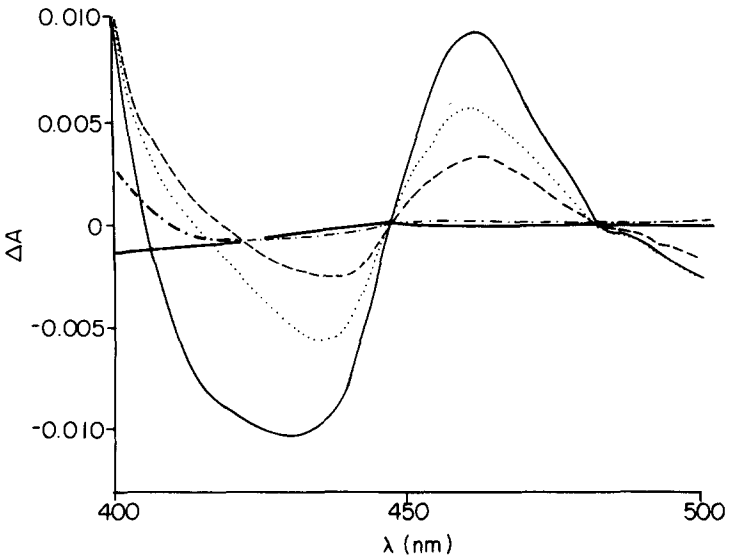

Fig. 1. NADPH-difference spectra of granules under various conditions. Granules (1.8 mg protein) of resting leukocytes were incubated in 1 ml of 65 mM sodium-potassium phosphate buffer, pH 5.5 or 7.0, containing 0.17 M sucrose at 37°C. The control spectrum of granules without NADPH was fed into the memory of the computer. The difference spectrum between the memorized spectrum and the spectrum with NADPH was plotted by feeding back from the computer. The reference wavelength was 480 nm. For details see in the text. The difference spectrum between granules with and without NADPH was recorded by adding 0.15 mM NADPH. 1 mM hydroquinone or 1 mM KCN was added to the medium. ——, pH 5.5; - - - -, pH 7.0; — —, with hydroquinone at pH 5.5; — - — -, with KCN at pH 5.5.

Mn^{2+}, KCN and hydroquinone on the formation of the NADPH-difference spectrum were studied to identify it further with compound III [17,18]. As previously reported, the oxidation of NAD(P)H by leukocyte granules was markedly stimulated at pH 5.5 by Mn^{2+} whereas appreciable stimulation was

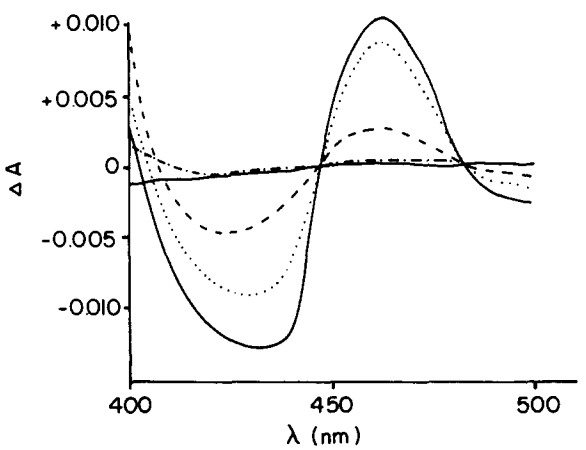

Fig. 2. NADPH-difference spectra of myeloperoxidase under various conditions. Myeloperoxidase (65 μg protein) was incubated in 1 ml of 65 mM sodium-potassium phosphate buffer, pH 5.5. The difference spectra were recorded by adding 0.15 mM NADPH as in Fig. 1. 0.5 mM $MnCl_2$ or 1 mM hydroquinone or 1 mM KCN was added to the medium. ——, with $MnCl_2$; — — —, with hydroquinone; — - — -, with KCN; - - - -, control.

not observed at neutral pH [19]. As shown in Fig. 1 the formation of the absorbance maximum was higher at acidic pH than that at neutral pH, and inhibited by KCN and hydroquinone. Fig. 3 shows the time course of the absorbance changes at 460 nm in the NADPH difference spectrum of granules. In the presence of Mn^{2+} at acidic pH, the absorbance maximum appeared to a greater extent but soon thereafter decayed. KCN completely inhibited the formation of compound III of both granules of resting and phagocytizing leukocytes. The effect of Mn^{2+} on the formation of the absorbance maximum was also the same in both granules. The effects of those reagents on granules were identical with the results of myeloperoxidase as shown in Fig. 2. Fig. 4 shows the oxidation of NADPH by both granules under the same conditions as shown in Fig. 3. The absorbance maximum of compound III disappeared just at the time when NADPH in the reaction mixture was consumed. In the presence of KCN, the absorbance maximum was not observed in both granules, however, NADPH was oxidized by granules of phagocytizing cells but not oxidized by granules of resting cells. The same spectral changes as shown in Figs. 1 and 3 were observed by using NADH.

Fig. 5 shows KCN difference spectra of cellular and subcellular fractions of resting cells. The spectrum of intact leukocytes has absorbance maxima around 460 and 430 nm, and an absorbance minimum around 410 nm. In a

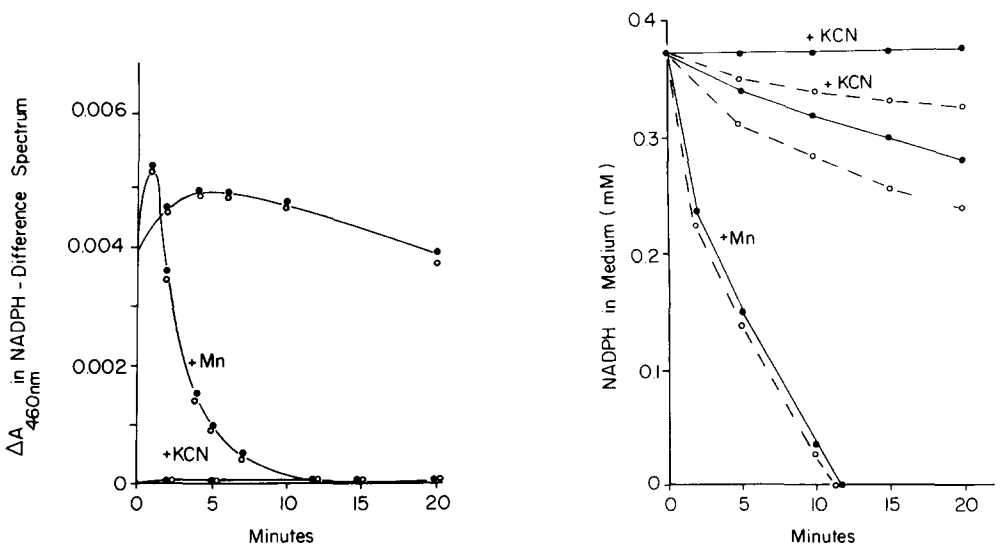

Fig. 3. Formation of the absorption maximum in NADPH-difference spectrum. Granules were prepared from both resting and phagocytizing leukocytes. Either granules (0.9 mg protein) were incubated in 1 ml of 65 mM sodium-potassium phosphate buffer, pH 5.5, containing 0.17 M sucrose at 37°C. The difference spectrum was recorded in the presence or the absence of 0.5 ml $MnCl_2$ or 1 mM KCN by adding 0.38 mM NADPH. Absorption changes at 460 nm were recorded at time intervals. Closed circle, granules of resting leukocytes; open circle, granules of phagocytizing leukocytes.

Fig. 4. Oxidation of NADPH by granules with and without Mn^{2+} or KCN. Experimental conditions were the same as described in Fig. 3. NADPH in the medium was measured in a Hitachi double-beam spectrophotometer Model 356. Closed circle, granules of resting cells; open circle, granules of phagocytizing cells.

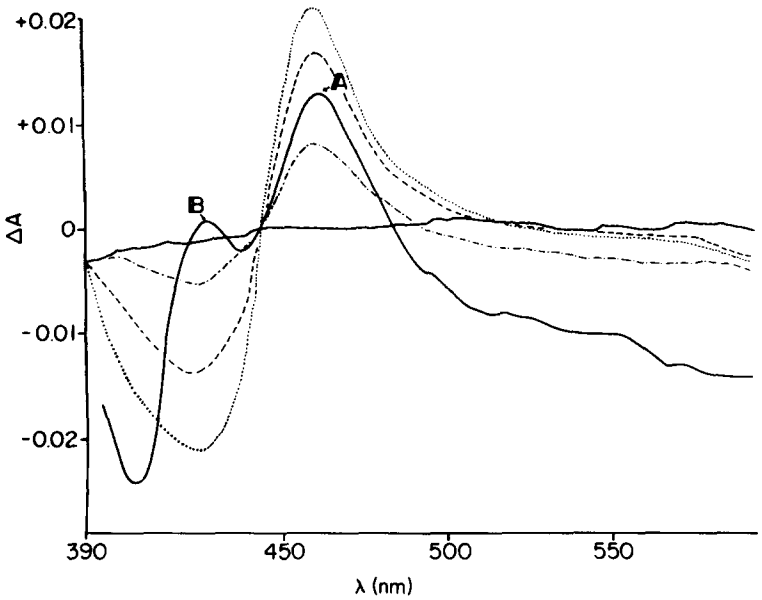

Fig. 5. KCN-difference spectra of cellular and subcellular fractions. KCN-difference spectrum of each fractions was recorded at 37°C by adding 1 mM KCN, after the base line of the reference spectrum had been fed into the memory. 1 ml of leukocyte suspension ($5 \cdot 10^7$ cells) in Krebs-Ringer phosphate buffer, pH 7.4, was incubated in the cuvette. Granules (2.6 mg) were incubated in 1 ml of 65 mM sodium-potassium phosphate buffer, pH 5.5. An aliquot of the soluble fraction isolated from granules (0.34 mg) was incubated in the same buffer as that of granules. Myeloperoxidase (63 μg) was incubated in the same buffer. ——, intact leukocytes; — — —, granules; — · — ·, soluble fraction; · · · · ·, myeloperoxidase.

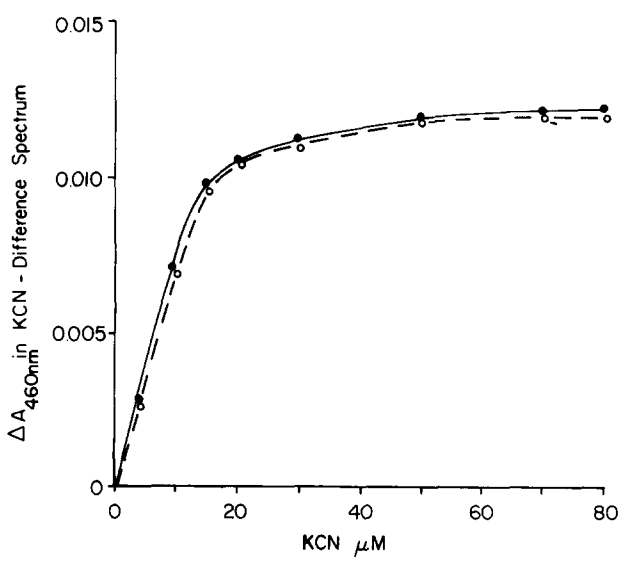

Fig. 6. Formation of the absorption maximum in KCN-difference spectrum. Granules (2.6 mg protein) of either resting or phagocytizing cells were incubated in 1 ml of 65 mM sodium-potassium phosphate buffer, pH 5.5. Formation of the absorption maximum at 460 nm was recorded by adding various concentrations of KCN. Closed circle, granules of resting cells; open circle, granules of phagocytizing cells.

subsequent experiment, the KCN-difference spectrum of granules was obtained and plotted in the same figure. An absorbance maximum at 430 nm (B) as shown in intact cells was not seen in the difference spectrum of granules whereas an absorbance maximum (A) was found at 460 nm. Therefore the absorbance maximum (B) is characteristic of a cytoplasmic component, probably catalase. The other absorbance maximum (A) is characteristic of granules. The KCN difference spectrum of a soluble fraction of granules was also similar to the spectrum of granules. As shown in Fig. 5, the absorbance maxima of cellular and subcellular fractions were in good agreement with KCN difference spectrum of myeloperoxidase [20]. The absorption maximum at 460 nm in the KCN difference spectrum was measured with various concentrations of KCN by using both granules of resting and phagocytizing leukocytes. As shown in Fig. 6, no marked difference between both granules was found in the formation of the absorbance maximum. The KCN difference spectrum of intact cells as shown in Fig. 5 was compared with that of phagocytizing cells. No marked difference between both leukocytes was also found under various concentrations of KCN (not shown).

The cytosol fraction did not show this difference spectrum using either KCN or NAD(P)H. The insoluble residue of granules precipitated after the treatment with cetavlon also did not show the difference spectra as described above.

Discussion

The first recording of the spectrophotometry of intact leukocytes was reported by Chance [21]. A microspectrophotometry was also applied to intact cells by Thorell and Åkerman [22]. Since their reports, no spectrophotometric studies were done on the metabolic stimulation of phagocytizing leukocytes. On the basis of the results presented here, the NAD(P)H oxidase in granules of resting leukocytes seems to be identical with myeloperoxidase. Granules isolated from phagocytizing leukocytes also seem to contain the same oxidase as observed in granules of resting cells. The difference spectrum between granules with and without H_2O_2 was also identical compound II of myeloperoxidase (not shown). As shown in Fig. 3, 1 mM KCN completely inhibited the formation of NADPH-difference spectrum of both granules, but did not inhibit total oxidation of NADPH by granules of phagocytizing leukocytes as shown in Fig. 4. Therefore the KCN-insenstitive oxidation of NAD(P)H has not been identical with myeloperoxidase. As shown in Fig. 6, the affinity of KCN for myeloperoxidase was the same in granules of resting and phagocytizing leukocytes. Therefore the KCN-insensitive oxidation cannot be attributed to a change in KCN affinity for granules after phagocytosis [19]. Compound III formation was induced by either NADH or NADPH. Thus the substrate specificity of the granule-NADPH oxidase as reported by Rossi and co-workers [7–9] was not verified in myeloperoxidase. In a preliminary experiment, the difference spectrum between resting and phagocytizing leukocytes was measured. The difference spectrum exhibited an increase of absorbance maximum around the Soret region but the absorbance maximum at 460 nm in the difference spectrum of compound III was not observed during phagocytosis. If compoud III of myeloperoxidase had formed in leukocytes during phagocytosis, it would

have been shown in the difference spectrum of intact cells.

The questions are how and what kind of enzyme plays the role of KCN-insensitive oxidation of NAD(P)H in granules after phagocytosis. We have found that O_2^- radical formation in granules of phagocytizing cells was higher than that in granules of resting cells by a modified ferricytochrome *C* (unpublished). The high activity to generate O_2^- radicals seems to be related with the KCN-insensitive oxidation of NAD(P)H. However, myeloperoxidase-oxidase reaction of NAD(P)H also produces O_2^- radicals. For the study of NAD(P)H oxidase, myeloperoxidase must be separated from granules and further spectrophotometric studies have to be done with subgranular fractions isolated from intact granules. Furthermore the granule fraction isolated from homogenate in this experiment should be separated from contaminating enzymes.

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