KINETIC STUDIES ON THE $H_2O_2(O_2^-)$-FORMING ENZYME IN GUINEA PIG LEUKOCYTES

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1. Introduction

When exposed to phagocytic particles, polymorphonuclear leukocytes (PMN) exhibit a respiratory burst that results in the production of reduced forms of O_2, including O_2^-, H_2O_2 and $OH^{\cdot}$ [1–4]. Studies of the enzyme mechanism underlying the activation of the oxidative metabolism in PMN of several species have regarded NADH or NADPH as an electron donor for the reduction of O_2. Some have postulated that the increase of oxygen uptake is supported by an increase of the oxidation of NADH [5–7], while others have suggested NADPH oxidase from observations of an increase in NADPH oxidase activity upon phagocytosis [8–11].

We have obtained evidence that a granule-rich fraction isolated from phagocytosing PMN exhibited a marked increase in the activity to generate H_2O_2 upon addition of NADPH as compared to the activity of a similar fraction from resting PMN [12], in agreement with [8,9]. In [12] we employed a spectrophotometric assay of the enzyme–substrate complex of cytochrome *c* peroxidase (CCP) for the detection of H_2O_2, since the complex of CCP provides a sensitive and reliable method to assay the rates of H_2O_2 generation in subcellular fractions containing hydrogen donors, such as NADH and NADPH [12]. Here we analyze further the kinetic properties of NADH and NADPH oxidase activities by following the rates of H_2O_2 generation by the granule-rich fractions isolated from resting and phagocytosing PMN through the use of the CCP–H_2O_2 method and compare the results to data obtained with other methods.

2. Materials and methods

2.1. *Preparation of the granule-rich fraction*

Guinea pig PMN were isolated from the peritoneal cavity 16 h after injection of sterilized 2% caseinate in saline as in [13]. The collected cells, which contained ~98% PMN, were resuspended in chilled Ca^{2+}-free Krebs Ringer phosphate buffer (pH 7.4) (KRP) before use. A leukocyte suspension containing 2×10^7 cells/ml was incubated, after temperature equilibration, for 5 min at 37°C with heat-killed *Escherichia coli* at ~100 bacteria/cell ratio, or without any phagocytic particles in Ca^{2+}-free KRP containing 5 mM glucose. Following the incubation, both resting and phagocytosing PMN were centrifuged down and homogenized in 0.34 M sucrose with a Teflon pestle to isolate the granule-rich fractions as in [14]. The homogenate was diluted 10 times with 0.34 M sucrose and centrifuged at $480 \times g$ for 10 min to remove cell debris and nuclei. The resulting supernatant was centrifuged at $20\,000 \times g$ for 15 min and the pellet containing granules was suspended in a small amount of 0.34 M sucrose. All procedures were carried out at 0–4°C.

2.2. *H_2O_2 and O_2^- production by the granule-rich fraction*

The rates of H_2O_2 production were measured by following the formation of the enzyme–substrate (H_2O_2) complex of CCP recording the $A_{419-407}$ (E_{mM} = 50 l/mmol [15]) increase as in [12]. The reaction medium contained various concentrations of NADH or NADPH, 2.5 μM CCP, and 0.17 M sucrose in 65 mM sodium potassium phosphate buffer (pH 5.8). The formation of CCP–H_2O_2 complex was initiated by adding an aliquot of the granular fraction to give 0.1–1.0 mg/ml. The rates of O_2^- generation were measured by recording the reduction of acetylated cytochrome *c* as in [14]. The assay medium contained 10 μM 69%-acetylated cytochrome *c*, 0.1 mM NADH or NADPH, 5 μg catalase/ml, and 0.17 M sucrose in 65 mM sodium potassium phosphate buffer pH 7.0

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or 5.8. The reduction of acetylated cytochrome *c* was initiated by the addition of the granular fraction to give 0.1–1.0 mg/ml. The rates of NADH or NADPH oxidation, coupled with the O_2^- and H_2O_2 generation, were assayed by recording $A_{349-400}$ decrease [16]. The assay medium contained various concentrations of NADH or NADPH and 0.17 M sucrose in 65 mM sodium potassium phosphate buffer (pH 5.8). The reaction was started by adding an aliquot of the granular fraction as above. All spectrophotometric assays were performed at 37°C in a Hitachi model 556 double-beam spectrophotometer.

2.3. *Protein and enzyme assays*

The protein concentration of each granular fraction was assayed following [17] with the modification in [12]. Myeloperoxidase (MPO) activity was assayed with the guaiacol test [18].

2.4. *Materials*

Male guinea pigs (400–500 g) were used for the isolation of PMN. *Escherichia coli* were cultured, killed by autoclaving and prepared as in [12]. CCP, purified from baker's yeast by the method in [19], was kindly donated by Professor T. Yonetani, Dept. Biochem. Biophys., Univ. Pennsylvania. Acetylated cytochrome *c* was prepared from native ferricytochrome *c* as in [14]. Superoxide dismutase was purchased from Sigma Chem. Co. Ferricytochrome *c*, catalase, NADH and NADPH were purchased from Boehringer. Other chemical reagents were of analytical grade.

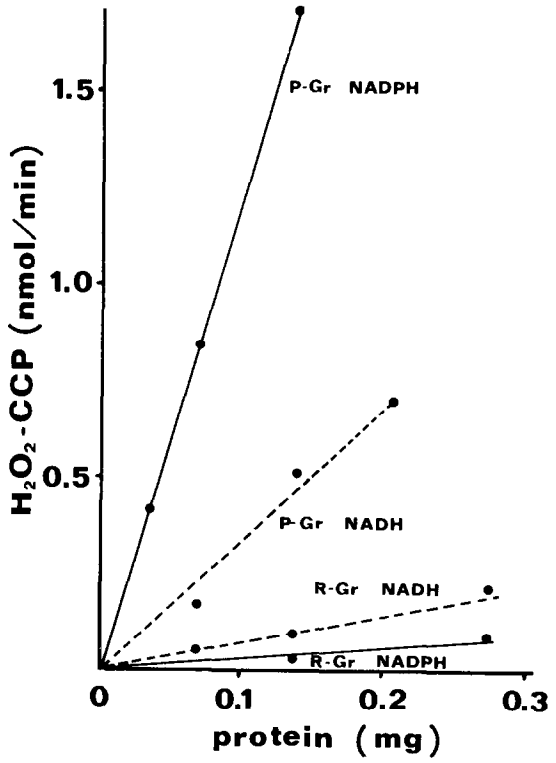

Fig.1. Production of H_2O_2 in the granule-rich fractions isolated from phagocytosing and resting PMN (abbreviated as P-Gr and R-Gr, respectively). The rates of H_2O_2 production were assayed by following the enzyme–substrate complex of CCP at 419–407 nm. The reaction mixture contained 0.1 mM NADH or NADPH, 2.5 μM CCP, 0.17 M sucrose and an aliquot of P-Gr or R-Gr suspension in 65 mM Na-K-phosphate buffer (pH 5.8). The H_2O_2–CCP formation was calculated from the initial rate upon addition of the granular fraction.

3. Results

Fig.1 shows the rates of H_2O_2 production by the granular fractions isolated from resting and phagocytosing PMN, which were abbreviated as R-Gr and P-Gr, respectively, plotted as a function of added protein. The P-Gr fraction exhibited a much higher activity than the R-Gr fraction upon addition of NADPH. The P-Gr fraction generated H_2O_2 at a rate of 9–12 nmol . min^{-1} . mg $protein^{-1}$ in the presence of 0.1 mM NADPH, while it generated about 3.0 nmol . min^{-1} . mg $protein^{-1}$ in the presence of 0.1 mM NADH. The ability of the R-Gr fraction to produce H_2O_2 was far lower than the former in the presence of either NADH or NADPH as shown in fig.1.

In parallel experiments, the rates of O_2^- production by both granular fractions were measured in the presence of NADH or NADPH. Fig.2 shows the reduction rates of acetylated cytochrome *c* by the O_2^- generated in the reaction mixture at pH 7.0, plotted as a function of the amount of added protein. The P-Gr fraction showed a considerably higher O_2^- producing activity in the presence of NADPH, but not in the presence of NADH, in agreement with the same specificity for NADPH to produce H_2O_2. The rates of O_2^- generation by both granular fractions diminished at the same pH as in fig.1. Kinetic studies were performed by following the rates of H_2O_2 generation.

Fig.3a shows the rates of H_2O_2 generation by both granular fractions, plotted against the concentration of added NADPH. The rate of H_2O_2 formation by the P-Gr fraction was progressively higher at increasing NADPH concentration until a maximum was reached

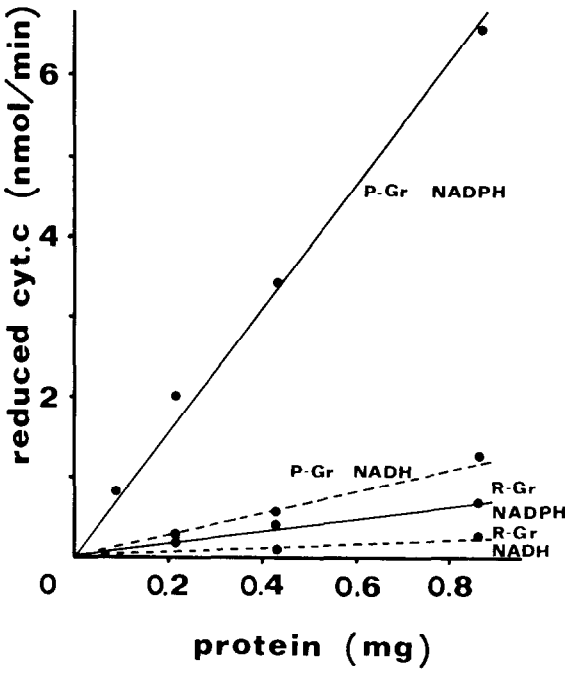

Fig.2. Production of O_2^- by the P-Gr and R-Gr fractions upon addition of NADH or NADPH. The rates of O_2^- formation were assayed by following the reduction of acetylated cytochrome *c* at 550–540 nm. The assay medium contained 10 μM acetylated cytochrome *c*, 5 μg catalase/ml, 0.1 mM NADH or NADPH, 0.17 M sucrose, and various concentrations of the granular fractions in 65 mM Na-K-phosphate buffer (pH 7.0). The O_2^- production was calculated from the initial rate of the reduction of acetylated cytochrome *c*.

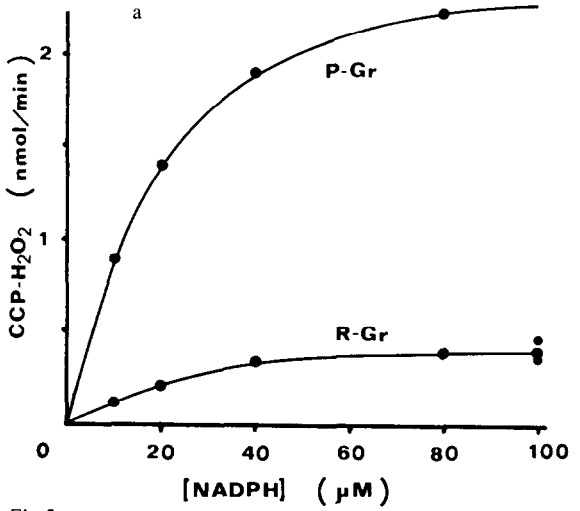

Fig.3a.

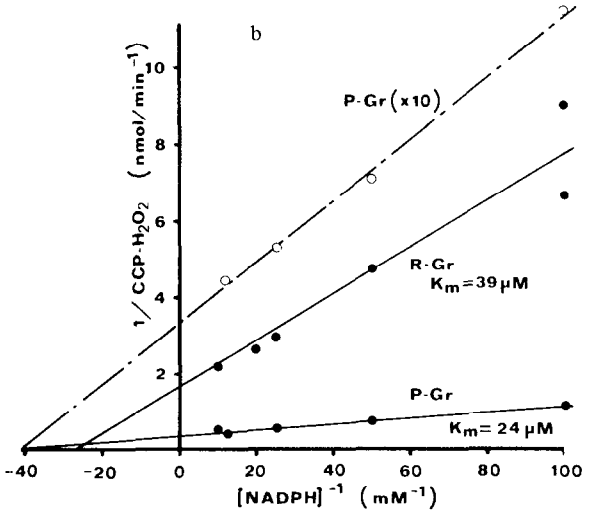

Fig.3.(a). The rates of H_2O_2 production by both P-Gr and R-Gr fractions plotted against the NADPH concentration added. The reaction medium was as in fig.1 except the use of an aliquot of the granular fractions, containing 0.35 mg protein. (b) Lineweaver-Burk plot of H_2O_2 producing activity in the R-Gr and P-Gr fractions as a function of the NADPH concentration added. The reciprocal plot of the CCP–H_2O_2 formation by the P-Gr fraction was 10-times magnified to obtain a precise K_m value.

at 0.1 mM. In contrast, the H_2O_2 generating activity of the R-Gr fraction reached a plateau at a lower concentration of NADPH (0.04–0.05 mM). Fig.3b shows a Lineweaver-Burk plot of the H_2O_2 generation rates as a function of NADPH concentration from the data of fig.3a. The K_m values for NADPH were 24 μM (P-Gr) and 39 μM (R-Gr), respectively.

On the other hand, a double reciprocal plot of NADPH oxidation rates was figured as a function of the concentration of NADPH added (data not shown): the K_m value of P-Gr for NADPH was 20–23 μM, in good agreement with the K_m value as obtained through the assay of H_2O_2 generation, whereas a precise K_m value of the R-Gr fraction was not obtained because of its low activity and a wide variation of the plots.

Table 1 represented the K_m values for NADH and NADPH obtained through the detection of the H_2O_2 production. The K_m of the P-Gr fraction for NADPH was ~1/2 the K_m of the R-Gr fraction. In contrast, there was no marked difference in the K_m value for NADH between the R-Gr and P-Gr fractions. The K_m for NADH was of the order of 10^2 in the case of either P-Gr or R-Gr fraction.

Table 1
K_m values (μM) for NADH and NADPH[a]

	R-Gr	P-Gr
NADPH	57 (39–86)	29 (24–42)
NADH	134 (71–187)	124 (78–167)

[a] Expressed as the mean of 5 expt. The figures in parentheses show the minimum and maximum K_m values in each experiment

4. Discussion

In view of the results obtained by our kinetic studies, we concluded that the ability of activated PMN to generate O_2^- and H_2O_2 is coupled with an increase of NADPH oxidation rather than NADH oxidation. A decrease in the K_m of the granule-rich fraction for NADPH upon phagocytosis was repeated by measuring the rates of NADH and NADPH oxidation with a spectrophotometric or polarographic method [9]. We obtained a similar result to [9] here, with respect to the decrease of K_m for NADPH, but the K_m values of both granular fractions obtained by us were 1/10th lower than the values in [9]. In our preliminary experiment, we obtained the molar ratios of the oxidized NADH or NADPH to the H_2O_2 generated as follows: P-Gr, 0.77(NADPH) and 0.76(NADH); R-Gr, 0.19(NADPH) and 0.27(NADH). The ratios suggest that a portion of the oxidized NADH or NADPH is not concerned with the H_2O_2 generating reaction, probably being mediated by contaminating mitochondrial or microsomal NAD(P)H oxidation systems. Furthermore, the rate of autoxidation of NAD(P)H is progressively higher in lower pH, so that the direct measurements of NAD(P)H oxidation may provide no reliable K_m value, if the rate of NAD(P)H oxidation is less than the rate of its autoxidation.

For the detection of O_2^- generated in subcellular fractions, acetylated cytochrome *c* is useful for its non-reducibility by mitochondrial or microsomal cytochrome *c* reductases, while the reducibility is retained [14]. We also observed a marked high activity of O_2^- production in the P-Gr fraction upon the addition of NADPH. We could not obtain so large a difference in the K_m value for NADPH between the two fractions as obtained by measuring the end product of H_2O_2, probably for the following reasons. The electron of O_2^- is well known to transfer to various kinds of electron acceptors (AH_2), such as NADH and NADPH, to form AH$^\cdot$ at a rate whose constant depends highly on a given pH and a electron acceptor [20–22]. In addition, the molar ratio of the cytochrome *c* (native) reduced to the O_2^- added in the assay medium (pH 7.0) was 0.48 and 0.34 in the presence and absence of catalase, respectively, using the xanthine–xanthine oxidase system. The ratio further decreased at lower pH through the use of acetylated cytochrome *c*. In contrast, we found that the molar ratio of the CCP–H_2O_2 complex formed to the H_2O_2 added was nearly 1.0 (0.79–0.88) (pH 5.8), because of the characteristic high affinity of the enzyme for H_2O_2 as substrate [12]. In view of the above rationale, this assay method may provide more reliable K_m values for NADH and NADPH than other methods.

Here we calculated the O_2^- and H_2O_2 generating activities of the P-Gr fraction on the basis of the protein concentration of the R-Gr fraction, isolated from the same number of cells as the P-Gr fraction, to avoid a miscalculated increase in the specific activity of the P-Gr fraction whose lysosomal proteins decreased after phagocytosis. The P-Gr and R-Gr fractions used here contained ~42 and 56% of the total cellular MPO activity, respectively. Even though the H_2O_2 and O_2^- generation was much higher in the P-Gr than R-Gr fraction, MPO activity in the P-Gr fraction was a little lower than in the R-Gr fraction. This fact suggests that the enhanced H_2O_2(O_2^-)-forming enzyme in the P-Gr fraction is not due to MPO-mediating activity found in the subcellular fraction.

References

[1] Sbarra, A. J. and Karnovsky, M. L. (1959) J. Biol. Chem. 234, 1355–1362.
[2] Iyer, G. Y. N., Islam, M. F. and Quastel, J. H. (1961) Nature 192, 535–541.
[3] Babior, B. M., Kipnes, R. S. and Curnutte, J. T. (1973) J. Clin. Invest. 52, 741–744.
[4] Tauber, A. I. and Babior, B. M. (1977) J. Clin. Invest. 60, 374–379.
[5] Evans, W. H. and Karnovsky, M. L. (1962) J. Biol. Chem. 236, 30–32.
[6] Cagan, R. H. and Karnovsky, M. L. (1964) Nature 204, 255 257.
[7] Baehner, R. L., Gilman, N. and Karnovsky, M. L. (1970) J. Clin. Invest. 49, 692–700.

[8] Rossi, F., Romeo, D. and Patriarca, P. (1972) J. Reticuloendothel. Soc. 12, 127–149.
[9] Patriarca, P., Cramer, R., Moncalvo, S., Rossi, F. and Romeo, D. (1971) Arch. Biochem. Biophys. 145, 255–262.
[10] Iverson, D., DeChatelet, L. R., Spitsnagel, J. K. and Wang, P. (1977) J. Clin. Invest. 59, 282–290.
[11] Holrn, D. C. and Lehrer, R. I. (1975) J. Clin. Invest. 55, 707–713.
[12] Kakinuma, K., Boveris, A. and Chance, B. (1977) FEBS Lett. 74, 295–299.
[13] Kakinuma, K. (1970) J. Biochem. 68, 177–185.
[14] Kakinuma, K. and Minakami, S. (1978) Biochim. Biophys. Acta 538, 50–59.
[15] Yonetani, T. (1965) J. Biol. Chem. 240, 4509–4514.
[16] Kakınuma, K. and Chance, B. (1977) Biochim. Biophys. Acta 480, 96–103.
[17] Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.
[18] Chance, B. and Maehly, A. C. (1955) Methods Enzymol. 2, 764–775.
[19] Yonetani, T. (1967) Methods Enzymol. 10, 336–339.
[20] Yamazaki, I. and Yokota, K. (1973) Mol. Cell. Biochem. 2, 39–52.
[21] Yokota, K. and Yamazaki, I. (1977) Biochemistry 16, 1913–1920.
[22] Land, E. J. and Swallow, A. J. (1971) Biochim. Biophys. Acta 234, 34–42.