Vol. 142, No. 1, 1987 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 15, 1987 Pages 213-220

A REASSESSMENT OF THE PRODUCT SPECIFICITY OF THE NADPH:O_2 OXIDOREDUCTASE OF HUMAN NEUTROPHILS

Terrence R. Green and Katherine L. Pratt

Clinical Pathology Service,
Veterans Administration Medical Center,
and Department of Biochemistry,
Oregon Health Sciences University,
Portland, OR 97201

Received December 3, 1986

Native ferricytochrome c, but not acetylated ferricytochrome c, stimulates the flow of electron equivalents passing through the neutrophil NADPH:O_2 oxidoreductase complex. At 28 uM it increases NADPH oxidase activity by 157 $\pm$ 15% (n=5) over that measured in its absence. Enhanced activity is predominantly seen in oxidoreductase-rich 27,000 x g membrane preparations obtained from phorbol myristate acetate activated cells. Superoxide formation is also enhanced. Although some of the stimulatory activity seen with addition of native ferricytochrome c to oxidoreductase-rich membrane suspensions might have been explained in terms of mitochondrial contamination, this was ruled out. Comparable membrane preparations from resting cells were devoid of NADPH oxidase activity. Azide, a well-known inhibitor of the electron transport chain, did not block the enhancing effect of native ferricytochrome c. These results indicate that native ferricytochrome c is not a suitable scavenger of superoxide in quantitating the product specificity of the oxidoreductase since it amplifies the apparent rate of superoxide formation with respect to measured rates of NADPH oxidation conducted in its absence. By using acetylated ferricytochrome c in place of native ferricytochrome c in quantitating the product specificity of the oxidoreductase we show that no more than 70% of the electron equivalents donated by NADPH to the oxidoreductase are involved in superoxide formation. The remaining 30% of the electron equivalents given up by NADPH to the oxidoreductase appear to be involved in direct formation of hydrogen peroxide.

The NADPH:O_2 oxidoreductase of the neutrophil is essential to enable the body to ward off infections (1,2). It appears to be a transmembrane complex, unaffected by exposure to either azide or cyanide. It catalyzes oxidation of NADPH on the cytosolic side of the membrane and reduction of O_2 principally to superoxide (O_2-) on the external surface of the cell (1-5). Subcellular fractions rich in oxidoreductase activity have been isolated from stimulated neutrophils whereas comparable fractions obtained from unstimulated (resting)

Abbreviations: cyt. c, ferricytochrome c; PMA, phorbol myristate acetate; SOD, superoxide dismutase; O_2-, superoxide.

0006-291X/87 $1.50

neutrophils are devoid of enzyme activity (6). Several studies have been made using subcellular membrane fractions rich in oxidoreductase activity in an effort to characterize its product specificity in reducing O_2, and have generally led to the conclusion that this enzyme system catalyzes the univalent reduction of O_2, and that H_2O_2 is formed through dismutation of O_2^- (7-11). In conducting stoichiometric measurements, O_2^- is normally measured by the superoxide dismutase (SOD) inhibitable ferricytochrome c (cyt. c) trapping assay at 550 nm (12). NADPH oxidation is usually measured by monitoring its oxidation spectrally at 340 nm in the absence of cyt. c. Implicit is the assumption that the oxidoreductase is unaffected by the presence of cyt. c used in trapping O_2^-.

We show here that native cyt. c stimulates electron flow through the oxidoreductase complex causing an increase in both NADPH oxidase and O_2^- generating activity. On the other hand, acetylated cyt. c traps O_2^- quantitatively without stimulating electron flux through the oxidoreductase complex. Using acetylated cyt. c in place of native cyt. c in quantitating O_2^- formation we demonstrate that the general presentation of the oxidoreductase as a multienzyme complex catalyzing the formation of two moles of O_2^- for every mole of NADPH oxidized is not an accurate description of its true product specificity.

Materials and Methods

CM-Sephadex, cyt. c (horse heart, Type VI), phorbol myristate acetate (PMA), superoxide dismutase, catalase, NADPH, deoxycholate, xanthine, and xanthine oxidase were all purchased from Sigma Chemical Co., St. Louis, MO. Acetic anhydride was purchased from American Scientific and Chemical Co., Portland, OR. All other common laboratory reagents were of the best grade available.

Acetylated cyt. c was prepared according to the method of Minakami, Titani and Ishikura (13).

The isolation of neutrophils from whole blood and preparation of 27,000 x $\bar{g}$ oxidoreductase-rich membrane fractions derived from PMA-stimulation of the isolated neutrophils, and the preparation of comparable membrane fractions from resting cell suspensions, were as previously described (14). All enzyme assays were conducted on freshly isolated membrane preparations. NADPH oxidase activity was measured by recording spectral changes occuring after mixing enzyme with substrate at 340 nm on a double beam Shimadzu UV-250 spectrophotometer. The sample cuvette contained in a total volume of 1.3 ml: 50 ug SOD, 6 x 10^3 units catalase, 0.06% deoxycholate, 0.48 umoles $CaCl_2$, 0.33 umoles $MgCl_2$, 200 nmoles DTPA, 200 nmoles NADPH and 50 umoles Tris-HCl buffer, pH 7.6. The reference cuvette contained the same reagents except NADPH was omitted from the reaction mixture. Enzyme assays were initiated by addition of 10 to 30 ul of enzyme to the sample and reference cuvettes. Initial rates were calculated using the

millimolar absorptivity coefficient for NADPH of 6.22. Native or acetylated cyt. c were also included in the final reaction mixtures at varying concentrations as indicated in the text. Superoxide generating activity was monitored under the same conditions except the wavelength was set at 550 nm, SOD was excluded from the sample cuvette, and native or acetylated cyt. c (see text) was included in all reaction mixtures at a final concentration of 75 uM. Enzyme activity was calculated from the initial rate of cytochrome c reduction using the millimolar absorptivity coefficient for the difference in absorptivity between reduced and oxidized cytochrome c of 21.1 (15). All assays were conducted at room temperature. One milliunit (mU) of enzyme activity corresponds to the consumption of substrate or formation of product equivalent to 1 nmole min^{-1}.

The O_2^- trapping efficiency of native and acetylated cyt. c was measured by varying the concentration of either native or acetylated cyt. c in cuvettes set up for assay of O_2^- generating activity but which contained 200 uM xanthine in place of NADPH and to which was then added a fixed aliquot of xanthine oxidase (6 mU).

Protein was measured by the method of Lowry et al. (16).

Results

Table I shows the effect of native cyt. c in stimulating NADPH oxidation of membrane preparations from PMA-stimulated and resting neutrophils. Resting cells were devoid of NADPH oxidase activity in the absence of cyt. c; in its presence low, albeit detectable, NADPH oxidase activity was observed (cf., Table I).

Table I. Effect of Cytochrome c on NADPH:O_2 Oxidoreductase Activity Recovered in the 27,000 x g Membrane Fraction Derived from PMA-Stimulated and Resting Human Neutrophils

Enzyme Source	Oxidized NADPH [a] ($mU\ ml^{-1}$)	
	- Cyt. c	+ Cyt. c
PMA-Stimulated Cells	97 ± 21 (n = 5)	152 ± 15 (n = 5)
Resting Cells	1 ± 3 (n = 4)	18 ± 3 (n = 4)

[a] NADPH oxidase activity was measured at 340 nm in the absence (-) and presence (+) of native cytochrome c as described in Materials and Methods. Enzyme preparations obtained from PMA-stimulated and resting cells were adjusted to the same protein concentrations (approx. 3 mg ml^{-1}) prior to assaying for enzyme activity. Values are expressed as the mean ± 1 SD with the numbers of individual determinations indicated in parentheses. The final concentration of native cytochrome c used in these assays was 28 uM.

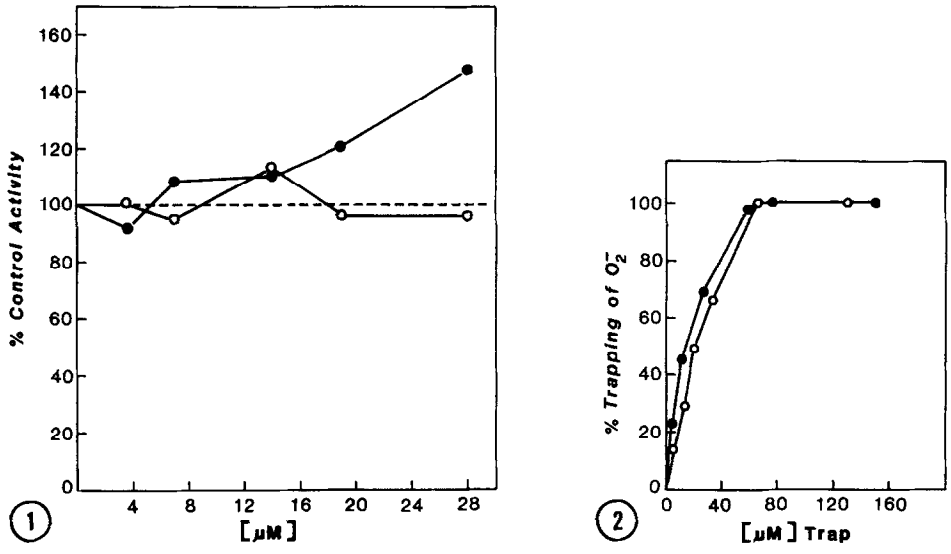

Fig. 1. Effect of increasing concentrations of native (●) and acetylated (○) cyt. c on NADPH oxidase activity compared to that measured in the absence of cyt. c. The dashed line represents the control activity measured on 27,000 x g membrane suspensions obtained from PMA-stimulated cells and assayed in the absence of cyt. c.

Fig. 2. Titration curves measuring recovery of O_2^- trapped by native (●) and acetylated (○) cyt. c v. concentration of each in the final assay mixtures. Xanthine oxidase served as the source in generating O_2^- and 100% trapping of O_2^- represents the maximum recovery of O_2^- detected at the highest concentration of O_2^- scavenger employed. There was no measurebale difference between the maximum O_2^- trapped at the highest concentrations of native or acetylated cyt. c employed in the assays.

Mitochondrial oxidase activity was absent and was not the source of the enhanced enzyme activity seen with inclusion of native cyt. c in the final assay mixtures. Azide at 100 uM in the final reaction mixtures failed to block the enhanced NADPH oxidase activity in either preparation. NADPH mediated cyt. c reductase activity (14) was also negligible at less than 10% of the total oxidase activity detected in membrane fractions obtained from PMA-activated cells.

Unlike native cyt. c, acetylated cyt. c did not stimulate NADPH oxidation. Fig. 1 compares the effect of varying concentrations of native and acetylated cyt. c in the final reaction mixtures. Due to the high background absorbance of cyt. c at 340 nm it was not feasible to follow the effect of concentrations in excess of 28 uM.

Fig. 2 compares the ability of acetylated and native cyt. c to scavenge O_2^- using xanthine oxidase as the source in generating a constant supply of O_2^-.

Table II. Effect of Native v. Acetylated Cytochrome c on the Apparent Molar Ratio of O_2^- Production to NADPH Oxidized in Oxidoreductase-Rich 27,000 x g Membrane Fractions Derived from PMA-Stimulated Human Neutrophils [a]

	Oxidized NADPH (mU ml^{-1})	O_2^- Detected (mU ml^{-1})	
		with native Cyt. c	with acetylated Cyt. c
Enzyme Activity	116 ± 16 (n = 7)	205 ± 22 (n = 7)	160 ± 21 (n = 5)
O_2^-/NADPH Ratio		1.8	1.4

[a] NADPH oxidase and O_2^- generating activities were measured as described in Materials and Methods. Values represent the mean ± 1 SD with the number of individual determinations indicated in parentheses. The final concentration of native and acetylated Cyt. c used to trap O_2^- was 75 uM.

Although acetylated cyt. c was less efficient in trapping O_2- on a molar basis compared to native cyt. c, both quantitatively trapped all of the O_2- generated when employed at concentrations in excess of 60 uM. Each was adjusted to a final concentration of 75 uM in measuring O_2- generating activity of the oxidoreductase. It can be seen from an inspection of the data in Table II and comparison to that shown in Table I that native cyt. c not only stimulates NADPH oxidase activity (Table I), but the outcome of this stimulatory effect is also evident in an increased rate of O_2- formation (Table II). This latter result can be seen by noting in Table II the average yield of O_2- generating activity detected with native cyt. c in comparison with that detected using excess acetylated cyt. c as the O_2- trapping agent.

With native cyt. c as the trapping agent for O_2- the calculated ratio of O_2- formation compared to NADPH oxidation (cf., Table II) averaged 1.8. With acetylated cyt. c the ratio averaged 1.4.

Discussion

We recently demonstrated that H_2O_2 is a direct product of the oxidoreductase in addition to O_2-, and that the ability of the oxidoreductase to

conduct univalent and divalent reduction of O_2 is substantially affected by a number of factors including the age of the enzyme preparation, the pH of the reaction mixture, the availability of NADPH, and the extent of exposure to detergents (17). In freshly prepared enzyme suspensions the molar ratio of O_2^- formation to NADPH oxidized using native cyt. c as the trapping agent in detecting O_2^- was found in our laboratory and by other investigators studying this problem to range from approximately 1.8 to 2.0 (7-11,17). Since NADPH is a 2-electron donor, ratios of O_2^- production to NADPH oxidized approaching the theoretical limit of 2.0 account for all of the possible electron equivalents available from NADPH. Thus the oxidoreductase has generally been viewed as an enzyme catalyzing principally univalent reduction of O_2. H_2O_2 formation has been assumed to arise through dismutation of O_2^-, and thus it has not been regarded as a significant direct product of the oxidoreductase.

To our knowledge the effect of native cyt. c in stimulating electron flow through the oxidoreductase complex has not previously been addressed. Its mechanism of stimulation of the oxidoreductase remains to be resolved. Azzi, Montecucco and Richter (18) used acetylated cyt. c to study O_2^- formation in the mitochondrial electron transport chain due to interferring cyt. c reductase and oxidase activities intrinsic to the electron transport chain which precluded the use of native cyt. c as a trapping agent. Acetylated cyt. c has previously been used by Kakinuma and Minakami (19) in studying O_2^- formation induced by saturated fatty acids and by Nasrallah et al. (20) in detecting O_2^- production in rabbit alveolar macrophages. The question of stoichiometry of product formation was not addressed in either of the latter two studies.

In the present study the increased NADPH oxidase activity observed with inclusion of native cyt. c in the assay mixtures designed to quantitate neutrophil oxidoreductase activity cannot be attributed to mitochondrial contamination of the enzyme preparations. Resting cell membrane preparations are devoid of oxidase activity. In addition, oxidase activity seen with inclusion of native cyt. c in the reaction mixtures was not blocked by azide (cf., Table I and Results). NADPH dependent cyt. c reductase activity intrinsic to the

oxidoreductase (14) accounts for no more than approximately 10% of the enhanced activity. Nonspecific diaphorase activity associated with the membrane is also negligible. Hence, native cyt. c, but not acetylated cyt. c, appears to stimulate the flow of electron equivalents through the oxidoreductase complex (cf., Fig. 1). Because of this phenomenom, native cyt. c is not a suitable scavenger of O_2^- where the yield of O_2^- is to be compared to NADPH oxidized by the oxidoreductase.

By using acetylated cyt. c as a scavenger of O_2^- formation it is evident that the true stoichiometric ratio of O_2^- formation with respect to NADPH oxidized is approximately 1.4 (cf., Table II), a value markedly less than the theoretical ratio of 2.0 expected for a univalent O_2 reducing enzyme system. Since we have previously shown that the only other principal product of the oxidoreductase is H_2O_2 (17), a more accurate representation of the stoichiometric reactions catalyzed by the oxidoreductase can be expressed as shown in equation 1 where m and n represent the mole fraction of O_2 committed to O_2^- and H_2O_2 formation, respectively.

$$(1)\quad (m+n)\text{NADPH} + (2m+n)O_2 \longrightarrow (m+n)\text{NADP}^+ + 2mO_2^- + nO_2^{-2} + (m+n)H^+$$

The molar ratio of O_2^- formation to NADPH oxidized of 1.4 indicates that 70% of the electron equivalents given up by NADPH are involved in univalent reduction of O_2 yielding O_2^-; 30% are involved in direct divalent reduction of O_2 to yield H_2O_2 as a direct product of the oxidoreductase. These results indicate that O_2^- is not an essential precursor of H_2O_2 formation, but rather that the oxidoreductase is fully capable of producing simultaneously O_2^- and significant quantities of H_2O_2 as NADPH is oxidized.

Acknowledgement

This work was supported by the Veterans Administration.

References

1. DeChatelet, L.R. (1975) J. Reticulo. Endothel. Soc. 24, 73-91.

2. Rossi, F., Patriarca, P., and Romeo, D. (1980) in The Reticuloendothelial System: A Comprehensive Treatise (Sbarra, A.J., and Strauss, R.R., eds.) Vol. 3, pp. 153-188, Plenum Press, NY.
3. Green, T.R., Schaefer, R.E., and Makler, M.T. (1980) Biochem. Biophys. Res. Comm. 94, 262-269.
4. Babior, G.L., Rosin, R.E., McMurrich, B.J., Peters, W.A., and Babior, B.M. (1981) J. Clin. Invest. 67, 1724-1728.
5. Briggs, R.T., Robinson, J.M., Karnovsky, M.L., and Karnovsky, M.J. (1986) Histochemistry 84, 371-378.
6. Babior, B.M. (1978) N. Engl. J. Med. 298, 659-668.
7. Babior, B.M., Curnutte, J.T., and McMurrich, B.J. (1976) J. Clin. Invest. 58, 989-996.
8. McPhail, L.C., DeChatelet, L.R., and Shirley, P.S. (1976) J. Clin. Invest 58, 774-780.
9. Gabig, T.G., and Babior, B.M. (1979) J. Biol. Chem. 254, 9070-9074.
10. Bellavite, P., Serra, M.C., Davoli, A., Bannister, J.V., and Rossi, F. (1978) Mol. Cell. Biochem. 52, 17-25.
11. Wakayma, H., Takeshige, K., Takayanagi, R., and Minakami, S. (1982) Biochem. J. 205, 593-601.
12. Flohe, L., and Otting, F. (1984) Meth. Enzymol. 105, 93-104.
13. Minakami, S., Tatani, K., and Ishikura, H. (1958) J. Biochem. (Tokyo) 64, 341-352.
14. Green, T.R., and Wu, D.E. (1985) FEBS Lett. 179, 82-86.
15. Van Gelder, B.F., and Slater, E.C. (1962) Biochim. Biophys. Acta 58, 593-595.
16. Lowry, O.H., Rosebrough, J.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
17. Green, T.R., and Wu, D.E. (1986) J. Biol. Chem. 261, 6010-6015.
18. Azzi, A., Montecucco, C., and Richter, C. (1975) Biochem. Biophys. Res. Comm. 65, 597-603.
19. Kakinuma, K., and Minakami, S. (1978) Biochim. Biophys. Acta 38, 50-59.
20. Nasrallah, V.N., Pamela, S.S., Myrvik, Q., and Waite, M. (1983) J. Immunol. 131, 2104-2106.