Superoxide-forming Enzyme From Human Neutrophils: Evidence for a Flavin Requirement

By Bernard M. Babior and Ruby S. Kipnes

The superoxide-forming activity of 27,000-g particles prepared from homogenates of zymosan-treated human neutrophils is lost if the assay is conducted in the presence of 0.045% Triton X-100. This loss in activity in the presence of detergent is prevented by 40 μM flavin adenine dinucleotide (FAD), but not by flavin mononucleotide, riboflavin, adenosine 5′-diphosphate, or adenosine 5′-monophosphate. With resting particles or particles from zymosan-treated chronic granulomatous disease neutrophils, no superoxide-forming activity is detectable even in the presence of FAD; this is true whether or not detergent is present in the assay. Particles extracted with detergent prior to assay are fully active if assayed in the presence of FAD, but show little activity if FAD is omitted from the assay mixture. These results suggest that the superoxide-forming enzyme from human neutrophils is a FAD-requiring enzyme.

THE EXPOSURE of neutrophils to an appropriate stimulus induces in these cells a series of far-reaching changes in oxygen metabolism.[1-4] These changes, collectively termed the "respiratory burst," are manifested by marked increases in oxygen consumption,[2] superoxide (O_2^-)[5-9] and H_2O_2[3,10,11] production, and hexose monophosphate (HMP) shunt activity.[2] The purpose of the respiratory burst appears to be to provide a group of highly reactive products of the partial reduction of oxygen which the cell can use for the destruction of invading microorganisms.[1,8]

Recent evidence indicates that the respiratory burst results from the activation of a particulate enzyme which catalyzes the reduction of oxygen to O_2^- using NADPH as the electron donor.[12-15] This O_2^--forming activity is missing from the neutrophils of patients with chronic granulomatous disease (CGD),[14,15] a condition characterized by the failure of these cells to display a respiratory burst in response to stimuli.[4,16,17]

We are currently engaged in the characterization of the O_2^--forming enzyme from human neutrophils. In the course of these studies, we have been able to demonstrate a requirement for flavin on the part of the enzyme. The evidence for this conclusion is the subject of the present report.

MATERIALS AND METHODS

FAD,* FMN,* riboflavin, AMP,* ADP,* NADPH* (preweighed vials), zymosan and horse heart cytochrome C (type VI) were purchased from Sigma Chemical, St. Louis. Bovine erythrocyte

*Abbreviations used in this paper: FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; AMP, adenosine 5′-monophosphate; ADP, adenosine 5′-diphosphate; NADPH, reduced nicotinamide adenine dinucleotide phosphate; CGD, chronic granulomatous disease.

From the Hematology Service, New England Medical Center Hospital, and the Department of Medicine, Tufts University School of Medicine, Boston, Mass.

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Address for reprint requests: Bernard M. Babior, M.D., Tufts University School of Medicine, New England Medical Center Hospital, Department of Medicine/Hematology, 171 Harrison Ave., Boston, Mass. 02111.

superoxide dismutase was obtained from Truett Laboratories, Dallas, and Triton X-100 from New England Nuclear, Boston. Other reagents were the best grades commercially available and were used without further purification.

Human neutrophils were prepared and activated with opsonized zymosan by a previously published method,[15] except that 1 m*M* NaN_3 was present during all steps, and the exposure of the cells to opsonized zymosan lasted 10 min. Particles were obtained after 27,000-*g* centrifugation as described elsewhere.[15] Apart from the experiment presented below in Table 5 (the assay conditions for which are given in the legend to that table) particulate O_2^--forming activity was assayed as previously described,[15] except that all assays were conducted at pH 7.0 and all solutions in which the cells or granules were suspended, including the final assay mixtures, contained 1 m*M* NaN_3. Assay mixtures contained particles equivalent to 0.1 mg protein (assayed by the method of Lowry et al.[18]), 0.1 m*M* NADPH, and other cofactors and detergent as indicated, the particles being added last. The assays were carried out at 25°C. In every case, the figures reported are initial rates.

Pretreatment of Particles With Detergent

Certain of the experiments were performed with particles which were exposed to detergent prior to assay. Pretreatment with detergent was accomplished as follows. Portions (0.6 ml) of particle suspension (1 mg particle protein/ml in 20 m*M* potassium phosphate buffer containing 1 m*M* NaN_3) were added to equal volumes of extraction buffer with or without FAD [0.45% Triton X-100 with or without 2 m*M* FAD/0.1 *M* potassium phosphate buffer (pH 7.0)/0.1 *M* NaN_3 = 1/4/0.05 (v/v)]. They were permitted to stand at room temperature for 5 min. The two mixtures (the one containing FAD and the one not containing FAD) were then centrifuged at 27,000 *g* for 10 min at 4°C. The supernatants, designated "Triton extracts," were reserved on ice. The two sets of detergent-treated particles were washed once with 20 m*M* potassium phosphate buffer, pH 7.4, containing 1 m*M* NaN_3; then each was suspended in 0.8 ml of the same buffer. The protein concentrations of the detergent-treated particle suspensions were determined, and the suspensions were diluted to 1 mg particle protein/ml. A portion of the original (i.e., unextracted) particle suspension was set aside to serve as control.

RESULTS

Previous studies have shown that the O_2^--forming activity from homogenates of zymosan-stimulated human neutrophils is found in particles sedimenting at 27,000 *g*.[14,15] Table 1 shows that the activity of such particles was abolished when the assay was conducted in the presence of 0.045% Triton X-100, but that virtually full activity was exhibited if 0.2 m*M* FAD was present in addition to the detergent. This finding suggests that the O_2^--forming enzyme is a flavoprotein.

Table 1. Effect of FAD on the O_2^--forming Activity in Particles From Zymosan-treated Normal and CGD Neutrophils Assayed in the Presence and Absence of Detergent

Conditions		O_2^- Production (nmol/min/mg protein)			
		Experiment 1		Experiment 2	
Triton X-100	FAD	Normal	CGD	Normal	CGD
−	−	36.4	0.4	15.0	0.5
−	+	29.7	0.5	19.6	1.6
+	−	1.4	0.7	0.6	0.8
+	+	21.8	0.7	19.9	0.3

Incubations were conducted as described in Materials and Methods. FAD (0.2 mM) and Triton X-100 (0.045%) were present as indicated. Particles from a different CGD patient were used for each of the two experiments. In each case, particles from normal and CGD cells were prepared and assayed on the same day.

Table 2. Effect of FAD on the O_2^--forming Activity of Particles From Resting and Zymosan-treated Neutrophils Assayed in the Presence of Detergent

Conditions	O_2^- Production (nmoles/min/mg protein)	
	Resting Particles	Zymosan-activated Particles
FAD absent	0.6	1.3
FAD present	0.9	9.2

Incubations were conducted as described in Materials and Methods. The concentration of Triton X-100 was 0.045%. FAD (0.2 mM) was present as indicated.

This conclusion is supported by a number of additional observations. One is that FAD failed to confer the ability to generate O_2^- onto a particulate fraction from CGD neutrophils, a preparation which has been shown previously[14,15] to lack the O_2^--forming activity (Table 1). Another is that FAD-dependent O_2^- production was seen with particles from zymosan-stimulated but not resting neutrophils (Table 2); this finding is in accord with previous work regarding the occurrence of O_2^--forming activity in neutrophil particles.[14,15] The possibility that FAD-dependent O_2^- formation represented the unmasking of a new O_2^--forming enzyme rather than the restoration of the activity of a detergent-inhibited enzyme is unlikely in view of the fact that FAD had little effect on O_2^- production in the absence of detergent [rates of O_2^- production by nine particle preparations were 19.7 ± 3.0 (SE) and 17.4 ± 2.9 (SE) nmoles/min/mg protein in the presence and absence of 0.2 m*M* FAD, respectively]. Finally, the systematic omission of the various constituents of the assay mixture has shown that O_2^- production in the presence of Triton X-100 requires both NADPH and unheated particles, in addition to FAD (Table 3).

It was of interest to determine whether cofactors other than FAD could preserve the O_2^--forming activity. Although FAD was used in the initial experiments, it was possible that under the conditions employed the flavin requirement would be rather nonspecific. Alternatively, it was conceivable that the adenine rather than the isoalloxazine moiety was responsible for the preservation of enzyme activity. This possibility might occur if, for example, the enzyme were subject to regulation by adenosyl nucleotides, in which case a binding site for such a nucleotide would be present, the occupation of which could stabilize the enzyme against the effect of the detergent.

Table 3. Requirements for FAD-dependent O_2^- Production by Zymosan-activated Particles Assayed in the Presence of Detergent

Assay Mixture	Spectral Change ($10^3 \times \Delta A_{550}$/min)	O_2^- Production (nmoles/min/mg protein)
Complete	56	30.3
Omit particles	2.8	1.5
Boiled particles*	5.0	2.7
Omit NADPH	0.9	0.5
Omit cytochrome C	0.9	—
Omit dismutase	−2.6	—

Incubations were conducted as described in Materials and Methods, with modifications as noted. FAD (0.2 mM) and Triton X-100 (0.045%) were present in all assay mixtures.

*Heated 2 min in boiling water, then centrifuged at low speed to remove precipitated protein.

Table 4. Specificity of the Cofactor Requirement

Cofactor	O_2^- Production (nmoles/min/mg protein)
None (control)	27.4
FAD	32.6
FMN	5.6
Riboflavin	3.2
ADP	1.0
AMP	2.5

Incubations were conducted as described in Materials and Methods. Cofactors (0.04 mM) were present as indicated. Triton X-100 (0.045%) was present in all assay mixtures except the one to which no cofactor was added (the control).

The results of experiments with several cofactors are shown in Table 4. It is apparent that of the compounds tested, FAD was by far the most effective in preserving the activity of the enzyme. Some activity was seen with FMN, while riboflavin,* AMP, and ADP were virtually without effect. Substantial preservation of the O_2^--forming activity was seen at FAD concentrations of 4 μM and higher (Fig. 1); at concentrations below 4 μM, the preservation of activity by FAD was slight. These findings strongly suggest that the cofactor required by the O_2^--forming enzyme is FAD.

The dependence of O_2^- formation on the concentration of detergent is illustrated in Fig. 2. As shown in the left-most panels, the rate of O_2^- production in the absence of detergent fell slowly with time regardless of whether or not FAD was present in the reaction mixture. Triton X-100 seemed to accelerate the loss in the O_2^--forming capacity of particles incubated without FAD; with 0.045% detergent, this capacity was lost rapidly enough so that O_2^- production did not occur under the conditions of the incubation. With FAD present, however, stimulation as well as inhibition of O_2^- production could be seen, depending on the concentration of detergent. In the experiment shown, the initial rate of O_2^- production in the presence of 0.022% Triton X-100 was almost twice the rate measured in its absence. At higher concentrations, however, the effect was similar to that seen in the absence of FAD—that is, an acceleration in the spontaneous loss of O_2^--generating activity.

The studies described thus far were all conducted with particles which had

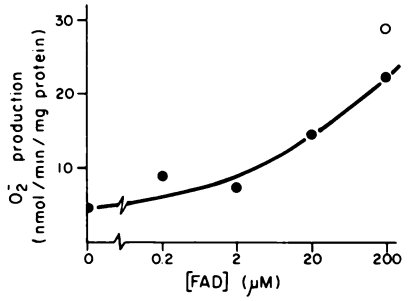

Fig. 1. O_2^--forming activity in the presence of detergent as a function of FAD concentration. Incubations were conducted as described in Materials and Methods. FAD concentrations were as shown. Concentration of Triton X-100, 0.045% (•) or 0 (○).

*Riboflavin showed some activity at higher concentrations (not shown).

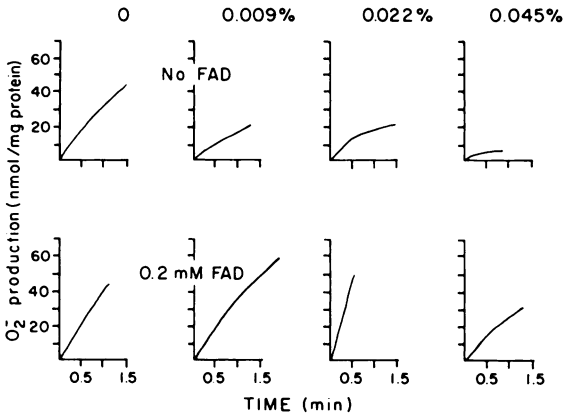

Fig. 2. O_2^--forming activity as a function of detergent concentration in the presence and absence of FAD. Incubations were conducted as described in Materials and Methods. Upper figures represent incubations carried out in the absence of FAD; lower figures, those carried out in its presence. Triton X-100 concentrations indicated above each column.

not been exposed to Triton X-100 except during the assay. To ascertain the reason for the loss in activity of the particulate O_2^--forming system in the presence of detergent, experiments were conducted in which the particles were pretreated with Triton X-100, then assayed in the presence of FAD at detergent concentrations low enough not to affect O_2^- production (Table 5). These experiments showed that, as long as the assay was carried out in the presence of FAD, the activity of detergent-extracted particles was roughly comparable to the activity of particles never exposed to detergent. This situation occurred

Table 5. O_2^--forming Activity of Particles Preincubated With Detergent

	O_2^- Production (nmoles/min)		
Assay Conditions	Particles Treated With FAD-containing Extraction Buffer	Particles Treated With FAD-free Extraction Buffer	Control Particles
Complete reaction mixture	2.32 ± 0.22	1.77 ± 0.06	2.06 ± 0.34
Omit particles	0.52 ± 0.10	0.41 ± 0.05	
Omit Triton extracts	1.74 ± 0.26	0.97 ± 0.21	
Omit particles and extracts	0.05 ± 0.05	0.05 ± 0.04	

Particles were treated with detergent as described in Materials and Methods. The O_2^--forming activity of the detergent-treated particles and the Triton extracts were determined as follows. Into each of two 1-ml cuvettes (1-cm path length) were placed 0.1 ml detergent-treated particles (or 0.1 ml 20 mM potassium phosphate buffer, pH 7.0, containing 1 mM NaN_3), 0.2 ml Triton extracts (or the corresponding extraction buffer), 0.6 ml assay buffer [0.13 M potassium phosphate buffer (pH 7.0)/2 mM FAD/0.1 M NaN_3 = 5/1/0.06 (v/v) for assays containing Triton extracts or extraction buffer without FAD; 0.13 M potassium phosphate buffer (pH 7.0)/1.2 mM FAD/0.1 M NaN_3 = 5/1/0.06 (v/v) for assays containing Triton extracts or extraction buffer with FAD)], 0.1 ml of 1 mM NADPH, and 0.01 ml ferricytochrome C solution (0.1 g/ml). To the reference cuvette was added 10 μl superoxide dismutase (3 mg/ml), while sample cuvettes received 10 μl of water. Dismutase-inhibitable cytochrome C reduction was then followed at 25° C in a Cary 118C recording spectrophotometer. Rates of cytochrome C reduction were calculated as previously described.[15]

Control assays were carried out with particles which had never been exposed to Triton X-100 (see Materials and Methods). For these determinations, the cuvettes received 0.1 ml particle suspension, 0.2 ml H_2O, 0.6 ml 20 mM potassium phosphate buffer, pH 7.0, containing 1 mM NaN_3, plus NADPH, ferricytochrome C, and either superoxide dismutase or water as described above. Dismutase-inhibitable cytochrome C reduction was measured as described above.

The data shown represent the mean ± SE from three experiments, each conducted with particles from a different preparation of neutrophils.

whether or not FAD was present during pretreatment of the particles with Triton X-100. If FAD was omitted from the assay, however, the activity of detergent-extracted particles was found to be greatly diminished as compared with the control (data not shown). The last finding was in accord with the observations reported above that 0.045% Triton X-100 in the assay mixture virtually abolished O_2^- production unless FAD was present as well. It thus appears that the effect of Triton X-100 is to release the flavin from the active site without causing a major alteration in the activity of the enzyme.

It is of interest that while most of the O_2^--forming activity in the detergent-treated preparation remained with the particles, a small but significant fraction was released into the extraction buffer. This finding suggests that detergent treatment might provide a basis for the solubilization of the O_2^--forming system from the activated particles. Experiments are currently under way to examine this possibility further.

DISCUSSION

Despite the fact that the present studies have been conducted with a crude system rather than with an enzyme purified to homogeneity, we believe that the evidence supporting the flavoprotein nature of the O_2^--forming enzyme is compelling. In the first place, O_2^- production in the presence of Triton X-100 required both FAD and "live" (i.e., unheated) particles, indicating that under these conditions both the flavin and an enzyme were necessary for this reaction to take place. The enzyme appears to be identical to the O_2^--forming enzyme which participates in the respiratory burst, since both require a pyridine nucleotide as electron donor, both are found in particles from zymosan-activated but not resting cells, and both are missing from particles isolated from zymosan-activated CGD neutrophils. Second, FAD is strongly preferred over other flavins. This observation supports the notion that O_2^- formation in the presence of Triton X-100 requires the participation of the flavin as an enzyme-bound prosthetic group rather than a nonspecific electron carrier, a function for which FMN and riboflavin would be equally suitable.

Third, a role for the flavin as a prosthetic group which transports reducing equivalents from NADPH to oxygen is suggested by the fact that virtually every flavoprotein described to date[19] has been a redox enzyme which requires the flavin for electron transport from substrate to oxidizing agent. Indeed, in a few cases it has been shown spectroscopically that the enzyme-bound flavin undergoes reduction and oxidation at a rate equal to or greater than the turnover number of the enzyme.[20] Furthermore, several of these flavoproteins, including xanthine oxidase,[21] dihydroorotic acid dehydrogenase,[22] lipoyl dehydrogenase,[23] and glutathione reductase,[23] produce some O_2^- during the course of their activity. From these considerations it seems highly likely that the O_2^--forming enzyme of the neutrophil requires a flavin, probably FAD, as a prosthetic group.

Although the present studies have demonstrated a qualitative requirement for FAD on the part of the O_2^--forming system from neutrophils, it is difficult to draw quantitative conclusions regarding the nature of the interaction between the cofactor and the enzyme. Flavin-requiring enzymes can be divided

into two categories: those to which the cofactor is bound noncovalently, and those to which the cofactor is attached through a covalent bond.[19] Either could display the behavior described here. In the case of an enzyme with noncovalently attached flavin, the detergent could weaken the interaction between the flavin and the enzyme without destroying the active site, so that the flavin originally bound to the active site diffuses into solution, and supplementary flavin must be added to supply the necessary cofactor. A similar situation could exist for an enzyme with covalently attached flavin, except that in this case the flavin would not diffuse away into solution, but would merely leave the active site, remaining attached to the enzyme at the point of the covalent bond but otherwise assuming a random orientation.

The significance of the relationship between the O_2^--forming activity and the concentration of FAD is similarly obscure. While it seems unlikely that the binding of FAD to the enzyme would be weaker than that corresponding to a K_m of 20 μM (the lowest FAD concentration yielding substantial preservation of activity compared with the control, i.e., FAD = 0; see Fig. 1), it is possible that the binding is much stronger, and that the affinity indicated by the concentration dependence data is artifactually reduced by the action of the detergent on the enzyme.

The demonstration of a cofactor requirement for the neutrophil O_2^--forming enzyme raises the possibility that specific therapy may be available for the treatment of CGD. In this condition, the O_2^--forming activity is lacking in the neutrophils. Although FAD did not restore O_2^--forming capacity to the neutrophil particles from the two CGD patients studied here, there may be other patients whose defect could be corrected by FAD. It is conceivable, for example, that in some patients an abnormality in the association of FAD with the enzyme is responsible for the defect in O_2^- formation, a situation analogous to that described in a patient with a deficiency of the flavin enzyme glutathione reductase.[24] In such patients, it might be possible to correct the defect through the administration of pharmacologic doses of riboflavin or FAD.

REFERENCES

1. Klebanoff SJ: Antimicrobial mechanisms in neutrophilic polymorphonuclear leukocytes. Semin Hematol 12:117-142, 1975

2. Sbarra AJ, Karnovsky ML: The biochemical basis of phagocytosis. I. Metabolic changes during the ingestion of particles by polymorphonuclear leukocytes. J Biol Chem 234: 1355-1362, 1959

3. Iyer GYN, Islam MF, Quastel JH: Biochemical aspects of phagocytosis. Nature 192: 535-541, 1961

4. Karnovsky ML: Chronic granulomatous disease—Pieces of a cellular and molecular puzzle. Fed Proc 32:1527-1533, 1973

5. Babior BM, Kipnes RS, Curnutte JT: Biological defense mechanisms: The production by leukocytes of superoxide, a potential bactericidal agent. J Clin Invest 52:741-744, 1973

6. Curnutte JT, Babior BM: Biological defense mechanisms: The effect of bacteria and serum on superoxide production by granulocytes. J Clin Invest 53:1662-1672, 1974

7. Weening RS, Wever R, Roos D: Quantitative aspects of the production of superoxide radicals by phagocytizing human granulocytes. J Lab Clin Med 85:245-252, 1975

8. Johnson RB, Keele BB, Misra HP, Lehmeyer JE, Webb LS, Baehner RL, Rajagopalan KV: The role of superoxide anion generation in phagocytic bactericidal activity: Studies with normal and chronic granulomatous disease leukocytes. J Clin Invest 55:1357-1372, 1975

9. Goldstein IM, Roos D, Kaplan HB, Weissmann G: Complement and immunoglobulins stimulate superoxide production by human

leukocytes independently of phagocytosis. J Clin Invest 56:1155-1163, 1975

10. Homan-Müller JWT, Weening RS, Roos D: Production of hydrogen peroxide by phagocytizing human granulocytes. J Lab Clin Med 85:198-207, 1975

11. Root RK, Metcalf J, Oshino N, Chance B: H_2O_2 release from human granulocytes during phagocytosis. I. Documentation, quantitation, and some regulating factors. J Clin Invest 55:945-955, 1975

12. Patriarca P, Cramer R, Moncalvo S, Rossi F, Romeo D: Enzymatic basis of metabolic stimulation in leukocytes during phagocytosis: The role of activated NADPH oxidase. Arch Biochem Biophys 145:255-262, 1971

13. Hohn DC, Lehrer RI: NADPH oxidase deficiency in X-linked chronic granulomatous disease. J Clin Invest 55:707-713, 1975

14. Curnutte JT, Kipnes RS, Babior BM: Defect in pyridine nucleotide dependent superoxide production by a particulate fraction from the granulocytes of patients with chronic granulomatous disease. N Engl J Med 293:628-632, 1975

15. Babior BM, Curnutte JT, McMurrich BJ: The particulate superoxide-forming system from human neutrophils. Properties of the system, and further evidence supporting its participation in the respiratory burst. J Clin Invest 58:989-996, 1976

16. Holmes B, Page AR, Good RA: Studies of the metabolic activity of leukocytes from patients with a genetic abnormality of phagocyte function. J Clin Invest 46:1422-1432, 1967

17. Baehner RL, Nathan DG: Leukocyte oxidase: Defective activity in chronic granulomatous disease. Science 155:835-836, 1967

18. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ: Protein measurement with the Folin phenol reagent. J Biol Chem 193:265-275, 1951

19. Mahler HR, Cordes EH: Biological Chemistry (ed 2). New York, Harper & Row, 1971, pp 645-656

20. Yagi K: Reaction mechanism of D-amino acid oxidase. Adv Enzymol 34:41-78, 1971

21. McCord JM, Fridovich I: The reduction of cytochrome *c* by milk xanthine oxidase. J Biol Chem 243:5753-5760, 1968

22. Forman HJ, Kennedy J: Superoxide production and electron transport in mitochondrial oxidation of dihydroorotic acid. J Biol Chem 250:4322-4326, 1975

23. Massey V, Strickland S, Mayhew SG, Howell LG, Engel PC, Matthews RG, Schuman M, Sullivan PA: The production of superoxide anion radicals in the reaction of reduced flavins and flavoproteins with molecular oxygen. Biochem Biophys Res Commun 36:891-897, 1969

24. Staal GEJ, Helleman PW, DeWael J, Veeger C: Purification and properties of an abnormal glutathione reductase from human erythrocytes. Biochem Biophys Acta 185:63-69, 1969