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Registry No. MgATP, 1476-84-2; $[Fe_4S_4(SCyh)_4]^{3-}$, 98652-71-2; $[Fe_4S_4(SBz)_4]^{3-}$, 63138-11-4; nitrogenase, 9013-04-1.

References

Aasa, R., & Vanngard, T. (1970) *J. Chem. Phys. 52*, 1612–1616.

Aasa, R., & Vanngard, T. (1975) *J. Magn. Reson. 19*, 308–314.

Anderson, G. L., & Howard, J. B. (1984) *Biochemistry 23*, 2118–2122.

Braaksma, A., Haaker, H., Grande, H. J., & Veeger, C. (1982) *Eur. J. Biochem. 121*, 483–491.

Braaksma, A., Haaker, H., & Veeger, C (1983) *Eur. J. Biochem. 133*, 71–76.

Burgess, B. K. (1984) in *Advances in Nitrogen Fixation* (Veeger, C., & Newton, W. E., Eds.) pp 103–114, Martinus Nijhoff/Dr. W. Junk Publishers, The Hague and Pudoc, Wageningen.

Burgess, B. K., Jacobs, D. B., & Stiefel, E. I. (1980) *Biochim. Biophys. Acta 614*, 196–209.

Hausinger, R. P., & Howard, J. B. (1983) *J. Biol. Chem. 258*, 13486.

Laskowski, E. J., Reynolds, J. G., Frankel, R. B., Foner, S., Papaefthymiou, G. C., & Holm, R. H. (1979) *J. Am. Chem. Soc. 101*, 6562–6570.

Lindahl, P. A., Orme-Johnson, W. H., Kent, T. A., Day, E. P., & Münck, E. (1985) *Rev. Port. Quim. 27*, 191.

Lindahl, P. A., Day, E. P., Kent, T. A., Orme-Johnson, W. A., & Munck, E. (1985) *J. Biol. Chem. 260*, 11160.

Lowe, D. J., Smith, B. E., & Eady, R. R. (1980) in *Recent Advances in Nitrogen Fixation* (Rao, S., Ed.) pp 34–87, Oxford and IBH Publishing, New Delhi.

Mortenson, L. E., & Thorneley, R. N. F. (1979) *Annu. Rev. Biochem. 48*, 387–418.

Orme-Johnson, W. H., Hamilton, W. D., Jones, T. L., Tso, M.-Y. W., Burris, R. H., Shah, V. K., & Brill, W. J. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 3142.

Orme-Johnson, W. H., Davis, L. C., Henzl, M. T., Averill, B. A., Orme-Johnson, N. R., Munck, E., & Zimmerman, R. (1977) in *Recent Developments in Nitrogen Fixation* (Newton, W. E., Postgate, J. R., & Rodriguez-Barrueco, C., Eds.) pp 131–178, Academic Press, London.

Palmer, G., Multani, J. S., Cretney, W. C., Zumft, W. G., & Mortenson, L. E. (1972) *Arch. Biochem. Biophys. 153*, 325.

Randolph, M. L. (1972) in *Biological Applications of Electron Spin Resonance* (Swartz, H. M., Bolton, J. R., & Borg, D. C., Eds.) pp 128–129, Wiley-Interscience, New York.

Watt, G. D. (1979) *Anal. Biochem. 99*, 399.

Yates, M. G., & Planque, K. (1975) *Eur. J. Biochem. 60*, 467–476.

Zumft, W. G., Mortenson, L. E., & Palmer, G. (1974) *Eur. J. Biochem. 46*, 525–535.

Purification and Properties of an O_2^{-}·-Generating Oxidase from Bovine Polymorphonuclear Neutrophils†

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ABSTRACT: A membrane-associated O_2^{-}·-generating oxidase has been purified from activated bovine polymorphonuclear neutrophils (PMN). The oxidase was extracted with Triton X-100 from a PMN membrane fraction largely devoid of lysosomal granules. The Triton extract was purified by a series of steps, including ion-exchange chromatography on DE-52 cellulose, gel filtration on Sephadex G-200, and isoelectric focusing. The O_2^{-}·-generating oxidase activity was assayed as a superoxide dismutase inhibitable cytochrome *c* reductase. The activity of the purified enzyme was strictly dependent on NADPH as electron donor. The purification factor with respect to the phorbol myristate acetate activated PMN was 75, and the recovery was about 6%. The reactivity of the purified oxidase was increased by 3–4-fold after incubation with asolectin. The minimum molecular weight of the oxidase, deduced from migration in sodium dodecyl sulfate–polyacrylamide gel electrophoresis, was 65 000 ± 3000. The optimum pH of the oxidase was 7.5, its $K_{M,NADPH}$ was $\simeq$30 μM, and its isoelectric point was at pH 5.0. The enzyme was inhibited by low concentrations of mersalyl (half-inhibition $\simeq$ 10 μM) and Cibacron Blue (half-inhibition < 10 μM). It was insensitive to 1 mM cyanide. Rapid loss of activity occurred at 0–2 °C, concomitantly with a decrease in sensitivity to superoxide dismutase: both activity and sensitivity to superoxide dismutase could be restored by addition of asolectin. The purified oxidase contained no spectrophotometrically detectable cytochrome *b*, and enzymatic assay failed to detect FAD in oxidase preparations subjected to heat treatment or trypsin digestion.

Polymorphonuclear neutrophils (PMN)[1] respond to a number of membrane stimulants by a sharp increase in superoxide (O_2^{-}·) production. The O_2 uptake corresponding to the respiratory burst is catalyzed by a cyanide-resistant NADPH

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[1] Abbreviations: PMN, polymorphonuclear neutrophils; PMSF, phenylmethanesulfonyl fluoride; PMA, phorbol myristate acetate; SOD, superoxide dismutase; LAPAO, (laurylamido)-*N*,*N*-dimethylpropylamine oxide; EDTA, ethylenediaminetetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate.

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oxidase according to the reaction

$$NADPH + 2O_2 \rightarrow NADP^+ + 2O_2^{-}\cdot + H^+$$

The respiratory system is most probably located in the plasma membrane of PMN. The NADPH-dependent, O_2^{-}·-generating oxidase has been extracted from human PMN by Triton X-100 (Gabig et al., 1978; Gabig & Babior, 1979; Babior & Peters, 1981) or deoxycholate (Tauber & Goetzl, 1979) and from guinea pig PMN by deoxycholate (Bellavite et al., 1983a,b; Serra et al., 1984) or a mixture of deoxycholate and Tween 20 (Wakeyama et al., 1982, 1983) or deoxycholate and Lubrol (Bellavite et al., 1984; Cross et al., 1984). A flavoprotein that transfers electrons from NADPH to artificial electron acceptors, but does not reduce O_2 to O_2^{-}·, has been suggested to be a component of the oxidase system (Gabig, 1983; Gabig & Lefker, 1984). A low potential cytochrome *b*, which has per se no NADPH-dependent O_2^{-}·-generating activity, and which is thought to be a component of the oxidase (Segal & Jones, 1978), has been purified from human PMN (Harper et al., 1984; Lutter et al., 1985) and bovine PMN (Pember et al., 1984). This paper reports the purification of a strictly NADPH-dependent O_2^{-}·-generating oxidase from bovine PMN and documents some of the properties of this oxidase.

Materials and Methods

Materials. Ferricytochrome *c* (horse heart grade VI), superoxide dismutase, and phorbol myristate acetate were from Sigma; NADPH and NADH were from Boehringer; sucrose was from Merck, DE-52 cellulose was from Whatman, and sterile Percoll was from Pharmacia. Stock solutions of phorbol myristate acetate (PMA) were made up to 1 mg/mL in dimethyl sulfoxide.

Preparation of a Particulate Fraction of Activated PMN Enriched in Oxidase. The preparation of bovine PMN described hereafter departs from that originally reported by Carlson & Kaneko (1973) and is therefore reported in some detail. Blood was routinely collected from 2-month old cattle, since in adult cattle, blood frequently contained a large percentage of eosinophils. We usually started from 5 L of blood that were mixed with 50 mL of 0.5 M EDTA. The red cells were lysed by dilution with 5 L of cold distilled water and mixed by inversion. After a contact of 45–60 s, isotonicity was reestablished by rapid addition of 0.5 L of 9% NaCl. All subsequent steps were carried out at 4 °C. The leukocytes were sedimented by centrifugation at 300*g* for 15 min. The pellet was resuspended in 400 mL of 2.7 mM KCl, 136.7 mM NaCl, 1.5 mM KH_2PO_4, and 8.1 mM Na_2HPO_4, pH 7.4 (phosphate–saline medium), and the suspension was filtered through gauze. A second hypotonic lysis was performed for complete removal of red cells. After reestablishment of isotonicity, the leukocytes were again sedimented by centrifugation at 200*g* for 15 min and resuspended in the minimal volume of saline medium (less than 100 mL). A third hypotonic treatment was sometimes required. The cell suspension was then centrifuged through 40% Percoll in phosphate–saline buffer. Ten-milliliter aliquots of the cell suspension were placed on 25 mL of the Percoll medium in 50-mL plastic tubes, and centrifugation was carried out at 200*g* for 30 min. The PMN sedimented at the bottom of the tubes whereas monocytes and thrombocytes were retained above the Percoll layer. After resuspension in phosphate–saline medium, the PMN were pelleted by centrifugation at 200*g* for 10 min. A second washing was required to eliminate residual Percoll. From 5 L of blood, about 1 g of PMN protein could be recovered. The PMN suspension, at a concentration of about 60 mg/mL in the phosphate–saline medium, could be stored overnight at 4 °C without damage.

For activation, the PMN suspension was diluted twice with the phosphate–saline medium supplemented with 40 mM glucose. The temperature was brought to 37 °C, and PMA was added to a concentration of 0.3 μg/mg of protein. After 2 min of incubation at 37 °C with gentle stirring, the PMN suspension was diluted with 1 volume of cold 25% ethylene glycol in 20 mM potassium phosphate, pH 7.4. Aliquots of 20–30 mL of the suspension were subjected to sonic oscillations for four periods of 15 s separated by 15-s intervals by using a Branson sonifier at 40-W output. During the course of sonication the temperature was maintained at 2–6 °C.

The sonicated homogenate was fractionated into three fractions by centrifugation at 1000*g* for 10 min, 10000*g* for 15 min, and 100000*g* for 90 min at 2–4 °C. The 100000*g* fraction, enriched in O_2^{-}·-generating oxidase (specific activity about 3 times higher than that of the PMN and recovery of 50–60%), was used for solubilization of the oxidase. Depending upon the preparation, 100–120 mg of protein was recovered in the 100000*g* fraction starting from 1 g of PMN protein.

Solubilization of NADPH Oxidase Activity. The 100000*g* particulate fraction was suspended in 12% ethylene glycol and 10 mM potassium phosphate, pH 7.4, to a final concentration of 5 mg of protein/mL. To assay the solubilizing efficiency of a given detergent, the 100000*g* fraction was mixed with the detergent, incubated for 20 min at 2–4 °C under constant stirring, and centrifuged at 140000*g* for 120 min at 2 °C. Both the pellet, resuspended in the initial volume of ethylene glycol–phosphate buffer, and the supernatant were assayed for oxidase activity. In the purification procedure described here, Triton X-100 at a final concentration of 0.25% was used.

DE-52 Cellulose and Sephadex Chromatography. DE-52 cellulose was equilibrated in 12% ethylene glycol, 10 mM potassium phosphate, and 0.1% Triton X-100 final, pH 7.4.

Sephadex G-200 (fine grade) was equilibrated with the same buffer as that used for the DE-52 cellulose chromatography.

Isoelectric Focusing. A linear 0–30% sucrose gradient was made in 12% ethylene glycol, 10 mM phosphate, 0.1% Triton X-100, and 0.4% (w/v) LKB ampholines, pH 4–8, in a 110-mL column at 4 °C. The soluble protein extract containing 10–20 mg of protein was applied to the middle of the ampholine column. The electrode reservoir at the cathode (bottom of the column) was filled with 5 mM NaOH in 50% sucrose (w/v) and that at the anode with 5 mM sulfuric acid. Electrofocusing was carried out at 4 °C for 15 h at 400 V and for 30 h at 1200 V at an intensity ranging between 1 and 2 mA. After completion of electrophoresis, 3-mL fractions were collected and assayed for pH and protein content. The O_2^{-}·-generating oxidase activity was assayed on the fractions after adjustment of pH to a value of about 7.4.

Protein Assay. Protein was determined as described by Bradford (1976) with bovine serum albumin as standard.

$NaDodSO_4$–Polyacrylamide Gel Electrophoresis. Prior to electrophoresis, proteins in Triton X-100 were precipitated by 0.1 N perchloric acid, and the precipitate was washed with cold acetone and redissolved in 0.2 M sodium phosphate, pH 7.5, and 1% $NaDodSO_4$. After heat denaturation (3 min at 100 °C) and reduction by β-mercaptoethanol, proteins were analyzed by polyacrylamide gel electrophoresis in the presence of $NaDodSO_4$ as described by Weber & Osborn (1969).

Measurement of O_2^{-}·. The rate of generation of O_2^{-}· by NADPH oxidase was calculated from the difference between the rate of reduction of ferricytochrome *c* at 550 nm (ϵ_{550} =

21.1 mM^{-1} cm^{-1}) in the absence and presence of superoxide dismutase at 25 °C. The assay was carried out in the phosphate–saline medium supplemented with 50 μM cytochrome *c* and 200 μM NADPH or NADH, final volume 2.0 mL. After reduction of cytochrome *c* had been recorded for 2–4 min, 100 μg of superoxide dismutase (SOD) was added to determine the SOD-inhibitable fraction of the NAD(P)H oxidase activity. Highly purified cytochrome *c* was required, since low-grade cytochrome *c* has been found to contain an impurity, possibly SOD, responsible for spontaneous disappearance of O_2^{-}·. In the determination of the rate of reduction of cytochrome *c*, correction was made for the spontaneous reduction of cytochrome *c* by NAD(P)H. This correction was negligible for low concentrations of NAD(P)H but became quite significant at or above 200 μM.

Assays of Cytochrome b and Flavins. Determination of cytochrome *b* was based on the use of absorption coefficients for reduced minus oxidized peaks of 21.6 mM^{-1} cm^{-1} at 558 nm and 106 mM^{-1} cm^{-1} at 425 nm. FMN and FAD were determined by the method of Faeder & Siegel (1973) with some modifications. This method is based on the differential fluorescence level of the two flavins at acidic and alkaline pH. Calibrated samples of FMN and FAD were prepared in the same medium as the assayed proteins. To all samples, including standards, sodium phosphate, pH 7.3, was added to the final concentration of 70 mM. The samples were heated at 95–100 °C in a water bath for 5 min; the tubes were rapidly cooled by immersion in crushed ice, and a calibrated volume of 4 N perchloric acid was added to give a pH of 2.3. After a 20000*g* centrifugation for 10 min to remove the denatured proteins, a 2.5-mL aliquot of the supernatant was transferred to a fluorometer cuvette. Fluorescence emission at 535 nm was recorded, the excitation wavelength being 450 nm. A calibrated volume of 5 N NaOH was then added to bring the pH to 7.8, and fluorescence was again recorded. A final fluorescence measurement was made after addition of a few grains of dithionite; this latter value corresponded to the fluorescence background independent of pH. The specific fluorescence of the flavins was corrected for the background fluorescence at each pH value. Calculations were carried out by using the relationships (Faeder & Siegel, 1973):

$$F_{7.8} = N_{7.8}[\text{FMN}] + D_{7.8}[\text{FAD}]$$

$$F_{2.3} = N_{2.3}[\text{FMN}] + D_{2.3}[\text{FAD}]$$

where *N* and *D* are the fluorescence constants for FMN and FAD determined by measurement of standard flavin solutions.

Alternatively, FAD was determined by an enzymatic method using the flavoenzyme D-amino acid oxidase deprived from its bound FAD by treatment with diluted H_2SO_4 in ammonium sulfate, followed by dialysis, as described by Friedmann (1963). In this method, the rate of O_2 uptake was determined by the slope of the oxygraphic trace, using an expanded scale. Amounts of FAD as low as 1 or 2 pmol in 1 mL could be detected with good accuracy. In the enzymatic method, a 0.5-mL aliquot of the oxidase preparation in 12% ethylene glycol, 10 mM phosphate, pH 7.4, and 0.1% Triton X-100 was either heated for 5 min at 100 °C or digested by trypsin. Digestion by trypsin was performed as follows; the oxidase preparation was first heated as described above, then supplemented with 20 μg of trypsin in 0.1 mL of 0.1 M phosphate buffer, pH 8.0, and left to incubate overnight at 25 °C; this was followed by heating at 100 °C for 3 min to denature trypsin.

Incubation with Asolectin. Asolectin in 12% ethylene glycol and 10 mM P_i, pH 7.4, was dispersed by sonic irradiation to a final concentration of 10 mg/mL until the suspension was totally clarified. The Triton extract (1 mg of protein/mL) in 12% ethylene glycol, 10 mM P_i, and 0.25% Triton X-100 pH 7.4, was incubated for 30 min at 0 °C with 1 mg of the dispersed asolectin, and the mixture was subjected to two successive ultrasonic irradiations in a Branson sonifier at a power of 40 W for 10 s each time. Treatment of purified fractions was identical except that the protein concentration was at least 10 times lower.

Results

Properties of the 100000g Fraction as Starting Material for Purification of the NADPH Oxidase. The 100000*g* fraction obtained after sonic disruption of activated bovine PMN and elimination of lysosomal granules by previous centrifugation at 10000*g* was enriched by about 3-fold in the NADPH-dependent O_2^{-}·-generating oxidase (assayed by the SOD-inhibitable reduction of cytochrome *c*; cf. Materials and Methods). The absence of oxidase activity in the 100000*g* supernatant indicated that the oxidase was a membrane-bound protein.

With the 100000*g* fraction, cytochrome *c* was reduced by both NADPH and NADH in the absence of SOD, with about the same activities for both electron donors, i.e., 65 ± 21 nmol of cytochrome *c* reduced min^{-1} (mg of protein)$^{-1}$ with NADPH as substrate and 75 ± 25 nmol cytochrome *c* reduced min^{-1} (mg of protein)$^{-1}$ with NADH as substrate. The two activities differed by their sensitivities to SOD and detergents and by their K_M with respect to NADH and NADPH. Nearly 90% of the NADPH–cytochrome *c* reductase activity was sensitive to SOD, compared to 15% of the NADH–cytochrome *c* reductase. In other words, 85% of the NADH–cytochrome *c* reductase had a diaphorase-like activity (insensitive to SOD), whereas 90% of the NADPH–cytochrome *c* reductase activity was directed to the formation of O_2^{-}·. The NADPH-dependent cytochrome *c* reductase activity was stimulated by low concentrations of deoxycholate (0.06%), whereas the NADH-dependent activity was inhibited by the same concentrations of deoxycholate. The $K_{M,NADH}$ of the SOD-insensitive NADH–cytochrome *c* reductase activity was 1 μM, while the $K_{M,NADPH}$ of the SOD-inhibitable NADPH–cytochrome *c* reductase activity was 30 μM. These data are summarized in Table I.

Solubilization by Detergents of the Membrane-Bound NADPH-Dependent O_2^{-}·-Generating Oxidase. Detergents were required to extract the SOD-sensitive NADPH-dependent cytochrome *c* reductase from the 100000*g* fraction of PMA-activated PMN homogenate, pointing to the intrinsic nature of the enzyme. Triton X-100, Emulphogen, LAPAO, and sodium deoxycholate had virtually the same efficiency (Table II). Because the purification procedure included a step of isoelectric focusing, deoxycholate being a charged molecule was not utilized. More than 50% of the NADPH–cytochrome *c* reductase activity of the 100000*g* fraction was extracted by 0.25% Triton X-100 at 2–4 °C under the conditions described under Materials and Methods; two-thirds of the extracted activity was inhibited by SOD and therefore corresponded to the O_2^{-}·-generating oxidase activity.

As shown in Figure 1, increasing amounts of SOD-sensitive NADPH–cytochrome *c* reductase were solubilized with increasing concentrations of Triton X-100 up to 0.25%; this was accompanied, however, by a net loss of total activity. Optimal extraction was achieved with Triton concentrations ranging between 0.20% and 0.25%.

The extract contained not only a SOD-sensitive NADPH–cytochrome *c* reductase activity but also an NADH–cytochrome *c* reductase activity, mostly SOD insensitive and 4–5

Table I: Maximal Rate and K_M of the SOD-Sensitive and SOD-Insensitive Cytochrome *c* Reductase of the 100000*g* Membrane Fraction of a Bovine PMN Homogenate. Effect of Deoxycholate[a]

parameters	NADPH cytochrome *c* reductase [nmol of cyt *c* reduced min^{-1} (mg of protein)$^{-1}$]			NADH cytochrome *c* reductase [nmol of cyt *c* reduced min^{-1} (mg of protein)$^{-1}$]		
	total act.[b] (SOD sensitive plus SOD insensitive)	SOD sensitive	SOD insensitive	total act.[c] (SOD sensitive plus SOD insensitive)	SOD sensitive	SOD insensitive
velocity [nmol of cyt *c* min^{-1} (mg of protein)$^{-1}$]	65 ± 21	52	13	75 ± 25	10	65
K_M (μM)		30	2		ND[d]	1
effect of 0.06% deoxycholate	270–300% increase			61–75% decrease		

[a] The enzyme activities were tested as described under Material and Methods. Bovine PMN were activated by PMA (cf. Materials and Methods). [b] In the presence of deoxycholate. [c] In the absence of deoxycholate. [d] ND, not determined.

Table II: Effect of Detergents on the Solubilization of the SOD-Sensitive NADPH-Dependent Cytochrome *c* Reductase Activity of the 100000*g* Membrane Fraction of PMA-Activated PMN

detergent used[a]	fraction assayed	protein (mg)	total NADPH–cyt *c* reductase act.[c]	SOD sensitive NADPH–cyt *c* reductase act.[c]
none	100000*g* fracton[b]	5.0	58	51
0.2% Triton X-100	soluble	1.7	31	20
	pellet	3.3	11	11
0.5% Emulphogen	soluble	1.9	26	17
	pellet	3.1	3	1
0.5% LAPAO	soluble	1.7	24	18
	pellet	3.3	2	1
0.5% deoxycholate	soluble	2.1	23	13
	pellet	2.9	6	5

[a] The different detergents were added to 5 mg of the 100000*g* fraction in 1 mL of 12% ethylene glycol and 10 mM P_i, pH 7.4, at their optimal concentration for extraction of activity. The mixtures were stirred for 20 min at 0 °C and then centrifuged for 2 h at 140000*g* in a Spinco Rotor 40. The supernatant (soluble fraction) and the pellet resuspended in the initial volume of 12% ethylene glycol and 10 mM P_i, pH 7.4, were assayed for NADPH cytochrome *c* reductase activity. [b] The 100000*g* fraction prepared from PMA-activated bovine PMN (cf. Table I) was suspended in 0.06% deoxycholate, which enhanced the activity of the NADPH oxidase to a maximal value. The 100000*g* fraction is therefore referred to as the control. [c] Units: nmol of cytochrome *c* reduced min^{-1} (mg of protein)$^{-1}$.

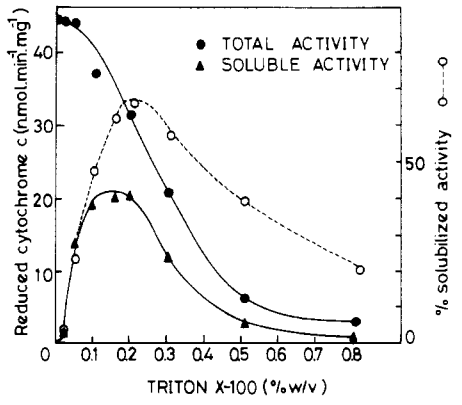

FIGURE 1: Effect of the concentration of Triton X-100 on solubilization of the SOD-sensitive NADPH–cytochrome *c* reductase from the 100000*g* particulate fraction of bovine PMN homogenate. Conditions are described under Materials and Methods.

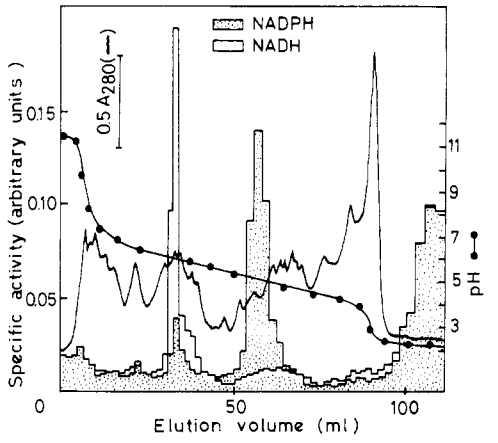

FIGURE 2: Demonstration by isoelectric focusing of the presence of two distinct NADH- and NADPH–cytochrome *c* reductase activities in the Triton extract of the 100000*g* particulate fraction of bovine PMN homogenate. Isoelectric focusing was carried out at 4 °C on 10 mg of protein, in a 110-mL sucrose gradient (5–40% sucrose) containing 0.4% (w/w) ampholines. Conditions are described under Materials and Methods. The protein content of the eluate was monitored at 280 nm. The enzyme activity was assayed on 0.1 mL of each fraction; the specific activity refers to the increase in absorbance at 550 nm, reflecting reduction of cytochrome *c* in the presence of 200 μM NADPH or NADH in phosphate–saline medium supplemented with 0.1 M phosphate buffer, pH 7.

times higher than the NADPH-dependent enzyme activity. The presence of two specific NADH- and NADPH-dependent activities was ascertained by electrofocusing (Figure 2). The NADH activity focused at pH 6.2 and the NADPH activity at pH 5.0. The K_M's of the two enzymes for NADH and NADPH were 1 μM and 30 μM, respectively, corroborating the results obtained with the 100000*g* particulate fraction. There was a small overlapping of the two activities, suggesting that, although the NADH enzyme preferentially uses NADH as electron donor, it can also accept NADPH, but with low efficiency. The reductase activity found at the acidic electrode was not heat sensitive and did not depend on NADH or NADPH; it was clearly nonenzymatic.

After extraction, the SOD-sensitive, NADPH–cytochrome *c* reductase lost its activity rapidly at 0–4 °C (25–40%/day). The K_M value of the residual active enzyme was not altered. Antiproteases like PMSF or diisopropyl fluorophosphate had a negligible protective effect. In contrast, the enzyme remained virtually unaltered for 1 week when kept at −80 °C.

Enzyme Purification. The data in Table III illustrate the course of a typical purification of the SOD-sensitive NADPH–cytochrome *c* reductase contained in the Triton extract of the 100000*g* particulate fraction described above. Total and SOD-inhibitable activities of NADPH- and

Table III: Purification of the $O_2^{\cdot-}$-Generating Oxidase from the 100000*g* Fraction of Activated Bovine PMN[a]

steps	fraction tested	protein (mg)	NADPH–cyt *c* reductase act.[c] total[e]	NADPH–cyt *c* reductase act.[c] SOD sensitive	NADH–cyt *c* reductase act.[c] total[e]	NADH–cyt *c* reductase act.[c] SOD sensitive	FAD[b] (nmol/mg)	cyt *b* (nmol/mg)
Triton extraction of the 10000–100000*g* fraction	extract	58	7.0	4.5	51.0	15.9	0.29, 0.52	0.13
DE-52 cellulose chromatography	peak I (DE-I)	14	24.0	12.3	126.1	19.0	0.18, 0.19	0.19
	peak II (DE-II)	5.8	76.0	53.1	8.1	2.3	0.11, 0.12	0.04
concn on Amicon membrane	DE-II after concn	5.6	45.1	20.0	7.0	0.2	0.08, 0.10	ND[d]
Sephadex chromatography	peak after the void volume	5.2	86.0	34.0	1.0	1.0	0.02, 0.02	0.02
isoelectric focusing	fraction pH 5	0.6	342.4	222.0	0.2	0.2	0.01, 0.02	0.02

[a] Conditions of assays are given under Materials and Methods. Because of spontaneous loss of activity upon aging, all values of activity have been calculated from measurements carried out at the same time on samples recovered at the different steps of the purification and kept until the end of the purification, i.e., 4–5 days after the initial step of extraction of the 100000*g* fraction. For example, the value of 7.0 nmol of cytochrome *c* reduced min^{-1} (mg of protein)$^{-1}$ in the presence of NADPH corresponding to the Triton extract of the 100000*g* fraction is that measured on an aliquot sample kept at 2 °C until the last stage of purification; at the onset of the purification procedure, its activity was 40 nmol of cytochrome *c* reduced min^{-1} (mg of protein)$^{-1}$. [b] The FAD content of the different fraction was determined by enzymatic assay on a heated sample (first value) and a heated plus trypsin-digested sample (second value). [c] Units: nmol of cytochrome *c* reduced min^{-1} mg^{-1}. [d] ND, not determined. [e] Total activity corresponds to the sum of SOD-sensitive and SOD-insensitive activities.

NADH–cytochrome *c* reductases were determined. The specific contents in FAD and cytochrome *b* were also measured.

As mentioned in the above section, an active extract loses its activity when left for hours or days at 2–4 °C. Because of the length of the purification procedure which lasted for 4–5 days, a spontaneous decrease in activity occurred, which had to be taken into account in the assessment of the purification factor. For the sake of clarity, we reported in Table III measurements made on aliquot samples saved at different stages of the purification procedure and analyzed at the final step. For example, the Triton extract had a NADPH–cytochrome *c* reductase activity corresponding to 35 nmol of cytochrome *c* reduced min^{-1} mg^{-1} at the onset of the purification and only to 7 nmol of cytochrome *c* reduced min^{-1} mg^{-1} when assayed at the end of the purification. The latter value is given in the table and is used for the calculation of the purification factor.

The first step in the purification procedure consisted in a chromatography on a DE-52 cellulose column (1.5 × 50 cm) equilibrated with 12% ethylene glycol, 10 mM phosphate, pH 7.4, and 0.1% Triton X-100. In the experiment referred in Table III, 29 mg of protein extract from the 100000*g* fraction in 12% ethylene glycol, 10 mM phosphate, pH 7.4, and 0.25% Triton X-100 was applied to the DE-52 cellulose column. After washing with 3 volumes of the above buffer, a continuous 0–0.3 M sodium sulfate gradient in the ethylene glycol medium was applied. The proteins were eluted by fractions of 3 mL. They were assayed for NADPH- and NADH–cytochrome *c* reductase activities. Two peaks were clearly differentiated. The first one, DE-I, enriched in NADH–cytochrome *c* reductase activity was eluted with 80–110 mM sodium sulfate; the second, DE-II, enriched in NADPH–cytochrome *c* reductase activity was eluted with 140–170 mM sodium sulfate (Figure 3A). Since SOD sensitivity was found essentially in the DE-II peak, the fractions corresponding to this peak were pooled and used for further purification. The pooled fractions were concentrated by filtration on a PM30 Amicon membrane to a final volume of 1 mL. Some apparent loss of activity resulted from concentration, possibly due to the concomitant increase in concentration of Triton X-100. In fact, when the concentrated solution was applied to a Sephadex G-200 column (1.6 cm × 36 cm) equilibrated with the ethylene glycol–phosphate–Triton X-100 buffer and eluted with the same buffer, a substantial recovery of activity was obtained. The reductase activity appeared just after the void volume (Figure 3B).

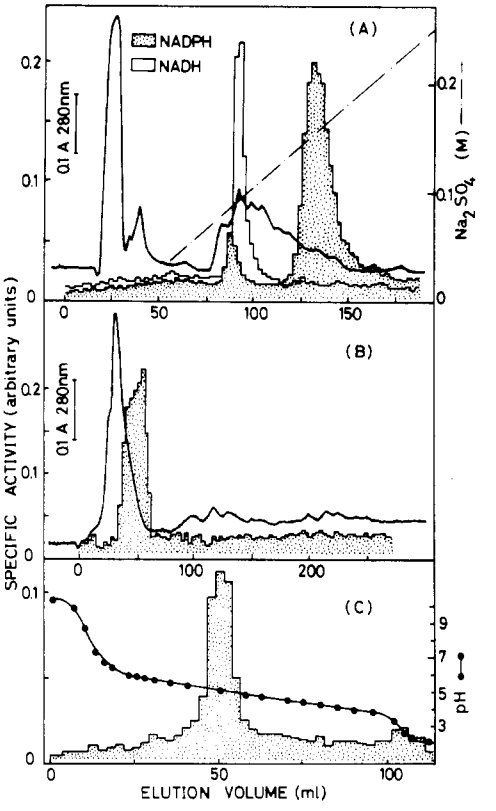

FIGURE 3: Elution patterns corresponding to steps 2, 4, and 5 of the purification procedure (see Table III). In step 2 (DE-52 chromatography), the two NADH- and NADPH-dependent activities were present. Steps 4 (Sephadex chromatography) and 5 (isoelectric focusing) concern the purification of the only NADPH-dependent reductase activity. Details are given under Results. The enzyme activity was assayed on 0.1 mL of each fraction as described under Materials and Methods; the specific activity refers to the increase in absorbance at 550 nm, reflecting reduction of cytochrome *c* in the presence of 200 μM NADPH or NADH.

The enzyme recovered from Sephadex chromatography in a volume of about 10 mL was finally subjected to isoelectric focusing (cf. Materials and Methods). The elution pattern (Figure 3C) indicated that the p*I* of the enzyme is around pH 5 as previously shown in the electrofocusing experiment carried out on a crude extract.

In the experiment illustrated in Table III, the NADPH-dependent SOD-sensitive cytochrome *c* reductase after electrofocusing was purified about 50-fold with respect to the initial Triton extract, and the recovery was close to 50%; as explained

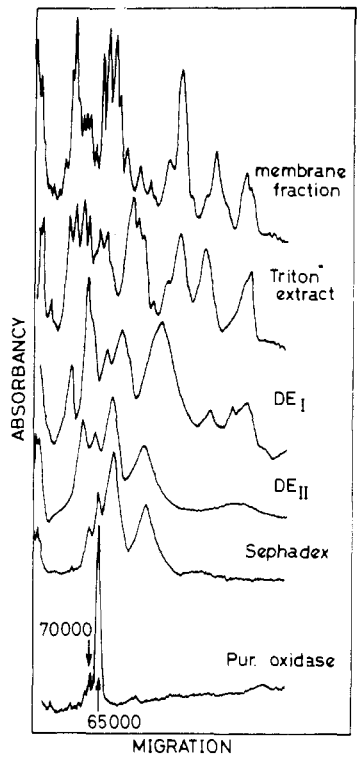

FIGURE 4: Analysis by $NaDodSO_4$–polyacrylamide gel electrophoresis of the protein species present at the different steps of the purification procedure (see text and Table III). The eluates containing the enzymatic activity were treated with 0.1 N perchloric acid. The precipitate after centrifugation (20–200 μg of protein) was washed with cold acetone to remove bound Triton X-100 and was finally solubilized in 0.1 M sodium phosphate and 1% $NaDodSO_4$ before being subjected to gel electrophoresis as described under Materials and Methods.

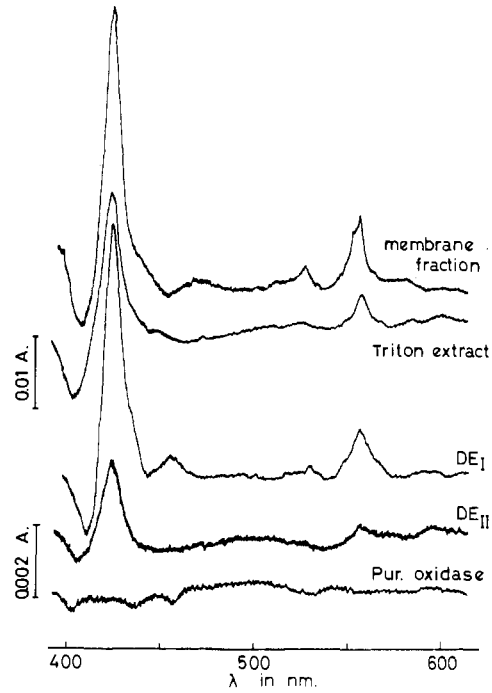

FIGURE 5: Dithionite difference spectra at 77 K of the active fractions recovered at the different steps of the purification procedure (see Figure 4). The difference spectra (reduced vs. oxidized) were recorded with a double-beam Perkin-Elmer 557 spectrophotometer. The optical path of the cuvettes was 2 mm. The corresponding protein concentrations were as follows: 100000*g* membrane fraction 4 mg/mL; Triton extract 1 mg/mL; DE-52 cellulose chromatography, DE-I 3.5 mg/mL and DE-II 0.3 mg/mL; purified oxidase after isoelectric focusing 0.3 mg/mL.

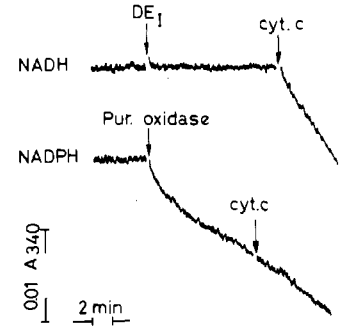

FIGURE 6: Compared oxidation of NADPH and NADH by the NADPH- and NADH-dependent oxidases in the presence or absence of cytochrome *c*. The assay was carried out with the purified NADPH-dependent enzyme after isoelectric focusing (purified oxidase) (3 μg of protein) and with the NADH-dependent enzyme eluted from DE-52 cellulose (DE-I) (12 μg of protein). The medium was the phosphate–saline buffer, pH 7.4, at 25 °C described under Methods. The final concentration of NADH and NADPH was 200 μM, and that of cytochrome *c* was 50 μM. The absorbancy at 340 *nm* was recorded.

above, these values take into account the spontaneous decay of activity of the oxidase through the different steps of the purification procedure; without correction, the recovery would be 10% and the purification 10-fold.

Throughout the purification process, the enrichment in given protein species was assessed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. A major protein characterized by a molecular weight of 65 000 ± 3000 with a minor contaminant of M_r 70 000 was recovered at the last step of the purification procedure (Figure 4).

Cytochrome *b* and flavin were assayed at each step of the purification procedure. More than 95% of the flavin in the crude Triton extract of the 100000*g* fraction consisted of FAD, as shown by differential fluorometry. Because Triton X-100 interfered with the differential fluorometric technique at low flavin concentrations, the enzymatic assay was preferred for FAD determination in the purified fractions. In contrast to the crude extract that contained substantial amounts of FAD, only traces of FAD were detected in the purified $O_2^{\cdot-}$-generating oxidase, after heat treatment or trypsin digestion of the enzyme to release the putative bound FAD. However, one cannot exclude the possibility that the heat or trypsin treatments are not appropriate for releasing a putative flavin, which would be very firmly bound to the enzyme.

Difference spectra (dithionite reduced minus oxidized) at 77 K of fractions recovered at the different steps of the purification process showed typical peaks of cytochrome *b* at 425, 527, and 557 nm in the crude extract but only traces of cytochrome *b* in the purified enzyme (Figure 5).

Alternative Procedure of Purification. When the amount of protein in the Triton extract of the 100000*g* fraction was less than 10 mg, it was found advantageous to reverse the order of the purification procedure and to start with the isoelectric focusing step. The fractions corresponding to the peak of activity of the NADPH–cytochrome *c* reductase at pH 5 were pooled and further purified by chromatography on DE-52 cellulose and Sephadex G-200, as described in Table III.

Properties of the SOD-Sensitive NADPH–Cytochrome c Reductase. The stoichiometry of the SOD-sensitive NADPH–cytochrome *c* reductase reaction was measured with the purified NADPH-dependent enzyme recovered after isoelectric focusing. The NADPH/$O_2^{\cdot-}$ ratio found was 0.5 in accordance with the expected stoichiometry (see the introduction).

The purified enzyme was able to oxidize NADPH in the presence of O_2 and the absence of cytochrome *c* (Figure 6).

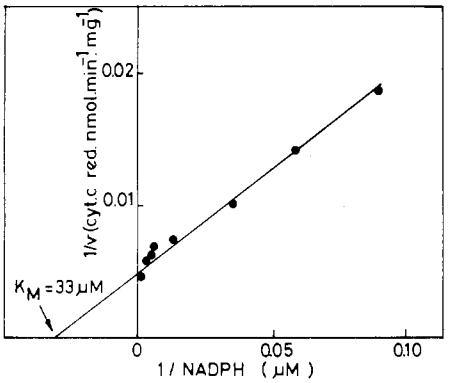

FIGURE 7: Double-reciprocal plot of the SOD-sensitive NADPH–cytochrome *c* reductase activity of the purified oxidase after isoelectric focusing (15 μg of protein). Conditions are described under Materials and Methods.

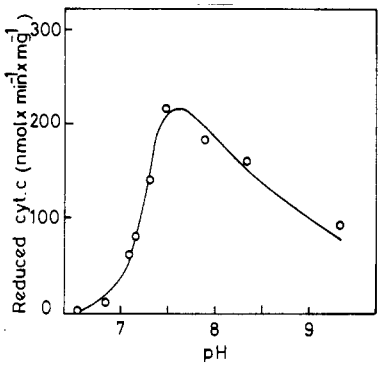

FIGURE 8: pH dependence of the SOD-sensitive NADPH–cytochrome *c* reductase activity of the purified oxidase after isoelectric focusing (10 μg of protein). Conditions are described under Materials and Methods.

In contrast, the fraction enriched in NADH-dependent cytochrome *c* reductase after DE-52 cellulose chromatography (DEI) could oxidize NADH only in the presence of cytochrome *c*. In the first case, electrons are transferred from NADPH to O_2, and the generated O_2^{-}· reduces cytochrome *c*; in the second, electrons are directly transferred from NADH to cytochrome *c*.

The purified oxidase had a K_M for NADPH of 30 μM (Figure 7), and the maximum activity was found around pH 7.5 (Figure 8). Its isoelectric point was at pH 5.0. The O_2^{-}· production by the purified oxidase was resistant to 1 mM cyanide. It should be pointed out that the respiratory activity of the 100000*g* fraction in the presence of NADPH was also insensitive to 1 mM cyanide; in contrast, the respiratory activity of the 100000*g* fraction in the presence of NADH was inhibited by about 50% by 1 mM cyanide. The purified oxidase was totally inactivated after 3 min at 100 °C. It was inhibited by mersalyl, a relatively specific thiol reagent and by Cibacron Blue, a compound capable of interacting with the respiratory burst in a membrane fraction from guinea pig PMN (Yamaguchi & Kakinuma, 1982). Half-inhibition was obtained with 7 μM mersalyl and 10 μM Cibacron Blue (Figure 9). These inhibitory concentrations are similar to those found with the membrane-bound oxidase in the 100000*g* fraction of PMN homogenate.

Effect of Asolectin. Asolectin enhanced the activity of the NADPH oxidase of the crude Triton extract and the purified fraction. Routinely, asolectin at the concentration of 10 mg/mL in 12% ethylene glycol–10 mM phosphate, pH 7.4, was subjected to sonic oscillation using a Branson sonifier at 40-W output until full clarification was obtained. In the experiment of Figure 10A, the clarified asolectin preparation

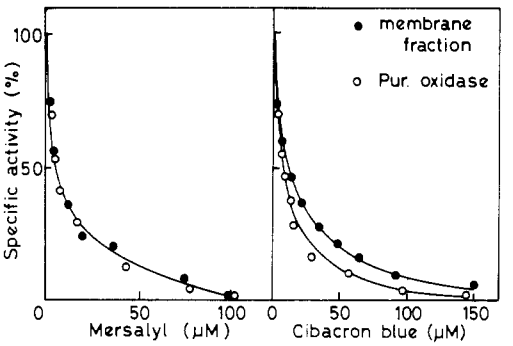

FIGURE 9: Effect of mersalyl and Cibacron Blue on the SOD-sensitive NADPH–cytochrome *c* reductase activity of the 100000*g* membrane fraction (100 μg of protein) and the purified oxidase after isoelectric focusing (30 μg of protein). Mersalyl and Cibacron Blue at the final concentration indicated were added to the reaction medium; the inhibition was established in less than 5 s.

was added to the 100000*g* Triton extract to obtain final concentrations of 1 mg of asolectin, 1 mg of protein, and 2.5 mg of Triton X-100 per mL. The mixture was incubated for 30 min at 0 °C and then subjected to two successive sonications for 10 s each at 40 W at 2–4 °C. The NADPH–cytochrome *c* reductase activity was increased by about 30%. Besides the increase in activity, asolectin has two other effects: (1) it enhanced considerably the sensitivity to SOD; (2) it reduced the lag that occasionally occurred before a steady-state reductase activity was expressed. This lag was more apparent with the aged enzyme or with the enzyme in the presence of an excess of detergent (Figure 10A). To ascertain that the effect of asolectin was related to O_2^{-}·-generating oxidase activity, the rate of NADPH oxidation was compared to that of O_2^{-}· generation. In the purification procedure illustrated in Table III, the rate of the SOD-sensitive NADPH–cytochrome *c* reduction was 222 nmol of cytochrome *c* reduced min^{-1} mg^{-1}, and the corresponding rate of NADPH oxidation was 118 nmol of NADPH oxidized min^{-1} mg^{-1}; in the presence of asolectin, the rates were 740 and 335 nmol min^{-1} mg^{-1}, respectively. Consequently, both in the absence and in the presence of asolectin, the NADPH/O_2^{-}· ratio was close to 0.5.

We have compared the effect of increasing concentrations of asolectin on the oxidase activity of the Triton extract (2.5 mg of Triton/mL) of the 100000*g* fraction of PMN homogenate and on the purified oxidase. The half-maximal stimulatory effect was obtained with less than 1 mg of asolectin/mL and full enhancement at 3–4 mg of protein/mL with the 100000*g* fraction extract. In the case of the purified oxidase, the optimal concentrations (mg/mL) of Triton X-100, asolectin, and purified protein in the sonication medium were in the ratio 1/1/0.1 (Figure 10B).

Discussion

General Comments on the Purification Procedure. This paper describes the extraction and purification of a strictly NADPH-dependent, O_2^{-}·-generating oxidase from bovine PMN activated by PMA. This hydrophobic protein was extracted by Triton X-100 from an enriched particulate fraction obtained by centrifugation at 100000*g* of a PMN homogenate, after a preliminary centrifugation at 10000*g*. The removal of the bulk of the lysosomal enzymes contained in the 10000*g* fraction prevented major contamination of the extract by lysosomal proteases. The further purification steps consisted of a DE-52 cellulose chromatography followed by concentration on Amicon membrane, filtration on Sephadex, and isoelectric focusing. The excess of salt after DE-52 cellulose chromatography and the excess Triton X-100 after concentration were removed by Sephadex filtration. All media used

contained 0.1% Triton X-100. Being neutral, Triton X-100 did not interfere with the migration of protein species during the course of isoelectric focusing in the purification procedure; this is not the case with deoxycholate.

Extraction of the oxidase activity by deoxycholate required concentrations equal to or higher than 0.3%; however, at much lower concentrations, namely, 0.04–0.08%, deoxycholate enhanced markedly the oxidase activity of the membrane-bound enzyme, for example, that of the 100000*g* fraction, without a solubilizing effect. This is simply due to facilitation of the access of NADPH to its specific site on the membrane-bound enzyme. We took advantage of this unmasking effect of deoxycholate to assay the O_2^{-}·-generating oxidase activity of the 100000*g* particulate fraction (cf. Table I). The same effect was obtained with a nonlysing concentration of Triton X-100 (less than 0.02%).

In the routine procedure adopted in this work, the O_2^{-}·-generating oxidase activity was elicited by incubation of the bovine PMN with PMA. It is noteworthy that, in the absence of PMA, Triton X-100 per se could elicit a noticeable oxidase activity in resting PMN (not shown). This is in accordance with the reported activating effect of digitonin on guinea pig PMN (Cohen & Chovaniec, 1978).

The purification procedure summarized in Table III started with Triton extraction of the particulate fraction recovered by centrifugation of the PMN homogenate between 10000*g* and 100000*g*. The purification factor of the O_2^{-}·-generating oxidase with respect to the crude extract of the 100000*g* fraction was 50, and the recovery was close to 50% (Table III). It should be recalled that the values given in Table III are corrected for the spontaneous loss of activity (see Results) occurring during the course of the purification. Should these corrections not be taken into account, the calculated recovery would be 10% and the purification factor only 10. Improvement of the purification method should be directed to devising appropriate conditions allowing maximal protection of the oxidase against aging.

The specific oxidase activity of the 100000*g* particulate fraction was about 3 times higher than that of the crude PMN homogenate (see Materials and Methods), but it decreased by more than 2-fold after extraction by Triton X-100, and the recovery was only 13% (cf. Table II). Combining these data with those of Table III, we can calculate a purification factor of about 75 for the O_2^{-}· generation oxidase with respect to the initial homogenate of PMN and a recovery of 6%. It is clear that the detergent extraction step was responsible for a severe loss of oxidase activity.

Comparison of Purification Procedures Reported in the Literature. Most of the purification procedures published to date made use of deoxycholate either alone or associated with Tween or Lubrol (see the introduction). A rapid survey of the most significant data shows that, in crude extracts or in partially purified preparations, the oxidase has a 10 times higher affinity for NADPH than for NADH (Gabig & Babior, 1979; Wakeyama et al., 1982; Cross et al., 1984). Our preparation from bovine PMN was essentially specific for NADPH; it had a specific activity of 222 nmol of O_2^{-}· produced min^{-1} (mg of protein)$^{-1}$ (not corrected for reactivation by asolectin) and 740 after treatment with asolectin (see Results). These values corresponded to 1–1.5 μmol of O_2^{-}· produced min^{-1} (mg of protein)$^{-1}$ after correction for the spontaneous loss of activity occurring during the course of purification, and it could attain 3–5 μmol of O_2^{-}· produced min^{-1} (mg of protein)$^{-1}$ in the presence of asolectin. It compares favorably to specific activities of crude preparations reported in the literature, expressed per minute per milligram of protein: 1 μmol in a crude extract of pig PMN (Wakeyama et al., 1982), 0.36 μmol in a crude extract of pig PMN (Cross et al., 1984), 0.15 μmol after gel filtration of an extract of pig PMN (Bellavite et al., 1984), and 0.29 μmol after gel filtration and glycerol gradient centrifugation of a crude extract of guinea pig PMN (Serra et al., 1984). The purification of a NADPH–cytochrome *c* reductase capable of catalyzing menadione-dependent O_2^{-}· formation in guinea pig PMN with a specific activity of 0.68 μmol/mg of protein was recently reported (Sakane et al., 1984); this enzyme differs from the bovine oxidase described here by its molecular mass, 87 000 daltons, and its requirement for menadione as electron acceptor.

A criterion of specificity for the O_2^{-}·-generating oxidase not often used in literature is the direct oxidation of NADPH by O_2 in the absence of cytochrome *c*. By use of the decrease in absorbancy at 340 nm, it was found here that the purified oxidase preparation is capable of oxidizing NADPH into $NADP^+$ by O_2 in the absence of cytochrome *c*. In contrast, the NADH–cytochrome *c* reductase in the crude extract, which is separated from the NADPH–cytochrome *c* reductase either by DE-52 cellulose chromatography or electrofocusing (see Results) could not oxidize NADH unless cytochrome *c* was added to the medium. Clearly the NADH cytochrome *c* reductase behaved as a diaphorase.

Are the Properties of the Purified Oxidase Compatible with a Function in the Respiratory Burst of Activated PMN? The purified oxidase has in common with the membrane-bound enzyme the following properties: (a) it is insensitive to cyanide, (b) it is inhibited by mersalyl and Cibacron Blue, (c) it generates O_2^{-}· at the expense of O_2, and (d) it is highly specific for NADPH and has the same affinity ($K_M \simeq 30$ μM) as the membrane-bound enzyme.

That the O_2^{-}·-generating oxidase is a membrane-bound enzyme is evidenced by the need of detergent to extract the enzyme. The enhancing effect of phospholipids (asolectin) on the oxidase activity and its sensitivity to SOD (Figure 10) are readily explained by substitution of lipids for Triton X-100 in the micelles containing the oxidase, lipids providing a more appropriate environment to the enzyme. The protective or stimulatory effect of phospholipids on crude extracts of PMN was already mentioned by Gabig & Babior (1979).

The purified bovine oxidase contained only traces of cytochrome *b*. Further, there was no spectral evidence for bound flavin, and no FAD was found in heat extract or trypsin digest of the enzyme. The data in the literature concerning the presence of FAD and cytochrome *b* in partially purified O_2^{-}·-generating oxidase are controversial. An FAD component in the O_2^{-}·-generating oxidase of activated PMN was suggested on the following basis: (a) In crude extracts of activated PMN, added FAD stimulated the O_2^{-}·-generating activity with K_M values of 61 nM (Babior & Peters, 1981) and 77 nM (Wakeyama et al., 1982). (b) Flavin analogues, like 5-carba-5-deaza-FAD, inhibited the oxidase activity (Light et al., 1981). Recently, however, an NADPH oxidase purified from pig neutrophils was reported to be virtually devoid of FAD (Bellavite et al., 1984). The FAD to cytochrome *b* ratio has been reported to vary, depending on the stage of the purification. For example, in a crude extract of activated pig PMN, the amounts of cytochrome *b* and FAD per milligram of protein were 331 and 431 pmol, respectively (Cross et al., 1984). After partial purification of the oxidase by gel filtration, the cytochrome *b* to FAD ratio, which was close to 1 in the crude extract, rose to nearly 20, and it was concluded

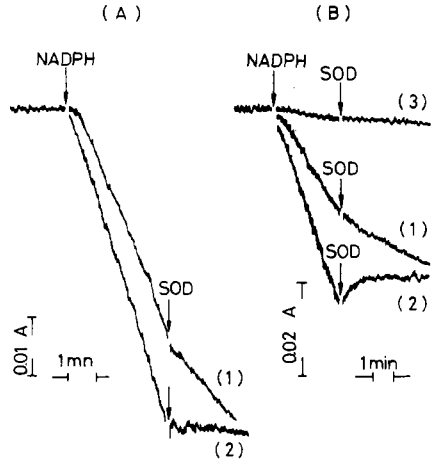

FIGURE 10: Effect of asolectin on the activity and SOD sensitivity of the crude and purified oxidase. (A) NADPH–cytochrome *c* reductase activity of the Triton extract from the 100000*g* fraction; (B) purified oxidase after isoelectric focusing. Conditions are described under Materials and Methods. NADPH, cytochrome *c*, and SOD were used at the final concentrations of 200 μM, 50 μM, and 100 μg, respectively. When present, asolectin was preincubated with the active fractions as described under Materials and Methods. In (A), trace 1 (control) corresponds to the Triton extract (150 μg of protein) without asolectin and trace 2 to the Triton extract pretreated by asolectin. In (B), trace 1 (control) corresponds to the purified oxidase (2 μg of protein) without asolectin, trace 2 to the purified oxidase pretreated with asolectin, and trace 3 to the heat-denatured oxidase with asolectin.

that cytochrome *b* is a genuine component of the oxidase (Bellavite et al., 1984). Scrutiny of these data indicated, however, that the buffer that equilibrated the Ultragel column was not supplemented with detergent and that the oxidase was eluted in the void volume; this could explain the presence of cytochrome *b* aggregates together with the oxidase in the void volume. Along this line, it is interesting to recall that cytochrome *b* has been claimed to be a component of the O_2^{-}·-generating oxidase (Segal & Jones, 1978). This claim was based in part on the correlation between genetic defects of cytochrome *b* and decrease in the respiratory burst; it was not supported, however, by a number of experimental data including the very slow reduction of cytochrome *b* in anaerobiosis during the respiratory burst (Babior, 1983; Morel & Vignais, 1984) and the absence of effect of CO on O_2 uptake (Morel & Vignais, 1984). At face value, the isolation of an O_2^{-}·-generating oxidase devoid of cytochrome *b* indicates that cytochrome *b* is not directly involved in the generation of O_2^{-}·; however, it remains possible that cytochrome *b* exerts some control on the course of the respiratory burst. We would like also to point out that the lack of demonstration of a flavin component in the heat extract of our purified oxidase preparation does not preclude the presence of a firmly bound flavin in the enzyme.

The purified protein of M_r 65000 described in the present paper is probably the O_2^{-}·-generating oxidase. One cannot entirely exclude, however, the possibility that this molecule is the core protein with a redox center of an oligomeric complex which would contain additional redox components capable of reacting rapidly with O_2 to give O_2^{-}·. Possible candidates are a flavoprotein and, less probably, the *b* type cytochrome. In our preparation, these putative redox components could be present in spectroscopically undetectable amounts, but their turnover would be high enough to account for the rapid production of O_2^{-}· by the enzymatic preparation. Only systematic reconstitution experiments may definitely solve the problem of the presence of catalytic or regulatory subunits in the O_2^{-}·-generating oxidase.

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Registry No. NADPH, 53-57-6; NADPH oxidase, 9032-22-8; O_2^{-}·, 11062-77-4.

REFERENCES

Babior, B. M. (1983) in *Advances in Host Defense Mechanisms* (Gallin, J. I., & Fauci, A. S., Eds.) Vol. 3, pp 91–119, Raven Press, New York.

Babior, B. M., & Peter, W. A. (1981) *J. Biol. Chem. 256*, 2321–2323.

Bellavite, P., Serra, M. C., Davoli, A., Bannister, J. V., & Rossi, F. (1983a) *Mol. Cell. Biochem. 52*, 15–25.

Bellavite, P., Cross, A. R., Serra, M. C., Davoli, A., Jones, O. T. G., & Rossi, F. (1983b) *Biochim. Biophys. Acta 746*, 40–47.

Bellavite, P., Jones, O. T. G., Cross, A. R., Papini, E., & Rossi, F. (1984) *Biochem. J. 223*, 639–648.

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248–254.

Carlson, G. P., & Kaneko, J. J. (1985) *Proc. Soc. Exp. Biol. Med. 142*, 853–856.

Cohen, H. J., & Chovaniec, M. E. (1978) *J. Clin. Invest. 61*, 1081–1087.

Cross, A. R., Parkinson, J. F., & Jones, O. T. G. (1984) *Biochem. J. 223*, 337–344.

Faeder, E. J., & Siegel, L. M. (1973) *Anal. Biochem. 53*, 332–336.

Friedmann, H. C. (1963) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., Ed.) pp 596–598, Academic Press, New York.

Gabig, T. G. (1983) *J. Biol. Chem. 258*, 6352–6356.

Gabig, T. G., & Babior, B. M. (1979) *J. Biol. Chem. 254*, 9070–9071.

Gabig, T. G., & Lefker, B. A. (1984) *Biochem. Biophys. Res. Commun. 118*, 430–436.

Gabig, T. G., Kipnes, R. S., & Babior, B. M. (1978) *J. Biol. Chem. 253*, 6663–6665.

Harper, A. M., Dunne, M. J., & Segal, A. W. (1984) *Biochem. J. 219*, 519–527.

Light, D. R., Walsh, C., O'Callaghan, A. M., Goetzl, E. J., & Tauber, A. I. (1981) *Biochemistry 20*, 1468–1476.

Lutter, R., Van Schaik, M. L. J., Van Zwieten, R., Wever, R., Roos, D., & Hamers, M. N. (1985) *J. Biol. Chem. 260*, 2237–2244.

Morel, F., & Vignais, P. V. (1984) *Biochim. Biophys. Acta 764*, 213–225.

Pember, S. O., Heyl, B. L., Kinkade, J. M., & Lambeth, J. D. (1984) *J. Biol. Chem. 259*, 10590–10595.

Sakane, F., Takabashi, K., & Koyama, J. (1984) *J. Biochem. (Tokyo) 96*, 671–678.

Segal, A. W., & Jones, O. T. G. (1978) *Nature (London) 276*, 515–517.

Serra, M. C., Bellavite, P., Davoli, A., Bannister, J. V., & Rossi, J. (1984) *Biochim. Biophys. Acta 788*, 138–146.

Tauber, A. I., & Goetzl, E. J. (1979) *Biochemistry 18*, 5576–5584.

Wakeyama, H., Takeshige, K., Takayanagi, R., & Minakami, S. (1982) *Biochem. J. 205*, 593–601.

Wakeyama, H., Takeshige, K., & Minakami, S. (1983) *Biochem. J. 210*, 577–587.

Weber, K., & Osborn, M. (1969) *J. Biol. Chem. 244*, 4406–4412.

Yamagashi, T., & Kakinuma, K. (1982) *Biochem. Biophys. Res. Commun. 104*, 200–206.