Structural and Catalytic Properties of the Solubilized Superoxide-Generating Activity of Human Polymorphonuclear Leukocytes. Solubilization, Stabilization in Solution, and Partial Characterization†

Alfred I. Tauber and Edward J. Goetzl*

ABSTRACT: Purified human polymorphonuclear (PMN) leukocytes that had been incubated with opsonized zymosan particles or with phorbol myristate acetate to achieve optimal activation of the superoxide-generating activity were homogenized and centrifuged to recover the subcellular particles that contained 92–95% of the superoxide-generating activity of the homogenates. Centrifugation of the particle-associated superoxide-generating activity on 10–50% sucrose gradients resolved the predominant activity from established enzymatic and other markers for the lysosomal granules, mitochondria, nuclei, cytosol, and plasma membranes. Incubation of the particle-associated superoxide-generating activity with 0.25 g/100 mL deoxycholate in 0.34 M sucrose solubilized a mean of 12.4 and 13.8% of the total activity in the particles derived from PMN leukocytes that had been activated by opsonized zymosan and phorbol myristate acetate, respectively. The specific activity of the solubilized superoxide-generating activity was one-tenth to one-fifth that of the particle-associated superoxide-generating activity. Nonetheless, both preparations were similar with respect to the pH range of 6.4–7.6 for optimal activity, the K_m value for NADPH which was lower than that for NADH, the resistance to inhibition by sodium azide, and the susceptibility to inhibition by *p*-(chloromercuri)-phenylsulfonic acid. The rapid loss of activity of solubilized superoxide-generating activity at 25 °C was reduced at 4 °C, and the half-time for decay at 4 °C was extended from 18 h to over 50 h by the addition of glycerol, ethylene glycol, or dimethyl sulfoxide. Filtration of solubilized superoxide-generating activity on Sephacryl S-200 at 4 °C for 10 h resolved two peaks of M_r 150 000 and >300 000 that maintained the initial level of specific activity. Isoelectric focusing of solubilized superoxide-generating activity for 18–24 h at 4 °C in a glycerol gradient containing ethylene glycol defined a p*I* of 7.6–8.3. Human PMN leukocyte superoxide-generating activity thus can be solubilized and purified in quantities sufficient for studies of its biochemical characteristics.

Human polymorphonuclear (PMN) leukocytes engaged in the phagocytosis of opsonized particles (Sbarra & Karnovsky, 1959) or exposed to soluble stimuli, such as phorbol myristate acetate (DeChatelet et al., 1976) or fluoride (Curnutte et al., 1979), exhibit a respiratory burst which is characterized by increases in oxygen consumption and glucose oxidation through the hexose monophosphate shunt and by increases in the production of metabolites of oxygen including superoxide ($O_2^{-}\cdot$),[1] hydroxyl radical (OH·), and hydrogen peroxide (H_2O_2) (Tauber & Babior, 1977, 1978). An activity which specifically catalyzes the reduction of oxygen to $O_2^{-}\cdot$ has been identified in a 27000*g* particulate preparation derived from homogenates of PMN leukocytes engaged in phagocytosis (Babior et al., 1976) or pretreated with phorbol myristate acetate (Dewald et al., 1979). The ability of the particulate preparation to catalyze the reduction of oxygen to $O_2^{-}\cdot$ is dependent on the donation of electrons from NADPH or NADH, and the respective K_m values indicate that the former cofactor is the preferred electron donor (Babior, 1978). That the NADPH-dependent superoxide-generating activity in the particulate preparation is central to the expression of the respiratory burst in intact PMN leukocytes has been suggested by the concomitant generation of H_2O_2 (Kakinuma et al., 1977) and $O_2^{-}\cdot$ (Babior et al., 1976) as well as OH· (Tauber et al., 1979a) by the cell-free particles, the dependence of the activation of particle-associated superoxide-generating activity on the prior stimulation of a respiratory burst in the cells that were subjected to homogenization, and the comparable susceptibility to inhibition by pharmacological agents of the respiratory burst in intact cells and the superoxide-generating activity of the particulate preparation (Babior et al., 1976). Further, superoxide-generating activity was not detectable in the particulate preparations from PMN leukocytes of patients with chronic granulomatous disease where the intact cells failed to manifest a respiratory burst (Curnutte et al., 1975; Hohn & Lehrer, 1975).

The superoxide-generating activity of human PMN leukocytes is considered to be localized in the plasma membrane based on the recovery of the superoxide-generating activity in crude membrane preparations (Dewald et al., 1979), the indirect cytochemical detection of the production of H_2O_2 at the cell surface (Briggs et al., 1975), and the inhibition by impermeant sulfhydryl-reactive agents of the elaboration of $O_2^{-}\cdot$ by stimulated PMN leukocytes (Goldstein et al., 1977). However, conflicting data have associated PMN leukocyte superoxide-generating activity with the lysosomal granules (Patriarca et al., 1976; Tsan, 1978) or have failed to support any relationship between particle-associated superoxide-generating activity and markers for defined subcellular compartments (Iverson et al., 1978). Particle-associated superoxide-generating activity that was solubilized in 0.8% Triton X-100 exhibited a molecular weight of less than ~300 000, as assessed by passage through filters which retained purified IgM, but was too unstable to be characterized in detail (Gabig

† From the Howard Hughes Medical Institute Laboratory, Harvard Medical School, and the Departments of Medicine, Harvard Medical School and the Robert B. Brigham Hospital and Peter Bent Brigham Hospital Divisions of the Affiliated Hospitals Center, Inc., Boston, Massachusetts 02115. *Received June 22, 1979; revised manuscript received September 25, 1979.* A.I.T. was supported by Grant AI-15116 from the National Institutes of Health.

* Address correspondence to this author at Harvard Medical School.

[1] Abbreviations used: $O_2^{-}\cdot$, superoxide; OH·, hydroxyl radical; H_2O_2, hydrogen peroxide; Me_2SO, dimethyl sulfoxide; 0.34 M sucrose–Tris, 0.34 M sucrose buffered with 10 mM Tris (pH 7.1).

0006-2960/79/0418-5576$01.00/0

et al., 1978). The ability to solubilize human leukocyte superoxide-generating activity in deoxycholate and to stabilize the activity of the solubilized preparation with ethylene glycol or dimethyl sulfoxide has facilitated the development of techniques for the purification of superoxide-generating activity and the definition of its biochemical characteristics.

Materials and Methods

Materials. Ferricytochrome *c* (type VI), superoxide dismutase (sp act. 2900 units/mg), NADPH, NADH, zymosan, phorbol 12-myristate 13-acetate, sodium deoxycholic acid, sodium cholic acid, nitrophenyl phosphate, flavin adenine dinucleotide, sulfanilic acid, sodium azide, *p*-(chloromercuri)phenylsulfonic acid, *p*-(hydroxymercuri)phenylsulfonic acid, phenolphthalein glucuronic acid, lactic acid dehydrogenase, pyruvate, egg white lysozyme, 2,4-dinitrophenylhydrazine, purine, xanthine oxidase, and ATP (Sigma Chemical Co., St. Louis, MO), DNase (Calbiochem, San Diego, CA), blue dextran, aldolase, Ficoll–Hypaque, and Macrodex (6%, w/v) (Pharmacia Fine Chemicals, Inc., Piscataway, NJ), and [γ-^{32}P]ATP and [^{35}S]sulfanilic acid (New England Nuclear, Inc., Boston, MA) were obtained as noted.

Activation and Assessment of PMN Leukocyte Superoxide-Generating Activity. Thirty-milliliter portions of venous blood from normal human subjects was mixed with 4 mL of 0.15 M sodium citrate (pH 5.2) and 5 mL of 5 g/100 mL dextran in 0.15 M NaCl in 50-mL polypropylene tubes and held at 25 °C for 45 min. The supernatant leukocyte-rich plasma was aspirated into clean polypropylene tubes and centrifuged at 100*g* for 10 min at 4 °C. The cell pellets were resuspended in 3 mL of distilled water and incubated at room temperature for 30 s to lyse residual erythrocytes, after which 1 mL of 0.6 M KCl was added to restore isotonicity as described (Curnutte & Babior, 1974). The mixed leukocytes were recovered by centrifugation at 250*g* for 5 min at 4 °C and were washed twice and resuspended in 0.02 M sodium phosphate buffered 0.13 M sodium chloride, pH 7.0 (phosphate-buffered saline). Three-milliliter portions were layered on 3 mL of Ficoll–Hypaque cushions and centrifuged at 400*g* for 20 min at 4 °C. The upper cell layer and the cushion were decanted from each tube, and the PMN leukocyte pellet was resuspended and washed once in phosphate-buffered saline; the purity of the PMN leukocytes equalled or exceeded 98%. Zymosan powder was opsonized by incubation in autologous serum and washed as described (Hohn & Lehrer, 1975) and was resuspended at a concentration of 12 mg/mL in phosphate-buffered saline containing 0.9 mM $CaCl_2$ and 0.49 mM $MgCl_2$ (phosphate-buffered saline with calcium and magnesium). Phorbol myristate acetate was dissolved in dimethyl sulfoxide (Me_2SO) at a concentration of 1 mg/mL and diluted in phosphate-buffered saline with calcium and magnesium to a final concentration of 1.5 μg/mL.

Portions of 2 × 10^8 PMN leukocytes in 2 mL of phosphate-buffered saline and 4 mL of the standard preparations of opsonized zymosan or phorbol myristate acetate were prewarmed to 37 °C, mixed, and incubated with shaking for 3 min at 37 °C to achieve maximal activation of superoxide-generating activity (Dewald et al., 1979). An equal volume of phosphate-buffered saline at 4 °C was added, and the PMN leukocytes were recovered by centrifugation at 400*g* for 10 min at 4 °C. Superoxide-generating activity in intact cells was quantitated at 25 and 37 °C as described by measuring the superoxide dismutase inhibitable reduction of cytochrome *c* (Tauber et al., 1979b). The reduction of ferricytochrome *c* to ferrocytochrome *c* was assessed by the increase in OD_{550nm}, and the quantity of cytochrome *c* that had been reduced was calculated by utilizing a millimolar extinction coefficient of 18.5 (Margoliash & Frohwirt, 1959). Superoxide-generating activity in subcellular particles and solubilized preparations was quantitated similarly by measuring the superoxide dismutase inhibitable reduction of cytochrome *c*, except that paired cuvettes were employed, both of which contained 0.7 mL of 0.13 M potassium phosphate buffer (pH 7.0) with 0.1 mM NADPH and 0.1 mM cytochrome *c* and one of which contained 30 μg of superoxide dismutase. The difference in the increase in OD_{550nm} was recorded continuously for 5 min at 25 °C with a double-beam spectrophotometer (Perkin-Elmer Model 552, Oak Brook, IL); the quantity of cytochrome *c* reduced by $O_2^{-}\cdot$ was calculated as noted above. NADPH oxidation by the superoxide-generating activity in 27000*g* particulate preparations and fractions from sucrose gradients and by solubilized superoxide-generating activity was determined by measuring the difference in the rate of decrease in OD_{340nm} of paired cuvettes, both of which contained 0.8 mL of 0.13 M potassium phosphate buffer (pH 7.0) with 0.1 mM NADPH, following the addition of 0.1 mL of sample to one and 0.1 mL of buffer to the other. The rate of decrease in OD_{340nm} was also measured for the superoxide-generating activity preparation alone, and this value was subtracted from that obtained with NADPH. The oxidation of NADPH to NADP was calculated from the net decrease in OD_{340nm} by utilizing a millimolar extinction coefficient of 6.2 (*Sigma Technical Bulletin*, 1974). Superoxide dismutase activity was assessed by a modification of the method of Salin & McCord (1974) as described (Tauber et al., 1979b) by using a xanthine oxidase system to generate $O_2^{-}\cdot$.

Preparation of Subcellular Particle Associated Superoxide-Generating Activity of PMN Leukocytes and Analysis of the Subcellular Distribution of Superoxide-Generating Activity. Portions of (2–8) × 10^8 PMN leukocytes that had been incubated with opsonized zymosan or phorbol myristate acetate were centrifuged at 400*g*, resuspended in 2 mL of 0.34 M sucrose that was buffered with 10 mM Tris-HCl (pH 7.1) (0.34 M sucrose–Tris), and homogenized in glass tubes with a Teflon pestle (Tri-R Instruments, Inc., Rockville Centre, NY) for 5 min at 4 °C (Figure 1). The homogenate was centrifuged at 400*g* for 10 min at 4 °C. The 400*g* supernatant was harvested for the preparation of particle-associated superoxide-generating activity and solubilized superoxide-generating activity and for studies of the subcellular distribution of superoxide-generating activity. The 400*g* pellet, which contained unbroken cells, nuclei, and opsonized zymosan phagolysosomes, was resuspended in 1.6 mL of 0.34 M sucrose–Tris, sonicated at 100W for 20 s at 4 °C, and recentrifuged at 400*g*; the supernatant was recovered and transferred to a clean test tube. Particle-associated superoxide-generating activity was prepared by centrifuging the 400*g* supernatant from homogenates of (2–8) × 10^8 PMN leukocytes at 27000*g* for 20 min at 4 °C (Figure 1). The pellet was suspended in 4 mL of 0.34 M sucrose–Tris, recentrifuged at 27000*g*, and resuspended in 2 mL of 0.34 M sucrose–Tris.

In studies of the subcellular distribution of the superoxide-generating activity-containing particles, the 400*g* supernatant from the homogenates of PMN leukocytes (Figure 1) or from the sonicated 400*g* pellet from homogenates was layered on 12-mL linear gradients of 10–50 g/100 mL sucrose that was buffered with 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.1) and contained 10 μg/mL DNase. The gradients were centrifuged at 45000*g* for 45 min at 4 °C. Fourteen 1-mL fractions were harvested from each gradient. Each fraction was assayed for superoxide-

generating activity, for NADPH-oxidizing activity, and for established subcellular enzymatic markers. The location of plasma membranes in the gradient was determined by assaying portions of each fraction for magnesium-dependent ATPase (Harlan et al., 1977) and alkaline *p*-nitrophenyl phosphatase (Bretz & Baggiolini, 1974) activity. In some experiments, PMN leukocytes also were pretreated with the covalent membrane-labeling reagent ^{35}S-diazotized sulfanilic acid (Goldstein et al., 1977) at concentrations of 3 mM for 15 min at 37 °C and washed twice with phosphate-buffered saline prior to homogenization; the radioactivity in each fraction of the sucrose gradient was determined. β-Glucuronidase (Stahl & Touster, 1971), found in the azurophilic granules (Bretz & Baggiolini, 1974), lysozyme (Baggiolini et al., 1970), a marker for azurophilic and specific granules (Bretz & Baggiolini, 1974; West et al., 1974), and lactic acid dehydrogenase (*Sigma Technical Bulletin*, 1974), found in the cytosol (Kane & Peters, 1975), were assayed as described. Cytochrome *c* oxidase, a mitochrondrial enzyme marker, was assayed by measuring the rate of oxidation of reduced cytochrome *c* at OD_{550nm} (Smith, 1955). One unit of enzymatic activity was defined as that quantity of β-glucuronidase which liberated 1 μg of phenolphthalein from phenolphthalein glucuronide per h, of lysozyme which hydrolyzed a quantity of *M. lysodeikticus* equivalent to that hydrolyzed by 1 μg of egg white lysozyme in 1 h, of alkaline *p*-nitrophenyl phosphatase which hydrolyzed 1 μmol of *p*-nitrophenyl phosphate per h, and of cytochrome oxidase which oxidized 1 nmol of cytochrome *c* per min. Protein concentration was determined by the Lowry (Lowry et al., 1951) or Bio-Rad (Bradford, 1976) method.

Solubilization and Stabilization of Superoxide-Generating Activity from PMN Leukocytes. Particle-associated superoxide-generating activity in the 27000*g* pellets from homogenates of (2–8) × 10^8 PMN leukocytes was resuspended in 2 mL of 0.34 M sucrose–Tris containing a detergent, homogenized with a Teflon pestle for 1 min at 4 °C, incubated for 20 min at 4 °C, and centrifuged at 100000*g* for 1 h at 4 °C (Figure 1). The supernatant was transferred to a clean test tube, and the pellet was resuspended in 1 mL of 0.34 M sucrose–Tris. Agents potentially capable of stabilizing the solubilized superoxide-generating activity were added to replicate portions of the 100000*g* supernatant prior to incubation at 4 and 25 °C and assessment of superoxide-generating activity at subsequent intervals up to 84 h.

Characterization of the Biochemical Properties of Superoxide-Generating Activity. The pH optimum of solubilized superoxide-generating activity was determined by diluting replicate portions of several preparations in a series of 0.13 M potassium phosphate buffers that ranged in pH from 5.0 to 9.0. Superoxide-generating activity was quantitated by assaying the rates of cytochrome *c* reduction and NADPH oxidation continuously for 5 min. K_m values were calculated by a linear regression technique (Cleland, 1967) from the results of experiments in which the concentration of NADPH was varied from 200 to 10 μM and that of NADH from 2000 to 100 μM (Babior et al., 1976). For analysis of the effects of specific enzymatic inhibitors on the superoxide-generating activity of intact cells, 10^6 PMN leukocytes were preincubated with various concentrations of an inhibitor for 10 min at 37 °C prior to the addition of standard concentrations of opsonized zymosan or phorbol myristate acetate. For subcellular particle associated or solubilized superoxide-generating activity, the same concentration of inhibitors was added to the cuvettes immediately prior to initiating the continuous recording of OD_{550nm} for 5 min as described above.

Purification of Solubilized Superoxide-Generating Activity. Superoxide-generating activity in the 100000*g* supernatant from homogenates of 8 × 10^8 PMN leukocytes was filtered on a 1.6 × 70 cm column of Sephacryl S-200 that was equilibrated and developed in 10 mM Tris-HCl (pH 7.1) at a flow rate of 15 mL/h. Fractions of 1.4 mL, amounting to 1% of bed volume, were collected, and portions were assayed for protein concentration and superoxide-generating activity. Isoelectric focusing was performed with a system (Isco, Inc., Model 212, Lincoln, NB) that holds a 35-mL density gradient in a jacketed column between two electrodes. The linear gradient was composed of 10–60 mL of glycerol, 15 mL of ethylene glycol, and 5 mL of ampholytes (40 mg/100 mL, pH range 3–11; Bio-Rad Laboratories, Richmond, CA) per 100 mL in distilled water. The electrode reservoirs were filled with 0.3 M formic acid (anode) and 0.1 M NaOH (cathode). The system was prefocused at 900 V, 4 °C, for 48 h. The superoxide-generating activity sample then was diluted to 1.5 mL with a 30-mL glycerol/100-mL ethylene glycol–ampholyte solution and applied at the midpoint of the density gradient, and the isoelectric focusing was continued for an additional 18–24 h at 900 V at 4 °C to a time at which the current had dropped to below 0.2 mA. The gradient then was collected in 2-mL fractions. The pH was determined for each fraction, 1 mL of 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.0) was added, 0.1 M NaOH or 0.1 M HCl was added dropwise to adjust the pH to 6.8–7.1, and distilled water was added to adjust the volume of each fraction to 4 mL.

Results

Activation of Subcellular Particle Associated Superoxide-Generating Activity. The incubation of portions of 2 × 10^7 PMN leukocytes with either opsonized zymosan particles or phorbol myristate acetate stimulated superoxide-generating activity in a time-dependent manner to a maximal rate that was a function of the concentration of the stimulus. Optimal concentrations of opsonized zymosan or phorbol myristate acetate stimulated a linear increase in superoxide-generating activity that reached a peak rate at 3 min of 56 ± 27 nmol of $O_2^{-}\cdot$ per min per 2 × 10^7 PMN leukocytes (mean ± SD, n = 6) with 2 mg/mL opsonized zymosan and 51 ± 22 nmol of $O_2^{-}\cdot$ per min per 2 × 10^7 PMN leukocytes (n = 4) with 1 μg/mL phorbol myristate acetate at 37 °C. At 25 °C, the same concentrations of opsonized zymosan and phorbol myristate acetate achieved peak rates of activation of superoxide-generating activity at 3 min that ranged from 27 to 32% of the corresponding values at 37 °C. Homogenates of (2–8) × 10^8 PMN leukocytes that had been incubated with opsonized zymosan or phorbol myristate acetate for 3 min at 37 °C were centrifuged at 400*g* for 10 min at 4 °C (Figure 1). The recovery of superoxide-generating activity in the 400*g* supernatant relative to that in the whole homogenate was 41–60% (n = 3) when opsonized zymosan was the stimulus and 85–94% (n = 3) when phorbol myristate acetate was the stimulus. Of the superoxide-generating activity that was recovered in the 400*g* supernatants from opsonized zymosan and phorbol myristate acetate stimulated PMN leukocytes, a mean of 92 and 95%, respectively, was sedimented by centrifugation at 27000*g* for 20 min at 4 °C, and the 27000*g* pellet was resuspended in 0.34 M sucrose–Tris (Figure 1). The mean specific activities at 25 °C (±SD, n = 5) of the particle-associated superoxide-generating activity recovered in the 27000*g* pellets from opsonized zymosan and phorbol myristate acetate stimulated PMN leukocytes were 54 ± 19 and 69 ± 21 nmol/(min·10^8 PMN leukocytes) or 28 ± 8 and 21 ± 8 nmol/(min·mg of protein), respectively.

PMN (2-8 x 10^8) + Opsonized Zymosan
or Phorbol Myristate Acetate

↓ 400g, 4°C, 10 min

Cell pellet resuspended in 0.34 M sucrose - 10 mM Tris-HCl (pH 7.1)

↓ homogenization
400g, 4°C, 10 min

Supernatant

↓ 27,000g, 4°C, 20 min

Pellet suspended in 0.34 M sucrose-Tris + 0.25% Na deoxycholate

↓ 100,000g, 4°C, 1 h

Solubilized superoxide-generating activity in supernatant

FIGURE 1: Activation, solubilization, and partial purification of PMN leukocyte superoxide-generating activity.

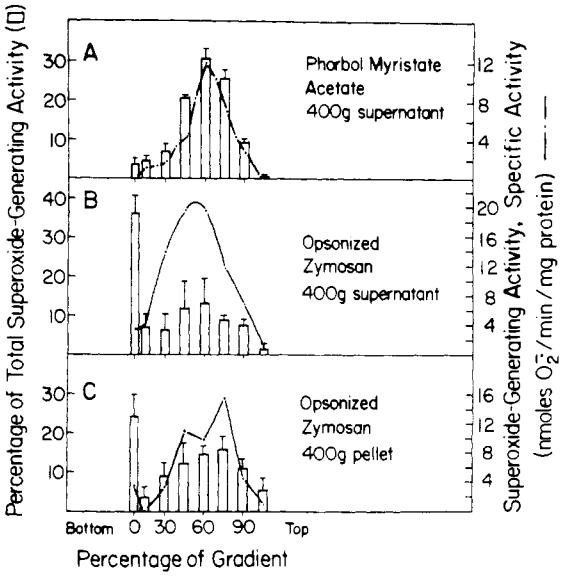

FIGURE 2: Subcellular distribution of PMN leukocyte superoxide-generating activity. The bars and brackets represent the mean percent ± SD (n = 4) of the total superoxide-generating activity recovered in each 1-mL fraction from the sucrose gradient, and each point (·) depicts the mean specific activity of the superoxide-generating activity. Total superoxide-generating activity recovered ranged from 85 to 93% of the total activity initially applied to the gradient. (A) Superoxide-generating activity derived from the 400g supernatant of homogenates of PMN leukocytes that had been treated with phorbol myristate acetate. Four preparations of (1–2) × 10^8 cells from different donors were incubated for 3 min at 37 °C with 1 μg/mL phorbol myristate acetate. (B) Superoxide-generating activity derived from the 400g supernatant of homogenates of PMN leukocytes that had phagocytosed opsonized zymosan. Four preparations of (1–2) × 10^8 cells from different donors were incubated with opsonized zymosan for 3 min at 37 °C. (C) Superoxide-generating activity derived from the 400g pellet of homogenates of PMN leukocytes that had phagocytosed opsonized zymosan. The pellets were derived from the same cell preparations that supplied the 400g supernatants (B).

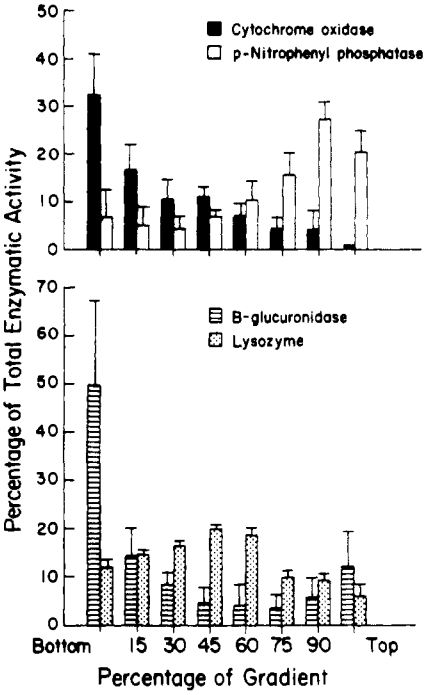

FIGURE 3: Distribution of PMN leukocyte enzymes on sucrose gradients. The 400g supernatants from homogenates of PMN leukocytes that had been incubated with opsonized zymosan or phorbol myristate acetate were centrifuged on sucrose gradients, and portions of each fraction were assayed for the enzymatic activities depicted; each bar and bracket is the mean ± SD of values for three to seven preparations. The mean total enzymatic activity (100%) applied to the gradients was 10 units of cytochrome oxidase (n = 3), 267 units of alkaline phosphatase (n = 5), 259 units of β-glucuronidase (n = 7), and 37 units of lysozyme (n = 4) per 10^8 PMN leukocytes. Neither the total activity nor the distribution of any enzyme was a function of the specific stimulus.

Subcellular Localization of the Superoxide-Generating Activity of PMN Leukocytes. For assessment of the subcellular distribution of superoxide-generating activity in PMN leukocytes that had been incubated with opsonized zymosan or phorbol myristate acetate, the 400g supernatants of homogenates were layered on sucrose gradients and centrifuged at 45000g for 45 min at 4 °C. Each of the portions of the gradient was analyzed for superoxide-generating activity and for previously defined enzymatic and other markers associated with the subcellular compartments. The superoxide-generating activity from PMN leukocytes treated with phorbol myristate acetate (Figure 2A) was distributed in several adjacent fractions and exhibited a peak at ~60% from the bottom of the gradient which did not cosediment with the peaks of activity of the granule markers, β-glucuronidase and lysozyme, or the mitochondrial marker, cytochrome oxidase, and which was resolved from the peaks of the conventional plasma membrane markers, alkaline p-nitrophenyl phosphatase

(Figure 3) and magnesium-dependent ATPase, and from the radioactivity associated with the plasma membrane determinants labeled by ^{35}S-diazotized sulfanilic acid. The 400*g* supernatant from homogenates of PMN leukocytes that had phagocytosed zymosan (Figure 2B) exhibited two peaks of superoxide-generating activity in sucrose gradients, one of which cosedimented with the peak obtained from cells treated with phorbol myristate acetate. The lesser portion of the superoxide-generating activity found in the 400*g* pellet from such cells (Figure 2C) had two peaks that were comparable to those found for the 400*g* supernatant. While a substantial portion of the total superoxide-generating activity was found in the peak at the bottom of the gradients for homogenates of PMN leukocytes that had phagocytosed zymosan, the specific activity in the 400*g* supernatant and pellet had a single peak and a bifid peak, respectively, that were distributed in the same region as those of the 400*g* supernatant from phorbol myristate acetate treated cells (Figure 2). Electron microscopic examination of the fractions from the midzone of two of the gradients, which contained the bulk of the superoxide-generating activity, revealed predominantly membrane vesicles, some granules, and rarely other subcellular structures.

That the low specific activity of the superoxide-generating activity which pelleted through the gradients was not attributable to the presence of an inhibitor was suggested by the failure of portions of this material to suppress the superoxide-generating activity in the major peak. The possibility that superoxide-generating activity might be present at the top of the gradients in a region containing superoxide dismutase which would mask the expression of superoxide-generating activity was investigated by pooling the fractions from the upper one-fourth of the gradients and centrifuging the pool at 100000*g* for 1 h at 4 °C to resolve the particle-associated superoxide-generating activity from superoxide dismutase. The 100000*g* pellet, which was resuspended in 0.34 M sucrose–Tris, contained a mean of 93% of the superoxide-generating activity (n = 3), while a mean of 95% of the superoxide dismutase remained in the supernatant. The total superoxide-generating activity in the 100000*g* pellet that was free of endogenous superoxide dismutase was only a mean of 1.5-fold higher than the superoxide-generating activity present in the initial pool of fractions from the upper one-fourth of the gradients. Thus, the presence of superoxide dismutase does not account for the lack of coincidence of the distribution patterns of the plasma membrane markers and the superoxide-generating activity. An independent assessment of NADPH-oxidizing activity indicated that it was distributed in the sucrose gradients in the same pattern as that seen for superoxide-generating activity that had been assayed by the NADPH-dependent reduction of cytochrome *c*.

Solubilization of Superoxide-Generating Activity. Incubation of the particle-associated superoxide-generating activity in the 27000*g* pellet (Figure 1) with a variety of detergents liberated a portion of the superoxide-generating activity in a soluble form that remained in the supernatant after centrifugation at 100000*g*. The extent of solubilization of superoxide-generating activity was a function of the concentration of each detergent (Table I). A mean of 12.4% of the particle-associated superoxide-generating activity from PMN leukocytes that had phagocytosed opsonized zymosan was solubilized in 0.25 g/100 mL deoxycholate, as compared to the mean spontaneous release of 0.7% of the activity. In four additional experiments with 0.25 g/100 mL deoxycholate, the comparable yield from the particulate fraction of PMN leukocytes that had been treated with 1 μg/mL phorbol myristate acetate was 13.8 ± 6.1% (mean ± SD). The extent of solubilization of superoxide-generating activity was less at lower and higher concentrations of deoxycholate, while cholate was a less effective solubilizing detergent than deoxycholate at equal concentrations (Table I).

Table I: Solubilization of Superoxide-Generating Activity in Detergents[a]

solubilization conditions	% solubilization (mean ± SD)[b]
buffer alone	0.7 ± 0.8 (n = 4)[c]
deoxycholate (g/100 mL)	
0.10	6.9 ± 0.9 (n = 2)
0.25	12.4 ± 7.1 (n = 4)
0.50	8.0 ± 5.0 (n = 3)
cholate (g/100 mL)	
0.25	8.5 ± 4.2 (n = 3)
0.50	6.4 ± 3.8 (n = 4)

[a] The 27000*g* pellet from homogenates of (4–8) × 10^8 PMN leukocytes that had phagocytosed opsonized zymosan (Figure 1) was resuspended in detergent in 0.34 M sucrose–Tris buffer and centrifuged at 100000*g* prior to assaying the superoxide-generating activity in the supernatant. [b] Percent solubilization was calculated by relating the total superoxide-generating activity recovered in the 100000*g* supernatant to the total activity present in the 27000*g* pellet suspended in buffer without detergent. [c] n represents the number of replicates that are reflected in each of the mean values.

Table II: Physical and Biochemical Characteristics of Solubilized Superoxide-Generating Activity from PMN Leukocytes

	opsonized zymosan[a]	phorbol myristate acetate[a]
sp act.[b] [nmol/(min·mg of protein)]	2.6 ± 1.5	1.7 ± 1.2
thermal lability, $t_{1/2}$[c]	8.5 ± 1.2 h (n = 4) (4 °C)	9 h (4 °C)
(0.34 M sucrose–Tris, pH 7.1)	24 ± 3 min (n = 3) (25 °C)	25 min (25 °C)
pH optimum[c]	6.8–7.1 (n = 3)	7.0
K_m, NADPH[c]	41.4 ± 29.9 μM (n = 4)	22.7 μM

[a] Portions of (4–8) × 10^8 PMN leukocytes were incubated with opsonized zymosan or phorbol myristate acetate; superoxide-generating activity was solubilized in 0.25 g/100 mL deoxycholate in 0.34 M sucrose–Tris buffer (Figure 1). [b] The levels of specific activity are the mean ± SD for five preparations of solubilized superoxide-generating activity for each stimulus. [c] Data presented are the mean ± 1 SD or the range for n preparations from phagocytosing cells and for one representative preparation from cells treated with phorbol myristate acetate.

The mean specific activity of the superoxide-generating activity solubilized in 0.25 g/100 mL deoxycholate was approximately one-tenth to one-fifth the level that was determined for the particle-associated superoxide-generating activity in the 27000*g* pellet of the same ten preparations (Table II). The solubilized superoxide-generating activity from PMN leukocytes that had been exposed to opsonized zymosan or phorbol myristate acetate was labile at 25 °C, with a mean $t_{1/2}$ of 24 min, while storage at 4 °C prolonged the survival of the solubilized superoxide-generating activity by approximately 20-fold. The rate of reduction of cytochrome *c* by solubilized superoxide-generating activity was maximal at pH levels of 6.4–7.6 in 0.13 M potassium phosphate buffer (Figure 4), and a comparable pH optimum of 6.4–7.9 was found based on the assessment of NADPH oxidation. In two additional experiments, superoxide-generating activity assayed by cytochrome *c* reduction was optimal at pH 6.5–7.1 and 6.6–7.3, respectively, and that assayed by NADPH oxidation was optimal at pH 6.8–7.9 and 6.8–7.4. The mean K_m value for NADPH of 41.4 μM was similar to that characteristic of

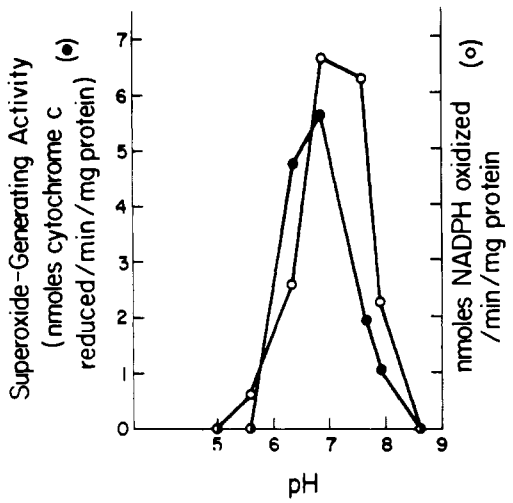

FIGURE 4: pH optimum of solubilized superoxide-generating activity. The superoxide-generating activity solubilized from the 27000*g* particulate fraction of 7×10^8 PMN leukocytes that had been treated with phorbol myristate acetate was assessed in 0.13 M potassium phosphate buffers.

Table III: Effect of *p*-(Chloromercuri)phenylsulfonic Acid on Superoxide-Generating Activity

	% of control	
superoxide-generating activity[a]	opsonized zymosan[b]	phorbol myristate acetate[c]
whole cells	61.6 ± 11.9	100
particle associated	3.0 ± 4.2	0
solubilized	5.0 ± 5.0	0

[a] 10^6 whole cells were preincubated with 20 μM *p*-(chloromercuri)phenylsulfonic acid or buffer for 10 min at 37 °C and then activated with opsonized zymosan or phorbol myristate acetate for 20 min. Cell-free superoxide-generating activity was assayed as described under Materials and Methods and was not preincubated with the inhibitor. [b] All values are the mean ± SD for superoxide-generating activity in three separate experiments. Control levels (100%) for whole cells ranged from 13.5 to 40.2 nmol/(20 min·10^6 cells). [c] All values are the mean for superoxide-generating activity in three separate experiments. Control values (100%) for whole cells ranged from 37.3 to 55.2 nmol/(20 min·10^6 cells).

particle-associated superoxide-generating activity (Babior et al., 1976) and was approximately 1/20 that of 700 and 900 μM found for NADH with two of the four preparations. No solubilized superoxide-generating activity was recovered from resting PMN leukocytes, and the total activity of solubilized superoxide-generating activity was proportional to the degree of expression of superoxide-generating activity in the donor PMN leukocytes. The rates of both cytochrome *c* reduction, a pyridine nucleotide dependent reaction, and NADPH oxidation were linear with protein concentrations from 0.1 to 1 mg/mL protein, and both activities were abolished by prior boiling of the solubilized preparations for 5 min.

As for the particle-associated superoxide-generating activity, the activity of solubilized superoxide-generating activity was neither enhanced by 0.2–2.0 mM FAD nor inhibited by 0.1 mM sodium azide. Both solubilized and particle-associated superoxide-generating activity were inhibited by 20 μM *p*-(chloromercuri)phenylsulfonic acid (Table III) and by 20 μM *p*-(hydroxymercuri)phenylsulfonic acid, which are plasma membrane impermeant agents that react with free sulfhydryl groups. In contrast, the superoxide-generating activity of intact PMN leukocytes that had phagocytosed zymosan was only partially inhibited by *p*-(chloromercuri)phenylsulfonic acid and

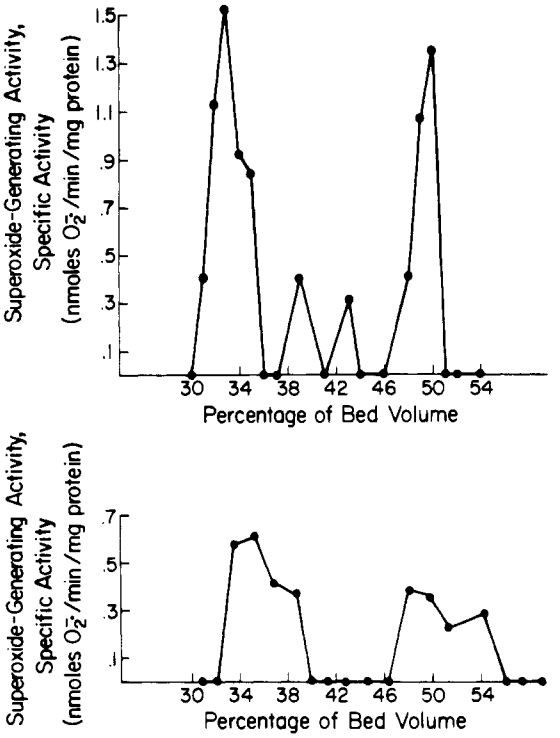

FIGURE 5: Filtration of solubilized superoxide-generating activity on Sephacryl S-200. Particle-associated superoxide-generating activity, contained in the 400*g* supernatant of homogenates of PMN leukocytes that had been pretreated with phorbol myristate acetate (top frame) or had phagocytosed opsonized zymosan (bottom frame), was pelleted by centrifugation at 27000*g* for 20 min at 4 °C. The superoxide-generating activity in the pellets was solubilized in 2 mL of 0.25 g/100 mL sodium deoxycholate in 0.34 M sucrose–Tris, and the insoluble material was removed by centrifugation at 100000*g* for 1 h at 4 °C prior to gel filtration of the supernatant. The markers employed were blue dextran ($M_r \simeq 2\,000\,000$) and aldolase (M_r 158 000), which eluted in the void volume and at 52% bed volume, respectively. (Top frame) 6×10^8 PMN leukocytes were activated by treatment with 1 μg/mL phorbol myristate acetate; the solubilized superoxide-generating activity applied to Sephacryl S-200 had a total activity of 17.5 nmol/min and a specific activity of 1.68 nmol/(min·mg of protein). (Bottom frame) 8×10^8 PMN leukocytes were incubated with opsonized zymosan; the solubilized superoxide-generating activity applied to Sephacryl S-200 had a total activity of 22.5 nmol/min and a specific activity of 0.68 nmol/(min·mg of protein).

that of cells treated with phorbol myristate acetate was unaffected.

Stabilization and Partial Purification of Solubilized Superoxide-Generating Activity. Solubilized superoxide-generating activity from both PMN leukocytes stimulated with opsonized zymosan and with phorbol myristate acetate was filtered on Sephacryl S-200 for 10 h at 4 °C, which resolved the superoxide-generating activity into two peaks, one of which appeared in the void volume, with a molecular weight of over 300000, and the other of which appeared just after the aldolase marker, with an apparent molecular weight of 150000 (Figure 5). The same pattern was observed with five consecutive preparations of solubilized superoxide-generating activity obtained from PMN leukocytes treated with phorbol myristate acetate where the mean specific activity (±SD) of the peak in the void volume was 2.4 ± 2.1 nmol/(min·mg of protein) and that of the peak of M_r 150000 was 1.2 ± 1.1 nmol/(min·mg of protein). In view of the lability of solubilized superoxide-generating activity in 0.34 M sucrose–Tris, a variety of stabilizing agents were explored in order to permit further studies of the physical characteristics of the superoxide-generating activity by procedures that required longer than 10 h to complete. Neither antioxidants such as dithiothreitol

Table IV: Stabilization of Solubilized Superoxide-Generating Activity

incubation conditions[a]	$t_{1/2}$ of solubilized superoxide-generating activity (h, 4 °C)[b]
0.34 M sucrose–0.25% deoxycholate	18
0.34 M sucrose–Tris–0.25% deoxycholate	10
glycerol (mL/100 mL)	
15	58
ethylene glycol (mL/100 mL)	
2	23
15	65
Me_2SO (mL/100 mL)	
3.5	42
7.5	50
15	68

[a] Each of the additives noted was dissolved in 0.34 M sucrose containing 0.25% deoxycholate. [b] The values presented are the mean of samples from three experiments.

nor inhibitors of PMN leukocyte proteases and esterases such as TPCK, TLCK, or PMSF (Aswanikumar et al., 1976) affected the rate of decay of superoxide-generating activity at 4 °C. However, the addition of glycerol, ethylene glycol, or dimethyl sulfoxide (Me_2SO) significantly improved the stability of the solubilized superoxide-generating activity (Table IV). Optimal concentrations of glycerol, ethylene glycol, and Me_2SO extended the $t_{1/2}$ at 4 °C from ~18 h in 0.34 M sucrose–0.25% deoxycholate alone to 58, 65, and 68 h, respectively. Solubilized superoxide-generating activity from PMN leukocytes that had been treated with phorbol myristate acetate was stabilized by the addition of 15% ethylene glycol (v/v) and subjected to isoelectric focusing for 18–24 h at 4 °C in a prefocused gradient of 10–60 g/100 mL of glycerol containing 15% ethylene glycol (v/v) and 2% ampholytes (w/v) exhibiting a pH range of 3–11 (Figure 6). The recovery of solubilized superoxide-generating activity in three consecutive experiments ranged from 35 to 65% of that applied to the prefocused column. The bulk of the solubilized superoxide-generating activity focused with an isoelectric point ranging from 7.6 to 8.3 (Figure 6). The mean specific activity (±SD) of the fractions with peak superoxide-generating activity from the isoelectric focusing of the three preparations was 0.95 ± 0.56 (±SD) nmol/(min·mg of protein).

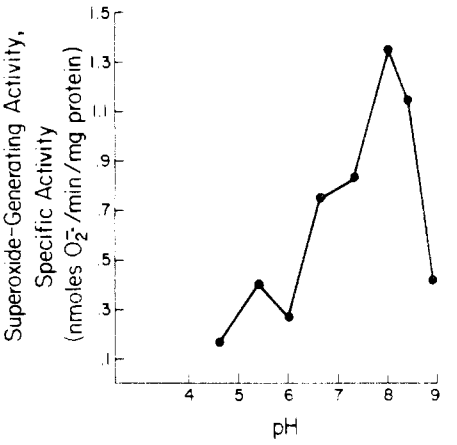

FIGURE 6: Isoelectric focusing of solubilized superoxide-generating activity. Superoxide-generating activity solubilized from the 27000*g* particulate fraction of 6×10^8 PMN leukocytes that had been treated with phorbol myristate acetate was subjected to isoelectric focusing for 18 h at 4 °C; recovery was 65% of the solubilized superoxide-generating activity that had been applied.

Discussion

The activation of the superoxide-generating activity of human PMN leukocytes is manifested only in intact cells that have been stimulated by exposure to phagocytosable particles or to soluble agents capable of perturbing cellular membranes (Babior, 1978). Further understanding of the mechanisms of activation, the intracellular distribution, and the regulation of expression of PMN leukocyte superoxide-generating activity is dependent on the elucidation of the structure, biochemical characteristics, and susceptibility to specific inhibition of the superoxide-generating activity isolated from previously stimulated cells. Subcellular particle associated preparations of superoxide-generating activity from PMN leukocytes that had been stimulated by opsonized zymosan or phorbol myristate acetate reproducibly retained optimal levels of activity and of specific activity and thus served as a source of superoxide-generating activity for further purification and biochemical characterization (Figure 1).

Although some data have suggested that the superoxide-generating activity of human PMN leukocytes is associated with the plasma membrane (Dewald et al., 1979; Goldstein et al., 1977), the superoxide-generating activity in subcellular particulate preparations sedimented independently of defined markers of the plasma membranes, mitochondria, and lysosomal granules when subjected to isopycnic centrifugation in sucrose gradients (Figures 2 and 3). Some previous analyses of the subcellular localization of the superoxide-generating activity of human PMN leukocytes have provided analogous results. Velocity or isopycnic centrifugation of homogenates of activated PMN leukocytes demonstrated that both NADH and NADPH oxidase activities were distributed in a band that exhibited a density greater than that of the azurophil granules but was distinct from either membrane or granule markers (Iverson et al., 1978). The identity of the oxidase activities and superoxide-generating activity was not established. The results of other studies have suggested that the distribution of NADPH and NADH oxidase activities within resting cells may be dependent on the concentration of pyridine nucleotide that is employed (Segal & Peters, 1977). The NADPH-dependent cytochrome *c* reducing activity was localized broadly in the lightest portions of the gradient containing plasma membrane, mitochondria, and cytosol when the NADPH concentration was 2.5 mM. In contrast, at an NADPH concentration of 1 mM, the NADPH/NADH oxidizing activities were localized in a portion of the gradient containing the azurophilic granules. Other approaches to the resolution of the subcellular constituents of human neutrophils that had been stimulated with either opsonized zymosan or phorbol myristate acetate led to a recovery of the superoxide-generating activity with a crude membrane fraction, whereas no significant activity was associated with specific or azurophilic granules (Dewald et al., 1979). While the present studies suggested that the subcellular localization of the superoxide-generating activity may be distinct from the cellular membranes and the lysosomal granules (Figures 2 and 3), the unique sedimentation pattern of superoxide-generating activity may be a function of membrane aggregation or other alterations induced by the local generation of high concentrations of $O_2^-\cdot$. That the cellular components may deteriorate during isolation is supported by the failure to increase the specific activity of the superoxide-generating activity in the relevant particles that were resolved in sucrose gradients from other constituents in the homogenate (Figure 2). However, the sedimentation pattern was not influenced by the presence of

DNase or EDTA, which minimize the aggregation of plasma membranes (Goetzl & Hoe, 1979). That a concentration of the impermeant sulfhydryl-reactive agent *p*-(chloromercuri)-phenylsulfonic acid, which totally inactivated particle-associated and soluble superoxide-generating activity, had no effect on the superoxide-generating activity of intact PMN leukocytes (Table III) suggests that the superoxide-generating activity is sequestered on the internal surface of the plasma membranes or in another intracellular compartment.

The superoxide-generating activity in the particulate preparations from previously stimulated PMN leukocytes was solubilized in part by 0.25 g/100 mL deoxycholate, which was more effective as a solubilizing agent than cholate (Table I). The solubilized superoxide-generating activity was not influenced by the addition of FAD or azide and exhibited a pH optimum and K_m for NADPH that were comparable to those for the particle-associated superoxide-generating activity (Table II; Figure 4; Babior et al., 1976). The yield and specific activity of solubilized superoxide-generating activity from particulate preparations of PMN leukocytes that were treated with Triton X-100 (Gabig et al., 1978) were similar to the values obtained for superoxide-generating activity solubilized by deoxycholate (Table II). However, the former preparations of superoxide-generating activity differed from those solubilized by deoxycholate since the activity exhibited a dependence on FAD and was not linearly related to the protein concentration in serial dilutions of the standard material (Gabig et al., 1978). Particle-associated superoxide-generating activity and solubilized superoxide-generating activity were equally susceptible to inhibition by sulfhydryl-reactive agents (Table III). The mean level of specific activity of the solubilized superoxide-generating activity consistently was only one-tenth to one-fifth that of the superoxide-generating activity in the particle-associated preparations, but both the particle-associated superoxide-generating activity and the solubilized superoxide-generating activity deteriorated at a comparable rate in a variety of buffers. The temperature-dependent lability of the solubilized enzymes in buffer thus cannot be attributed to the detergent solubilization procedure. Attempts to alter the rate of decay and the level of specific activity by dialysis, gel filtration, or ultrafiltration of solubilized superoxide-generating activity or by the addition of protease inhibitors, reducing agents, or albumin were unsuccessful. While phospholipids, especially phosphatidylethanolamine, can enhance by up to twofold the expression of the activity of solubilized superoxide-generating activity (Gabig & Babior, 1979), the basis for this effect has not been established.

The inherent instability of the solubilized superoxide-generating activity initially restricted purification efforts to gel filtration on Sephacryl S-200 at 4 °C (Figure 5). Solubilized superoxide-generating activity from PMN leukocytes activated by opsonized zymosan or phorbol myristate acetate was recovered from Sephacryl S-200 in two discrete peaks of M_r 150 000 and >300 000, respectively, both of which demonstrated a specific activity similar to that of the crude solubilized superoxide-generating activity. Neither peak was inhibitable by azide, and each exhibited physical and biochemical properties similar to those of the solubilized superoxide-generating activity prior to gel filtration. The finding that Me_2SO, ethylene glycol, and glycerol stabilized the activity of the solubilized superoxide-generating activity (Table IV) permitted the application of isoelectric focusing in glycerol gradients containing ethylene glycol, a technique that required 18–24 h at 4 °C to attain equilibrium. The solubilized superoxide-generating activity focused with one predominant band of activity at pH 7.6–8.3 (Figure 6). The total level of superoxide-generating activity recovered from either Sephacryl S-200 filtration or isoelectric focusing was too low to employ the other procedure in a sequential purification effort. The solubilized superoxide-generating activity thus possesses a unique profile of biochemical characteristics including a bimodal molecular weight distribution of 150 000 and greater than 300 000, a p*I* of 7.6–8.3, an apparent preference for NADPH over NADH as the electron donor, and a susceptibility to inhibition by *p*-(chloromercuri)phenylsulfonic acid but not by azide.

References

Aswanikumar, S., Schiffman, E., Corcoran, B. A., & Wahl, S. M. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 2439.

Babior, B. M. (1978) *N. Engl. J. Med. 298*, 659, 721.

Babior, B. M., Curnutte, J. T., & McMurrich, B. J. (1976) *J. Clin. Invest. 58*, 989.

Baggiolini, M., Hirsch, J. G., & De Duve, C. (1970) *J. Cell Biol. 45*, 586.

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

Bretz, U., & Baggiolini, M. (1974) *J. Cell Biol. 63*, 251.

Briggs, R. T., Drath, D. B., Karnovsky, M. L., & Karnovsky, M. J. (1975) *J. Cell Biol. 67*, 566.

Cleland, W. W. (1967) *Adv. Enzymol. Relat. Areas Mol. Biol. 29*, 1.

Curnutte, J. T., & Babior, B. M. (1974) *J. Clin. Invest. 53*, 1662.

Curnutte, J. T., Kipnes, R. S., & Babior, B. M. (1975) *N. Engl. J. Med. 293*, 628.

Curnutte, J. T., Babior, B. M., & Karnovsky, M. L. (1979) *J. Clin. Invest. 63*, 637.

DeChatelet, L. R., Shirley, P. S., & Johnson, R. B. (1976) *Blood 47*, 545.

Dewald, B. M., Baggiolini, M., Curnutte, J. T., & Babior, B. M. (1979) *J. Clin. Invest. 63*, 21.

Gabig, T. G., & Babior, B. M. (1979) *Clin. Res. 27*, 460A.

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

Goetzl, E. J., & Hoe, K. Y. (1979) *Immunology 37*, 407.

Goldstein, I. M., Cerqueira, M., Lind, S., & Kaplan, H. B. (1977) *J. Clin. Invest. 59*, 249.

Harlan, J., DeChatelet, L. R., Iverson, D. B., & McCall, C. E. (1977) *Infect. Immun. 15*, 436.

Hohn, D. C., & Lehrer, R. I. (1975) *J. Clin. Invest. 55*, 707.

Iverson, D. B., Wang-Iverson, P., Spitznagel, J. K., & DeChatelet, L. R. (1978) *Biochem. J. 176*, 175.

Kakinuma, K., Boveris, A., & Chance, B. (1977) *FEBS Lett. 74*, 295.

Kane, S. P., & Peters, T. S. (1975) *Clin. Sci. Mol. Med. 49*, 171.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265.

Margoliash, E., & Frohwirt, N. (1959) *Biochem. J. 71*, 570.

Patriarca, P., Cramer, R., & Dri, P. (1976) in *Movement, Metabolism and Bactericidal Mechanisms of Phagocytes* (Rossi, F., Patriarca, P., & Romeo, D., Eds.) p 167, Piccin Medical Books, Padua, Italy.

Salin, M. L., & McCord, J. M. (1974) *J. Clin. Invest. 54*, 1005.

Sbarra, A. J., & Karnovsky, M. L. (1959) *J. Biol. Chem. 234*, 1355.

Segal, A. W., & Peters, T. S. (1977) *Clin. Sci. Mol. Med. 52*, 429.

Sigma Technical Bulletin (1974) No. 500, Sigma Chemical Co., St. Louis, MO.

Smith, L. (1955) in *Methods in Enzymology II* (Colowick, S. P., & Kaplan, N. O., Eds.) p 732, Academic Press, New York.
Stahl, P. D., & Touster, O. (1971) *J. Biol. Chem. 246*, 5398.
Tauber, A. I., & Babior, B. M. (1977) *J. Clin. Invest. 60*, 374.
Tauber, A. I., & Babior, B. M. (1978) *Photochem. Photobiol. 28*, 701.
Tauber, A. I., Gabig, T. G., & Babior, B. M. (1979a) *Blood 53*, 666.
Tauber, A. I., Goetzl, E. J., & Babior, B. M. (1979b) *Inflammation (N.Y.) 3*, 261.
Tsan, M.-F. (1978) *RES, J. Reticuloendothel. Soc. 23*, 205.
West, B. C., Rosenthal, A. S., Gelb, N. A., & Kimball, H. R. (1974) *Am. J. Pathol. 77*, 41.

Structure and Enzymatic Functions of Thioredoxin Refolded by Complementation of Two Tryptic Peptide Fragments†

Ivan Slabý‡ and Arne Holmgren*

ABSTRACT: The physicochemical and catalytic properties of thioredoxin-T′ are described. This complemented protein structure consists of a 1:1 complex between the inactive fragments thioredoxin-T-(1–73) and thioredoxin-T-(74–108). These are generated by selective trypsin cleavage at Arg-73 in lysine-modified and denatured *Escherichia coli* thioredoxin. Thioredoxin-T′ was a slowly formed but stable complex with an apparent K_D below 10^{-8} M. The tryptophan fluorescence spectrum and the CD spectrum were very similar to those of native thioredoxin; some conformational differences were detected by gel chromatography and radioimmunoassay. Thioredoxin-T′-S_2 was a substrate for NADPH and thioredoxin reductase and had 1–2% of the activity of native thioredoxin. This low relative activity was the result of a major increase in the K_m value. Thioredoxin-$(SH)_2$ was a hydrogen donor for *E. coli* ribonucleotide reductase with about 3% relative activity. These results for thioredoxin-T′ are correlated with the known three-dimensional structure of thioredoxin. The microenvironment around Arg-73 that is close to the active disulfide appears to be of critical importance for the interactions of thioredoxin with thioredoxin reductase and ribonucleotide reductase.

Thioredoxin-S_2[1] contains an intramolecular cystine disulfide bridge (–Cys_{32}-Gly-Pro-Cys_{35}–) as the catalytically important group (Holmgren, 1968). This disulfide is reduced to a dithiol with NADPH in a reaction catalyzed by the specific flavoprotein thioredoxin reductase [for a review, see Holmgren (1980)]. Thioredoxin-$(SH)_2$ is an efficient disulfide reductase and is reoxidized in reactions such as those shown in Figure 1. The assays for thioredoxin activity are thus based on the cyclic oxidoreduction of thioredoxin in the presence of NADPH and thioredoxin reductase and a disulfide acceptor. A novel and so far largely unknown function of *Escherichia coli* thioredoxin is to be the host-coded essential subunit of phage T7 DNA polymerase (Mark & Richardson, 1976), required for virus DNA replication in vivo and in vitro.

Previous studies of the structure and function of thioredoxin have been aimed at an understanding of its molecular mechanism of action. Thus, the complete primary structure of the 108 amino acid residues of thioredoxin-S_2 from *E. coli* has been determined (Holmgren, 1968). The three-dimensional structure of thioredoxin-S_2 has been solved to 2.8-Å resolution by X-ray crystallographic techniques (Holmgren et al., 1975). In addition, we previously identified two systems of peptide fragments that can be used to reconstitute thioredoxin-S_2 by noncovalent complementation (Holmgren, 1972a; Slabý & Holmgren, 1975). Cleavage of thioredoxin with cyanogen bromide at the single methionine residue (Met-37) yields the two peptide fragments thioredoxin-C-(1–37) and thioredoxin-C-(38–108) that upon mixing form thioredoxin-C′. Selective cleavage by trypsin of thioredoxin, where all the lysines have been blocked by citraconic anhydride, splits the molecule at the single arginine residue (Arg-73). The resulting peptide fragments, after removal of citraconyl groups by mild acid hydrolysis, thioredoxin-T-(1–73) and thioredoxin-T-(74–108), interact specifically to give thioredoxin-T′ (Slabý & Holmgren, 1975).

Thioredoxin-T′ and thioredoxin-C′ both give full immunoprecipitation activity with rabbit antibodies against native thioredoxin and have a low but significant enzymatic activity with thioredoxin reductase (Slabý & Holmgren, 1975). The component peptides of these complexes are enzymatically inactive. However, thioredoxin-T-(74–108) and also thioredoxin-C-(38–108) were strong inhibitors of the precipitation reaction of native thioredoxin-S_2 with its antibodies. This demonstrated that one of the major antigenic determinants of thioredoxin is contained in the thioredoxin-T-(74–108) amino acid sequence. Furthermore, the results show that this COOH-terminal fragment has the capacity of nucleated folding into a structure similar to that which it normally occupies in native thioredoxin. This conclusion has been strongly supported by the three-dimensional structure of thioredoxin-S_2

† From the Department of Chemistry, Karolinska Institutet, S-104 01 Stockholm, Sweden. *Received June 27, 1979; revised manuscript received September 26, 1979.* This investigation was supported by grants from the Swedish Medical Research Council Projects 13X-3529 and 13P-4292, Magnus Bergvalls Stiftelse, and by a short-term EMBO fellowship to I.S.

‡ Present address: Department of Medical Chemistry, Charles University, Karlovarska 48, Plzen, Czechoslovakia.

[1] Abbreviations used: thioredoxin-S_2 (T-S_2) and thioredoxin-$(SH)_2$ [T-$(SH)_2$], the oxidized and reduced forms of thioredoxin, respectively; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid). Peptide fragments have been designated by an adoption of the rules of the IUPAC–IUB Commission on Biochemical Nomenclature. Fragments obtained after CNBr cleavage are denoted by C and after selective cleavage with trypsin are denoted by T. The reconstituted noncovalent complexes are denoted thioredoxin-C′ and thioredoxin-T′ (Slabý & Holmgren, 1975).

0006-2960/79/0418-5584$01.00/0