Mechanism of the superoxide-producing oxidase of neutrophils

O_2 is necessary for the fast reduction of cytochrome *b*-245 by NADPH

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A soluble oxidase from phorbol-stimulated pig neutrophils contained FAD and cytochrome *b*-245. A typical preparation produced 13.03 mol of superoxide ($O_2^{\cdot -}$) $\cdot s^{-1} \cdot mol$ of cytochrome *b* $^{-1}$ (348 nmol $\cdot min^{-1} \cdot mg$ of protein $^{-1}$). In the aerobic steady state, cytochrome *b* was 8.9% reduced. Steady-state cytochrome *b* reduction was absent from extracts of unstimulated cells; K_m values for NADPH, for $O_2^{\cdot -}$ production and cytochrome *b* reduction were similar. The calculated aerobic rate of cytochrome *b* reduction was equal to the measured rate of $O_2^{\cdot -}$ production in a variety of preparations and in the presence of a range of inhibitors. Under anaerobic conditions the rate was slow: O_2 is apparently required for rapid electron flow into the oxidase complex. Cytochrome *b* is shown to be kinetically competent to act as part of the $O_2^{\cdot -}$ -generating complex.

Superoxide ($O_2^{\cdot -}$) is produced by neutrophils and other phagocytic leucocytes in response to particulate or soluble stimuli. The $O_2^{\cdot -}$ and derived species ($OH^{\cdot}$ and H_2O_2) (Babior, 1978), are involved in killing infecting micro-organisms and, possibly, developing tumours (Adams & Nathan, 1983). Inappropriate stimulation of leucocytes leads to tissue damage in rheumatoid arthritis and gout (Dieppe & Doherty, 1982). The $O_2^{\cdot -}$ -generating oxidase is located in the plasma membrane (Dewald *et al.*, 1979) and requires NADPH as electron donor (Curnutte & McMurry, 1976). Solubilized oxidase (Cross *et al.*, 1984) contains FAD and a cytochrome *b* with a midpoint oxidation–reduction potential at pH 7 ($E_{m,7.0}$) of $-245 mV$ (cytochrome *b*-245) (Cross *et al.*, 1981). Both are reduced when NADPH is added anaerobically to extracts of activated cells but not of resting cells; reduced cytochrome *b* is oxidized quickly on aeration (Cross *et al.*, 1982; Gabig *et al.*, 1982). Neutrophils from X-chromosome-linked chronic granulomatous patients lack cytochrome *b*-245 and fail to produce $O_2^{\cdot -}$ on stimulation (Segal *et al.*, 1983); these patients have low resistance to infection. This and other evidence suggests an electron-transport system of the type shown in Scheme 1.

The anaerobic rate of cytochrome *b* reduction (v_1) is too slow to support the known rate of $O_2^{\cdot -}$ production (Cross *et al.*, 1984); we show here that, under steady-state aerobic conditions, this anomaly disappears. Oxygen apparently induces electron transfer to take place within the active oxidase complex.

Materials and methods

Neutrophils were isolated from whole blood, stimulated with phorbol 12-myristate 13-acetate (Sigma) and harvested as described by Cross *et al.* (1984). The cells were resuspended in disruption buffer, which had the following composition: sucrose (8.6%, w/v), $MgSO_4$ (4 mM), NaN_3 (1 mM), $CaCl_2$ (1 mM), deoxyribonuclease 1 (10 $\mu g \cdot ml^{-1}$),

Scheme 1. Sequence of electron-transport components postulated to be involved in NADPH-dependent $O_2^{\cdot -}$ production

v_1 is the steady-state rate of electron flow from NADPH to cytochrome *b*-245; v_2 is the steady-state rate of reduction of oxygen by reduced cytochrome *b*-245; k_2 is the first-order rate constant for the latter reaction.

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silicone antifoam (0.01%, w/v) (BDH) and phenylmethanesulphonyl fluoride ($5 \mu\text{g} \cdot \text{ml}^{-1}$); chymotrypsin, leupeptin, antipain, pepstatin and 7-amino-1-chloro-3-L-tosylamidoheptan-2-one ('TLCK') (all supplied by Sigma) were each added at $1 \mu\text{g} \cdot \text{ml}^{-1}$. The suspension was disrupted by nitrogen cavitation after stirring at 500 lbf/in² (3.45 MPa) for 20 min in a nitrogen bomb (Parr Instrument Co.) at 0°C. The suspension was centrifuged at 500g for 7 min, and the postnuclear supernatant collected and re-centrifuged at 100000g for 30 min. The membrane pellet was suspended in 20 mM-glycine buffer, pH 8.0, containing 25% (v/v) glycerol, 1 mM-MgSO₄, 0.5 mM-CaCl₂, 1 mM-Na₃N₃, 0.25% (v/v) Lubrol PX (Sigma), 0.25% (w/v) sodium deoxycholate (BDH) and the same mixture of proteinase inhibitors as were put in the disruption buffer. The suspension was sonicated three times for 20 s at 100 W, with 1 min cooling between treatments, in a Branson sonifier, and re-centrifuged at 100000g. The clear supernatant contained 40% of the activity of the postnuclear supernatant.

NADPH- or NADH-dependent superoxide generation was measured as the superoxide-sensitive rate of reduction of added ferricytochrome *c* at 20°C as described by Cross *et al.* (1982). Absorption spectra of 0.4 ml samples were recorded in a rapid-scanning spectrophotometer (Cross *et al.*, 1984). The cuvette was stirred, except during data collection (duration 12 s), to maintain aerobiosis.

Results and discussion

Oxidase solubilized from plasma membrane of phorbol-stimulated pig neutrophils varied some-

what in O₂^{-•}-generating activity from preparation to preparation (see Table 1). One such preparation contained both FAD (492 pmol·mg of protein⁻¹) and cytochrome *b*-245 (445 pmol·mg of protein⁻¹) (Cross *et al.*, 1984). Its maximum rate of O₂^{-•} generation was 348 nmol·min⁻¹·mg of protein⁻¹ when either NADPH or NADH was electron donor, but the *K_m* for NADPH was much lower (45 μM) than that for NADH (460 μM). This measured maximum rate of O₂^{-•} production was 13.03 s⁻¹·mol of cytochrome *b*⁻¹. The rates of postulated intermediate reactions, *v*₁ and *v*₂ (Scheme 1), should equal this steady-state rate if Scheme 1 is correct. In previous experiments (Cross *et al.*, 1982) we have shown that the rate of oxidation of reduced cytochrome *b*-245 by oxygen has a *t*_{1/2} of 4.7 ms, giving a first-order rate constant of 147 s⁻¹. These experiments repeated on our solubilized preparations (Fig. 1) showed that it had the same kinetic properties.

The reduction of cytochrome *b* under conditions where oxygen is absent is slow, and only 50% complete after several minutes in either whole cells (Cross *et al.*, 1982) or in soluble extracts (Cross *et al.*, 1984). A thorough search by anaerobic stopped-flow spectroscopy for a fast-reacting cytochrome *b* component failed to detect such an entity. This result is unexpected, since there is good experimental evidence implicating cytochrome *b*-245 in this pathway, and yet the measured anaerobic rate is less than 10% of that required by Scheme 1.

We have now determined the rate at which cytochrome *b*-245 is reduced by NADPH under aerobic conditions. A sample of soluble oxidase was placed in each cuvette of a sensitive split-beam

Table 1. Rates of superoxide production and steady-state cytochrome *b*-245 reduction of different preparations of solubilized superoxide-producing oxidase

Abbreviations used: cyt., cytochrome, pCMB, *p*-chloromercuribenzoate; N.D., none detected.

Enzyme preparation	Cyt. <i>b</i> -245 reduced by NADPH (%)	Specific rate of cyt. <i>b</i> -245 reduction (<i>v</i> ₁) (mol of cyt. <i>b</i> ·s ⁻¹ ·mol of cyt. <i>b</i> ⁻¹)	Specific rate of O ₂ ^{-•} production (mol of O ₂ ^{-•} ·s ⁻¹ ·M·mol of cyt. <i>b</i> ⁻¹)
A	8.8 ± 0.72 (<i>n</i> = 5)	12.99 ± 1.06	13.03
B	4.1 ± 0.14 (<i>n</i> = 3)	6.03 ± 0.21	5.73
C	1.0 (<i>n</i> = 1)	1.47	1.13
D	4.2 (<i>n</i> = 1)	6.17	5.9
A + pCMB (10 μM)	N.D.	0	0
A 'aged' by incubation at 25°C for 30 min	N.D.	0	0
E (enzyme prepared from unstimulated cells)	N.D.	0	0.4

spectrophotometer (Cross *et al.*, 1984) and a baseline was recorded. NADPH was added to the sample cuvette and the steady-state spectrum was recorded under conditions where O_2 was not limiting. Cytochrome *b*-245 was partly reduced (Fig. 2); it was then fully reduced by the addition of a few grains of sodium dithionite. An extract of membrane from unstimulated neutrophils submitted to the same procedure showed no reduction of the cytochrome *b* on adding NADPH (Fig. 3). The reducibility of cytochrome *b*-245 by NADPH requires pre-treatment of the whole cells with stimulant before extraction.

The specific rate of electron flow from cytochrome *b*-245 in the steady state (v_2) is given by the rate constant $k_2 \times$ proportion of reduced cytochrome *b*:

$$\begin{aligned} v_2 &= k_2 \times \frac{[\text{Reduced cytochrome } b\text{-245}]}{[\text{Total cytochrome } b\text{-245}]} \text{ mol of cytochrome } b^{-1} \\ &= 147 \times 0.089 \text{ s}^{-1} \cdot \text{mol of cytochrome } b^{-1} \\ &= 13.08 \text{ s}^{-1} \cdot \text{mol of cytochrome } b^{-1} \end{aligned}$$

In the steady state, $v_1 = v_2 = 13.08 \text{ s}^{-1} \cdot \text{mol of cytochrome } b^{-1}$.

The calculated rate of reduction of cytochrome *b* here is very close to the measured overall rate of NADPH-dependent O_2 -production of catalysed by this oxidase preparation ($13.03 \text{ mol} \cdot \text{s}^{-1} \cdot \text{mol of cytochrome } b\text{-245}^{-1}$), confirming the role of cytochrome *b*-245 in the oxidase complex (see also results from other preparations in Table 1).

Effect of inhibitory treatments

The oxidase is notoriously unstable at room temperature (Cross *et al.*, 1984; Light *et al.*, 1981) ($t_{1/2}$ around 2–4 min). After soluble extract had been left at room temperature for 30 min, the aerobic steady-state reduction of cytochrome *b* by NADPH was abolished. The addition of $10 \mu\text{M}$ -p-

Fig. 1. Kinetics of oxidation of cytochrome *b*-245 of the solubilized oxidase of pig neutrophils

Stopped-flow experiments were performed as described previously (Cross *et al.*, 1982), with the following modifications. The solubilized oxidase was incubated for 40 min under an atmosphere of argon in the presence of 20 mM-glucose, glucose oxidase (2.5 units·ml⁻¹) and catalase (2.5 units·ml⁻¹) before 90% reduction with sodium dithionite and anaerobic transfer to one flow line of a Hi-Tech SF-3L stopped-flow spectrophotometer thermostatically controlled at 20°C. The tubing of the spectrophotometer was immersed in a bath of 100 mM-sodium dithionite and was flushed with buffer containing 20 mM-glucose, glucose oxidase (25 units·ml⁻¹) and catalase (25 units·ml⁻¹) immediately before use. The oxidation of cytochrome *b*-245 after mixing with air-saturated buffer was measured by monitoring ΔA_{559} . The mean result from four traces is shown by the continuous line. The digitized record was transferred to an ITT 2020 microcomputer and analysed by a non-linear least-squares technique (Edsall & Gutfreund, 1983), shown by the broken line, giving a rate constant of 147 s^{-1} .

Fig. 2. Difference absorption spectra showing the aerobic steady-state reduction of cytochrome *b*-245 by NADPH in a detergent-solubilized preparation of the O_2 -generating oxidase prepared from stimulated neutrophils

(a) After spectra of the oxidized enzyme had been recorded, $20 \mu\text{l}$ of 20 mM-NADPH was added (final concn. 1 mM), spectra recorded within 1 min, and the oxidized spectrum subtracted. The absorbance scale of the spectrum has been expanded 4-fold for clarity. (b) Complete reduction of cytochrome *b*-245 by the addition of sodium dithionite to (a). A few crystals of sodium dithionite were added to fully reduce the preparation and spectra recorded. To determine the total amount of cytochrome *b* present, reduction was estimated by $\Delta A_{559-540} = 21.6 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ (Cross *et al.*, 1982).

Fig. 3. Difference absorption spectra showing (a) the absence of reduction of cytochrome *b*-245 in the aerobic steady state by NADPH in the solubilized enzyme prepared from unstimulated neutrophils, and (b) complete reduction of cytochrome *b*-245 by the addition of a few crystals of sodium dithionite to (a)

The methods were as described in Fig. 1, except that a portion of the cells was not stimulated with phorbol 12-myristate 13-acetate but were otherwise treated identically. The absorbance scale of the spectrum has been expanded 4-fold for clarity.

chloromercuribenzoate abolished both oxidase activity and the steady-state reduction of the cytochrome *b*-245 by NADPH (Table 1).

The $O_2^{\cdot-}$ -generating oxidase, unlike mitochondrial oxidases, is insensitive to 1 mM-KCN (Sbarra & Karnovsky, 1959). Addition of 1 mM-KCN to the soluble oxidase had no effect on the steady-state level of reduction of cytochrome *b*-245 by NADPH.

Approximate K_m for cytochrome *b*-245 reduction

The steady-state level of reduction of the cytochrome by 50 μ M-NADPH is approx. 65% of that given by 100 μ M-NADPH (results not shown).

Reduction of cytochrome *b*-245 by 100 μ M-NADH was too slight to be measured. At 1 mM-NADH the reduction was approximately the same as that given by 100 μ M-NADPH. Reduction of the cytochrome *b* has a much lower K_m for NADPH than for NADH, the values being similar to those for $O_2^{\cdot-}$ production (Cross *et al.*, 1984; Wakeyama *et al.*, 1982).

These effects of activation, anaerobiosis, inhibitors and the favoured K_m for NADPH show that the cytochrome being studied is unconnected with mitochondrial or microsomal cytochrome *b* contamination. Confirmation of this was obtained by potentiometric titration, which did not detect high-potential *b* cytochromes in the oxidase extract.

Effect of O_2 on the $O_2^{\cdot-}$ -generating oxidases

The observation that the anaerobic rate of electrons into the oxidase system is very slow is strikingly similar to the slow reduction of mammalian cytochrome *c* oxidase by reduced cytochrome *c* (Andreasson *et al.*, 1972) or the reaction where pulsed cytochrome *c* oxidase (which has reacted with oxygen) oxidizes reduced cytochrome *c* at a much higher rate than resting oxidase (Antonini *et al.*, 1977). These non-orthodox kinetics are likely to result from a conformational change produced on binding oxygen or on electron transfer within the complex. They explain why previous attempts to demonstrate the kinetic competence of cytochrome *b*-245 in the oxidase system have been unsuccessful.

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