NADPH oxidase of guinea-pig macrophages catalyses the reduction of ubiquinone-1 under anaerobic conditions

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The stimulation-specific NADPH-dependent reduction of ubiquinone-1 (Q-1) in guinea-pig macrophages was studied. The activity was due neither to any modified product of the phagocytosis-specific NADPH oxidase nor to non-specific diaphorases of the cells, since the activity was measured in sonicated or detergent-disrupted cells by subtracting the activity in the resting cells from that in cells activated by phorbol 12-myristate 13-acetate. The activity was not mediated by superoxide anions, since strict anaerobic conditions were employed. The anaerobic reduction of Q-1 was NADPH-specific, like superoxide formation under aerobic conditions, and its maximal velocity was also essentially the same as that of superoxide formation. The oxidase does not directly reduce Q-1 under aerobic conditions [Nakamura, Murakami, Umei & Minakami (1985) FEBS Lett. **186**, 215–218], and the electron transfer from NADPH to cytochrome *c* by the oxidase under aerobic conditions was not enhanced by the addition of Q-1. The observations indicate that the phagocytosis-specific NADPH oxidase reduces Q-1 and that oxygen competes with the reduction of Q-1. Q-1 seems to accept electrons not from the intermediary electron carriers of the oxidase but from the terminal oxygen-reducing site of the enzyme.

INTRODUCTION

Neutrophils and macrophages release superoxide anions ($O_2^{\cdot-}$) on exposure to particulate or soluble stimuli (Babior *et al.*, 1973; Drath & Karnovsky, 1975; Dechatelet *et al.*, 1976). The $O_2^{\cdot-}$ is generated by one-electron reduction of molecular O_2 catalysed by phagocytosis-specific NADPH oxidase (Babior *et al.*, 1976). Flavoprotein and cytochrome *b* have been proposed to be components of the NADPH oxidase, and ubiquinone has also been suggested to be implicated. The NADPH oxidase has not been purified so far, so artificial electron acceptors have been used to characterize the speculated components.

Reduction of DCIP by the phagocytosis-specific NADPH oxidase has been shown (Green & Schaefer, 1981; Wakeyama *et al.*, 1983). The activation of the NADPH oxidase by ubiquinone (Crawford & Schneider, 1982), the reduction of ubiquinone-1 (Q-1), a short-chain derivative of ubiquinone-10, by the oxidase under aerobic conditions (Takeshige *et al.*, 1984) and the reduction of endogenous ubiquinone (Gabig & Lefker, 1985) have also been shown. These observations seem to suggest that ubiquinone shuttles electron flow between flavoprotein and cytochrome *b*. Bellavite *et al.* (1984), however, have shown that the reduction of DCIP by the oxidase is mediated by $O_2^{\cdot-}$ and we reported that the reduction of Q-1 by the oxidase under aerobic conditions could be inhibited by SOD when sufficiently high concentrations of SOD were used (Nakamura *et al.*, 1985). These observations raise a question on the involvement of ubiquinone in the NADPH oxidase.

In the present paper we show that Q-1 is reduced by

the phagocytosis-specific NADPH oxidase under anaerobic conditions. Q-1 seems to accept electrons from the O_2 -reducing site of the enzyme and not from other components of the respiratory chain.

EXPERIMENTAL

Materials

Cytochrome *c* (horse heart, type VI), SOD (EC 1.15.1.1; from bovine blood), were purchased from Sigma Chemical Co. NADPH was obtained from Oriental Yeast Co. (Tokyo, Japan), PMA from LC Service Corp. (Woburn, MA, U.S.A.) and PEG 400 from Wako Pure Chemical Industries (Osaka, Japan), and Q-1 was generously given by Eisai Pharmaceutical Co. (Tokyo, Japan). All other chemicals were of the best grade available.

Macrophage preparation

Elicited macrophages (EMs) were obtained from peritoneal cavities of guinea pigs to which mineral oil (15–20 ml/animal) had been injected 1–5 weeks before the animals were killed. EMs were washed three times with PBS, treated hypo-osmotically to lyse erythrocytes if necessary, and were suspended in a HEPES/saline buffer, pH 7.5 (17 mM-HEPES/120 mM-NaCl/5 mM-KCl/1 mM-MgSO₄/5 mM-glucose) at a concentration of 10⁸ EMs/ml. The purity of macrophages was 97% or more.

Preparation of macrophage homogenates

Macrophages in the HEPES/saline buffer were pre-incubated for 20 min at 25 °C, stimulated with 10 µg of

Abbreviations used: Q-1, ubiquinone-1; PMA, phorbol 12-myristate 13-acetate; DCIP, 2,6-dichlorophenol-indophenol; SOD, superoxide dismutase; PEG 400, poly(ethylene glycol) 400; EMs, elicited macrophages; PBS, phosphate-buffered saline (140 mM-NaCl/2.7 mM-KCl/8.1 mM-Na₂HPO₄/1.5 mM-KH₂PO₄ buffer, pH 7.2); Me₂SO, dimethyl sulphoxide.

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PMA/10⁸ EMs for 5 min in the presence of 10 mM-NaN₃, cooled in ice, and centrifuged at 400 *g* for 5 min. Stimulated cells were resuspended in an equal volume of 25% (w/v) ethylene glycol and disrupted by sonication at 0 °C (15 pulses and seven pulses at 15 W interrupted by a 40 s cooling period) in a Branson model 200 sonifier fitted with a microtip. The sonicated material was stored at -70 °C in Eppendorf plastic tubes in 100 μ l portions. Superoxide-forming activity was stable for several weeks at -70 °C. Except for replacement of PMA by Me₂SO, the preparation of homogenates from unstimulated cells was essentially the same as that of stimulated cells. Samples were thawed at 0 °C, supplemented with PEG 400 at a final concentration of 25% (w/v) and sonicated for dispersion. Assays were performed within 2 h of thawing.

Assays

O₂^{•-}-forming activity was measured by the reduction of cytochrome *c* in the presence of reduced nicotinamide coenzymes as described by Nakagawara & Minakami (1975). A cuvette containing 1 ml of reaction mixture [consisting of a Hepes/acetate buffer, pH 7.5 (17 mM-Hepes/120 mM-potassium acetate/5 mM-sodium acetate/1 mM-MgSO₄), 100 μ M-cytochrome *c*, 10 mM-NaN₃ and 200 μ M-NADPH] was placed in a thermostatically controlled cuvette holder of a Hitachi 557 dual-wavelength spectrophotometer at 25 °C. The assay was started by the addition of a sample and the increase in *A*₅₅₀ with reference to 540 nm (*A*₅₅₀ - *A*₅₄₀) was monitored on a recorder. The O₂^{•-}-forming activity was calculated from the initial velocity of the reduction of cytochrome *c* by using a molar absorption coefficient of 20.0 × 10³ M⁻¹·cm⁻¹, and the stimulation-dependent activity was obtained from the activity of the stimulated sample after subtracting that of the unstimulated one. Q-1-reducing activity of macrophage homogenates was measured under anaerobic conditions by monitoring the rate of cytochrome *c* reduction in the presence of Q-1.

Anaerobic conditions

Anaerobic conditions were achieved in a Thunberg cuvette with a 1 cm light-path. It contained 2 ml of the same reaction mixture as the aerobic assay in the main compartment and a sample equivalent to 8 × 10⁵ EMs mixed with 100 μ l of 10% (w/v) PEG 400 in the side arm. The cuvette was evacuated and filled with argon three times. After preincubation at 25 °C for 5 min, the sample was mixed with the reaction mixture and the activity was measured spectrophotometrically. For assay of detergent-lysed macrophages, 3.5 × 10⁵ EMs in 100 μ l of the Hepes/saline buffer was stimulated by 2 μ g of PMA at 37 °C for 5 min, mixed with 25 μ l of ice-cold ethylene glycol and 25 μ l of PEG, and put in the side arm of a Thunberg cuvette. Renex 30 was added to the reaction mixture to a final concentration of 0.05% in the main compartment for the lysis of macrophages.

RESULTS

Stimulation-dependent reduction of Q-1 under anaerobic conditions

We have previously shown that essentially all reduction of Q-1 by the phagocytosis-specific NADPH oxidase under aerobic conditions was mediated by O₂^{•-} (Naka-

Table 1. O₂^{•-}-dependent cytochrome *c* reduction and stimulation-dependent Q-1 reduction under anaerobic conditions

The reduction of cytochrome *c* and Q-1 was measured under aerobic or anaerobic conditions. Assays were performed as described in the Experimental section. Expt. 1: the reduction of cytochrome *c* was measured in the absence of Q-1. Expt. 2: Q-1 reduction was measured by monitoring the reduction of cytochrome *c* in the presence of 150 μ M-Q-1. The concentration of cytochrome *c* was 100 μ M. Values are expressed as nmol of electrons/min per 10⁶ EMs, and represent the means ± S.D. (*n* = 3).

Q-1	O ₂	Activity (nmol of electrons/min per 10 ⁶ EMs)		
		Stimulated	Unstimulated	Net increase
Expt. 1				
—	—	2.8±0.1	2.8±0.3	0.0±0.2
—	+	19.9±2.1	3.1±0.5	16.8±1.5
Expt. 2				
+	—	7.6±0.3	4.9±0.6	2.7±0.4
+	+	17.8±0.6	4.8±0.7	13.0±0.5

mura *et al.* 1985). This, however, does not necessarily mean that Q-1 is not reduced directly by the oxidase, because the reduction may possibly be inhibited by O₂ competitively and the low activity may be hidden by the high activity of reducing O₂. We therefore conducted experiments to ascertain conclusively whether the Q-1-reducing activity is present in or absent from the NADPH oxidase under anaerobic conditions.

The following procedure was used in the present investigation to determine the activities of the phagocytosis-specific NADPH oxidase. Two portions of the cell suspension were incubated with or without PMA, then sonicated, and the difference of the activities in both preparations are given as net increases corresponding to the activities of the phagocytosis-specific oxidase. Although we can purify the preparation from the stimulated cells, we cannot obtain a control preparation from the resting cells, so that we are unable to exclude the activities of other diaphorases if we use a purified preparation. Moreover, artificial diaphorase activities might be produced by the purification procedure. The anaerobic conditions of the present experiments were sufficient to abolish the formation of O₂^{•-} by the NADPH oxidase, as shown in Table 1 (column of net increase in Expt. 1).

Under anaerobic conditions a significant stimulation-dependent reduction of Q-1 was observed and the rate was about 20% of that under aerobic conditions (Table 1, Expt. 2). In contrast with the activity assayed under aerobic conditions (Nakamura *et al.*, 1985), the reduction of Q-1 under anaerobic conditions was not inhibited even by quite high concentrations of SOD (3000 units/ml), indicating the absence of the O₂^{•-}-mediated reduction. The net increases were 10.1 ± 1.2 and 10.0 ± 1.1 nmol of electrons/min per 10⁶ EMs in the absence and in the presence of 3000 units of SOD/ml respectively. An essentially similar result was obtained with DCIP as an electron acceptor (M. Murakami, M. Nakamura & S. Minakami, unpublished work).

The anaerobic NADPH:Q-1 reductase activity was

Fig. 1. Effect of the cell density of macrophage homogenates on the rate of Q-1 reduction under anaerobic conditions

The rate of Q-1 reduction was measured at various homogenate cell densities as described in the Experimental section. Δ , PMA-stimulated; $\blacktriangle$, unstimulated; $\circ$, net increase. The concentration of Q-1 was $610 \mu\text{M}$.

also found in the sample disrupted by a detergent (Renex 30). The net increase was 6.9 ± 1.5 nmol of electrons/min per 10^6 EMs in the presence of $590 \mu\text{M}$ -Q-1. The lower activity in the detergent-lysed sample compared with that in the sonicated sample may be attributed to the lability of the enzyme in detergent.

Characterization of the stimulation-dependent Q-1-reducing activity

The rate of the Q-1 reduction under anaerobic conditions increased proportionally with the increase in the cell density of the homogenate (Fig. 1). In subsequent experiments, the concentration of the sample was fixed at 4×10^5 cells/ml.

We measured the rate of the Q-1 reduction at various Q-1 concentrations (Fig. 2). The stimulation-dependent Q-1-reducing activity increased hyperbolically with increase in the concentration of Q-1. The apparent K_m of the activity for Q-1 was $590 \mu\text{M}$ and the $V_{\max}$ was 20.8 nmol of electrons/min per 10^6 cells. The $V_{\max}$ was essentially the same as the rate of the stimulation-dependent $\text{O}_2^{\cdot-}$ formation of the same sample (20.2 nmol of electrons/min per 10^6 cells). The rate of $\text{O}_2^{\cdot-}$ formation under aerobic conditions can be considered as the $V_{\max}$ because the O_2 concentration is about 10 times the K_m value for O_2 (Kakinuma & Kaneda, 1982).

It is necessary to know whether Q-1 was reduced to hydroquinone (Q-1H₂) or to its semiquinone (Q-1H $\cdot$). If Q-1 is directly reduced to Q-1H₂ to some extent, the $V_{\max}$ obtained for the Q-1 reduction would have been less than the true value, because cytochrome *c* accepts electrons from the semiquinone and not from the hydroquinone form under our assay conditions. Yamazaki's group (Nakamura *et al.*, 1978) has demonstrated that hydroquinone could be formed at a low concentration of benzoquinone by xanthine oxidase. We therefore measured the oxidation of NADPH and compared it with the

Fig. 2. Effect of Q-1 concentration on the rate of Q-1 reduction under anaerobic conditions

The reduction of Q-1 was measured at various concentrations of Q-1 under anaerobic conditions. The net increase of Q-1 reduction is plotted against Q-1 concentration. Results are means $\pm$ s.d. ($n = 3$). The inset shows a Lineweaver-Burk plot for the same experiment.

univalent reduction of Q-1 in the phagocytosis-associated activity and obtained the ratio of the Q-1 reduction to the NADPH oxidation as 2.0 ± 0.3 ($n = 3$). The result indicates that Q-1 was exclusively reduced to its semiquinone and that the $V_{\max}$ of the anaerobic NADPH:Q-1 reductase activity was comparable with that of the NADPH-dependent $\text{O}_2^{\cdot-}$ formation.

The phagocytosis-specific oxidase is known to be specific to NADPH (Patriarca *et al.*, 1971). We measured Q-1 reduction under anaerobic conditions with NADPH and NADH, both at a concentration of $200 \mu\text{M}$, and observed that the NADH-dependent reduction was about 20% of the NADPH-dependent reduction (Table 2). The ratio is in good agreement with that of the phagocytosis-specific $\text{O}_2^{\cdot-}$ formation calculated from the reported K_m values (Cohen *et al.*, 1980; Nakamura & Baxter, 1981).

$\text{O}_2^{\cdot-}$ formation in the presence of Q-1

If the NADPH:Q-1 reductase activity of the stimulated cells is catalysed by the NADPH oxidase and Q-1 accepts electrons at the O_2 -reducing component of the oxidase,

Table 2. Nicotinamide nucleotide specificity of the stimulation-dependent Q-1-reducing activity

The rate of cytochrome *c* reduction was measured in the presence of $610 \mu\text{M}$ of Q-1 and $200 \mu\text{M}$ -NADPH or -NADH under anaerobic conditions. Values are expressed as nmol of electrons/min per 10^6 EMs and represent the means $\pm$ s.d. ($n = 3$).

	Activity (nmol of electrons/min per 10^6 EMs)		
	Stimulated	Unstimulated	Net increase
NADPH	13.6 ± 1.0	3.4 ± 0.1	10.2 ± 0.7
NADH	18.7 ± 0.9	16.8 ± 0.3	1.9 ± 0.7

Table 3. Cytochrome *c* reduction in the presence or in the absence of Q-1 under aerobic conditions

The assays were performed as described in the Experimental section. A 10 μ l volume of 118 mM-Q-1 or of ethanol was added to the reaction mixture. Values are expressed as nmol of electrons/min per 10⁶ EMs and represent the means $\pm$ S.D. ($n = 3$).

Q-1	Activity (nmol of electrons/min per 10 ⁶ EMs)		
	Stimulated	Unstimulated	Net increase
+	18.1 $\pm$ 1.5	4.9 $\pm$ 0.5	13.2 $\pm$ 0.7
-	16.0 $\pm$ 1.0	3.6 $\pm$ 0.5	12.5 $\pm$ 0.5

the extent of electron flow from NADPH to cytochrome *c* should be constant regardless of the presence or absence of Q-1. Moreover, if the activity were catalysed by an enzyme different from the oxidase or Q-1 accepted electrons from intermediary carriers of the enzyme, the reduction of cytochrome *c* should increase by the addition of Q-1. Table 3 shows that the added Q-1 did not increase the overall electron flow to cytochrome *c*. We may therefore conclude that the stimulation-dependent NADPH:Q-1 reductase activity is catalysed by the O₂^{•-}-forming oxidase and that Q-1 receives electrons at the site situated after the rate-limiting steps of the electron flow, possibly at the site that gives electrons to O₂.

DISCUSSION

The following three findings presented in this paper provide evidence for the supposition that the phagocytosis-specific NADPH oxidase has Q-1 reductase activity under anaerobic conditions. (1) The activity appeared after phagocytotic stimulation. (2) The activity, like O₂^{•-} formation, was NADPH-specific. (3) The $V_{\max}$ of the activity was essentially the same as that of the O₂^{•-} formation. The supposition was further confirmed by the observation that the enzyme under aerobic conditions was not increased by the addition of Q-1 at a concentration that could by itself give two-thirds of the maximal activity. We therefore propose that Q-1 and O₂ competitively accept electrons from the oxidase at the same site or at sites that cannot be separated kinetically from each other.

We have previously shown that the phagocytosis-specific NADPH oxidase is essentially unable to reduce Q-1 directly under aerobic conditions (Nakamura *et al.*, 1985). This finding can be explained by the competition between O₂ and Q-1. If O₂ is a competitive inhibitor for the reduction of Q-1, the K_i for Q-1 reduction should be equal to the K_m of the oxidase for O₂ (27 μ M; Kakinuma & Kaneda, 1982). By using the K_m values and the concentrations of O₂ and Q-1, we can calculate the amount of Q-1 directly reduced under aerobic conditions where the reduction of O₂ is not rate-limiting. About 3% of the total reduction of ferricytochrome *c* may be due to the direct reduction of Q-1 and the remaining 97% may be mediated by O₂^{•-}, which explains the previous finding

that SOD almost completely inhibited the Q-1 reduction under aerobic conditions.

Crawford & Schneider (1982) and Gabig & Lefker (1985) have suggested that ubiquinone-10 shuttles the two components of the NADPH oxidase, a flavoprotein and a *b*-type cytochrome, when phagocytes are stimulated. We used Q-1, a short-chain derivative of the ubiquinone, to confirm their supposition, because it is an artificial electron acceptor that can mimic the oxidoreduction of the endogenous ubiquinone in the mitochondrial respiratory chain. We observed the Q-1 reductase activity of the oxidase, but could not support the supposition of a shuttling role for the ubiquinone, although Q-1 may differ from the endogenous ubiquinone. Q-1 seems to accept electrons at the terminal stage of electron transfer and does not appreciably stimulate the ferricytochrome reduction of both the stimulated and unstimulated cells (Table 3). The ubiquinone content in neutrophils has been studied by several authors (Crawford & Schneider, 1982; Cunningham *et al.*, 1982; Cross *et al.*, 1983; Lutter *et al.*, 1984; Gabig & Lefker, 1985), but the existence of the ubiquinone and its subcellular localization in neutrophils remain controversial. Cross *et al.* (1983) reported that ubiquinone was present only in mitochondria, and Lutter *et al.* (1984) could not detect it in the cells.

The phagocytosis-specific NADPH oxidase does reduce Q-1, but the physiological role of ubiquinone-10 in the phagocytic respiratory burst remains to be elucidated.

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REFERENCES

- Babior, B. M., Kipnes, R. S. & Curnutte, J. T. (1973) *J. Clin. Invest.* **52**, 741-744
- Babior, B. M., Curnutte, J. T. & McMurrich, B. J. (1976) *J. Clin. Invest.* **58**, 989-996
- Bellavite, P., della Bianca, V., Serra, M. C., Papini, E. & Rossi, F. (1984) *FEBS Lett.* **170**, 157-161
- Cohen, H. J., Chovanec, M. E. & Davies, W. A. (1980) *Blood* **55**, 355-363
- Crawford, D. R. & Schneider, D. L. (1982) *J. Biol. Chem.* **257**, 6662-6668
- Cross, A. R., Jones, O. T. G., Garcia, R. & Segal, A. W. (1983) *Biochem. J.* **216**, 765-768
- Cunningham, C. C., Dechatelet, L. R., Spach, P. I., Parce, J. W., Thomas, M. J., Lees, C. J. & Shirley, P. S. (1982) *Biochim. Biophys. Acta* **682**, 430-435
- Dechatelet, L. R., Shirley, R. S. & Johnston, R. B., Jr. (1976) *Blood* **47**, 545-551
- Drath, T. G. & Karnovsky, M. L. (1975) *J. Exp. Med.* **141**, 257-262
- Gabig, T. G. & Lefker, B. A. (1985) *J. Biol. Chem.* **260**, 3991-3995
- Green, T. R. & Schaefer, R. E. (1981) *Biochemistry* **20**, 7483-7487
- Kakinuma, K. & Kaneda, M. (1982) in *Biochemistry and Function of Phagocytes* (Rossi, W. & Patriarca, P., eds.), pp. 351-360, Plenum Press, New York
- Lutter, R., VanZwieten, R., Weening, R. S., Hammers, M. N. & Roos, D. (1984) *J. Biol. Chem.* **259**, 9603-9606
- Nakagawara, A. & Minakami, S. (1975) *Biochem. Biophys. Res. Commun.* **64**, 760-767

- Nakamura, M. & Baxter, C. R. (1981) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **40**, 1619
- Nakamura, M., Kurebayashi, H., & Yamazaki, I. (1978) *J. Biochem. (Tokyo)* **83**, 9–17
- Nakamura, M., Murakami, M., Umei, T. & Minakami, S. (1985) *FEBS Lett.* **186**, 215–218
- Patriarca, P., Cramer, R., Moncalvo, S., Rossi, F. & Romeo, D. (1971) *Arch. Biochem. Biophys.* **145**, 255–262
- Takeshige, K., Wakeyama, H. & Minakami, S. (1984) *Biochim. Biophys. Acta* **798**, 127–131
- Wakeyama, H., Takeshige, K. & Minakami, S. (1983) *Biochem. J.* **210**, 577–581

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