NADPH-dependent reduction of 2,6-dichlorophenol-indophenol by the phagocytic vesicles of pig polymorphonuclear leucocytes

Hiroshi WAKEYAMA, Koichiro TAKESHIGE and Shigeki MINAKAMI
Department of Biochemistry, Kyushu University School of Medicine, Fukuoka 812, Japan

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NADPH-dependent 2,6-dichlorophenol-indophenol (DCIP) reductase activity in the homogenate of phagocytosing pig polymorphonuclear leucocytes was twice that of the resting cells and the activity in the phagocytic vesicles corresponded to the activity increment due to phagocytosis. The apparent K_m value of the reductase activity in the vesicles for NADPH was $30\mu\text{M}$, which is similar to that of the NADPH-dependent superoxide (O_2^-) formation. Increasing the DCIP reductase activity by increasing the DCIP concentration caused a decrease in the O_2^- -forming activity, the NADPH oxidation rate being constant and independent of the dye concentration. *p*-Chloromercuribenzoate and cetyltrimethylammonium bromide at low concentrations inhibited the O_2^- -forming activity of the vesicles without inhibiting the DCIP reductase. Quinacrine inhibited both O_2^- formation and DCIP reduction. The DCIP reductase activity could be extracted with a mixture of deoxycholate and Tween-20, which extracts the O_2^- -forming activity. The reductase activity in the extract was enhanced 2-fold by the addition of FAD, and its apparent K_m was $0.085\mu\text{M}$. These results indicate that the NADPH-dependent DCIP reductase activity of the phagocytic vesicles is catalysed by a flavin-containing component of the O_2^- -forming system.

The respiratory burst of phagocytosing polymorphonuclear leucocytes is associated with the formation of superoxide anions (O_2^-). It is catalysed by an activation of O_2^- -forming oxidase (Babior, 1978; Newburger *et al.*, 1980), which is dormant in resting cells (DeWald *et al.*, 1979). The oxidase is considered to be membrane-bound (Briggs *et al.*, 1975; DeWald *et al.*, 1979) and to contain FAD (Babior & Kipnes, 1977). Association of a quinone reductase activity has been suggested for the NADH-dependent O_2^- -forming activity (Badwey & Karnovsky, 1980) and for the NADPH-dependent one (Green & Schaefer, 1981), but the preparation solubilized by Triton X-100 is reported to lack the dye reductase activity (Babior & Peters, 1981). Involvement of a *b*-type cytochrome (Segal & Jones, 1979) and ubiquinone (Crawford & Schneider, 1982) in the system has also been suggested.

We have previously reported the preparation of phagocytic vesicles with high NADPH-dependent O_2^- -forming activity and extraction of the activity with a mixture of deoxycholate and Tween-20 (Wakeyama *et al.*, 1982). In the present study we

characterized the NADPH-dependent DCIP reductase activity of the vesicles and examined the relation between the dye-reducing activity and the O_2^- -forming activity. The DCIP reductase activity seems to be catalysed by a flavin-containing component of the O_2^- -forming system.

Experimental

Isolation of phagocytic vesicles

Pig polymorphonuclear leucocytes and phagocytic vesicles were prepared as described previously (Wakeyama *et al.*, 1982).

Assay procedures

DCIP reductase activity was measured in 1 ml of a reaction mixture consisting of 0.1 mM-DCIP, 25 EC units of superoxide dismutase (Fe-type), 0.25 mM-NADPH (or 1.5 mM-NADH), 1 mM-KCN and 50 mM-potassium phosphate buffer, pH 7.5. We added KCN to inhibit peroxidase, which will reoxidize the reduced dye, and superoxide dismutase (Fe-type) to scavenge superoxide, which, in the presence of KCN, reduces the dye. The assay was started by the addition of the vesicle samples, and the absorbance change ($A_{600} - A_{800}$) was

Abbreviation used: DCIP, 2,6-dichlorophenol-indophenol.

measured at 20°C on a Hitachi 557 dual-wavelength spectrophotometer equipped with a recorder. The reductase activity was calculated from the initial velocity of the reduction by using a molar absorption coefficient of $21 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$ and was corrected for by subtracting the non-enzymic rate of reduction of DCIP, obtained by omission of the leucocyte-vesicle sample. The ferricyanide-reductase activity was measured similarly by replacing DCIP by 0.75 mM-ferricyanide and measuring the absorbance change $A_{420} - A_{500}$.

The NADPH-dependent O_2^- -forming activity was measured by the absorbance change $A_{550} - A_{540}$ in a reaction mixture consisting of 50 μM -cytochrome *c*, 0.25 mM-NADPH and 50 mM-potassium phosphate buffer, pH 7.5, at 20°C. The oxidation of NADPH was measured at 340 nm in the above reaction mixture at 20°C by omitting cytochrome *c* and decreasing NADPH concentration to 0.125 mM. Protein was determined by the method of Lowry *et al.* (1951) after the oil was removed by centrifugation at 250 *g* for 10 min.

Reagents

Superoxide dismutase (Fe-type) was prepared from *Escherichia coli* by the method of Yost & Fridovich (1973). Ferricytochrome *c* (type III) was obtained from Sigma Chemical Co. St. Louis, MO, U.S.A. Iothalamic acid (Conray) was generously given by the Daiichi Pure Chemical Co., Tokyo, Japan. DCIP, potassium ferricyanide, cetyltrimethylammonium bromide and quinacrine hydrochloride were purchased from Nakarai Chemicals, Kyoto, Japan. *p*-Chloromercuribenzoate and Tween-20 were obtained from Wako Pure Chemical Industries, Osaka, Japan. Deoxycholate was from Difco Laboratories, Detroit, MI, U.S.A. FAD was purchased from Wakamoto Chemicals, Tokyo, Japan. NADPH and NADH were obtained from Oriental Yeast Co., Tokyo, Japan. Other reagents were of analytical grade.

Results

Dye reductase activities in homogenate

When homogenates of pig polymorphonuclear leucocytes, both resting and phagocytosing oil droplets, were compared for their activities of NADPH:DCIP reductase and NADPH:ferricyanide reductase, the activities in the homogenate of the phagocytosing cells were about twice those of the resting cells (Table 1). Phagocytosis caused about 30% activation of the NADH:DCIP reductase activity. The enhancement of the dye reductase activities was not so large as that of the O_2^- formation (40-fold; Wakeyama *et al.*, 1982), because various NAD(P)H dehydrogenases exist in the cells.

Dye reductase activities in subcellular fractions

Distribution of the reductase activities in homogenates of the resting and the phagocytosing cells is shown in Table 2. About 40% of the NADPH-

Table 1. *Dye reductase activities in homogenates of resting and phagocytosing pig polymorphonuclear leucocytes*

Leucocytes were incubated for 5 min at 37°C with (phagocytosing) or without (resting) oil droplets in the presence of 1 mM-KCN and homogenized in 0.34 M-sucrose buffered with 5 mM-Hepes, pH 7.5, at 4°C. Values represent the means $\pm$ S.D. for four independent experiments.

	Specific activity (nmol of electrons/min per mg of protein)	
	Resting	Phagocytosing
NADPH:DCIP reductase	15.2 $\pm$ 1.2	34.0 $\pm$ 1.2
NADH:DCIP reductase	30.9 $\pm$ 2.0	42.0 $\pm$ 2.8
NADPH:ferricyanide reductase	19.9 $\pm$ 1.1	40.8 $\pm$ 1.9

Table 2. *Dye reductase activities in subcellular fractions of pig leucocytes*

The cells were incubated as described in Table 1. Three fractions were obtained by centrifugation at 76 500 *g* for 1 h at 4°C: one floating (vesicle), one remaining in 0.34 M-sucrose (cytosol) and one sedimenting at the bottom (sediment). Values represent the means $\pm$ S.D. for four independent experiments. Values are expressed as nmol of electrons/min; specific activities (activity/mg of protein) are given in parentheses.

	Activity				
	Resting		Phagocytosing		
	Sediment	Cytosol	Vesicle	Sediment	Cytosol
NADPH:DCIP reductase	727 $\pm$ 39 (32 $\pm$ 2)	202 $\pm$ 23 (5.4 $\pm$ 0.4)	693 $\pm$ 37 (65 $\pm$ 4)	861 $\pm$ 42 (47 $\pm$ 3)	228 $\pm$ 21 (7.2 $\pm$ 0.4)
NADH:DCIP reductase	1560 $\pm$ 76 (64 $\pm$ 3)	390 $\pm$ 20 (11 $\pm$ 1.2)	283 $\pm$ 19 (23 $\pm$ 1)	1560 $\pm$ 82 (93 $\pm$ 4)	537 $\pm$ 24 (17 $\pm$ 1.4)
NADPH:ferricyanide reductase	975 $\pm$ 39 (41 $\pm$ 2)	251 $\pm$ 14 (7 $\pm$ 1)	980 $\pm$ 32 (91 $\pm$ 4)	1020 $\pm$ 44 (59 $\pm$ 3)	264 $\pm$ 15 (9 $\pm$ 1)

dependent reductase activities in the homogenate of the phagocytosing cells were recovered in the vesicle fraction, hence the activities in the fraction roughly correspond to the activity increments due to phagocytosis. The activities in the vesicle fraction are neither those of mitochondria (Hatefi & Hanstein, 1973) nor those of endoplasmic reticulum (Williams & Kamin, 1962; Masters *et al.*, 1965), because contamination by mitochondrial and microsomal marker enzymes in the fraction are negligible (Wakeyama *et al.*, 1982). The activities of the dye reductases in the homogenate of the resting cells were mostly found in the sediment fraction, which contains both mitochondrial and endoplasmic reticulum. The phagocytic-vesicle fraction was used in subsequent studies.

Properties of the dye reductase activity in the vesicles

The optimal pH for activity was 7.5, which agrees with that for the O_2^- -forming activity. Apparent K_m values of the DCIP reductase and ferricyanide reductase activities for NADPH were $25\mu M$ and $104\mu M$, and the values for NADH were 0.56 mM and 1.75 mM respectively. These properties of the dye reductase activities were similar to those of the O_2^- -forming activity, which has K_m values of $35\mu M$ for NADPH and 0.4 mM for NADH (Wakeyama *et al.*, 1982). The reductase activities were extremely labile, and the half-lives of the reductase activities were about 15 min at 20°C in 0.34 M -sucrose buffered with 5 mM -Hepes [4-(2-hydroxymethyl)-1-piperazine-ethanesulphonic acid], pH 7.5.

Quinacrine, an analogue of flavin, non-competitively inhibited both O_2^- -forming and DCIP reductase activities, as shown in Fig. 1. The K_i values for both activities were essentially the same ($800\mu M$), suggesting that a flavoprotein is involved in both reactions. Similar inhibition was observed for ferricyanide reductase activity.

Stoichiometry of electron transfer

DCIP inhibited O_2^- formation, as reported by Green & Schaefer (1981). As shown in Table 3, an increase in DCIP concentration caused an increase in the rate of reduction of DCIP and a decrease in that of O_2^- . The sum of the electrons transferred to both DCIP and O_2^- equalled the electrons released by NADPH oxidation, measured separately, and was independent of the DCIP concentration. These stoichiometric relations in the vesicle fraction indicate that the reduction of DCIP is catalysed by the O_2^- -forming system and no other NADPH-oxidizing systems exist in the vesicles. O_2^- was formed even at high concentration of DCIP (0.1 mM).

Fig. 1. Inhibition of the DCIP reductase and O_2^- -forming activities of the vesicles by quinacrine

The NADPH-dependent DCIP reductase (○, ●) and O_2^- -forming (□, ■) activities were measured at various concentrations of quinacrine and expressed as nmol of electrons/min per mg of protein. The concentrations of NADPH were 0.25 mM (open symbols) and 0.1 mM (closed symbols). Each point represents the mean result for three experiments.

Table 3. Stoichiometry of electron-transport activities in the vesicles of pig leucocytes

Rates of the NADPH-dependent reduction of O_2^- and DCIP by the vesicles of pig leucocytes were measured and the sum of the electrons donated to both acceptors was compared with the electrons released by the oxidation of NADPH. Values are the means $\pm$ S.D. for four experiments.

[DCIP] (μM)	Electron flow (nmol of electrons/min per mg of protein)			
	To O_2^- (a)	To DCIP (b)	Sum (a + b)	From NADPH
0	87 ± 3	—	87 ± 3	90 ± 4
8	78 ± 5	12 ± 4	90 ± 5	84 ± 3
19.5	61 ± 3	30 ± 6	90 ± 4	82 ± 5
33	43 ± 2	44 ± 6	87 ± 3	92 ± 5
54	35 ± 1	50 ± 4	85 ± 3	92 ± 3
72	27 ± 2	58 ± 6	85 ± 2	98 ± 6
108	24 ± 1	64 ± 4	88 ± 2	88 ± 4

Differential inhibition of dye reductase and O_2^- -forming activities

p-Chloromercuribenzoate inhibited 50% of the O_2^- -formation at about $4\mu M$ without inhibiting the reduction of DCIP and ferricyanide. O_2^- formation was completely inhibited by $20\mu M$ -*p*-chloromer-

Fig. 2. Differential inhibition of the dye reductase and O_2^- -forming activities

The NADPH-dependent activities of DCIP reduction (O), ferricyanide reduction (Δ) and O_2^- formation ($\square$) were measured at various concentrations of *p*-chloromercuribenzoate (a) and cetyltrimethylammonium bromide (b). Control activities (nmol of electron/min per mg of protein) were 64.8 for DCIP reductase, 88.9 for ferricyanide reductase and 85.7 for O_2^- formation. Each point represents the mean result for four experiments.

curibenzoate, whereas more than half of the reductase activities remained at this concentration of inhibitor (Fig. 2a). A similar differential inhibition

Table 4. NADPH-linked electron-transport activities in a detergent extract of pig polymorphonuclear leucocytes

A mixture containing the vesicle fraction (1 mg of protein/ml), 0.5% (v/v) Tween-20, 0.5% (w/v) deoxycholate and 25% (v/v) ethylene glycol in 50 mM-glycine buffer, pH 8.5, was kept in ice for 30 min and centrifuged at 117000*g* for 1 h at 4°C. The activities of the NADPH-dependent O_2^- formation and DCIP reduction by the vesicle fraction were 84.1 and 62.4 nmol of electrons/min per mg of protein.

Reaction	Specific activity (nmol of electrons/min per mg of protein)
NADPH-dependent O_2^- formation	978 $\pm$ 44
NADPH:DCIP reductase	764 $\pm$ 32
+ 1 μM -FAD	1626 $\pm$ 56
NADPH:ferricyanide reductase	1051 $\pm$ 63
NADPH oxidation	980 $\pm$ 44

was observed with cetyltrimethylammonium bromide, a cationic detergent, which inhibited 50% of the O_2^- formation at about 0.01% detergent concentration without inhibiting the reductase activities. O_2^- formation was completely inhibited at 0.1% detergent, whereas more than half of the reductase activities remained at that concentration (Fig. 2b).

Extraction of the dye-reductase activities

The NADPH-dependent DCIP and ferricyanide reductase activities could be extracted with a mixture of deoxycholate and Tween-20. The activities were enhanced about 1.7-fold by the treatment with the detergent mixture, and about 84% ($n=3$) of the activities were extracted by repeating the extraction four times, in agreement with the results for the extraction of the O_2^- -forming activity (Wakeyama *et al.*, 1982). The specific activities of the dye reductases increased more than 10-fold on extraction with a mixture of deoxycholate and Tween 20, as was the case for the O_2^- -forming activity. When the activities were expressed as mol of electrons transferred/min, the ferricyanide reductase activity was essentially the same as the O_2^- -forming and the NADPH oxidase activities, and the DCIP reductase showed about 80% of this activity (Table 4). The lower DCIP reductase activity is explained by the observation that some O_2^- formation remains in the presence of DCIP used for the assay (Table 3). The DCIP reductase activity of the extract was enhanced about 2-fold by 1 μM -FAD, as in case of the O_2^- -forming activity (Wakeyama *et al.*, 1982), with an apparent K_m value for FAD of 0.085 μM . The DCIP reductase activity of the vesicles was not enhanced by FAD.

Discussion

The results reported in the present paper show that the NADPH-dependent DCIP- and O_2 -reducing activities of the phagocytic vesicles have the following common properties. (1) Both activities in the homogenate were enhanced by phagocytosis and the increased activities were found in the vesicles. (2) Properties such as optimal pH and K_m values for NADPH and NADH were essentially the same. (3) Quinacrine, a flavin analogue, inhibited both activities with essentially the same K_i value. (4) Both activities could be extracted by a detergent mixture and the specific activities of both enzymes were essentially the same. (5) The activities of the extract were enhanced by FAD with similar K_m values. Moreover, a stoichiometric study has shown that the sum of the electrons transferred to DCIP and O_2 agreed with the amount of electrons released from NADPH. These observations strongly suggest that the NADPH-dependent reduction of DCIP by the vesicles is catalysed by the O_2 -forming system responsible for the respiratory burst of the cells.

Some differences in properties, however, exist between the reduction of dyes and O_2 . *p*-Chloro-mercuribenzoate and cetyltrimethylammonium bromide inhibited the O_2 -formation without inhibiting the reduction of dyes, suggesting that the former reaction is catalysed by a system consisting of more than two components and that the dyes accept electrons from a component containing FAD. Although the possibility that a single enzyme reduces O_2 and dyes at different reaction sites cannot be ruled out, we may speculate that a flavoprotein reduces both dyes and a *b*-type cytochrome proposed by Gabig *et al.* (1982) and Cross *et al.* (1982), and the reduced cytochrome donates electrons to O_2 , thus forming O_2^- .

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