Intrinsic Dichlorophenolindophenol Reductase Activity Associated with the Superoxide-Generating Oxidoreductase of Human Granulocytes†

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ABSTRACT: NADPH-dependent dichlorophenolindophenol (DCIP) reductase activity cosediments with NADPH-dependent O_2^--generating activity in subcellular particulate fractions of zymosan-stimulated human polymorphonuclear leukocytes (PMN's). Subcellular fractions derived from unstimulated PMN's were devoid of both activities, as were fractions from zymosan-stimulated PMN's of a patient known to have chronic granulomatous disease. NADPH-dependent DCIP reduction associated with the oxidoreductase-rich subcellular fractions was unaffected by addition of excess superoxide dismutase sufficient to abolish all traces of O_2^- production as measured by conversion of ferricytochrome *c* to its ferrous form. Moreover, DCIP inhibited NADPH-dependent production of O_2^- in subcellular fractions derived from normal donors. In contrast to the subcellular studies, whole cell suspensions were ineffective in reducing extracellular DCIP despite their capacity to generate O_2^-, albeit at a lesser rate, in the presence of this electron-accepting dye. These results demonstrate that DCIP reductase activity is associated with the oxidoreductase complex and suggest that it is located on the inner side of the PMN's plasma membrane. The stability of the oxidoreductase complex is markedly improved by storage in glycerol. Both overall O_2^--generating activity and DCIP reductase activity exhibit a similar pH optimum of 7.0. The K_m of the oxidoreductase complex for DCIP is 33 μM.

Superoxide (O_2^-)[1] formation is an essential component in the armada of complex reactions employed by phagocytes in effecting bacterial killing. In human polymorphonuclear leukocytes (PMN's) its formation is catalyzed by a membrane-bound NAD(P)H-dependent O_2^--generating oxidoreductase complex (Badwey & Karnovsky, 1980; Babior, 1978). Several indirect lines of evidence suggest that a *b* type cytochrome may be a component of the oxidoreductase complex (Segal & Jones, 1978–1980; Millard et al., 1979). Cytochrome *b* type redox systems are generally associated with quinone reductases (Von Jagow & Sebald, 1980; Crane, 1977). This property and recent studies indicating that a flavoprotein is also a component of the oxidoreductase complex (Light et al., 1981; Gabig & Babior, 1979) suggest that it should be feasible to detect intrinsic quinone reductase activity in the oxidoreductase complex.

We wish to report that the NADPH-dependent O_2^--generating oxidoreductase does contain intrinsic quinone (DCIP) reductase type activity as assessed by the electron-accepting dye dichlorophenolindophenol (DCIP). We have not ascertained whether the DCIP reductase type activity associated with the oxidoreductase is a true quinone reductase like that associated with *b* type cytochromes or whether this activity is associated with the flavoprotein component of the oxidoreductase. Badwey & Karnovsky (1980) have recently noted the discovery of quinone reductase activity associated with an NADH-dependent O_2^--generating oxidoreductase derived from human PMN's whereas Babior & Peters (1981) have suggested that the NADPH-dependent oxidoreductase lacks quinone reductase type activity from studies on partially purified extracts of the oxidoreductase. We present here evidence which establishes that the DCIP reductase component is involved in the redox reactions culminating in O_2^- production. Some of the basic properties of the DCIP reductase are also presented along with information on stabilization of the oxidoreductase in crude subcellular extracts recovered from activated cells. The purity of our enzyme extracts is essentially the same as that of Light et al. (1981) and Babior & Peters (1981) except the oxidoreductase-rich fractions in this study were not disrupted by detergents before assessment of enzyme activity.

Materials and Methods

Materials. Ferricytochrome *c*, superoxide dismutase, NADPH, concanavalin A (Con A), zymosan, and DCIP were all obtained from Sigma Chemical Co., St. Louis, MO. Sodium diatrizoate–ficoll was purchased from Bionetics Laboratory Products, Kensington, MD. All other chemicals were of the best grade available.

Isolation of PMN's. Purified PMN's were isolated from whole blood by brief centrifugation, collection of the buffy coat, and subsequent centrifugation on diatrizoate–ficoll density gradients as described elsewhere (Green et al., 1980). The final cell pellets were resuspended in Hank's buffered saline solution (HBSS), pH 7.4, and counted on a Coulter particle counter.

Preparation of PMN Stimulants. Stock Con A solution was made up to 1.5 mg/mL in distilled water. Zymosan was boiled in water and suspended in 0.02% NaN_3 to a final concentration of 20 mg/mL (dry weight). Serum-treated zymosan (STZ) was prepared fresh by gently agitating 2 mg of zymosan with 17% autologous serum in HBSS in a final volume of 1.3 mL at 37 °C for 10 min immediately before stimulation of cells.

Preparation of Subcellular Particulate Fractions. PMN's from individual donors were suspended in 1 mL of HBSS, and the cell suspensions were divided into two 0.5-ml aliquots. To one aliquot was added 1.3 mL of STZ suspension. As a control, to the second aliquot of cells was added 1.2 mL of 17%

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[1] Abbreviations: CGD, chronic granulomatous disease; Con A, concanavalin A; cyt *c*, cytochrome *c*; DCIP, dichlorophenolindophenol; HBSS, Hank's buffered saline solution; PBS, phosphate-buffered saline; PMN's, polymorphonuclear leukocytes; STZ, serum-treated zymosan; O_2^-, superoxide.

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autologous serum in HBSS. The cells were then centrifuged at 400*g* for 5 min. After removal of the cell-free supernatant, the cell pellets were incubated for 5 min at 37 °C. The STZ-fed and unfed cells were then pooled separately in 0.34 M sucrose made up in 131 mM NaCl and 8 mM sodium potassium phosphate buffer, pH 7.0 (PBS–sucrose buffer) and lysed by sonication. Cell lysis was confirmed by light microscopy. Crude cell lysates were subfractionated by differential centrifugation as noted in the text. Particulate fractions recovered from the lysates were routinely resuspended in PBS–sucrose buffer, pH 7.0, at a final concentration of 2×10^8 cell equiv/mL and stored on ice until assayed.

Measurement of O_2^--Generating Oxidoreductase Activity. O_2^- formation was quantitated by modification of the cytochrome *c* assay of McCord et al. (1973). All assays were conducted on a continuous recording double-beam Cary 219 spectrophotometer at 550 nm. Whole cell assays were conducted at room temperature in HBSS, pH 7.4, made up in cytochalasin B (4.6 μg/mL) in the presence or absence of Con A (104 μg/mL) and in the presence of ferricytochrome *c* (63 μM). Superoxide dismutase (182 units/mL) was included in the reference cuvette. Approximately 4×10^6 PMN's were added to the sample and reference cuvettes, and O_2^--generating oxidoreductase activity was then initiated by addition of Con A to both cell suspensions. The total reaction volume was 2.6 mL.

Subcellular oxidoreductase activity was measured in a similar fashion except PBS–sucrose buffer, pH 7.0, containing also 0.5 mM NaCN was used in place of HBSS and Con A and cytochalasin B were omitted from the reaction mixture. NADPH (final concentration 0.1 mM) was used to initiate oxidoreductase activity following addition of 10–20 μL of enzyme to both reference and sample cuvettes. The total reaction volume was 2.4 mL.

Whole cell and subcellular particulate assays for O_2^--generating oxidoreductase activity in the presence of DCIP were conducted in the same manner at 550 nm as described above, respectively, except DCIP (final concentration 0.1 mM) was also included in the assay mixture.

Measurement of DCIP Reductase Activity. The capacity of whole cells to express extracellular DCIP reductase activity upon stimulation by Con A was assessed as follows: Cells (4×10^6 PMN's) were suspended in the sample and reference cuvettes of the Cary 219 spectrophotometer in HBSS, pH 7.4, containing DCIP (final concentration 0.1 mM). Con A (104 μg/mL) was included in the *reference* cuvette to activate the cells. The total reaction volume was 2.4 mL. Reductase activity was assessed at 600 nm by continuous recording of the absorbance change induced with inclusion of Con A in the reference cuvette.

Subcellular DCIP reductase activity was measured as follows: DCIP (final concentration 0.1 mM) was premixed in PBS–sucrose buffer, pH 7.0. Immediately before the assay, NADPH (final concentration 0.1 mM) was added to the mixture, and 3 mL of this mixture was then transferred to the sample and reference cuvettes. Following a 10–15-s trace recording to ensure a stable base line, approximately 20 μL of enzyme was transferred to the *reference* cuvette. Reductase activity assessed at 600 nm appeared as a positive increase in absorbance, reflecting enzymatic reduction (bleaching) of DCIP in the reference cuvette. No change in the absorbance of DCIP occurred when NADPH was omitted from the reaction mixture.

pH Optima Studies. The pH optimum for NADPH-dependent O_2^--generating and DCIP reductase activities was ascertained by conducting the assays as described above, except in PBS–sucrose buffers adjusted to the pH values noted in the text. pH corrections were made in the molar absorptivity coefficients for cytochrome *c* and DCIP by measuring the relative change in absorbance of each at the specific pHs used in evaluating enzyme activity to that at pH 7.0. Corrected absorptivity coefficients were then obtained for calculation of the enzyme activity at varying pH values by using reference millimolar absorptivity coefficients of 21.1 and 16.1 mM^{-1} cm^{-1} for cytochrome *c* and DCIP, respectively, at pH 7.0 (Van Gelder & Slater, 1962; Dawson et al., 1969).

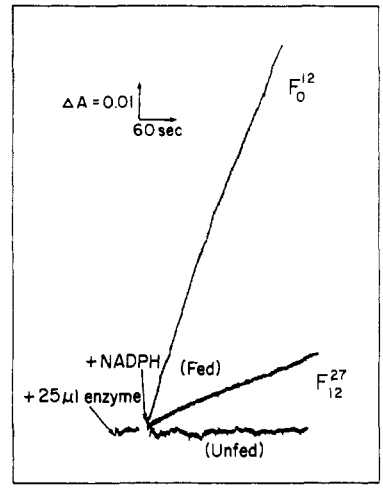

FIGURE 1: Spectrophotometric tracings illustrating NADPH-dependent O_2^--generating activity as measured by the cytochrome *c* assay in subcellular F_0^{12} and F_{12}^{27} fractions (see text) derived from STZ-fed PMN's (upper two curves) and unfed controls (lower curve). Only the F_{12}^{27} fraction derived from the unfed control is shown in the lower curve to contrast experimental and control preparations. The F_0^{12} fraction derived from unfed PMN's was also devoid of activity.

Protein Measurements. Protein was determined by the method of Lowry et al. (1951).

Results

Figure 1 contrasts NADPH-dependent O_2^--generating oxidoreductase activity seen in the 12000*g* (F_0^{12}) and 12000–27000*g* (F_{12}^{27}) particulate fractions recovered from STZ-fed and unfed PMN's. Approximately 80% of the total activity recovered in the particulate fractions sedimented at less than 12000*g* following 10-min centrifugation. Neither the F_0^{12} nor the F_{12}^{27} fractions recovered from unfed cells exhibited any oxidoreductase activity. For routine isolation of oxidoreductase-rich fractions, we found it convenient to subject the crude lysates to a low-speed 400*g* centrifugation step for 10 min to remove contaminating STZ particles before subsequent differential centrifugation of the remaining supernatants. This step did not appreciably alter the yield of oxidoreductase recovered in the F_0^{12} and F_{12}^{27} fractions. The specific activity in terms of O_2^--generating activity for these two subcellular fractions averaged 20 and 28 nmol min^{-1} (mg of protein)$^{-1}$, respectively.

NADPH-dependent DCIP reductase activity was present in the same proportions in these particulate fractions whereas it was absent in fractions prepared from unfed PMN's. Figure 2 contrasts activity in the F_0^{12} fraction recovered from STZ-fed cells to the absence of any apparent activity in the F_0^{12} fraction recovered from the same lot of cells unexposed to STZ.

Inclusion of 0.1 mM DCIP in the cytochrome *c* assay for O_2^- resulted in approximately a 3-fold decrease in O_2^- production (Figure 3). Whole cell suspensions activated by Con A were ineffective in reducing DCIP despite their obvious

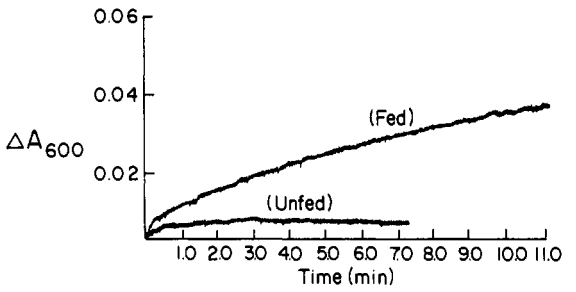

FIGURE 2: Spectrophotometric tracings of NADPH-dependent DCIP reductase activity measured in the F_0^{12} particulate fractions recovered from STZ-fed (upper curve) and unfed (lower curve) PMN's. Assays were conducted on the same lot of cells divided into equal aliquots for preparation of STZ-fed and unfed control F_0^{12} fractions as described under Materials and Methods. The final pellets were resuspended to a final concentration of 2×10^8 cell equiv/mL in PBS–sucrose, pH 7.0.

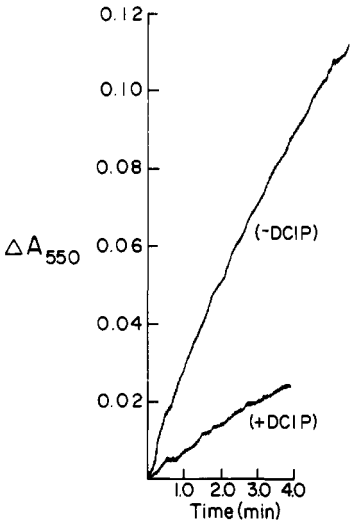

FIGURE 3: Effect of DCIP on subcellular NADPH-dependent O_2^--generating oxidoreductase activity. DCIP (0.1 mM) was excluded (–DCIP) or included (+DCIP) in the standard cytochrome *c* assay for O_2^- as described under Materials and Methods. The assay was initiated by addition of 20 μL of F_0^{12} from STZ-fed PMN's to both cuvettes in the Cary 219 double-beam spectrophotometer.

capacity to express O_2^--generating activity under the same experimental conditions (Figure 4). In these experiments, we sought specifically to assess the capacity of whole cells to reduce DCIP from the extracellular side of their plasmalemma by maintaining an equivalent number of cells suspended in both the reference and sample cuvettes. This allowed us to blank out intracellular DCIP reduction occurring with uptake of DCIP into the cell. Con A was used in place of STZ to minimize light scattering caused with inclusion of STZ in the assay mixture, thus enabling us to make direct spectroscopic measurements of O_2^--generating and DCIP reductase activities in the whole cell suspensions. Despite no apparent reduction of DCIP from the external side of the cell, the apparent rate of O_2^- production in the whole cell suspensions decreased to 44% of that seen in the absence of DCIP. These latter results were thus in reasonable accord with our observations on the effect of DCIP in suppressing NADPH-dependent O_2^- production in the subcellular F_0^{12} fractions as cited above.

The molar ratio of NADPH-dependent DCIP reductase activity to O_2^- production at pH 7.0 in PBS–sucrose buffer containing 0.5 mM NaCN was approximately 0.70 ($n = 8$). Figure 5 demonstrates that the overall pH optimum in PBS–sucrose buffer supplemented with 0.5 mM NaCN for O_2^- production is much broader than that for DCIP reductase activity, although both activities exhibited a pH optimum near

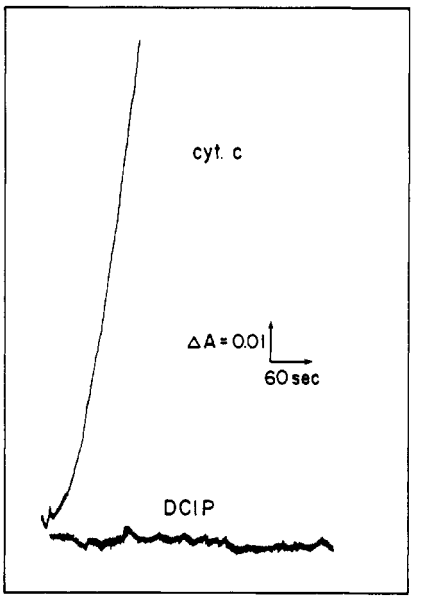

FIGURE 4: Spectrophotometric tracings of Con A induced O_2^- production and extracellular DCIP reduction (none present) expressed in whole cell suspensions (4×10^6 PMN's) in HBSS, pH 7.4. Cytochrome *c* reduction (O_2^--generating activity) was measured at 550 nm, and extracellular DCIP reductase activity was measured at 600 nm as described under Materials and Methods.

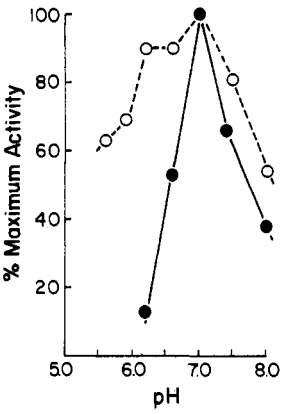

FIGURE 5: pH optimum of NADPH-dependent O_2^--generating (○) and DCIP reductase (●) activities assessed in PBS–sucrose buffers with oxidoreductase-rich F_0^{12} derived from STZ-fed cells as described in the text. Each point is the average of duplicate assays.

7.0 under the conditions of these assays. Cyanide was included in the assay buffers to inhibit peroxidase activity present as a contaminant in the crude enzyme extracts. Without cyanide in the assay buffers, we observed less apparent DCIP reductase activity. Further examination revealed that this was caused by peroxidase-catalyzed reoxidation of reduced DCIP apparently caused by concomitant formation of H_2O_2 through dismutation of O_2^- formed in situ upon addition of NADPH to the enzyme mixtures. Even at pH 7.0, both activities were extremely labile when stored at room temperature in PBS–sucrose buffer (Figure 6). Although DCIP reductase activity was initially more labile than O_2^--generating activity, after the first 30 min, the decay curves of both enzyme activities appeared to parallel one another. Approximately 65% of the initial O_2^--generating activity was lost during the first 100-min storage at room temperature compared to approximately 90% loss of DCIP reductase activity. On the other hand, both activities were markedly stabilized by glycerol, with no detectable loss in enzyme activity under the same conditions when the PBS buffer was supplemented with 15% glycerol in place of sucrose over the first 100-min storage at room temperature.

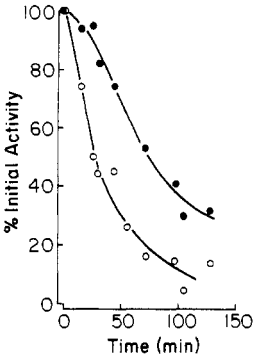

FIGURE 6: Colability studies on storage of oxidoreductase-rich F_0[12] fractions at room temperature in PBS–sucrose, pH 7.0. NADPH-dependent O_2^--generating activity (●); NADPH-dependent DCIP reductase activity (○). Both activities were completely stable at room temperature when stored in 15% glycerol in PBS, pH 7.0, as described in the text (data not shown).

Table I: Detection of NADPH-Dependent O_2^--Generating and DCIP Activities in Whole Cell and Subcellular Fractions of a Normal and CGD Donor

	oxidoreductase act. (nmol/min)	
expt	O_2^- detected	DCIP reduced
I, whole cells, normal donor (4×10^6 PMN's)	9.6[a]	0.0[a]
II, 20 μL of F_0[12], normal donor	4.9	2.8
III, 20 μL of F_0[12], CGD donor	0.0	0.0

[a] Stimulated with Con A (125 μg/mL) in HBSS, pH 7.4, at room temperature. Whole cells of the CGD donor were devoid of oxidoreductase activity. Assays were conducted as described under Materials and Methods. In whole cell assays, only extracellular DCIP reduction was assessed as described in the text.

There was no difference in the measureable rate of NADPH-dependent reductase activity associated with the oxidoreductase-rich subcellular fractions whether superoxide dismutase (364 units) was included or omitted from assay mixtures. The quantity of superoxide dismutase employed in these experiments was sufficient to completely abolish detection of O_2^- under similar assay conditions when ferricytochrome *c* was used in place of DCIP to assess O_2^- production. Thus the mechanism of DCIP reduction was not secondary to O_2^- formation. The K_m of the NADPH-dependent DCIP reductase assayed at pH 7.0 in PBS–sucrose buffer containing 0.5 mM NaCN was approximately 33 μM for DCIP by Lineweaver–Burk double-reciprocal plots.

Lastly, we observed a concomitant absence of both NADPH-dependent DCIP reductase and O_2^--generating activities in fractions recovered from STZ-fed PMN's of a patient known to have chronic granulomatous disease (CGD) in opposition to that of normal PMN donors. Table I contrasts the relationship between whole cell and subcellular O_2^--generating oxidoreductase and DCIP reductase activity and compares typical findings in cells derived from a normal donor with those of the patient with CGD examined in this study.

Discussion

The biochemical defect in CGD is not known, but functionally there is a complete absence of O_2 uptake and concomitant O_2^- production when various stimulants known to elicit a "burst response" in normal PMN's are presented to PMN's of donors with CGD (Babior, 1978). This defect is believed to be caused by an inborn error caused by chromosome lesions in the gene (or genes) responsible for assembly and/or expression of native oxidoreductase activity (Borregaard et al., 1979; Cheson et al., 1977; Quie et al., 1968). Hence, it is of considerable significance that not only O_2^--generating activity was absent from the particulate fractions of the CGD donor, as expected, but also DCIP reductase activity was absent. These results imply that the gene(s) responsible for normal expression of oxidoreductase activity must also be involved in controlling DCIP reductase activity. The simplest explanation of these findings is that both activities are biochemically associated with the same multienzyme complex. That DCIP reductase activity is a component of the oxidoreductase is further supported by the data demonstrating (i) that NADPH-dependent O_2^--generating and DCIP reductase activities cosediment and are distributed to the same extent in the same subcellular fractions, (ii) that both activites are expressed in subcellular fractions recovered from stimulated but not unstimulated PMN's, (iii) that DCIP inhibits the initial rate of O_2^- production in a manner consistent with the concept that it competes for electrons destined for transfer to O_2 (cf. Figure 3), and (iv) that both activities exhibit similar lability profiles at room temperature in PBS–sucrose buffer while both are stabilized by PBS supplemented with glycerol. It is also noteworthy that the catalytic activities of each are approximately equivalent.

The marked difference in the pH profiles illustrated in Figure 5 for overall O_2^--generating activity compared to DCIP reductase activity, and the initially more rapid loss of DCIP reductase activity in specimens stored at room temperature in the absence of glycerol (cf. Figure 6), indicates that there must be at least two catalytic steps involved in shuttling electrons between NADPH and O_2. From the data in Figure 6, it is clear that the catalytic site for DCIP reduction is not the rate-limiting step in overall O_2^- production from NADPH. The decay rate of the reductase initially exceeds that of overall O_2^- production. The parallel decay curves seen later on in Figure 6 following initially large losses of DCIP reductase activity most likely reflect the fact that the reductase has become the rate-limiting step in overall O_2^- production.

The fact that the apparent molar ratio of DCIP reductase activity to O_2^- production is approximately 0.7 is not in opposition to the conclusion that the DCIP reductase does not initially limit the rate of O_2^- production. DCIP reduction requires two electrons whereas conversion of O_2 to O_2^- requires only a one-electron transfer. Therefore, in terms of electron flux, the ratio of DCIP reduction to O_2^- formation is 1.4, indicating that the shunting of electrons to DCIP is in excess of that to O_2. It should be borne in mind, however, that artificial electron-accepting dyes such as DCIP are at best analogue substrates which may or may not reflect optimal catalytic activity. They are useful in measuring relative changes in the apparent activity. Limitations in attempting to interpret stochiometric turnover rates with artificial electron-accepting dyes have been reviewed elsewhere (Crane, 1977). Nevertheless, the apparent excess rate of DCIP reduction over O_2^- production in terms of electron flux, the initially distinct lability curves of DCIP reductase activity compared to O_2^--generating activity, differences in the pH activity profiles, and, last, the failure of superoxide dismutase to abolish NADPH-dependent DCIP reduction all indicate that this latter activity is intrinsic to the oxidoreductase complex and not a consequence of O_2^- production.

The inability of whole cells to reduce extracellular DCIP, and the full expression of NADPH-dependent activity in particulate fractions, demonstrates that the DCIP reductase

component of the oxidoreductase complex is only accessible to experimental manipulation from the cytosolic side of the PMN's plasmalemma. This suggests that the reductase component measured in these experiments is asymmetrically positioned in the plasmalemma to interact with substrate from the cytosolic side of the membrane. The decreased rate of O_2^- production seen with exposure of intact cells to DCIP most likely reflects the fact that DCIP can still cross the cell membrane where it can divert electrons otherwise destined for O_2 by interacting directly with the oxidoreductase complex at a redox site preceding reduction of O_2. The decreased rate of O_2^- production may also reflect, in part, intracellular depletion of NADPH required by the oxidoreductase for production of O_2^-. It is well-known that electron-accepting dyes such as DCIP can deplete cells of intracellular NADPH (Babior, 1978; Cheson et al., 1977; Brin & Yonemoto, 1958). Nevertheless, the fact that DCIP inhibits O_2^- production in subcellular fractions establishes that the mechanism of inhibition cannot be solely attributed to depletion of NADPH required by the oxidoreductase for activity.

The stabilization of the oxidoreductase complex by glycerol should allow further fractionation of its components. This has heretofore not been feasible because of the extreme lability of this multienzyme complex (Light et al., 1981; Babior & Peters, 1981). Studies are currently underway in solubilizing and further fractionating the components of the oxidoreductase complex in accordance with the results presented in this study.

Acknowledgments

We thank Drs. Robert Bigley and Lawrence Wolff for their helpful discussions and support in securing blood from the CGD donor examined in this study.

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