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DELINEATION OF THE CATALYTIC COMPONENTS OF THE NADPH-DEPENDENT O_2^- GENERATING OXIDOREDUCTASE OF HUMAN NEUTROPHILS[1]

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SUMMARY: Four catalytic components of the NADPH-dependent O_2^- generating oxidoreductase of human neutrophils have been identified. DCIP reductase, cytochrome c reductase and a chromophore 450-455 reductase are present in phorbol myristate acetate stimulated neutrophils and absent in resting cells and phorbol myristate acetate stimulated chronic granulomatous disease cells. Quinol dehydrogenase activity has also been demonstrated in activated and resting cells. Furthermore, a chromophore absorbing in the reduced state at 450-455 nm participates in superoxide production. This chromophore is reduced by NADPH or duroquinol and is missing in cell lysates derived from a patient with chronic granulomatous disease.

When resting neutrophils come into contact with opsonized bacteria, particulate or soluble stimuli, they exhibit a marked increase in O_2^- and H_2O_2 production (1,2). This burst in O_2^- and H_2O_2 production is associated with the expression of oxidoreductase activity in the plasmalemma of the cell (3-8). Neutrophils from patients with chronic granulomatous disease (CGD)[2] lack the capacity to express NADPH-dependent O_2^- generating activity when exposed to stimuli known to elicit a response in normal cells (9). DCIP reductase has previously been identified in association with the oxidoreductase and this component was missing in a young male child with CGD (7). In this study the differences in oxidoreductase activities expressed in resting, activated and CGD cells have been used to delineate catalytic components of the oxidoreductase from which a model of the enzyme complex is presented. One of these, a redox chromophore absorbing in the reduced

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[2]Abbreviations: O_2^-, superoxide; DCIP, dichlorophenolindophenol; CGD, chronic granulomatous disease; PMA, phorbol myristate acetate; cyt. c, cytochrome c; cyt. b, cytochrome b; Q, quinone; QH, quinol.

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state at 450-455nm, is missing in a male CGD patient presented in this study. This patient also lacks the DCIP reductase component of the oxidoreductase complex.

MATERIALS AND METHODS: Ferricytochrome c (cyt. c), superoxide dismutase, NADPH, duroquinone, sodium borohydride, sodium dithionite, dimethyl sulfoxide, phorbol myristate acetate (PMA), and dichlorophenolindophenol (DCIP) were all obtained from Sigma Chemical Co., St. Louis, MO. All other chemicals were of the best grade available.

Purified myeloperoxidase was a generous gift of Drs. Seymour Klebanoff and Henry Rosen, Department of Medicine, University of Washington, Seattle, WA.

Duroquinol was prepared fresh by sodium borohydride reduction of duroquinone as described by Hare and Crane (10).

Purified neutrophils were prepared as previously described (7). Oxidoreductase-rich fractions were prepared by resuspending purified neutrophils (approx. 1-2 x 10^8 cells) in 5 ml Hank's buffer, pH 7.4, to which was added PMA to a final concentration of 10 ug/ml (stock PMA, 1 mg/ml in dimethyl sulfoxide). Following a 5 min incubation at 37°, the cells were centrifuged at 400 x $\bar{g}$. The cell pellet was resuspended in 8 to 10 ml of ice-cold 10 mM Tris-HCl, pH 7.0, lysed by sonication, then centrifuged at 27,000 x $\bar{g}$ for 30 min. The 27,000 x $\bar{g}$ pellet was resuspended in ice-cold 1 mM Tris-HCl, 15% glycerol, pH 8.6. Except for omission of PMA, preparation of the final enzyme pellet from resting cells was the same as that of activated cells.

Continuous enzyme assays and difference spectra were made on a Varian Cary 219 double beam spectrophotometer at room temperature. The assay buffer, except where noted differently, was 8 mM Tris-HCl, pH 7.6, made up in 0.32 mM $CaCl_2$ and 0.20 mM $MgSO_4$. Immediately before assaying, stock enzyme was diluted 10% by volume in 2% triton X-100. NADPH-dependent O_2^- generating activity was assayed as previously described (7), except the final reaction volume was reduced to 1.25 ml. NADPH-dependent cyt. c reductase activity was similarly assayed, except water was added in place of NADPH to the reference cuvette and superoxide dismutase (50 ug) was included in both the sample and reference cuvettes. NADPH oxidase activity was assayed at 340 nm following addition of 0.1 mM NADPH to the assay buffer in a total reaction volume of 1.2 ml. Enzyme activity was calculated using an absorbtivity coefficient of 6.22 $mM^{-1}cm^{-1}$ for NADPH. NADPH-dependent DCIP reductase activity was assayed as previously described (7), but in the present buffer system supplemented with 0.4 mM NaCN in a total reaction volume of 1 ml.

NADPH and duroquinol dependent difference spectra were obtained as follows: to the sample and reference cuvettes were added 0.5 to 1.0 ml of enzyme and 10 mM Tris-HCl, pH 7.0, made up in 0.4 mM $CaCl_2$ and 0.26 mM $MgSO_4$ to a final volume of 2 ml. Baseline scans were then obtained by scanning from 600 to 340 nm (2 nm/sec). After the sample and reference cuvettes were balanced, substrate was added to the sample cuvette and overlay scans were then recorded between 600 and 340 nm at 2 min intervals. Dithionite difference spectra were similarly obtained by addition of a few crystals of dithionite to the sample cuvettes, except scans were restricted between 600 and 400 nm due to the high absorbance of dithionite below 400 nm.

Protein was determined by the method of Lowry et al. (11).

RESULTS AND DISCUSSION: In addition to NADPH-dependent O_2^- generating activity, PMA-stimulated neutrophils also possess three other NADPH-dependent activities including DCIP reductase, cyt. c reductase and chromophore 450-455 reductase as shown in Table I. All of the NADPH-dependent catalytic activities are markedly less active in particulate fractions from unstimulated control and PMA-treated CGD cells. Difference spectra upon addition of duroquinol to enzyme suspensions from either resting or stimulated neutrophils show a major absorbtion peak at 450 nm (Fig. 1).

Table I. Catalytic Components Associated with the NADPH-Dependent O_2^- Generating Oxidoreductase of Human Neutrophils.

	Catalytic Activity (mU/mg protein)[a]		
	Normal Neutrophils		CGD Neutrophils
	PMA-Stimulated	Resting	PMA-Stimulated
NADPH-Dependent:			
1. O_2^- generating activity	48	1.4	0.38
2. Oxidase activity	23	1.4	N.A.[b]
3. DCIP reductase activity	29	1.8	0.46
4. cyt. c reductase activity	6.3	1.4	0.76
5. chromophore 455 reductase[c]	+	-	-
Duroquinol-Dependent:			
1. Chromophore 450 reductase[c]	+	+	N.A.
Dithionite Difference Spectra:			
1. Chromophore 455[c]	+	+	-

[a]All assays were conducted as described in Materials and Methods. One mU equals 1 nmole/min product formed or substrate consumed as indicated.

[b]N.A. = not assayed.

[c]Substrate generated difference spectra. (+), spectrum seen following substrate addition; (-), spectrum not seen following substrate addition (cf., Figs. 1 and 2).

Duroquinol was oxidized with concomitant formation of H_2O_2 under these conditions. The magnitude of the difference spectra, the rate of duroquinol oxidation, and the catalytic production of H_2O_2 are markedly influenced by both pH and the presence of divalent metal ions. Further details on the chemistry of these reactions will be presented elsewhere (12).

Enzyme suspensions from activated (cf., Fig. 2) or resting cells (data not shown) exhibited three distinct reduction peaks at 474, 455 and 429 nm upon addition of dithionite to the sample cuvettes. The 474 nm peak was confirmed to be associated with myeloperoxidase by dithionite difference spectroscopy on purified myeloperoxidase under the same assay conditions. A dithionite difference spectrum on enzyme derived from the CGD patient presented in this study showed no evidence of the 455 nm peak (cf., Fig. 2). In addition, the 429 nm cyt. b type peak was markedly diminished compared to that of normal cells.

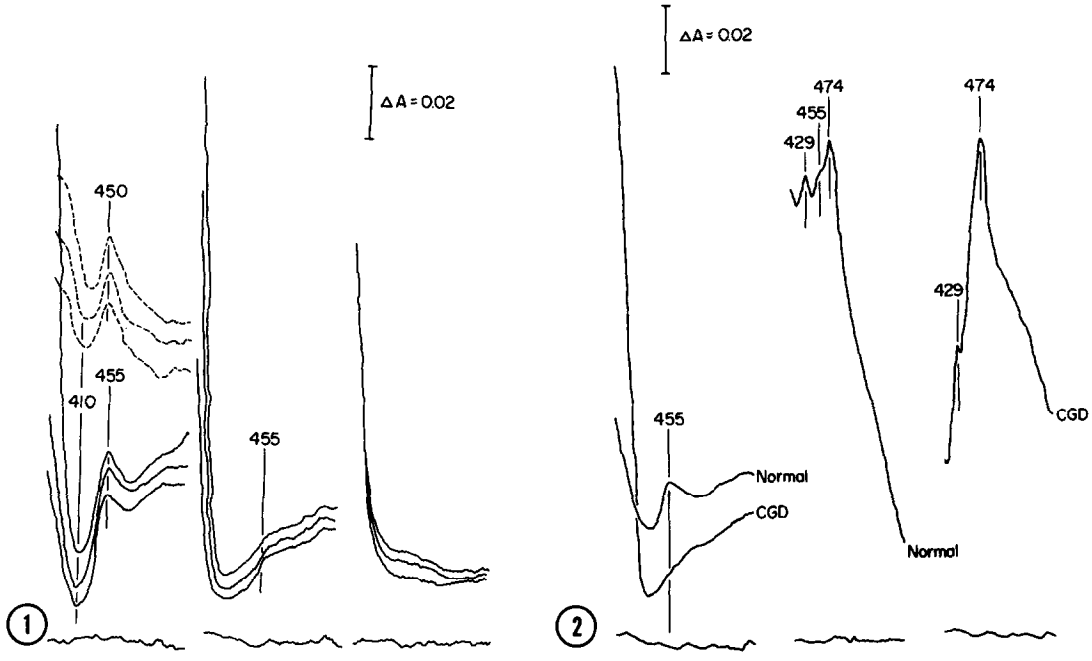

Fig. 1. Substrate-dependent difference spectra on 27,000 x $\bar{g}$ particulate fractions from PMA-stimulated neutrophils (left and middle scans), and similar fractions from resting neutrophils (right scan). (-----), spectrum generated following addition of duroquinol (initial concentration, 0.48 mM) to fractions recovered from PMA-stimulated cells; (———), spectra generated following addition of NADPH (final concentration, 36 uM) to the same fraction from which the duroquinol-dependent spectrum was generated in the absence of cyanide (left scan), in the presence of 0.4 mM cyanide (middle scan), and in fractions from resting neutrophils in the absence of cyanide (right scan). Baseline tracings before addition of substrates are shown beneath each series of scans. Final protein concentrations, 1.5 mg/ml. The earliest scan is shown at the top of each series taken immediately after substrate addition, and thereafter at 2 min intervals.

Fig. 2. Comparative difference spectra on particulate fractions derived from PMA-stimulated normal and CGD cells. Left, difference spectrum 2 min after addition of NADPH (final concentration, 36 uM) to 27,000 x $\bar{g}$ particulate fractions recovered from PMA-stimulated normal and CGD cells. Final protein concentration, 1.4 and 1.7 mg/ml, respectively. Middle and right scans, dithionite difference spectra on the same fractions.

CN^- blocked NADPH-dependent formation of the 455 nm reduction spectrum (cf., Fig. 1). It did not significantly alter O_2^- generating, DCIP reductase, cyt. c reductase nor duroquinol dehydrogenase activities. Dithionite difference spectra run in the presence of CN^- showed the appearance of only the 429 and 474 nm peaks (cf., Fig. 3). Hence, CN^- appears to have either shifted the absorbtion maximum of the reduced 455 nm peak, or, alternatively, to have inhibited its reduction without markedly effecting overall O_2^- production.

Fig. 3. Dithionite difference spectra on the same particulate 27,000 x $\bar{g}$ fraction from PMA-stimulated neutrophils in the absence (lower scan) and presence (upper scan) of 0.4 mM NaCN. The final protein concentration in both experiments was 1.7 mg/ml.

A model consistent with all of the above findings is illustrated in Fig. 4. The effect of CN^- can be explained assuming that there are alternate mechanisms of generating O_2^- upon binding of CN^- to this chromophore. In this model we have depicted the 450-455 nm chromophore as the terminal oxidase. Alternate routes of O_2^- production are also suggested. Likely alternate routes would be through auto-

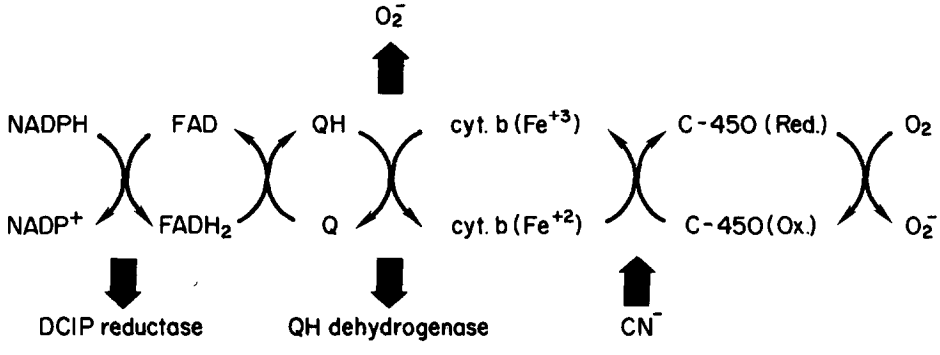

Fig. 4. Hypothetical model of the NADPH-dependent O_2^- generating oxidoreductase complex. Linked redox reactions consistent with observations on the properties of the oxidoreductase are presented. Alternate routes of O_2^- production are depicted at the quinol/quinone junction (QH/Q). Chromophore 450-455 (C-450) missing in the CGD patient along with DCIP reductase, is depicted as the terminal oxidase.

oxidation of semiquinones formed through the action of either quinone reductase or quinol dehydrogenase, or through auto-oxidation of the cyt. b type chromophore previously suggested to be a component of the oxidoreductase (4,13-14). In addition to the cyt. b component, this model is also consistent with observations by Schneider and co-workers on the role of quinones in O_2^- generation (15-17), and with earlier work by Babior and co-workers (3,18) suggesting the presence of a flavoprotein as an essential component of the oxidoreductase.

The NADPH-dependent DCIP reductase component of the oxidoreductase complex has now been partially purified (19). It can be dissociated from the membrane by detergent extraction with concomitant loss of O_2^- generating activity. The solubilized reductase lacks NADPH-dependent duroquinone, menadione and cyt. c reductase activities. Thus it is most likely associated with the primary NADPH oxidase site (cf., Fig. 4). Since it is inactive in particulate fractions from resting cells (cf., Table I), regulation of the oxidoreductase complex apparently occurs at this catalytic site, or at a step preceding this site. Thus it is not surprising that none of the other NADPH-dependent catalytic components cited in Table I are detectable in resting cell lysates. On the other hand, duroquinol can donate electrons to the complex "downstream" from the NADPH oxidase site, thus generating O_2^- and H_2O_2 despite the absence of NADPH-dependent oxidoreductase activity in resting cell lysates.

In conclusion, the above observations on the three catalytic activities (DCIP reductase, cyt. c reductase and chromophore 450-455 reductase) are consistent with current findings on the properties of the oxidoreductase. These are: (i) presence in stimulated cell lysates; (ii) absence or depressed activity in resting cell lysates; and (iii) absence in lysates from CGD neutrophils. The absence of the 450-455 nm chromophore in lysates from the CGD patient presented in this study is of great interest because it demonstrates a biochemical lesion associated with this genetic disorder which can be characterized and identified. It is not yet clear whether the other lesion seen in this patient resulting in failure to express DCIP reductase activity is at the reductase site, or, alternatively, precedes expression

of this site at a point in the activation of the oxidoreductase complex. Work is currently underway in identifying the chemical nature of the 450-455 nm chromophore.

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