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CATALYTIC PROPERTIES OF THE RESOLVED FLAVOPROTEIN AND CYTOCHROME B COMPONENTS OF THE NADPH DEPENDENT O_2^-. GENERATING OXIDASE FROM HUMAN NEUTROPHILS

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The resolved flavoprotein and cytochrome b_{559} components of the NADPH dependent O_2^-. generating oxidase from human neutrophils were the subject of further study. The resolved flavoprotein, depleted of cytochrome b_{559}, was reduced by NADPH under anaerobic conditions and reoxidized by oxygen. NADPH dependent O_2^-. generation by the resolved flavoprotein fraction was not detectable, however it was competent in the transfer of electrons from NADPH to artificial electron acceptors. The resolved cytochrome b_{559}, depleted of flavoprotein, demonstrated no measureable NADPH dependent O_2^-. generating activity and was not reduced by NADPH under anaerobic conditions. The dithionite reduced form of the resolved cytochrome b_{559} was rapidly oxidized by oxygen, as was the cytochrome b_{559} in the intact oxidase.

Human neutrophils contain an oxidase system that kills ingested bacteria by delivering potent oxidant species into the phagolysosome. Control of oxygen activation for this process is enzymatically mediated by a specifically activatable NADPH dependent (1) O_2^-. generating system that requires flavin adenine dinucleotide (2,3) as an essential cofactor. Other evidence suggests that cytochrome b_{559} is an essential component of this system (4). The subcellular oxidase fraction from stimulated normal neutrophils contains a flavoprotein component that can be completely resolved from the cytochrome b_{559} (5). In the present report, certain catalytic properties of the resolved flavoprotein or cytochrome b_{559} containing components from normal neutrophils are described.

METHODS. Cytochrome c (horse heart, type VI), superoxide dismutase (bovine erythrocyte), flavin adenine dinucleotide, reduced nicotinamide adenine dinucleotide phosphate, deoxycholic acid, cholic acid and phorbol myristate acetate were obtained from Sigma. Protein was measured by the dye binding technique as described in the instruction manual to the Bio-Rad Protein Assay

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Kit, Bio-Rad Laboratories, Richmond, CA. All other reagents were of the best commercially available grade and used without further purification.

Isolation, stimulation and fractionation of neutrophils. Neutrophils from normal volunteers were isolated from fresh blood, stimulated with phorbol myristate acetate, sonically disrupted then fractionated by differential isopycnic sedimentation identically to a previously reported method (6), then fractionated into resolved flavoprotein and cytochrome b_{559} components as previously described (5).

Enzymatic assays. All enzymatic assays were performed at 25°C as continuous spectrophotometric assays in a Cary 219 spectrophotometer and reported as initial rates. NADPH dependent O_2^- generation was measured as previously described (6). NADPH oxidation (E^{340nm}=6,220 $M^{-1}cm^{-1}$), 2, 6 dichlorophenol indophenol reduction (E^{600nm}=21,000 $M^{-1}cm^{-1}$)(7), and ferricyanide reduction (E^{420nm}=1,040 $M^{-1}cm^{-1}$) (8) were monitored spectrophotometrically. Standard assays of 2,6 dichlorophenol indophenol or ferricyanide reduction were initiated in microcuvettes by the addition of 10 µl of the enzyme fraction to the following components: 70 µl of 0.1 M potassium phosphate (pH 7.0), 10 µl of 1 mM NADPH and 10 µl of 1 mM electron acceptor (ferricyanide or 2,6 dichlorophenol indophenol). The reference cuvette for each reaction contained only 10 µl of 1 mM 2,6 dichlorophenol indophenol or ferricyanide (as appropriate) and 90 µl of 0.1 M potassium phosphate buffer (pH 7.0). Additions to or deletions from the standard assay are as indicated in table I. Reported rates are corrected for blank values of dye reduction in which the appropriate buffer blank was substituted for enzyme solution. Spectrofluorimetric measurements were performed on a Perkin Elmer MPF 44B spectrofluorimeter and corrected for intensity of excitation light. Anaerobic conditions were achieved in specially designed 1 cm path length Thunberg type fluorimetric or spectrophotometric cuvettes the details of which have been reported (6).

RESULTS

Fluorescence excitation and emission spectra of the resolved flavoprotein fraction from stimulated normal neutrophils in air saturated buffer and anaerobically at various times after the addition of NADPH are shown in figure 1. Prior to addition of NADPH, these fluorescent characteristics were typical of certain oxidized flavoproteins (9) and were unchanged after the sample was made anaerobic. Immediately following addition of NADPH to the sample under anaerobic conditions fluorescence at excitation wavelengths below 410 nm was increased due to fluorescence of the added NADPH, but fluorescence at excitation wavelengths above 410 nm, unobscured by NADPH, decreased to near baseline levels over time. Disappearance of the excitation peak at 450 nm following anaerobic addition of NADPH was interpreted to be due to reduction of enzyme bound FAD (E-FAD $\longrightarrow$ E-$FADH_2$) (9). Following the addition of air saturated buffer the excitation maximum at 450 nm reappeared, and at thirty minutes was identical to that prior to the addition of NADPH (not shown).

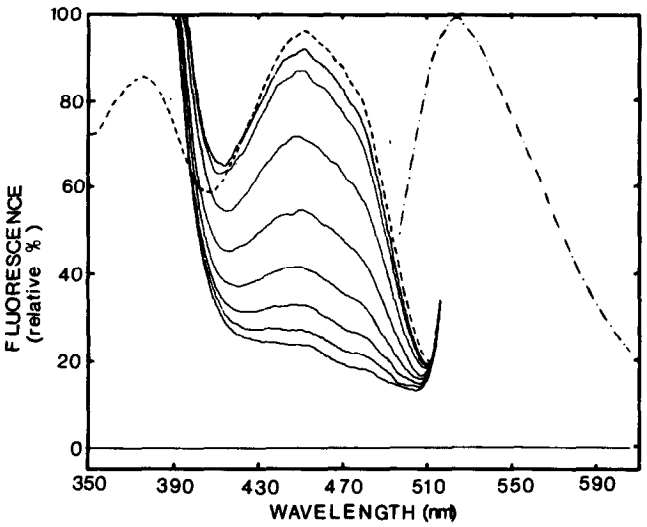

Figure 1. Fluorescence spectra of the resolved flavoprotein fraction. The resolved flavoprotein fraction (700-900 pmol FAD/ml, 1-1.2 mg protein/ml) was prepared from normal stimulated neutrophils as described (5). Immediately prior to each experiment, 1M KH_2PO_4 was added to the sample to titrate the pH to 6.9-7.1. (approximately 42 μl KH_2PO_4 to each 1 ml of sample). At 25°C, fluorescence excitation spectra (350-510 nm) of the preparation were recorded while emission was monitored at 530 nm, and emission spectra (500-590 nm) were recorded while the samples were constantly excited at 450 nm. Excitation (- - - -) and emission (-·-·-·-) spectra under aerobic conditions were as shown, and were unchanged after the sample was made anaerobic (not shown). Under anaerobic conditions, 10 μl of anaerobic 1 mM NADPH solution was added to the 1.0 ml sample and repetitive excitation scans were performed from 390-510 nm (————).

The kinetics of disappearance of the 450 nm fluorescence excitation peak following the anaerobic addition of NADPH and reappearance of this peak following addition of oxygenated buffer are shown in figure 2. Between measurements the sample was kept dark.

Table I shows that, in the presence of 100 μM NADPH, the intact oxidase produced approximately 260 nmol O_2^-./min/mg protein and consumed 136 nmol NADPH/min/mg protein. Under identical conditions, O_2^-. production and NADPH consumption by the resolved flavoprotein or cytochrome b_{559} fractions were unmeasureable. The resolved flavoprotein fraction mediated the NADPH dependent reduction of two artificial electron acceptors in the presence or absence of superoxide dismutase. Reduction of 2,6 dichlorophenol indophenol, a two electron acceptor, resulted in simultaneous NADPH consumption in an approximate 1:1 molar ratio. Reduction of the single electron acceptor ferricyanide, resulted in simultaneous NADPH consumption in an approximate 1:2

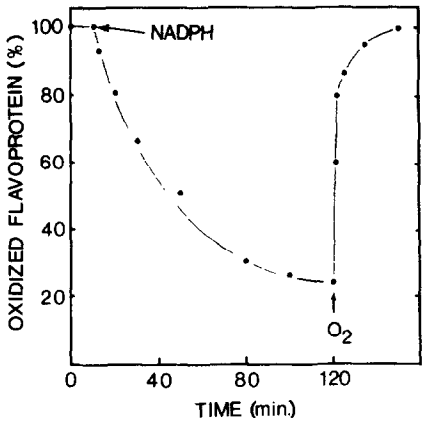

Figure 2. Kinetics of NADPH reduction and oxygen reoxidation of the resolved flavoprotein. Sample preparation and experimental conditions were identical to those shown in figure 2. Fluorescence emission at 530 nm (excitation 450 nm) was recorded at the indicated times (•) after the sample was made anaerobic. At the times indicated by the arrows, 10 µl of anaerobic 1 mM NADPH solution (NADPH) or 10 µl of air saturated buffer (O_2) was added to the 1.0 ml sample of flavoprotein. Relative fluorescence emission of the sample at 530 nm prior to the addition of NADPH was arbitrarily assigned the value of 100% oxidized flavoprotein.

molar ratio. Exposure of the resolved flavoprotein fraction to p-chloromercuribenzoate resulted in complete inhibition of NADPH dependent 2,6 dichlorophenol indophenol or ferricyanide reduction. The resolved cytochrome b_{559} fraction demonstrated no measureable amounts of O_2^-. production, 2,6 dichlorophenol indophenol or ferricyanide reduction in the presence of NADPH even when assayed in the presence of FAD (10-60 µM in assay).

Table II shows that 20 % of the intrinsic cytochrome b_{559} in the intact oxidase was reduced at 2 minutes after the anaerobic addition of NADPH, whereas reduction of the resolved cytochrome b_{559} by NADPH under anaerobic conditions did not occur, even in the presence of exogenous FAD (10 µM). In both preparations of the cytochrome b_{559}, reoxidation of the dithionite reduced form by oxygen was complete in less than 5 seconds (rate limited by hand mixing).

DISCUSSION

The relevance of the resolved flavoprotein to the NADPH dependent oxidase of normal human neutrophils was supported by its reactivity with NADPH, the generally accepted physiological substrate of the oxidase in vivo (10). Two

Table I.

Preparation	NADPH dependent O_2^-.production	Aerobic NADPH Oxidation	NADPH dependent 2,6 dichloro-phenol indophenol reduction	NADPH dependent ferricyanide reduction
	(nmol/min/mg protein, mean ± SE, n=4)			
Intact particulate oxidase				
standard assay	260 ± 4	136 ± 8		
+S.O.D.			4 ± 2	7 ± 2
p-chloromercuri-benzoate treated enzyme	<0.05	<0.2	<0.05	<1
Resolved flavoprotein fraction				
standard assay	<0.05	<0.2	19.5 ± 1.7	30 ± 4
+S.O.D.			17.3 ± 2	26 ± 4
-NADPH			<0.05	<1
p-chloromercuri-benzoate treated enzyme			<0.05	<1
Resolved cytochrome b_{559} fraction				
standard assay	<0.05	<0.2	<0.05	<1

NADPH-dependent electron transport by subcellular neutrophil fractions. Subcellular fractions were prepared from normal stimulated neutrophils, and each fraction was assayed for the indicated enzyme activities in the standard assays described in the METHODS. Deletion of NADPH (-NADPH) or addition of superoxide dismutase, 30 ug/ml assay (+S.O.D.), were as indicated. Exposure of the neutrophil fractions to 25 μM p-chloromercuribenzoate (as indicated) was for 15 minutes at 0-4°C prior to assay (2.5 μM in assay).

lines of evidence were presented that support the reactivity of the resolved flavoprotein with NADPH: direct demonstration of E-FAD reduction by NADPH and mediation of NADPH dependent reduction of artificial electron acceptors. Electron turnover of the intact oxidase preparation from stimulated neutrophils based on FAD content (5) could be estimated at 1,040 min^{-1}. By comparison, the resolved flavoprotein fraction transferred electrons from NADPH to 2,6 dichlorophenol indophenol with a turnover number based on FAD content (5) of 54 min^{-1}. This 20-fold discrepancy may reflect partial denaturation of the flavoprotein component during resolution, or that neither

Table II.

Preparation	Anaerobic NADPH reduction of cytochrome b_{559} (% at 2 min, mean ± SE, n=4)	Oxygen reoxidation of dithionite reduced cytochrome b_{559} (% at 5sec, mean ± SE, n=3)
Intact oxidase	20 ± 4	100 ± 1
Resolved cytochrome b_{559} fraction	< 1	100 ± 1

Cytochrome b_{559} reduction by NADPH and oxidation by oxygen. NADPH reduction or oxygen reoxidation of the intrinsic cytochrome b_{559} in the intact particulate oxidase fraction from stimulated neutrophils (4-5 mg protein/ml, 1,000-1,500 pmols cytochrome b_{559}/ml) or in the resolved cytochrome b_{559} fraction from the same cells (0.5-1.0 mg protein/ml, 600-1,300 pmols cytochrome b_{559}/ml) was measured identically to the previously described method (6). The intact oxidase and resolved cytochrome b_{559} fraction were prepared as described (5).

2,6 dichlorophenol indophenol nor ferricyanide are as effective as the intrinsic electron acceptor(s) in the particulate complex. The apparent initial rate of electron flow from NADPH to FAD estimated from figure 2 was slow (0.07 neq/min/nmol FAD). In other well studied flavoprotein systems such as lipoamide dehydrogenase (11,12), this rate is extremely slow and does not reflect catalytic turnover because all electrons accepted from physiological substrate initially pass through the FAD cofactor, but are rapidly transferred to a redox active disulfide site on the enzyme. Thus the experiments shown in figures 1 and 2 may in fact represent the equilibrium accumulation of E-$FADH_2$ rather than the initial rate of electron transfer to E-FAD.

A component of the oxidase mediating NADPH dependent 2,6 dichlorophenol indophenol reduction has been described by two groups of investigators (13,14) but was not detected by another (2). We hypothesize that the flavoprotein component of the oxidase mediates this process, and discrepancies reported by other investigators may be dependent upon the degree of dissociation of flavoprotein from the remainder of the oxidase complex under different conditions of preparation or assay.

The above results were consistent with the previously proposed hypothesis that the NADPH dependent O_2^-. generating oxidase is a complex of a

flavoprotein and cytochrome b_{559}. Since the flavoprotein component and essential sulfhydryl group(s) (6) are proximal to the cytochrome b_{559}, we further propose that E-FAD is the initial two electron acceptor from NADPH, and analogous to lipoamide dehydrogenase, a redox active disulfide near the FAD site may transfer these electrons from E-$FADH_2$ to the cytochrome b_{559}. The cytochrome b_{559} may participate in the complex as an essential terminal electron donor to oxygen, and the differences in catalytic properties of the resolved vs. unresolved cytochrome b_{559} may therefore be explainable by depletion of flavoprotein. The participation of other components or redox active centers in the complex is possible.

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