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MINI-REVIEW

Isolation of the Respiratory Burst Oxidase: The Role of a Flavoprotein Component

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Abstract

The article reviews the enzymatic and electron transfer properties of a low-potential FAD-dependent flavoprotein that is a component of the NADPH-dependent O_2^- · -generating respiratory burst oxidase of phagocytes. Current methods available for isolation of the respiratory burst oxidase and the flavoprotein component of the complex are also reviewed. These studies and data obtained from affinity-labeling of respiratory burst oxidase components, suggest that the flavoprotein has a molecular weight of 65–67 kD. The prevailing evidence suggests that the flavoprotein functions as a dehydrogenase/electron transferase and can directly catalyse NADPH-dependent O_2^- · formation when isolated. However, in neutrophil plasma membranes, the prevailing evidence suggests that the flavoprotein functions primarily to transfer electrons from NADPH to cytochrome b_{-245} and that this latter redox component is the catalytic side of O_2^- · formation. A working model for the arrangement of the flavoprotein and cytochrome b_{-245} components of the respiratory burst oxidase in neutrophil membranes is proposed.

Key Words: Phagocytes; superoxide; NADPH; oxidoreductase; FAD; cytochrome *b*; chronic granulomatous disease.

Historical Perspective

Historically, the term "respiratory burst" referred to a set of metabolic changes associated with phagocyte stimulation: a rise in O_2 consumption (Baldridge and Gerard, 1933), increased glucose oxidation via the hexose monophosphase shunt (Sbarra and Karnovsky, 1959) and production of extracellular H_2O_2, a potential microbicidal agent (Iyer *et al.*, 1961). These studies

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suggested that phagocyte stimulation led to activation of NAD(P)H-dependent oxidases that catalyzed the reduction of O_2 to H_2O_2. The activity of such enzymes would result in increased $NADP^+$ levels, thereby explaining activation of the hexose monophosphate shunt, which is limited by the availability of $NADP^+$ (Beck, 1958).

The crucial role played by the respiratory burst in the microbicidal functions of phagocytes was apparent from studies on neutrophils of patients with chronic granulomatous disease (CGD), a syndrome associated with susceptibility to recurrent, chronic, and often fatal infections (Holmes *et al.*, 1967). CGD neutrophils appeared to have all the functions of normal neutrophils except that they were unable to mount a respiratory burst when challenged with a variety of known phagocyte stimuli.

With the discovery that stimulated neutrophils produced the superoxide anion radical, $O_2^-\cdot$ (Babior *et al.*, 1973), it became apparent that the respiratory burst was designed to provide the stimulated phagocyte with a potent array of antimicrobial oxidants. The chemical reactivities of $O_2^-\cdot$ and H_2O_2 are such that their simultaneous release into the phagolysosome could give rise to hypochlorous acid, HOCl, the hydroxyl radical, $OH\cdot$, and singlet oxygen, $^1O_2$ [reviewed in Babior (1978)]. Given the central role played by phagocytes in host defense, it was no surprise that considerable interest was provoked by these findings and intense efforts were made to identify the enzyme(s) responsible for the respiratory burst and whose absence gave rise to CGD.

Several candidate respiratory burst oxidases were identified [see Babior, (1978), Badwey and Karnovsky (1980), and Rossi *et al.* (1982) for reviews], but a clear favorite emerged: a membrane-bound NADPH-oxidase that catalyzed the reduction of O_2 to $O_2^-\cdot$ according to the stoichiometry

$$2O_2 + NADPH \rightarrow 2O_2^-\cdot + NADP^+ + H^+ \quad (1)$$

Dormant in resting cells, the enzyme was activated in stimulated neutrophils and, importantly, was absent in membranes isolated from CGD neutrophils (Curnette *et al.*, 1975; Babior *et al.*, 1976). Since neutrophil $O_2^-\cdot$ production could account for nearly all the O_2 consumed during the respiratory burst (Root and Metcalf, 1977) and the activity of NADPH-oxidase could also account for total O_2 consumption during the respiratory burst (Gabig and Babior, 1979), it was suggested that NADPH-oxidase was the respiratory burst oxidase. The primary event of the respiratory burst was proposed to be activation of NADPH-oxidase to form $O_2^-\cdot$, with subsequent dismutation of $O_2^-\cdot$ to form H_2O_2 as follows:

$$2O_2 + NADPH \rightarrow 2O_2^-\cdot + NADP^+ + H^+ \quad (2)$$

$$2O_2^-\cdot + 2H^+ \rightarrow H_2O_2 + O_2 \quad (3)$$

Since the late 1970s, the terms respiratory burst oxidase and NADPH-oxidase have become synonymous. Early attempts to solubilize the enzyme

in Triton X-100 led to the important observation that flavin adenine dinucleotide (FAD) was an essential cofactor for activity, suggesting that NADPH-oxidase was a FAD-linked oxidase, as shown in Scheme 1 below (Babior and Kipnes, 1977; Gabig and Babior, 1979; Light *et al.*, 1981).

Scheme 1

$$\text{NADPH} \rightarrow \text{FAD} \rightarrow \text{O}_2$$

However, it was also shown that human neutrophils contained a unique low-potential *b*-type cytochrome, with oxidase-like properties, that was absent from the plasma membranes of neutrophils obtained from CGD patients with an X-linked mode of disease inheritance (Segal *et al.*, 1978; Segal and Jones, 1978; Cross *et al.*, 1981). Reduction of this cyrochrome b_{559} during neutrophil stimulation under anaerobic conditions was shown to be a characteristic of normal neutrophils (Segal and Jones 1979). In autosomal CGD patients whose neutrophils had a cyrochrome-b_{559}-positive phenotype, stimulation under anaerobic conditions did not result in reduction of cytochrome b_{559}, suggesting absence of the cytochrome's reductase (Segal and Jones, 1980). These and subsequent observations suggested that NADPH-oxidase was an electron transport complex arranged as shown in Scheme 2 below.

Scheme 2

$$\text{NADPH} \rightarrow \text{FAD} \rightarrow \text{cytochrome } b_{559} \rightarrow \text{O}_2 \qquad (4)$$

This simple model has been the subject of considerable controversy, with investigators supporting the role of cytochrome b_{559} as terminal oxidase in the enzyme complex and other suggesting that the site of $O_2^- \cdot$ production was at the level of the flavoprotein. The recent purification to homogeneity of cytochrome b_{559} (Segal, 1987; Teahan *et al.*, 1987; Parkos *et al.*, 1987) and cloning of the gene responsible for the X-linked cytochrome-b_{559}-negative CGD phenotype (Royer-Pokora *et al.*, 1987) have demonstrated, unequivocally, that cytochrome b_{559} is an essential component of the NADPH-oxidase enzyme complex. Cytochrome b_{559} appears to be a heterodimer of 22-kD and 91-kD polypeptides; the 91-kD polypeptide appears to be a structural polypeptide of the protein and it is the gene for this polypeptide that is aberrant in the X-linked form of CGD (Dinauer *et al.*, 1987). The role of cytochrome b_{559} in the NADPH-oxidase complex is the subject of another review in this series to which the reader is referred.

The subject of the present review will be the properties of a FAD-linked flavoprotein found in phagocyte plasma membranes that has been proposed to be a component of the NADPH-oxidase complex, and its role in isolated preparations of NADPH-oxidase.

Properties of the Flavoprotein Component of NADPH-oxidase

FAD Is Required for NADPH-oxidase Activity

Reconstitution of NADPH-oxidase activity by addition of exogeneous FAD to Triton X-100-containing solubilization buffers provided the first evidence that NADPH-oxidase contained a flavoprotein component (Babior and Kipnes, 1977; Gabig and Babior, 1979). Studies with FAD analogues (Light *et al.*, 1981) showed that reconstituted NADPH-oxidase activity was correlated with the oxidation-reduction midpoint potential (E_m) of the flavin moiety; 8-Cl-FAD ($E_m = -152$ mV) > FAD (-208 mV) > 1-deaza-FAD (-280 mV). These results suggested that reduction of FAD was partially rate determining during turnover of NADPH-oxidase. Similar results that confirmed these findings were obtained more recently with the analogues 8-S-FAD, 8-phenyl-S-FAD, and 8-F-FAD (Parkinson and Gabig, 1988). Interestingly, 5-deaza-FAD, an analogue that is only competent for 2-electron transfers, was found to inhibit NADPH-oxidase activity, providing evidence that the flavoprotein component mediated electron transfer to an obligate 1-electron acceptor (Light *et al.*, 1981). However, since reduced 5-deaza-FAD is inert to reoxidation by O_2, this observation could not distinguish as to whether the flavoprotein was directly involved in $O_2^-\cdot$ formation or whether it was transferring electrons to a 1-electron acceptor such as cytochrome b_{559}.

The dissociation constant of Triton X-100-solubilized NADPH-oxidase for FAD was estimated by saturation kinetics to be 61 nM (Babior and Peters, 1981). Similar results for FAD (78 nM) and FAD analogues (62–69 nM) have been obtained recently (Parkinson and Gabig, 1988).

Spectral, Biophysical, and Catalytic Properties of the Flavoprotein

Analysis of membranes isolated from neutrophils revealed substantial quantities of noncovalently bound FAD, but very little flavin mononucleotide (FMN) or riboflavin (Cross *et al.*, 1982; Bellavite *et al.*, 1983; Gabig, 1983; Gabig and Lefker, 1984a,b). FAD content was 100–500 pmol · mg protein^{-1}, depending on the neutrophil source and purity of isolated membranes. Comparison to the cytochrome b_{559} content revealed that these two proposed components of NADPH-oxidase were present in approximately equimolar amounts.

Fluorescence spectroscopy of neutrophil plasma membranes revealed the presence of a fluorescent flavoprotein with characteristic excitation maxima at 380 and 460 nm and an emission maximum at 520 nm (Cross *et al.*, 1982). Membranes isolated from three autosomal CGD patients had normal FAD levels, whereas FAD levels and the presence of the fluorescent

flavoprotein were diminished to ~40% of normal in membranes from three X-linked CGD patients, suggesting association between the fluorescent flavoprotein and cytochrome b_{559}. The flavoprotein and cytochrome b_{559} of membranes isolated from stimulated cells could be resolved from each other by selective bile salt extraction, with concomitant loss of NADPH-oxidase activity (Gabig, 1983). In a CGD patient with normal levels of cytochrome b_{559}, the resolved flavoprotein fraction contained only 8% of normal FAD levels and the fluorescent flavoprotein was absent. Conversely, in a patient with spectrally abnormal cytochrome b_{559}, the FAD levels and fluorescence spectrum of the flavoprotein fraction were normal. In a further study with CGD patients of the cytochrome-b_{559}-negative phenotype, the resolved flavoprotein fraction obtained from two patients were found to contain levels of FAD that were 20 and 12% of normal and correspondingly lower levels of the fluorescent flavoprotein were observed (Gabig and Lefker, 1984a).

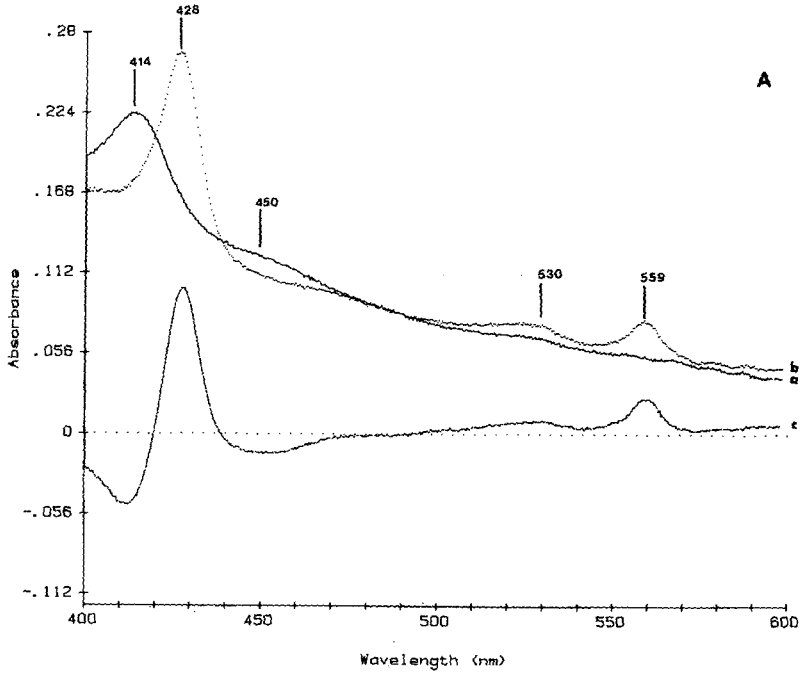
The catalytic properties of the resolved flavoprotein and cytochrome b_{559} fractions were also examined. Anaerobic addition of NADPH to the resolved flavoprotein resulted in bleaching of the FAD fluorescence, consistent with reduction of enzyme-bound FAD to $FADH_2$ (Gabig and Lefker, 1984b). Reduced $FADH_2$ could be reoxidized by O_2, but the product of reoxidation was not determined. In contrast to NADPH-oxidase in intact membranes, the resolved flavoprotein did not catalyze NADPH-dependent $O_2^-\cdot$ production. However, it could mediate electron transfer from NADPH to artificial acceptors such as 2,6-dichlorophenolindophenol and ferricyanide. Turnover number, based on FAD content, was only 54 min^{-1} for the resolved flavoprotein as compared to 1040 min^{-1} for intact NADPH-oxidase. These differences suggested that the resolved flavoprotein was denatured or that it mediated electron transfer to its intrinsic acceptor in the NADPH-oxidase complex more efficiently than to artificial acceptors. The cytochrome b_{559} fraction was also devoid of NADPH-dependent $O_2^-\cdot$ production and, in contrast to intact NADPH-oxidase preparations, was not reduced by NADPH under anaerobic conditions. Cytochrome b_{559} in both intact NADPH-oxidase preparations and when resolved from the flavoprotein could be chemically reduced with dithionite and was rapidly (< 5 sec) reoxidized by air. Significantly, anaerobic NADPH-dependent reduction of cytochrome b_{559} was absent in membranes from stimulated neutrophils of a CGD patient with demonstrated absence of the fluorescent flavoprotein (Gabig and Lefker, 1984a). Attempts to reconstitute NADPH-oxidase activity by combining the resolved flavoprotein and cytochrome b_{559} fractions in the presence of phospholipids and added FAD succeeded in restoring only 3–6% of NADPH-oxidase activity.

These studies suggested the flavoprotein component of the NADPH-oxidase complex mediated electron transfer from NADPH to cytochrome

b_{559} in a manner consistent with Scheme 2 shown above, but the precise site of $O_2^- \cdot$ production within the complex could not be identified unequivocally. These studies also suggested that the CGD phenotype could arise from a number of genetic defects. Clearly, in relation to the flavoprotein and cytochrome b_{559} components of NADPH-oxidase, three distinct CGD phenotypes could be demonstrated: (a) absence of cytochrome b_{559} with normal levels of the fluorescent flavoprotein, (b) diminished levels of both cytochrome b_{559} and the fluorescent flavoprotein, and (c) normal levels of cytochrome b_{559} with absence of the fluorescent flavoprotein.

Improved methods for obtaining solubilized preparations of NADPH-oxidase enabled NADPH-dependent FAD reduction to be determined by absorbance spectroscopy (Cross *et al.*, 1984). Figure 1A shows spectra that were obtained from a preparation of Lubrol-PX/deoxycholate-solubilized pig neutrophil NADPH-oxidase. The air-oxidized spectrum shows a broad maximum at 414 nm due to oxidized cytochrome b_{559} and a pronounced shoulder at 450 nm due to oxidized FAD. Dithionite reduction produced peaks at 428, 530, and 559 nm, due to reduced cytochrome b_{559}, and bleaching of the absorbance at 450 nm due to reduction of FAD. The dithionite-reduced-minus-air-oxidized difference spectrum clearly showed the three peaks of cytochrome b_{559} and a trough at 450 nm due to FAD. Difference spectra obtained after addition of NADPH to the preparation under anaerobic conditions are shown in Fig. 1B. The kinetics of NADPH-dependent reduction of cytochrome b_{559} and FAD could be estimated from absorbance changes at 559 and 450 nm, respectively, using appropriate isosbestic points, and the results are shown in Fig. 2. The kinetics of NADPH-dependent reduction of both proposed redox centres of the NADPH-oxidase complex were slow; after prolonged incubation, FAD reduction reached $\sim 70\%$ and cytochrome b_{559} reduction $\sim 40\%$. Substantial quantities of both components were reduced before measurements could be taken, but reduction kinetics were too slow to account for the measured rate of NADPH-dependent $O_2^- \cdot$ production. The kinetics of FAD reduction observed in this solubilized preparation of NADPH-oxidase were very similar to those reported for the resolved flavoprotein (Gabig and Lefker, 1984b).

Fig. 1. Spectral properties and NADPH-dependent reduction of FAD and cytochrome b_{559} in solubilized preparations of NADPH-oxidase. (A) Air-oxidized spectrum (a), dithionite-reduced spectrum (b), and dithionite-reduced-minus-air-oxidized difference spectrum (c) of a crude preparation of NADPH-oxidase solubilized from plasma membranes of stimulated pig neutrophils with 0.25% Lubrol-PX–0.25% deoxycholate. The absorbance maxima indicated are for FAD (450 nm, oxidized) and cytochrome b_{559} (414 nm, oxidized; 428, 530, and 559 nm, reduced). (B) NADPH-reduced-minus-air-oxidized difference spectra obtained after anaerobic addition of 1 mM NADPH. FAD and cytochrome b_{559} reduction were estimated from the absorbance changes at 450 and 559 nm, respectively.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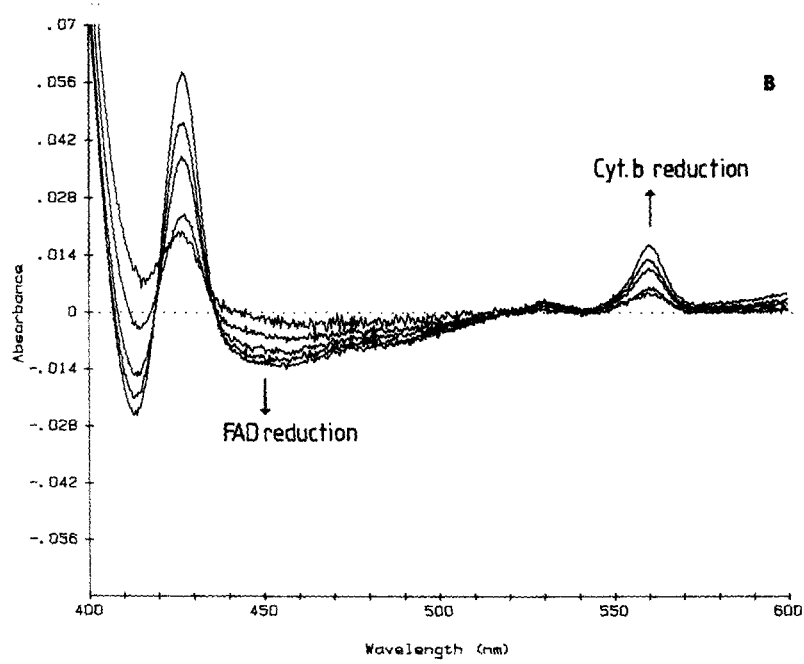
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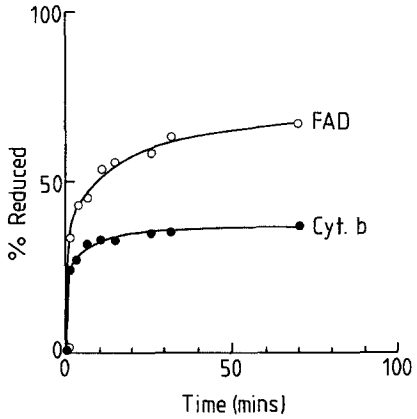

Fig. 2. Kinetics of NADPH-dependent reduction of FAD and cytochrome b_{559} in solubilized NADPH-oxidase under anaerobic conditions. The extent of FAD and cytochrome b_{559} reduction were determined from difference spectra (see Fig. 1) obtained at various time points after the addition of 1 mM NADPH to solubilized NADPH-oxidase under aerobic conditions. FAD and cytochrome b_{559} reduction are given as % of total FAD and cytochrome b_{559} in the preparation.

These independent observations regarding the anomalous kinetic behavior of the flavoprotein and cytochrome b_{559} components of NADPH-oxidase under anaerobic conditions were a cause for some concern. However, NADPH-dependent reduction of both components was reevaluated under aerobic steady-state conditions (Cross *et al.*, 1985). Figure 3 shows a difference spectrum of a solubilized preparation of NADPH-oxidase in the presence of 1 mM NADPH under aerobic conditions (expanded fourfold for clarity), together with a spectrum of the sample after complete reduction by dithionite. Under aerobic steady-state conditions, FAD reduction determined from the trough at 450 nm was estimated at 45%, and cytochrome b_{559} reduction determined from absorbance at 559 nm was 8.9%. An identical experiment performed on a solubilized sample obtained from resting neutrophils that had no NADPH-oxidase activity showed no NADPH-dependent reduction of either FAD or cytochrome b_{559}.

The pseudo-first-order rate constant for the reaction of reduced detergent-solubilized cytochrome b_{559} with O_2 was measured at 147 sec^{-1}, enabling calculation of the rate of cytochrome b_{559} reduction under steady-state conditions. The value obtained, 13.08 mol cytochrome b_{559} reduced · sec^{-1} · mol total cytochrome b_{559}^{-1} was very close to the calculated rate of O_2^- · production by the same sample 13.03 mol O_2^- · · sec^{-1} · mol total cytochrome b_{559}^{-1}. Furthermore, for a series of solubilized preparations with varying levels of NADPH-oxidase activity, a linear relationship was found between the rate of O_2^- · production and the amount of both FAD and cytochrome b_{559} reduction under these aerobic steady-state conditions (Parkinson, 1985). A plot of the calculated rate of cytochrome b_{559} reduction and the rate of O_2^- · production

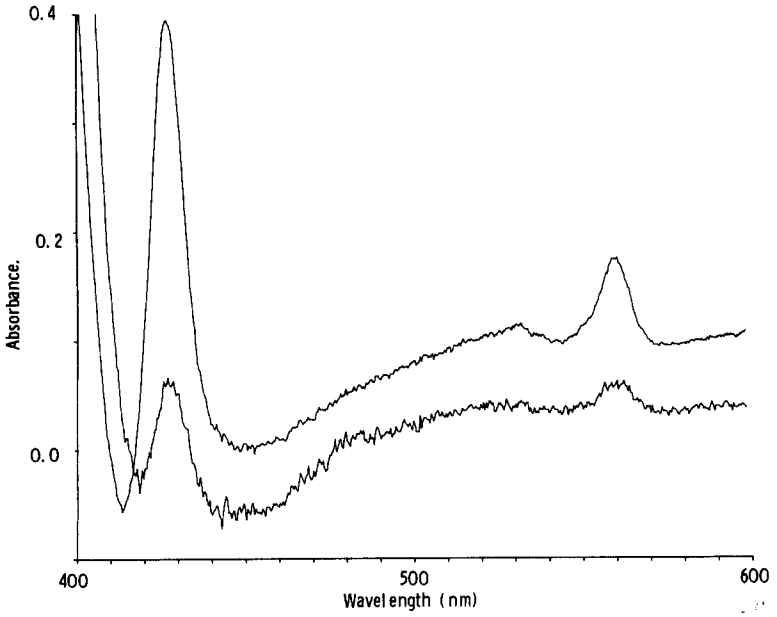

Fig. 3. Aerobic steady-state NADPH-dependent reduction of FAD and cytochrome b_{559} in a preparation of solubilized NADPH-oxidase. Computerized difference spectra obtained from a crude preparation of solubilized NADPH-oxidase after addition of 1 mM NADPH under aerobic steady-state condtions (lower trace, expanded fourfold for clarity) and after complete reduction with dithionite (upper trace). NADPH-dependent FAD reduction determined from the trough at 450 nm was 45%, and cytochrome b_{559} reduction determined from the peak at 559 nm was 8.9%.

was found to be linear with a slope of 1. These observations were of significance because they demonstrated not only that the kinetics of FAD and cytochrome b_{559} reduction under aerobic steady-state conditions were rapid, but also that electron transfer from NADPH to both components was dependent on neutrophil stimulation prior to membrane isolation and solubilization. These properties would be expected of proposed redox components of the NADPH-oxidase complex. The results also strongly supported the proposal that the flavoprotein and cytochrome b_{559} were arranged in the NADPH-oxidase complex as shown in Scheme 2, with FAD acting as a mediator of electron transfer from NADPH to cytochrome b_{559}.

The most comprehensive study of the biophysical propertoes of the flavoprotein component of NADPH-oxidase used electron paramagnetic resonance (EPR) techniques to exploit the paramagnetic properties of the FAD semiquinone free radical, FADH · (Kakinuma *et al.*, 1986). Potentiometric titration of plasma membranes isolated from both stimulated and resting neutrophils revealed an EPR signal at $g = 2.004$, with a peak-to-peak

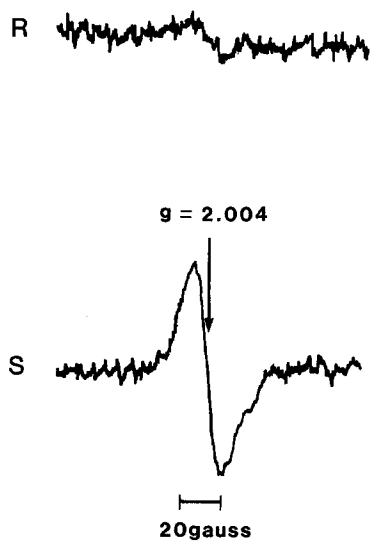

Fig. 4. EPR spectra of isolated neutrophil membranes incubated with NADPH under anaerobic conditions. Plasma membranes were isolated from resting (R) or stimulated (S) pig neutrophils. After anaerobic incubation in the presence of 1 mM NADPH for 60 min, samples were transferred to EPR tubes and low-temperature EPR spectra were obtained at 197 K. An EPR spectrum typical of a blue neutral flavin semiquinone (g = 2.004, peak-to-peak line width = 19 G) was only obtained in the plasma membrane sample isolated from stimulated cells.

line width of 19 G that is typical of the neutral "blue" form of flavin semiquinone radicals. The titration revealed an E_m at pH 7.0 of −280 mV. The NADPH dependence of formation of this EPR signal was determined under anaerobic conditions in the presence of 1 mM NADPH. As shown in Fig. 4, the EPR signal at g = 2.004 was only observed in the membrane sample obtained from stimulated neutrophils. The power saturation behavior of the EPR signal in membranes isolated from resting and stimulated neutrophils suggested that the FAD free radical was in the vicinity of a transition metal center and that the distance between the two species was decreased following neutrophil stimulation. It was inferred that the transition metal center was likely to be that of Fe^{3+} in the heme moiety of cytochrome b_{559}.

The E_m value of the flavoprotein, −280 mV, was sufficiently low to propose that the flavoprotein might react directly with O_2 to form $O_2^-\cdot$, as had been observed for other flavoproteins (Massey *et al.*, 1971). However, it was also noted that this midpoint potential was between that of the NADPH/$NADP^+$ couple (−320 mV) and that of cytochrome b_{559} which had previously been determined at −245 mV (Cross *et al.*, 1981). Since the

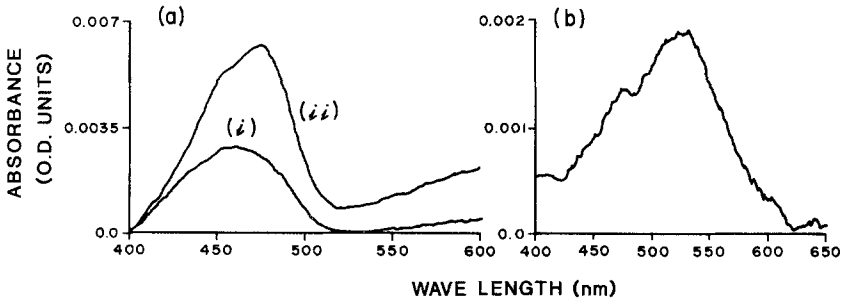

Fig. 5. Absorbance spectra of solubilized NADPH-oxidase reconstituted with 8-substituted FAD analogues. NADPH-oxidase was solubilized from human neutrophil plasma membranes using 0.2% Triton X-100. (a) Spectrum of 8-F-FAD-reconstituted NADPH-oxidase before (i) and 20 min after (ii) reaction with thiophenol. (b) Spectrum of 8-S-FAD-reconstituted NADPH-oxidase.

midpoint potential of the $O_2^- \cdot/O_2$ couple has been determined at -160 mV [this value is calculated using the physiological concentration of O_2 in solution and not the partial pressure of O_2, which gives a misleading value of -330 mV (Wood, 1974)], a bioenergetically sound model of the redox reactions of the NADPH-oxidase complex can be proposed as shown in Scheme 3 below.

Scheme 3

$$\underset{-320\,\text{mV}}{\text{NADPH/NADP}^+} \rightarrow \underset{-280\,\text{mV}}{\text{FADH}_2\text{/FAD}} \rightarrow \underset{-245\,\text{mV}}{\text{cyt.b}^{2+}\text{/cyt.b}^{3+}} \rightarrow \underset{-160\,\text{mV}}{O_2^- \cdot/O_2}$$

The most recent study on the spectral and biophysical properties of the flavoprotein component of NADPH-oxidase used 8-substituted FAD analogues as active site probes (Parkinson and Gabig, 1988). Triton X-100-solubilized NADPH-oxidase activity was reconstituted with 8-F-FAD, 8-S-FAD, and 8-phenyl-S-FAD (see above). The ability of 8-F-FAD to react with nucleophiles was used to probe the solvent accessibility of the flavin in the binding site of NADPH-oxidase. 8-S-FAD was used as a spectral probe since this flavin is bound by different classes of flavoproteins in distinct spectral forms. The results from these spectral studies are shown in Fig. 5. Reaction of 8-F-FAD reconstituted with NADPH-oxidase and thiophenol was evidenced by a shift from the absorbance maximum of 8-F-FAD at 438 nm to 475 nm, with a shoulder at 455 nm, and an increase in extinction coefficient of the bound flavin. The resulting spectrum was characteristic of that of 8-S-phenyl-FAD, indicating that the 8-position of bound 8-F-FAD was readily accessible to solvent. Reconstitution of 8-S-FAD with solubilized NADPH-oxidase resulted in a small shift of the absorbance maximum at

520 nm of the free flavin to ~530 nm, indicating that NADPH-oxidase stabilized the red "thiolate" form of 8-S-FAD.

The solvent accessibility of the 8-position of FAD bound to the flavoprotein component of NADPH-oxidase was similar to results obtained with other flavoproteins that use NADPH or NADH as substrates, which have all been shown to have the 8-position of the flavin exposed to solvent (Ghisla and Massey, 1986). The stereospecificity of hydride transfer from the reduced pyridine nucleotide to the *re*-face of enzyme-bound flavins appears to be absolute in the flavoproteins studied so far (Manstein *et al.*, 1986), and it seems likely that the orientation of the flavin binding site in such enzymes is a conserved motif. There is a correlation between the spectral form of 8-S-flavins bound by flavoproteins and the type of flavin semiquinone radical stabilized by these flavoproteins. That NADPH-oxidase appeared to bind the red thiolate form of 8-S-FAD was of interest; this form of 8-S-flavin is typically bound by flavoproteins of the electron transferase class that also stabilize the blue neutral form of the flavin semiquinone radical (Ghisla and Massey, 1986). In contrast, flavoproteins of the oxidase class that bind the blue "paraquinoid" form of 8-S-flavins, which have resolved three-banded spectra in the 600-nm region, are found to stabilize the red anionic form of the flavin semiquinone radical. The spectral studies with reconstituted solubilized NADPH-oxidase appeared to confirm the EPR studies described above, and indicated that the flavoprotein component of NADPH-oxidase was likely to be a flavoprotein of the electron transfer class rather than a simple oxidase.

Since the properties of the flavoprotein component reviewed in this section were obtained in crude membrane or solubilized preparations of NADPH-oxidase, their unequivocal assignment remains to be confirmed in preparations of purified NADPH-oxidase and/or the flavoprotein component itself. However, these studies have provided an important frame of reference with which to compare the properties of preparations of isolated NADPH-oxidase, which will be the subject of the next section of this review.

Isolation of NADPH-oxidase

Due to the extreme lability of NADPH-oxidase in detergent solutions [$t_{1/2}$ = 24 min @ 25° C (Light *et al.*, 1981)], progress in isolating the enzyme has been depressingly slow. Methods have been reported for solubilization and isolation of NADPH-oxidase from a variety of mammalian phagocyte sources. In this section, an attempt is made to given an overview of the relative successes of the described methods and to evaluate the role of the proposed flavoprotein component in these isolated preparations of the enzyme.

Bellavite and coworkers reported partial purification of NADPH-oxidase from guinea pig and pig neutrophils and also guinea pig macrophages (Bellavite *et al.*, 1983, 1984; Serra *et al.*, 1984; Berton *et al.*, 1985). Similar results were obtained using either 0.3% deoxycholate or a mixture of 0.4% deoxycholate and 0.4% Lubrol-PX for detergent solubilization. The solubilized enzyme was subjected to gel-filtration chromatography on a column of Ultrogel-AcA22 using a glycerol/phosphate detergent-free buffer. NADPH-oxidase (70–80%) was eluted from this column as a high-molecular-weight aggregate ($> 2 \times 10^6$ kD) in the void volume, and was concentrated by filtration. The enzyme was further purified by centrifugation through a 25–35% glycerol density gradient. The specific activity of the final preparation was 209 nmol $O_2^- \cdot \cdot \min^{-1} \cdot$ mg protein^{-1}, which represented a fivefold purification with respect to the crude solubilized extract (Serra *et al.*, 1984). Attempts to purify the enzyme further by treating the fraction isolated from the glycerol gradient with dissociating agents such as Triton X-100, Lubrol-PX, urea, or KCl resulted in total loss of activity, although treatment with 0.4 M NaCl did provide a sedimentable form of the enzyme that was slightly purified with respect to the glycerol gradient fraction. The final yield of NADPH-oxidase activity was ~9% with respect to the solubilized extract.

In both neutrophils and macrophages, analysis for cytochrome b_{559} and FAD content of fractions obtained from the Ultrogel-AcA22 column revealed copurification of cytochrome b_{559} with NADPH-oxidase activity, whereas the majority of FAD was retarded by the column and associated with NADH-dependent diaphorase activities (Bellavite *et al.*, 1984; Berton *et al.*, 1985). In contrast to the approximately equimolar ratio of these redox components found in solubilized extracts, the partially purified enzyme from neutrophils had a cytochrome b_{559} to FAD ratio of 19 : 1 and that from macrophages had a ratio of 5 : 1. These studies strongly supported the role of cytochrome b_{559} as a component of NADPH-oxidase and further suggested that the NADPH-oxidase complex might be similar to the complex of cytochrome P-450 with cytochrome P-450 reductase where the ratio has been found to be in the order of 10 to 25 : 1 (Yang, 1977; Gut *et al.*, 1983). However, in view of the known stabilizing effect of FAD on solubilized preparations of the enzyme reported by this (Bellavite *et al.*, 1983) and other groups (Gabig and Babior, 1979; Light *et al.*, 1981), the possibility that the low yields obtained were due to progressive loss of FAD during isolation could not be excluded.

An alternative strategy for NADPH-oxidase isolation from stimulated bovine neutrophils has been described (Doussiere and Vignais, 1985). The enzyme was solubilized in 0.25% Triton X-100 and purified as follows: DE-52 cellulose ion-exchange chromatography with elution by a 0 to 0.3 M

linear Na_2SO_4 gradient, Sephadex G-200 gel-filtration chromatography, and preparative isoelectric focusing (pH 4.0–8.0) in a 0–30% sucrose gradient column.

DE-52 cellulose chromatography efficiently resolved NADPH-oxidase from contaminating diaphorase activities, and NADPH-oxidase activity eluted from the Sephadex G-200 column just after the void volume. Isoelectric focusing indicated that the enzyme had a pI of 5.0. Correcting for spontaneous loss of activity, the final preparation was obtained in ~50% yield, was 50-fold purified with respect to the crude extract, and had a specific activity of 222 nmol $O_2^- \cdot \cdot \cdot min^{-1} \cdot mg\ protein^{-1}$. Analysis by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) revealed one major polypeptide of 65 kD with a minor contaminant of 70 kD. The enzymatic properties of the purified bovine enzyme were similar to those reported for the membrane-bound enzyme in other mammalian phagocytes: K_m for NADPH = 30 μM, very low activity with NADH as substrate, NADPH oxidation to $O_2^- \cdot$ production ratio = 0.5, and insensitivity to cyanide. The enzymic activity could be enhanced threefold by the addition of asolectin, in keeping with previous observations of a phospholipid requirement for NADPH-oxidase activity (Gabig and Babior, 1979; Serra *et al.*, 1984).

FAD content (0.02 nmol · mg $protein^{-1}$) and cytochrome b_{559} content (0.02 nmol · mg $protein^{-1}$) of the isolated enzyme were very low, raising doubts as to the role of both of these components in this preparation. However, it was noted that recovery of NADPH-oxidase activity after Triton X-100 extraction of membranes was only 13%, giving a yield in the final preparation of only 6%.If spontaneous loss of NADPH-oxidase activity was not corrected for, the true yield and purification factors obtained by the method described were very low. This suggested that Triton X-100 treatment of the bovine enzyme, and purification in Triton X-100-containing buffers, led to irreversible dissociation of components essential for activity from the enzyme complex. The small amount of NADPH-oxidase activity obtained in the final preparation might therefore have been accounted for by the trace amounts of FAD and cytochrome b_{559} that were observed.

Isolation of human NADPH-oxidase was reported using chromatography of the deoxycholate-solubilized enzyme through Red Sepharose CL-6B (Markert *et al.*, 1985). Enzyme bound to the affinity gel matrix in a buffer containing 0.125% Lubrol-PX/0.125% deoxycholate was eluted by a front of detergent-free buffer rather than by ligand displacement. The enzyme obtained by this one-step procedure was purified 48-fold with respect to isolated membranes. Although the enzyme was obtained in very low yield (1.6% compared to the starting membrane fraction and allowing for spontaneous loss of activity), it was found to have a high specific NADPH-oxidase activity of 5100 nmol $O_2^- \cdot \cdot \cdot min^{-1} \cdot mg\ protein^{-1}$. SDS-PAGE analysis

showed a major polypeptide at 65 kD and several minor polypeptides. The polypeptide composition was studied in more detail using preparations obtained by a slightly improved method (Glass *et al.*, 1986). SDS-PAGE showed three major polypeptides at 65, 48, and 32 kD. One major band was observed on nondenaturing gels and, when this was excised and analyzed by SDS-PAGE, the 65-, 48-, and 32-kD polypeptides were also observed. These three polypeptides were inferred to be subunit components of the NADPH-oxidase complex. Due to the instability of the preparation, attempts to identify and isolate the enzyme by activity staining of nondenaturing gels were unsuccessful. The efficacy of this Red Sepharose method was subsequently confirmed by an independent study (Bellavite *et al.*, 1986).

However, these independent studies differed markedly concerning the presence of cytochrome b_{559} and FAD in the isolated enzyme. In one study, the FAD content was found to be 20 nmol · mg protein^{-1} (Glass *et al.*, 1986), whereas 0.12 nmol · mg protein^{-1} was found in the other (Bellavite *et al.*, 1986). One study estimated cytochrome b_{559} content at only 0.1 nmol · mg protein^{-1} (Glass *et al.*, 1986), whereas the other study found 2.6 nmol · mg protein^{-1}, and a difference absorbance spectrum identical to that of cytochrome b_{559} was readily observed (Bellative *et al.*, 1986). The latter study also found that the elution profiles of NADPH-oxidase activity, cytochrome b_{559}, and protein from the Red Sepharose column were exactly superimposable. To the impartial observer, these disparate findings, which were obtained using ostensibly identical methods, would seem to be impossible to reconcile. The finding that the isolated enzyme contained FAD, but very little cytochrome b_{559}, suggested one of two possibilities (Glass *et al.*, 1986). First, that NADPH-oxidase was a flavoprotein that directly catalyzed NADPH-dependent O_2^- · production without the involvement of cytochrome b_{559}. Second, that the isolated preparation contained only the flavoprotein dehydrogenase component of the NADPH-oxidase complex. The second interpretation implies that the ability of the flavoprotein to react directly with O_2 might be an artifact of purification similar to altered electron acceptor specificities observed during isolation of mitochondrial dehydrogenases.

That the second of these two interpretations might have been correct was supported by a recent report of the purification of the FAD component of NADPH-oxidase from stimulated pig neutrophils (Kakinuma *et al.*, 1987). NADPH-oxidase was solubilized from membranes with 0.75% octyglucoside and subjected to isoelectric focusing (pH 4–8) in polyacrylamide gels. Gels were stained for NADPH-oxidase activity with nitroblue tetrazolium (NBT), a yellow dye that forms an insoluble blue formazan precipitate upon reaction with O_2^- · (Baehner and Nathan, 1968). With samples obtained from stimulated cells, a strong NADPH-dependent NBT-staining band was observed at pI 5.0. Staining of this band was much weaker in samples obtained from

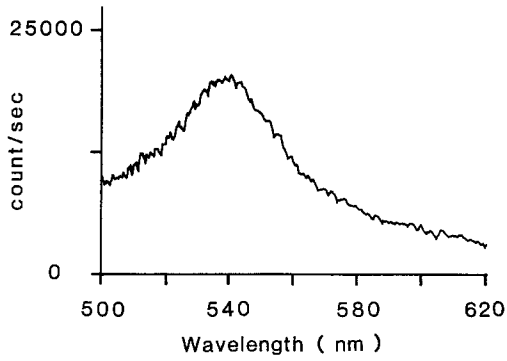

Fig. 6. Fluorescence spectrum of a purified flavoprotein component of NADPH-oxidase. NADPH-oxidase was solubilized from stimulated pig neutrophil membranes in 0.75% octylglucoside. An extract of isoelectric focusing gels in the region of pI 5.0 was used for photo-counting fluorescence spectroscopy. The figure shows an oxidized-minus-reduced difference fluorescence emission spectrum of the pI 5.0 extract, using 450 nm as excitation wavelength.

resting cells. Also, the NBT stain was much less when using NADH as substrate, was markedly inhibited by superoxide dismutase (SOD), and was insensitive to cyanide, properties which suggested that the enzymic activity responsible for NBT reduction was a component of NADPH-oxidase. The enzyme focusing at pI 5.0 was extracted from a large number of gels by electrophoretic elution of freeze-thawed gel slices. As shown in Fig. 6, the extract showed an emission spectrum of a fluorescent flavoprotein with an emission maximum at 540 nm. The preparation contained 1048 pmol · mg protein^{-1} FAD, and similar results were obtained with extracts prepared from resting neutrophils. On the basis of FAD content, the flavoprotein component of NADPH-oxidase was ~ 13-fold purified with respect to the membrane preparation used as starting material.

Characterization of the enzymatic properties of this extract provided some intriguing results. The freshly extracted enzyme, which was very unstable, catalyzed NADPH-dependent cytochrome *c* reduction that was insensitive to SOD. Since the enzyme was anionic and cytochrome *c* is known to be cationic, this suggested that an electrostatic interaction between the enzyme and cytochrome *c* might have interfered with electron transfer from the flavoprotein to O_2. This was supported by the observation that NADPH-dependent cytochrome *c* reduction occurred under anaerobic conditions. Most intriguingly, when the enzyme was freeze-thawed slowly in the assay buffer, subsequent addition of NADPH revealed decreased NADPH oxidation, but the oxidase activity was now SOD sensitive. Moreover, stoichiometric analysis of NADPH-oxidation, O_2^-· production, and H_2O_2 production revealed a 1 : 2 : 1 relationship, in agreement with the known catalytic properties of NADPH-oxidase [cf. Eqs. (2) and (3)]. Both the fresh

and freeze-thawed forms of the enzyme had affinities for NADPH (K_m = 26–43 μM) that were similar to values previously determined for NADPH-oxidase. These findings strongly suggested that the isolated enzyme was the flavoprotein component of NADPH-oxidase.

Freeze-thawing of the preparation was interpreted to induce clustering of the hydrophobic enzyme, which would be unstable in water, to form more stable micellar structures, thereby altering the specificity of the enzyme for cytochrome *c* and O_2. These studies provided strong evidence that the isolated flavoprotein could directly interact with O_2 to catalyze $O_2^-\cdot$ formation. Since simple flavoprotein oxidases have not been found to react with O_2 to form $O_2^-\cdot$, whereas flavoprotein electron transferases have (Massey and Ghisla, 1983), these findings suggested that the isolated flavoprotein was of the electron transferase class. It was suggested that the flavoprotein might be identical to that previously shown, in neutrophil membranes, to stabilize the blue neutral flavin semiquinone radical, another characteristic of flavoprotein electron transferases [see above; Kakinuma *et al.*, (1986)]. Although the interaction of the isolated flavoprotein with cytochrome b_{559} was not examined in this study, the isoelectric focusing experiments suggested that the two components had opposite charges. Heme-staining experiments revealed the absence of any cytochromes in the region of pI 5.0, but strong staining at pI > 8.0, presumably due to cytochrome b_{559}, was observed. It was suggested that, in neutrophil plasma membranes, the flavoprotein (pI 5.0) might form a stable electrostatic complex with cytochrome b_{559} (pI > 8.0). In such a complex, the flavoprotein might mediate 2 to 1-electron transfer from NADPH either directly to O_2 or to cytochrome b_{559}, but neither possibility could be excluded.

Nondenaturing two-dimensional electrophoresis of the solubilized preparation followed by NBT staining was used to estimate the size of the flavoprotein. A major band at ~67 kD was observed, very similar in size to the 65-kD polypeptide components of isolated NADPH-oxidase described previously (Doussiere and Vignais, 1985; Glass *et al.*, 1986). Moreover, the isoelectric point of the flavoprotein isolated in this study was identical to that reported for a preparation of isolated bovine NADPH-oxidase (Doussiere and Vignais, 1985). Taken together, these findings would suggest that the flavoprotein component of NADPH-oxidase has been identified as a 65 kD polypeptide. Further evidence that this may be the case has been provided by affinity-labeling studies in preparations of NADPH-oxidase, and these studies are the subject of the next section of this review.

Components of NADPH-oxidase: Affinity-Labeling Studies

Affinity-labeling has been used as a powerful tool to characterize the molecular components of many enzyme systems. Surprisingly, relatively few

attempts have been made to identify components of NADPH-oxidase using this technique. However, the few affinity-labeling studies that have been reported have provided useful information. These findings are reviewed in this section according to the type of affinity label used.

NADPH Analogue Affinity Labels

An NADPH analogue, 2′,3′-dialdehyde NADPH (*o*-NADPH), was prepared by periodate oxidation of NADPH, and the effect of this analogue on solubilized NADPH-oxidase activity was determined (Umei *et al.*, 1986). Solubilized NADPH-oxidase used *o*-NADPH as a substrate in $O_2^-\cdot$ production; the K_m for *o*-NADPH was 31 μM compared to 33 μM for NADPH, V_{max} was about half that for NADPH, and *o*-NADPH was a competetive inhibitor of NADPH-dependent $O_2^-\cdot$ production. A 91-min incubation of NADPH-oxidase with 1.2 mM *o*-NADPH resulted in 68% loss of NADPH-oxidase activity compared to control. The inhibition was partially reversed by 5 mM NADPH, but not by NAD^+, NADH, or $NADP^+$, suggesting affinity labeling of the NADPH-binding site of NADPH-oxidase.

Periodate-oxidized nucleotides inhibit other nucleotide-binding proteins through formation of a Schiff's base at active-site lysine residues (Mas and Colman, 1983). Formation of an irreversible inhibitor–enzyme complex between *o*-NADPH and NADPH-oxidase was therefore attempted by chemical reduction of the Schiff's base with sodium cyanoboro[^{3}H]hydride. Radiolabeled proteins were analyzed in the preparation by SDS-PAGE and scintillation counting of gel slices. This treatment resulted in specific labeling of a 66-kD polypeptide in the presence of 0.1 mM *o*-NADPH, and label incorporation was decreased in the presence of excess NADPH.

Further studies showed that the 66-kD polypeptide was labeled in membranes isolated from resting neutrophils and monocytes, but not from lymphocytes, erythrocytes, or platelets, suggesting phagocyte-specific expression of the NADPH-binding protein (Umei *et al.*, 1987). Analysis of membranes from CGD neutrophils demonstrated that the NADPH-binding component was present in four X-linked patients with the cytochrome-b_{559}-negative phenotype, an autosomal cytochrome-b_{559} positive patient, and an autosomal cytochrome-b_{559}-negative patient. All of the patients studied had lower than normal plasma membrane levels of FAD, suggesting a defect in the flavoprotein dehydrogenase component of NADPH-oxidase. If the NADPH-binding protein identified by these studies was identical to the flavoprotein described by others, the data obtained from CGD patients suggested that the defect in the dehydrogenase component was not in the NADPH-binding domain of the protein.

Another pyridine nucleotide analogue, *N*-4-azido-2-nitrophenyl-amino-butyryl-NADPH (NAP_4-NADPH), has been used as a photoaffinity label of

the NADPH-binding component of bovine NADPH-oxidase (Doussiere *et al.*, 1986). This analogue was not a substrate of NADPH-oxidase, but photoirradiation of NADPH-oxidase in the presence of the analogue resulted in NADPH-oxidase inhibition. Kinetic analysis of this light-dependent inhibition by NAP_4-NADPH demonstrated a competitive-type inhibition with respect to NADPH. Similar results were obtained with NAP_4-$NADP^+$ and inhibition was only partially reversible in the presence of high NADPH concentrations, suggesting that photoirradiation in the presence of NAP_4-$NADP^+$ resulted in irreversible photoinactivation of NADPH-oxidase. Photoirradiation of neutrophil membranes in the presence of [^{3}H]NAP_4-NADPH resulted in affinity labeling of several polypeptides when analyzed by SDS-PAGE. A decrease in labeling was observed for a number of these polypeptides in the presence of excess NADPH and particularly for ones of 65 and 23 kD. The 65-kD polypeptide was also found to be labeled to a much lesser extent (20%) in membranes isolated from resting cells. To investigate the relation of this photoaffinity-labeled polypeptide to the 65-kD polypeptide found in purified preparations of bovine NADPH-oxidase (Doussier *et al.*, 1985), fractions obtained during the isolation of bovine NADPH-oxidase were photoirradiated in the presence of [^{3}H]NAP_4-NADPH and analyzed by SDS-PAGE. A correlation was found between the successive purification of NADPH-oxidase and labeling of the 65-kD polypeptide. In the final NADPH-oxidase preparation, the 65-kD polypeptide was the only polypeptide labeled by this photoaffinity probe, in keeping with the previous finding that the major polypeptide component of this preparation was a 65-kD protein.

8-N_3-FAD

The recent synthesis of 8-N_3-FAD provided a useful photoaffinity label with which to study flavoproteins (Fitzpatrick *et al.*, 1985). Since FAD analogues have been readily reconstituted with Triton X-100-solubilized preparations of NADPH-oxidase, 8-N_3-[^{32}P]FAD was synthesized and used as a potential photoaffinity label of the flavoprotein component of crude solubilized NADPH-oxidase preparations (Parkinson and Gabig, 1988). However, light irradiation of NADPH-oxidase preparations reconstituted with the analogue resulted in very low levels of incorporation of radiolabel, consistent with nonspecific labeling. This was likely due to the exposure of the 8-position of the flavin to solvent where the nitrene radical formed by photoirradiation will react predominantly with water. That the 8-position of FAD in NADPH-oxidase may be exposed to solvent was suggested by the reactivity of 8-F-FAD reconstituted with the enzyme to nucleophiles (see above). The possibility that alternative FAD analogues, such as 6-N_3-FAD, might be used more successfully to label the flavoprotein component of

NADPH-oxidase should not be excluded, since some flavoproteins that do not label with 8-N_3-FAD have been labeled with 6-N_3-FAD (Massey *et al.*, 1986).

Diphenyleneiodonium

The inhibitory effects of diphenyleneiodonium (DPI), and structurally related compounds, on NADPH-oxidase activity have been well documented (Cross and Jones, 1986; Cross, 1987; Hancock and Jones, 1987; Ellis *et al.*, 1988). At DPI concentrations equimolar to the FAD content of crude solubilized NADPH-oxidase preparations, 36% inhibition was observed (Cross and Jones, 1986). Inhibition was time dependent and resembled closely the inhibitory effect of DPI on NADPH–ubiquinone reductase, a mitochondrial flavoprotein electron transferase (Ragan and Bloxham, 1977). At a DPI concentration of 2 μM, NADPH-oxidase was 91% inhibited and NADPH-dependent aerobic steady-state reduction of both FAD and cytochrome b_{559} was almost completely abolished. This suggested that DPI was acting within the NADPH-oxidase complex at, or near to, the level of the flavoprotein component. Using [^{125}I]-labeled DPI as an affinity label, SDS-PAGE revealed the presence of one major labeled polypeptide of $\sim$45 kD in preparations obtained from both stimulated and resting cells. Labeling of the 45-kD polypeptide was decreased in the presence of NADPH in both preparations, suggesting that the 45-kD polypeptide and the NADPH-binding site of NADPH-oxidase were in close proximity within the enzyme complex and that the mechanism of NADPH-oxidase activation did not involve alteration in the ability to bind NADPH. Similar results were obtained in macrophages, in which a 45-kD polypeptide component of NADPH-oxidase preparations was also labeled by incubation with [^{125}I]DPI (Hancock and Jones, 1987).

The role of this 45-kD polypeptide has not been established. However, a polypeptide of similar size (45–48 kD) is phosphorylated in stimulated, but not resting, neutrophils (Gennaro *et al.*, 1985; Heyworth and Segal, 1986). In a number of autosomal CGD patients with the cytochrome-b_{559}-positive phenotype, in which the primary defect in NADPH-oxidase may be due to either malfunction of the flavoprotein component or an inability of the cell to effect NADPH-oxidase activation, phosphorylation of this polypeptide was found to be defective (Segal *et al.*, 1985). This suggested that the phosphorylated polypeptide was either a regulatory component of NADPH-oxidase itself or an essential component in the pathway of NADPH-oxidase activation. It is an intriguing speculation to suggest that the 48-kD polypeptide observed in purified NADPH-oxidase preparations (Glass *et al.*, 1986) might be the same as the DPI-labeled polypeptide or even the polypeptide that is phosphorylated during neutrophil stimulation.

A Working Model of NADPH-oxidase and Future Studies

Substantial evidence now exist to support the proposal that the flavoprotein component of NADPH-oxidase is a low-potential NADPH-dependent electron transferase that mediates 2-electron transfer from NADPH to a 1-electron acceptor. In isolated membranes and crude detergent-solubilized preparations of NADPH-oxidase, the prevailing evidence suggests that this 1-electron acceptor is cytochrome b_{559}. However, two groups appear to have recently isolated the flavoprotein component in an active form that is not associated with cytochrome b_{559}, but that directly reacts with O_2 to form O_2^-. (Glass *et al.*, 1986; Kakinuma *et al.*, 1987). This property of the isolated flavoprotein is in keeping with its low midpoint potential (-280 mV) and the known ability of isolated flavoprotein electron transferases to catalyze $O_2^- \cdot$ production. However, both isolated NADPH-oxidase and the isolated flavoprotein are very unstable and are only obtained in low yields by conventional detergent-solubilization and chromatographic purification methods. The most plausible explanation for the inability of any group in the past decade to obtain convincing yields and stable preparations of NADPH-oxidase is that detergent-solubilization of the NADPH-oxidase complex results in irreversible dissociation of essential components of the complex. It is reasonable to assume that the substantial losses of activity reported by all of these studies are caused by detergent-induced FAD depeletion and/or cytochrome b_{559} depletion of the NADPH-oxidase complex. This implies that the components of the activated NADPH-oxidase complex are loosely associated and that isolation of the enzyme is likely to continue to be an exercise in tireless perseverance and saintly patience.

A model for the organization of the NADPH-oxidase complex in the plasma membrane of neutrophils, which is consistent with the data reviewed in this article, is shown in Fig. 7. In the model, the flavoprotein and cytochrome b_{559} components of the NADPH-oxidase complex are arranged vectorially within the membrane in a 1 : 1 complex. NADPH oxidation by the flavoprotein occurs at the cytoplasmic face of the membrane and electrons are then transferred to cytochrome b_{559}, which catalyzes reduction of O_2 to $O_2^- \cdot$ at the extracellular (intraphagolysosomal) face of the membrane. Based on the labeling and SDS-PAGE analyses described, the flavoprotein has been tentatively assigned a molecular weight of 65 kD. The 22-kD and 91-kD polypeptides of cytochrome b_{559} are based on the properties of the isolated protein and the cloned gene for X-linked CGD (see review in this series). The 22-kD polypeptide is assumed to be the heme-binding polypeptide and the catalytic site of $O_2^- \cdot$ production, and is shown in association with the structural 91-kD polypeptide. On a more speculative level, 32-kD and 45-kD polypeptides are also shown. Inclusion of the 32-kD polypeptide is based on

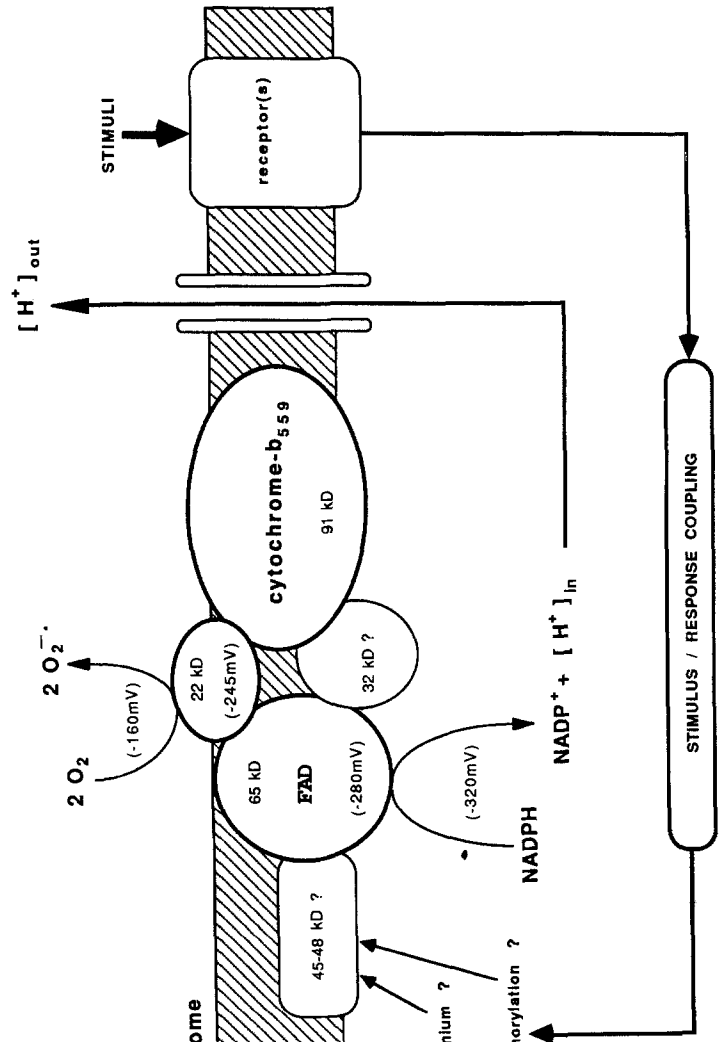

Fig. 7. Organization of the NADPH-oxidase enzyme complex in the plasma membrane of stimulated neutrophils. The model shows a 1 : 1 complex of the flavoprotein (65-kD polypeptide) and cytochrome b_{559} (22- and 91-kD polypeptides) arranged vectorially within the plasma membrane of neutrophils. The flavoprotein is shown to function as an electron transferase, mediating 2-electron transfer from NADPH to cytochrome b_{559}. Compensation for the electrogenic activity of the NADPH-oxidase complex is mediated by a proton pump that transfers H^+ released at the cytoplasmic face of the membrane into the phagolysosome. On the basis of SDS-PAGE analyses, 45- to 48-kD and 32-kD polypeptides are also shown. The 45- to 48-kD polypeptide is indicated as a potential phosphorylation site and/or the site of diphenyleneiodonium inhibition. The complex is shown to be activated via receptor-mediated, stimulus–response coupling mechanisms, which remain to be elucidated.

SDS-PAGE analysis of NADPH-oxidase preparations (Glass *et al.*, 1986; Serra *et al.*, 1984). The 45-kD polypeptide is included on the basis of studies described in this review and is indicated as a potential site of phosphorylation and/or the site of action of DPI. Protons released on the cytoplasmic face of the plasma membrane as a result of NADPH oxidation are shown to be pumped across the membrane by a proton channel (Henderson *et al.*, 1988). The activity of this channel compensates for net translocation of a negative charge and neutrophil membrane depolarization that is caused by the activation of the electrogenic NADPH-oxidase complex (Henderson *et al.*, 1987).

Several features of this model may be directly addressed by future studies. It should be a relatively simple matter to settle the identity of the flavoprotein polypeptide since this polypeptide should also be the NADPH-binding site of the enzyme complex. Isolated preparations of NADPH-oxidase or the flavoprotein component could be affinity labeled using either the cyanoboro[^{3}H]hydride/*o*-NADPH or the NAP_4-NADPH method. Along the same lines, affinity labeling with [^{125}I]DPI in such preparations should also be initiated. These studies would establish the presence of the 45- to 48-kD polypeptide in the complex and would facilitate identifying its relation to a similar-sized polypeptide whose phosphorylation is defective in CGD patients. Since little is known regarding the precise molecular mechanism utilized by stimulated phagocytes to activate NADPH-oxidase, identification of any regulatory polypeptide within the complex would represent a major step forward in our understanding of this crucial enzyme.

Providing sufficient quantities of the flavoprotein component can be obtained by present and improved methods, it should be possible to study the electron transfer properties of this component in more detail. In particular, it would be of considerable interest to determine the rate constants for reduction and oxidation of enzyme-bound FAD. The fluorescent properties of the flavoprotein might be used for this purpose. Knowledge of these kinetic constants would be invaluable for determining the validity of the proposed model, and methods already described for crude preparations of solubilized NADPH-oxidase, in which FAD and cytochrome b_{559} reduction are readily measurable, could be used for such studies (Cross *et al.*, 1985). Finally, reconstitution experiments might establish the interaction of the isolated flavoprotein with purified preparations of cytochrome b_{559}. In this respect, the recent development of a cell-free system for NADPH-oxidase activation and reliable methods for the isolation of cytochrome b_{559} promise to be of great value (see reviews in this series).

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