Phagocyte NADPH-oxidase

STUDIES WITH FLAVIN ANALOGUES AS ACTIVE SITE PROBES IN TRITON X-100-SOLUBILIZED PREPARATIONS*

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NADPH-oxidase of stimulated human neutrophil membranes was solubilized in Triton X-100 and activity reconstituted with FAD, 8-F-FAD, 8-phenyl-S-FAD, and 8-S-FAD. The enzyme had similar affinities for all the flavins with $K_m$ values in the 60–90 mM range. $V_{max}$ was found to increase 4-fold with increasing redox midpoint potential of the flavin. 8-F-FAD reconstituted with the enzyme was reactive toward thiophenol, suggesting exposure of the 8-position to solvent, a finding supported by unsuccessful attempts to label the enzyme with the photoaffinity probe 8-Na-[32P]FAD. Solubilized oxidase stabilized the red thiolate form of 8-S-FAD, a characteristic of flavoproteins of the dehydrogenase/electron transferase classes which stabilize the blue neutral form of the flavin semiquinone radical.

Phagocytes contain a unique membrane-associated NADPH-oxidase enzyme, dormant in resting cells, which becomes activated in stimulated cells and catalyzes the 1e− reduction of molecular oxygen to the superoxide anion $O_2^-$ (1). The central role played by the oxidase in the microbicidal functions of phagocytes has long been recognized, and absence of oxidase activity is associated with chronic granulomatous disease (CGD), a syndrome characterized by recurrent, chronic, and often fatal infections (2). A major thrust of phagocyte research in the past decade has thus been to characterize the molecular nature of the oxidase and the cellular mechanisms evoked for its activation (3, 4).

Early reconstitution experiments with Triton X-100-solubilized NADPH-oxidase demonstrated a requirement for FAD as a cofactor (5, 6). This solubilized form of the enzyme could also be reconstituted with 8-chloro- and 1-deaza-FAD and was strongly inhibited by the obligate 2e− donor, 5-deaza-FAD (7). At about the same time it was also demonstrated that phagocytes contained a unique low-potential 6-type cytochrome (cytochrome $b_{245}$) which had oxidase-like properties (8, 9) and was absent from the phagocytes of the majority of CGD patients (10, 11). These and numerous subsequent studies (12–14) led to the proposal that NADPH-oxidase was likely to be an electron transport enzyme complex of an FAD-linked flavoprotein and cytochrome $b_{245}$, arranged as shown in Scheme 1.

\[
\text{NADPH} \rightarrow 2e^- + 2H^+ \rightarrow \text{FAD} \rightarrow 2e^- + \text{cytochrome } b_{245} \rightarrow 1e^- + O_2 \\
\text{Scheme 1}
\]

The rewards of many years of persistent research have recently led to the purification, by two independent groups, of cytochrome $b_{245}$ (15–17) and to the cloning of the putative gene responsible for the X-linked form of CGD (18). Cytochrome $b_{245}$ purified from human neutrophils appears to be a heterodimer comprising a small 22-kDa polypeptide and a heavily N-glycosylated polypeptide of approximately 90 kDa. This latter polypeptide was recently shown by amino acid sequencing studies (16) and immunologic studies using synthetic peptides derived from the cloned X-CGD gene (19) to be that encoded by the X-CGD gene. Immunologic studies with antibodies raised against purified cytochrome $b_{245}$ indicate that the 22-kDa polypeptide is likely to contain the binding site for protoporphyrin-IX (17), in agreement with the lack of homology between the X-CGD gene and genes of other cytochromes (18).

In contrast to the detailed knowledge that has been obtained about cytochrome $b_{245}$, our knowledge of the flavoprotein component of NADPH-oxidase is scant. The protein has not been unequivocally purified nor has its gene been identified and cloned. It is known that the plasma membranes of neutrophils contain noncovalently protein-bound FAD at approximately equimolar concentrations to cytochrome $b_{245}$ and that the flavoprotein is fluorescent with spectral characteristics similar to other fluorescent flavoproteins (12, 20). The flavoprotein can be resolved from cytochrome $b_{245}$ (with subsequent loss of NADPH-oxidase activity) by selective bile salt extraction of neutrophil plasma membranes, and this flavoprotein may be missing in the neutrophils of some CGD patients (21).

The biophysical properties of the flavoprotein have been studied using anaerobic EPR techniques which detect signals generated by flavin semiquinone radicals at $g$-values of approximately 2.004 (22). The 19 G peak-to-peak spectral line width of the neutrophil flavin radical indicated that the flavoprotein stabilizes a neutral "blue" semiquinone. Potentiometric titration of the EPR signal revealed a redox midpoint potential ($E_m$) of $-280$ mV, sufficiently low for the flavoprotein to act as physiological reductant for cytochrome $b_{245}$. Interestingly, although the flavoprotein from both stim-
ulated and unstimulated cells gave a semiquinone EPR signal upon chemical reduction with dithionite, only the flavoprotein from stimulated cells gave rise to an EPR signal upon the addition of NADPH.

In the present studies we have attempted to characterize the flavoprotein component of NADPH-oxidase further by making use of 8-substituted FAD analogues in reconstitution experiments with Triton X-100-solubilized preparations of the human neutrophil enzyme. The use of such analogues as active-site probes of flavoproteins has recently been reviewed (23, 24). 8-N2-FAD has been used as a photoaffinity label for flavoproteins (25). The chemical reactivity of 8-halogen-substituted flavins toward thiols can be used to assess the solvent accessibility of this position of the isoalloxazine ring (26). 8-S-FAD can be used as a spectral probe: various spectrally distinct, resonance-stabilized forms of this analogue exist and are differentially bound by certain classes of flavoproteins (24, 27).

MATERIALS AND METHODS

Ferricytochrome c (type VI), superoxide dismutase, phorbol 12-myristate 13-acetate, NADPH, NADH, NaN3, FMN, FAD, and Naja naja snake venom were from Sigma. Triton X-100 was from RPI Corp., Grove Village, IL. Thiophenol was from Aldrich. All other reagents were of highest purity commercially available. 8-F-FAD was a very generous gift of Dr. Vincent Massey, University of Michigan, Ann Arbor. FAD-synthetase, partially purified from *Brevibacterium ammonigenes* was a very generous gift of Drs. Dietmar J. Manstein and Edna E. Fai, Max-Planck-Institute for Medical Research, Heidelberg, West Germany. [32P]ATP (3000 Ci/mmol) was from Du Pont-New England Nuclear. Perkin-Elmer λ-UV/Vis and MFP-66 Fluorescence Spectrophotometer linked to a Perkin-Elmer 7500 Professional computer were used for spectroscopic measurements.

Isolation and Stimulation of Human Neutrophils— Buffy coat preparations from normal healthy donors were used as a source of human neutrophils. Neutrophils were isolated by dextran sedimentation, Ficoll-hypaque centrifugation, and hypotonic lysis of erythrocytes, as described (28). Isolated neutrophils were suspended in PBS supplemented with 1 mM MgCl2, 0.5 mM Ca2+, 4% v/v ethylene glycol, 0.3% v/v Triton X-100, and 1 mM NaN3 at approximately 5 × 10^6 cells/ml. The cell suspension was brought to 37 °C in a shaking water bath and stimulated for 20 min by the addition of 1 µg/ml of PMA. The reaction was stopped by flow of a 1 ml/min. Purified 8-F-[32P]FAD was lyophilized and converted to 8-N2-F-[32P]FAD by reaction with NaN3 as described (29).

Reconstitution Assays—NADPH-oxidase in plasma membranes isolated from phorbol 12-myristate 13-acetate-stimulated cells was solubilized with Triton X-100, reconstituted with FAD or appropriate analogues and assayed as described (7). Plasma membranes (2 mg/ml in PBS-sucrose) were mixed with an equal volume of 0.4% v/v Triton X-100, 50% v/v ethylene glycol, 20 mM Tris, pH 8.0, and sonicated as described above. Aliquots were then reconstituted with FAD or 8-substituted FAD analogues, left on ice for 1 h and assayed for NADPH-oxidase activity. Assays were performed at 20 °C in 300-μl microcuvettes in a buffer containing 100 μM ferricytochrome c, 50 mM triethanolamine-HCl, pH 8.0 (31), supplemented with 1 mg/ml phosphatidylethanolamine (6).

Flavin Assay—Fluorimetric assays of FAD content were performed as described (32).

Cytochrome b5—Cytochrome b5 content was assayed by dithionite-reduced-minus-air-oxidized difference absorbance spectroscopy as previously described (9).

Protein Assay—Protein assays were performed using the Pierce BCA Protein Assay Kit, using bovine serum albumin as standard (50).

RESULTS

Kinetic Data—As previously described by others (6, 7), solubilization of neutrophil plasma membranes with Triton X-100 resulted in substantial loss of NADPH-oxidase activity. The comparative ability of FAD and the 8-substituted FAD analogues to reconstitute NADPH-oxidase activity when added to solubilization buffers was investigated, and the results are summarized in Fig. 1 and Table I. All the flavins tested exhibited similar affinities for the enzyme with apparent Km values in the range 60–80 mM; the apparent Kc of Triton X-100-solubilized NADPH-oxidase for FAD has previously been determined at 61 nm (34). 8-F-FAD (Em = -152 mV) and 8-phenyl-S-FAD (Em = -171 mV) were found to have Vmax values approximately 2–3-fold greater than FAD (Em = -219 mV); the low-potential 8-S-FAD (Em = -290 mV) gave a Vmax value of only 60% relative to that of FAD. The stimulatory effects of relatively high-potential flavin analogues on reconstituted NADPH-oxidase activity has previously been observed for 8-Cl-FAD and 1-deaza-FAD (7). These results were interpreted to suggest that reduction of FAD by NADPH during turnover of NADPH-oxidase is partially rate-determining.

Spectral Data—Observation of the spectral characteristics of FAD analogues added to solubilized NADPH-oxidase preparations proved to be difficult, as the absorbance levels were very low. These problems were largely overcome by using a computer-linked spectrophotometer capable of storing and averaging a large number of spectra. The reaction of thiophenol with 8-F-FAD that had been reconstituted with solubilized NADPH-oxidase is shown in Fig. 2a. The characteristic absorbance spectrum of 8-F-FAD (Em' = 11.7 nm2 cm−1, 438 nm) was observed to shift dramatically after addition of thiophenol to that characteristic of 8-phenyl-S-FAD with an absorbance maximum at approximately 475 nm (Em' = 23.6 nm2 cm−1) and a pronounced shoulder at 455 nm (29). This reaction, which was complete 8-
FIG. 1. Double-reciprocal plots of reconstituted solubilized NADPH-oxidase activity \( (V = \text{nanomoles } O_2/\text{min/mg}) \) as a function of the concentration of added FAD or 8-substituted FAD analogues \( (S = \text{nm}) \). Plasma membranes isolated from phorbol 12-myristate 13-acetate-stimulated human neutrophils were solubilized in 0.2% v/v Triton X-100 and reconstituted with exogenous flavins as described under “Materials and Methods.” NADPH-oxidase activity was measured as the initial rate of superoxide dismutase-ligated in 0.2% v/v Triton X-100 and reconstituted with exogenous flavodoxin, as opposed to that for the anionic paraquinoid thiolate form of 8-S-FAD that is preferentially stabilized by 20 min at 15°C, suggested that the 8-position of 8-F-FAD was exposed to solvent. The additional evidence that the flavoprotein component functional incorporation of the flavoprotein component of NADPH-oxidase provided the initial impetus for the present study.

Further spectral data were obtained by reconstitution of 8-S-FAD with solubilized NADPH-oxidase, as shown in Fig. 2b. The spectral characteristics of 8-S-FAD and 8-phenyl-S-FAD are similar to that published for 8-S-riboflavin, and 8-F-FAD to that published for 8-Cl-FAD. The spectral characteristics of 8-S-flavins bound to a number of flavoproteins have been described (27). The spectrum resembles most closely that described for the anionic thiolate form of 8-S-FAD that is preferentially stabilized by flavodoxin, as opposed to that for the anionic paraquinoid form of 8-S-FAD that is stabilized by glucose oxidase and which has a resolved three-banded spectrum in the region of 600 nm.

**Photoaffinity Labeling Studies**—The reactivity of 8-F-flavins toward nucleophiles has recently been exploited to make 8-N\(_3\)-flavins which have proven to be, like other arylazides that produce highly reactive nitrene radicals upon photoirradiation, useful photoaffinity labels (25). Due to the low concentrations of flavins used in this study it was not possible to determine the extent of covalent incorporation of photoirradiated 8-N\(_3\)-FAD into samples by spectroscopic means. Synthesis of radiolabeled 8-N\(_3\)-FAD was required and the method described in the present studies provided 8-N\(_3\)-[\(^{32}\)P]FAD labeled to a specific activity of 50–200 cpm/pmol, sufficiently high to permit scintillation counting as a means for determining the extent of covalent incorporation.

To prevent interference by endogenous FAD, solubilized oxidase preparations were subjected to Sephadex G-25 chromatography before reconstitution with 8-N\(_3\)-[\(^{32}\)P]FAD. The radiolabeled photoaffinity probe was then added to the sample of solubilized NADPH-oxidase, in the dark, at an equimolar concentration to the cytochrome b\(_{559}\) concentration of the sample and left on ice to allow reconstitution. The sample was then photoirradiated in a melting ice-water bath as described (35), and noncovalently bound 8-N\(_3\)-[\(^{32}\)P]FAD removed by precipitation in trichloroacetic acid and dialysis against 8 m guanidine hydrochloride overnight (25). The percent incorporation of 8-N\(_3\)-[\(^{32}\)P]FAD was determined by scintillation counting of the dialysate.

Light irradiation of solubilized oxidase that had been incubated on ice with the photoaffinity label for 4 h and overnight resulted in very low levels of covalent incorporation of the radiolabel: 0.4 and 0.6%, respectively. These low levels of incorporation are consistent with nonspecific labeling and a further indication that the 8-position of the flavin may well be exposed to solvent, where the nitrene radical generated by photoirradiation will react predominantly with water (25). These results precluded any further investigations by electrophoresis to determine the identity of any polypeptides that may have been labeled by the probe.

**DISCUSSION**

Although Triton X-100 has not proven to be a useful detergent for purification of NADPH-oxidase, it has enabled reconstitution experiments to be performed that have revealed FAD as an essential electron transfer component of the enzyme (6). Reconstitution with FAD analogues has also been achieved using this approach and has provided some information regarding the catalytic role of FAD in the transfer of electrons from NADPH to O\(_2\) (7). The present study generally confirms and expands upon these earlier reports and provides independent evidence that the flavoprotein component functions as a dehydrogenase.

The chemical reactivity of the 8-CH\(_3\) position of the flavin isoalloxazine ring has enabled the introduction of several functional groups to this position and has provided a variety of useful active-site probes of flavoproteins (24). 8-F-flavins, which have greater reactivity toward nucleophiles than 8-Cl-flavins, have only recently been synthesized (36) and have been used to make 8-N\(_3\)-flavins as photoaffinity labels of flavoproteins (25). The possibility that radiolabeled 8-N\(_3\)-FAD might be used to label directly and thereby identify the flavoprotein component of NADPH-oxidase provided the initial impetus for the present study.

Photoirradiation of solubilized NADPH-oxidase reconstituted with 8-N\(_3\)-[\(^{32}\)P]FAD did not result in significant covalent incorporation of the probe, indicating that the 8-position of the FAD was likely to be exposed to solvent. The nonspecificity of the reaction suggested that further analysis of samples by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography would not reveal any relevant information regarding the identity of the flavoprotein compo-

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**TABLE I**

<table>
<thead>
<tr>
<th>Flavin</th>
<th>( K_m ) (nM) mean ± S.D.</th>
<th>( V_{max}^{*} ) (µM) mean ± S.D.</th>
<th>( E_{0}^{*} ) (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FAD</td>
<td>78 ± 5</td>
<td>63 ± 7</td>
<td>-219</td>
</tr>
<tr>
<td>8-S-FAD</td>
<td>69 ± 10</td>
<td>38 ± 6</td>
<td>-290</td>
</tr>
<tr>
<td>8-Phenyl-S-FAD</td>
<td>62 ± 12</td>
<td>158 ± 9</td>
<td>-171</td>
</tr>
<tr>
<td>8-F-FAD</td>
<td>66 ± 7</td>
<td>140 ± 15</td>
<td>-152</td>
</tr>
</tbody>
</table>

\( V_{max}^{*} \) has units of nanomoles O\(_2\)/min/mg of protein.

\( E_{0}^{*} \) is the midpoint potential of the flavin as described under "Materials and Methods."
Fig. 2. Absorbance spectra of solubilized NADPH-oxidase reconstituted with 8-substituted FAD analogues. Triton X-100-solubilized NADPH-oxidase samples were passed through a column of Sephadex G-25 to remove endogenous FAD and immediately used for spectral studies. Computer-derived difference absorbance spectra were obtained by subtracting an averaged base-line spectrum (n = 32) from spectra obtained after the addition of FAD analogues (n = 32). Spectra were recorded in 200-μl microcuvettes thermostatted at 10°C. a, the cytochrome b$_{245}$ content in the preparation was 220 pmol/ml; i, difference spectrum after reconstitution with 211 pmol/ml 8-F-FAD; ii, difference spectrum 20 min after the addition of 1 μl of a 1:1000 dilution of thiophenol in methanol (27). b, the cytochrome b$_{245}$ content of the preparation was 70 pmol/ml. Difference spectrum obtained after reconstitution with 65 pmol/ml 8-S-FAD.

The absorbance spectrum obtained for 8-S-FAD reconstituted with solubilized NADPH-oxidase (absorbance maximum 530 nm) was similar to 8-S-FMN reconstituted with M. eldenii flavodoxin and with NADPH-cytochrome P-450 reductase (27). This spectrum is characteristic for the ionized form of 8-S-FAD in which the negative charge is localized at the 8-position of the flavin and is referred to as the red "thiolate" form. This contrasts to the other extreme resonance-stabilized form of 8-S-flavin in which the charge is localized at the N(1)→C(2) = 0 locus. This form, observed on reconstitution of 8-S-FAD with glucose oxidase and D-amino acid oxidase, is characterized by a resolved three-banded spectrum centered at 600 nm and is referred to as the blue "paraquinoid" form.

That Triton X-100-solubilized NADPH-oxidase appears to bind the red thiolate form of 8-S-FAD is of interest since there is a general correlation between the form of 8-S-flavin bound by flavoproteins and the type of flavin semiquinone radical that they stabilize. Flavoproteins of the dehydrogenase/electron transferase classes (e.g. flavodoxin and NADPH-cytochrome P-450 reductase) and which bind the red thiolate form of 8-S-flavin are also found to stabilize the blue neutral semiquinone radical (23, 24, 27). This contrasts to flavoproteins of the oxidase class (e.g. glucose oxidase) which preferentially bind the blue paraquinoid form of the flavin semiquinone radical.

The present studies would appear to be in agreement with a previous report that plasma membranes isolated from stimulated neutrophils contain a flavoprotein that stabilizes a blue neutral flavin semiquinone radical when reduced either chemically by the addition of dithionite or enzymatically by the addition of NADPH, the physiological electron donor for NADPH-oxidase (22). Taken together, these findings would place the flavoprotein component of NADPH-oxidase in the dehydrogenase/electron transferase class of flavoproteins and would logically lead to certain predictions that future studies might address.

First, a characteristic of flavoprotein oxidases is that the N(5)→N(4) = 0 of the flavin ring is exposed to solvent rendering it susceptible to nucleophilic addition by sulfite, a reaction characterized by bleaching of the normal flavin absorbance and fluorescence spectra (27, 30). The flavoprotein component of the NADPH-oxidase of neutrophil membranes should not form this sulfate adduct. Secondly, 6-N$_{3}$FAD has recently been synthesized (39) and might be used as an alternative photoaffinity label to identify the polypeptide containing the FAD-binding domain of the NADPH-oxidase complex. Two flavoproteins, Old Yellow Enzyme and D-Amino Acid Oxidase, previously shown to have an exposed 8-position of the flavin ring, exhibit extensive covalent labeling using 6-N$_{3}$-flavins as photoaffinity labels (40).

A discussion of the data presented in this study would not be complete without considering problems that arise when working with crude systems. This question is of particular importance with regard to the conclusions drawn from the spectroscopic studies that have been described. It is quite possible that the spectra obtained represent composites of flavins reconstituted with both NADPH-oxidase and other undefined flavoproteins that may be present in the plasma membrane fraction used for this study. Neutrophils are known to contain NADH-cytochrome b$_{5}$ reductase activity (41). However, this enzyme, in contrast to NADPH-oxidase, is stable to treatment with both Triton X-100 and Triton X-114, a property which has facilitated its purification to homogeneity from neutrophil plasma membranes (42). This study also revealed that NADH-cytochrome b$_{5}$ reductase only contributes 5% of the total FAD content of neutrophil plasma membranes.

Comparison of the cytochrome b$_{245}$ and FAD content of plasma membranes isolated from normal and CGD neutrophils has been made (12, 20, 21, and 43). These studies reveal several phenotypic variants of CGD that can broadly be characterized as (a) those in which cytochrome b$_{245}$ levels are essentially zero but where the FAD levels are normal, (b)...
those in which the levels of both cytochrome b$_{558}$ and FAD are substantially lower than normal, and (c) those in which cytochrome b$_{558}$ levels are normal but the level of FAD is less than 10% of normal. Decreased levels of FAD in CGD neutrophil plasma membranes has been shown to correlate to decreases in the amount of a fluorescent flavoprotein that is present in normal neutrophil plasma membranes and that the spectral data obtained will require pure preparations of oxidase and/or the flavoprotein component. It should be noted that, even with readily available neutrophil plasma membranes, the present studies were performed at the sensitivity limits of absorbance spectroscopy. Current methods available for purification of NADPH-oxidase do not provide preparations of sufficient quantity or concentration to allow these studies to be performed.

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REFERENCES

38. Deleted in proof