Use of an Affinity Label to Probe the Function of the NADPH Binding Component of the Respiratory Burst Oxidase of Human Neutrophils*

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The respiratory burst oxidase of neutrophils can be activated in a cell-free system in which solubilized membranes, cytosol, and Mg++ are required and in which sodium dodecyl sulfate is used to convert the dormant oxidase to an active form. The 2',3'-dialdehyde analog of NADPH was used as an affinity label for the cytosolic NADPH-binding component of the respiratory burst oxidase from human neutrophils. When treated with this affinity label in the presence of sodium cyanoborohydride to reduce Schiff bases, neutrophil cytosol was shown to lose at least 90% of its activity in the cell-free system. In contrast to normal cytosol, treated cytosol had lost its ability to abolish the lag time required for activation of the oxidase, suggesting that the treated cytosol was no longer able to participate in the rate-limiting activation step. Furthermore, the treated cytosol had lost its ability to convert the oxidase from a form with a high K_m to a form with a low K_m for NADPH. The ability of dialdehyde-treated cytosol to activate the oxidase could be restored by untreated cytosol with a concentration dependence suggesting that only one kinetically active component of the oxidase was inhibited by treatment with the NADPH analog. Like the dialdehyde-treated cytosol, cytosols from patients with chronic granulomatous disease caused by a deficiency in a cytosolic protein (pp47) fail to participate in the biochemical processes leading to activation can be identified. Experiments using these cell-free systems have demonstrated that activation requires the simultaneous presence of components contributed by cell membranes (either particulate or solubilized) and components contributed by the cytosol. In these systems, activation of the oxidase is initiated by the addition of a detergent (e.g. arachidonate (3-6) or SDS† (7)) or of protein kinase C plus ATP (8).

Studies validating these cell-free oxidase activation systems have been performed using constituents prepared from the cells of patients with chronic granulomatous disease (CGD), a condition characterized by the inability of phagocytes from affected individuals to generate O_2 (2, 9, 10) owing to a defect in one of the components of the respiratory burst oxidase or its activating apparatus. These studies have demonstrated that in some forms of CGD, the defective component is in the membrane (3, 7, 11, 12), while in others it is in the cytosol (13, 14). Cytosol from the latter group of CGD patients is incapable of supporting oxidase activation in the cell-free system (13-16).

Although cytosol factor activity has been eluted from a gel filtration column in fractions corresponding to an apparent molecular mass of 240 kDa (12), attempts to isolate this activity as a single unitary component have not been successful. Studies of the kinetics of activation of the respiratory burst oxidase in a cell-free system have accounted for this failure by demonstrating a nearly third order relationship between the concentration of cytosol and oxidase activity (14, 17). These findings suggest a mechanism in which at least three kinetically distinct cytosolic factors are required for the activation process (17). This mechanism has recently been confirmed by work showing that (a) the cytosol "faster" can be separated into at least four different components (18), and

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The abbreviations used are: SDS, sodium dodecyl sulfate; CGD, chronic granulomatous disease; PIPES, 1,4-piperazinediethanesulfonic acid; EGTA, (ethylenebis(oxyethylenenitrilo)tetraacetic acid.
(b) CGD can be caused by a deficiency in either of two of these cytosolic constituents (15, 16, 18). The functions of these cytosolic constituents and the nature of their participation in the activity of the oxidase, however, have not yet been defined.

Using periodate-oxidized NADPH ("NADPH dialdehyde") as an affinity label, we have obtained evidence strongly suggesting that in resting neutrophils, one of the cytosolic constituents is the NADPH binding component of the respiratory burst oxidase (19). In the present paper, we describe experiments using cytosol in which the NADPH binding component of the oxidase has been irreversibly inactivated by NADPH dialdehyde. This inactivated cytosol was employed as a reagent to probe the kinetic role that the NADPH binding component plays in the activation of the oxidase in a cell-free system. Our studies show that the NADPH binding component participates both in the rate-limiting step in oxidase activation and in the modulation of the affinity of the oxidase for NADPH. Results obtained by mixing the inactivated cytosol with cytochrome from patients with cytosol-defective CGD (type II, Scripps classification (11)) further suggest that in resting neutrophils, the NADPH binding component occurs in the form of a slowly dissociating complex that contains at least one other cytosolic oxidase component, and that this complex participates in oxidase activation as a single kinetic species.

MATERIALS AND METHODS

Reagents were the best grade commercially available and were used without further purification. SDS (electrophoresis purity grade) and sodium deoxycholate (Ultrol grade) were obtained from Bio-Rad and Calbiochem, respectively. Neutrophils and neutrophil subcellular fractions from normal donors were prepared as described previously (18). The protein contents of the subcellular fractions have been previously reported as follows (17): cytosol, 250 ± 42 (S.D.) μg/10⁷ cell eq; deoxycholate-solubilized membranes, 25.7 ± 7.4 μg/10⁷ cell eq. The cytosolic preparations were adjusted prior to aliquoting so that they contained 9 × 10⁷ cell eq/ml.

Cell-Free Activation of the Respiratory Burst Oxidase—Activation of the oxidase in the cell-free system was monitored by following the superoxide dismutase-inhibitable reduction of cytochrome c with time at 550 nm in a thermostatted dual beam recording spectrophotometer, using slight modifications of published methods (17). Unless otherwise noted, assay mixtures contained 0.1 mM cytochrome c, 6.5 mM sodium deoxycholate (Ultrol grade) were obtained from Bio-Rad and Calbiochem, respectively. Neutrophils and neutrophil subcellular fractions from normal donors were prepared as described previously (18). The protein contents of the subcellular fractions have been previously reported as follows (17): cytosol, 250 ± 42 (S.D.) μg/10⁷ cell eq; deoxycholate-solubilized membranes, 25.7 ± 7.4 μg/10⁷ cell eq. The cytosolic preparations were adjusted prior to aliquoting so that they contained 9 × 10⁷ cell eq/ml.

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In the assays in which the concentration of cytosol was varied systematically, incubations were carried out in 96-well plates as previously described (18). Reactions were initiated by the simultaneous addition of SDS to all wells after a 1-min equilibration period at 37 °C. O₂ production was followed by measuring A₅₅₀ at 5-s intervals with a kinetic microplate reader (Vmax, Molecular Devices Corp., Menlo Park, CA), calculating the maximum rate of absorbance change with a kinetic software package (Softmax Release 2.0, Molecular Devices Corp.) that monitored dVₕ/dt as a function of time. With the exception of the cytosol content, the compositions of the mixtures were as described above, but the reaction volume was 150 μl. For each sample, Vₚ, the activity of the oxidase, was calculated from the difference between the maximum rates of cytochrome c reduction in the sample and an otherwise identical reaction in an otherwise identical reaction containing superoxide dismutase (extinction coefficient 20.5 mmol⁻¹ cm⁻¹) (18). Reaction orders were calculated by nonlinear least squares regression, fitting the observed data to the equation

\[ V_p/p = \left( \frac{V_m}{V_m - V_{II}} \right) \left( \frac{[S]}{K_s + [S]} \right) \]

where p represents a computer-derived proportionality constant.

Preparation of NADPH Dialdehyde and Its Incorporation into the Cytosolic Component—NADPH Dialdehyde Synthesis—NADPH dialdehyde was synthesized according to a modification of the procedure of Mas and Colman (20). The NADPH dialdehyde content of the final preparation was calculated assuming an extinction coefficient \( \epsilon_{230} = 6.22 \text{ mM}^{-1} \text{ cm}^{-1} \). NADPH dialdehyde was identified as the peak eluting at 0.15 M triethanolamine HCl. The fractions containing this peak were pooled, reduced to NADPH dialdehyde with isocitric acid and isocitric dehydrogenase, and subjected to ion exchange chromatography to isolate the dialdehyde as described in the original method (20). The NADPH dialdehyde content of the final preparation was calculated assuming an extinction coefficient \( \epsilon_{230} = 6.22 \text{ mM}^{-1} \text{ cm}^{-1} \), and aliquots were stored in 0.3 M triethanolamine buffer (pH 8.0) at −70 °C until use. The final preparation of NADPH dialdehyde yielded a single UV-absorbing peak on TLC analysis (19) and contained a single peak (96% purity) absorbing at both 260 and 340 nm on fast protein liquid chromatography analysis with a Mono Q column.

Covalent linkage of the NADPH dialdehyde to the NADPH binding site of the oxidase was performed by incubation of neutrophil cytosol with 0.1 mM NADPH dialdehyde for 30 min at 4 °C, then adding NaCNBH₃ (0.5 mM final concentration) and continuing the incubation for an additional 24 h. As described previously (19), this procedure typically results in the loss of more than 90% of the activity of the oxidase as assayed in the cell-free activation system when compared to cytosol incubated for similar periods with buffer alone, or with buffer and NaCNBH₃. Following the 24-h incubation, the dialdehyde-treated cytosol (2–10 ml) was dialyzed at 4 °C against 1-liter portions of buffer containing 100 mM KCl, 3 mM NaCl, 3.5 mM MgCl₂, and 10 mM PIPES (pH 7.3), using Mₚ = 3500 exclusion membranes (Spectraperm, Spectrum Medical Industries, Los Angeles, CA) and changing the buffer hourly × 4. The cytosol (protein concentration 260 ± 19.8 μg/10⁷ cell eq (n = 41)) was divided into aliquots and stored at −70 °C until use. In the sample used for competition analysis (see below), more complete inactivation of the NADPH binding component was accomplished by treating the cytosol with 0.2 mM NADPH dialdehyde and 1.0 mM NaCNBH₃, according to the foregoing protocol.

Patients—The neutrophils from three patients with Type II cytosol-positive CGD were used to provide cytosol deficient in defined oxidase components (11, 13). Other work (15, 16, 18, 21–23) suggests that these cytosols are probably all deficient in a Mₚ = 47,000 phosphoprotein that has been shown to be closely related to the respiratory burst oxidase; accordingly, they will be designated as pp47-deficient cytosols. Each of the three patients was known to have a severe deficiency of cytosol factor activity (<4% in each case). One of the pp47-deficient type II CGD patients (J. C.) has been previously described (13, 14, 15).

RESULTS

O₂ Production by Cell-free Systems Containing NADPH Dialdehyde-treated Cytosol in the Absence and Presence of Untreated Cytosol—Little oxidase activity was generated by a cell-free system containing dialdehyde-treated cytosol (Fig. 1A, O), while oxidase activity (Vₚ) in a system containing normal cytosol increased as a power of the cytosol concentration (Fig. 1A, O), as previously observed:

\[ V_p = [\text{cytosol}]^r \]

In earlier studies, the exponent r, equal to the reaction order for cytosol, was found to be 2.5 (14, 17). This value has been interpreted in terms of a requirement for at least three cyto-
NADPH Binding Component of the Respiratory Burst Oxidase

The effect of dialdehyde-treated cytosol on the kinetics of the respiratory burst oxidase

Cell-free oxidase activation assays were carried out over a range of NADPH concentrations with the indicated quantities of normal and dialdehyde-treated cytosol as described under "Materials and Methods." Kinetic constants were calculated by nonlinear least squares regression using the equation $V = V_{\text{max}}[\text{NADPH}]/(K_\text{m} + [\text{NADPH}])$. The data shown represent the mean ± S.E. of the calculated parameters for each set of cytosol combinations. The number of assays for each set of data is shown in parentheses.

| Cytosol        | Untreated | Treated | $K_m$ (µM) | $V_{\text{max}}$ (nmol O$_2^-$ min$^{-1}$/10$^7$ cell eq membrane) |
|----------------|-----------|---------|------------|---------------------------------------------------------------|
| cell eq × 10$^{-7}$/assay | µM        |          |            |                                                               |
| 0.36           | None      | 6        | 114 ± 22.5 | 6.5 ± 1.0                                                      |
| None           | 1.0       | 2        | 118 ± 0.8  | 10.7 ± 1.1, 117                                              |
| 1.26           | None      | 1        | 36.7       |                                                               |
| 0.36           | 0.9       | 3        | 81.6 ± 8.2 | 29.8 ± 11.3                                                   |

Previous studies with the cell-free activation system, we found that oxidase generated at low cytosol concentrations showed a $K_m$ for NADPH of ≈130 µM, while at high cytosol concentrations, the $K_m$ for the same substrate shifted to ≈30 µM (14, 17). The pp47-deficient cytosols could mediate the same shift in $K_m$ when used to supplement a cell-free system containing limiting amounts of normal cytosol, even though by themselves the defective cytosols supported little oxidase activity at any concentration (14). This finding suggests that the CGD cytosols possessed normal quantities of the cytosolic factor(s) responsible for this shift. In similar experiments, we tested the ability of the dialdehyde-treated cytosol to alter the $K_m$ of the low affinity form of the oxidase. The results (Table I) show that, like oxidase generated using low concentrations of normal cytosol, the residual oxidase formed with dialdehyde-treated cytosol alone had a low affinity for NADPH (i.e. a high $K_m$). Furthermore, the low affinity oxidase generated using limiting amounts of normal cytosol was not converted to a high affinity form when the reaction mixture was supple-
The ability of untreated or dialdehyde-treated cytosol to abolish the lag in activation of the oxidase was examined using minor modifications of a previously described method (14). The cytosol or cytosol equivalent to be tested (1 \times 10^{12} cell eq of treated or untreated cytosol, or buffer) was combined with solubilized neutrophil membrane and all the components of the cell-free oxidase activation assay except NADPH. At \( t = 3.5 \) min, SDS (37 \( \mu \)M during preincubation, 32 \( \mu \)M during assay) was added, and the mixture was allowed to incubate at 25 \(^\circ\)C. At \( t = 0 \), \( O_2 \) generation was initiated by the addition of NADPH together with an aliquot of a second cytosol or cytosol equivalent (1 \times 10^{12} cell eq of treated or untreated cytosol or buffer; final volume, 0.75 ml), and \( O_2 \) generation was measured by following the reduction of cytochrome c at 550 nm as described under “Materials and Methods.” The results shown are representative of three experiments performed using different preparations of treated and untreated cytosol. Curve A shows the course of \( O_2 \) generation when normal cytosol is added first and treated cytosol second. Curve B and C show \( O_2 \) generation when treated cytosol or buffer, respectively, are added first and normal cytosol second. In all experiments, curve A showed no lag in \( O_2 \) production, while the usual lag was seen in curves B (dialdehyde-treated cytosol first) and C (buffer first). The final rate of \( O_2 \) generation (i.e., \( V_{max} \)) was always greater in incubations containing dialdehyde-treated cytosol than in incubations containing buffer, probably reflecting the ability of the unaffected oxidase components in the dialdehyde-treated cytosol to affect the final amount of oxidase generated, despite their failure to abolish the lag.

**Effect on the Lag**—When the oxidase in the cell-free system in activated in the presence of substrate, there is an appreciable delay between the addition of the activating agent (SDS in our assays) and the attainment of full \( O_2 \) generation. This lag, a manifestation of the first stage of oxidase activation, can be abolished by activating the enzyme before adding the substrate. (Activation is accomplished by incubating cytosol, membranes, Mg^{2+}, and SDS for 3.5 min in the absence of substrate.) An earlier study showed that cytosols from pp47-deficient CGD patients were unable to support the first stage of oxidase activation (i.e., the conversion of incompetent to competent oxidase precursor). To evaluate whether dialdehyde-treated cytosol was capable of eliminating the lag, a cell-free system containing the inactivated cytosol was incubated with SDS in the absence of substrate. \( O_2 \) generation was then initiated by the addition of NADPH together with an aliquot of normal cytosol. The results (Fig. 2) demonstrate that the lag is eliminated by preincubation with untreated cytosol, but not by an identical incubation with dialdehyde-treated cytosol or with buffer alone. These findings suggest that the NADPH binding subunit, like pp47, participates in the first stage of oxidase activation.

**Competition for Membrane Binding Sites**—The foregoing experiments suggest that the cytosolic NADPH binding component interacts directly with the membrane-associated component(s) of the oxidase. If so, it is possible that the dialdehyde-inactivated NADPH binding component would be able to compete with the active component for membrane binding sites. Competition was tested by examining the effect of the dialdehyde-treated cytosol on \( O_2 \) production by the cell-free system under conditions in which the membrane-associated oxidase components were limiting. The results are shown in Fig. 3. It is seen that when normal cytosol was used, \( O_2 \) generation increased progressively with cytosol concentration until the cytosol/membrane ratio reached 10 cell eq of cytosol/eq of membrane. The decline in the slope of the curve at \( \sim 2.0 \times 10^{12} \) cell eq/ml of cytosol suggests that at this point, the concentration of membrane in the assay begins to limit the amount of oxidase generated at full activation. Therefore, a cytosol concentration of \( 1.8 \times 10^{12} \) cell eq/ml was chosen as the starting point to test the ability of the dialdehyde-treated cytosol to compete with untreated cytosol for limited quan-

**Panel A**

![Superoxide production](chart1.png)

**Panel B**

![Competition for membrane oxidase components between NADPH-dialdehyde-treated and untreated neutrophil cytosol](chart2.png)

**Panel C**

![Superoxide generation](chart3.png)
TABLE II
Complementation between dialdehyde-treated cytosol and pp47-deficient cytosol

Assays were performed as described under “Materials and Methods.” Incubations contained 2.66 × 10^7 cell eq of dialdehyde-treated cytosol alone (“dialdehyde-treated”) or CGD cytosol alone (“-”) or 1.33 × 10^7 cell eq of each of the dialdehyde-treated and CGD cytosols (“+”). Note that in every case, O_2 production by reaction mixtures containing a combination of pp47-deficient cytosol and dialdehyde-treated cytosol exceeded O_2 production by reaction mixtures containing either cytosol alone (see text).

| Experiment | Dialdehyde-treated | Patient deficient in pp47 |
|------------|-------------------|--------------------------|
|            | G. P.             | J. C.                    | J. J.                    |
| 1          | 6.9               | 7.7                      | 14.8                     | 5.5                       | 16.9                     | 1.8                       | 8.4                       |
| 2          | 13.5              | 11.5                     | 20.9                     | 8.8                       | 19.2                     | 2.9                       | 15.0                      |
| 3          | 9.1               | 11.5                     | 15.9                     | 7.5                       | 19.2                     |                           |                           |
| 4          | 8.6               | 2.8                      | 15.6                     |                           |                          |                           |                           |

The kinetics of activation of the respiratory burst oxidase in the cell-free system has been previously explained according to the following model (17):

\[
M + 5 \overset{5,5'}{\underset{\text{Mg}^2+}{\rightarrow}} MS \overset{k}{\rightarrow} [MS]^* \overset{[MS]^*}{\rightarrow} \overset{C_1}{\rightarrow} \overset{C_2}{\rightarrow} \overset{C_3}{\rightarrow} \overset{C_4}{\rightarrow} \text{Oxidase}
\]

where M represents the membrane-associated components of the resting oxidase; S is a stabilizing cytosolic component that combines with the membrane-associated components to form the incompetent precursor complex, MS; [MS]^* is the competent precursor complex, generated by treatment of the incompetent precursor complex with SDS in the presence of Mg^2+; C_1, C_2, C_3, and C_4 are additional cytosolic factors (possibly identical) that convert the competent precursor complex into catalytically active forms of the oxidase; and [MS]^*C_1, [MS]^*C_2C_3, and [MS]^*C_4 are, respectively, the high and low K_m forms of the catalytically active oxidase.

It seems likely that the oxidase component inactivated by NADPH dialdehyde is the NADPH-binding subunit of the enzyme (19). The kinetic studies have furnished information as to how the NADPH binding component of the oxidase fits into the above model. The inability of the NADPH dialdehyde-treated cytosol to abolish the lag in activation of the oxidase demonstrates that this cytosol lacks a component required for the first stage of the oxidase activation process. Similarly, the failure of cytosols from pp47-deficient neutrophils to abolish the lag indicates that this component is needed for the first stage of the activation process. If the component inactivated by NADPH dialdehyde is different from pp47, the number of cytosolic components needed for the first stage of activation rises to at least 2. Earlier kinetic studies have shown, however, that the cytosol contributes only a single component to the first stage of the activation reaction (14, 17). To reconcile these observations, it is necessary to postulate that the cytosolic components required for this stage of the activation reaction (i.e. pp47 and the dialdehyde-inactivated component, as well as others (18)) must exist in the form of a complex. In the model, this complex corresponds to component S.

Evidence for the existence of such a complex has been provided by earlier studies. Gel filtration of concentrated neutrophil cytosol has yielded a single peak of M, = 240,000 that contains all the components needed to interact with membrane in the cell-free activation assay (12). A cytosolic preparation capable of supporting oxidase activation in the cell-free system has been obtained by affinity chromatography of neutrophil cytosol over 2',5'-ADP-agarose (24) and GTP-agarose (15). Although other data clearly indicate the presence of multiple oxidase components in neutrophil cytosol, the ability of each of these purification methods to furnish a single fraction that is capable of supporting oxidase activation suggests that these components exist as a complex.

The complementation studies were carried out to determine the relationship between the dialdehyde-inactivated component and pp47. Interpretation of these studies, however, is not straightforward. Because the reaction order for cytosol is greater than 1, the usual criterion for judging complementation—namely, that the activity in a reaction mixture containing both components be greater than the sum of activities in reaction mixtures containing each component individually—does not necessarily apply. Complementation can be identified regardless of reaction order, however, by using the criterion that the activity of a reaction mixture containing the two cytosols at equal volumes v must exceed the activities of each of the reaction mixtures in which the cytosols are used alone, but at a volume of 2v. This criterion is fulfilled by the complementation studies carried out with dialdehyde-treated cytosol and pp47-deficient cytosol, indicating that pp47 is unlikely to be the NADPH binding component of the oxidase.

Oxidase activity in the complementing reaction mixtures,
however, was much lower than expected from the activity of reaction mixtures containing normal cytosol at the same concentration (i.e. 2u). We propose that this result may be attributable to a sluggish rate for the dissociation of S into its components. In such a case, the pp47 in the dialdehyde-inactivated cytosol is locked up in an inactive S complex and is therefore unavailable to restore activity to the pp47-deficient cytosol. The observed levels of activity, although significantly greater than expected in the absence of complementation, are only a small fraction of the rates expected if the normal oxidase components present in the two defective cytosols were able to interchange freely.

The inability of the dialdehyde-treated cytosol to convert the oxidase from its low affinity to its high affinity form provides evidence that the NADPH binding component of the oxidase participates in that transformation. This finding provides information about the mechanism by which the affinity of the oxidase is regulated. In the resting cell, the NADPH dialdehyde is known to inhibit the oxidase through occupation of a high affinity binding site (19). Thus, the oxidase must either have a second, low affinity NADPH binding site which is not being inhibited under the incubation conditions used in this study, or the affinity of a single NADPH binding site must initially decrease when the NADPH binding protein is translocated to the membrane and then increase again as additional cytosolic factors combine to form the fully active oxidase. If the first hypothesis is correct, the dialdehyde-treated cytosol might be unable to alter the affinity of the oxidase for NADPH because all of the available high affinity binding sites are occupied by NADPH dialdehyde. In the second instance, the inhibited NADPH binding protein contributed by the dialdehyde-treated cytosol may sequester the available cytosolic factors that regulate substrate affinity, preventing them from acting on any uninhibited NADPH binding protein.

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