Subcellular Localization of the Human Neutrophil NADPH Oxidase

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Human neutrophils were fractionated by nitrogen cavitation and Percoll density centrifugation, and the subcellular localization of FAD-flavoprotein, \( \beta \)-cytochrome, NADH-cytochrome \( b \)-reductase, and NADPH-dependent cytochrome \( c \) reductase were determined in normal cells, cells from two patients with chronic granulomatous disease (CGD), and normal cells that had been stimulated with phorbol myristate acetate. In normal cells, a FAD-flavoprotein is found in a 1:2 molar ratio, with cytochrome \( b \) in the fractions containing the specific granules. Triton X-114 phase distribution indicates that the \( \beta \)-cytochrome but not the \( \beta \)-cytochrome-associated flavoprotein is an integral membrane protein. 80% of this flavoprotein, as well as all the \( \beta \)-cytochrome, was absent in these fractions from 2 CGD patients, although these patients had normal quantities of FAD in the fractions containing plasma membranes and cytosol. During stimulation the \( \beta \)-cytochrome-associated flavoprotein of the granules translocates with the \( \beta \)-cytochrome to the plasma membrane where NADPH oxidase is localized. Definition of the role of these NADPH oxidase constituents may provide a molecular description of the normal neutrophil respiratory burst and the molecular defect(s) in CGD.

Human neutrophils and other phagocytes possess a non-mitochondrial oxygen-consuming biochemical pathway which generates superoxide anions (\( O_2^\cdot \) ) (1). This pathway, the so-called respiratory burst of phagocytosis, is activated upon stimulation with a variety of both particulate and soluble stimuli (1-3). Superoxide is probably generated in the plasma membrane or the phagosomal membrane (4-6) and dissipates to form hydrogen peroxide, \( H_2O_2 \) (4, 7). Other free radical oxygen species, e.g. the hydroxyl radical (OH-*) are formed by nonspecified reduction reactions (8, 9). The generation of these reactive species is essential for normal bactericidal (10, 11), fungicidal (12), fungicidal (13), and tumoricidal (14) activities of phagocytes, as illustrated by studies with scavengers of these reactants (10-14) and by CGD, \(^1\) where these patients suffer from recurrent disease due to the absence of this phagocytic respiratory burst (15, 16). While this pathway is not yet fully elucidated, the current model designates the hexose monophosphate shunt as the source of reduced pyridine nucleotide, NADPH; this electron source donates, in a hydride transfer, two electrons to a membrane-bound (plasma membrane or phagosomal membrane) oxidase, which is functionally defined as an NADPH-dependent superoxide dismutase-inhibitable cytochrome \( c \) reductase. This NADPH oxidase was first characterized in a particulate preparation from activated normal neutrophils but is absent when harvested from resting neutrophils (17, 18) or activated neutrophils from CGD patients (19, 20). This oxidase has been described as a flavoprotein, since its activity, when solubilized in Triton X-100, is enhanced by FAD (21) and the substitution of flavin analogs incapable of 1-electron transfer inhibits oxidase activity (22).

We and others have provided indirect evidence that a unique \( \beta \)-cytochrome participates in the electron transport that reduces oxygen to \( O_2^- \). 1) This cytochrome is absent in neutrophils from patients with X-linked CGD, whose phagocytes are incapable of generating a respiratory burst (23). 2) The cytochrome is reduced endogenously in normal cells when stimulated under anaerobic conditions (24, 25). 3) The reduction does not take place in the neutrophils from those CGD patients containing normal amounts of \( \beta \)-cytochrome (the autosomal recessive form of CGD) (23, 26). Thus, the above presented data are consistent with the hypothesis that the NADPH oxidase is a multicomponential system functioning as an electron transport chain, the terminal component of which is the \( \beta \)-cytochrome and a proximal component of which is a flavoprotein which accepts electrons from NADPH.

We have recently described a new method for the subcellular fractionation of human neutrophils using Percoll density gradients. With this method, we have demonstrated that \( \sim 90\% \) of the \( \beta \)-cytochrome resides in the membrane of the specific granules (or of granules with the same density) of unstimulated human neutrophils and that the \( \beta \)-cytochrome translocates to the plasma membrane, in proportion to the degradation of the specific granules when the cells are stimulated (27). We speculate that this translocation is essential to the formation of an electron transport chain which generates \( O_2^- \). This present study was initiated to localize and characterize flavin components possibly associated with the \( \beta \)-cytochrome in an effort to further define the constituents of the NADPH oxidase.

MATERIALS AND METHODS

Isolation of Neutrophils—Healthy donors were phlebotomized with 450 ml of blood which was processed as described in detail (27). Briefly, erythrocytes were sedimented in the presence of Dextran T-500 (Pharmacia Fine Chemicals) and the neutrophils centrifuged through Ficoll-Hypaque (Ficoll, Pharmacia Fine Chemicals; Hypaque, Winthrop Laboratories, New York, NY) as described by Boyum (28). Residual erythrocytes were lysed in a two-step procedure (27), and the neutrophils were resuspended in ice-cold relaxation medium.

\(^1\) The abbreviations used are: CGD, chronic granulomatous disease; PMA, phorbol myristate acetate; EGTA, ethylene glycol bis(\( \beta \)-aminoethyl ether)-N,N,N'\(^\prime\),N'-tetraacetic acid.

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buffer minus EGTA: 100 mM KCl, 3 mM NaCl, 1 mM ATP (Na+), (Sigma), 3.5 mM MgCl₂, 10 mM piperazine-N₂,N' -bis-[ethanesulfonic acid], pH 7.3, if centrifugation was to follow, or in Krebs-Ringer phosphate buffer: 119 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO₄, 0.75 mM CaCl₂, 15 mM NaH₂PO₄/Na₂HPO₄, pH 7.4, containing 5.5 mM glucose, if the cells were to be stimulated before centrifugation.

Subcellular Fractionation—Subcellular fractionation was performed as described in "Materials and Methods." Nuclei and unbroken cells were pelleted (P₁) by centrifugation at 500 × g for 10 min at 4 °C. The supernatant (S₁) was decanted and loaded onto discontinuous Percoll (Pharmacia Fine Chemicals) gradients and centrifuged at 4 °C for 20 min at 20,000 rpm (48,000 g) in an SS 34 rotor (DuPont Company, Wilmington, DE). The supernatants from these density gradients (S₂) and the sedimented material, the α, β, and γ bands (27), were aspirated by hand using a plastic Pasteur pipette, or alternatively, the gradients were fractionated into 1.2-ml portions by aspiration from the bottom of the gradient. Percoll was removed from the α, β, and γ bands by centrifugation at 45,000 rpm (220,000 × g) in an SW 50.1 rotor (Beckman Instruments) and the biological material resuspended in 2 ml of phosphate-buffered saline: 140 mM NaCl, 15 mM NaH₂PO₄/Na₂HPO₄, pH 7.0.

Triton X-114 Phase Separation—An 11.4% Triton X-114 solution (Sigma) was made as follows: 50 ml of 2% Triton X-114, 15 mM glycine, 3 mM Tris-HCl, pH 8.6, was prepared at 0 °C and then heated to 37 °C for the Triton solution to cloud. The Triton phase was then sedimented by centrifugation at 1500 × g for 20 min at 37 °C, the supernatant was siphoned off, and the sediment resuspended to 50 ml by adding 4°C buffer: 15 mM glycine, 3 mM Tris-HCl, pH 8.6. The solution was again heated to 37 °C and the Triton was sedimented again by centrifugation (1500 × g, 20 min). This procedure was repeated 3 times and the final Triton solution was used as the 11.4% Triton X-114 stock. For phase separation of proteins, 1 ml of sample from the resuspended α, β, and γ bands or from S₃ (1–4 mg of protein/ml) was added to 4 ml of 0.7% Triton X-114 (from 11.4% stock) in 150 mM NaCl, 15 mM Tris-HCl, pH 7.4, 110 mM α-cytosine, and placed on ice for 20 min before mixing. The amounts of Percoll and nuclear fragments, was then pelleted by centrifugation in an SW 50.1 rotor at 45,000 rpm for 30 min at 4 °C. The supernatant from this centrifugation was decanted into plastic tubes and heated to 37 °C for the solution to cloud, and the Triton was sedimented at 1500 × g for 20 min at 37 °C. The Triton-rich sediment (volume ~250 μl) was diluted to the original volume (5 ml) in 150 mM NaCl, 15 mM Tris-HCl, pH 7.4, 0.3% Triton X-100 (Sigma). The addition of Triton X-100 was required to increase the cloud point of the solutions so that enzyme assays and spectroscopy could be performed in the turbid sample. The Triton X-100 concentration was reduced to 0.7% Triton X-113 at 0 °C and heated to 37 °C. The centrifugation was repeated and the resultant supernatant was assayed.

Spectroscopy—Absorption spectra from 400–600 nm of dithionite reduced versus unreduced samples were measured and recorded in the turbid sample compartment of a Perkin-Elmer 575 ST spectrophotometer (Coleman Instruments, Oak Brook, IL) as described (27). Spectra (except for Triton phase separation) were recorded in the absence and presence of 0.2% Triton X-100. Cytochrome b was quantitated using an absorption coefficient for the 553-nm peak of 21.62 mM·cm⁻¹ (28). Myeloperoxidase was quantitated using an absorption coefficient for the 472-nm peak of 75 mM·cm⁻¹ (30). Determination of Flavin—1.5-ml samples (0.5–2 mg/ml of protein) to which 0.1% Triton X-100 was added were routinely boiled for 5 min, cooled to 0 °C, and then incubated for 20 min at 0 °C in the presence of 10% trichloroacetic acid. The samples were then centrifuged at 1000 × g for 20 min at 4 °C and the supernatants assayed; the trichloroacetic acid was extracted in 6 ml of ethyl ether six times. The pH was then adjusted to 7.0 with 0.2 N NaOH, and emission spectra were measured and recorded from 480–600 nm with constant excitation at 450 nm on a Perkin-Elmer 650-10S fluorescence spectrophotometer (Coleman Instruments). The fluorescence spectrophotometer was recorded after adjusting the samples to pH 2.75 with 0.2 N HCl (31). Preliminary experiments were performed in which the samples were incubated with 10 mg/ml of trypsin (Sigma) for 20 min at room temperature prior to boiling to estimate the amounts of covalently and noncovalently bound flavin (32). Standards of FAD and FMN (Sigma) (0.05–1.0 μM) were always run in parallel and used to quantitate the amount of flavin in the samples. In order to see whether the flavin was protein bound, 1-ml samples were dialyzed overnight against 500 ml of phosphate-buffered saline at 4 °C with constant stirring. The amounts of flavin in the dialysis bags were then determined as described above and compared to identical samples that had not been dialyzed.

Enzymes—Alkaline phosphatase (EC 3.1.3.1) was assayed as described (27). NADH-cytochrome b₅ reductase (EC 1.6.2.2) was assayed as described (33) by continuously tracing the reduction of 1 mM ferricyanide at 25 °C using an absorption coefficient of the reduced cytochrome b₅ of 0.211 mM⁻¹·cm⁻¹ (34) in the presence of 0.1 mM NADH (Sigma) in a Perkin-Elmer 559A spectrophotometer (1 unit of enzyme liberates 1 μmol of product/min). NADPH-dependent O₂ generation was measured by continuously following the superoxide dismutase (bovine erythrocyte, Sigma) inhibitable cytochrome c (horse heart type VI, Sigma) reduction at 25 °C in a Perkin-Elmer 559A spectrophotometer, as described (35) using an absorption coefficient for the reduced cytochrome c of 21.1 mM⁻¹·cm⁻¹ (36). Vitamin B₉₇-binding protein was measured essentially as described by Gottlieb et al. (37) with details as given in Ref. 27. Protein was determined as described by Lowry et al. (38).

RESULTS

Neutrophils, 1–2 × 10⁶ cells, in 20 ml of buffer were disrupted by nitrogen cavitation as described under "Materials and Methods." Nuclei and the unbroken cells in the cavitate were pelleted (P₁) by centrifugation, and the supernatant (S₁) was decanted and layered on two discontinuous Percoll gradients, density 1.05 g/ml over 1.12 g/ml. The gradients were centrifuged and the resulting α, β, and γ bands and the supernatant, S₂, were collected, and the Percoll removed as described under "Materials and Methods." Table I gives the content of cytochrome b and FAD in the cavitate, post-nuclear supernatant, and the supernatant of the Percoll gradient of human neutrophils. The flavin was identified as FAD by a pH-dependent fluorescence (excitation at 450 nm, with peak fluorescence at 530 nm), which was identical with that of FAD standards (pH 2.75/pH 7.00 = 3.07 mean of 8 determinations; range, 2.51–3.41) and different from that of FMN which fluoresces less at low pH (31). Thus, of the extractable flavins in human neutrophils, the vast majority is FAD. The FAD was noncovalently bound to protein since 85% was retained in the dialysis bag after overnight dialysis (see under "Materials and Methods") and since the flavin was liberated from the protein by boiling and trichloroacetic acid precipitation.

Table I

| b-Cytochrome | FAD | Protein |
|--------------|-----|---------|
| pmol/mg protein | % of cavitate | |
| Cavitate | 105 | 95 | 100 |
| S₁ | 86 | 86 | 86 |
| S₂ | Undetectable | 21 | 62 |
Human Neutrophil Flavoprotein and b-Cytochrome

Subcellular fractions from Percoll density gradients were obtained as described under "Materials and Methods." Specific activity is quantity or activity/mg of protein. The percentage distribution of recovered activities is given. Recoveries ranged from 65-95% of the material loaded on the gradients. Figures are the mean of the number of experiments given in parentheses. The ranges are given within parentheses.

|                | Specific activity | Percent | Total | Specific activity | Percent | Total | Specific activity | Percent | Total | Specific activity | Percent | Total |
|----------------|-------------------|---------|-------|-------------------|---------|-------|-------------------|---------|-------|-------------------|---------|-------|
| Alkaline phosphatase (units/mg protein) | Undetectable | 0 | 0.010 (5) | 1.5 | 1.05 (5) | 96.2 | 0.002 (5) | 3.3 |
| Vitamin B12-binding protein (ng B12 bound/mg protein) | Undetectable | 0 | 34.9 (5) | 98.5 | 0.48 (5) | 1.2 | 0.04 (5) | 1.3 |
| Myeloperoxidase (pmol/mg protein) | 951 (5) | 84.8 | 267 (5) | 16.4 | Undetectable | 0 | Undetectable | 0 |
| NADH b5-reductase (units/mg protein) | Undetectable | 0 | 810-1220 | 178-287 | 225 | 15.7 | Undetectable | 0 |
| b-Cytochrome (pmol/mg protein) | 1.2 | (2.5-8.4) | 341 (5) | 47.4 | 338 (5) | 28.2 | 21.5 (5) | 23.2 |
| FAD (pmol/mg protein) | 5.3 (5) | 1.2 | (305-416) | (271-442) | (18.0-31.3) |

**TABLE III**

Disappearance of FAD, b-cytochrome, and vitamin B12-binding protein from the b band during activation

| Experiment | b-Cytochrome | FAD | Vitamin B12-binding protein |
|------------|--------------|-----|-----------------------------|
| I          | 30.5         | 27.3 | 28.5                        |
| II         | 42.6         | 38.0 | 50.0                        |
| III        | 56.8         | 45.9 | 62.1                        |

**Fig. 1.** The percent distribution of b-cytochrome and FAD in b and g bands from Percoll density gradients of cavitated human neutrophils which were either preincubated with PMA (2 μg/ml in dimethyl sulfoxide) for 3 min at 37 °C or with dimethyl sulfoxide alone prior to cavitation. Bars are means and ranges for 3 independent experiments (same as reported in Table III). The amounts of FAD in B plus g bands are taken as 100%. The amount of FAD in the supernatants from the Percoll density gradients S0 did not differ between samples from control and activated cells (data not shown).

The subcellular distribution of cytochrome b5, flavoproteins (FAD), NADH-cytochrome b5 reductase, and the markers alkaline phosphatase (plasma membranes (5, 39)), vitamin B12-binding protein (specific granules (39, 40)), and myeloperoxidase (azurophil granules (41)) is given in Table II. The table confirms our previous report on the distribution of azurophil granules, specific granules, and plasma membranes on the Percoll gradients and the predominant co-localization of the b-cytochrome with the specific granules. The table further shows that a flavoprotein, the NADH-cytochrome b5 reductase (42), is present almost exclusively in the membranes of the g band.

The flavoproteins are distributed in three sites: approximately 50% in the b band containing the specific granules, 30% in the membranes of the g band, and 20% in the cytosol (S0). The FAD in the cytosol was present as a flavoprotein, and not merely FAD dissociated from the membranes or the specific granules, since it was retained in the dialysis bag during overnight dialysis (data not shown). It is noteworthy that the flavoprotein from the b band exists in a 1:2 molar ratio with the b-cytochrome, whereas there is approximately 50% more flavoprotein than b-cytochrome in the g band. A close association between the b-cytochrome and the flavoprotein in the b band is indicated by the finding (Fig. 1 and Table III) that both translocate to the same extent to the g band containing the plasma membrane following PMA stimulation of the neutrophils prior to cavitation. Also, this association is further suggested by the demonstration (Table IV) that neutrophils from two CGD patients, both totally deficient in b-cytochrome, have a loss of ~40% of total neutrophil FAD that is entirely accounted for by loss of ~80% of the FAD in the b band, with normal amounts of flavin in the azurophil granules, membranes of the g band, and the cytosol. These data strongly indicate that the flavin and b-cytochrome of the g band are functionally related and show that the flavoprotein in the cytosol and the g band are different from that of the b band.

Further evidence of the heterogenous nature of neutrophil flavoproteins is given by Triton X-114 phase distribution. A dilute solution of Triton X-114 has a cloud point around 20 °C (43). At and above this temperature, the Triton micelles associate into large micelle aggregates (44) which sediment at low speed centrifugation (45). This separation between a
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Triton-rich phase which sediments and a Triton-poor phase which does not is a convenient method of separating integral membrane proteins, that is proteins solubilized by the Triton and maintained in the detergent phase, from water-soluble hydrophilic proteins that are excluded from the Triton micelles (45). As shown in Table V, the b-cytochrome is an integral membrane protein, as are the NADH-cytochrome b, reductase and alkaline phosphatase, whereas myeloperoxidase and vitamin B_{12}-binding protein are excluded from the Triton phase in accordance with the intragranular location of these proteins. Of the flavoproteins of the b band, 85% are excluded from the Triton phase and 15% are retained in the Triton sediment. This finding indicates that the flavoprotein of the b band consists of at least two flavoproteins, one of which is hydrophilic and represents 85% of the flavoprotein of this band, and the remaining flavoprotein which is amphiphilic. This is in accordance with the finding in two CGD patients, where 80% of the flavoprotein in the b band is missing. Lack of material precluded examination of whether the flavoprotein left in the b band of the neutrophils from these patients was amphiphilic, i.e. integrated in the membrane. The FAD from the normal cells, that was excluded from the Triton phase, remained protein bound since it was retained in the dialysis bag during overnight dialysis (data not shown).

Of the flavoprotein in the y fraction, a higher percentage was amphiphilic compared to the flavins of the b band, 30%. One of these amphiphilic flavoproteins is the NADH-cytochrome b, reductase (Table V). This enzyme has also been characterized as an integral membrane protein in other tissues (46). As noted in the legend to Table IV, the b, reductase activity was normal in both CGD patients. Since the turnover number is not known for this enzyme in neutrophils, it could not be calculated how much of the FAD in the membranes of the y band derives from this protein.

The data presented above localizes electron carriers of potential importance for the respiratory burst activity in the granules of the resting neutrophils (the specific granules or granules with the same density as the specific granules). We were, therefore, interested in determining the subcellular localization of the NADPH-dependent superoxide dismutase-inhibitable cytochrome c reductase activity. As seen in Fig. 2, which shows one of two essentially identical experiments, NADPH oxidase activity is found only in the light fraction.

**TABLE IV**

Subcellular distribution of FAD in neutrophils from 2 CGD patients

| Cells | b Band | b Band | y Band | S2 |
|-------|--------|--------|--------|----|
| Patient I | 55 (92) | 4.5 (4.9) | 91 (335) | 298 (291) | 19 (22) |
| Patient II | 52 (91) | 4.6 (4.1) | 82 (365) | 262 (271) | 24 (21) |

**TABLE V**

Triton X-114 phase separation of markers and electron carriers in fractions of human neutrophils

Human neutrophils were fractionated as described under "Materials and Methods" with the exception that the cells were pretreated with diisopropylfluorophosphate (5 mM) (Sigma) for 5 min prior to cavitation. The figures are the mean of two essentially identical independent experiments. The figures are expressed as percentage of total activity in the 0.7% Triton X-114 supernatant obtained after centrifugation at 4°C prior to phase separation at 37°C (see under "Materials and Methods").

| Distribution | Myeloperoxidase | Vitamin B\textsubscript{12}-binding protein | Alkaline phosphatase | b-Cytochrome | NADH-b, reductase | FAD | FAD | FAD |
|--------------|----------------|--------------------------------------|---------------------|-------------|-----------------|-----|-----|-----|
| Triton X-114 supernatant | 95.4 | 90.5 | 0.7 | 1.2 | 0.9 | 86 | 72 | 95 |
| Triton X-114 pellet | 1.9 | 1.5 | 79.3 | 92.8 | 85.1 | 14.2 | 25 | 3.2 |

**FIG. 2.** Distribution of markers and electron carriers on Percoll gradients. 1.8 × 10⁸ neutrophils were divided and half incubated with PMA, 2 μg/ml, for 3 min at 37°C. The other half of the cells served as control. After cavitation the postnuclear supernatants were layered on four discontinuous Percoll gradients, two for each condition. Fractions of 1.2 ml each were collected by aspiration from the bottom of each gradient until only the supernatant, S2, was left. The fractions from the two corresponding gradients were combined and assayed. Spectra for quantitating b-cytochrome and myeloperoxidase were obtained in the absence and presence of 0.2% Triton X-100 on samples diluted 6-fold with phosphate-buffered saline. S2 indicates supernatant from the gradient. □, assays of control fractions; , assays of samples from stimulated cells.
that constitutes the γ band and co-sediments with alkaline phosphatase activity (plasma membranes). The NADPH oxidase activity is present in the same fractions from stimulated cells as those to which the b-cytochrome has translocated during the stimulation. The presence of Percoll precluded extraction of FAD from these fractions, but as seen in Fig. 1 and Table III, the FAD as well as the b-cytochrome from the granules of the β band translocate to the γ band during stimulation. It should be noted that the NADH-cytochrome b₅ reductase does not change its location nor its activity during activation (Fig. 2).

**DISCUSSION**

The results reported herein confirm our previous study of the subcellular localization of the preponderant portion of human neutrophil b-cytochrome to the specific granules, or granules with the same density as the specific granules, and the translocation of the cytochrome to the plasma membrane upon stimulation (27). We have now shown that the b-cytochrome is an integral membrane protein as demonstrated by Triton X-114 phase separation. We have further shown that flavoproteins in human neutrophils mainly contain FAD and are localized to three subcellular fractions; approximately 50% is in the β band (which contains the specific granules), 30% is in the membranes of the γ band (which contains plasma membrane), and 20% remains in the cytosol. Less than 3% is in the α band which contains the azurophilic granules. Although we have not identified these flavoproteins as distinct, two CGD patients lacked approximately 40% of the total neutrophil flavoprotein pool, and this loss was accounted for by a specific absence of the flavoprotein content in the β band. This suggests that the β band flavoprotein is distinct from that of the γ band and cytosol fractions. A close association (and probable structural relationship) between this flavoprotein and the b-cytochrome is indicated by their presence in the same subcellular fraction, the β band, their absence from the neutrophils from two CGD patients, and the demonstration that they both translocate to the γ band during stimulation of normal neutrophils.

Mounting evidence supports the hypothesis that the NADPH oxidase is a multicomponent system, consisting of a flavoprotein and b-cytochrome constituents (47-50). Based on this study and previous work (22), a simple model for this oxidase is a two-component system (Fig. 3), in which the flavoprotein oxidizes NADPH in a 1:1 stoichiometry; the 2-electron reduction of FAD is followed by sequential 1-electron transfer steps to oxygen via the b-cytochrome. The association of 2 molecules of b-cytochrome with 1 molecule of the flavoprotein reflects the ratio found in the granules of the β band and affords a model mechanism for two 1-electron transfer steps in the formation of O₂⁻. It is, however, quite conceivable that more components, e.g. a quinone (51) or another flavoprotein (47), are constituents of this electron transport chain. But if a second flavoprotein is incorporated in this electron chain, it must derive from the plasma membrane, since the amount of flavoprotein in the cytosol does not change during activation.

The hypothetical model depicted in Fig. 3 would indicate that the granules of the β band are the site of origin of the two components of the NADPH oxidase. However, it is clear from the experiments and those described by Dewald et al. (5) that NADPH oxidase activity is found only in the γ fraction, containing the plasma membranes of activated neutrophils. It, therefore, follows that if the flavoprotein and the b-cytochrome complex of the granules participates in NADPH oxidase activity, it can only be that fraction which has translocated to the plasma membrane during stimulation that is active. This is in agreement with the finding of Badwey et al. (52) that treatment of human neutrophils with p-diazobenzzenesulfonic acid prior to stimulation does not affect subsequent respiratory burst activity, but such treatment completely inhibits O₂⁻ generation when added to cells which have already been stimulated.

Recently two reports on the close association of a flavoprotein and the b-cytochrome in the NADPH oxidase have been published. Gabig (50) reported that a flavoprotein and the b-cytochrome were present in a 1:1 molar ratio in a membrane preparation from stimulated human neutrophils. However, such a preparation consists of a mixture of membranes from granules and the original plasma membrane, each of which have different flavin to b-cytochrome ratios. Cross et al. (47) also reported a 1:1 molar ratio of flavin to b-cytochrome in their subcellular fractions of human neutrophils. However, these preparations are characterized by gross overlap between plasma membranes and specific granules which obfuscates the distribution pattern of the b-cytochrome and flavoprotein. Their finding that the 1:1 molar ratio of flavin to b-cytochrome was constant during a 110-fold purification indicated to the authors that the flavin was attached to the cytochrome (47). However, their demonstration that in neutrophils from 3 CGD patients, all of whom lacked the b-cytochrome, only one-half of the membrane flavin normally associated with the b-cytochrome was absent, contradicts a flavin and b-cytochrome linkage in a 1:1 molar ratio. Instead this finding indicates that their membrane preparation contains membranes from both the specific granules and the plasma membrane, since in the two similar CGD patients reported here, the flavin defect can be accounted for by the absence of a specific flavoprotein in the granules of the β band. Further, our finding that the flavoprotein and the b-cytochrome of the β band can be almost completely dissociated by Triton X-114 clearly shows that the flavoprotein and the b-cytochrome cannot reside in the same protein. This is in agreement with Gabig’s finding (50) that flavoproteins may be dissociated from the membranes without loss of the bound b-cytochrome.

Clearly, further studies are needed to define the function of the flavoproteins localized in three distinct subcellular sites, but these findings, however, suggest a methodologic approach for a thorough investigation of the interaction of the specific granule flavoprotein and the b-cytochrome in defining the electron transport chain responsible for O₂⁻ generation. Definition of the role of these NADPH oxidase constituents may provide a molecular description of the normal neutrophil respiratory burst and the molecular defect in CGD.

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