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Structural changes are induced in human neutrophil cytochrome *b* by NADPH oxidase activators, LDS, SDS, and arachidonate: intermolecular resonance energy transfer between trisulfopyrenyl-wheat germ agglutinin and cytochrome b_{558}

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Abstract

Anionic amphiphiles such as sodium- and lithium dodecyl sulfate (SDS, LDS), or arachidonate (AA) initiate NADPH oxidase and proton channel activation in cell-free systems and intact neutrophils. To investigate whether these amphiphiles exert allosteric effects on cytochrome *b*, trisulfopyrenyl-labeled wheat germ agglutinin (Cascade Blue®–wheat germ agglutinin, CCB-WGA) was used as an extrinsic fluorescence donor for resonance energy transfer (RET) to the intrinsic heme acceptors of detergent-solubilized cytochrome *b*. In solution, cytochrome *b* complexed with the CCB-WGA causing a rapid, saturable, carbohydrate-dependent quenching of up to ~55% of the steady-state fluorescence. Subsequent additions of SDS, LDS, or AA to typical cell-free oxidase assay concentrations completely relaxed the fluorescence quenching. The relaxation effects were specific, and not caused by dissociation of the CCB-WGA–cytochrome *b* complex or alterations in the spectral properties of the chromophores. In contrast, addition of the oxidase antagonist, arachidonate methyl ester, caused an opposite effect and was able to partially reverse the activator-induced relaxation. We conclude that the activators induce a cytochrome *b* conformation wherein the proximity or orientation between the hemes and the extrinsic CCB fluorescence donors has undergone a significant change. These events may be linked to NADPH oxidase assembly and activation or proton channel induction.

Keywords: Structural change; Cytochrome *b*; NADPH oxidase activator

1. Introduction

Neutrophils and other phagocytes of the immune system participate in protection of the host by engulfing and destroying pathogens [1]. Central to this process is the assembly of the NADPH oxidase complex at the neutrophil plasma membrane. Currently, the oxidase is known to comprise the cytosolic proteins, $p40^{phox}$, $p47^{phox}$, $p67^{phox}$, the membrane associated Rac1/2, Rap1A, and the transmembrane flavocytochrome *b* (a.k.a. flavocytochrome b_{558}, cytochrome b_{559}, flavocytochrome b_{-245}, and cytochrome *b*) [2,3]. Flavocytochrome *b* serves as a locus for assembly of the oxidase complex at the plasma membrane. In the active NADPH oxidase complex, flavocytochrome *b* functions as the terminal electron carrier for reduction of extracellular molecular oxygen to the highly reactive superoxide anion (O_2^-) [2]. Superoxide then reacts with constituents of the surrounding milieu to form a number of microbicidal agents that are targeted to invading organisms.

The importance of a functioning NADPH oxidase in the host immune response is demonstrated by individuals with chronic granulomatous disease (CGD). Phagocytes from these individuals are unable to mount an oxidative burst, and in the absence of superoxide are unable to kill certain types of organisms. This deficiency results in recurrent, life-

Abbreviations: AA, arachidonic acid, arachidonate; AA-ME, arachidonate methyl ester; AFM, atomic force microscopy; CCB, Cascade Blue®; CGD, chronic granulomatous disease; CMC, critical micelle concentration; CNBr, cyanogen bromide; DAG, diacylglycerol; GlcNAc, *N*-acetylglucosamine; NeuNAc, sialic acid, *N*-acetyl-neuraminic acid; O_2^-, superoxide anion; PA, phosphatidic acid; PAGE, polyacrylamide gel electrophoresis; PAO, phenyl arsine oxide; phox, phagocyte oxidase; RET, resonance energy transfer; SDS, sodium dodecyl sulfate; WGA, wheat germ agglutinin

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threatening infections for the host. Genetic anomalies in $p47^{phox}$, $p67^{phox}$, or flavocytochrome *b* are sufficient to impair NADPH oxidase function [4].

The principal oxidase component is flavocytochrome *b*, a bis-heme, heterodimeric protein composed of an extensively glycosylated 91-kDa large subunit, $gp91^{phox}$, and a 22-kDa non-glycosylated small subunit, $p22^{phox}$ [5]. The intrinsic noncovalently bound heme moieties [6] of flavocytochrome *b* are localized to the NH_2-terminal transmembrane domains [7–9] and are believed to be essential for transmembrane electron transfer [10,11] as well as proton conduction [8]. Flavocytochrome *b* binds both FAD [12] and NADPH [13], suggesting that all of the necessary elements of the electron transport chain are intact within cytochrome *b* alone, and that the cytosolic subunits serve a regulatory role. This proposition is supported by the work of Koshkin and Pick [14], and later Doussière et al. [15], who showed that purified, relipidated flavocytochrome *b* in the presence of NADPH, FAD, and anionic amphiphile could sustain limited superoxide production in the absence of the cytosolic subunits.

Since the introduction of the cell-free oxidase assay [16–18], several studies have been undertaken to identify the mechanisms that control oxidase activation. Under assay conditions, recombining of cytosolic and membrane fractions is generally insufficient to elicit oxidase activity. However, upon addition of anionic amphiphiles such as sodium dodecyl sulfate (SDS), lithium dodecyl sulfate (LDS), or arachidonate (AA), the oxidase is activated in a concentration-dependent manner. Although this system has been used extensively to study the oxidase, the effects of the amphiphiles are poorly understood and currently under significant scrutiny.

Observations that the anionic amphiphiles induce conformational changes in the cytosolic subunits concurrent with oxidase activation [19,20], combined with the discovery of SH3 binding domains in both $p47^{phox}$ and $p67^{phox}$ [21], led to the notion that the anionic amphiphiles might function to expose certain protein domains. Once exposed, interprotein interactions between the cytosolic subunits of the oxidase as well as between the cytosolic subunits and flavocytochrome *b*, were postulated to activate the oxidase. In support of this hypothesis, truncated [22] or fusion constructs [23] of $p47^{phox}$ and $p67^{phox}$, or PKC-phosphorylated $p47^{phox}$ [24], were all shown able to partially activate the oxidase in the absence of exogenous anionic amphiphile, calling into question *any* role for the negatively charged lipids. However, in all cases, oxidase activation was substantially less than that attainable when using wild-type cytosolic subunits activated with SDS, suggesting that the amphiphile-independent systems are incomplete. Moreover, in the "amphiphile-independent" systems, reconstitution of cytochrome *b* into lipid vesicles is accomplished with either poorly defined lipid mixtures, known to be contaminated by anionic lipids [25], or by purposely including acidic lipids such as phosphotidylinositol (PI) or phosphotidylserine (PS) [23] in the reconstitution mixture. Since these systems are not truly amphiphile-free, the argument that the activity is amphiphile independent is weakened considerably.

In situ interactions between the oxidase subunits, cofactors, and lipids are even less clear, but have been postulated to act in concert to alter the spatial relationships between the cofactors, hemes, and the side chains of vicinal aromatic amino acids of flavocytochrome *b* [26–28]. Together, these studies suggest that oxidase activation within the cell involves multiple protein–protein interactions that are regulated through a combination of phosphorylation and interactions with anionic lipids. Interestingly, practically all notions of the activation process incorporate assumptions that conformational changes in flavocytochrome *b* are ultimately responsible for establishing electron flow from NADPH to oxygen via through-protein electron tunneling [29,30] or more conventional means of electron transfer.

Therefore, though it appears that anionic amphiphiles directly affect the cytosolic subunits, such interactions do not preclude the existence of important functional effects they might have on membrane components of the oxidase. Although the induction of an active state geometry of flavocytochrome *b* has not been demonstrated directly, a substantial amount of indirect evidence exists, deriving from inhibitor, kinetic, oxygen affinity, electron spin resonance, and atomic force microscopy studies. Paclet et al. [31] used atomic force microscopy (AFM) to measure the size of flavocytochrome *b*-containing liposomes before and after addition of AA. Their data suggested a change in the conformation of flavocytochrome *b* during the AA-mediated transition from the inactive to the active state. Other studies have shown that the accessibility of the flavocytochrome *b* hemes to arsenic and cyanide derivatives [11] was influenced by SDS and AA in parallel with a transition in the spin-state of the heme iron [32]. These effects were concurrent with the activation state of the oxidase in both cell-free systems, utilizing membrane-bound flavocytochrome *b*, and intact neutrophils [11,33]. Further analysis suggested the presence of an amphiphile binding site on flavocytochrome *b* that, once occupied, simultaneously decreased the affinity of phenyl arsine oxide (PAO) for the hemes of flavocytochrome *b* and increased the affinity of O_2 [15]. Conformational changes in flavocytochrome *b* have also been inferred from kinetic studies of oxidase activation. Cross et al. [34], in their study of oxidase activation by SDS and AA, proposed that the lag in activity during the transition from what appeared to be an intermediate state of oxidase activation to the fully activated state could be explained by a change in the conformation of flavocytochrome *b*.

Additional studies support direct interactions between flavocytochrome *b* and anionic amphiphiles. The lag period prior to the onset of oxidase activity was shown to decrease when cytosol and membrane fractions were preincubated together with anionic activators, but not when incubated separately [35,36]. Independently, Doussière et al. [15]

observed that preincubation of membrane fractions with AA increased both the rate and amount of superoxide produced, while preincubation with the cytosolic fraction alone actually impeded oxidase activation. Also, the cytosol-independent oxidase activity observed by Koshkin and Pick [14] and Doussière et al. [15] showed a strict dependence on anionic amphiphile. Collectively, these observations suggest that the amphiphiles interact directly with flavocytochrome *b* and are involved with regulating oxidase activity.

To directly test the hypothesis that the oxidase activators mediate the induction of an active-state geometry of cytochrome *b*, we employed a system that was validated by our laboratory for measurement of the distance between a specific, fluorescently labeled, surface-exposed lysine and the heme of cytochrome *c* [37]. In the present cytochrome *b* system, we utilized wheat germ agglutinin (WGA), conjugated with the acetyl azide derivative of the trisodium salt of 1,3,trisulfo-8-pyrenyloxyacethydrazide, or Cascade Blue® acetyl azide (CCB) [38] as an extrinsic fluorescence donor (CCB-WGA). Since WGA binds tightly to cytochrome *b* via the $gp91^{phox}$ glycan [5,39], the derivatized form, CCB-WGA, should bind in the same manner and serve as an extrinsic fluorescence donor for RET spectroscopic analyses of detergent-solubilized, partially purified cytochrome *b*. When prepared in this manner, the cytochrome *b* is depleted of the optical cofactors, NADPH, and FAD [5], thus allowing the intrinsic cytochrome *b* hemes to be used as the sole fluorescence acceptors. This system can be used to detect minute changes in the spatial relationships between the donors and acceptors. Since all NADPH oxidase reconstitution assays require anionic amphiphiles for full activity, we used this RET system to directly measure their effects on cytochrome *b* structure. Our results indicate that CCB-WGA is able to form a stable complex with the $gp91^{phox}$ glycan in solution and partially quench the probe fluorescence. This quenching is relaxed by addition of the oxidase activators SDS, LDS, or AA, suggesting that they interact directly with cytochrome *b* to impart changes in the molecular geometry of the protein. Moreover, these effects approached saturation at activator concentrations consistent with maximal activity in cell-free oxidase assays, and did not occur when the methyl ester of arachidonate (AA-ME), an oxidase antagonist [16], was substituted for the activators in our RET system. Collectively, these observations suggest that the structural changes induced in cytochrome *b* by the activators are specific and saturable in a concentration range consistent with their role in activation of the NADPH oxidase.

2. Experimental procedures

2.1. Materials

KCl, NaCl, EDTA, NaN_3, gelatin, [ethylenebis(oxyethylenenitrilo)]tetra acetic acid (EGTA), syringe filters for filtering buffers for fluorescence work (Whatman, 25-mm diameter, polyethersulfone membrane, 0.2-μm pore size), and sulfuric acid were purchased from Fisher Scientific (Tustin, CA). *N*-acetylglucosamine (GlcNAc), *N*-*N*′-diacetylglucosamine (chitobiose), heparin–sepharose 4B beads, *N*-formyl-Met-Leu-Phe, dihydrocytochalasin B, Na_2 ATP, chymostatin, WGA, diisopropylfluorophosphate (DFP), sodium dithionite, quinine sulfate, arachidonate or the methyl ester suspended in chloroform, bovine serum albumin (BSA), fetuin, ovalbumin (grade VI), proteinase K, Hanks balanced salts, TRIZMA–HCl (Tris–HCL), 30% hydrogen peroxide, ABTS, LDS, goat α-mouse IgG conjugated to HRP, and Triton X-100 detergent were from Sigma Chemical Co (St. Louis, MO). [*N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid)] (HEPES) and ultrapure SDS were obtained from United States Biochemical (Cleveland, OH). *N*-octyl-β-D-glucopyranoside (OG, octylglucoside), dithiothreitol (DTT, Cleland's reagent),and phenylmethylsulfonyl fluoride (PMSF) were from Calbiochem-Novabiochem Corp. (La Jolla, CA). CNBr-activated Sepharose 4B beads were purchased from Pharmacia Biotech AB (Uppsala, Sweden). Cylindrical, UV-transparent microcuvettes (6-mm outside diameter) were purchased from Sienco, Inc. (Färgelanda, Sweden). CCB-WGA conjugate was purchased in lyophilized form from Molecular Probes (Eugene, OR). The BCA protein assay, supplied in kit form, and Reacti-Bind® 96-well EIA polystyrene plates were from Pierce, Inc. (Iselin, NJ).

2.2. Buffers

Buffer A consisted of 150 mM NaCl, 10 mM Na_2PO_4, 0.72 mM Triton X-100 (0.045%, or 3 × critical micelle concentration (CMC)), pH 7.4 and 0.02% NaN_3, in deionized water, filtered through a 0.2-μm pore size membrane filter. Membrane resuspension buffer, MRB(−), was made as per the method of Parkos, et al. [5], and consisted of 10 mM NaCl, 100 mM KCl, 10 mM HEPES, 1 mM EDTA, and 0.1 mM dithiothreitol, pH 7.4, in deionized water. MRB(+) was MRB(−) supplemented with 1 mM PMSF and 10 μg/ml chymostatin immediately before use. Phosphate buffered saline (PBS) (10 mM) contained 10 mM NaH_xPO_4, 150 mM NaCl, 0.02% NaN_3, pH 7.4, in deionized water. Digest buffer contained 50 mM Tris–HCl, pH 7.4, 10 mM $MgCl_2$, and 0.02% NaN_3 in deionized water. Hanks balanced salt solution was purchased as a premeasured dry powder and mixed as per manufacturer's instructions. Relax(+) consisted of 10 mM NaCl, 100 mM KCl, 10 mM HEPES, 3.5 mM $MgCl_2$, and 1.0 mM ATP, pH 7.4. Buffers used for ELISA experiments were: 100 mM Phosphate Coupling Buffer (100 mM NaH_xPO_4 and 150 mM NaCl in distilled water, pH 7.2), Blocking Buffer (100 mM phosphate buffer supplemented with 0.05% Tween 20 and 3% BSA), Wash Buffer (10 mM HEPES, 150 mM NaCl in distilled water, pH 7.4), and ABTS reagent (50 mM sodium citrate in distilled water, pH 4.2 supplemented just before

use with ABTS and hydrogen peroxide to final concentrations of 455 μM and 0.1%, respectively).

2.3. Purification of cytochrome b

Isolation of human PMNs and purification of cytochrome *b* were carried out as described by the method of Parkos et al. [5]. Cytochrome *b* heme quantitation prior to heparin purification was determined from the reduced *minus* oxidized spectrum at 558 nm using $\Delta\varepsilon_{558} = 29.3$ (mM cm)$^{-1}$ [40], blanked against control buffer, and reduced by addition of sodium dithionite, mixed in deionized water immediately prior to use, to a final concentration of 10 mM. After heparin purification, cytochrome *b* heme quantitation was carried out using $\varepsilon_{414} = 130.8$ (mM cm)$^{-1}$ [40] blanked against buffer. For RET experiments, the cytochrome *b* was purified only to the heparin step. At this stage of the purification the primary glycoprotein contaminant is Mac-1 (CD11b/CD18). In preliminary studies, using more highly purified preparations devoid of Mac-1 contamination (but with significantly lower yields) [5,41], we found no measurable difference in the degree of quenching or the stoichiometry of CCB-WGA:cytochrome *b* required to achieve maximum quenching. We concluded that the Mac-1 did not contribute a significant degree of competitive binding for CCB-WGA. The absorbance was measured on either a Hewlett Packard HP 8452A diode-array spectrophotometer or a Molecular Dynamics Spectra-Max 250, environmentally controlled, microtiter plate spectrophotometer. When necessary, the samples were sonicated using a Fisher 50 probe style Sonic Dismembrator, model XL2005.

2.4. CCB-WGA probe

The CCB-WGA probe was stored in the dark either in lyophilized form at −20 °C with desiccant, or suspended at 1 mM concentration in 10 mM PBS, pH 7.4, aliquoted, stored at −20 °C, and thawed immediately prior to use. Quantitation of CCB-WGA was accomplished using $\varepsilon_{400} = 31{,}400$ (cm M)$^{-1}$ (Molecular Probes) for the CCB, and the WGA content back-calculated using the labeling stoichiometry average of 4.6:1, CCB/WGA (information supplied by Molecular Probes). Immediately prior to use, the probe was diluted in Buffer A to a working concentration of 10 or 20 nM, based on WGA content, and filtered through a 0.2-μm pore size syringe filter to remove particulate contamination. To prevent moisture condensation on cuvettes, the working solution was brought to room temperature before use.

2.5. Arachidonate

AA and AA-ME stocks in chloroform were stored at −20 °C, in a sealed, dark container that had been purged with argon. Working solutions were prepared by transferring aliquots from the stock to a fresh tube where the chloroform was removed by evaporation in a stream of ultra high purity argon at room temperature. The amphiphiles were resuspended in deionized water by brief probe sonication, purged with argon, and kept on ice prior to use. All working solutions of AA and AA-ME were used the same day as prepared.

2.6. Fetuin

The protein was weighed dry, suspended in buffer A, and the concentration determined using the BCA method as described by Pierce, compared to BSA standards. Fetuin was prepared as an affinity ligand for the CCB-WGA probe as described previously [42] except CNBr-activated Sepharose 4B beads were purchased from Pharmacia Biotech, and the ligand was coupled at 4 mg/ml as per manufacturer's instructions.

2.7. ELISA

Assays were conducted in 96-well maleic anhydride-activated polystyrene Reacti-Bind® plates. A 100-μl volume containing 2–10 μg of WGA or CCB-WGA dissolved in 100 mM phosphate Coupling Buffer was added to each well and allowed to react overnight at room temperature (the amount of WGA or CCB-WGA that bound to the wells was determined by BCA assay to saturate at ∼0.5 μg/well for both ligands). The wells were emptied and blocked by exposure of each well to 200 μl of Blocking Buffer for 2 h at room temperature. The wells were then rinsed five times with 250 μM LDS in Buffer A to displace any weakly bound ligand, followed by five rinses with Buffer A. Partially purified cytochrome *b*, 0.2–0.5 μg heme (above), in 100 μl of Buffer A was then added to each well and incubated overnight at 4 °C. The plate was then rinsed five times with Buffer A and the different concentrations of LDS or AA dissolved in Buffer A were added. The plate was allowed to incubate for 20 min at room temperature, and then rinsed five times with Buffer A. One hundred microliters of primary antibody, mAb 54.1 [43], at a concentration of 2 μg/ml in Blocking Buffer was added to each well. The plate was then incubated at room temperature for 2 h and rinsed five times with Wash Buffer. One hundred microliters of goat α-mouse horse radish peroxidase conjugated IgG secondary antibody was added per well at a 1:1000 dilution in Blocking Buffer and incubated for 1 h at room temperature. The plate was then rinsed five times with Wash Buffer and developed by adding 100 μl of ABTS reagent per well. Absorbance values were measured at 405 nm.

2.8. Fluorescence measurements

All reagents containing CCB-WGA were kept in foil-wrapped containers and solutions containing cytochrome *b* or lipids were kept on ice prior to use. Experiments were conducted at ambient temperature in cylindrical 6-mm out-

side-diameter 500-μl continuously stirred UV transparent microcuvettes. Fluorescence measurements were recorded using a Photon Technologies Inc. Quanta-Master QM-1 steady state fluorometer with excitation monochromators set at 376 nm and the emission monochromator set at 418 nm for kinetic measurements. The slit width for both monochromators was set at 2 mm, corresponding to 4- and 8-nm bandpass for the excitation and emission monochromators, respectively. All fluorescence spectra were corrected for the wavelength-dependent instrument response using the software functions provided by the manufacturer (Felix software ver 1.1, 1996 PTI, Inc). For all experiments the CCB-WGA working solution (10 or 20 nM) was introduced to a continuously stirred cuvette, and the fluorescence monitored for a short period prior to addition of other constituents. Cytochrome *b* was added with either a Hamilton syringe or a Pipetteman micropipetter directly to the stirred cuvette. All experimental data shown represent averages or typical results from at least two independent experiments on different preparations or from different days. All fluorescence values were corrected for dilution effects from changes in cuvette volume due to reagent addition. Fluorescence quenching curves were transformed by first subtracting the baseline fluorescence, and then the absolute values of the fluorescence change were fit to a "one-phase exponential association" curve by nonlinear regression using the equation $Y = Y_{max}(1 - e^{-kx})$, where Y_{max} represents the fluorescence at saturation of the quenching effect. Fluorescence relaxation curves were also transformed by subtracting the baseline fluorescence, and the resulting values were fit to a "1-site binding hyperbola" curve from which the binding parameters of the anionic amphiphiles were calculated. All curve-fitting analyses were performed using GraphPad Prism version 3.02 for Windows (GraphPad Software, San Diego, CA USA, www.graphpad.com., Copyright (c) 1994–1999 by GraphPad Software. All rights reserved).

2.9. RET distance calculations

Förster distance calculations were determined as described [44,45] assuming a K^2 value of 2/3, and a refractive index of 1.44. R_0 for the CCB-WGA probe was calculated at 47 Å allowing a useful range of approximately 25 to 90 Å for distance estimates between the donor and acceptor molecules. The quantum yield of the CCB-WGA molecule was determined by the method outlined by Parker and Rees [46] using quinine sulfate in 0.1 N H_2SO_4 as a reference standard, quantitated using $\varepsilon_{346} = 1.09 \times 10^4$, with a quantum yield = 0.51 ± 0.03 if $A_{346} < 0.02$ AU (< 1 μM) [47]. Since the CCB labeling sites on the WGA are unknown, we assumed a spherical shape with a diameter of 50 Å, corresponding to an average axial dimension of the WGA molecule as determined from X-ray crystal coordinates [48]. Due to the intrinsic planar symmetry of the heme molecule, the orientation relative to the donor dipole imposes at most a 20% error in any distance approximations [49].

3. Results

3.1. Resonance energy transfer (RET) between trisulfopyrenyl-labeled WGA and the heme(s) of cytochrome b

A fluorescent conjugate of WGA, CCB-WGA, was used as a nonperturbing, extrinsic fluorescence donor molecule for RET to the intrinsic heme ligands of cytochrome *b*. This system was used to monitor changes in the structure of detergent-solubilized cytochrome *b* during exposure to the oxidase activators AA, SDS, and LDS. The efficiency of the energy transfer, or in this instance, the degree of steady-state fluorescence quenching, allowed estimation of the average radial distance separating the donor–acceptor pairs based on Förster theory [44] (see Section 2). Fig. 1 shows the spectral overlap between the normalized CCB-WGA fluorescence emission spectrum ($\lambda_{ex} = 376$ nm, solid line) and the Soret absorbance profile of the oxidized heme prosthetic groups of cytochrome *b* (dashed line). The spectrally matched properties of the donor–acceptor pair satisfy the criteria required for RET [44,50] characterized by an R_0 value of 47 Å, the distance at which 50% of the donor fluorescence would be quenched.

Addition of partially purified cytochrome *b* (~ 7.5 nM) in a single aliquot to Buffer A containing 20 nM CCB-WGA conjugate, resulted in partial quenching of the CCB fluorescence (Fig. 2A). Titration of solutions containing CCB-WGA with aliquots of cytochrome *b* (Fig. 2B) caused a saturable, concentration-dependent quenching of up to 53.9 ± 1.8% ($n = 4$) of the initial fluorescence. This value was somewhat variable (~ 20%) among blood donors and was inversely dependent on the age of the membrane extract. Based on Förster calculations, this degree of quenching is consistent with an average CCB to heme radial

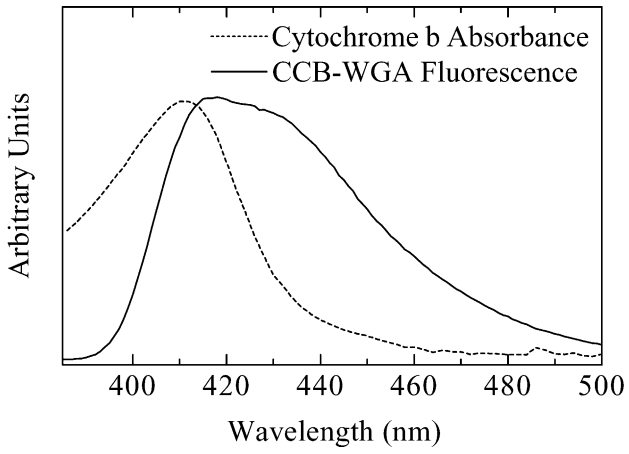

Fig. 1. Overlap of Cascade Blue® fluorescence and cytochrome *b* absorbance spectra. Normalized oxidized Soret absorbance spectrum of cytochrome *b* (dotted line) in buffer A with λ_{max} at 414 nm. Emission spectrum of Cascade Blue® dye when covalently bound to wheat germ agglutinin (CCB-WGA) (solid line) in buffer A, with $\lambda_{ex} = 376$ nm and λ_{em} maximum at 418 nm.

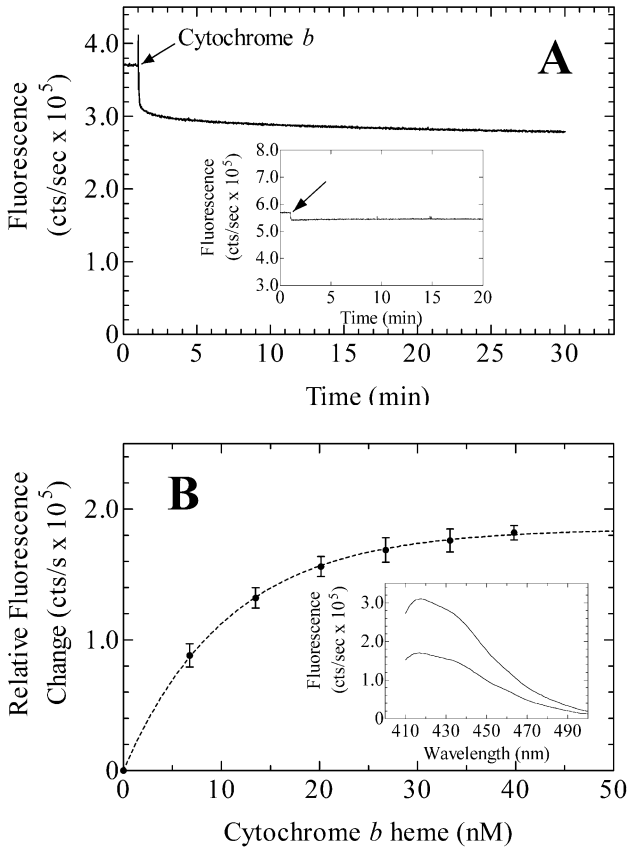

Fig. 2. Quenching of CCB-WGA fluorescence by addition of cytochrome *b*. (A) The effect of addition of ~ 7.5 nM cytochrome *b* on the fluorescence of 20 nM CCB-WGA in buffer A (λ_{ex} = 376 nm, λ_{em} = 420 nm). Time 0–1 min is the initial CCB-WGA fluorescence in the absence of cytochrome *b*. At 1 min, partially purified cytochrome *b* is added to the stirred cuvette and the fluorescence is monitored for a total of 30 min. (Inset) A control consisting of cytochrome *b* added to free CCB dye. Time 0–1 min is the initial fluorescence of 20 nM CCB. At 1 min a single 20-μl aliquot of cytochrome *b* (0.5 μM heme) is added (final concentration of ~ 20 nM) to the cuvette. The fluorescence is decreased by ~ 4%, consistent with the dilution from the change in cuvette volume. (B) Concentration dependence of fluorescence quenching of 20 nM CCB-WGA by cytochrome *b*. Single 2-μl aliquots of partially purified cytochrome *b* (0.85 μM) were added to a stirred cuvette containing 20 nM CCB-WGA. Following each addition of cytochrome *b*, the effects were allowed to equilibrate and the fluorescence was measured. These data were transformed by subtracting the baseline fluorescence and then plotting (●) the resultant absolute values ± S.E.M (n = 4). The transformed values were fit to a curve (· · ·) as described in Section 2. At saturating concentrations of cytochrome *b*, the CCB-WGA fluorescence would be quenched a maximum of 53.9 ± 1.8%, corresponding to an average heme to CCB moiety distance of 45.8 ± 0.5 Å. (Inset) The shape of the CCB-WGA emission spectrum remains unchanged after addition of cytochrome *b*. CCB-WGA fluorescence emission spectrum (20 nM) (λ_{ex} = 376 nm) is recorded before (upper trace) and after addition of 20 nM cytochrome *b* (lower trace).

distance of 45.8 ± 0.5 Å. Reverse titration of cytochrome *b* with CCB-WGA produced an analogous effect (not shown). Experimental concentrations of both the CCB-WGA and the cytochrome *b* were 10 to 40 nM, well below the level where any significant inner-filter effects would occur (for λ_{ex} = 376 nm, A_{376} < 0.001 AU for both CCB and cytochrome b) [45].

Also, addition of 10 nM cytochrome *b* to solutions containing an equimolar amount of an CCB-ethanolamine conjugate (Fig. 2A, inset) caused only a slight reduction in the fluorescence intensity, reflecting only dilution effects from cytochrome *b* addition. This control result indicated that any inner-filter effects were inconsequential to the observed fluorescence quenching and that the charged trisulfonic acid moieties of the CCB molecule did not promote nonspecific ionic interactions between the molecules. Addition of CCB-WGA to cytochrome *b* had no effect on the heme absorbance spectrum (not shown) or the shape of the CCB-WGA fluorescence emission spectrum (Fig. 2B, inset), indicating that formation of the CCB-WGA–cytochrome *b* complex neither perturbed the heme environment nor changed the intrinsic spectral properties of the CCB. Together, these data suggested that the fluorescence quenching effect was due to RET between the donor–acceptor pairs, similar to our previous work with cytochrome *c* [37].

3.2. The interaction between the CCB-WGA conjugate and cytochrome b is a lectin–carbohydrate specific interaction

Native WGA has been shown to have both high affinity and specificity for GlcNAc and its multimers, and to a lesser extent, *N*-acetyl-neuraminic acid (sialic acid, NeuNAc) [51]. To confirm that the same ligand specificity was retained between CCB-WGA and cytochrome b, we examined the effects of GlcNAc or the dimeric form, *N*-*N*′-diacetylglucosamine (chitobiose), or NeuNAc in both competition (Fig. 3) and inhibition assays (not shown). Addition of chitobiose to a final concentration of 400 μg/ml (942 μM) to solutions containing 20 nM CCB-WGA previously complexed with cytochrome *b* resulted in a rapid, total reversal of the fluorescence quenching (Fig. 3). Complete inhibition of

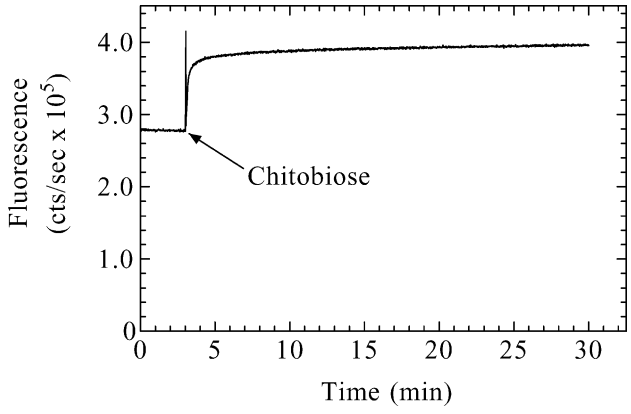

Fig. 3. Chitobiose reverses CCB-WGA fluorescence quenching by cytochrome *b*. The effect of chitobiose on the fluorescence of 20 nM CCB-WGA in the presence of 7.5 nM cytochrome *b*. At 3 min, chitobiose dissolved in Buffer A is added in a single aliquot to a final concentration of 400 μg/ml (942 μM) to the stirred cuvette. Fluorescence quenching is rapidly reversed indicating competitive dissociation of the CCB-WGA, cytochrome *b* complex.

quenching was also observed when chitobiose was added to CCB-WGA prior to cytochrome *b* (not shown). In contrast, concentrations of GlcNAc or NeuNAc, up to 1 mM, were ineffective in either reversal or inhibition of the quenching of the CCB-WGA fluorescence by the cytochrome. Moreover, addition of either ligand to the CCB-WGA alone had no effect on the fluorescence (not shown). These observations reflect the differences in the IC_{50} concentrations for chitobiose (0.15 mM), GlcNAc (12.5 mM), and NeuNAc and sialic acid (25 mM) for inhibition of agglutinating activity by WGA [52]. Since cytochrome *b* has a high GlcNAc content [39], these results suggest that formation of the CCB-WGA–cytochrome *b* complex and subsequent quenching is due to a lectin–carbohydrate interaction between the CCB-WGA and multimeric GlcNAc on $gp91^{phox}$.

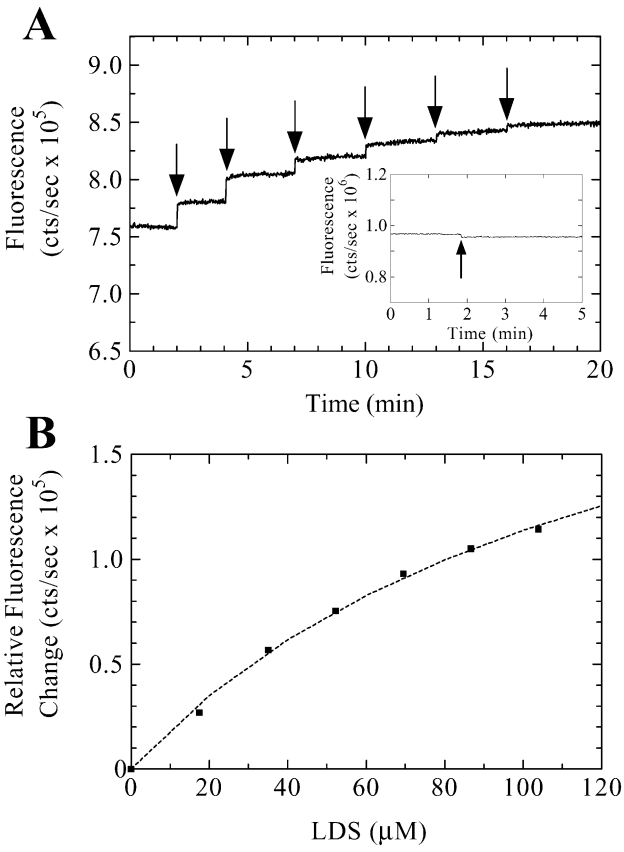

Fig. 4. LDS relaxes CCB-WGA fluorescence quenching by cytochrome *b*. The effect of addition of LDS to 20 nM CCB-WGA in Buffer A previously quenched by addition of 20 nM cytochrome *b*. (A) Time 0–2 min is the cytochrome *b*-quenched CCB-WGA fluorescence. Beginning at 2 min, successive 1-μl aliquots (arrows) of 10 mM LDS (~ 17.5 μM final concentration) were added to the stirred cuvette to a final concentration of ~ 100 μM. (Inset) Addition of a single aliquot of LDS (arrow) to a final concentration of ~ 120 μM to CCB-WGA alone in Buffer A has no measurable effect on the probe fluorescence. (B) The fluorescence intensity was measured immediately before each successive addition of LDS from panel A. These data were transformed by subtracting the baseline fluorescence, and the values were plotted (■). The resulting plot was fit to a 1-site binding isotherm (· · ·) as described in Section 2. The K_d for LDS was calculated to be 129.2 ± 17.4 μM ($R^2 = 0.9977$), and extrapolation to the saturating concentration of LDS indicates that the fluorescence quenching would be completely relaxed.

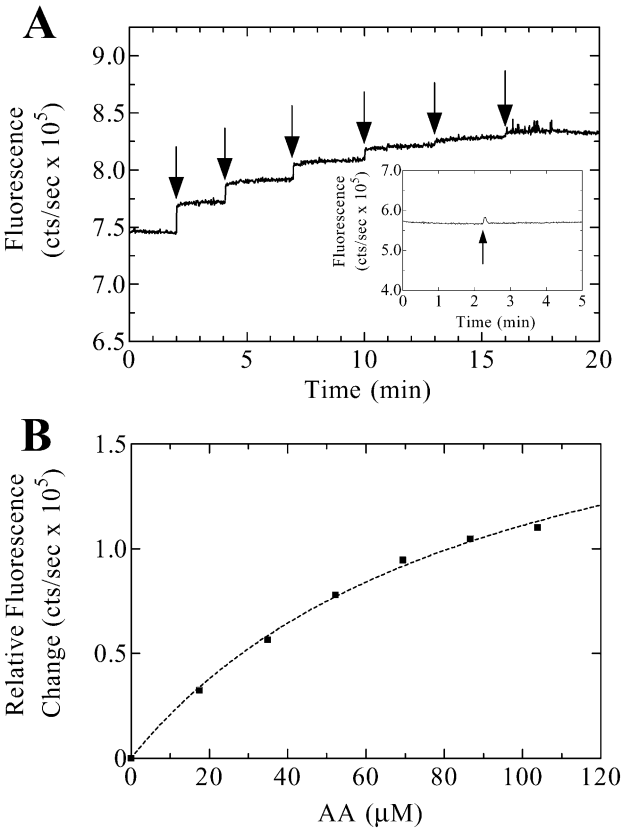

Fig. 5. AA relaxes fluorescence quenching of CCB-WGA by cytochrome *b*. The effect of addition of AA to 20 nM CCB-WGA in buffer A previously quenched by addition of 20 nM cytochrome *b*. (A) Time 0–2 min is the cytochrome *b*-quenched CCB-WGA fluorescence. Beginning at 2 min, successive 1-μl aliquots (arrows) of 10 mM AA (~ 17.5 μM final concentration) were added to the stirred cuvette to a final concentration of ~ 100 μM. (Inset) Addition of a single aliquot of AA (arrow) to a final concentration ~ 300 μM to CCB-WGA alone in Buffer A has no measurable effect on the probe fluorescence. (B) Fluorescence intensity measurements made immediately before each successive addition of AA were transformed by subtracting the baseline fluorescence, and the values were plotted (■). The resulting plot was fit to a 1-site binding isotherm (· · ·) as described in Section 2. The K_d for AA was calculated at 93.1 ± 11.5 μM ($R^2 = 0.9965$), and extrapolation to the saturating concentration of AA indicates that the fluorescence quenching would be completely relaxed.

Reversal of quenching by addition of chitobiose was accompanied by an ~ 10% increase in the fluorescence beyond the initial amount prior to quenching (Fig. 3). The increase was attributed to relaxation of a small amount of intramolecular quenching in the conjugated WGA (4.6 CCB per WGA dimer) upon its binding to complex sugars based on experiments utilizing other glycoproteins and proteinase K-digested cytochrome *b* [41]. Together, these experiments suggest that the amount of intermolecular quenching of CCB-WGA fluorescence by cytochrome *b*, described above, might be a slight underestimate.

3.3. Fluorescence quenching of CCB-WGA by cytochrome b is relaxed by SDS, LDS, and AA

Most cell-free NADPH oxidase assays require addition of either SDS or LDS to initiate activity [53]. AA is another potent activator of O_2^- production and proton channel conductance by the NADPH oxidase when introduced either exogenously to intact phagocytes [54,55], or in cell-free systems [16], where it has been shown to interact directly with cytochrome *b* [15]. We thus exploited our system to investigate whether interactions between the activators and cytochrome *b* induce structural changes in the cytochrome that would be apparent as changes in the RET efficiency. Addition of aliquots of LDS (Fig. 4A, arrows), SDS (not shown), or AA (Fig. 5A, arrows) to CCB-WGA previously quenched by cytochrome *b* resulted in a concentration-dependent relaxation of the fluorescence quenching, with the effect approaching saturation at concentrations consistent with maximal cell-free oxidase system activity. The fluorescence values resulting from each successive addition of the activators were recorded after equilibrium was reached (Figs. 4B and 5B) and fit to a binding isotherm modeling a 1-site binding interaction (see Section 2). Under these conditions, the K_d values for SDS, LDS, and AA were calculated to be 44.6 ± 4.8, 129.2 ± 17.4, and 93.1 ± 11.5 μM, respectively. Controls of SDS (not shown), LDS, or AA (Figs. 4A and 5A, insets), added to CCB-WGA alone had no effect on the fluorescence intensity of the probe. Additional experiments with SDS additions to 1.75 mM (SDS CMC = 1.33 mM) to the CCB-WGA also had no effect on the probe fluorescence (not shown). These results indicate that the CCB-WGA fluorescence is insensitive to high concentrations of anionic detergent.

The methyl ester of arachidonate (AA-ME) is unable to elicit NADPH oxidase activity in either whole cells or cell-free assays, and has been shown to competitively counteract AA-mediated oxidase activation [16,56]. If the activator-induced relaxation effects were linked to oxidase activation, then addition of AA-ME to our RET system should cause dissimilar effects, and possibly be able to counteract those of AA. Indeed, in contrast to AA, LDS, or SDS, addition of AA-ME to CCB-WGA previously quenched with cytochrome *b* failed to relax the quenching and actually caused a slight decrease in the steady-state fluorescence (Fig. 6A). Furthermore, when AA-ME was added after AA to the previously quenched CCB-WGA, the fluorescence relaxation effects from the AA were partially reversed (Fig. 6B).

3.4. Relaxation of fluorescence quenching is not due to dissociation of the complex or alterations in heme spectrum

ELISA experiments were conducted next to ensure that the activator-induced fluorescence relaxation was not due to dissociation of the CCB-WGA complex. Cytochrome *b* was added to wells containing covalently bound CCB-WGA (Fig. 7) or WGA (not shown). Different concentrations of LDS or AA were added to the wells in buffer conditions analogous to the RET experiments and over the same time frame. The wells were then probed with α-gp91^{phox} mAb 54.1 [43]. Exposure of the WGA or CCB-WGA–cytochrome *b* complex to either LDS or AA at concentrations up to 100 μM had little effect on the ELISA signal. At activator concentrations between 100 and 150 μM, both activators caused a partial loss of signal. Additionally, 120 μM SDS had no effect on the ability of CCB-WGA to either bind immobilized fetuin, or to be eluted by chitobiose from this affinity matrix (not shown). These results indicate that the activator-induced quenching relaxation (Figs. 4 and 5) was not due to dissociation of the CCB-WGA–cytochrome *b* complex.

Interactions between the activators and cytochrome *b* might also perturb the heme environment thereby affecting the Soret absorbance spectrum. The consequent changes in the spectral overlap between the donor–acceptor pair would

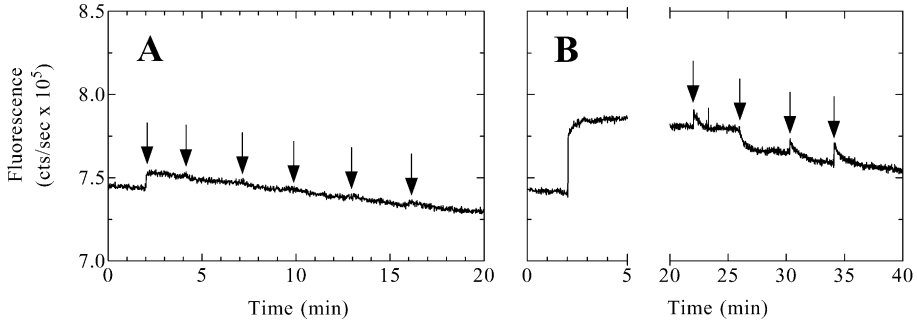

Fig. 6. AA-ME has the opposite effect of AA on fluorescence quenching. (A) The effects of addition of AA-ME on the fluorescence of CCB-WGA previously quenched by cytochrome *b*. Time 0–2 min is the fluorescence of 20 nM CCB-WGA previously quenched by exposure to 20 nM cytochrome *b* for 20 min. Beginning at 2 min, successive 1-μl aliquots (arrows) of 10 mM AA-ME (~ 17.5 μM final concentration per aliquot) were added to the stirred cuvette to a final concentration of ~ 100 μM. The fluorescence intensity is diminished slightly with each addition, an effect opposite of that observed with LDS, SDS, or AA. (B) The reversal of the AA-induced relaxation effects by addition of AA-ME. Time 0–2 min is the fluorescence of 20 nM CCB-WGA previously quenched by exposure to 20 nM cytochrome *b* for 20 min. At 2 min a single 2-μl aliquot of 10 mM AA (~ 35 μM final concentration) was added to the stirred cuvette and allowed to equilibrate for 20 min. Beginning at 22 min, successive 2-μl aliquots (arrows) of 10 mM AA-ME (~ 35 μM per aliquot) were added to the stirred cuvette to a final concentration of ~ 140 μM. The AA-induced quenching effects are partially reversed by addition of AA-ME.

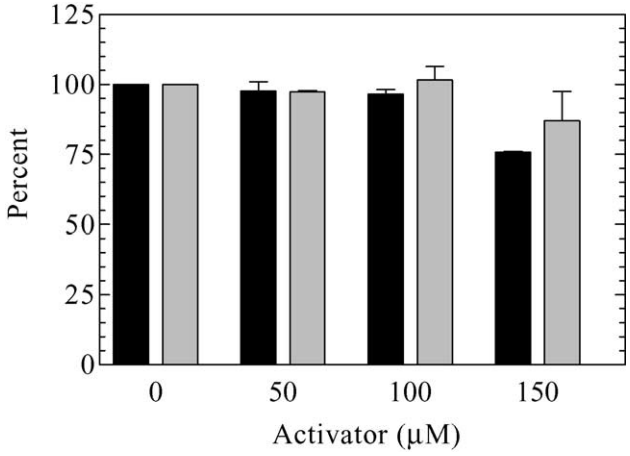

Fig. 7. ELISA of cytochrome *b* bound to immobilized CCB-WGA after exposure to LDS and AA. Cytochrome *b* was bound to immobilized CCB-WGA on an ELISA plate under essentially the same conditions and buffers as in the RET assays. The complexes were then exposed to varying concentrations of AA or LDS and probed with an α-cytochrome *b* mAb as described in Section 2. Shown are the relative amounts of cytochrome *b* bound to the immobilized CCB-WGA ± S.D. from two separate experiments with each concentration of either LDS (black bars) or AA (gray bars) done in triplicate. Data are expressed as percent of control wells that contained only Buffer A. No significant change in the amount of cytochrome *b* bound by the CCB-WGA is observed at the 100 μM experimental concentrations of either activator. Activator concentrations of 150 μM reduce the amount bound by approximately 25% for LDS and 14% for AA.

contribute to the observed quenching relaxation. However, exposure of cytochrome *b* to 100 μM SDS or AA had no effect on the Soret absorbance spectrum over a 20-min period in buffer conditions identical to the RET experiments (not shown). In contrast, at SDS or AA concentrations greater than ~ 160 μM, the Soret absorbance maximum was blue-shifted from 414 to 408 nm, concomitant with a reduction in the overall absorbance, and a loss of the 558 nm α-band following dithionite reduction (not shown). These observations suggested that activator concentrations greater than ~ 160 μM perturbed the native heme environment, probably by nonspecific detergent-like interactions. These results correlated well with previous studies that showed maximum oxidase activity occurring at approximately 100–120 μM SDS or AA with higher concentrations corresponding to a sharp decline [16,57].

4. Discussion

Our RET system utilized Triton X-100-solubilized, partially purified human neutrophil cytochrome *b* that was free of spectral interference from flavin or NADPH cofactors, both of which have intrinsic fluorescence and absorbance spectral overlaps with CCB. Detergent solubilization also circumvented the complexities of inter-cytochrome *b* RET due to aggregation or close proximity within the liposome, or orientation relative to the bilayer. By eliminating these confounding variables from our system, the RET effects that we observed could be interpreted within the context of a simplified donor–acceptor system consisting solely of CCB-WGA and monodisperse cytochrome *b*. At this degree of purity, the cytochrome *b* is capable of reconstituting defective respiratory burst activity in detergent extracts of neutrophil membranes obtained from patients with the autosomal and X-linked forms of CGD [58]. In addition, it is able to bind the low molecular weight G protein rap1A in a phosphorylation-dependent manner [59]. Most importantly, it displays both oxidized and reduced heme absorbance spectra identical to that of lipid-bound flavocytochrome *b*, indicating a native heme environment. Thus, the detergent-solubilized cytochrome *b* used for these experiments represents a reasonable model of membrane-bound flavocytochrome *b*.

Our results suggest that functionally relevant structural rearrangements do indeed occur within cytochrome *b* concurrent with exposure to activators at concentrations routinely used to initiate NADPH oxidase activity in vitro. The results derive from a novel RET system that exploits the high affinity lectin–carbohydrate interaction between CCB-WGA and the carbohydrate of $gp91^{phox}$ to form a complex. Within this complex, the CCB moieties are positioned close enough to the intrinsic cytochrome *b* hemes to allow direct transfer of electronic excitation energy. Consequently, up to ~ 55% of the CCB fluorescence is quenched, and subsequent additions of the anionic amphiphiles LDS, AA, or SDS to the complex relax the quenching, implying a change in the spatial relationships (distance, orientation) between the donor–acceptor pairs.

The different anionic amphiphilic oxidase activators cause very similar effects on RET in the CCB-WGA/cytochrome *b* heme system; they completely restore the CCB-WGA fluorescence from the cytochrome *b*-quenched state. The complete relaxation of quenching at the calculated saturating concentrations of LDS, SDS, or AA to this RET system, corresponds to an apparent displacement between the CCB-WGA and a cytochrome *b* heme of roughly a minimum of 20–30 Å, based on Förster distance calculations (see Section 2), assuming there is a 5–10% residual quenching after addition of the anionic amphiphiles. Since these effects appear to occur without perturbation of any of the chromophores of the system, they probably arise from rearrangements between inter- or intra-cytochrome *b* domains in combination with reorientation of the heme dipoles [8,60] relative to the CCB donors. Such large rearrangements have been measured in other heme-bearing systems such as nitric oxide synthase oxygenase and the Reiske ironsulfur protein of the mitochondrial cytochrome bc_1 complex [61,62].

The changes we observe might also be due to the activators interacting with domains of the protein that are not directly linked to oxidase or proton transport activity. However, the inability of AA-ME (Fig. 6), an antagonist of AA in cell-free assays [15], to induce effects similar to AA

would argue against this premise. Also, the activator-induced effects (Figs. 4 and 5) approach saturation at concentrations consistent with those shown to elicit maximal activity in cell-free oxidase assays [16,57]. This saturable behavior further suggests specific binding, thus corroborating previous observations [15].

Anionic amphiphile-induced structural changes in cytochrome *b* may also relate to events observed in the cell. Specifically, AA has been shown to be liberated from the phagocyte membranes following receptor-mediated activation, suggesting it may mediate both oxidase [63,64] and proton channel activity [65]. AA appears to be liberated from phospholipids by the action of $cPLA_2$ [66] under the control of a MAP kinase [67]. A human myeloid cell line deficient in $cPLA_2$ was unable to activate the oxidase in response to various stimuli until supplemented with exogenous AA [68], and inhibitors of $cPLA_2$ were also shown to inhibit the oxidative burst [67]. Synthetic analogues of endogenous neutrophil lipids other than AA also have been shown to either synergize with SDS and AA, or act alone as activators in cell-free oxidase systems [69,70].

A second body of evidence suggests that membrane-phospholipid-derived AA may also be involved with regulation of the proposed $gp91^{phox}$ proton channel function [65,71]. Most recently, proton channel activation has been proposed to be directly linked to release of heme from one of its putative ligands, His^{115} of $gp91^{phox}$ [8]. Changes in histidine ligation during oxidase activation might provide a mechanism that would couple electron and proton transport activities. These lines of investigation strongly implicate the anionic amphiphiles in induction of structural changes in flavocytochrome *b*. Such changes may promote productive interactions between the cytosolic subunits and flavocytochrome b, thereby enabling full oxidase activation, or possibly directly affect the function of flavocytochrome *b*.

Preliminary results from testing certain lipid effectors on our system, chosen based on their ability to elicit superoxide generation in vitro [72], showed that certain phosphatidic acids (PAs) but not diacylglycerols caused effects similar to SDS, LDS, and AA [41]. Thus, LDS, SDS, AA, and specific PAs possess similar characteristics that may define the requirements for effector action within our system: an anionic head group and either an unsaturated, or restricted length saturated fatty acid component, paralleling previous observations [16]. The similar effects imparted by these different activators on the RET system correlates well with their biochemical effects within the context of oxidase activation. Thus, we believe they represent specific, functionally relevant effects.

Numerous investigators have postulated that structural changes in flavocytochrome *b* are a key step in oxidase activation [15,23,25,31,34,73–79]. Our observations corroborate this supposition, offering new insight into the complexities of oxidase activation and providing a foundation for further investigation of the link between flavocytochrome *b* structural changes and regulation of transmembrane electron and proton conduction. The deficiencies of the cytosol- or amphiphile-independent oxidase systems might very well be reconciled in a system where phosphorylation events and amphiphile interactions with several of the oxidase subunits combine to effect full activity. The RET system also provides a means to monitor the induction of functionally relevant structural changes in cytochrome *b* arising from interactions with specific compounds other than those tested in this work, or possibly, the oxidase subunits.

In preliminary efforts [41] we have extended the scope of these experiments to include utilization of fluorescently labeled, cytochrome *b*-specific monoclonal antibodies with known epitope interactions as donor molecules, allowing investigation of changes in the spatial relationships between specific epitopes and the hemes, potentially in intact cells or isolated neutrophil membranes. In such systems, we have observed similar quenching effects from extracellular and intracellular binding sites on both detergent-solubilized cytochrome *b* and membrane-bound flavocytochrome *b*.

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References

[1] S.J. Klebanoff, R.A. Clark, The Neutrophil: Function and Clinical Disorders, Elsevier, Amsterdam; North-Holland Pub. Co., New York, 1978.
[2] F.R. DeLeo, M.T. Quinn, Journal of Leukocyte Biology 60 (1996) 677.
[3] J.H.W. Leusen, A.J. Verhoeven, D. Roos, Journal of Laboratory and Clinical Medicine 128 (1996) 461.
[4] A.J. Thrasher, N.H. Keep, F.B. Wientjes, A.W. Segal, Biochimica et Biophysica Acta 1227 (1994) 1.
[5] C.A. Parkos, R.A. Allen, C.G. Cochrane, A.J. Jesaitis, Journal of Clinical Investigation 80 (1987) 732.
[6] A.R. Cross, J. Rae, J.T. Curnutte, The Journal of Biological Chemistry 270 (1995) 17075.
[7] T.R. Foubert, J.B. Bleazard, J.B. Burritt, J.M. Gripentrog, D. Baniulis, R.M. Taylor, A.J. Jesaitis, The Journal of Biological Chemistry 276 (2001) 38852.
[8] A. Maturana, S. Arnaudeau, S. Ryser, B. Banfi, J.P. Hossle, W. Schlegel, K.H. Krause, N. Demaurex, The Journal of Biological Chemistry 276 (2001) 30277.
[9] K.J. Biberstine-Kinkade, F.R. DeLeo, R.I. Epstein, B.A. LeRoy, W.M. Nauseef, M.C. Dinauer, The Journal of Biological Chemistry 276 (2001) 31105.
[10] M.T. Quinn, M.L. Mullen, A.J. Jesaitis, The Journal of Biological Chemistry 267 (1992) 7303.
[11] J. Doussière, J. Gaillard, P.V. Vignais, Biochemistry 38 (1999) 3694.
[12] J. Doussière, G. Buzenet, P.V. Vignais, Biochemistry 34 (1995) 1760.

[13] J. Doussière, G. Brandolin, V. Derrien, P.V. Vignais, Biochemistry 32 (1993) 8880.
[14] V. Koshkin, E. Pick, FEBS Letters 338 (1994) 285.
[15] J. Doussière, F. Bouzidi, A. Poinas, J. Gaillard, P.V. Vignais, Biochemistry 38 (1999) 16394.
[16] Y. Bromberg, E. Pick, Cellular Immunology 88 (1984) 213.
[17] D. Rotrosen, C.L. Yeung, J.P. Katkins, The Journal of Biological Chemistry 268 (1993) 14256.
[18] L.C. McPhail, P.S. Shirley, C.C. Clayton, R. Snyderman, Journal of Clinical Investigation 75 (1985) 1735.
[19] J.H. Lee, J.W. Park, Journal of Protein Chemistry 19 (2000) 643.
[20] S.D. Swain, S.L. Helgerson, A.R. Davis, L.K. Nelson, M.T. Quinn, The Journal of Biological Chemistry 272 (1997) 29502.
[21] P. Finan, Y. Shimizu, I. Gout, J. Hsuan, O. Truong, C. Butcher, P. Bennett, M.D. Waterfield, S. Kellie, The Journal of Biological Chemistry 269 (1994) 13752.
[22] K. Hata, T. Ito, K. Takeshige, H. Sumimoto, The Journal of Biological Chemistry 273 (1998) 4232.
[23] K. Ebisu, T. Nagasawa, K. Watanabe, K. Kakinuma, K. Miyano, M. Tamura, The Journal of Biological Chemistry 276 (2001) 24498.
[24] L.R. Lopes, C.R. Hoyal, U.G. Knaus, B.M. Babior, The Journal of Biological Chemistry 274 (1999) 15533.
[25] Y. Gorzalczany, N. Sigal, M. Itan, O. Lotan, E. Pick, The Journal of Biological Chemistry 275 (2000) 40073.
[26] V. Koshkin, Biochimica et Biophysica Acta 1232 (1995) 225.
[27] A.R. Cross, J.T. Curnutte, The Journal of Biological Chemistry 270 (1995) 6543.
[28] V. Koshkin, O. Lotan, E. Pick, Biochimica et Biophysica Acta 1319 (1997) 139.
[29] R.A. Marcus, N. Sutin, Biochimica et Biophysica Acta 811 (1985) 265.
[30] D. Beratan, S. Skourtis, Current Opinion in Chemical Biology 2 (1998) 235.
[31] M.H. Paclet, A.W. Coleman, S. Vergnaud, F. Morel, Biochemistry 39 (2000) 9302.
[32] J. Doussière, J. Gaillard, P.V. Vignais, Biochemistry 35 (1996) 13400.
[33] J. Doussière, A. Poinas, C. Blais, P.V. Vignais, European Journal of Biochemistry 251 (1998) 649.
[34] A.R. Cross, R.W. Erickson, J.T. Curnutte, The Journal of Biological Chemistry 274 (1999) 15519.
[35] B.M. Babior, R. Kuver, J.T. Curnutte, The Journal of Biological Chemistry 263 (1988) 1713.
[36] M.E. Kleinberg, H.L. Malech, D. Rotrosen, The Journal of Biological Chemistry 265 (1990) 15577.
[37] R.M. Taylor, B. Lin, T.R. Foubert, J.B. Burritt, J. Sunner, A.J. Jesaitis, Analytical Biochemistry 302 (2002) 19.
[38] J.E. Whitaker, R.P. Haugland, P.L. Moore, P.C. Hewitt, M. Reese, Analytical Biochemistry 198 (1991) 119.
[39] A.M. Harper, M.F. Chaplin, A.W. Segal, Biochemical Journal 227 (1985) 783.
[40] R. Lutter, M.L.J. Van Shaik, R. Van Zwieten, R. Wever, D. Roos, M.N. Hamers, The Journal of Biological Chemistry 260 (1985) 2237.
[41] T.R. Foubert, Human Neutrophil Cytochrome *B*: Structure and Function, Montana State University, 2002.
[42] B. Sela, J.L. Wang, G.M. Edelman, The Journal of Biological Chemistry 250 (1975) 7535.
[43] J.B. Burritt, M.T. Quinn, M.A. Jutila, C.W. Bond, A.J. Jesaitis, The Journal of Biological Chemistry 270 (1995) 16974.
[44] Th. Förster, Annalen Der Physik 2 (1948) 55.
[45] P. Wu, L. Brand, Analytical Biochemistry 218 (1994) 1.
[46] C.A. Parker, W.T. Rees, Analyst 87 (1962) 83.
[47] J.W. Eastman, Photochemistry and Photobiology 6 (1967) 55.
[48] C.S. Wright, J. Jaeger, Journal of Molecular Biology 232 (1993) 620.
[49] J.M. Vanderkooi, P. Glatz, J. Casadei, G.V. Woodrow III, European Journal of Biochemistry 110 (1980) 189.
[50] B.W. Van Der Meer, G. Coker III, S.-Y.S. Chen, Resonance Energy Transfer: Theory and Data, VCH Publishers, Inc., New York, 1994.
[51] A.K. Allen, A. Neuberger, N. Sharon, Biochemical Journal 131 (1973) 155.
[52] V.P. Bhavanandan, A.W. Katilc, The Journal of Biological Chemistry 254 (1979) 4000.
[53] S. Sphungin, I. Dotan, A. Abo, E. Pick, The Journal of Biological Chemistry 264 (1989) 9195.
[54] L.M. Henderson, G. Banting, J.B. Chappell, The Journal of Biological Chemistry 270 (1995) 5909.
[55] M.J. Steinbeck, G.G. Hegg, M.J. Karnovsky, The Journal of Biological Chemistry 266 (1991) 16336.
[56] E. Ligeti, J. Doussière, P.V. Vignais, Biochemistry 27 (1988) 193.
[57] Y. Bromberg, E. Pick, The Journal of Biological Chemistry 260 (1985) 13539.
[58] M.T. Quinn, J.T. Curnutte, C.A. Parkos, M.L. Mullen, P.J. Scott, R.W. Erickson, A.J. Jesaitis, Blood 79 (1992) 2438.
[59] M.T. Quinn, C.A. Parkos, L.E. Walker, S.H. Orkin, M.C. Dinauer, A.J. Jesaitis, Nature 342 (1989) 198.
[60] M.D. Esposti, M. Crimi, C. Kortner, A. Kroger, T. Link, Biochimica et Biophysica Acta 1056 (1991) 243.
[61] B.R. Crane, A.S. Arvai, D.K. Ghosh, C. Wu, E.D. Getzoff, D.J. Stuehr, J.A. Tainer, Science 279 (1998) 2121.
[62] E.A. Berry, M. Guergova-Kuras, L.S. Huang, A.R. Crofts, Annual Review of Biochemistry 69 (2000) 1005.
[63] M.C. Chabot, L.C. McPhail, R.L. Wykle, D.A. Kennerly, C.E. McCall, Biochemical Journal 286 (1992) 693.
[64] Y. Bromberg, E. Pick, Cellular Immunology 79 (1983) 240.
[65] L.M. Henderson, S. Thomas, G. Banting, J.B. Chappell, Biochemical Journal 325 (1997) 701.
[66] A. Lowenthal, R. Levy, The Journal of Biological Chemistry 274 (1999) 21603.
[67] R. Dana, H.L. Malech, R. Levy, Biochemical Journal 297 (Pt. 1) (1994) 217.
[68] R. Dana, T.L. Leto, H.L. Malech, R. Levy, The Journal of Biological Chemistry 273 (1998) 441.
[69] M. Baggiolini, F. Boulay, J.A. Badwey, J.T. Curnutte, FASEB Journal 7 (1993) 1004.
[70] D.E. Agwu, L.C. McPhail, S. Sozzani, D.A. Bass, C.E. McCall, Journal of Clinical Investigation 88 (1991) 531.
[71] B. Banfi, A. Maturana, S. Jaconi, S. Arnaudeau, T. Laforge, B. Sinha, E. Ligeti, N. Demaurex, K.H. Krause, Science 287 (2000) 138.
[72] D. Qualliotine-Mann, D.E. Agwu, M.D. Ellenburg, C.E. McCall, L.C. McPhail, The Journal of Biological Chemistry 268 (1993) 23843.
[73] N. Alloul, Y. Gorzalczany, M. Itan, N. Sigal, E. Pick, Biochemistry 40 (2001) 14557.
[74] A.R. Cross, R.W. Erickson, J.T. Curnutte, Biochemical Journal 341 (1999) 251.
[75] T.J. Mankelow, L.M. Henderson, Biochemical Journal 358 (2001) 315.
[76] Y. Nisimoto, S. Motalebi, C.H. Han, J.D. Lambeth, The Journal of Biological Chemistry 274 (1999) 22999.
[77] M.H. Paclet, A.W. Coleman, J. Burritt, F. Morel, European Journal of Biochemistry 268 (2001) 5197.
[78] P. Ravel, F. Lederer, Biochemical and Biophysical Research Communications 196 (1993) 543.
[79] D.J. Uhlinger, S.R. Tyagi, J.D. Lambeth, Biochemistry 34 (1995) 524.