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The Active Form of the Ferric Heme in Neutrophil Cytochrome b_{558} Is Low-Spin in the Reconstituted Cell-Free System in the Presence of Amphophil[1]

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The spin state of the heme in superoxide ($O_2^{\bullet-}$)-producing cytochrome b_{558} purified from pig neutrophils was examined by means of room-temperature magnetic circular dichroism (MCD) under physiological conditions. Cytochrome b_{558} with varying amounts of low-spin and high-spin heme was prepared by either pH adjustment or heat treatment, and the $O_2^{\bullet-}$-forming activity in a cell-free system was found to correlate with the low-spin heme content. The possibility that the $O_2^{\bullet-}$-forming activity results from a transient high-spin ferric heme form that is induced during activation by anionic amphophils has also been investigated. EPR spectra of cytochrome b_{558} activated by either arachidonic acid or myristic acid, showed that a transient high-spin ferric species accounting for approximately 50% of the heme appeared in the presence of arachidonic acid, but not in the presence of myristic acid. Hence the appearance of a transient high-spin ferric heme species on activation with an amphophil does not afford a common activation mechanism in the NADPH oxidase system. The EPR results for cytochrome b_{558} activated with arachidonic acid showed that the transient high-spin ferric heme can bind cyanide. However, the high-spin ferric heme does not contribute to the $O_2^{\bullet-}$ production of cytochrome b_{558} in cell-free assays in the presence of cyanide.

Key words: cytochrome b_{558}, EPR, low-spin heme, NADPH oxidase, superoxide.

The phagocyte NADPH oxidase catalyzes the generation of superoxide anions ($O_2^{\bullet-}$) in response to invading microorganisms (for reviews see Refs. *1* and *2*). The $O_2^{\bullet-}$-generating NADPH oxidase is dormant in resting cells and is activated by various stimulants interacting with membrane receptors. Activation of the NADPH oxidase requires the assembly of cytosolic regulatory proteins ($p47^{phox}$, $p67^{phox}$, and GTP-binding protein, Rac 1 or Rac 2) and the membrane-bound protein, cytochrome b_{558}. Cytochrome b_{558} is a heterodimer composed of a large glycosilated subunit, $gp91^{phox}$ (91 kDa) and a small subunit, $p22^{phox}$ (22 kDa). Through many studies on chronic granulomatous disease (CGD), cytochrome b_{558} has been shown to be an essential component of the $O_2^{\bullet-}$-generating NADPH oxidase (*3, 4*).

The $O_2^{\bullet-}$-producing reaction in neutrophils is catalyzed by an NADPH oxidase complex located in the plasma membrane of phagocytic cells. Electron transfer from NADPH to the heme prosthetic group of cytochrome b_{558} is generally thought to be mediated by a FAD moiety. A variety of flavoproteins, such as 77 kDa protein (*5*), nitro blue tetrazolium reductase (*6*), and NADPH-cytochrome *c* reductase (*7*), have been suggested to be possible intermediate electron carriers. However, the addition of these flavoproteins to purified cytochrome b_{558} resulted in low $O_2^{\bullet-}$-generation activity, as compared to that of the intact NADPH oxidase. Several lines of indirect evidence, including the ability of cytochrome b_{558} to bind FAD and the presence of an FAD binding site determined from the sequence homology with ferredoxin-$NADP^+$ reductase (*8-11*), have subsequently suggested that cytochrome b_{558} itself is a flavoprotein. At present the concept of a flavocytochrome b_{558} as the redox core of the NADPH oxidase is widely accepted, even though the FAD-bound form of cytochrome b_{558} has yet to be purified.

Cytochrome b_{558} was first identified as the essential component of the multi-component NADPH oxidase in the early 1980s, and it has been the subject of numerous biochemical and physicochemical studies. The heme group in cytochrome b_{558} was shown to have properties consistent with a role as the terminal oxidase site in $O_2^{\bullet-}$ production, *i.e.*, low redox potential ($E_m = -245$ mV) (*12*), CO-binding capability (*12-14*), NADPH-dependent reduction under anaerobic conditions (*15*), and rapid reoxidation of the reduced heme (*16*). However, studies in this laboratory have shown that the CO-binding capability of intact heme is very poor (*17*), and that no difference in the NADPH-dependent reduction rate of the heme under anaerobic condi-

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tions is apparent for the NADPH oxidase prepared from both stimulated and resting cells (*18, 19*). The combination of EPR and near-IR magnetic circular dichroism (MCD) studies at a cryogenic temperature has also shown that the heme group in resting cytochrome b_{558} is low-spin with bis-histidyl axial ligation (*20, 21*). Moreover, EPR and room temperature MCD studies indicated that the low-spin form of the ferric heme is essential for NADPH oxidase activity to produce $O_2^{\bullet -}$ (*20*). Consequently, it seems unlikely that $O_2^{\bullet -}$ is produced from O_2 bound directly to a vacant site on the reduced heme in cytochrome b_{558}, and the specific role of the heme group in mediating electron transfer to O_2 remains to be determined. This conclusion was recently questioned by the discovery that arachidonic acid, an anionic amphophil which elicits NADPH oxidase activation, induces a low-spin to high-spin transition in cytochrome b_{558} (*22*), thereby providing a vacant coordination site on the heme for O_2 binding.

In the present study, we have prepared oxidized cytochrome b_{558} samples with different low-spin/high-spin heme ratios by either varying the pH or partial thermal-denaturation. Using these preparations, we have reexamined the relationship between the spin-state of the ferric heme of cytochrome b_{558} in the NADPH oxidase and its $O_2^{\bullet -}$-generation activity under physiological conditions by room temperature MCD spectroscopy. In addition, we have used EPR and room temperature MCD to assess the spin state of the ferric heme in cytochrome b_{558}, after activation by an anionic amphophil, arachidonic acid or myristic acid, and attempted to correlate the NADPH oxidase activity with the spin state of ferric heme in activated samples.

EXPERIMENTAL PROCEDURES

Materials—Sodium myristate, myristic acid, and diisopropyl fluorophosphate were obtained from Wako Pure Chemicals, Tokyo. DEAE-Sepharose CL-6B and heparin-Sepharose 6B were obtained from Pharmacia LKB Biotechnology. Heptylthioglucoside and EGTA were purchased from Dojindo Laboratories, Kumamoto. NADPH was purchased from Oriental Yeast, Tokyo. Superoxide dismutase (SOD), cytochrome *c* (type VI, from horse heart), and arachidonic acid were purchased from Sigma, St. Louis. Guanosine 5′-(γ-thio)triphosphate (GTPγS) was a product of Boehringer Mannheim. Other chemical reagents were of analytical grade.

Purification of Cytochrome b_{558}—Resting or myristic acid-stimulated neutrophils were obtained from pig blood as described previously (*23*), and then treated with 2 mM diisopropyl fluorophosphate for 20 min on ice. A membrane fraction was obtained from the sonicated neutrophils, and the NADPH oxidase containing cytochrome b_{558} was solubilized from the membrane fraction with heptylthioglucoside according to the previously described method (*17*). Cytochrome b_{558} was purified from solubilized membranes according to the method reported previously (*20*).

Assaying of Cell-Free $O_2^{\bullet -}$ Production—The $O_2^{\bullet -}$-generation activity of the NADPH oxidase was determined at 25°C in a reconstituted cell-free assay system according to a reported method (*6*) with slight modification. The pH of the purified cytochrome b_{558} preparation was adjusted to a desired value by mixing with small amounts of 0.5 M NaOH or 0.5 M HCl, and the preparations were kept for 10 min on ice. The pH of such preparations was re-adjusted to 7.0 by the addition of 0.5 M NaOH or 0.5 M HCl prior to the assay. Cytochrome b_{558} (2 pmol) was incubated for 5 min at 25°C with cytosol (130–150 μg) from resting cells, 20 μM GTPγS, and an appropriate amount of an amphophil, such as sodium myristate or myristic acid, or arachidonic acid. The assay medium was composed of 50 mM phosphate buffer (pH 7.0), 2 mM NaN_3, 1 mM EGTA, and 1 mM $MgCl_2$, and 30–50 μM cytochrome *c* was added to give a final volume of 0.8 ml. $O_2^{\bullet -}$ production was measured with a Hitachi dual-wavelength spectrophotometer (Model 556) by recording the reduction of cytochrome *c* at 550 nm, with and without SOD after the addition of 125 μM NADPH.

Spectroscopic Measurements—Absorption spectra were measured at 25°C in the range of 400 and 600 nm in a microcuvette (10 mm light path, 3 mm width) in a Unisoku Biospectrophotometer US-401 (Unisoku, Osaka), interfaced to a NEC PC9801 computer. The volume of the sample used in the microcuvette was 120 to 150 μl. Room temperature MCD spectra were recorded using a JASCO J-500 spectropolarimeter interfaced to a Jasco MCD-1B electromagnet. Spectra were recorded in the wavelength range of 350–600 nm, at a magnetic field of 1 T and a temperature of 25°C. EPR spectra were recorded using a Jeol (JES-FE) X-band EPR spectrometer equipped with an LTR Heli-Trans liquid helium flow cryostat (Air Products and Chemicals). The conditions for measurements were as follows: microwave power, 1–10 mW; modulation amplitude, 10 G at 100 kHz; response time, 0.3 s; sweep time, 4 min; and temperature, 5 to 30 K. CuEDTA (1 mM) in water was employed as a concentration standard. The pH of the concentrated purified cytochrome b_{558} preparation was adjusted with aliquots of 0.5 M NaOH or 0.5 M HCl at 25°C, and then measured with a Cosmo pH Boy-C1 at 25°C.

RESULTS

EPR Spectra of Purified Cytochrome b_{558}—Cytochrome b_{558} was purified from pig blood neutrophils without any denaturation, and exhibited high $O_2^{\bullet -}$-generation activity in the cell-free system. The catalytic activity of the purified cytochrome b_{558} preparation used in this study was over 45 mol of $O_2^{\bullet -}$ produced per second per mol of heme in cytochrome b_{558}. Figure 1 shows EPR spectra of the purified cytochrome b_{558} recorded at temperatures in the range of 5–20 K in the low field region. In accord with in previous studies (*21*), the spectra are dominated by two low-spin ferric heme resonances: a fast-relaxing resonance, $g=$ 3.23, 2.10, 1.39, accounting for 70% of the heme, and a slower relaxing resonance, $g=3.00$, $\sim$2.2, $\sim$1.6, accounting for $\sim$30% of the heme. A very weak $g=6$ resonance from an axial high-spin ferric heme is also apparent at temperatures below 10 K, but this is a very minor species accounting for $<5\%$ of the heme at pH 7. In addition, there is a derivative-shaped feature centered at $g=4.3$ that is attributed to a minor component of an adventitiously bound, high-spin Fe(III) ion. Since changes in ferric heme axial ligation and spin-state can occur on freezing (*24*), it is desirable to estimate the ratio of low-spin to high-spin heme in cytochrome b_{558} under physiological conditions, rather than by EPR studies at cryogenic temperatures. In this work, we have utilized MCD spectroscopy to assess the low-spin-to-high-spin ratio for the heme in purified sam-

ples of cytochrome b_{558} at room temperature (*25*).

Room temperature MCD spectra of purified cytochrome b_{558}, obtained at pH values from 3 to 7, were recorded in the range at 350-600 nm (Fig. 2). Since the peak-to-trough Soret-band MCD signal intensity of the low-spin ferric heme is at least 20 times greater than that of the high-spin heme at room temperature (*25*), the percentage of the low-spin heme can be determined from the peak-to-trough Soret band MCD intensity. In this assessment, the Soret band intensity for the purified cytochrome b_{558} at pH 7, which did not show any significant high-spin EPR signal at 5 K, was assumed to correspond to 100% low-spin heme. With this criterion, the percentage of low-spin heme in samples adjusted to pH 3.1, 4.2, 5.7, and 7 was estimated to be 9, 36, 77, and 100%, respectively. These results are consistent with those of previous EPR studies on frozen solutions of cytochrome b_{558} (*20*). The high-spin species is attributed to an irreversibly denatured form, since a return to pH 7 did not restore the MCD or EPR spectrum of the low-spin heme (*20*).

To determine if the low-spin or acid-induced high spin form of the ferric heme in cytochrome b_{558} is more competent in NADPH oxidase assays, the $O_2^{\cdot -}$-generation activity of the purified cytochrome b_{558} after pH adjustment was assessed in the cell-free system. The catalytic activities are plotted in Fig. 3 as a function of the percentage of the low-spin heme, as determined by room temperature MCD. The activity was found to correlate directly with the percentage of low-spin heme, indicating that the acid-induced high-spin heme is not competent for $O_2^{\cdot -}$ generation by the NADPH oxidase. Similar experiments were conducted on samples in which the low-spin to high-spin heme ratio was perturbed by thermal denaturation of cytochrome b_{558} at 40-45°C, in order to exclude the effect of the change in pH on the protein structure. The observed activities of heat-treated cytochrome b_{558} preparations are also plotted in Fig. 3 as a function of the percentage of low-spin heme. Once

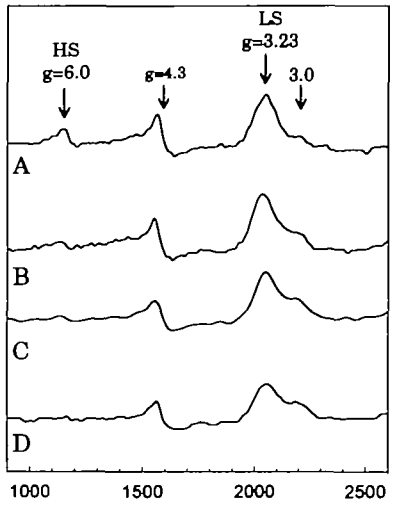

Fig. 1. **EPR spectra of oxidized cytochrome b_{558} recorded at 5-20 K.** Purified cytochrome b_{558} was concentrated to 73 μM and the medium was 50 mM phosphate buffer, pH 7.0. The EPR spectra labeled A, B, C, and D were recorded at 5, 10, 15, and 20 K, respectively. HS and LS indicate high-spin and low-spin heme resonances, respectively. The EPR instrumental settings were as follows: microwave frequency, 9.04 GHz; microwave power, 10 mW; modulation amplitude, 10 gauss; and response time, 0.3 s.

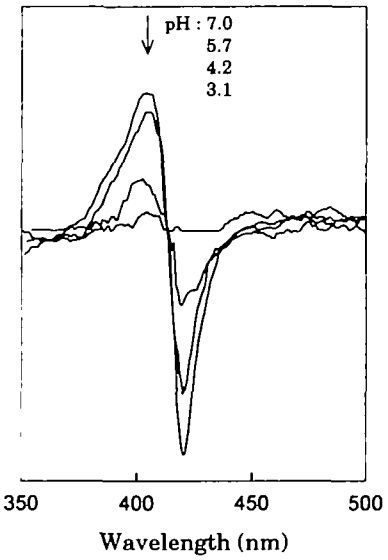

Fig. 2. **Effect of pH on the room-temperature MCD spectra of oxidized cytochrome b_{558}.** Purified cytochrome b_{558} was concentrated to 45 μM, and the pH was adjusted by addition of aliquots of 0.5 M NaOH or 0.5 M HCl at 0°C. The pH values were as follows (from top to bottom); pH 7.0, 5.7, 4.2, and 3.1. The MCD spectra were recorded at 25°C. The cytochrome b_{558} sample used in this experiment did not exhibit a significant high-spin EPR signal at pH 7.0 in spectra measured at 5 K. The percentage of low-spin heme in cytochrome b_{558} at pH 7.0 was therefore assumed to be 100%, and the percentage of the low-spin heme in cytochrome b_{558} at each pH was calculated based on the Soret-band MCD intensity.

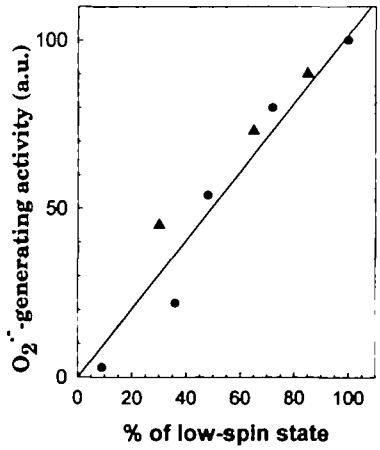

Fig. 3. **Effect of the percentage of the low-spin heme in cytochrome b_{558} on the $O_2^{\cdot -}$-generation activity of the NADPH oxidase system.** Samples of cytochrome b_{558} with different ratios of low-spin to high-spin heme were prepared by adjusting the pH (●) and by heat-treatment at 40°C (▲). In both cases, the percentages of low-spin heme were calculated based on MCD spectra measured at 25°C. The $O_2^{\cdot -}$-generation activity of these preparations was measured in the cell-free system at pH 7.0. The reaction mixture contained cytochrome b_{558} (2 pmol), cytosol (135 μg), and 30 μM cytochrome *c* in 800 μl of phosphate buffer (50 mM, pH. 7.0).

again, the $O_2^{\bullet -}$-generation activity correlates with the percentage of low-spin heme, suggesting that the low-spin form is the catalytically competent species in the NADPH oxidase.

Spin-State of the Ferric Heme in Cytochrome b_{558} Activated by Anionic Amphophils—The above experiments demonstrate that the oxidized resting heme in active cytochrome b_{558} is low-spin ferric. To determine if a low-spin-to-high-spin conversion occurs during the activation process in a cell-free assay system, the effects of the addition of two typical amphophils, arachidonic and myristic acid, were investigated. First, dose-effect experiments were performed to examine the effects of increasing concentrations of each amphophil on the $O_2^{\bullet -}$-generation activity of the NADPH oxidase in the reconstituted cell-free system. As shown in Table I, on a per heme basis, 4-5 times more myristic acid is required to induce maximal activity than in the case of arachidonic acid. Furthermore, when cytochrome b_{558} prepared from porcine neutrophils was used, the $O_2^{\bullet -}$-generation activity of the oxidase elicited by myristic acid was about 20-30% higher than that induced by arachidonic acid.

The effects of arachidonic and myristic acid, at concentrations that induce optimal $O_2^{\bullet -}$-generation activity, on the EPR properties of oxidized cytochrome b_{558} at 10 K are shown in Fig. 4. The preparation of cytochrome b_{558} used for these EPR experiments contained both the "$g=3.23$" and "$g=3.00$" low-spin species, and a minor high-spin "$g=6$" species. Room temperature MCD studies indicated that 93% of the heme is in the low-spin form in this preparation. In accord with the results of Doussière *et al.* (*22*), activation with arachidonic acid induces a low-spin-to-high-spin transition, as evidenced by the dramatic increase in the "$g=6$" resonance and the concomitant decrease in the "$g=3.23$" resonance. This low-spin-to-high-spin transition was irreversible. However, both EPR quantitation and room temperature MCD studies indicated that the resulting sample is an approximate 50:50 mixture of high-spin and low-spin forms. The addition of optimal amounts of cytosol to further activate the oxidase did not perturb the spin-state mixture. Hence it is unclear if it is the low-spin or high-spin form that is responsible for the activity in these activated preparations.

Very different EPR results were obtained for samples of purified cytochrome b_{558} activated with myristic acid, which is widely used as a stimulant of the oxidase (*26, 27*). No increase in the high-spin component was observed, even when an appropriate amount of myristic acid was added to obtain the optimal oxidase activation. However, the ratio of the "$g=3.23$" and "$g=3.00$" low-spin species was slightly perturbed in favor of the latter species. This result clearly demonstrates that generation of a transient high-spin heme is not required during activation by anionic amphophils.

Properties of the High-Spin Ferric Heme in Cytochrome b_{558}—In order to clarify whether or not the high-spin ferric heme in arachidonic acid-activated cytochrome b_{558} is responsible for $O_2^{\bullet -}$ generation, the properties of the high-spin heme in the activated oxidase were further investigated. First, the ability of the high-spin ferric heme to bind cyanide was assessed by EPR spectroscopy. Figure 5, A and B, shows EPR spectra in the presence of arachidonic acid before and after addition of cyanide. The EPR spectrum of the arachidonic acid-treated sample used in this study was dominated by the $g=6$ resonance of the high-spin species. Upon the addition of 2 mM cyanide, the high-spin signal at $g=6$ dramatically decreased and a new low-spin EPR signal appeared with a low-field component at $g=2.55$. This resonance is attributed to cyanide binding to the high-spin heme. In addition, a weak $g=3.2$ resonance for the unperturbed low-spin heme was now clearly visible. These EPR spectra show that the high-spin heme can form a complex with cyanide, and in general the properties are analogous to those of high-spin ferric hemes in hemoglobin and denatured forms of cytochrome b_{558} (*20*). It has long been known that activated NADPH oxidase in neutrophils

TABLE I. **Effect of arachidonic acid (AA) or myristic acid (MA) on the $O_2^{\bullet -}$-generation activity of the NADPH oxidase system in the reconstituted cell-free system.** An aliquot of purified cytochrome b_{558} (2 pmol) was incubated with cytosol (135 μg), 10 μM GTPγS, and several amounts of arachidonic acid (AA) or myristic acid (MA) in 0.1 ml of phosphate buffer (50 mM, pH 7.0). The assay was carried out in the absence of added FAD at 25°C. The amount of AA or MA added to the reaction mixture is shown in μmol per nmol of the heme in the reaction mixture.

[AA] (μmol)	[MA] (μmol)	$O_2^{\bullet -}$-generation activity (mol of $O_2^{\bullet -}$/s/mol of heme) ($n=3$)
0.5	–	21.2±2.3
1.0	–	32.5±4.1
2.3	–	60.4±8.7
3.0	–	34.3±5.2
–	4.8	12.4±1.8
–	7.3	49.5±6.6
–	10.2	72.1±10.5
–	17.5	23.8±3.8

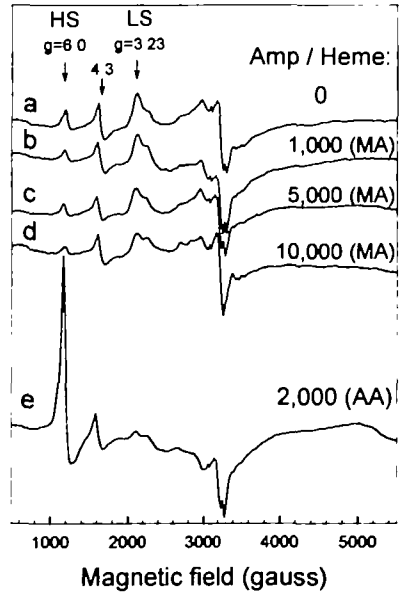

Fig. 4. **Effect of amphophil (Amp), arachidonic acid (AA) or myristic acid (MA), on the EPR spectra of purified cytochrome b_{558}.** Purified cytochrome b_{558} was concentrated to 75 μM, and the medium was 50 mM phosphate buffer, pH 7.0. Trace a, oxidized cytochrome b_{558}, as purified; traces b-d, cytochrome b_{558} incubated for 5 min at 25°C with 1, 5, and 10 μmol of myristic acid (MA) per nmol of the heme, respectively; trace e, cytochrome b_{558} incubated for 5 min at 25°C with 2 μmol of arachidonic acid per nmol of the heme. The instrumental conditions for all spectra were identical to those in Fig. 1, and all spectra were recorded at 10 K.

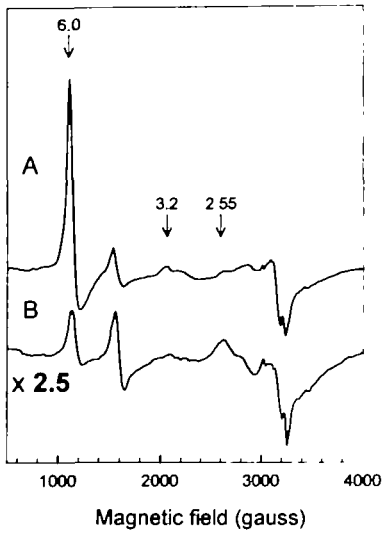

Fig. 5. **Effect of cyanide on the EPR spectrum of oxidized cytochrome b_{558} in the presence of arachidonic acid.** Purified cytochrome b_{558} was concentrated to 75 μM, and the medium was 50 mM phosphate buffer, pH 7.0. Spectrum A: oxidized cytochrome b_{558} was incubated for 5 min at 25°C, with 2 μmol of arachidonic acid per nmol of the heme. Spectrum B: the sample used in spectrum A was incubated with 2 mM cyanide for 5 min at 25°C. The receiver gain in spectrum B was 2.5 times higher than that in spectrum A. The instrumental conditions for all spectra were identical to those used in Fig. 1.

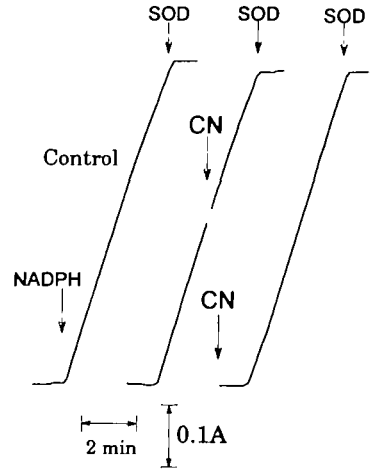

Fig. 6. **Effect of cyanide on the $O_2^{\bullet-}$ generation in the reconstituted NADPH oxidase system.** Purified cytochrome b_{558} was incubated with arachidonic acid, as described in Fig. 5, and the $O_2^{\bullet-}$-generation activity of this preparation was assayed in a cell-free system, as described in Fig. 3. Left trace, control experiment, with NADPH (125 μM) added to start the reaction. Middle trace, 2 mM cyanide (CN) was added, subsequent to initiating the reaction with NADPH, at the time indicated by the arrow. Right trace, the sample was incubated with 2 mM cyanide for 2 min before the reaction was initiated by the addition of NADPH.

exhibits a cyanide-insensitive respiratory burst (*28*). Since the high-spin form of the heme in cytochrome b_{558} can bind cyanide, we conclude that it is unlikely to function as the $O_2^{\bullet-}$-generating site.

The $O_2^{\bullet-}$-generation activity of cytochrome b_{558} activated by arachidonic acid was also measured in the cell-free system in the presence and absence of cyanide. Figure 6 shows the time-course of NADPH-dependent cytochrome *c* reduction catalyzed by the arachidonic acid-activated NADPH oxidase in the absence (left trace) and presence of cyanide (middle and right traces). In the absence of cyanide, the activated NADPH oxidase showed $O_2^{\bullet-}$-generation activity of 24 ± 2.5 mol of $O_2^{\bullet-}$ produced per s/mol of cytochrome b_{558} in the cell-free system. When cyanide was added to the activated oxidase, the $O_2^{\bullet-}$-generation activity, 21 ± 1.8 mol of $O_2^{\bullet-}$, was the same as that of the control, *i.e.* no inhibition or enhancement of the $O_2^{\bullet-}$-generation activity of the activated oxidase was observed. These results strongly indicate that the high-spin heme in the activated cytochrome b_{558} does not contribute to the $O_2^{\bullet-}$ generation of the NADPH oxidase in the cell-free system.

DISCUSSION

Our previous EPR studies on purified cytochrome b_{558}, prepared from either stimulated or resting neutrophils, showed that undenatured cytochrome b_{558} is predominantly in the low-spin state (*20, 21*). These studies also suggested that the low-spin state of the heme is essential for $O_2^{\bullet-}$ generation and that the heme remains in the low-spin state throughout the redox cycle (*20, 21*). This conclusion was recently questioned by the observation that arachidonic acid, an anionic amphophil which induces oxidase activation in a cell-free system, converts the six-coordinate low-spin ferric heme to a five-coordinate high-spin ferric heme (*22*). The pentacoordinated form of the reduced heme was proposed to react directly with O_2 to generate $O_2^{\bullet-}$ (*22*). This prompted us to reinvestigate the relationship between the spin state of the ferric heme and the $O_2^{\bullet-}$-generation activity, by both EPR at cryogenic temperatures and room temperature MCD. The latter approach is capable of determining a spin-state mixture under physiological conditions, thereby ruling out the possibility of freezing artifacts. In addition, the ferric heme spin states have been assessed in samples activated with two anionic amphophils, arachidonic and myristic acid. Finally, the possibility that the high-spin ferric form, induced by arachidonic acid, is responsible for $O_2^{\bullet-}$ production, has been addressed by investigating the effects of cyanide on the EPR properties and oxidase activity.

The results show a direct correlation between the low-spin heme content and $O_2^{\bullet-}$-generation activity for samples containing varying amounts of the high-spin heme induced by irreversible thermal denaturation or exposure to acidic pH. Although the EPR properties of the high-spin ferric heme in irreversibly denatured samples are indistinguishable from those of the high-spin heme that is induced by arachidonic acid (*22*), these results leave open the possibility that a transient, reversibly generated high-spin heme species is responsible for $O_2^{\bullet-}$ production in cytochrome b_{558}. However, EPR studies with myristic acid-activated samples clearly demonstrated that transient production of a high-spin heme is not a general requirement for $O_2^{\bullet-}$-generation activity. Moreover, arachidonic acid-activated samples were shown to contain both high-spin and low-spin ferric hemes in an approximate 50:50 mixture, and the effects of cyanide on the EPR properties and activity argue strongly against a role for the high-spin form in the $O_2^{\bullet-}$-

generation activity. Cyanide was shown to bind at the vacant coordination site of the high-spin heme to give a low-spin species, but has no effect on the $O_2^{\bullet -}$-generation activity of arachidonic acid-activated samples. Hence these results indicate that it is the low-spin heme component in arachidonic acid-activated samples that is responsible for the catalytic activity. From all of these results, we conclude that the appearance of the high-spin ferric state of the heme is not related to the activation of the NADPH oxidase in the cell-free system. We cannot yet rule out the possibility that it is a pentacoordinate high-spin ferrous form that reacts with O_2 in amphophil-activated samples, *i.e.* that there is redox-dependent spin-state change for the heme in activated samples. Variable-temperature MCD and resonance Raman experiments on reduced samples in the presence of amphophils are planned to address this question.

Although all the components of the NADPH oxidase have been identified, little is known about the mechanism for their assembly leading to activation of the oxidase, especially the mechanism and function of amphophil stimulants such as arachidonic acid, myristic acid and sodium dodecyl sulfate (SDS). Sumimoto *et al.* (*29*) reported that activation by arachidonic acid or SDS is induced by a conformational change of cytosolic proteins, *i.e.*, the exposure of the Src homology 3 (SH3) domains of $p47^{phox}$, which had been masked by the C-terminal region of this protein in a resting state. This unmasking by amphophils plays a crucial role in the assembly of the oxidase components, because the unmasking allows $p47^{phox}$ to interact with the target protein, cytochrome b_{558}. In addition, amphophils can interact directly with proteins, and this interaction is dependent on their hydrophilic-hydrophobic balance. Therefore, arachidonic acid tends to bind not only $p47^{phox}$ but also cytochrome b_{558}, and the binding of arachidonic acid to cytochrome b_{558} seems to induce the conformational change in the protein structure that leads to the appearance of the high-spin heme. The absence of a low-spin-to-high-spin transition with myristic acid is therefore interpreted in terms of weaker binding interaction between myristic acid and cytochrome b_{558} compared with arachidonic acid. This interpretation is tentatively supported by the EPR results for cytochrome b_{558} plus arachidonic acid in the presence of cytosol. The addition of cytosol did not induce any change in the high-spin heme content, suggesting that arachidonic acid binds to cytochrome b_{558} more tightly than to cytosolic proteins.

In the present study, we conclude that the low-spin ferric form of the heme is essential for the $O_2^{\bullet -}$ generation of the NADPH oxidase, but this conclusion does not necessarily mean that the low-spin heme in cytochrome b_{558} functions as the terminal oxidase of the NADPH oxidase. For example, it should be emphasized that we have been studying the physical properties of the oxidized heme in cytochrome b_{558}. We have yet to obtain either direct or indirect evidence of $O_2^{\bullet -}$ generation from reduced heme in cytochrome b_{558}, and the spin state of the reduced heme in amphophil-activated samples has yet to be determined. Hence a pentacoordinate high-spin ferrous heme might be responsible for the binding of O_2 in activated samples of cytochrome b_{558}. Once electron transfer from Fe(II) to O_2 has occurred, rebinding of a histidine axial ligand to give the low-spin ferric species may assist the removal of bound $O_2^{\bullet -}$. On the basis of the results of spectroscopic studies, Isogai *et al.* (*16*) hypothesized that electrons are transferred from the reduced heme edge of cytochrome b_{558} to oxygen without the formation of an oxygenated complex of the heme. If this is the case, some electron acceptors might be capable of intercepting an electron that is transferred from the reduced heme edge to O_2. However, studies involving a range of electron acceptors, with redox potentials ranging from -250 to 0 mV, have failed to reveal evidence of inhibition of $O_2^{\bullet -}$ generation by the NADPH oxidase (*17, 30*). Alternatively, the heme group in cytochrome b_{558} might not contribute to the electron transfer, but function only in maintaining the proper conformational structure. A precedent for such a role is the heme *b* prosthetic group in Complex II (succinate:ubiquinone oxidoreductase) from *Escherichia coli* (*31*). Clearly there is much that remains to be learned concerning the role of the heme prosthetic group in cytochrome b_{558}.

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