Binding of FAD to Cytochrome b\textsubscript{558} Is Facilitated during Activation of the Phagocyte NADPH Oxidase, Leading to Superoxide Production

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The superoxide-producing phagocyte NADPH oxidase can be reconstituted in a cell-free system. The activity of NADPH oxidase is dependent on FAD, but the physiological status of FAD in the oxidase is not fully elucidated. To clarify the role of FAD in NADPH oxidase, FAD-free full-length recombinant p47\textsubscript{phox}, p67\textsubscript{phox}, and p40\textsubscript{phox}, and Rac were prepared, and the activity was reconstituted with these proteins and purified cytochrome b\textsubscript{558} (cyt b\textsubscript{558}) with different amounts of FAD. A remarkably high activity, over 100 μmol/s/μmol heme, was obtained in the oxidase with purified cyt b\textsubscript{558} ternary complex (p47\textsubscript{phox}-p67\textsubscript{phox}-p40\textsubscript{phox}), and Rac. From titration with FAD of the activity of NADPH oxidase reconstituted with purified FAD-devoid cyt b\textsubscript{558} dissociation constant \(K_a\) of FAD in cyt b\textsubscript{558} of reconstituted oxidase was estimated as nearly 1 nm. We also examined addition of FAD on the assembly process in reconstituted oxidase. The activity was remarkably enhanced when FAD was present during assembly process, and the efficacy of incorporating FAD into the vacant FAD site in purified cyt b\textsubscript{558} increased, compared when FAD was added after assembly processes. The absorption spectra of reconstituted oxidase under anaerobiosis showed that incorporation of FAD into cyt b\textsubscript{558} recovered electron flow from NADPH to heme. From both \(K_a\) values of FAD and the amount of incorporated FAD in cyt b\textsubscript{558} of reconstituted oxidase, in combination with spectra, we propose the model in which the \(K_a\) values of FAD in cyt b\textsubscript{558} is changeable after activation and FAD binding works as a switch to regulate electron transfer in NADPH oxidase.

The phagocyte NADPH oxidase catalyzes the generation of superoxide anions (O\textsubscript{2}\textsuperscript{−}) in response to invading microorganisms. Superoxide anions are precursors of a variety of reactive oxygen species that are utilized in killing bacterial and fungal pathogens (1, 2). The physiological significance of the phagocyte NADPH oxidase in host defense is illustrated by the severe recurrent bacterial and fungal infections that occur in patients with chronic granulomatous disease whose phagocytes are unable to generate O\textsubscript{2}\textsuperscript{−} (3, 4). This NADPH oxidase complex consists of membrane-bound flavocytochrome b\textsubscript{558}, a heterodimer composed of gp91\textsubscript{phox} and p22\textsubscript{phox}, four cytosolic proteins, p40\textsubscript{phox}, p47\textsubscript{phox}, p67\textsubscript{phox}, and the small GTPase Rac. In the resting cells, p40\textsubscript{phox}, p47\textsubscript{phox}, and p67\textsubscript{phox} exist as a heterotrimetric complex that contains one copy of each protein (5). Activation of the oxidase is initiated by phosphorylation, which might induce the conformational changes by modulating intra- and intermolecular interactions in the p47\textsubscript{phox}-p67\textsubscript{phox} complex. With these interactions, the activated oxidase is formed via assembly of the cytosolic regulatory proteins with cyt b\textsubscript{558}.

Activation of phagocyte NADPH oxidase can be mimicked in a cell-free system reconstituted with cyt b\textsubscript{558}, p47\textsubscript{phox}, p67\textsubscript{phox}, p40\textsubscript{phox}, and Rac. These cytosolic proteins bind to cyt b\textsubscript{558} to form an active oxidase complex, which can generate O\textsubscript{2}\textsuperscript{−} in the presence of NADPH. The activated NADPH oxidase is highly labile because of the dissociation of each subunit of the active complex (6). Recently, interactions among each subunit protein have been extensively studied; protein-protein interactions mediated by Src homolog 3 (SH3), tetratricopeptide repeat domain, and switch I have been reported (7–9). In the cytoplasm of resting cells, p40\textsubscript{phox}, p47\textsubscript{phox}, and p67\textsubscript{phox} exist as a tight complex that can be purified by gel chromatography with an apparent molecular mass of 250–300 kDa (10–11). Sedimentation equilibrium and dynamic light-scattering experiments disclosed that the p47\textsubscript{phox}-p40\textsubscript{phox} complex contains one copy of each protein, and the apparent high molecular weight of this complex, as estimated by gel filtration studies, is because of an extended, nonglobular shape (5).

gp91\textsubscript{phox} in cyt b\textsubscript{558} represents the only catalytic component in NADPH oxidase, containing both redox centers, FAD, and two nonidentical hemes. Many studies of the heme in cyt b\textsubscript{558} have been reported as follows: (i) two hemes are located in gp91\textsubscript{phox} (12); (ii) two nonidentical hemes with midpoint redox potentials of −265 and −225 mV (13); (iii) the heme has a low spin six coordination site (14); and (iv) the low spin state of the heme is essential for O\textsubscript{2}\textsuperscript{−} generation in reconstituted NADPH oxidase (15). In contrast to the extensive studies on the heme, a relatively small amount of information on FAD (16–18) has been described for the heme.”

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**The abbreviations used are: cyt b\textsubscript{558}, cytochrome b\textsubscript{558}; HTG, N-heptetyl-D-thio-glucoside; TC, ternary complex of recombinant cytosolic proteins p47\textsubscript{phox}-p67\textsubscript{phox}; GTP\textsubscript{S}, guanosine 5'-[y-thio]triphosphate; PBS, phosphate-buffered saline; Ni-NTA, nickel-nitriiyoacetic acid; AEBSF, 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride; SH3, Src homolog 3; gp, glycoprotein.**
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been obtained, including such as the contents of flavins (FAD) and FMN) in the oxidase and reoxidation in flavocytochrome b₅₅₈. One plausible model is that FAD is involved in O₂ generating in NADPH oxidase and that exogenous FAD can restore O₂ generating activities in the cell-free system reconstructed with FAD-depleted purified cyt b₅₅₈ and cytosolic proteins. Detailed information on the behavior of FAD molecules in the FAD-binding site of the NADPH oxidase and its physiological status is requisite to elucidate the electron transfer reactions among the two redox centers, but the behavior of FAD in intact cells is still unclear. Therefore, a simplified in vitro experiment excluding unfavorable side reactions and thermal instability of participating proteins is desired and required.

In the present work we describe the reconstitution of NADPH oxidase activity in a cell-free system with FAD-depleted purified cyt b₅₅₈ and recombinant FAD-free cytosolic regulatory proteins, i.e. the ternary complex (p47-p67-p40) and Rac. We report the effects of exogenously added FAD on the reconstituted activity of NADPH oxidase, and we show that enhancement of the activity by FAD is dependent on the assembly of the reconstituted oxidase. Furthermore, we show that electron transfer from NADPH to heme under anaerobicosis is restored after incorporating exogenous FAD into FAD-depleted cyt b₅₅₈. These analyses provide the direct evidence that the electron flow from NADPH to heme via FAD observed is directly related to the O₂-generating reaction in phagocyte NADPH oxidase.

EXPERIMENTAL PROCEDURES

Materials—Sodium pyrurate and diisopropyl fluorophosphates were obtained from Wako Pure Chemicals. DEAE-Sepharose CL-6B, CM-Sepharose, heparin-Sepharose, glutathione-Sepharose 4B, Superdex 75 column, Superdex 200 column, pGEX 6P, and PreScission protease were obtained from Amersham Biosciences. N-Hexyl-β-thioglucoside (HTG) and EGTA were purchased from Dojindo Laboratories. NADPH was from Oriental Yeast. Superoxide dismutase, FAD, FMN, GTP, S-glucose oxidase, arachidonic acid, phospholipid, 12-myristate 13-acetate, and cytochrome c (type VI from horse heart) were purchased from Sigma. Ni-NTA was obtained from Qiagen. pProEx HTB and TEV protease were purchased from Invitrogen. Centriprep YM-10 was obtained from Millipore. 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (AEBSF) was purchased from Nacalai Tesque. Kodakaraensis DNA polymerase for PCR and ligase high for subcloning were obtained from Toyobo. Restriction enzymes, EcoRI and NdeI, were purchased from Takara. The oligonucleotide primers were synthesized by Sigma-Genosys.

Purification of Cyt b₅₅₈—Neutrophils were obtained from pig blood, as described previously (19), and then treated with 2 mM diisopropyl fluorophosphate for 20 min on ice. Cyt b₅₅₈ was solubilized from the membrane fraction with HTG and was purified by using DEAE-Sepharose, CM-Sepharose, and heparin-Sepharose as described previously (14, 20). The specific content of heme in the purified cyt b₅₅₈ preparation was 10.6–12.4 nmol per mg of protein.

Preparation of Recombinant Proteins—the hexahistidine tag was introduced into the vector pGEX-6P-1. Two oligonucleotides with the sequences 5′-TGCATCTCACCATCATCATATACGGC-3′ and 5′-GGCCGATATGTCCCT-3′ were annealed and phosphorylated. The product was ligated into SalI- and NotI-digested pGEX-6P-1 (pGEX-His).

The full-length p47phox was cloned by PCR from the plasmid of human p47phox (7, 21), using 5′-GGGATCCATGCGGCCAGCGTTGC-3′ as the forward primer and 5′-GGGTGAGCCAGCTCTGCAGTGTTTC-3′ as the reverse primer. The PCR product was digested with BamHI and SalI, gel-purified, and ligated into BamHI- and SalI-digested pGEX-His. The plasmids of the full-length p47phox with amino-terminal glutathione S-transferase-tagged and carboxyl-terminal His-tagged constructs were transformed and overexpressed, as shown in the preparation for the full-length p47phox. From obtained cells, the full-length p47phox was similarly purified, as shown above.

The full-length p40phox was amplified by PCR from the plasmid of human p40phox (22), using 5′-CCCCATCGTGTGGCGCCAGCAGCGTGC-3′ as the forward primer and 5′-CCGAATTCATATGCGCCATTGCTGTTGAGCCATATGTTG-3′ as the reverse primer. The PCR product was digested with NcoI and EcoRI, gel-purified, and ligated into BamHI and SalI-digested pGEX-His. The PCR product was cloned into pProEx HTB (pPro-p40). The plasmid pP-Trx was a generous gift from Dr. S. Ishii (Laboratory of Molecular Genetics, The Institute of Physical and Chemical Research, RIKEN) (23). These plasmids of pPro-p40 and pP-Trx were co-transformed in E. coli BL21(DE3) and were overexpressed. The cells were disrupted by sonication at 4 °C in 25 mM Tris buffer, pH 7.8, and 500 mM NaCl. The protein was applied on a Ni-NTA column equilibrated with 25 mM Tris buffer, pH 7.8, 500 mM NaCl, and 5 mM imidazole. The bound protein was eluted with 25 mM Tris buffer, pH 7.8, 500 mM NaCl, and 250 mM imidazole. Fractions containing proteins were purified on a Superdex 75 gel filtration column and eluted with 25 mM Tris buffer, pH 8.5, and 150 mM NaCl. The protein was concentrated by Centriprep YM-30 to around 10 mg/ml.

The DNA encoding Rac2 constitutively active form was obtained, as described previously (8), and was subcloned into pProEx HTB (pPro-Hac2). The plasmid pProRac2 was transformed in E. coli BL21(DE3) and was overexpressed. The cells were disrupted by sonication at 4 °C in PBS including 0.1 mM AEBSF, pH 7.4. The protein was applied on a Ni-NTA column equilibrated with PBS and 5 mM imidazole, pH 7.4. After washing with a 20-fold column volume of PBS and 10-fold column volume of 25 mM Tris buffer, pH 7.4, including 5 mM imidazole, the bound protein was eluted with 500 mM NaCl and 250 mM imidazole. Fractions containing proteins were purified on a Superdex 75 gel filtration column and eluted with 25 mM Tris buffer, pH 8.0, 150 mM NaCl.

Preparation of Binary and Ternary Cytosolic Complexes—To obtain binary complexes of p47-p67phox and p67-p40phox, p67phox was mixed with a 2-fold molar excess of p47phox or p40phox. After the mixtures had been incubated for more than 10 min at 4 °C, the binary complexes were purified from excess uncomplexed protein (p47phox or p40phox) by gel filtration on a Superdex 200 column using a buffer containing 50 mM Tris buffer, pH 7.4, and 150 mM NaCl. Fractions containing the binary complexes were concentrated by Centriprep YM-50 to around 10 mg protein/ml.

The ternary complex of p47-p67-p40 was obtained by mixing the binary complex p47-p67phox with a 2-fold molar excess of p40phox. After the mixtures had been incubated for more than 10 min at 4 °C, the binary complexes were purified from excess uncomplexed protein (p47phox or p40phox) by gel filtration on a Superdex 200 column using a buffer containing 50 mM Tris buffer, pH 7.4, and 150 mM NaCl. Fractions containing the ternary complex were concentrated by Centriprep YM-50 to around 10 mg protein/ml.

Preparation of Truncated Binary Complex—Fusion protein between truncated p67phox (1-242) and truncated p47phox (151-286) (p67-p40phox truncated binary complex) was constructed by a two-step PCR technique using four primer sets. The first step was a PCR with the spheroplast of Staphylococcus aureus, and the truncated p47phox gene. The DNA fragment encoding p67phox (1-242) was amplified from the full-length p67phox gene by PCR using primers as follows: 5′-primer (primer A) was 5′-GGCCATATGCGTTGAGCCATACG-3′, including NdeI digestion site at the 5′-
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RESULTS

Ternary Complex Used in This Study—The soluble full-length proteins of p47^phox, p67^phox, p40^phox, and p44^phox were obtained in relatively high yields (>20 mg of protein/liter), as described under “Experimental Procedures.” Fig. 1A shows the gel filtration chromatogram of purified proteins; binary (p47-p67^phox) and ternary (p47-p67-p40^phox) complexes were fractionated without contamination of monomer components. The molecular weight of purified ternary complex was calibrated by the analytical gel filtration, and the obtained value was around 250 kDa, which was similar to that in the previous report (5). Fig. 1B shows silver-stained SDS-PAGE analysis of purified proteins. The purity of each sample is demonstrated by SDS-PAGE, full-length p40^phox (lane 1), p47^phox (lane 2), p67^phox (lane 3), binary (p47-p67^phox), and ternary (p47-p67-p40^phox) complexes are shown in Fig. 1B.

Reconstituted Activity of NADPH Oxidase—Cyt b_{558} was purified from neutrophil membranes without any modification of the heme environment and was used for evaluation of O_2 generating activity in the cell-free system. Heme and FAD associated with the cytochrome preparations at each purification step were measured and are summarized in Table I. Crude membrane contained the heme and FAD of cyt b_{558} in a molar ratio of about 2.5:1. During the purification, the specific content of FAD decreased, whereas that of the heme increased. These purified cyt b_{558} exhibited high O_2 generating activity in the cell-free system with native cytosol, which shows that cyt b_{558} used in this study was highly purified without any irreversible protein denaturation.

The O_2 generating activity of the oxidase was reconstituted in the cell-free system with recombinant, full-length FAD-free cytosolic proteins (Rac2, p47^phox, p40^phox, and p44^phox) and purified cyt b_{558}. Incubation of the cytosolic component individually and in combination or the TC with purified cyt b_{558} was examined, and the purified cyt b_{558} showed cytosolic protein-dependent superoxide production. In the cell-free reconstituted system, the activity was totally dependent on the addition of both FAD and anionic amphiphilic activators (such as myristic acid or arachidonic acid). Replacement of FAD with FMN abolished the stimulation of superoxide production. The remarkably high O_2 generating activity of the reconstituted oxidase, over 100 μmol of O_2/s/μmole of heme, was obtained in the system with TC. The reconstituted oxidase activity with TC (p47-p67-p40^phox) was compared with the binary complex p47-p67^phox. The activity with TC was 9.9 ± 2.8% (p < 0.05) higher than with p47-p67^phox, showing that p40^phox is not essential for reconstitution of the activity. Previous findings (28, 29) were also confirmed in the cell-free reconstituted system with the recombinant TC. In the case of the other binary complexes, p47-p40^phox or p67-p40^phox, any superoxide generating activity was not detected under similar reaction conditions.

Stability of the Reconstituted NADPH Oxidase Activity—The purified ternary complex, p47-p67-p40^phox, exists at a tight complex of 1:1:1 stoichiometry in solutions (5) and is stable for at least 24 h without any dissociation of each component, as judged from the gel filtration experiment of purified ternary complex. Fig. 2 shows the stability of the oxidase activity reconstituted with TC at 25°C. When the reconstituted oxidase was left for 60 min at 25°C, the activity of the oxidase with TC was about 90% of its initial level, and its stability was remarkably elongated compared with that in the native cytosol. The half-lives of the activity with TC and the native cytosol were about 10 min and >12 h, respectively. Because the lability of the oxidase activity is caused by the dissociation of each protein in the oxidase complex (6), the long stability of the reconstituted activity with TC might be attributable to the stable structure of the reconstituted oxidase complex.

Titration with FAD of Superoxide Generating Activity of the Reconstituted NADPH Oxidase—In earlier experiments using the cell-free system, native cytosolic fractions were used with either purified or crude cyt b_{558}. There have been several reports on the titration of reconstituted oxidase activity with FAD, but an accurate stoichiometric analysis of FAD for activity was quite difficult because of the presence of free FAD or loosely bound FAD to the cytosolic proteins. Therefore, in this study, a titration study of reconstituted activity was carried out, employing the FAD-free p47-p67-p40^phox complex and Rac and purified cyt b_{558} with different FAD contents. Fig. 3 shows the effect of exogenously added FAD on the production of superoxide by purified cyt b_{558} in the presence of different con-
centrations of FAD-free TC. The purified cyt \( b_{558} \) lost most of its FAD, compared with that of partially purified cyt \( b_{558} \), and the content of heme and FAD was at a molar ratio of about 1:0.01–1:0.03 (94–98% of FAD was lost), assuming that the ratio of heme to FAD was 2:1. The reconstituted oxidase activity was enhanced remarkably by the addition of exogenous FAD and reached a plateau when the ratio of FAD to heme exceeded 2.0. The minimal amount of exogenous FAD required to manifest optimal activity did not vary with increasing amounts of TC. Replacement of FAD with FMN abolished the stimulation of superoxide production. These results indicate that the enhancement of the activity by the addition of FAD depends on the ratio of added FAD to heme present in cyt \( b_{558} \) but not on that of added FAD to TC.

**Effects of Addition of FAD on the Assembly Process of the NADPH Oxidase**—The effects of the addition of FAD on the assembly of proteins in the reconstituted oxidase were examined in NADPH oxidase reconstituted with purified cyt \( b_{558} \) and cytosolic protein complex, TC. The results for the reconstituted oxidase with TC and purified cyt \( b_{558} \) are shown in Fig.

| TABLE I: Purification of cyt \( b_{558} \) from resting neutrophil membranes |
|-----------------------------------------------|
|                  | Heme | FAD | Heme/FAD | Activity* |
|-------------------|------|-----|----------|-----------|
| Crude membrane    | 238 ± 15 \( (n = 15) \) | 95 ± 13 \( (n = 15) \) | 2.51 | 14.2 ± 2.5 \( (n = 15) \) |
| Crude extract     | 1650 ± 123 \( (n = 15) \) | 432 ± 72 \( (n = 15) \) | 3.82 | 64.5 ± 8.3 \( (n = 15) \) |
| Purified cyt \( b_{558} \) | 11,600 ± 950 \( (n = 12) \) | 201 ± 94 \( (n = 12) \) | 57.7 | 60.8 ± 6.7 \( (n = 12) \) |

* Superoxide generating activity was evaluated in a cell-free system reconstituted with native cytosol and membrane fractions or purified cyt \( b_{558} \) at 25 °C.
4A in the presence and absence of FAD. In the reconstituted oxidase system with purified cyt b$_{558}$ devoid of FAD, the activity was enhanced remarkably by the addition of FAD, but the time of addition of FAD to the system was reflected in the resultant superoxide generating activity. When FAD was added to the mixture of cytosolic proteins and purified cyt b$_{558}$ before treatment of such a mixture with anionic amphiphiles, i.e., FAD was present during the assembly process, the oxidase activity increased nearly 10 times compared with the activity without FAD. However, the activity increased only 1.4 times when the same amount of FAD was added after the assembly process, which suggests that FAD was not fully incorporated into the reconstituted oxidase, probably because of the steric hindrance at the FAD-binding site of cyt b$_{558}$ in the activated oxidase complex.

**Determination of FAD Incorporated into Cyt b$_{558}$ of Reconstituted NADPH Oxidase**—In order to confirm the hypothesis shown above, the amount of FAD incorporated into the purified cyt b$_{558}$ was determined before and after cell-free activation, i.e., the treatment with anionic amphiphiles, in the presence of exogenous FAD with or without TC. Table II shows the ratio of FAD to heme in purified cyt b$_{558}$, together with the resultant activity of the reconstituted oxidase. In these measurements, not only FAD but also FAD weakly bound to cyt b$_{558}$ and/or TC were separated from cyt b$_{558}$ by the gel filtration technique. Thus, only FAD molecules, which were tightly bound to cyt b$_{558}$, were evaluated (Table II). Before incubation with exogenous FAD, the ratios of FAD to heme in purified cyt b$_{558}$ used in this study were in the range of 0.01–0.05:1. The amount of incorporated FAD did not increase merely by the incubation with FAD, unless treated with anionic amphiphiles in the presence of TC, which indicates that incubation of cyt b$_{558}$ with FAD is not sufficient to incorporate FAD into the FAD-binding site in cyt b$_{558}$. On the contrary, when exogenous FAD was added before the assembly process, the amount of FAD incorporated into cyt b$_{558}$ was remarkably enhanced, compared with the results when FAD was added after the assembly process. A similar experiment was carried out in the absence of TC. Even in the absence of TC, exogenous FAD molecules were incorporated into cyt b$_{558}$ by the treatment with amphiphiles, but the amount of incorporated FAD was only one-third that in the presence of TC. These results, shown in Table II, strongly indicate that the treatment of the oxidase by the anionic amphiphile is requisite for the incorporation of exogenous FAD into FAD-depleted cyt b$_{558}$. During the treatment with anionic amphiphiles, intra- and inter-molecular conformational change took place, which might have induced the structural change in the FAD-binding site in cyt b$_{558}$ and the protein-protein interaction of cyt b$_{558}$ and TC, followed by the tight binding of FAD to cyt b$_{558}$.

In order to test this view, the dissociation constant ($K_d$) of FAD in cyt b$_{558}$ of reconstituted oxidase was estimated. Under the assumption that the superoxide generating activity is proportional to the amount of FAD incorporated cyt b$_{558}$-TC complex, the reconstituted activity was plotted as functions of added FAD (Fig. 5). The $K_d$ value of FAD in cyt b$_{558}$ of the reconstituted oxidase was estimated from Fig. 5 as 0.94 nM, which indicates that FAD molecules were strongly bound to cyt b$_{558}$ after the treatment with amphiphiles in the presence of TC. It is important to study whether the tight binding of FAD to purified cyt b$_{558}$ observed in this cell-free reconstitution system has physiological relevance. Therefore, unbound and bound FAD in activated neutrophils was compared with resting cells (Table III). Table III shows that the total amount of FAD and heme in membranes did not change upon activation of cells with phorbol 12-myristate 13-acetate, but a distinct increase in the amount of bound FAD was observed, indicating that FAD binds to cyt b$_{558}$ tightly in the activated NADPH oxidase.
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FIG. 4. Effect of the addition of FAD on assembly processes of the reconstituted NADPH oxidase. A, the cell-free mixture containing purified cyt b₅₅₈ (1 pmol), TC (250 nm), and Rac2 (800 nm) was treated with an optimal amount of sodium myristate. The reconstituted NADPH oxidase activity was determined with or without FAD (10 pmol). In this experiment, FAD was added either before or after the assembly process. Graphs and error bars represent the means ± S.D. from three experiments. B, the same experiment was carried out in the reconstituted system with the fused protein (500 nm) instead of TC. In this reconstituted system, NADPH oxidase activities were measured with or without anionic amphiphile activator (sodium myristate). Other experimental conditions were the same as in A.

Next, we examined whether the conformational change in cyt b₅₅₈ occurring during the treatment of the oxidase proteins with amphiphiles affects the incorporation of FAD into cyt b₅₅₈ by using fused protein between truncated p67(phox)-(1–242) and truncated p47(phox)-(151–286). Because this fused protein has no polybasic region, the SH3 domain in p47(phox) of the fused protein can bind to the proline-rich region in p22(phox) of cyt b₅₅₈ without the addition of anionic amphiphile (7, 30), which results in the activation of the oxidase and the generation of superoxide. The results for the oxidase reconstituted with the fused protein and purified cyt b₅₅₈ are shown in Fig. 4B. The oxidase reconstituted with the fused protein can generate superoxide without anionic amphiphiles, but the amount of superoxide generated is greatly enhanced by the addition of anionic amphiphiles, suggesting that the conformational change in cyt b₅₅₈ by the treatment with anionic amphiphiles activator plays an important role in the incorporation of FAD into cyt b₅₅₈. It should be noted that in both cases of TC and the fused protein the reconstituted oxidase activity is not enhanced by FAD added after the treatment of the oxidase with anionic amphiphiles, i.e., after assembly processes. From the results in Table II showing that the efficacy of incorporating FAD into cyt b₅₅₈ depends not only on the treatment with amphiphiles but also on the coexistence of TC or the fused protein, it appears that cytosolic proteins (or TC) play an important role in retaining FAD at the FAD-binding site of cyt b₅₅₈, such as by masking the FAD-binding site of cyt b₅₅₈ in the activated oxidase.

**TABLE II**

| Cyt b₅₅₈ | TC | Amphiphiles | FAD | FAD/heme | Activity |
|----------|----|------------|-----|---------|---------|
| +        | +  | +         | +   | +       | Before*  |
|          |    |            |     |         | 0.41 ± 0.11 | 58.2 ± 8.73 |
| +        | +  | +         | +   | +       | After*   |
|          |    |            |     |         | 0.14 ± 0.00 | 8.9 ± 0.7   |
|          | -  | +         | +   | +       | Before*   |
|          |    |            |     |         | 0.18 ± 0.01 | ND#        |
|          | -  | +         | +   | +       | After*    |
|          |    |            |     |         | 0.06 ± 0.02 | ND#        |
|          | -  | -         | +   | +       | -        |
|          |    |            |     |         | 0.05 ± 0.02 | ND#        |

* "Before" indicates that FAD was added to the mixture of purified cyt b₅₅₈ and cytosolic proteins before treatment with amphiphiles. "After" indicates that FAD was added to the above mixture after treatment with amphiphiles, i.e., after assembly of the proteins.
# Nondetectable.

Fig. 5. Estimation of the Kₐ of FAD in purified cyt b₅₅₈ of reconstituted NADPH oxidase. The cell-free reconstitution system contained purified cyt b₅₅₈ (1 pmol), TC (250 nm), Rac2 (800 nm), and increasing amounts of FAD, and the reaction mixture was treated with an optimal amount of sodium myristate. The reconstituted activities were evaluated in a similar way to that in Fig. 3, and these values were plotted as a function of FAD concentration. The Kₐ value of FAD in purified cyt b₅₅₈ of activated oxidase is obtained by fitting reconstituted activities to the one-site saturation curve.

Electron Transfer Reaction in FAD-incorporated NADPH Oxidase—The electron transfer reactions in reconstituted NADPH oxidase were compared under both aerobic and anaerobic conditions. As shown in Figs. 2–5, under aerobic conditions the superoxide generating activity of reconstituted NADPH oxidase with cyt b₅₅₈ and TC was remarkably enhanced by the incorporation of FAD to cyt b₅₅₈. Electron transfer reactions in the reconstituted NADPH oxidase were also examined under anaerobic conditions (Fig. 6). Electron transfer from NADPH to
heme in purified cyt \(b_{558}\) was measured by the reduction of heme in cyt \(b_{558}\) under anaerobic conditions. When purified cyt \(b_{558}\) was reconstituted with TC in the absence of FAD, no reduction of heme in cyt \(b_{558}\) took place even in the presence of NADPH. The electron transfer path might have been broken by the removal of FAD from cyt \(b_{558}\). When purified cyt \(b_{558}\) was reconstituted with TC in the presence of FAD, however, a reduction of heme in reconstituted cyt \(b_{558}\) occurred because of the addition of NADPH. Fig. 6 shows the difference spectrum of native FAD-reconstituted cyt \(b_{558}\) obtained by subtracting the oxidized spectrum from the reduced spectrum with NADPH, and the kinetics of the reduction of the heme in cyt \(b_{558}\) are shown in the inset. The obtained spectrum is a typical cyt \(b_{558}\) reduced minus oxidized difference spectrum, showing the location of the \(a\) band (558–559 nm) and Soret band (426–427 nm) peaks. The appearance of reduced heme in FAD-reconstituted cyt \(b_{558}\) by NADPH strongly suggests that the electron transfer ability of the reconstituted oxidase is recovered after incorporation of FAD into FAD-depleted purified cyt \(b_{558}\). The inset in Fig. 6 demonstrates that the heme in FAD-reconstituted purified cyt \(b_{558}\) is reduced in a time-dependent manner by NADPH in anaerobiosis. Although the reduction rate of heme is slower than the superoxide-generating rate of the reconstituted NADPH oxidase (19, 31), the results indicate that electrons provided from NADPH are transferred via the same electron transfer chain in NADPH oxidase under aerobic and anaerobic conditions.

**DISCUSSION**

The superoxide-generating phagocyte NADPH oxidase is dormant in resting cells and becomes active upon cell stimulation by pathogens or amphiphiles in vitro. The switching from a resting to an activated state in the oxidase system is precisely controlled by several different mechanisms, for example by translocation of the cytosolic proteins to the membrane and the conformational change in cytosolic proteins induced by phosphorylation reaction during the activation process. In resting cells, three cytosolic proteins, \(p47^{phox}\), \(p67^{phox}\), and \(p40^{phox}\), exist as a tight complex, and recently these proteins were found to form a tight complex with 1:1:1 stoichiometry in vitro (5). In the present study, we prepared the ternary cytosolic protein complex, \(p47^{phox}\)-\(p67^{phox}\)-\(p40^{phox}\) (TC), together with several binary complexes, such as \(p47^{phox}\)-\(p67^{phox}\), \(p47^{phox}\)-\(p40^{phox}\), and \(p67^{phox}\)-\(p40^{phox}\), from each full-length recombinant cytosolic protein. By using these binary and ternary complexes, the reconstituted activity of NADPH oxidase was examined in the presence of purified cyt \(b_{558}\). The reconstituted activity with TC was higher and more stable than that with any binary complexes or the native cytosol.

Anionic amphiphiles, such as arachidonic acid (32) and myristic acid (33), can activate the phagocyte NADPH oxidase not only in intact cells but also in a cell-free system via assembly of the cytosolic regulatory proteins \(p47^{phox}\), \(p67^{phox}\), and Rac with the membrane-associated cyt \(b_{558}\) consisting of \(p22^{phox}\) and gp91\(^{phox}\). It is evident that the amphiphiles interact with each protein and induce conformational changes before assembly of the oxidase complex; anionic amphiphiles interact with \(p47^{phox}\) containing two SH3 domains, which specifically bind to a proline-rich region in the cytoplasmic tail of \(p22^{phox}\). The induced \(p47^{phox}\)-\(p22^{phox}\) interaction likely plays a crucial role in the activation of the NADPH oxidase. Besides these interactions, anionic amphiphiles also interact with other oxidase proteins, which appears to render the protein into a conformation capable of oxidase activity. The interaction between amphiphiles and proteins is dependent on their hydrophobic-hydrophilic
Regulated Incorporation of FAD to Cytochrome b_{558} 26385

![Diagram](image)

Fig. 7. A schematic illustration of a possible model for the FAD-binding site of cytochrome b_{558} in both the resting and activated states of NADPH oxidase. In the resting state, FAD is loosely bound to cytochrome b_{558}, and FAD is in equilibrium between being bound and unbound to cytochrome b_{558}. When the oxidase is treated with anionic amphiphile activators, the FAD-binding site in cytochrome b_{558} is slightly distorted, which induces fitting of FAD molecules into the FAD-binding site of cytochrome b_{558}. After the tight binding of FAD to cytochrome b_{558}, the FAD-binding site is masked with cytosolic protein complex; therefore, FAD is not easily released from the activated oxidase. Furthermore, common inhibitors of electron transfer reactions are unable to gain access to the redox centers, FAD and heme, in cytochrome b_{558}.

being unbound and bound to cytochrome b_{558} because of the weak binding capability of FAD in cytochrome b_{558} in the resting state.

Fig. 7 depicts a schematic illustration of a possible model for the FAD-binding site of cytochrome b_{558} in both the resting and activated states of NADPH oxidase. In the resting state, some of the FAD molecules are loosely bound to cytochrome b_{558}, i.e. the equilibrium between being bound and unbound to cytochrome b_{558}. Once the NADPH oxidase is treated with anionic amphiphile activators, the FAD-binding site of cytochrome b_{558} is modified so as to fit FAD tightly into its FAD-binding site, and free unbound FAD thus binds to cytochrome b_{558} tightly. Furthermore, this occupation of the FAD-binding site in cytochrome b_{558} is followed by the association of the cytosolic proteins. This masking and protection of the FAD-binding site by the cytosolic protein complex is important for several reasons. By making this structure, bound FAD is not easily released from cytochrome b_{558}. Therefore, the reconstituted oxidase activity is very stable even though the active oxidase is left at room temperature for many hours, as shown in Fig. 2. Furthermore, by masking the FAD site with the cytosolic protein complex, the FAD site might be protected from chemicals that are commonly used as inhibitors, such as cyanide, carbon monoxide, and pyridine (36).

From both the $K_d$ value of FAD and the ratio of heme to FAD in purified cytochrome b_{558} obtained in this study, we derived the model in which $K_d$ of FAD is changeable after activation of the NADPH oxidase (Fig. 7). The change in the $K_d$ value of FAD is physiologically important in terms of avoidance of accidental

balance, and common amphiphiles tend to bind not only to cytosolic proteins but also to membrane-bound cytochrome b_{558}. Anionic amphiphiles can bind to cytochrome b_{558} similar to other cytosolic proteins, and thus the binding of anionic amphiphiles induces a structural change in cytochrome b_{558} that leads to the appearance of high spin heme, as evidenced by low temperature EPR studies (15, 34). From these results, it is likely that not only the heme environment but also the flavin-binding site in cytochrome b_{558} is modulated by the treatment of cytochrome b_{558} with anionic amphiphiles in the cell-free superoxide-generating system. In the present study, a $K_d$ of nearly 1 mM was calculated for the binding of FAD to the purified FAD-depleted cytochrome b_{558}, which shows that FAD is tightly bound to cytochrome b_{558} of activated NADPH oxidase. If the $K_d$ value of FAD in cytochrome b_{558} of resting intact cells is the same as that in the activated oxidase and is in the nanomolar range, FAD should be tightly bound to cytochrome b_{558} in intact cells.

It is very important to understand the FAD-binding status in the NADPH oxidase in physiological conditions. There are at least two models to explain our results on the $K_d$ of FAD. For model 1, the $K_d$ value of FAD in cytochrome b_{558} is not different between the resting and activated states. Therefore, purified cytochrome b_{558} lost most of its FAD because of the change in its charge, lipid environment, etc. during the purification procedures. For model 2, the $K_d$ value of FAD in cytochrome b_{558} is decreased in the activated oxidase during the activation process, compared with that in the resting oxidase. In the resting state, FAD is in equilibrium between being bound and unbound to cytochrome b_{558}. However, once the oxidase is activated, the structure of the FAD-binding site is modified, resulting in the tight binding of FAD to cytochrome b_{558}. Cytosolic proteins also help maintain FAD at the FAD-binding site of activated oxidase.

Flavin contents and the ratio of FAD to heme in crude membranes from resting neutrophils have been reported (18, 26, 35–36). It is generally accepted that the ratio of heme to FAD ranges from 2:1 to 3:1, and in our laboratory the value of 2.5:1 was obtained, as shown in Table I. One should note that this ratio is obtained in the crude membranes and that this ratio does not indicate the ratio in cytochrome b_{558}. Segal et al. (35) reported that the FAD content of crude membranes from cells of patients with X-linked chronic granulomatous disease lacking cytochrome b_{558} was one-quarter that of normal subjects and also that these FAD molecules did not belong to NADPH oxidase, which exists even in uninduced HL60 cells. Yoshida et al. (37) also published similar results, although they claimed that one-third of all FAD in crude membranes of phagocytes is not related to NADPH oxidase. Several FAD proteins had been reported as possible candidates responsible for NADPH oxidase, but these candidates were negated when cytochrome b_{558} was found to be a flavocytochrome (35, 38). Therefore, one-third or one-quarter of FAD in phagocytes might be derived from flavoproteins that are not related to NADPH oxidase. Several FAD proteins had been reported as possible candidates responsible for NADPH oxidase, but these candidates were negated when cytochrome b_{558} was found to be a flavocytochrome (35, 38). Therefore, one-third or one-quarter of FAD in phagocytes might be derived from flavoproteins that are not related to NADPH oxidase. Thus, according to the above considerations, the ratio of heme to FAD in NADPH oxidase of crude membranes becomes 3.5:1–4:1. Furthermore, by using the same chemiluminescence method as Yoshida et al. (26), the ratio of free and bound FAD in crude membranes was found to be 6:4, suggesting that more than half of FAD in crude membranes is free and unbound, and more than half of the FAD sites in cytochrome b_{558} seems to be unoccupied. As shown in Table III, the amount of FAD bound to the oxidase in stimulated neutrophils increased, compared with the resting cells, although the amount of heme in the oxidase was constant regardless of the stimulation of cells. All these facts strongly suggest that the FAD-binding sites in cytochrome b_{558} of resting cells are not fully occupied with FAD; FAD is in equilibrium between bound and unbound to cytochrome b_{558} because of the weak binding capability of FAD in cytochrome b_{558} in the resting state.

Fig. 7 depicts a schematic illustration of a possible model for the FAD-binding site of cytochrome b_{558} in both the resting and activated states of NADPH oxidase. In the resting state, FAD is loosely bound to cytochrome b_{558}, and FAD is in equilibrium between being bound and unbound to cytochrome b_{558}. When the oxidase is treated with anionic amphiphile activators, the FAD-binding site in cytochrome b_{558} is slightly distorted, which induces fitting of FAD molecules into the FAD-binding site of cytochrome b_{558}. After the tight binding of FAD to cytochrome b_{558}, the FAD-binding site is masked with cytosolic protein complex; therefore, FAD is not easily released from the activated oxidase. Furthermore, common inhibitors of electron transfer reactions are unable to gain access to the redox centers, FAD and heme, in cytochrome b_{558}.

From both the $K_d$ value of FAD and the ratio of heme to FAD in purified cytochrome b_{558} obtained in this study, we derived the model in which $K_d$ of FAD is changeable after activation of the NADPH oxidase (Fig. 7). The change in the $K_d$ value of FAD is physiologically important in terms of avoidance of accidental
production of reactive oxygen species, because in the resting state it is unlikely that electrons will flow into the electron transfer chain, where FAD is loosely or improperly incorporated into cyt $b_{558}$. Further studies will be necessary to examine the mechanism by which FAD acts as the switch for activating NADPH oxidase.

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