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Cytochrome b_{-245} of the Neutrophil Superoxide-generating System Contains Two Nonidentical Hemes

POTENTIOMETRIC STUDIES OF A MUTANT FORM OF gp91^{phox}*

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Analysis of potentiometric titrations of the cytochrome b_{-245} from a X$^+$ chronic granulomatous disease patient with an Arg^{54} → Ser mutation in gp91^{phox} indicates that the mutant form of the cytochrome contains two nonidentical hemes with midpoint potentials of E_{m7} = −220 and E_{m7} = −300 mV. In the light of this information, reanalysis of redox titrations of wild-type cytochrome b_{-245} implies that it probably also contains two separate heme centers with midpoint potentials of E_{m7} = −225 and E_{m7} = −265 mV. The effect of the Arg^{54} → Ser substitution is to reduce the midpoint potential of one of the heme centers by approximately 35 mV and suggests possible interaction between Arg^{54} and a heme propionate side chain.

Neutrophils contain a multicomponent, NADPH-dependent, O_2^- generating system that is used as a powerful antimicrobial weapon in the process of host defense (recently reviewed in Ref. 1). The redox centers of this NADPH oxidase, FAD and heme, are both contained within a unique low potential flavocytochrome, cytochrome b_{-245} (also known as cytochrome b_{558} or cytochrome b_{559}) that also contains the NADPH binding domain (2–4). Cytochrome b_{-245} is composed of a heavily glycosylated large β-subunit (gp91^{phox}) and a small α-subunit (p22^{phox}), the products of two separately regulated genes. Each subunit is apparently unstable in the absence of its partner. The simplest model of cytochrome b_{-245} is that of a gp91^{phox}/p22^{phox} heterodimer containing 1 mol of heme and 1 mol of FAD. However, theoretical considerations and experimental evidence from purification (2, 5–8), proteolytic (9), and reconstitution[1] (10, 11) studies suggest that each mole of cytochrome b_{-245} probably contains 2 mol of heme and 1 mol of FAD. In addition, optical, EPR, CD, and resonance Raman spectra (7, 12–16) are consistent with the presence of multiple, bis-histidinyl, hexacoordinate, low spin hemes. Hitherto, the presence of only a single species of heme in cytochrome b_{-245} has been inferred from oxidation-reduction potentiometric studies, where the cytochrome titrates as a single component with a midpoint potential of −245 mV (17). Here we present evidence from the studies of the nonfunctional cytochrome from an X$^+$ CGD[2] patient with an Arg^{54} → Ser mutation in gp91^{phox} that clearly demonstrates the presence of two nonidentical hemes with midpoint potentials of E_{m7} = −220 mV and E_{m7} = −300 mV. In light of this information, reanalysis of redox titrations of wild-type cytochrome b_{-245} suggests the presence of two hemes with closely spaced midpoint potentials of E_{m7} = −225 mV and E_{m7} = −265 mV.

EXPERIMENTAL PROCEDURES

Materials—Anthraquinone 2,6-disulfonate, duroquinone, 2-hydroxy-1,4-naphthoquinone, and 2,3,5,6-tetramethylphenylenediamine were obtained from Aldrich. Anthraquinone and sodium dithionite were supplied by Fluka and Fisher, respectively; heptyl-β-D-thioglucopyranoside was from Calbiochem. All other reagents were purchased from Sigma. Pyocyanine was synthesized from phenazine methosulfate using the photochemical method described in Ref. 18.

Isolation of Neutrophils and Preparation of Subcellular Fractions—Membranes and cytosolic fractions were prepared from the unstimulated neutrophils of both normal and CGD donors as described previously (19) and stored in aliquots at −80 °C.

Solubilization and Purification of Cytochrome b_{-245}—Cytochrome b_{-245} was partially purified as described previously (20).

Measurement of Oxidation-Reduction Potentials—Potentiometric titrations were performed as described previously (17) using partially purified cytochrome b_{-245} preparations derived from 9 × 10^8 neutrophils in a total volume of 2.7 ml of 100 mM KCl, 50 mM MOPS, pH 7.0. The following mediators were used at 12.5 μM: phenazine methosulfate, phenazine ethosulfate, anthraquinone, anthraquinone 2-sulfonate, anthraquinone 2,6-disulfonate, 2-hydroxy-1,4-naphthoquinone, 2,3,5,6-tetramethylphenylenediamine. Pyocyanine was added at 6 μM. Spectra were recorded between 580 and 520 nm at a series of electrode potentials, using a Uvikon 810 spectrophotometer. The degree of reduction of cytochrome b_{-245} was estimated from the height of the α absorbance band. An Orion 720A meter (Orion Research Inc., Boston MA) was used to measure the half-cell potential relative to a saturated calomel reference electrode. The potential was adjusted by the addition of <μl volumes of solutions of sodium dithionite (reductive titrations) and potassium ferricyanide (oxidative titrations). The accuracy of the apparatus was checked by titration of a 5 μM solution of phenosafranin ($E_{m7.0}$ = −252 mV).

RESULTS

We previously showed that the Arg^{54} → Ser mutation in gp91^{phox} results in a cytochrome b_{-245} with a nonfunctional heme with a slightly shifted Soret band that is unable to accept electrons from the reduced flavin center (21). In contrast, the flavin domain of the cytochrome b_{-245} is fully functional, as it is capable of accepting electrons from NADPH and reducing the artificial dye acceptor iodonitrotetrazolium violet at rates equivalent to that of the wild-type cytochrome. To determine if the loss of function was due to an alteration in heme redox potential, we performed a series of oxidation-reduction potential measurements using cytochrome b_{-245} partially purified from neutrophil membranes from both normal and CGD patients. The results are shown in Fig. 1. The wild-type cyto-

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[1] A. R. Cross, unpublished results.

[2] The abbreviations used are: CGD, chronic granulomatous disease; MOPS, 4-morpholinepropanesulfonic acid; E_m, midpoint redox potential.

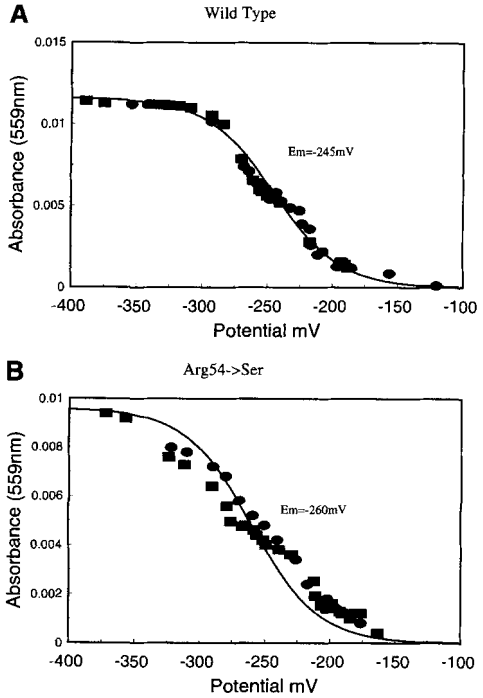

FIG. 1. **Potentiometric titration of wild-type and Arg^{54} → Ser cytochrome b_{-245}; one component, 1-electron transfer.** Oxidation-reduction potential measurements were performed as described under "Experimental Procedures." *Circles* represent oxidative titrations (potassium ferricyanide); *squares*, reductive titrations (sodium dithionite). *A*, titration of wild-type cytochrome b_{-245}; the *solid curve* is a theoretical line for a single species, 1-electron transfer process with a midpoint potential of −245 mV. *B*, titration of Arg^{54} → Ser cytochrome *b*; the *solid curve* is the theoretical curve for a single species, 1-electron transfer with a midpoint of −260 mV.

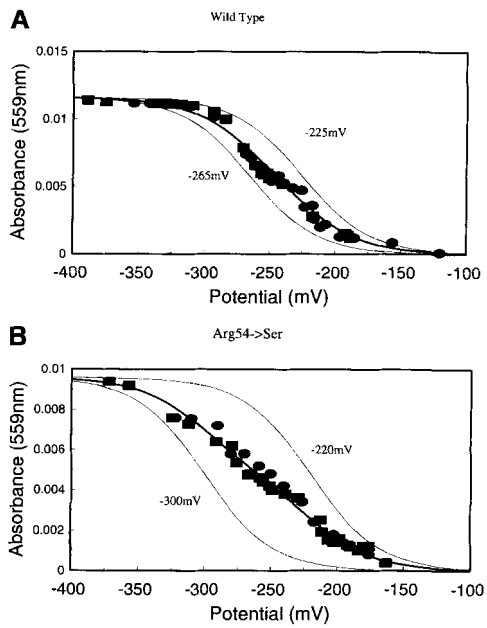

FIG. 2. **Potentiometric titration of wild-type and Arg^{54} → Ser cytochrome b_{-245}; two components, 1-electron transfer.** *A*, data as in Fig. 1*A* with the *solid curve* representing two species with midpoint potentials of −225 and −265 mV, each contributing 50% to the total absorbance change. *B*, data as in Fig. 1*B* with the *solid curve* representing two species with midpoint potentials of −220 and −300 mV, each contributing 50% to the total absorbance change. *Circles* represent oxidative titrations; *squares*, reductive titrations.

chrome titration could be fitted fairly well ($\Sigma_{(O-E)^2} = 1.1 \times 10^{-5}$) to a Nernst equation curve for a single component with a $E_{m7} = -245$ mV (Fig. 1*A*), the same midpoint potential as originally determined (17). In contrast, titration of the Arg^{54} → Ser cytochrome clearly did not fit the expected simple single component, 1-electron transfer process (Fig. 1*B*) ($\Sigma_{(O-E)^2} = 2.6 \times 10^{-5}$). By assuming two components are present, the data could be fitted to curves corresponding to two species with midpoint potentials of −220 and −300 mV, each contributing 50% to the absorbance at 559 nm (Fig. 2*B*) ($\Sigma_{(O-E)^2} = 9 \times 10^{-6}$). By inferring that two nonequivalent hemes are also present in the wild-type cytochrome b_{-245}, the data in Fig. 1*A* can be fitted in a similar fashion to a 2-component curve with midpoint potentials of $E_{m7} = -225$ and $E_{m7} = -265$ mV (Fig. 2*A*) producing an excellent fit ($\Sigma_{(O-E)^2} = 9 \times 10^{-6}$). Titrations of the cytochrome b_{-245} from a CGD patient with a Pro^{415} → His mutation in gp91^{phox} in which the flavin domain is nonfunctional (2, 21) were indistinguishable from that of the wild-type cytochrome, in accordance with a previous report (16) (data not shown).

DISCUSSION

From the data presented above, it appears that both wild-type and mutant cytochromes contain two separate, nonidentical hemes. Both contain a heme center with a midpoint potential around −220 mV contributing approximately 50% to the total absorbance change; in the wild-type cytochrome, there is a second, closely spaced center with $E_{m7} = -265$ mV. The similarity in redox potentials makes resolution of the two centers difficult in the wild-type cytochrome. In the Arg^{54} → Ser cytochrome, the potential of the second lower potential center has been shifted downward by 35 mV from −265 mV to −300 mV and can be clearly resolved. At the present time, the locations of the hemes within the cytochrome are not known with any certainty. p22^{phox}, which is reported to contain heme, contains only one invariant histidine, the latter lying within a sequence with homology to the heme-binding domains of a cytochrome oxidase subunit and the chromaffin granule cytochrome b_{561}. In view of the assignment of the axial ligands in cytochrome b_{-245} as bis-histidinyl, a single p22^{phox} subunit cannot wholly contain a heme group. gp91^{phox} contains 17 histidines, none lying within sequences known to be homologous with other heme proteins. Thus, one heme group is probably shared between a p22^{phox} and gp91^{phox} subunit (or two p22^{phox} subunits), and one heme is likely to be contained within gp91^{phox} itself.

It is thought that invariant arginine residues near the membrane surface of mitochondrial cytochromes *b* form hydrogen bonds with the negatively charged propionyl groups of the heme (22 and references therein). Hydropathy plots of gp91^{phox} predict Arg^{54} to be at the beginning of a membrane-spanning segment close to the membrane surface, and, therefore, this residue could perform a similar hydrogen bonding function in cytochrome b_{-245}. The adjacent membrane-spanning segment (amino acids 100–120) contains 3 potential heme-liganding histidine residues. Substitution of the positively charged Arg^{54} to an uncharged serine residue would decrease the electron withdrawing nature of this group and thereby lower the redox potential of the heme. Site-directed mutagenesis studies of the analogous situation in iso-1-cytochrome *c* have demonstrated such decreases in redox potential when glutamine (−30 mV), asparagine (−34 mV), or alanine (−50 mV) were substituted for the arginine (Arg^{38}) that forms a hydrogen bond with one of the heme propionate side chains (23).

In view of the fact that the redox potential of the heme in cytochrome b_{-245} is altered by less than 40 mV by the Arg^{54} → Ser substitution, it is perhaps surprising that the mutant form has no detectable O_2^- generating activity, particularly since we

have shown that the flavin center is fully functional in this mutation (20, 21). Further studies using site-directed mutagenesis and other naturally occurring mutant forms of cytochrome b_{-245} will be invaluable in dissecting the factors affecting electron transfer functions in cytochrome b_{-245}.

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