The Active N-terminal Region of p67phox

STRUCTURE AT 1.8 Å RESOLUTION AND BIOCHEMICAL CHARACTERIZATIONS OF THE A128V MUTANT IMPLICATED IN CHRONIC GRANULOMATOUS DISEASE*

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Upon activation, the NADPH oxidase from neutrophils produces superoxide anions in response to microbial infection. This enzymatic complex is activated by association of its cytosolic factors p67phox, p47phox, and the small G protein Rac with a membrane-associated flavocytochrome b558. Here we report the crystal structure of the active N-terminal fragment of p67phox at 1.8 Å resolution, as well as functional studies of p67phox mutants. This N-terminal region (residues 1–213) consists mainly of four TPR (tetratricopeptide repeat) motifs in which the C terminus folds back into a hydrophobic groove formed by the TPR domain. The structure is very similar to that of the inactive truncated form of p67phox bound to the small G protein Rac previously reported, but differs by the presence of a short C-terminal helix (residues 187–193) that might be part of the activation domain. All p67phox mutants responsible for Chronic Granulomatous Disease (CGD), a severe defect of NADPH oxidase function, are localized in the N-terminal region. We investigated two CGD mutations, G78E and A128V. Surprisingly, the A128V CGD mutant is able to fully activate the NADPH oxidase in vitro at 25 °C. However, this point mutation represents a temperature-sensitive defect in p67phox that explains its phenotype at physiological temperature.

The NADPH oxidase of phagocytic cells is responsible for the production of microbialidal superoxide anions. This enzymatic complex is activated at the onset of phagocytosis by association of its cytosolic factors p67phox, p47phox, and the small G protein Rac with a membrane-associated flavocytochrome b558. Here we report the crystal structure of the active N-terminal fragment of p67phox at 1.8 Å resolution, as well as functional studies of p67phox mutants. This N-terminal region (residues 1–213) consists mainly of four TPR (tetratricopeptide repeat) motifs in which the C terminus folds back into a hydrophobic groove formed by the TPR domain. The structure is very similar to that of the inactive truncated form of p67phox bound to the small G protein Rac previously reported, but differs by the presence of a short C-terminal helix (residues 187–193) that might be part of the activation domain. All p67phox mutants responsible for Chronic Granulomatous Disease (CGD), a severe defect of NADPH oxidase function, are localized in the N-terminal region. We investigated two CGD mutations, G78E and A128V. Surprisingly, the A128V CGD mutant is able to fully activate the NADPH oxidase in vitro at 25 °C. However, this point mutation represents a temperature-sensitive defect in p67phox that explains its phenotype at physiological temperature.

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The atomic coordinates and structure factors (code 1hh8) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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‡The abbreviations used are: CGD, chronic granulomatous disease; TPR, tetratricopeptide repeat; SAD, single wavelength anomalous dispersion; rmsd, root mean square deviation; GST, glutathione S-transferase; GTP-y-S, guanosine 5′-O-(thio)triphosphate.

EXPERIMENTAL PROCEDURES

Native and Selenomethionyl Protein Production—The cDNA for the N-terminal fragment of p67phox corresponding to amino acids 1–213 was obtained by polymerase chain reaction and cloned into pET-15b (Novagen). The protein was expressed in Escherichia coli BL21 (DE3) and purified by affinity chromatography in two steps: first on a Ni2+-column equilibrated in 20 mM Hepes, pH 7.5, 250 mM NaCl and eluted with a linear gradient of imidazole and second on an SP-Sephadose column equilibrated in 20 mM Hepes, pH 7.5 and eluted with a linear gradient of imidazole.
Structure and Stability of p67<sub>phox</sub>

The protein was then concentrated to 5–6 mg/ml with Centri-10 (Amicon). Seleno-l-methionine (Se-Met)-labeled protein was produced in a similar way. The protein was expressed in E. coli B834 (DE3). A 50-ml preculture in Luria-Bertani medium was used to inoculate 2 liters of a defined medium prepared as reported before (13) supplemented with 20 μg/ml of Se-Met. The last purification step was done in the presence of 10 mM dithiothreitol and 1 mM EDTA to avoid oxidation of selenomethionines. High resolution electrospore ionization mass spectrometry was consistent with 100% selenium incorporation.

**CGD Mutants of p67<sub>phox</sub>**—Mutants G75E and A128V of p67<sub>phox</sub>-(1–213) were constructed using site-directed mutagenesis (Stratagene kit) on the native DNA cloned in the pET-15b vector. The mutant A128V was expressed in E. coli BL21 (DE3) at 15 °C for 16 h instead of the 3 h at 37 °C employed for the native protein. The first steps of the purification were the same as for the native protein, but an additional purification step was carried out by gel filtration on a Superdex 200 Hida 16/60 column (Amersham Pharmacia Biotech) equilibrated in 20 mM Heps, pH 7.5 and 200 mM NaCl. The protein was concentrated to 5 mg/ml with Centri10.

**Limited Proteolysis**—Native p67<sub>phox</sub>-(1–213) and the A128V mutant at a concentration of 0.6 mg/ml were submitted to limited proteolysis for one hour at 25 °C by trypsin (Roche Molecular Biochemicals) at a protease/protein ratio of 1:200 (w/w).

**Circular Dichroism (CD)**—CD spectra were recorded on a Jasco V-570 spectropolarimeter with a 1-mm path length cell and a thermostated cell holder. The protein concentrations were 0.5 mg/ml. The spectra were recorded on one sample per protein from 15 to 35 °C by increments of 5 °C at each temperature, the sample was incubated for 15 min.

**NADPH Oxidase Activity**—The NADPH oxidase activating potency was assessed in a semi-recombinant cell-free system (14) containing 3.5 μg of membrane protein, 20 pmol of recombinant p47<sub>phox</sub>, 20–400 pmol of an N-terminal fragment of p67<sub>phox</sub>, 2 mM MgCl₂, and 10 pmol of Rac in a final volume of 200 μl. Rac was loaded with GTP·S in the presence of 4 mM EDTA, followed by addition of MgCl₂ to 20 mM. An optimal amount of arachidonic acid (5–20 nmol) was added with strong agitation. After a 10-min incubation, the elicited oxidase activity was assessed using the superoxide dismutase inhibitable cytochrome c reduction in the presence of 250 μM NADPH and 1 μM cytochrome c, followed at 550 nm using a Labystem EIMs microplate reader.

**Assay of Rac Binding**—Rac fused to GST or GST alone were immobilized on glutathione-Sepharose beads (Amersham Pharmacia Biotech). Rac was loaded with GTP·S·Y on the beads. The beads were washed and incubated for 2 h at 4 °C with a stoichiometric amount of p67<sub>phox</sub>-A128V mutant. After washing, proteins bound to the beads were analyzed by SDS-polyacrylamide gel electrophoresis.

**Crystallization**—Crystals of the native protein or the Se-Met protein were grown at 20 °C by vapor diffusion in hanging drops by mixing equal volumes of protein (5–6 mg/ml) and reservoir solutions (17% 4.5, 10% glycerol, and 10 mM dithiothreitol in the case of the Se-Met protein). Equal volumes of protein (5–6 mg/ml) and reservoir solutions (17% 4.5, 10% glycerol, and 10 mM dithiothreitol in the case of the Se-Met protein) were mixed and equilibrated in 2 liters of a defined medium prepared as reported before (13) at 37 °C employed for the native protein. The first steps of the purification were the same as for the native protein, but an additional purification step was carried out by gel filtration on a Superdex 200 Hida 16/60 column (Amersham Pharmacia Biotech) equilibrated in 20 mM Heps, pH 7.5 and 200 mM NaCl. The protein was concentrated to 5 mg/ml with Centri10.

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**Crystallization**—Crystals of the native protein or the Se-Met protein were grown at 20 °C by vapor diffusion in hanging drops by mixing equal volumes of protein (5–6 mg/ml) and reservoir solutions (17% polyethylene glycol monomethyl ether 2000, 100 mM sodium citrate, pH 4.5, 10% glycerol, and 10 mM dithiothreitol in the case of the Se-Met protein). The crystals grew as thin needles (20 × 200 × 200 μm) and belong to the trigonal space group P₃₂₁ (a = b = 67.7 Å, c = 50.2 Å) with one molecule in the asymmetric unit. Crystals exhibit various amounts of merohedral twinning; however, non-twinned crystals could be selected after analyzing the diffraction data.

**Data Collection and Processing**—A native data set was collected (beamline ID41-EH1, ESRF-Grenoble) to 1.8 Å resolution (Table 1), integrated with DENZO (15) and scaled with SCALA (16). A SAD data set was collected on a selenomethionyl-substituted crystal at the K absorption edge of selenium (beamline ID29, ESRF-Grenoble). Data were integrated and scaled with the DENZO/SCALEPACK programs. The program Shake and Bake (17) located 5 of 7 selenium atoms expected in the asymmetric unit.

**Structure Determination and Refinement**—Initial phases calculated with MLPHARE (16) using the 5 selenium sites allowed the location of the N terminal and 191–193 at the C terminus were added manually using the program O (19). The structure was refined with CNS (20) to a final crystallographic R<sub>f</sub> of 18.2% and an R<sub>free</sub> of 20.5% at 1.8 Å resolution (Table II). The model consists of residues 2–193 of p67<sub>phox</sub>, 160 water molecules, and one citrate anion, the citrate being essential for crystallization. All non-glycine residues are in the most favored or additionally allowed regions of the Ramachandran plot according to PROCHECK (21). The figures were prepared with MOLSCRIPT (22) and Raster3D (23).

**RESULTS AND DISCUSSION**

**Overview of the Structure and Physiological Relevance**—The N-terminal region of p67<sub>phox</sub> used in this study was shown to be fully competent in NADPH oxidase activation both in vitro and in vivo (24, 25). The electron density map obtained from SAD phasing was clearly interpretable (Fig. 1). p67<sub>phox</sub>-(1–213) consists of four TPR motifs followed by an extended loop, which inserts into the hydrophobic groove formed by the helical organization of the TFRs (Fig. 2). The first three TFRs are contiguous and 16 residues (105 to 120) forming two antiparallel β-strands are inserted between TFR3 and TFR4. A comparison with other recently reported TFR domain structures (9, 26) highlights a remarkable conservation of the overall structure. For example, the TPR1 domain of Hop (PDB accession code 1ELW), composed of three TPR motifs, could be superimposed on three consecutive TPR motifs of p67<sub>phox</sub> with rmsd values of 0.95 Å and 1.40 Å with the first three and the last three p67<sub>phox</sub> TPRs, respectively. This indicates that the fold of TPR domains is highly conserved despite a rather small sequence identity; in addition, the insertion of amino acids 105–120 in p67<sub>phox</sub> does not disrupt the superhelical structure of the TPR domain. Following TFR4, a helix (residues 156–166) terminates the TPR domain and an extended structure (residues 168–186) folds back into the internal hydrophobic groove of the superhelix. The structure ends with a short helix (residues 187–193),
mainly composed of polar residues. Residues 170–185 interact extensively with residues belonging to the A helices of TPR motifs or to the inserted β-strands (Fig. 2b), probably stabilizing the overall structure. In particular, Arg-181 interacts with Gln-115. In the Rac-p67<sup>phox</sup> complex, Arg-181 is replaced by Lys-181 and the same type of interaction is observed, consistent with the existence of a polymorphism (6). The sequence of p67<sup>phox</sup> (1–213) is extremely rich in basic amino acids with 22 lysine and 6 arginine residues located mainly on the external surface of the super-helix. These residues are uniformly distributed at the surface without forming a highly basic patch. Residues downstream from Leu-193 are not visible in the electron density map.

The recent structure of Rac-p67<sup>phox</sup> (1–203) (12) showed that all the residues of p67<sup>phox</sup> involved in the complex formation belong to the inserted β-strands (Arg-102, Asn-104, Leu-106, Asp-108) or to the loops connecting TPR1 to TPR2 (S37) and TPR2 to TPR3 (D67, H69). The structures of both the non-complexed and complexed forms of p67<sup>phox</sup> can be remarkably well superimposed with a rmsd of 0.57 Å on main chain atoms, showing that the interaction of p67<sup>phox</sup> (1–213) with Rac does not require structural rearrangements of this N-terminal region. In particular, the residues involved in the interaction with Rac are totally accessible in the non-complexed structure, their side chains pointing toward the solvent. Although the two constructs of p67<sup>phox</sup> (1–203 in the complex and 1–213 in this study) differ only by ten amino acids in length, the first was reported to be inactive whereas the second fully activates NADPH oxidase in vitro (24). This drastic difference could be related to the presence of the C-terminal α-helix (residues 187–193) present in our model that is not seen in the Rac-p67<sup>phox</sup> complex structure, although amino acids 1–203 of p67<sup>phox</sup> are present in the crystal. The folding of this helix is probably facilitated by the presence of amino acids up to 213. From functional studies of various truncated forms of p67<sup>phox</sup>, amino acids 199–210 of p67<sup>phox</sup> were defined as an NADPH oxidase activation domain (24), and this region was reported to be involved in the regulation of electron transfer (27). The comparison of the structure of p67<sup>phox</sup> (1–213) to that of p67<sup>phox</sup> (1–203) in the Rac-p67<sup>phox</sup> complex (12) suggests that the activation domain includes helix 187–193.

Mutations in p67<sup>phox</sup> That Cause Chronic Granulomatous Disease—CGD is an inherited disorder of neutrophil function characterized by an increased susceptibility to infection because of a defect in the NADPH oxidase components. Mutations involving p67<sup>phox</sup> are single cases and are located in the N-terminal region of the protein. An in-frame deletion of K58 was found to prevent Rac binding to p67<sup>phox</sup> (28). Deletion of amino acids 19–21 renders the protein inefficient for oxidase activation (29). These amino acids are located between helices A and B of TPR1 and are exposed to solvent. Various point mutations encountered in p67<sup>phox</sup> of CGD patients (R77Q, G78E, A128V, D160V/K161E) were studied and shown to be associated with the absence of the protein in vivo (6, 30). This observation accounts either for mRNA or protein instability or for an increased sensitivity to proteases. Amino acids Gly-78 and Ala-128 are located in similar positions in the α-helices of the TPR motifs (position 8 of helix A). Their mutations are likely to destabilize TPR packing. A sequence alignment of multiple TPRs shows that position 8 in helix A is restricted to glycine, alanine, or serine residues.

We produced G78E and A128V CGD mutants of p67<sup>phox</sup> (1–213). The G78E mutant was insoluble in bacteria. Because the A128V mutant showed lower solubility than the native protein, it was expressed at 15 °C instead of 37 °C. Surprisingly, this mutant was still able to bind Rac and to activate the NADPH oxidase in a cell-free system at 25 °C (Fig. 3), suggesting correct folding. Moreover, the CD spectra at 25 °C of the mutant and of the native proteins were identical and characteristic of α-helices, indicating no modification in the secondary structure (Fig. 4, inset). Altogether, these experiments do not explain the CGD phenotype of this mutant. To assess slight differences in the tertiary structure, the native protein and the A128V mutant were subjected to limited proteolysis by trypsin at 25 °C. As shown in Fig. 5, the native protein is poorly degraded up to 60 min, whereas the A128V mutant shows notable degradation starting at 15 min. Additionally, the CD spectrum as a function of the temperature shows that the native protein is still stable at 40 °C whereas the A128V mutant begins to lose its helical folding at 30 °C (Fig. 4) and precipitates at 40 °C.

The behavior of the two CGD mutants can be interpreted with respect to the structure of the native protein p67<sup>phox</sup> (1–213). The mutation G78E leads to a misfolding of the protein by steric hindrance within the TPR2 motif. In the less drastic
p67<sub>phox</sub> highlights a short α-helix that participates to the NADPH oxidase activation domain. Interestingly, most of the CGD mutants of p67<sub>phox</sub> are located in the N-terminal region of p67<sub>phox</sub>. The structural and functional studies reported here permit an elucidation of the instability of the G78E and A128V mutants and may be extended to mutations that affect TPR folding. The coordinates and the structure factors have been deposited in the Protein Data Bank with ID code 1hh8.

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