Heme Histidine Ligands within gp91phox Modulate Proton Conduction by the Phagocyte NADPH Oxidase*

Received for publication, November 17, 2000, and in revised form, April 25, 2001
Published, JBC Papers in Press, June 1, 2001, DOI 10.1074/jbc.M010438200

Andrés Maturana‡§, Serge Arnaudeau‡, Stephan Rysér‡, Botond Banfi‡, Johann Peter Hossle‡, Werner Schlegel§, Karl-Heinz Krause‡, and Nicolas Demaurex**

From the ‡Department of Physiology, University of Geneva Medical Center, 1211 Geneva 4, §Fondation pour Recherches Médicales, University of Geneva, 1211 Geneva 4, ¶Biology of Aging Laboratory, Department of Geriatrics, Geneva Medical School, 1225 Geneva, and ¶Division of Immunology and Hematology, University-Children’s Hospital Zürich, 8032 Zürich, Switzerland

The membrane subunit of the phagocyte NADPH oxidase, gp91phox, possesses a H+ channel motif formed by membrane-spanning histidines postulated to coordinate the two heme groups forming the redox center of the flavocytochrome. To study the role of heme-binding histidines on proton conduction, we stably expressed the gp91phox cytochrome in human embryonic kidney 293 cells and measured proton currents with the patch clamp technique. Similar to its shorter homologue, NADPH oxidase homologue 1, which is predicted not to bind heme, gp91phox-generated voltage-activated, pH-dependent, H+-selective currents that were reversibly blocked by Zn2+. The gp91phox currents, however, activated faster, deactivated more slowly, and were markedly affected by the inhibition of heme synthesis. Upon heme removal, the currents had larger amplitude, activated faster and at lower voltages, and became sensitive to the histidine reagent diethylpyrocarbonate. Mutation of the His-115 residue to leucine abolished both the gp91phox characteristic 558-nm absorbance peak and voltage-activated currents, indicating that His-115 is involved in both heme ligation and proton conduction. These results indicate that the gp91phox proton channel is activated upon release of heme from its His-115 ligand. During activation of the oxidase complex, changes in heme coordination within the cytochrome might increase the mobility of histidine ligands, thereby coupling electron and proton transport.

The NADPH oxidase catalyzes the one-electron reduction of molecular oxygen to superoxide, the precursor of a variety of toxic oxygen radicals generated by neutrophils, eosinophils, and macrophages, to kill invading microorganisms (1, 2). This enzyme is crucial in the host defense against microbial pathogens, and patients with chronic granulomatous disease (CGD)1,

who fail to assemble a functional oxidase, suffer from severe recurrent infections (3, 4). The NADPH oxidase is a multicomponent enzyme composed of at least three cytosolic subunits, p47phox, p67phox, and p40phox, and two membrane-associated subunits, p22phox and gp91phox (1, 5). Upon stimulation, the cytosolic components associate with the membrane-bound subunits, resulting in a functional oxidase complex that transfers electrons from cytosolic NADPH to extracellular oxygen (6, 7). The transfer of electrons across the plasma membrane generates a massive depolarization, that, if uncompensated, would prevent further electron transfer and the associated production of superoxide (8, 9). Because large amounts of acid equivalents are released in the cytosol during the hydrolysis of NADPH and its resynthesis by the hexose monophosphate shunt, the oxidase has been proposed to act as a H+ channel to preserve electroneutrality and allow the extrusion of the intracellular acid (10, 11). Accordingly, both the depolarization and the cytosolic acidification generated by the oxidase are potentiated by Zn2+, an inhibitor of H+ channels (9, 12). The proton channel function of gp91phox is still debated, although patch clamp studies in activated phagocytes confirmed the close coupling between H+ channels and the NADPH oxidase (9, 13). Activation of the NADPH oxidase induced the appearance of a new type of H+ currents in human eosinophils stimulated with GTPyS or Ca2+ (9). The oxidase-associated H+ currents had an unusually low threshold of voltage activation, enabling the entry of H+ ions and a cytosolic acidification. In addition, the new currents activated faster, deactivated more slowly, were more sensitive to Zn2+, and were blocked by the histidine-reactive agent DEPC (9). These unusual H+ currents were not strictly coupled to electron transport, as they persisted in the presence of diphenyliodonium (DPI), an inhibitor of the NADPH oxidase, or under anoxic conditions. However, they were absent in cells from CGD patients lacking either the gp91phox or the p47 subunit (9), indicating that channel activation requires a functional oxidase. Similar currents were induced by PMA in neutrophils voltage-clamped using the perforated patch configuration to preserve cellular integrity. Upon PMA stimulation, the H+ current amplitude increased progressively and did not correlate with the amplitude of the electron currents but instead strongly correlated with the amplitude of resting H+ currents (13). Block of the oxidase with DPI had no effect on the maximal H+ conductance.
but reversed the slowing of current deactivation, suggesting a complex interaction between oxidase activity and H$^+$ channels.

Different conclusions were drawn from these diverging, but not contradictory, observations. We concluded that phagocytes express two types of H$^+$ channels and that gp91$^{phox}$ is probably the low threshold channel associated with an active oxidase (9). In contrast, DeCoursey et al. (13) concluded that PMA modulates a pre-existing H$^+$ channel that is probably not gp91$^{phox}$ (13). Consistent with gp91$^{phox}$ being the H$^+$ channel itself, expression of full-length or N-truncated gp91$^{phox}$ generates H$^+$ currents and pH changes in CHO cells (14–16). Moreover, the recently cloned NOX-1S protein, generated by alternative splicing of the NADPH oxidase homologue gene NOX-1, also catalyzes voltage-gated H$^+$ currents when expressed into HEK-293 cells (17). The NOX-1S currents had a high threshold of voltage activation, unlike the H$^+$ currents observed in phagocytes with an active oxidase. Thus, gp91$^{phox}$ and NOX-1S are either two distinct H$^+$ channels, or, alternatively, they both function as channel modulators. If gp91$^{phox}$ is indeed the H$^+$ channel of phagocytes, its membrane expression should be sufficient to generate phagocyte-like H$^+$ currents in non-phagocytic cells. In contrast, if gp91$^{phox}$ is a channel modulator, the phagocytic currents are not likely to be reproduced by the heterologous expression of gp91$^{phox}$ in non-phagocytic cells, which display distinct ion channels and cannot assemble a functional oxidase.

In this study, we show that the stable expression of gp91$^{phox}$ at the plasma membrane of HEK-293 cells is associated with voltage-gated H$^+$ currents. Like all H$^+$ currents, the gp91$^{phox}$-associated currents were pH-dependent, H$^+$-selective, and reversibly blocked by micromolar concentrations of Zn$^{2+}$. However, they had faster activation kinetics than the currents observed in the same recipient cell line stably expressing the oxidase complex might thereby functionally couple electron heme coordination and/or spin state during the activation of the whole cell conductance. All recordings were performed using pClamp 6.0 software. Leak currents were small compared with the whole cell H$^+$ currents and were subtracted only to allow calculation of the whole cell conductance. All recordings were performed >3 min after achieving the whole cell configuration to allow the equilibration of the cytosol with the pipette solution.

Unless otherwise indicated, the bath solution contained (in mM): CsAsp 75, HEPES 100, CaSO4 50, MgCl$_2$ 1, EGTA 0.2, pH 7.5. Pipette solutions, contained for pH 5.7, CsAsp 90, MES 100, CsOH 20; for pH 6.3, CsAsp 35, PIPES 100, CsOH 132; for pH 7.3, CsAsp 75, HEPES 100, CsOH 50; and for pH 8.0, CsAsp 77.5, Tris 100, aspartic acid 44.2. All pipette solutions contained, in addition, 1 mM MgCl$_2$, 0.2 mM EGTA, and 2 mM ATP. For ion substitution experiments CsAsp was replaced by NMG-Asp or CsCl. All chemicals were obtained from Sigma or Fluka. DEPC was dissolved in 50% ethanol at 1.2 μL and used at a final concentration of 50% ethanol at 1.2 μL and used at a final concentration of 1.2 μM, such that the final ethanol concentration did not exceed 0.1%.

**Data Analysis**—Data analysis was performed using Origin software (MicroCal, Northampton, MA). For exponential fits, the first 5 ms of the whole cell recordings were fitted to a single exponential using the nonlinear least squares regression method. All currents were normalized to the peak current density at 0 mV.

**Immunofluorescence**—The gp91$^{phox}$ antibodies used were as follows: a rabbit polyclonal antibody directed against amino acid 562–569 of the C-terminal domain (kindly provided by Dr. F. Morel, Grenoble, France; see Figs. 1 and 4), the c-terminal monoclonal antibody (kindly provided by Dr. Dirk Roos, Amsterdam, The Netherlands; see Fig. 6), and the 7D5 monoclonal antibody directed against an external epitope of gp91$^{phox}$ (kindly provided by Dr. K. Nemet, Budapest, Hungary). A mouse IgG1 (anti-Myc) was used as isotype-matched control for the two monoclonal antibodies.

Cells plated in 12-mm coverslips were washed twice with phosphate-buffered saline (PBS), fixed with 3% paraformaldehyde, permeabilized with 0.3% Triton X-100, and incubated for 20 min in PBS supplemented with 1% BSA. Cells were then exposed to the rabbit peptide antibody (1/800), mAb48 (1/600), or mAb7D5 (1/800) for 1 h on a shaker at 0.2% Tween and 1% BSA. After three successive washes, unspecific labeling was blocked with 10% non-immune goat serum in PBS for 1 h. Cells were then incubated with the secondary antibody, goat anti-rabbit (1/800), goat anti-mouse (1/800) for 1 h in 0.2% Tween and 1% BSA PBS. Cells were then washed twice in 0.2% Tween and 1% PBS and two PBS and finally mounted on glass. Fluorescence images were acquired using a Zeiss LSM 510 confocal microscope (see Fig. 4) or with a cooled CCD camera mounted on an inverted Zeiss Axiosvert S100 TV microscope (see Figs. 1 and 6).
Spectral Analysis of gp91phox—Total cellular membranes were prepared from stable gp91phox clones, control HEK-293 cells, and human neutrophils as described (19). Briefly, 10^6 cells/ml in PBS + 2 mM EGTA were incubated for 20 min at 0 °C under gentle agitation in lysis buffer containing 1% Triton X-100, 10 mM HEPES, 3.5 mM MgCl₂, pH 7.4, and 2 tablets of Complete*ED protease inhibitor mixture (Roche Molecular Biochemicals). Cells were then centrifuged at 12000 x g for 30 min at 4 °C. The resulting supernatant was collected, and the protein concentration measured by the Bradford method. Absorbance spectra of the membrane preparations were recorded between 400 and 600 nm on a Bio-Tek spectrometer (Kontron) before (oxidized) and after (reduced) addition of a few grains of sodium dithionite. The differential spectra were obtained by subtracting the oxidized from the reduced measurement.

Western Blot—20 µg of membrane proteins solubilized in sample buffer (0.187 at Tris-HCl, pH 6.8, 7% SDS, 0.03% bromophenol blue) were loaded on an 8% SDS-polyacrylamide gel electrophoresis gel (4% stacking gel) and transferred to a polyvinylidene difluoride membrane in semi-dry conditions at 200 milliamperes for 45 min at room temperature. The polyvinylidene difluoride membrane was reversibly stained with red ponceau and incubated for 1h in the presence of 1% Triton X-100, 10 mM HEPES, 3.5 mM MgCl₂, pH 7.5 supplemented with 5% milk. The membrane was then exposed overnight at 4 °C to the mAb48 (1/1000) in 0.1% Tween TBS plus 5% milk, washed three times for 5 min in 0.1% Tween TBS, and exposed to a peroxidase-conjugated goat anti-mouse secondary antibody (1:20000; Bd Transduction Laboratories, Basel, Switzerland). The blot was revealed with enhanced chemiluminescence reagents (LumiGlo reagent and Peroxide reagent; BioConcept, Cell Signaling Switzerland, Luzern, Switzerland) and images taken with the luminescent image analyzer LAS-1000 plus (Fujifilm) using Aida 2.3 acquisition software.

RESULTS

Expression Induces Voltage-activated Currents—To investigate whether gp91phox functions as a proton channel, the membrane cytochrome was expressed in HEK-293 cells, the cell line used previously to characterize the H^+ channel, the membrane cytochrome was expressed in HEK-293 cells or in cells transfected with the empty pcDNA3 vector. Background currents were observed in non-transfected HEK-293 cells or in cells transfected with the empty pcDNA3 vector, as reported previously in CHO cells and remains properly targeted to the plasma membrane.

To assess the selectivity of the gp91phox currents, we measured the reversal potential of the tail currents, E_rev. Outward currents were activated by depolarizing cells to 60 mV for 2 s, and deactivating tail currents were recorded during repolarization to voltages ranging from −100 to 0 mV. Fig. 2 shows superimposed tail currents recorded at pH_i,pHₑ, 5.7:7.5 in symmetric CsAsp solutions (inset). An exponential fit to the tail currents was performed to extract the instantaneous current/voltage relationship and calculate the reversal potential, which averaged −72 ± 3 mV in these conditions (n = 15). This negative reversal potential indicates that the current is unlikely to be carried by Cs or Asp, whose equilibrium potential is close to zero, but rather by H^+, whose equilibrium potential is −105 mV. Accordingly, E_rev was not affected by the replacement of Cs^+ by NMG^+ or of Asp by Cl^− but changed to −44.6 ± 2.4 (n = 9) and −7.3 ± 2.4 mV (n = 6) when the pH value of the pipette was changed to 6.3 and 7.3, respectively (Fig. 2A). This corresponds to a change of 42 mV per pH unit, whereas the Nernst equation predicts a shift of 58 mV. This deviation most likely reflects the imperfect intracellular pH clamp and the inability of the pipette solutions to impose an outward pH gradient (pH_o,pHₑ, 5.7:7.5; inset). Currents were activated by 3-s depolarizing steps to voltages ranging from −40 to 80 mV. WT, wild-type cells. B, immunolocalization of gp91phox in stable HEK-293 transfectants. Cells were fixed with 3.7% paraformaldehyde, permeabilized with 0.3% Triton X-100, and exposed to a policlonal antibody directed against the C-terminal tail of gp91phox (kindly provided by Dr. F. Morel, Grenoble, France). Images are representative of three independent experiments. C, current densities of wild-type cells and of cells transfected with the empty pcDNA3 vector or with gp91phox. The currents measured at the end of a 3-s depolarizing pulse to 60 mV were divided by the cell capacitance.

The gp91phox-associated Current Is H^+-selective, pH-dependent, and Zn^2+-sensitive—Voltage-gated proton channels are extremely H^+-selective, strongly modulated by the pH gradient, and reversibly inhibited by polyvalent metal cations (22). To assess the selectivity of the gp91phox currents, we measured the reversal potential of the tail currents, E_rev. Outward currents were activated by depolarizing cells to 60 mV for 2 s, and deactivating tail currents were recorded during repolarization to voltages ranging from −100 to 0 mV. Fig. 2 shows superimposed tail currents recorded at pH_i,pHₑ, 5.7:7.5 in symmetric CsAsp solutions (inset). An exponential fit to the tail currents was performed to extract the instantaneous current/voltage relationship and calculate the reversal potential, which averaged −72 ± 3 mV in these conditions (n = 15). This negative reversal potential indicates that the current is unlikely to be carried by Cs or Asp, whose equilibrium potential is close to zero, but rather by H^+, whose equilibrium potential is −105 mV. Accordingly, E_rev was not affected by the replacement of Cs^+ by NMG^+ or of Asp by Cl^− but changed to −44.6 ± 2.4 (n = 9) and −7.3 ± 2.4 mV (n = 6) when the pH value of the pipette was changed to 6.3 and 7.3, respectively (Fig. 2A). This corresponds to a change of 42 mV per pH unit, whereas the Nernst equation predicts a shift of 58 mV. This deviation most likely reflects the imperfect intracellular pH clamp and the increased H^+ depletion occurring at acidic pipette pH, as consistently reported in previous H^+ channel studies (9, 23, 24). However, even assuming that the deviation is because of a finite permeability of the conductance to other ions, the relative H^+
Proton Currents Generated by the gp91phox Cytochrome

The gp91phox currents are H⁺-selective, pH-dependent, and Zn²⁺-sensitive. A, Erev measured at different pipette pH values. Currents were activated by a 2-s depolarization to 60 mV, and deactivating tail currents measured during repolarization at potentials ranging from −100 to −40 mV (inset, Erev = −64 mV in this experiment). The Erev measured in symmetrical solutions containing CsAsp (squares), NMG-Asp (circles), and CsCl (triangles) are plotted against the pipette pH values. Bath pH was 7.5 in all experiments. Values are means ± S.E. of four to fifteen experiments; the dotted line is the Erev predicted by the Nernst equation for a perfectly H⁺-selective conductance. B, families of currents recorded at different pipette pH values in gp91phox cells (left) and current-voltage relationship measured at a pipette pH value of 5.7 (squares; n = 31), 6.3 (triangles; n = 9), and 7.3 (circles; n = 9). Data are mean ± S.E. C, effect of Zn²⁺ on the currents elicited by a 2-s depolarization to 60 mV at pH 7.5. The current was rapidly and reversibly blocked by the addition of 3 mM Zn²⁺ to the bath solution. Data are representative of eight different cells.

permeability pH/PCO₂ calculated according to the Goldman-Hodgkin-Katz equation is >10⁶ given the very low concentration of H⁺ compared with the other ions. Thus, the gp91phox currents are very selective for H⁺.

Consistent with the known pH dependence of H⁺ channels, increasing the pH gradient by perfusing cells with more acidic pipettes increased the amplitude of the gp91phox current and shifted its threshold of voltage dependence to more negative voltages (Fig. 2B). Decreasing the pH gradient had the opposite effect, the threshold for voltage activation remaining above the H⁺ equilibrium potential, and only outward currents were observed in all conditions (Fig. 2B). As expected, addition of the divalent metal cation Zn²⁺ (3 mM free [Zn²⁺]) calculated to be −200 μM markedly reduced the outward currents (Fig. 2C).

The block was rapid and was fully reversible upon wash of Zn²⁺ from the bath solution, with a slight overshoot reflecting the build up of H⁺ ions near the plasma membrane during the block, as H⁺ extrusion is blunted under these conditions. The predominant effect of Zn²⁺ was to shift the voltage dependence of the current activation by 60 mV toward more positive voltages and to cause a marked slowing of the kinetics of activation (not shown). This is consistent with the known effects of Zn²⁺, which modulates H⁺ channel gating but not its instantaneous current-voltage relationship (25), an effect attributed to the binding of Zn²⁺ to external sites on the channel protein. Thus, the H⁺ currents observed in stable gp91phox transfectants have properties that are typical of voltage-gated H⁺ channels.

Comparison of gp91phox and NOX-1S H⁺ Currents—The gp91phox-associated H⁺ currents appear very similar to the H⁺ currents associated with the expression of NOX-1S (17). This was somewhat surprising, as the NOX-1 gene is expressed mostly in colon, whereas gp91phox expression is restricted to phagocytes. Because H⁺ channels in different tissues exhibit very distinct kinetics of voltage activation (22), these kinetic differences would be expected to persist in the HEK-293 background of the two proteins indeed function as H⁺ channels. If, on the other hand, the proteins act as channel modulators, as has been proposed (13), the kinetic properties of the currents would reflect the endogenous channel of the recipient cell line.

To test this possibility, we analyzed in detail the kinetics of activation of the gp91phox and NOX-1S currents. To minimize the effects due to differences in expression levels, stable gp91phox and NOX-1S clones displaying similar current densities were chosen for comparison. Fig. 3 shows that the H⁺...
currents activated faster in gp91phox than in NOX-1S cells. An exponential fit to the current activated by a 60-mV pulse yielded a time constant for activation ($\tau_{act}$) of $295 \pm 18\ ms$ for gp91phox cells ($n = 24$) and $370 \pm 27\ ms$ for NOX-1S cells ($n = 19, p < 0.02$). Faster activation was observed in gp91phox cells at all voltages but became significant only at voltages exceeding 60 mV (Fig. 3B). In addition, the $H^+$ currents deactivated more slowly in gp91phox cells than in NOX-1S cells (Fig. 3C). The time constant of deactivation ($\tau_{d}$) was determined by fitting an exponential curve to the tail currents measured at different repolarizing voltages following a pulse to 60 mV (Fig. 3D). At $-50\ mV$, $\tau_{d}$ was $379 \pm 38\ ms$ for the gp91phox cells ($n = 15$) and $237 \pm 27\ ms$ for the NOX-1S cells ($n = 12, p < 0.02$). Thus, proton currents activated faster and deactivated more slowly in gp91phox than in NOX-1S cells, suggesting that the underlying channel proteins have distinct properties.

**Heme Depletion Modulates gp91phox but Not NOX-1S $H^+$ Currents**—Unlike NOX-1S, gp91phox contains two non-identical heme groups embedded within the membrane that mediate the final steps of electron transfer to molecular oxygen. Both hemes are coordinated covalently by histidines in the axial positions, the most probable ligand pairs being His-101–His-209 and His-115–His-222 (26). His-209 and His-222 are located in the fifth transmembrane domain missing from the NOX-1S splice variant, which is thus predicted not to bind heme. In contrast, His-101 and His-115 are located in the third transmembrane domain, within the putative $H^+$ transport motif shared by gp91phox and NOX-1S. Importantly, His-115 is the central residue within the chain of histidines thought to conduct $H^+$ ions and has been shown by mutagenesis to be the residue most critical for $H^+$ channel activity (16, 21). The binding of heme to this residue within the full-length cytochrome might explain the kinetic differences between the gp91phox and NOX-1S currents.

To assess whether the presence of heme within the gp91phox cytochrome affects $H^+$ currents, we cultured the cells in the presence of succinyl acetone (SA), an inhibitor of heme synthesis. SA specifically inhibits the enzyme 5-aminolevulinic acid dehydratase that catalyzes the formation of porphobilinogen from 5-aminolevulinic acid, thereby preventing the synthesis of heme (27). Exposure of HEK-293 cells to 10 $\mu$g/ml SA for 4 days had no effects on the fluorescence intensity and staining pattern obtained with the gp91phox antibody, indicating that heme depletion does not alter the expression of gp91phox at the plasma membrane (Fig. 4B). The lack of heme incorporation was verified by spectroscopy, by measuring the reduced minus oxidized difference absorption spectra from membrane preparations of gp91phox cells treated with SA (see Fig. 6). The specific $\alpha$-band absorbance peak at 558 nm characteristic of neutrophils (orange line) was clearly visible in untreated gp91phox cells (red) but was almost completely abolished in SA-treated cells (green), indicating that the heme was not incorporated in the presence of SA. Consistent with the conserved membrane expression of gp91phox, no differences were observed between control and heme-depleted cells when $H^+$ currents were measured with acidic pipette solutions (current densities at 40 mV were as follows: $3.1 \pm 0.3$ versus $2.7 \pm 0.2$ pA/picofarad for control and SA-treated cells, respectively; $n = 31$ and 6; $pH_{pip} = 5.7:7.5$). However, in conditions favoring $H^+$ influx ($pH_{pip} = 8.0:7.5$), the currents were markedly altered in heme-depleted gp91phox cells (Fig. 4A). The currents in SA-treated gp91phox cells activated faster and had larger amplitude, slower deactivation kinetics, and a much lower threshold of voltage activation (Fig. 4B; $n = 18$). The threshold for voltage activation was shifted by $-50\ mV$ to more negative voltages, allowing inward steady-state $H^+$ currents at negative potentials (Fig. 4B). The currents reversed sign around 5 mV; i.e., $-15\ mV$ below $H^+$ equilibrium potential, a deviation likely reflecting the drop in pH below the plasma membrane caused by the inward $H^+$ currents. As expected, the $H^+$ currents in cells expressing the heme-devoid protein NOX-1S were not affected by SA, ruling out nonspecific effects of the heme depletion protocol (Fig. 4A). Thus, heme depletion modulates $H^+$ currents in gp91phox but not in NOX-1S cells. The modulated currents closely resemble the low threshold $H^+$ currents observed in activated phagocytes (9, 13), strongly suggesting that gp91phox is the oxidase-associated $H^+$ channel of phagocytes.

**Block by DEPC**—The large inward $H^+$ currents observed upon heme depletion suggest that removal of the heme molecules increases the mobility of protons flowing from the extracellular side. The heme coordinated by the His-115 residue is located near the extracellular side and transfers electrons to an external oxygen binding pocket where $O_2$ is formed (28). To test whether removal of heme from the cytochrome increases the accessibility of the His-115 residue to external protons, we assessed the sensitivity of $H^+$ currents to the histidine-reactive agent DEPC. As shown in Fig. 4A, 1.2 mM DEPC completely blocked the SA-induced increase in $H^+$ currents but had only minimal effects in cells that had not been exposed previously to SA. The effects of DEPC were rapid and progressive, and the decrease in steady-state current amplitude was paralleled by a decrease in the amplitude of tail currents (Fig. 5C). The 4-fold increase in current amplitude observed upon SA induction at alkaline pH was completely reversed by DEPC (Fig. 5B), sug-
suggesting that histidine residues mediate most of the effects of the heme depletion protocol. This suggests that the binding of heme to a critical histidine residue, likely His-115, modulates the conductive properties of the gp91phox H\(^+\) channel. The changes in heme properties occurring within gp91phox during oxidative activation might thus explain the appearance of low threshold H\(^+\) currents during activation of phagocytes.

Role of His-115—To verify that the His-115 residue mediates both H\(^+\) conduction and heme ligation, this residue was replaced by leucine, and the mutated gp91phox was stably expressed in HEK-293 cells. The H115L mutation did not affect the expression of gp91phox, as verified by immunofluorescence and Western blotting (Fig. 6A). The H115L gp91phox mutant was then tested for H\(^+\) conduction by the patch clamp technique as in Fig. 1. No voltage-activated currents could be elicited by depolarizing steps up to 80 mV (Fig. 6B), the current density being comparable with control, non-transfected cells (0.78 \pm 0.14 pA/picofarad; \(n = 10\)). This confirmed that the His-115 residue is critical for proton conduction as reported previously (16, 21). The ability of the H115L mutant to bind heme was then assessed by spectroscopy (Fig. 6C). The specific \(\alpha\)-band absorbance peak at 558 nm present in neutrophils (orange), gp91phox cells (red), gp91phox cells treated with SA (green), H115L cells (black), and control HEK-293 cells (blue). The absorbance peak at 558 nm specific of gp91phox hemes is shown. Traces are representative of three experiments from different membrane preparations.

Fig. 5. Heme removal exposes histidine residues. A, effect of the histidine-reactive agent DEPC on H\(^+\) currents in control and heme-depleted gp91phox cells. Conditions were as in Fig. 4. B, effects of DEPC on the currents induced by heme depletion. Data are mean \pm S.E. of the current amplitude measured at 20 mV; normalized to the currents measured in the absence of SA and DEPC. The number of recorded cells is indicated above the bars. * \(p < 0.001\) versus SA with out DEPC, from paired t test. C, time course of the block by DEPC. Cells were depolarized every 15 s to 20 mV to induce H\(^+\) currents (insets); when indicated, 1.2 mM DEPC was added to the bath solution.

Fig. 6. Histidine 115 is critical for proton conduction and heme insertion. A, effect of the His-115 \(\rightarrow\) Leu mutation on gp91phox expression. Immunofluorescence (left) and Western blot (right) representative of three independent experiments performed with the mAb48 monoclonal antibody are shown. Inset pictures show the staining of the isotype-matched control antibody. H115L cells stably expressing mutated gp91phox with histidine 115 replaced by leucine; gp91phox; stable gp91phox transfectants; PMN, human neutrophils; Control, non-transfected HEK-293 cells. B, currents elicited by depolarizing voltage pulses in H115L cells. Conditions were as in Fig. 1A. Traces are representative of 10 cells. C, differential absorption spectra (dithionite-reduced minus oxidized) of membranes prepared from neutrophils (polymorphonuclear; orange), gp91phox cells (red), gp91phox cells treated with SA (green), H115L cells (black), and control HEK-293 cells (blue). The absorbance peak at 558 nm specific of gp91phox hemes is shown. Traces are representative of three experiments from different membrane preparations.

DISCUSSION

Our results confirm that gp91phox, the membrane-associated subunit of the NADPH oxidase, functions as a voltage-gated H\(^+\) channel. Upon expression in non-phagocytic cells, gp91phox generated H\(^+\) currents similar to the currents generated by the oxidase homologue NOX-1S. The currents were strongly pH-dependent, extremely H\(^+\) selective, and reversibly blocked by micromolar concentrations of Zn\(^{2+}\). The gp91phox currents had slow kinetics of activation, resembling the currents of resting phagocytes but activated slightly faster and deactivated more slowly than the NOX-1S currents. Unlike NOX-1S currents, the gp91phox currents were markedly affected by the inhibition of heme synthesis, consistent with the different heme content of the two proteins. Upon heme depletion, the gp91phox currents

---

\(^2\) M. Dinauer, personal communication.
Proton Currents Generated by the gp91<sub>phox</sub> Cytochrome

Fig. 7. Proton conduction by gp91<sub>phox</sub>. Top, model of gp91<sub>phox</sub> topology showing the two heme groups coordinated non-covalently in the axial position by histidines in the third and fifth transmembrane domains, according to Ref. 26. A proton wire is formed by the histidine chain within the gp91<sub>phox</sub> third transmembrane domain. Bottom, the “hopping” of protons is limited by the reduced mobility of the His-115 residue, which is a heme ligand. The mobility of this residue is increased upon heme depletion or during oxidative activation, as the heme undergoes a transition from a low spin hexacoordinated state to a high spin pentacoordinated state. The change in heme coordination, by facilitating proton conduction through gp91<sub>phox</sub>, functionally couples electron and proton transport.

activated at lower voltages, had larger amplitude and distinct kinetics of activation and deactivation, and were blocked by the histidine-reactive agent DEPC. Thus, the presence of heme modulates the H<sup>+</sup> channel properties of the gp91<sub>phox</sub> cytochrome.

Heme insertion is critical for the formation of stable heterodimers between gp91<sub>phox</sub> and the non-glycosylated subunit p22<sub>phox</sub>, a process that is required for the insertion of the flavocytochrome in the plasma membrane of phagocytes (28, 29). Accordingly, heme depletion prevented the formation of stable heterodimers (29, 30) and strongly decreased the surface expression of gp91<sub>phox</sub> in PLB-985 cells, an effect that was reversible and prevented by the addition of exogenous heme (28). Heme removal did not affect the biogenesis of p65, the high mannose precursor of gp91<sub>phox</sub>, but the protein failed to mature to its fully glycosylated state, and p65 monomers were rapidly degraded by the cytosolic proteasome (29). Non-phagocytic cell lines appear more tolerant in allowing the expression of gp91<sub>phox</sub> and membrane insertion of gp91<sub>phox</sub> and NOX-1<sub>S</sub> in PLB-985 cells, an effect that was reversed and prevented by the addition of exogenous heme (28). Heme removal did not affect the biogenesis of p65, the high mannose precursor of gp91<sub>phox</sub>, but the protein failed to mature to its fully glycosylated state, and p65 monomers were rapidly degraded by the cytosolic proteasome (29). Non-phagocytic cell lines appear more tolerant in allowing the expression of gp91<sub>phox</sub> and membrane insertion of gp91<sub>phox</sub> subunits, possibly reflecting a difference in the proteolytic environment. Stable gp91<sub>phox</sub> cell lines have been generated in COS-7 and 3T3 cells (28), as well as in CHO (14) and HEK-293 cells (this study). In all cases, the expression of gp91<sub>phox</sub> was confirmed by Western blotting or immunofluorescence. The predominant species detected in immunoblots from COS-7 membranes appears to be the 58- or 65-kDa precursor. Despite the apparent lack of maturation of the gp91<sub>phox</sub> protein, the cells retained the characteristic spectral and redox properties of neutrophil flavocytochrome b558 (26). The heme spectral signature was also clearly visible in our gp91<sub>phox</sub> HEK-293 transfectants, but the size of the expressed protein was closer to 91 kDa even in the H115L mutant, which is unable to bind heme (Fig. 6, A and C). This suggests that, in the HEK-293 cell line, the gp91<sub>phox</sub> protein is able to mature to its fully glycosylated state even in the complete absence of heme insertion. A role of the p22<sub>phox</sub> subunit in the maturation process cannot be ruled out, as expression of p22<sub>phox</sub> could be detected in the HEK-293 cell line by reverse transcriptase polymerase chain reaction (data not shown). However, the lack of effect of heme depletion or of the His-115 mutation on the plasma membrane staining indicates that, regardless of its glycosylation state, gp91<sub>phox</sub> is stably expressed in HEK-293 cells in the absence of heme.

Whereas gp91<sub>phox</sub> expression was sufficient to reconstitute the currents observed in resting phagocytes, removal of the heme molecules produced very distinct currents. The currents generated by heme-depleted gp91<sub>phox</sub> mimicked the H<sup>+</sup> currents observed in phagocytes during the activation of the NADPH oxidase (9, 13), suggesting that gp91<sub>phox</sub> is the channel activated by PMA or GTP<sub>S</sub> in phagocytes. In neutrophils, a clear correlation was observed between the density of H<sup>+</sup> currents measured before and after stimulation with PMA, suggesting that most of the activated channels are already in the membrane (13). However, gp91<sub>phox</sub> is clearly not the only channel of phagocytes, because eosinophils from X91<sup>−/−</sup> mice express not gp91<sub>phox</sub> but probably also NOX-1<sub>S</sub>, consistent with the presence of NOX-1<sub>S</sub> mRNA in HL-60 cells (17). The relative contribution of the NOX-1<sub>S</sub> and gp91<sub>phox</sub> channels in resting and activated phagocytes, however, is difficult to assess for several reasons. 1) In the absence of stimulation, the gp91<sub>phox</sub> currents are undistinguishable from the currents generated by NOX-1<sub>S</sub>. 2) Although it did not display a shift in voltage activation, the channel in CGD cell was also activated by GTP<sub>S</sub> (9), making it difficult to attribute the increase in current amplitude to a specific channel. 3) CGD cells might compensate for the lack of gp91<sub>phox</sub> by expressing increased levels of NOX-1<sub>S</sub> or of other channels. 4) PMA and GTP<sub>S</sub> might activate the two channels in a different manner. The effects of PMA are largely mediated by the generation of arachidonic acid, which directly binds to the gp91<sub>phox</sub> protein (31) and induces its H<sup>+</sup> channel activity (15, 32). Accordingly, cells lacking cytosolic phospholipase A2 do not respond to PMA but respond to exogenous arachidonic acid (33). The effects of GTP<sub>S</sub> might be more complex, as it induces a massive exocytosis and also promotes the translocation of the cytosolic oxidase subunits (34, 35).

The shift in voltage activation of the gp91<sub>phox</sub> channel clearly does not require the redox function of the oxidase, as it can be induced in cells unable to mount a functional oxidase. Thus, H<sup>+</sup> fluxes are not strictly coupled to the flow of electrons, and the lack of correlation between electron and proton currents cannot be ascribed to the absence of the H<sup>+</sup> channel function of gp91<sub>phox</sub>. Indeed, earlier observations in a series of CGD variants suggested that the H<sup>+</sup> channel function of the oxidase required its assembly but not its redox function (36). Accordingly, the H<sup>+</sup> currents activated by GTP<sub>S</sub> in eosinophils persisted in the presence of DPI or in anoxic conditions (9). The gp91<sub>phox</sub> channel can be directly activated by arachidonic acid (16), which binds to the cytochrome and induces the transition of the heme iron from a low spin hexacoordinated state to a high spin pentacoordinated state (37). This change in heme coordination increases the affinity of the heme for O<sub>2</sub> and appears to reproduce several of the effects of heme depletion. In CHO cells expressing full-length or truncated gp91<sub>phox</sub>, arachidonic acid increased the current amplitude and caused a 23-mV shift in the current-voltage relationship, allowing steady-state
inward H⁺ currents (16). However, the currents in CHO transfec-
tants activated much faster than in our HEK-293 cells, and
arachidonic acid had no further effects on the kinetics of ac-
tivation or deactivation. This might reflect the higher expres-
sion levels achieved, as the average currents were ~50-fold higher
in CHO than in HEK-293 transfecants (3.4–4.2 nA at 80 mV,

\[ \text{pH}_i = 6.5:8.0 \] (16), versus 70 pA at 80 mV, \( \text{pH}_o : \text{pH}_i = 5.7:7.5 \)).

These high levels might have saturated the heme synthesis
pathway, leading to the production of gp91phox proteins lacking
heme. The absence of heme in a fraction of the expressed
gp91phox proteins would explain the increased current ampli-
tude and faster activation observed in approximately half the
cells (16), as well as the partial effects of arachidonic acid.

Taken together, however, the similar effects of arachidonic acid
and heme depletion indicate that residues at or near the heme-
binding site mediate the voltage-dependent H⁺ fluxes.

Strong evidence suggests that the residues His-111, His-115, and
His-118 form a proton-conducting channel within gp91phox.

This motif is retained in all known proteins that function as
voltage-gated proton channels, NOX-1S (17), NOX-1L,3
gp91phox (see Ref. 16 and this study), and a gp91phox truncation
mutant (16). These histidine residues are aligned along the
axis of an \( \alpha \)-helix (Fig. 6) and might function as a “proton wire”
by allowing the hopping of protons between pairs of hydrogen-
bonded donors and acceptor residues (38). Water molecules
might extend deep into the wire, allowing the same histidine to
be alternatively exposed to the intracellular and extracellular
side, as shown in a mutated Shaker channel (39).

Regardless of the mechanism, the reorientation or “turning” step of the donor
component requires a certain rotational mobility of the histi-
dines residues that form the wire. The central His-115 residue
appears critical, as the gp91phox currents were nearly abolished
when His-115 was mutated to leucine (see Fig. 6B and Ref. 16).

As His-115 functions both as a heme ligand and as a proton
donor/acceptor (Fig. 6), its mobility likely depends on the pre-
sence of heme (Fig. 7). Accordingly, in the absence of heme the currents in our gp91phox transfectants had larger amplitude,
faster activation, and increased voltage dependence and were
blocked by DEPC. This indicates that the removal of heme from its
histidine ligands increases proton conduction, as well as the
accessibility of these histidines to the external solvent.

The binding of heme to His-115 thus determines the H⁺ conduc-
tive properties of the oxidase and underlies the transitions between
the H⁺ currents observed in resting and activated phagocytes
(Fig. 6). This model has important implication for the function
of the oxidase. It implies that the protons pass close to the
heme-iron center of the flavocytochrome and that His-115 is a
labile heme ligand. The shifting accessibility of the His-115
residue during oxidase activation would thus functionally link
electron and proton transport.

---

3 N. Demaurex, unpublished observations.

Acknowledgments—We thank Cyril Castelhou for expert help in
cell culture and immunofluorescence, Isabelle Piuze and Elzbieta
Huggler for expert help in Western blot and neutrophils preparation,
and Dr. L. Bernheim for critical reading of the manuscript. We thank
Drs. F. Morel, Grenoble, France, M. Nakamura, Nagasaki, Japan, and
D. Roos, Amsterdam, The Netherlands for providing us with gp91phox
and p22 antibodies.

REFERENCES

1. Segal, A. W., and Shatwell, K. P. (1997) Ann. N. Y. Acad. Sci. 832, 215–222

2. Babior, B. M. (1999) Blood 93, 1464–1476

3. DeCoursey, T. E. (1998) Front. Biosci. 3, d477–82

4. DeCoursey, T. E., and Cherny, V. V. (1993) Biochim. Biophys. Acta 1171, 177–179

5. Ebert, P. S., Hess, R. A., Frykholm, B. C., and Tschudy, D. P. (1979) Biochem. J. 179, 272–278

6. Ebert, P. S., Hess, R. A., Frykholm, B. C., and Tschudy, D. P. (1979) Biochem. J. 179, 272–278

7. Ebert, P. S., Hess, R. A., Frykholm, B. C., and Tschudy, D. P. (1979) Biochem. J. 179, 272–278

8. Ebert, P. S., Hess, R. A., Frykholm, B. C., and Tschudy, D. P. (1979) Biochem. J. 179, 272–278

9. Ebert, P. S., Hess, R. A., Frykholm, B. C., and Tschudy, D. P. (1979) Biochem. J. 179, 272–278

10. Ebert, P. S., Hess, R. A., Frykholm, B. C., and Tschudy, D. P. (1979) Biochem. J. 179, 272–278

11. Ebert, P. S., Hess, R. A., Frykholm, B. C., and Tschudy, D. P. (1979) Biochem. J. 179, 272–278

12. Ebert, P. S., Hess, R. A., Frykholm, B. C., and Tschudy, D. P. (1979) Biochem. J. 179, 272–278

13. Ebert, P. S., Hess, R. A., Frykholm, B. C., and Tschudy, D. P. (1979) Biochem. J. 179, 272–278

14. Ebert, P. S., Hess, R. A., Frykholm, B. C., and Tschudy, D. P. (1979) Biochem. J. 179, 272–278

15. Ebert, P. S., Hess, R. A., Frykholm, B. C., and Tschudy, D. P. (1979) Biochem. J. 179, 272–278
Heme Histidine Ligands within gp91phox Modulate Proton Conduction by the Phagocyte NADPH Oxidase

Andrés Maturana, Serge Arnaudeau, Stephan Ryser, Botond Banfi, Johann Peter Hossle, Werner Schlegel, Karl-Heinz Krause and Nicolas Demaurex

J. Biol. Chem. 2001, 276:30277-30284.
doi: 10.1074/jbc.M010438200 originally published online June 1, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M010438200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 39 references, 22 of which can be accessed free at http://www.jbc.org/content/276/32/30277.full.html#ref-list-1