Minireview

Interactions between NADPH oxidase and voltage-gated proton channels: why electron transport depends on proton transport

Thomas E. DeCoursey*

Department of Molecular Biophysics and Physiology, Rush Presbyterian St. Luke's Medical Center, 1750 West Harrison, Chicago, IL 60612, USA

Received 25 August 2003; accepted 1 September 2003

First published online 7 October 2003

Edited by Gunnar von Heijne, Jan Rydström and Peter Brzezinski

Abstract **Leukocytes kill microbes by producing reactive oxygen species, using a multi-component enzyme complex, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. Electrons pass from intracellular NADPH through a redox chain within the enzyme, to reduce extracellular O_2 to O_2^-. Electron flux is electrogenic, and rapidly depolarizes the membrane potential. Excessive depolarization can turn off electron transport by self-inhibition, but this is prevented by proton flux that balances the electron flux. Although the membrane potential depolarizes by ~100 mV during the respiratory burst (NADPH oxidase activity), NADPH oxidase activity is independent of voltage in this range, which permits optimal function and prevents self-inhibition.**

Key words: Phagocyte; Respiratory burst; Ion channel; Membrane potential; Superoxide; pH

1. Introduction

Pathways for electron and proton transport occur in many molecules involved in the conversion of light or food into stored energy. The 'central dogma of membrane bioenergetics' [1] states that electron translocation creates a proton gradient (protonmotive force) across the mitochondrial inner membrane (or chloroplast thylakoid membrane, or bacterial plasma membrane) that is used to generate ATP [2,3]. Many of the membrane proteins involved in these bioenergetic functions incorporate pathways for both protons and electrons. This review describes proton and electron translocation in the system used by phagocytes (a subset of circulating white blood cells) to generate superoxide anion, O_2^-, which contributes to pathogen killing and also participates in intra- or intercellular signaling. This process is called the 'respiratory burst' because oxygen consumption increases dramatically [4]. In the phagocyte system (Fig. 1), distinct molecules mediate electron and proton transport. The nicotinamide adenine dinucleotide phosphate (NADPH) oxidase enzyme complex moves electrons across the membrane from intracellular NADPH to extracellular O_2 to generate O_2^-. Proton efflux is mediated by a distinct and separate pathway, the composition of which has yet to be identified [5,6].

2. NADPH oxidase is electrogenic and voltage-sensitive

Since NADPH oxidase was first proposed to be electrogenic by Lydia Henderson, Brian Chappell, and Owen Jones [7], it was assumed that the electron flux would have to be balanced to maintain electroneutrality. Henderson and colleagues observed electrogenic H^+ efflux during the respiratory burst, which they proposed to be the compensatory mechanism. Five years earlier, also at Bristol, Roger Thomas and Bob Meech had described the existence of voltage-gated proton channels in snail neurons [8]. Henderson et al. [7] suggested that a similar channel might exist in phagocytes and mediate charge compensation. Subsequently, voltage-gated proton channels were demonstrated directly by patch-voltage-clamp in human neutrophils [9] and other mammalian cells [10–12]. Recent studies have examined (a) the extent to which charge compensation is actually required, (b) whether it is mediated by H^+ efflux or K^+ efflux [13], and (c) whether H^+ efflux is mediated by voltage-gated proton channels or another pathway.

If NADPH oxidase is electrogenic, then at least one step in the electron transport process must be voltage-dependent [14]. Furthermore, the presumed requirement for charge compensation implies, by its obverse, that sufficient depolarization would prevent electrons from leaving the cell and thus inhibit enzyme function. Until recently, the voltage dependence of electron transport had been measured only over a narrow voltage range. Because electrons are transported across the entire membrane (unlike most components in electron transport chains of energy conversion), the activity of the enzyme can be measured directly as an electron current [15,16]. Activation of phagocytes by phorbol esters (PMA, phorbol myristate acetate) results in assembly and function of the NADPH oxidase complex [16], which can be studied using the permeabilized-patch variant [17] of the patch-clamp technique [18]. Fig. 2 illustrates the measurement of electron current (I_e) over a wide voltage (V) range [19]. These data confirm that NADPH oxidase activity is strongly voltage-dependent, and show that depolarization to +200 mV abolishes NADPH oxidase function. Surprisingly, the I_e–V relationship is highly

*Fax: (1)-312-942 8711.
E-mail address: tdecours@rush.edu (T.E. DeCoursey).

Abbreviations: FAD, flavin adenine dinucleotide; I_e, electron current; NADPH, nicotinamide adenine dinucleotide phosphate; V, voltage; $V_{threshold}$, the threshold voltage for activation of voltage-gated proton channels

0014-5793/03/$22.00
doi:10.1016/S0014-5793(03)01103-7

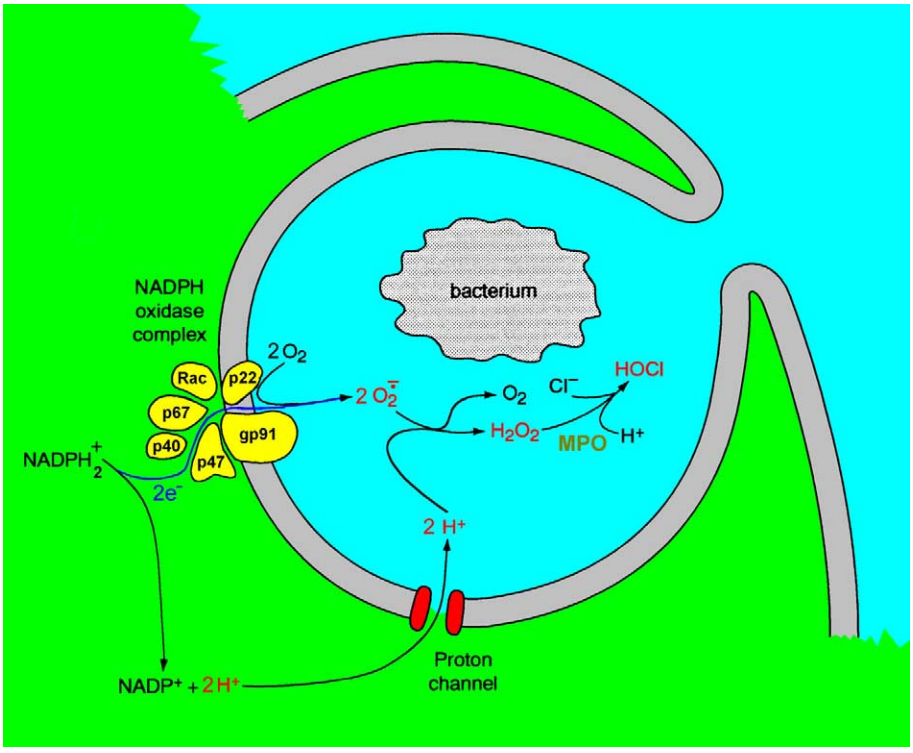

Fig. 1. Cartoon illustrating the NADPH oxidase complex in phagocytes and the related voltage-gated proton channel. In a resting phagocyte (neutrophil, macrophage, or eosinophil), gp91^{phox} and p22^{phox} are present in the membrane as a heterodimer, and the other four components are located in the cytosol. Upon stimulation by various agents, all the components assemble to form a functional complex. From [20].

non-linear (Fig. 2b,c). NADPH oxidase activity is steeply voltage-dependent at large positive voltages, but nearly independent of membrane potential at negative voltages. In intact neutrophils the membrane potential depolarizes from a resting potential of roughly −60 to −80 mV [20], to +58 mV during the respiratory burst [21]. Teleologically, then, the voltage-independent region is beneficial because it means that the oxidase does not inhibit itself throughout the physiological range of membrane potentials. The strong rectification of the I_e–V relationship suggests that two distinct steps in electron trans-

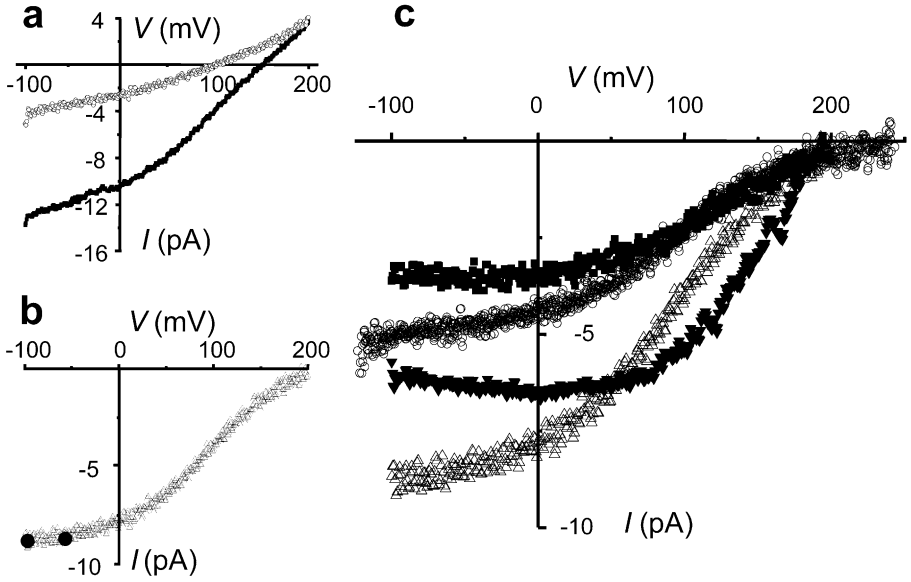

Fig. 2. Voltage dependence of electron current generated by NADPH oxidase during its function in a living human eosinophil. a: The voltage was ramped from −100 mV to +200 mV or more, after activation of NADPH oxidase by 60 nM PMA. Electron current generated by NADPH oxidase (I_e) was isolated by inhibiting NADPH oxidase function with diphenyleneiodonium (DPI). b: The residual current in the presence of DPI (open symbols) was subtracted from the total current (solid symbols) to give the net I_e. c: Examples of the I_e–voltage relationship in four cells. Experiments were done in the presence of 5 mM $ZnCl_2$ to inhibit H^+ currents, which would have been hundreds of picoamperes in amplitude in these cells. From [19].

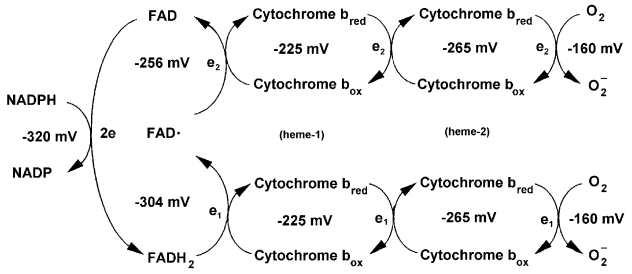

Fig. 3. Diagram showing the presumed electron pathways through the NADPH oxidase complex. The first electron removed from NADPH passes through the lower pathway, the second through the upper. Depicting two pathways is schematic; both electrons pass through the same two hemes, but at different times, and with slightly different energetics. The chemical midpoint potentials are given. This figure was generously provided by Andrew R. Cross.

port are rate-determining at different voltages [19]. We thus need to consider the electron pathway.

3. The electron pathway

Although the NADPH oxidase complex has many components, the probable electron pathway, illustrated in Fig. 3, is contained within a single subunit. The gp91^{phox} component of NADPH oxidase appears to contain both hemes (denoted cytochrome *b* in Fig. 3) as well as the binding sites for NADPH, flavin adenine dinucleotide (FAD), and presumably, O_2 [22]. The other components of the enzyme complex play various roles in assembly and regulation of function [23]. Five components (gp91^{phox}, p67^{phox}, p47^{phox}, p22^{phox}, and Rac) are sufficiently indispensable that their absence, due to mutation, results in chronic granulomatous disease (CGD) [4]. Without medical intervention, the severe impairment of microbial killing in CGD leads to significant morbidity and mortality secondary to recurrent infection. During the 'respiratory burst', the rapid increase in O_2 consumption that occurs during phagocytosis [24], two electrons are removed from NADPH in separate steps, and are translocated along the pathways diagrammed in Fig. 3. The chemical midpoint potentials in Fig. 3 indicate that the pathways generally favor electron translocation from intracellular NADPH to extracellular O_2.

4. What is the rate-limiting step?

Looking in more detail, we see that the two hemes in flavocytochrome b_{558} are *bis*-histidine-ligated and that the relevant His residues are 13 or 14 amino acid residues apart in cytochrome b_{558} of NADPH oxidase [22]. A very similar arrangement is seen in cytochrome bc_1 and in a synthetic multiheme protein in which the heme-ligating His are both 14 residues apart [25,26], which corresponds to a 20–21 Å separation between the redox centers [26,27]. Long-range electron transfer in proteins is well documented [28,29]. Although the intervening medium plays a profound role in electron transfer between redox metals [30], the dominant factor that regulates the rate of electron transfer within proteins is the distance between the participating redox donor and acceptor [31–33]. Electron transfer between redox centers within a protein separated by 20–21 Å (center-to-center) typically occurs at 10^3–10^6 s^{-1} [30]. Considering the mildly unfavorable energetics of heme$_1$ → heme$_2$ transfer in NADPH oxidase, the actual turnover rate of ~300–330 s^{-1} [34,35] falls quite reasonably at the lower end of the range of observed electron transfer rates. Taken together, the location of both His within the membrane based on hydropathy analysis [22], the relatively slow predicted rate of electron transfer over such a long distance, and the 'uphill' energetics of the heme$_1$ → heme$_2$ transfer (Fig. 3) strongly implicate this step as being the voltage-sensitive process that is rate-determining at large positive voltages (Fig. 2).

Even more interesting is the voltage-independent region, because this includes the entire physiological range of membrane potentials. We expect that due to the large electrical driving force, heme$_1$ → heme$_2$ electron transfer is rapid at negative voltages, and another step that is insensitive to membrane potential is rate-determining. At present, we can only speculate as to the identity of this step. The final reduction of O_2 to O_2^- seems an unlikely candidate, because O_2^- production is not limited by substrate O_2 concentration down to ~5% of atmospheric O_2 levels [36]. However, this evidence indicates only that the association of O_2 with the oxidase is not rate-determining. Future studies are needed to resolve this question.

5. Electron transport through NADPH oxidase in phagocytes requires proton efflux

The fact that depolarization alone can shut off NADPH oxidase activity (Fig. 2) graphically demonstrates that charge compensation is absolutely necessary for sustained oxidase function. Strong evidence implicates H^+ efflux through voltage-gated proton channels as the primary mechanism of charge compensation. Activation of NADPH oxidase results in profound depolarization if H currents, and consequent charge compensation, are inhibited by the divalent cations Cd^{2+} and especially Zn^{2+} (preventing charge compensation) [7,37–39]. Furthermore, H^+ current inhibitors block O_2^- production [19,40–44]. However, the evidence is not quite as straightforward as one might like, because in every study, the concentrations of metals that inhibit O_2^- production greatly exceed those required to inhibit H^+ channels. The resolution of this apparent contradiction is discussed below.

In Fig. 4a, the inhibition of O_2^- production by Zn^{2+} is illustrated for neutrophils and eosinophils. Although the latter

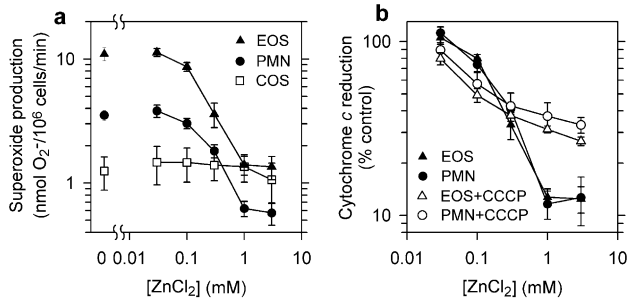

Fig. 4. a: The maximum rate of O_2^- release, assessed as cytochrome *c* reduction, is plotted in cells stimulated by PMA in the presence of various concentrations of $ZnCl_2$. Mean and S.E.M. are plotted for four batches of human eosinophils (▲), nine of human neutrophils (●), and four of COS$_{phox}$ cells (□). b: The human phagocyte data from panel a are replotted after normalization, along with measurements in the presence of PMA, $ZnCl_2$, and the protonophore CCCP, which partially restored function. From [19].

assemble more NADPH oxidase complexes in the plasma membrane [45] and generate O_2^- about three times faster than do stimulated neutrophils, the normalized concentration–response curves superimpose (Fig. 4b). Similar measurements in the non-phagocytic cell line COS-7, stably transfected with the essential NADPH oxidase components (which we term COS_{phox} cells, in Fig. 4a) [46] reveal insensitivity to Zn^{2+}. COS-7 cells were selected for study because they lack endogenous NADPH oxidase as well as voltage-gated proton channels [6,47]. Transfection with NADPH oxidase components invests them with the capacity to exhibit agonist-dependent superoxide generation (Fig. 4a), although to a lesser degree than that seen in phagocytes. However, in contrast to phagocytes, the superoxide generation in the heterologously transfected COS_{phox} cells is insensitive to Zn^{2+}. Insensitivity of oxidase activity in COS_{phox} cells to Zn^{2+} supports the idea that the effects of Zn^{2+} in phagocytes are due to inhibition of H^+ channels. The mechanism of charge compensation in COS_{phox} cells is not known. Nevertheless, the lack of inhibition of O_2^- production by Zn^{2+} in COS_{phox} cells indicates that Zn^{2+} acts on H^+ channels rather than, for example, some step in the assembly of the NADPH oxidase complex.

Additional evidence that H^+ efflux is required for NADPH oxidase activity is shown in Fig. 4b. In the presence of $ZnCl_2$, O_2^- production is partially restored by addition of the protonophore CCCP [19]. In contrast, the K^+ ionophore valinomycin does not restore O_2^- production in the presence of metal inhibitors [41].

It was mentioned above that the metal concentrations that inhibit O_2^- production (e.g. 0.1–0.3 mM, Fig. 4a) are orders of magnitude greater than those required to inhibit H^+ channels. Voltage-gated proton currents in phagocytes are clearly reduced by as little as 1 μM Zn^{2+} [38,48]. An appreciation of the mechanism by which Zn^{2+} inhibits H^+ currents is key to resolving this apparent discrepancy. Zn^{2+} does not 'block' the H^+ channel, in the classical sense of a cork plugging a bottle, but rather binds to a site on the external side of the channel where it slows opening and shifts the voltage dependence of gating to more positive voltages [49]. This shift in voltage sensitivity occurs by a mechanism first proposed by Andrew Huxley in discussions with Bernard Frankenhaeuser and Alan Hodgkin [50]. When a divalent cation binds at the external side of the membrane, a protein within the membrane will sense an altered local electrical field. Due to the greater positive charge at the extracellular side of the membrane (by the usual convention membrane potential is the voltage inside minus the voltage outside), the channel will behave as if the membrane potential is more negative than it actually is. Accordingly, a greater applied depolarization will be necessary to cause a depolarization-activated channel to open.

6. What does this mean to a phagocyte?

When the respiratory burst is activated in a phagocyte, the H^+ channel properties are modulated to enhance the probability that the channel is open. The voltage at which H^+ channels start to open, $V_{threshold}$, shifts about −40 mV, from roughly +20 mV in a resting cell to −20 mV after stimulation of NADPH oxidase [16]. The electrogenic action of NADPH oxidase will rapidly depolarize the membrane beyond $V_{threshold}$ and depolarization will continue until enough H^+ channels open and until the driving force ($V-E_H$, the difference between the membrane potential, V, and the Nernst potential for H^+, E_H) increases to the point at which proton efflux exactly balances electron efflux. Empirically, this occurs at +58 mV in human neutrophils [21] and +17 mV in human eosinophils [39]. We know that Zn^{2+} concentrations >0.1–0.3 mM are necessary to inhibit O_2^- production (Fig. 4a). The shift of $V_{threshold}$ that this concentration of Zn^{2+} will cause can be estimated to be 80–90 mV, based on the empirical relationship determined by Cherny and DeCoursey [49]: $\ln(1-P_{Zn})^{-1}\times 10$ mV, where P_{Zn} is the probability that the Zn^{2+} receptor on the H^+ channel is occupied by Zn^{2+}. Thus, $V_{threshold}$ will shift to +60 or +70 mV, into the region of the current–voltage relationship where NADPH oxidase activity is steeply voltage-dependent. The electron current amplitude will be reduced rapidly by any additional depolarization, hence O_2^- production will be reduced proportionately. Thus, the observed effect of Zn^{2+} on phagocyte NADPH oxidase activity reflects a metal-dependent shift in the sensitivity of the proton channel to membrane potential. What appeared for years to be a chink in the argument supporting a role for H^+ channels in the respiratory burst turns out to be completely predictable from the strong rectification of the I_e–V relationship.

Acknowledgements: This work was supported in part by the Heart, Lung and Blood Institute of the National Institutes of Health (research grants HL52671 and HL61437 to T.E.D.C.). I appreciate helpful discussions with Harry B. Gray and William M. Nauseef.

References

[1] Wikström, M. and Saraste, M. (1984) in: New Comprehensive Biochemistry, Bioenergetics (Ernerster, L., Ed.), pp. 49–94, Elsevier, Amsterdam.
[2] Mitchell, P. (1961) Nature 191, 144–148.
[3] Wikström, M.K. (1977) Nature 266, 271–273.
[4] Dinauer, M.C., Nauseef, W.M. and Newburger, P.E. (2001) in: The Metabolic and Molecular Bases of Inherited Disease, Vol. 3 (Scriver, C.R., Beaudet, A.L., Valle, D., Sly, W.S., Childs, B., Kinzler, K.W. and Vogelstein, B., Eds), pp. 4857–4887, McGraw-Hill, New York.
[5] DeCoursey, T.E., Cherny, V.V., Morgan, D., Katz, B.Z. and Dinauer, M.C. (2001) J. Biol. Chem. 276, 36063–36066.
[6] Morgan, D., Cherny, V.V., Price, M.O., Dinauer, M.C. and DeCoursey, T.E. (2002) J. Gen. Physiol. 119, 571–580.
[7] Henderson, L.M., Chappell, J.B. and Jones, O.T.G. (1987) Biochem. J. 246, 325–329.
[8] Thomas, R.C. and Meech, R.W. (1982) Nature 299, 826–828.
[9] DeCoursey, T.E. and Cherny, V.V. (1993) Biophys. J. 65, 1590–1598.
[10] DeCoursey, T.E. (1991) Biophys. J. 60, 1243–1253.
[11] Kapus, A., Romanek, R., Qu, A.Y., Rotstein, O.D. and Grinstein, S. (1993) J. Gen. Physiol. 102, 729–760.
[12] Demaurex, N., Grinstein, S., Jaconi, M., Schlegel, W., Lew, D.P. and Krause, K.-H. (1993) J. Physiol. 466, 329–344.
[13] Reeves, E.P., Lu, H., Jacobs, H.L., Messina, C.G.M., Bolsover, S., Gabella, G., Potma, E.O., Warley, A., Roes, J. and Segal, A.W. (2002) Nature 416, 291–297.
[14] Läuger, P. (1991) Electrogenic Ion Pumps, Sinauer Associates, Sunderland, MA.
[15] Schrenzel, J., Serrander, L., Bánfi, B., Nüsse, O., Fouyouzi, R., Lew, D.P., Demaurex, N. and Krause, K.-H. (1998) Nature 392, 734–737.
[16] DeCoursey, T.E., Cherny, V.V., Zhou, W. and Thomas, L.L. (2000) Proc. Natl. Acad. Sci. USA 97, 6885–6889.
[17] Lindau, M. and Fernandez, J.M. (1986) Nature 319, 150–153.
[18] Hamill, O.P., Marty, A., Neher, E., Sakmann, B. and Sigworth, F.J. (1981) Pflügers Arch. 391, 85–100.

[19] DeCoursey, T.E., Morgan, D. and Cherny, V.V. (2003) Nature 422, 531–534.
[20] DeCoursey, T.E. (2003) Physiol. Rev. 83, 475–579.
[21] Jankowski, A. and Grinstein, S. (1999) J. Biol. Chem. 274, 26098–26104.
[22] Biberstine-Kinkade, K.J., DeLeo, F.R., Epstein, R.I., LeRoy, B.A., Nauseef, W.M. and Dinauer, M.C. (2001) J. Biol. Chem. 276, 31105–31112.
[23] Babior, B.M. (1999) Blood 93, 1464–1476.
[24] Baldridge, C.W. and Gerard, R.W. (1933) Am. J. Physiol. 103, 235–236.
[25] Yun, C.H., Crofts, A.R. and Gennis, R.B. (1991) Biochemistry 30, 6747–6754.
[26] Robertson, D.E., Farid, R.S., Moser, C.C., Urbauer, J.L., Mulholland, S.E., Pidikiti, R., Lear, J.D., Wand, A.J., DeGrado, W.F. and Dutton, P.L. (1994) Nature 368, 425–432.
[27] Link, T.A., Schägger, H. and von Jagow, G. (1986) FEBS Lett. 204, 9–15.
[28] Winkler, J.R., Nocera, D.G., Yocom, K.M., Bordignon, E. and Gray, H.B. (1982) J. Am. Chem. Soc. 104, 5798–5800.
[29] Mayo, S.L., Ellis Jr., W.R., Crutchley, R.J. and Gray, H.B. (1986) Science 233, 948–952.
[30] Gray, H.B. and Winkler, J.R. (2003) in: The Porphyrin Handbook, Bioinorganic and Bioorganic Chemistry, Vol. 11 (Kadish, K.M., Smith, K.M. and Guilard, R., Eds.), pp. 51–73, Elsevier, Amsterdam.
[31] Marcus, R.A. and Sutin, N. (1985) Biochim. Biophys. Acta 811, 265–322.
[32] Moser, C.C., Keske, J.M., Warncke, K., Farid, R.S. and Dutton, P.L. (1992) Nature 355, 796–802.
[33] Page, C.C., Moser, C.C., Chen, X. and Dutton, P.L. (1999) Nature 402, 47–52.
[34] Cross, A.R., Parkinson, J.F. and Jones, O.T.G. (1985) Biochem. J. 226, 881–884.
[35] Koshkin, V., Lotan, O. and Pick, E. (1997) Biochim. Biophys. Acta 1319, 139–146.
[36] Gabig, T.G., Bearman, S.I. and Babior, B.M. (1979) Blood 53, 1133–1139.
[37] Nanda, A. and Grinstein, S. (1991) Proc. Natl. Acad. Sci. USA 88, 10816–10820.
[38] Bánfi, B., Schrenzel, J., Nüsse, O., Lew, D.P., Ligeti, E., Krause, K.-H. and Demaurex, N. (1999) J. Exp. Med. 190, 183–194.
[39] Bankers-Fulbright, J.L., Gleich, G.J., Kephart, G.M., Kita, H. and O'Grady, S.M. (2003) J. Cell Sci. 116, 3221–3226.
[40] Beswick, P.H., Brannen, P.C. and Hurles, S.S. (1986) J. Clin. Lab. Immunol. 21, 71–75.
[41] Henderson, L.M., Chappell, J.B. and Jones, O.T.G. (1988) Biochem. J. 255, 285–290.
[42] Simchowitz, L., Foy, M.A. and Cragoe, E.J. (1990) J. Biol. Chem. 265, 13449–13456.
[43] Kapus, A., Szászi, K. and Ligeti, E. (1992) Biochem. J. 281, 697–701.
[44] Lowenthal, A. and Levy, R. (1999) J. Biol. Chem. 274, 21603–21608.
[45] Lacy, P., Abdel Latif, D., Steward, M., Musat-Marcu, S., Man, S.F.P. and Moqbel, R. (2003) J. Immunol. 170, 2670–2679.
[46] Price, M.O., McPhail, L.C., Lambeth, J.D., Han, C.-H., Knaus, U.G. and Dinauer, M.C. (2002) Blood 99, 2653–2661.
[47] Maturana, A., Arnaudeau, S., Ryser, S., Bánfi, B., Hossle, J.P., Schlegel, W., Krause, K.-H. and Demaurex, N. (2001) J. Biol. Chem. 276, 30277–30284.
[48] DeCoursey, T.E., Cherny, V.V., DeCoursey, A.G., Xu, W. and Thomas, L.L. (2001) J. Physiol. 535, 767–781.
[49] Cherny, V.V. and DeCoursey, T.E. (1999) J. Gen. Physiol. 114, 819–838.
[50] Frankenhaeuser, B. and Hodgkin, A.L. (1957) J. Physiol. 137, 218–244.