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The Role of External and Matrix pH in Mitochondrial Reactive Oxygen Species Generation*

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Reactive oxygen species (ROS) generation in mitochondria as a side product of electron and proton transport through the inner membrane is important for normal cell operation as well as development of pathology. Matrix and cytosol alkalization stabilizes semiquinone radical, a potential superoxide producer, and we hypothesized that proton deficiency under the excess of electron donors enhances reactive oxygen species generation. We tested this hypothesis by measuring pH dependence of reactive oxygen species released by mitochondria. The experiments were performed in the media with pH varying from 6 to 8 in the presence of complex II substrate succinate or under more physiological conditions with complex I substrates glutamate and malate. Matrix pH was manipulated by inorganic phosphate, nigericine, and low concentrations of uncoupler or valinomycin. We found that high pH strongly increased the rate of free radical generation in all of the conditions studied, even when $\Delta pH = 0$ in the presence of nigericin. In the absence of inorganic phosphate, when the matrix was the most alkaline, pH shift in the medium above 7 induced permeability transition accompanied by the decrease of ROS production. ROS production increase induced by the alkalization of medium was observed with intact respiring mitochondria as well as in the presence of complex I inhibitor rotenone, which enhanced reactive oxygen species release. The phenomena revealed in this report are important for understanding mechanisms governing mitochondrial production of reactive oxygen species, in particular that related with uncoupling proteins.

The superoxide anion, a highly active compound that gives rise to hydrogen peroxide and other reactive oxygen species (ROS),[2] is produced by the mitochondria under normal physiological conditions as a side product of electron transport redox reactions in the respiratory chain. Disturbance of ROS production could result in the development of diverse pathologies, such as diabetes (1), neurodegeneration (2), heart failure (3), and chronic obstructive pulmonary disease (4). Understanding this process is important to develop therapeutic strategies. At present, the whole sequence of the reactions in the electron transport chain and coupling with proton transport through the membrane is not completely clarified; even less is known about the ROS production mechanism.

According to the current opinion, intermediate free radical species of mitochondrial electron transporters, which normally appear in the course of the redox reactions constituting electron-transport process, play an active role in this mechanism. They can interact with dissolved oxygen, converting it into superoxide anions (5).

The sites of superoxide generation are located in both complex I (6–11) and complex III (5, 11–13). Among all possible radicals arising as intermediates of one-electron transport, the semiquinones, emerging in several places of electron transport sequence, are considered the most capable to reduce molecular oxygen producing superoxide anion (14). The semiquinone anion radical (SQ^-) appears in respiratory complex I and complex III as a product of either one-electron ubiquinone reduction or one-electron ubiquinol oxidation followed by two protons released into the medium surrounding the inner mitochondrial membrane (15–17). The elementary reactions resulting in production of SQ^- are presented by Reactions 1 and 2.

Respiratory chain substrates (NADH or succinate) feed the electron flow and define the rate of ubiquinone reduction into SQ^-, which could take place equally in complex I or complex III.

$$Q + e^- \leftrightarrows SQ^-$$

REACTION 1

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[2] The abbreviations used are: ROS, reactive oxygen species; AFU, arbitrary fluorescence units; complex I, NADH dehydrogenase; complex II, succinate dehydrogenase; complex III, cytochrome *c* reductase; FCCP, carbonyl cyanid *p*-trifuoromethoxyphenylhydrazone; UCP, uncoupling protein; SQ^-, ubisemiqunone anion radical.

SQ^- remains bound with complex I or complex III until it receives the second electron and catches two protons from the medium.

$$SQ^- + e^- + 2H^+ \leftrightarrows QH_2$$

REACTION 2

The forward direction of Reaction 2 depends on medium pH. Reactions 1 and 2 are spatially organized in the inner membrane. If the electron transport chain operates in its normal mode, transferring protons from the matrix to the intermembrane space, the net reactions (Reactions 1 and 2) proceed in the forward direction for the species linked with the matrix side of the inner membrane and in the reverse direction for the species linked with the outer side of the membrane. In this way, only matrix pH would affect production of SQ^-. However, if the reactions are highly reversible, the forward reaction (Reaction 2) also affects SQ^- linked with the outer side of the inner membrane, so that its concentration depends on pH of the surrounding medium.

As an alternative to Reaction 2, the semiquinone anion radical could interact with oxygen, converting it into superoxide anion, although with much less probability.

$$SQ^- + O_2 \leftrightarrows Q + O_2^-$$

REACTION 3

The rate of this reaction according to mass action law is proportional to $[SQ^-]$. This concentration in turn depends on the rates of SQ^- production and utilization by the electron transport chain (Reactions 1 and 2).

Reactions 1 and 2 represent just elementary steps of the complex structurally organized process of electron transport through many transporters. The factors that are commonly accepted as main determinants of this process, and Reactions 1 and 2 as a part of it, are (i) the levels of oxygen, (ii) the levels of respiratory substrate, and (iii) transmembrane potential. Reaction 2 indicates that medium proton concentration is also one of the factors defining one of the principal steps of electron transport.

Thus, Reactions 1 and 2 define the concentration of semiquinone anion radical in the way that a decrease in proton concentration (pH increase) must decrease the rates of SQ^- reduction into ubiquinol and increase SQ^- concentration. Correspondingly, superoxide production in accordance with Reaction 3 must increase with alkalization of the matrix.

In situ, even if cytosolic pH is well controlled, several processes could contribute to the alkalization of matrix (*e.g.* cation uptake, such as Ca^{2+}, which decreases the transmembrane potential and thus induces the respective efflux of matrix protons). Operation of uncoupling proteins (UCPs) could be another way of *in situ* intramitochondrial pH regulation. UCPs possess an ability of passive proton conductance, which can be increased by activators (fatty acids) or decreased by inhibitors (purine nucleotides) (18, 19). A substantial body of evidence indicates that UCPs are involved in regulation of cellular level of free radicals (20–25), which could be interpreted as the result of affecting the electric and/or concentration component of the proton electrochemical potential.

The aim of the present study is to understand the role of proton concentration in ROS generation as one of the principal factors defining this important process contributing to normal cell operation or development of pathology. We have found that lack of protons in mitochondrial matrix favors ROS generation, whereas acidification of matrix strongly inhibits this process.

EXPERIMENTAL PROCEDURES

Isolation of Rat Brain Mitochondria—All procedures involving animals were approved by the Children's Hospital of Pittsburgh (Animal protocol number CHP UPMC ARCC 18–05) and were in compliance with principles of laboratory animal care and current laws of the United States. Rat brain mitochondria were isolated from the cortex of adult Sprague-Dawley rats. After removal, tissue was minced and homogenized in ice-cold isolation buffer I, which contained 225 mM mannitol, 75 mM sucrose, 5 mM HEPES buffer (pH adjusted to 7.3 with KOH), 0.1 mg/ml fatty acid-free bovine serum albumin, 1 mM tetrapotassium EDTA, and 12% Percoll. The homogenate thus obtained was carefully layered on the top of a discontinuous gradient of Percoll (24 and 42%) prepared using the same buffer. The preparation was then centrifuged at 16,000 × *g* for 10 min. The fraction containing the mitochondria located between 42 and 24% Percoll was carefully withdrawn by a syringe and washed from Percoll twice by pelleting in isolation buffer I. The resulting mitochondrial suspension was diluted in isolation medium II, which was similar to isolation buffer I, except for the concentration of EDTA (0.1 mM) and lack of albumin, and spun down at 12,000 × *g* for 10 min. The deposit of mitochondria was homogenized in isolation buffer II at a final protein concentration of ~20 mg/ml and stored on ice until use. The protein concentration in the mitochondrial samples was determined using a protein assay kit. Mitochondria prepared in this way were active for at least 5–6 h, as determined by their ability to maintain a stable transmembrane potential in the presence of oxidizable substrates.

Fluorescence Measurements—Measurements were performed in a stirred cuvette mounted in a Shimatzu RF-5301 spectrofluorimeter maintained at 37 °C. Mitochondria (0.2 mg/ml protein) were added to 1.5 ml of the basic incubation medium, which contained 125 mM KCl, 5 mM $MgCl_2$, 10 mM Tris, 10 mM HEPES (pH adjusted to a particular value between 6 and 8 with KOH or HCl), 10 μM EGTA, 5 mM succinate or 5 mM glutamate, and 5 mM malate as oxidizable substrates. Due to a tendency of precipitation at alkaline pH in the presence of inorganic phosphate, concentration of $MgCl_2$ was reduced to 0.5 mM. The incubation media were prepared freshly on each experimental day.

Hydrogen Peroxide—Hydrogen peroxide was measured using a 2 μM concentration of the fluorescent Amplex red dye in the presence of 1 unit/ml horseradish peroxidase as previously described (26). Measurements were carried out at excitation/emission wavelengths of 560 nm (slit 1.5 nm)/590 nm (slit 3 nm), respectively. H_2O_2 generation was calibrated by constructing calibration curves using known H_2O_2 concentrations in the standard incubation buffer together with Amplex red and horseradish peroxidase but without mitochondria at all pH values 6–8. At acidic pH, the response of the dye was lower than

that at alkali pH. The correction coefficients calculated from the titration curves for different medium pH were as follows: pH 6.0, 0.68; pH 6.5, 0.90; pH 7.0, 1.00; pH 7.5, 1.08; pH 8.0, 1.10. These correction coefficients were applied to compensate for a difference in H_2O_2 reporting system response when determining rates of ROS release at a given pH. The other medium components were without effect on the H_2O_2 reporting system.

Mitochondrial Transmembrane Potential—Mitochondrial transmembrane potential ($\Delta\Psi_m$) was estimated using fluorescence quenching of the cationic dye safranin O that is accumulated and quenched inside energized mitochondria. The excitation wavelength was 495 nm (slit 3 nm) and emission 586 nm (slit 5 nm), and the dye concentration used was 2.5 μM (11).

Respiration of Mitochondria—Respiration of mitochondria was measured by a Clark type oxygen electrode (Hansatech Co.; oxygen electrode unit DW1). The water-jacketed chamber was maintained at 37 °C and constantly stirred with a magnetic stirring bar.

Data Analysis—Data are presented as S.E. of 3–10 separate experiments. Statistical analysis was performed using Student's *t* test for comparison of two groups or one-way analysis of variance with Bonferroni's correction for multiple comparison implemented in Prism 4.0 software (GraphPad Software, San Diego, CA).

Materials—Amplex red was obtained from Molecular Probes, Inc. (Eugene OR). Reagents for the protein assay were purchased from Pierce. All other reagents and inhibitors were purchased from Sigma.

RESULTS

To study the pH-dependent component in the mechanism of reactive oxygen species generation in mitochondria, we used three types of incubation media with different impacts on the pH and electrical components of proton motive force through the inner membrane: condition 1, The medium without permeant anions, such as P_i; condition 2, the medium of condition 1 supplemented with P_i; condition 3, the medium of condition 2 supplemented with nigericin.

Rat brain mitochondria respiratory characteristics measured in condition 2 and pH variations from 6 to 8 with succinate or glutamate plus malate as substrates are presented in Fig. 1. These data show that our mitochondrial preparations are good in coupling between respiration and phosphorylation in physiological range of pH. At pH 7, the addition of oligomycin inhibits its respiration, whereas the addition of ADP (state 3 (*St 3*)) essentially stimulates it, so that the ratio of respiration rates (state 3/state oligomycin (*St 3*/*St Oligo*)) was 5.6 ± 0.6 ($n = 3$) for succinate and 6.5 ± 0.3 ($n = 6$) for glutamate plus malate. At alkaline pH, ADP stimulated respiration to a smaller degree, possibly because the lack of protons in the medium limits the process.

The first condition provides the maximal value of ΔpH (maximal pH in mitochondrial matrix (pH_{in}) at a given pH of incubation medium (pH_{out}) and minimal value of $\Delta\Psi$.

P_i co-transported with H^+ (or in exchange to OH^-) decreases ΔpH (and respectively increases $\Delta\Psi$). Therefore, the second condition stabilizes pH_{in} at a value less alkaline compared with the first condition; it provides the $\Delta\mu H^+$ distribution between electrical and chemical components similar to that in mitochondria of living cells.

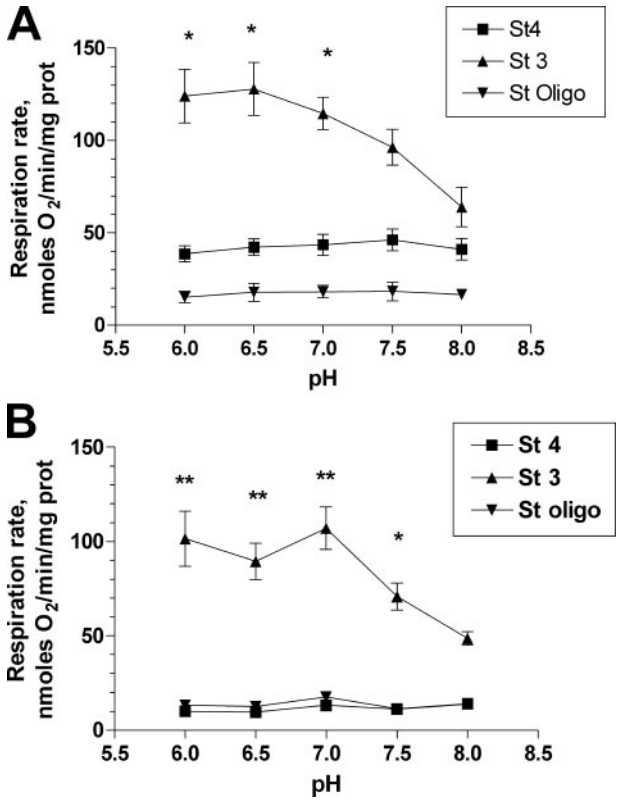

FIGURE 1. **pH dependence of mitochondrial respiration in state 3 (*triangles*), state 4 (*squares*), and state 4 with added oligomycin (*inverse triangles*), with succinate (*A*) and glutamate and malate (*B*) as substrates.** Basic incubation medium (see "Experimental Procedures") supplemented with 1 mM P_i was used. Statistical analysis was as follows. *A*, rates of respiration in state 3 taken at pH 8 were significantly different from that at pH 6–7 ($n = 3$–4; *, $p < 0.05$, *t* test). *B*, rates of respiration in state 3 taken at pH 8 were significantly different from that at pH 6–7.5; rates at pH 7 and 7.5 were significantly different from each other ($n = 6$; *, $p < 0.05$; **, $p < 0.001$, *t* test).

Nigericin as an H^+/K^+ exchanger adjusts ΔpH according to the K^+ gradient across the inner membrane. Since K^+ is the major osmogenic component, mitochondrial volume normally is stabilized when K^+ concentrations inside and outside are the same and therefore under the third condition $pH_{in} = pH_{out}$. Since the chemical component of $\Delta\mu H^+$ is close to zero, $\Delta\Psi$ could reach a higher value than under the other conditions (Fig. 2).

Fig. 3*A* (*squares*) shows that the rate of ROS production under condition 1 is 3 times increased when the pH of media changed from 6 to 7, whereas the levels of membrane potential decreased (Fig. 3*B*, *squares*), and the respiration rate slightly increased (Fig. 3*C*, *squares*). Usually, high $\Delta\Psi$ is considered to be a factor promoting ROS production (factor iii in the Introduction). Here the situation is controversial; ROS production is increased when $\Delta\Psi$ is decreased. Obviously, substrate and oxygen concentration (factors ii and i) remain the same for all conditions; therefore, medium pH is the only factor correlating with increase of ROS.

At more alkaline pH (7.5 and 8), mitochondria undergo spontaneous depolarization. As Fig. 4 demonstrates, the loss of membrane potential can be delayed by the presence in the

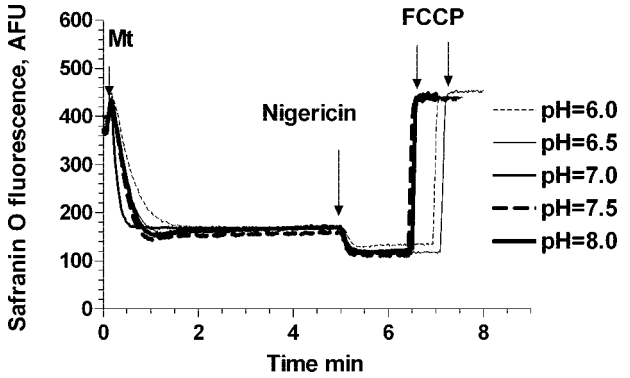

FIGURE 2. **Nigericin increases membrane potential of mitochondria oxidizing succinate at pH 6–8.** Basic incubation medium supplemented with 1 mM P_i was used. Additions at the time points indicated by the *arrows* were as follows: nigericin, 100 nM; FCCP, 200 nM.

medium of the agents known to inhibit permeability transition pore opening; cyclosporine A together with ADP and oligomycin. This fact indicates that the loss of membrane potential in this condition is due to permeability transition pore opening.

In the presence of 1 mM of P_i (condition 2), which makes the conditions more physiological, the behavior of the analyzed system is similar to that under condition 1. As Fig. 3*A* (*triangles*) shows, the rate of ROS production steeply increased upon increase of medium pH, reaching a maximum at pH 7.5, while membrane potential (Fig. 3*B*, *triangles*) remained constant. The decrease of ROS production at pH 8 correlates with a slight decrease of respiration rate (Fig. 3*C*, *triangles*).

When P_i and nigericin were present in the medium (condition 3), the rates of ROS production also increased along with the medium pH increase from 6 to 8 (Fig. 3*A*, *circles*), $\Delta\Psi$ was practically invariable (Fig. 3*B*, *circles*), and respiration rate (Fig. 3*C*, *circles*) repeated the pattern when only P_i was present (condition 2), reaching maximum at pH 7.5. Thus, if $\Delta pH = 0$, the whole pattern remains qualitatively similar to that observed in the presence of P_i (condition 2).

The pH dependences presented in Fig. 3 clearly show that pH itself, and not ΔpH, is an important determinant of ROS production. As can be seen, at pH ranging from 6 to 7, when mitochondria do not undergo permeability transition, ROS production under condition 1 is higher than under conditions 2 or 3, which could indicate an important role of matrix pH. The difference in ROS production can be seen when the conditions change in the course of the experiment, producing qualitatively the same outcome (*i.e.* the addition of P_i or nigericin always resulted in decrease of the ROS generation rate despite the increase in $\Delta\Psi$ (Fig. 5, *A* and *B*) because of trading the ΔpH component of proton motive force to the electric one).

The difference in ROS production between the three conditions presented in Fig. 3 resulted, evidently, from the difference in corresponding matrix pH. In addition to the use of P_i and nigericin, application of the ionophores FCCP and valinomycin can be instrumental in manipulating matrix pH to study its role in mitochondrial ROS generation. Both of these ionophores can decrease membrane potential; however, protonophore FCCP acidifies the matrix by increasing proton leak, whereas valinomycin causes increase of matrix pH by carrying K^+ into the matrix, decreasing $\Delta\Psi$ and, thus, facilitating the proton ejection by the electron transport chain. To stress the qualitatively different effects of these ionophores, we applied a small concentration of FCCP that did not substantially change $\Delta\Psi$ and a concentration of valinomycin that essentially depolarized mitochondria (Fig. 6*A*). As Fig. 6*B* shows, FCCP induced a dramatic decrease of ROS production, wheras valinomycin temporarily increased it.

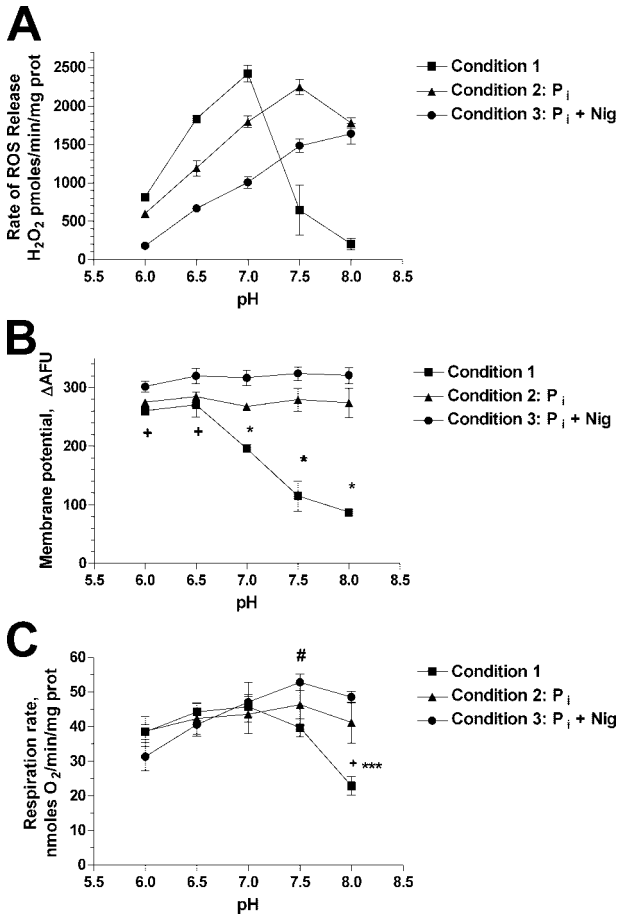

FIGURE 3. **Effect of medium pH on rate of ROS release (*A*), level of membrane potential (*B*), and rates of respiration of mitochondria oxidizing succinate (*C*).** Basic medium (see "Experimental Procedures") was used in all experiments with different supplementations: condition 1, basic medium alone (*squares*); condition 2, basic medium with 1 mM P_i (*triangles*); condition 3, basic medium with 1 mM P_i and 100 nM nigericin (*circles*). Statistical analysis was as follows. *A*, ROS rates taken at all conditions and all pH values were significantly different from each other ($n = 3–10$, $p < 0.05$), with an exception for conditions 2 and 3 at pH 8 (one-way analysis of variance test). Within the same condition, ROS rates taken at each pH were significantly different from that at the adjacent pH value ($n = 3–10$, $p < 0.05$), with the exceptions of the rates taken at pH 7.5 and 8 in condition 3 (*t* test). *B*, the levels of membrane potential measured at pH 7.0–8.0 for condition 1 were significantly different from that in condition 2 or 3 ($n = 3–8$; *, $p < 0.05$, analysis of variance test) and also from the membrane potential at pH 6.0–6.5 for the same condition ($n = 3$; +, $p < 0.05$, *t* test). *C*, at pH 8.0, rates of respiration measured in conditions 2 and 3 were significantly different from that in condition 1 ($n = 3–8$; +, $p < 0.05$, analysis of variance test). In condition 1, rates at pH 8.0 were significantly different from that at all other pH values ($n = 7–8$; ***, $p < 0.0001$, *t* test); in condition 3, rates at pH 7.5 were significantly different from that at pH 6.0 and 6.5 ($n = 3–4$; #, $p < 0.05$, *t* test).

In the experiments described above, succinate was applied as a substrate. Used in much higher concentrations than normally produced in Krebs cycle, it stimulates high ROS production rate, and this was convenient to study the process, although the ROS production rates were much higher than

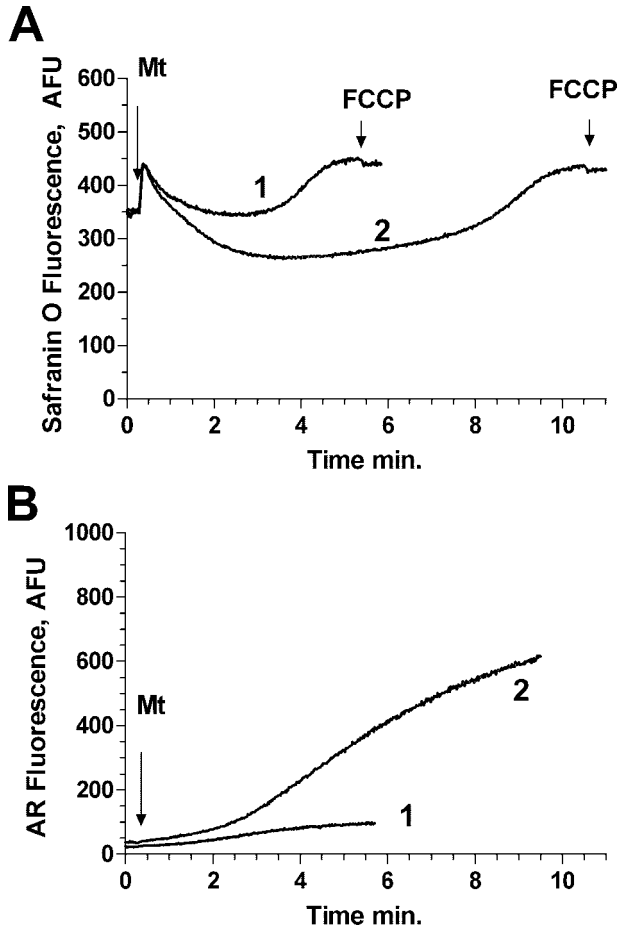

FIGURE 4. **Spontaneous depolarization of mitochondria takes place in the medium without P_i (condition 1) at pH 8.** *A*, membrane potential; *B*, ROS. Additions were as follows. 2 μM CsA, 200 μM ADP, and 2 μM oligomycin were present in the medium before the addition of mitochondria (*curve 2*).

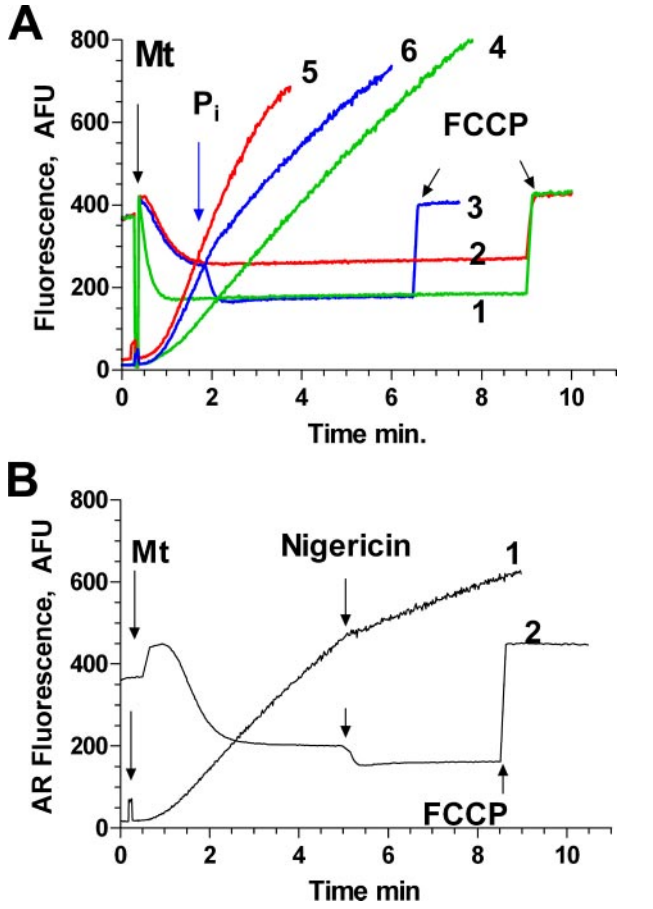

FIGURE 5. **Inorganic phosphate (*A*) and nigericin (*B*) increase the value of membrane potential but decrease the rate of ROS release.** The same relative scale of fluorescence was used for both dyes. *A*, membrane potential (*traces 1–3*) and ROS (*traces 4–6*). Basic incubation medium, pH 7, was used (see "Experimental Procedures"); 1 mM P_i was present (*traces 1* and *4*), or no P_i was present (*traces 2*, *3*, *5*, and *6*). The additions of mitochondria (*Mt*) (*all traces*), 1 mM P_i (*traces 3* and *6*) and 200 nM FCCP (*traces 1–3*) were made at the time points indicated by *arrows*. *B*, basic incubation medium without P_i was used; pH was adjusted to 6.5. *Trace 1*, ROS; *trace 2*, membrane potential. At the time points indicated by *arrows*, the following additions were made: mitochondria (*all traces*), 100 nM nigericin (*all traces*), and 200 nM FCCP (*trace 2*).

could be observed under physiological conditions. This high concentration of succinate could be observed, however, at pathologies like ischemia (27, 28). More physiological substrates, glutamate and malate, produce much smaller amounts of ROS (11, 29). However, as Fig. 7*A* illustrates, the rates of ROS release in this case also significantly increased with the increase of medium pH. These experiments were performed in the presence of both P_i and nigericin to ensure the conversion of the ΔpH component of proton motive force into the electric one (condition 3). Fig. 7, *B* and *C*, shows that, indeed, the addition of inorganic phosphate and nigericin did increase membrane potential the same way as it was observed with succinate as substrate.

Blocking complex I with an inhibitor rotenone completely depolarized mitochondria (Fig. 7*B*) and dramatically stimulated ROS generation (11, 29). Fig. 8 shows that upon application of rotenone, regardless of depolarization, the rate of ROS generation increased with the increase of medium pH. Thus, all presented data show the same pattern of ROS production increase in conjunction with the alkalization of medium.

DISCUSSION

In a study performed recently by Lambert and Brand (30), the authors came to the conclusion that pH gradient through the inner mitochondrial membrane determines ROS production. Our results clearly show that even in the absence of pH gradient (Figs. 3*A* (*circles*), 7*A*, and 8) medium pH is an essential factor defining ROS production by the respiratory chain, and pH increase itself induces the increase in ROS generation These data are in agreement with an early study presented by Turrens and Boveris (31), where a simpler model of submitochondrial particles was used. The authors have shown that the rate of superoxide generation increased in conjunction with an increase of medium pH from 7 to 9.2 in the presence of NADH or succinate as substrates and inhibitors rotenone or antimycin A, respectively.

The pH dependence of ROS production could be explained mechanistically, as outlined in the Introduction. Proton binding could be a limiting step in semiquinone radical-ubiquinol transformation; in this case, the decrease of proton concentration must stabilize semiquinone radical. Ohnishi and Trumpower (32) directly measured that semiquinone radical concentration in respiratory chain steeply increased with the

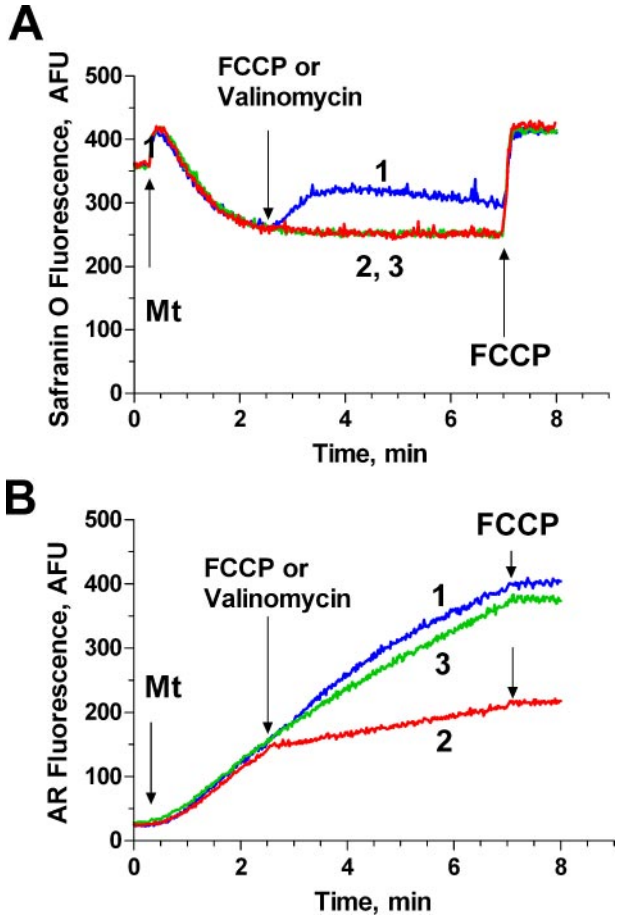

FIGURE 6. **Ionophores valinomycin and FCCP have opposite effect on the rate of ROS release by brain mitochondria.** *A*, recordings of membrane potential; *B*, recordings of ROS release. Basic medium without P_i (pH 6) was used. At the time points indicated by *arrows*, the following additions were made: mitochondria (*Mt*) (*all traces*), 20 pM valinomycin (*traces 1A* and *1B*), and 2.5 nM FCCP (*traces 2A* and *2B*). Concentration of K^+ ions in the medium was 125 mM. To achieve a complete depolarization, the final addition of FCCP at 7 min was 200 nM.

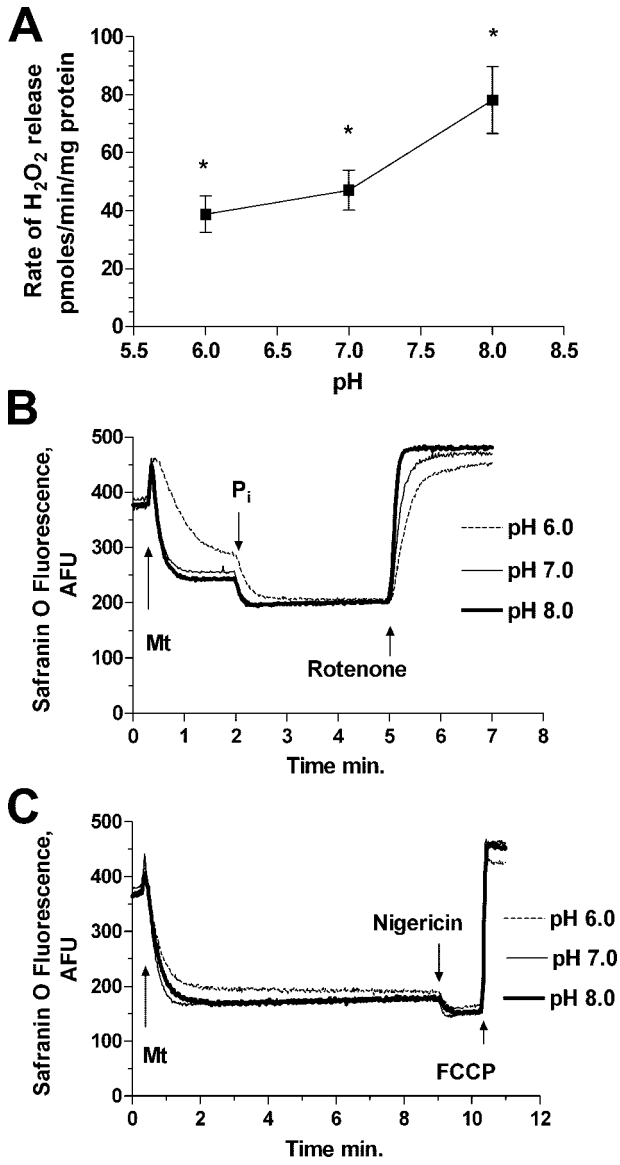

FIGURE 7. **Effect of medium pH on rate of ROS release (*A*) and level of membrane potential of mitochondria oxidizing complex I substrates glutamate and malate (*B* and *C*).** Basic incubation medium was supplemented with 1 mM P_i (*A* and *C*) and 100 nM nigericin (*A*). For *traces* of membrane potential (*B* and *C*), pH was adjusted to the values indicated by each trace. The additions of mitochondria (*Mt*), 1 mM P_i, 1 μM rotenone, 100 nM nigericin, and 200 nM FCCP were made at the time points indicated by the *arrows*. Statistical analysis was as follows. All data sets for ROS rates were statistically different from each other ($n = 4$; *, $p < 0.05$, *t* test).

increase of pH. This supports the proposed role of medium pH in mitochondrial ROS generation by respiratory chain.

The experimental results of (30) are in line with the ones presented here, and we believe that they could be better interpreted in terms of absolute value of matrix pH. Indeed, $\Delta pH = pH_{in} - pH_{out}$ could be increased by two ways (either by increasing pH_{in} or by decreasing pH_{out}) with a different outcome. Higher pH_{in} would stabilize semiquinone radical on the matrix side of the membrane, thus stimulating ROS production, and this was observed in the work (30) as well as in the present study. However, lowering outside pH (increase of outside proton concentration) would counteract the dissociation of protons from ubiquinol and formation of semiquinone radical (Reaction 2). In the latter case, we expect that higher pH gradient would decrease ROS production.

Comparison of the data presented in Fig. 3*A* indicates that in the same medium pH, ROS production is different, apparently because the matrix pH is different as defined by the respective conditions of incubation. The decrease of matrix pH induced by the addition of P_i and nigericin (switch from condition 1 to condition 2 or 3) is accompanied by an increase of $\Delta\Psi$ (Fig. 3*B*), which is expected to stimulate ROS production. However, the ROS generation rate decreased (Figs. 3*A* (*triangles* and *circles*) and 5), thus indicating that in these circumstances the effect of pH dominates over the opposite effect of $\Delta\Psi$.

The mentioned above comparison of the three conditions could give an approximate quantitative estimation of pH gradient and the role of matrix pH in ROS production. In the presence of nigericin, matrix pH is the same as external pH; therefore, subtracting the ROS production rate under condition 3 from that under two other conditions (as indicated in Fig. 9), we can deduce the role of matrix pH difference in ROS production. As Fig. 9 shows, at pH 6, the difference between ROS production rate under the condition when P_i and nigericin are absent

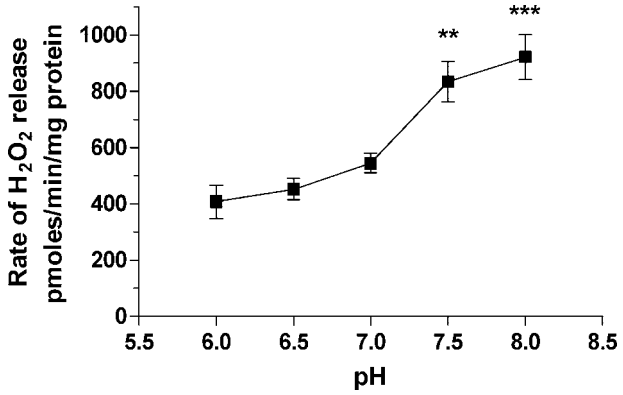

FIGURE 8. **Acidic pH inhibits rotenone-induced ROS generation by RBM in the presence of glutamate and malate.** Basic incubation medium supplemented with 1 mM P_i was used; concentration of rotenone was 1 μM. Statistical analysis was as follows. Data sets taken at pH 6.0, 6.5, and 7.0 were significantly different from that at pH 7.5 and 8.0 ($n = 5–8$; **, $p < 0.01$; ***, $p < 0.001$, *t* test).

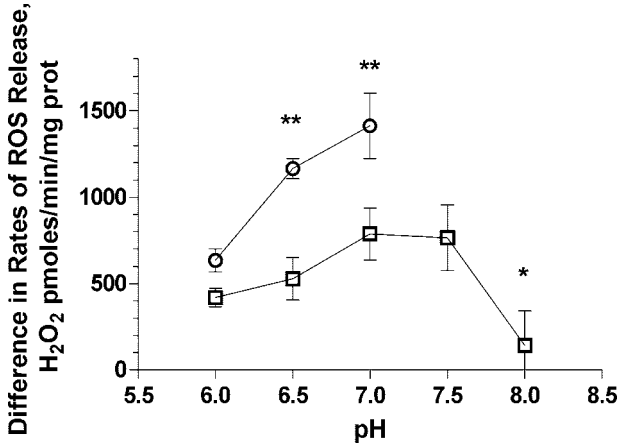

FIGURE 9. **Difference between the rates of ROS release calculated from the data presented in Fig. 3.** Results of subtraction of ROS release rates are plotted *versus* corresponding pH of the medium. *Circles*, condition 1 to condition 3; *squares*, condition 2 to condition 3. Statistical analysis was as follows. Values of difference between condition 1 and condition 3 (*circles*) at pH 6.5 and 7 were significantly different from that at pH 6 ($n = 3–10$; **, $p < 0.01$, *t* test); values of difference between condition 2 and condition 3 (*squares*) at pH 7 were significantly different from that at pH 8 ($n = 3–10$; *, $p < 0.05$, *t* test).

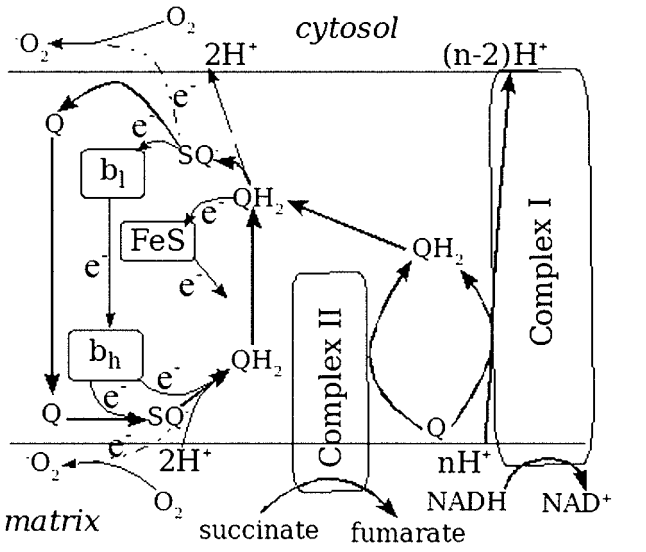

FIGURE 10. **Scheme complex I to complex III segment of mitochondrial respiratory chain.** Redox reactions in electron transport chain and proton translocation are fed by glycolysis and the Krebs cycle, which provide NADH, complex I substrate, and succinate, complex II substrate. Respiratory complex I accepts electrons from NADH, oxidizing it to NAD^+, and delivers these electrons to ubiquinone (Q). This delivery is coupled with proton transport from matrix to cytosol. Proton transport in complex III is coupled with electron transport in accordance with the generally accepted ubiquinone/ubiquinol (Q/QH_2) cycle mechanism, which is shown in more detail. The overall reaction performed by complex III is as follows, $QH_2 + 2Cyt\,C_{ox} + 2H^+n \leftrightarrow Q + 2Cyt\,C_{red} + 4H^+p$ (*i.e.* it oxidizes ubiquinol, reducing cytochrome *c* and releasing two H^+ to the cytosolic side (positive or p-side; this is reflected in the index of released H^+)). In addition, it translocates two protons from matrix (negative or n-side) to cytosol. Ubiquinol (QH_2) delivers its first electron to Fe^{3+}, releasing two protons at the p-side of the inner mitochondrial membrane and producing semiquinone radical (SQ^-). Then the latter gives its unpaired electron to cytochrome b_l, and produced ubiquinone (Q) dissociates from the complex. Free Q binds at the n-side and receives two electrons from cytochrome b_h, resulting from oxidation of two QH_2 molecules, thus producing subsequently SQ^- and QH_2, taking protons from the n-side. Dissociation of the produced QH_2 accomplishes a round of the cycle.

(condition 1) and when they are present (condition 3) is 650 pmol/min/mg protein. The reason for this difference is different matrix pH, which is higher in the absence of P_i and nigericin (condition 1). The same difference in ROS production (650 pmol/min/mg prot) could be induced by a 0.5-unit pH shift from pH 6 to 6.5 in the presence of both P_i and nigericin (under condition 3) (Fig. 3*A*, *circles*). Therefore, if under condition 1 matrix pH defines this difference in ROS production, it must be not less than 6.5 when pH of the medium is 6.

At pH 7, the difference between ROS production under conditions 1 and 3 is even higher (1300 pmol/min/mg protein (Fig. 9, *circles*)). Using the same reasoning as above, we can deduce that under condition 1 at pH 7, an even higher difference between external and internal pH should be expected. Thus, high ΔpH must result in ΔΨ decrease, and it was measured as Fig. 3*B* (*squares*) shows. Moreover, so high a pH difference assumes extremely low intramitochondrial proton concentrations; this could be a limiting factor restricting respiration rate when mitochondria are in state 3, as demonstrated by Fig. 1 (*triangles*). This indirect estimation of pH gradient by ROS production is in agreement with measurements made by conventional methods, 0.5–1.4 pH units (30, 33, 34).

ROS levels are physiologically important as a metabolic signal (35, 36). ROS overproduction is one of the main factors inducing apoptosis (37). Here we show that mitochondrial matrix pH is one of the factors controlling ROS signaling.

Higher matrix pH in the absence of P_i and nigericin (condition 1) at medium pH more than 7 resulted in spontaneous depolarization, probably due to permeability transition as Figs. 3*B* (*squares*) and 4*A* illustrate. In these conditions, mitochondria experience dramatic perturbations with the loss of all gradients across the inner membrane. This resulted in a substantial decrease of ROS release, which requires functional integrity of the mitochondrial membrane (Figs. 3*A* (*squares*) and 4*B*). It can be seen in Fig. 10 that SQ^- is not produced if the electron efflux from the FeS center is blocked. On the other hand, matrix pH was shown to be an essential factor in induction of permeability transition (38). Evidence from the literature indicates that the induction of permeability transition may occur in the presence of inorganic phosphate and/or Ca^{2+} (39). Since these compounds were not present in the medium of condition 1, our results demonstrate that pH itself could regulate permeability

transition; this proves the conclusion of our previous theoretical study (40).

The permeability transition was not observed under conditions 2 and 3; however, the alkalization-induced increase of respiration rate when $\Delta\Psi$ remained constant (Figs. 3*C*, *triangles* and *circles*) indicates that ROS affects membrane permeability, increasing leaks. In the presence of P_i and nigericin (under condition 3), respiration increased more steeply with pH increase despite slower ROS production (Fig. 3*C*, *circles*). This could be due to the fact that $\Delta\Psi$ in the presence of both P_i and nigericin (condition 3) is higher and that the leak strongly depends on $\Delta\Psi$. At pH 8, respiration decreases ($\Delta\Psi$ remains unchanged), apparently due to the decrease in proton leak, since external H^+ concentration decreases. Steep inhibition of respiration observed in condition 1 at pH 7.5 and 8, in all likelihood, is the consequence of the loss of cytochrome *c* in the course of permeability transition.

The data discussed above leave no doubts that change in matrix pH is vital; it controls such physiologically important processes as ROS production and permeability transition. The question is whether matrix pH could be changed considerably in physiological conditions. Proton translocation coupled with electron transport itself cannot change pH extensively, because a small amount of translocated protons results in high increase of electric potential, which stops the process. However, if proton translocation is coupled with an influx of some cations, such as Ca^{2+} or K^+, it could result in a considerable alkalization of matrix. K^+ permeability is regulated physiologically (41), and Fig. 6 illustrates that the modulation of K^+ permeability using valinomycin increments ROS production. Mitochondria are also permeable for Ca^{2+}, and it is possible that Ca^{2+} overload of cells and, consequently, mitochondria could take place under some stress conditions. Although proton leak normally is compensated by respiratory proton translocation, it could set a dynamic steady state with matrix less alkaline. Application of uncoupler FCCP shown in Fig. 6 modulates this situation. In cells, proton leak could be modulated by uncoupling proteins (20–25). The activation of UCPs can down-regulate the level of ROS by controlling the current of protons into the matrix without considerable compromise of mitochondrial ATP production.

To demonstrate the phenomenon of pH regulation of ROS production by the respiratory chain, mitochondria were studied in the conditions of artificially high succinate levels. In this case, they produce ROS with a higher rate than when respiring on physiological substrates glutamate and malate (although with similar pH dependence, as Figs. 3*A* and 7*A* show). pH, evidently, is not the only factor defining ROS production rate. If the levels of ubiquinol are high and, according to mass conservation, the levels of ubiquinone are low, the lack of electron acceptor ubiquinone results in a block of electron efflux from cytochrome b_h and, subsequently, b_l (see Fig. 10). In this situation, the levels of cytosolic side semiquinone radical and, consequently, the rate of ROS production must be high. Oxidation of succinate, available in excess, produces much higher levels of ubiquinol than would be produced by complex I, since ubiquinone reduction by respiratory complex II is not related with proton translocation (as in complex I) and is not restricted by the proton electrochemical gradient. We suggest that this is the main reason why mitochondria, when respiring on succinate, produce much more ROS than when they respire on complex I substrates.

It should be noted that the increase of matrix pH stabilizes not only matrix side but also cytosolic side semiquinone. The stabilization of matrix side semiquinone slows down the electron transport in the chain upstream, thus increasing the lifetime of semiquinone radical bound to cytosolic side. This rationale is in accordance with the finding that complex III of mitochondria respiring on succinate produces substantial amounts of ROS on the cytosolic side of the membrane (42, 43).

The whole set of experiments presented here supports the proposed pH-dependent mechanism of ROS generation in mitochondrial respiratory chain and its link with mitochondrial permeability transition. It allows us to understand the mechanism by which UCPs down-regulate the level of ROS in cells. The specific properties and quantitative data featuring mitochondrial ROS generation could be a basis for a more detailed study aimed at exploration of the mechanism of ROS production.

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