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EFFICIENCY OF PROTON EXTRUSION BY CHEMICALLY MODIFIED MITOCHONDRIA

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Bovine heart mitochondria were treated with limited amounts of iodoacetamide, 1-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline, phenylglyoxal, tetranitromethane and 1-fluoro-2,4-dinitrobenzene, respectively. Examination of the respiration and proton extrusion characteristics of the chemically modified mitochondria suggests that sulfhydryl and imidazole groups are not directly involved in proton pumping, but that some of the labeled carboxyl, amino, guanidinium and phenolic groups may participate in an indirect proton-extrusion process. Cross-linking mitochondria with glutaraldehyde drastically decreases the efficiency of proton extrusion, whereas treatment of mitochondria with valeraldehyde under similar conditions did not affect the proton-pumping efficiency significantly. The latter observations show that conformational change in the inner mitochondrial membrane may play a crucial role in the active translocation of protons coupled to electron transport. Comparison of the reactivities of the essential amino and carboxyl groups in mitochondria in different oxidation states suggests that these two types of essential functional groups are more exposed to water in the oxidized state. An indirect mechanism for proton pumping based on protein conformational change driven by electron transport based on the results of the present chemical modification studies is suggested.

Introduction

The active extrusion of protons from respiring mitochondria has been explained in terms of postulated redox loops which require a constant proton-to-oxygen atom ratio (H^+/O) of 2 per coupling site [1,2]. However, in view of the recent extensive studies on the true value of the H^+/O ratio [3,4], the observation of the proton-pumping properties of cytochrome *c* oxidase [5] and the change in H^+/O ratio caused by chemical reagents [6,7], there seems to be the need of an additional mechanism for proton pumping coupled to electron transport. A simple electrogenic mechanism can be ruled out, because the removal of membrane potential by the addition of valinomycin and K^+ accelerates rather than inhibits the active extrusion of protons from respiring mitochondria. On the other hand, the observed redox Bohr effects in isolated cytochrome b-c_1 complex and cytochrome *c* oxidase [8] suggest that the often mentioned indirect proton-pumping mechanism based on protein conformational change triggered by the oxidation and reduction of electron carriers may indeed deserve serious consideration. In order to operate effectively, such a proton pump requires both functional groups which change their pK_a upon oxidation and a built-in control mechanism which prevents these

Abbreviations: EEDQ, 1-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline; δ_{ss}, nmol H^+ extruded/mg mitochondrial protein; FDNB, 1-fluoro-2,4-dinitrobenzene; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; k_1, first-order rate constant for the backward leakage of extruded protons during respiration.

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groups from picking up or releasing protons from the same side of the membrane when the corresponding electron carrier is reduced.

In an attempt to gain some insight into the nature of the mitochondrial functional groups which participate in this additional proton-pumping mechanism, the oxidation-reduction and proton extrusion characteristics of chemically modified mitochondria have been examined in this work. Inasmuch as electron transport is the primary driving force, any chemical reaction which retards electron transport automatically also decreases the rate of proton extrusion, regardless of the pumping mechanism. However, the effect of chemical modification on the H^+/O ratio, which measures the efficiency of proton extrusion, will depend on the pumping mechanism. For the directly coupled proton pumping by redox loops, chemical modification should not decrease the H^+/O ratio. But those labeling reagents which affect the essential functional groups for proton pumping by the indirect mechanism may decrease the rate of proton extrusion more than the rate of electron transport, and consequently may lower the overall H^+/O ratio. With measured values of the H^+/O ratio as a guide, the present experimental data suggest that the list of functional groups which participate in indirectly coupled proton pumping may include the carboxyl, phenolic, amino and possibly guanidinium groups.

Experimental Procedures

Materials. Heavy bovine heart mitochondria were prepared from fresh beef heart as described previously [9]. The concentration of endogenous P_i in these mitichondria was very low and that the observed H^+/O ratio was found to be insensitive to the addition of *N*-ethylmaleimide. Consequently, in the subsequent experiments for labeling mitochondria with other reagents, *N*-ethylmaleimide was omitted. The chemicals used were all of the reagent grade.

Measurement of H^+/O ratio. The rate of oxygen consumption was monitored with an oxygen electrode in a thermostatically controlled and constantly stirred Gilson glass cell which was completely filled with 1.67 ml of a diluted suspension of mitochondria in the assay medium. The pH change in the mixture was monitored with a combination pH electrode (Beckman 39030) fitted to the top of the glass cell. The assay medium contained 0.4 M sucrose, 50 mM KCl, 1.5 mM Tris-HCl (pH 7.4) 1 mM $MgCl_2$, 4.4 μM rotenone, 4–8 mg mitochondrial protein/1.67 ml and 100 ng valinomycin/mg protein. The outputs from the oxygen and pH electrodes were recorded simultaneously by a two-channel strip-chart recorder. The oxygen electrode was calibrated by injecting a measured volume of oxygen-saturated water into the cell filled with anaerobic buffer. The pH electrode was calibrated by injecting a measured volume of standard HCl solution into the mitochondrial suspension after each experiment.

The experimental traces for oxygen consumption and proton extrusion in a typical measurement are shown in Fig. 1A. It may be noticed that the initial time lag in the response of the oxygen electrode is much longer than that of the pH electrode. Upon the injection of succinate substrate through a 2-mm port in the Teflon sleeve of the pH electrode, the pH of the medium decreased rapidly due to proton extrusion from respiring mitochondria, but it soon reached a steady-state value because of the backward diffusion of ex-

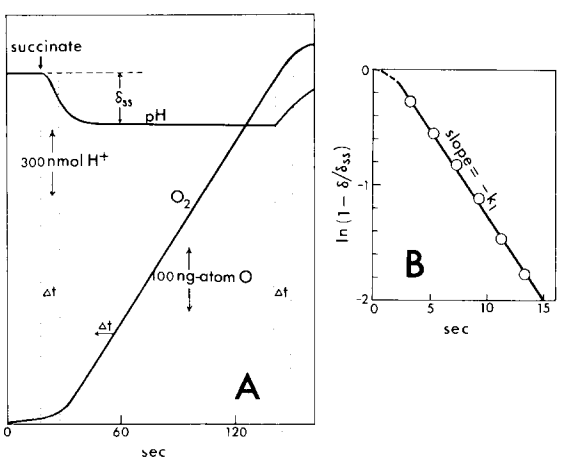

Fig. 1. Time dependence of medium pH and oxygen concentration in a typical measurement of proton extrusion and oxygen consumption by mitochondria. The details of the emasurements are shown in A and the calculation of proton-pumping rate from the linear plot in B are described in Experimental Procedures. Because of the slower response of the oxygen electrode, the O_2 trace should be shifted to the left by Δt before comparison with the pH trace.

truded protons. After a longer time lag ($\Delta t \approx 9$ s) following the injection of excess succinate (4.5 mM in the assay mixture), the oxygen concentration as indicated by the recorder was observed to decrease (in the upward direction in Fig. 1A). When the dissolved oxygen was depleted, the extrusion of protons stopped abruptly and the pH of the medium gradually rose again because of the backward diffusion of extruded protons.

The H^+/O ratio was obtained by dividing the steady-state rate of proton extrusion by the steady-state rate of oxygen consumption. The steady-state rate of oxygen consumption was obtained directly from the linear portion of the oxygen concentration trace. Because of the backward diffusion of extruded protons, the steady-state rate of proton extrusion could not be measured directly with precision, but was determined by a kinetic method described previously for chloroplasts [10]. As shown in Fig. 1B, a plot of $\ln(1 - \delta/\delta_{ss})$ against time usually gives a straight line represented by $\ln(1 - \delta/\delta_{ss}) = -k_1 t$, where δ and δ_{ss} denote nmol H^+ extruded/mg protein at time t and at the steady state, respectively, and k_1 represents the first-order rate constant for the backward leakage of extruded protons during respiration. It has been shown that k_1 differs from the first-order rate constants of the biphasic decay of the proton gradient after anaerobiosis [11]. Differentiating the equation with respect to t gives $d\delta/dt = k_1(\delta_{ss} - \delta)$. Since $\delta = 0$ at $t = 0$, we obtain the steady-state rate of proton extrusion as $R_0 = k_1\delta_{ss}$. Because k_1 and δ_{ss} can be measured directly from Fig. 1A and B, we can determine R_0 precisely in this way without the relatively large errors often introduced in the graphical determination of initial slopes.

Using succinate as the substrate, the observed H^+/O ratio of unmodified mitochondria from different preparations, which had been stored from 6 to 8 h at 0°C as control samples in the chemical modification experiments, ranged from 5.5 to 7.8.

Chemical modification of mitochondria. Mitochondria at a concentration of 60–80 mg/ml suspended in 0.25 M sucrose + 10 mM Hepes buffer at pH 7.1 were incubated with various labeling reagents at 0°C for 6–8 h. In each case, the labeling reagent was the limiting reactant and was essentially depleted at the end of the incubation period. Because of the high concentration of mitochondrial proteins and endogenous substrates, the mitochondria remained essentially in the reduced state throughout the incubation period. In the few experiments with oxidized mitochondria listed in Table I, 5 ml of mitochondria were put in thin dialysis tubing and dialyzed at 3°C for 5 h against 1500 ml of rapidly stirred, air-saturated buffer containing 0.44 M sucrose, 50 mM KCl, 125 ng valinomycin/mg mitochondrial protein and 10 mM Hepes at pH 7.1. After dialysis, the oxidized mitochondria sample was divided to 0.5 ml aliquots and incubated with various labeling reagents at 0°C for 6–8 h and assayed by the same procedures.

Results

Labeling with iodoacetamide and N-ethylmaleimide

At pH 7.1, iodoacetamide labels both sulfhydryl

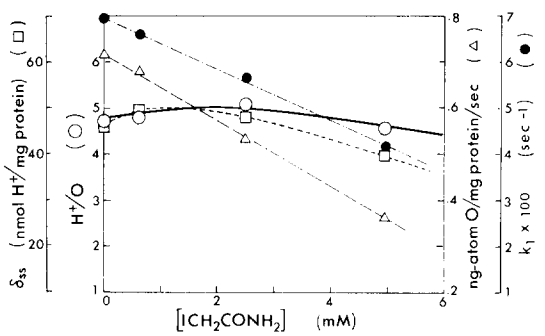

Fig. 2. Respiration and proton extrusion by mitochondria modified with iodoacetamide. Composition of each incubation mixture: mitochondrial protein, 75.5 mg/ml; sucrose, 0.25 M; Hepes, 10 mM at pH 7.1; ICH_2CONH_2, as indicated in the figure. After each mixture was incubated for 6–8 h at 0°C, an aliquot containing 3.78 mg protein was injected into 1.67 ml of the assay medium at 25°C for respiration and proton-extrusion measurements. Iodoacetamide was added to each incubation mixture (0.5 ml) as 1–6 μl of a stock solution of the reagent in methanol. Control mixtures with an equivalent amount of methanol but no iodoacetamide were also incubated at 0°C and assayed. It was found that up to 6 μl methanol/0.5 ml aqueous suspension, mitochondria were unaffected by the solvent, and that the respiration and proton-extrusion characteristics of the control mitochondria samples remained essentially unchanged throughout each series of measurements. The measurement of respiration rate, H^+/O ratio, δ_{ss} and k_1 are described in Experimental Procedures. Since even in the incubation mixture containing the highest concentration of iodoacetamide (5 mM) mitochondrial protein was present in great excess, the labeling reagent was depleted at the end of the reaction period.

and imidazole groups. The data in Fig. 2 show that limited labeling of mitochondria by iodoacetamide decreases both the rate of oxygen reduction and the rate of backward diffusion of extruded protons, but has little effect on the H^+/O ratio which can be used as a measure of the overall efficiency of proton extrusion coupled to electron transport. Similar results were obtained with *N*-ethylmaleimide as the labeling reagent. These results suggest that the labeled sulfhydryl and imidazole groups are not directly involved in any indirect proton-pumping mechanism coupled to electron transport. This conclusion is also consistent with the observation by Reynafarje et al. [3] that labeling mitochondria by *N*-ethylmaleimide did not change the H^+/O ratio measured in the absence of P_i.

Labeling with EEDQ

The effect of labeling mitochondria with limited amounts of the carboxylic acid reagent EEDQ is summarized in Fig. 3. The reaction with 5 mM EEDQ lowers the respiration rate by a factor of 2, but lowers the proton-pumping rate by a factor of 5, so that the pumping efficiency as measured by the H^+/O ratio was decreased by a factor of 2.5. This observed decrease in H^+/O ratio was not due to an artifact caused by possible uncoupling agents produced in the EEDQ reaction, because the backward leakage constant k_1 was also found to decrease with increasing EEDQ concentration. These results strongly suggest that certain mitochondrial carboxyl groups participate in this conformational pumping mechanism. The suggestion is also consistent with the observation that the proton-pumping activity of cytochrome *c* oxidase was inhibited by dicyclohexylcarbodiimide [12,13]. On the other hand, these data are also compatible with the postulated coexistence of a redox loop mechanism, since the observed H^+/O ratio did not drop to zero.

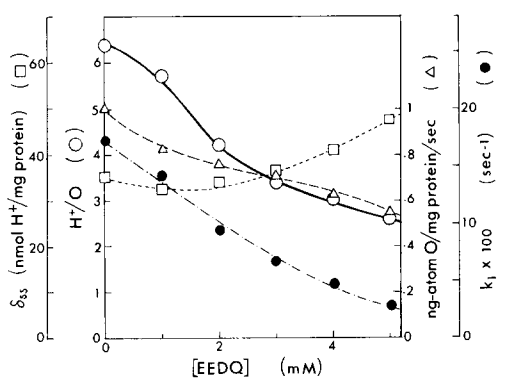

Fig. 3. Respiration and proton extrusion by mitochondria modified with EEDQ. Composition of each incubation mixture: mitochondrial protein, 69 mg/ml; sucrose, 0.25 M; Hepes, 10 mM at pH 7.1; EEDQ, as indicated in the diagram. The procedure for measuring respiration and proton extrusion is the same as that for Fig. 2.

Labeling with phenylglyoxal

Phenylglyoxal is known to react with guanidinium groups and to a lesser extent also with amino groups. The effect of labeling mitochondria with limited amounts of phenylglyoxal is summarized in Fig. 4. Although the reaction with 6 mM phenylglyoxal lowers the respiration rate only slightly, the H^+/O ratio was decreased almost by a factor of 2. This observation suggests that guanidinium groups may also be involved in proton pumping. Complication due to possible uncoupling agents produced by the phenylglyoxal reaction can again be ruled out by the observed rapid decrease in k_1 with increasing phenylglyoxal concentration.

Reaction with tetranitromethane

Fig. 5 shows that nitration of the tyrosine residues of mitochondria with limited amounts of tetranitromethane, $C(NO_2)_4$, caused a drastic decrease in both the H^+/O ratio and the backward leakage constant k_1. The experimental results

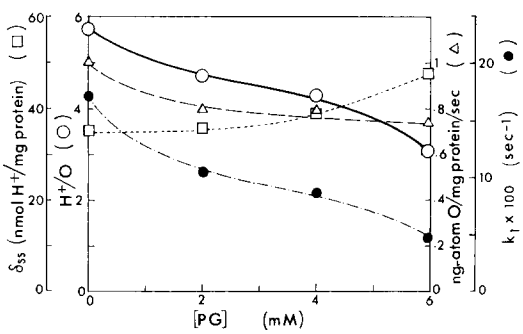

Fig. 4. Respiration and proton extrusion by mitochondria modified with phenylglyoxal. Composition of incubation mixture: mitochondrial protein, 69 mg/ml; sucrose, 0.25 M; Hepes, 10 mM at pH 7.1. The assay procedure is the same as that for Fig. 2.

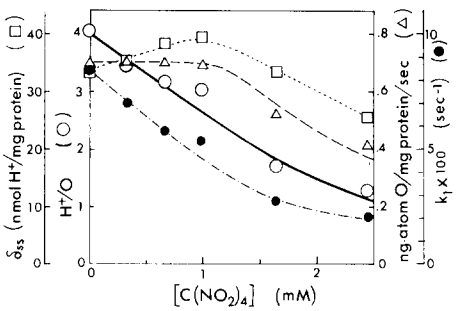

Fig. 5. Respiration and proton extrusion by mitochondria modified with tetranitromethane. Composition of the incubation mixture: mitochondrial protein, 70 mg/ml; sucrose, 0.25 M, Hepes, 10 mM at pH 7.1. The assay procedure is the same as that for Fig. 2.

strongly suggest the participation of phenolic groups in proton pumping coupled to electron transport.

Labeling with FDNB

At pH 7.1, FDNB may react with amino, sulfhydryl and imidazole groups. The data in Fig. 6 show that labeling mitochondria with limited amounts of FDNB has only a minor effect on respiration rate but decreases drastically the H^+/O ratio. Since the data in Fig. 2 show that sulfhydryl and imidazole groups are not involved in indirect proton pumping, Fig. 6 suggests that certain amino groups probably participate in indirect proton pumping.

Reaction with glutaraldehyde and valeraldehyde

Cross-linking of mitochondrial proteins with glutaraldehyde hinders conformational changes in the energy-transducing membrane and hence may be expected to inhibit both the rate of electron transport and the efficiency of proton pumping. As expected, Fig. 7 shows that reaction of mitochondria with limited amounts of glutaraldehyde effectively reduces both the leakage constant k_1 and the H^+/O ratio. Since the parallel experiments with valeraldehyde produced little effect on either the leakage constant or the H^+/O ratio, we must conclude that the large decrease in proton-extrusion efficiency caused by glutaraldehyde is essentially due to the cross-linking reaction, not due to the conversion of a small number of mitochondrial amino groups to the Schiff base.

Dependence of reactivity on the oxidation state of mitochondria

The oxidation- or reduction-triggered conformational change in certain electron-carriers could alter the pK_a as well as the reactivity of some of their function groups which are involved in the proton-pumping mechanism. In order to explore this possibility, mitochondria in a higher oxidized

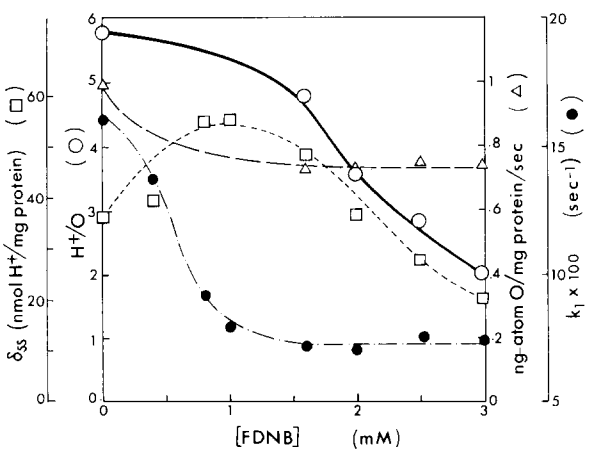

Fig. 6. Respiration and proton extrusion by mitochondria modified with FDNB. Composition of the incubation mixture: mitochondrial protein, 70 mg/ml; sucrose, 0.25 M; Hepes, 10 mM at pH 7.1. The assay procedure is the same as that for Fig. 2.

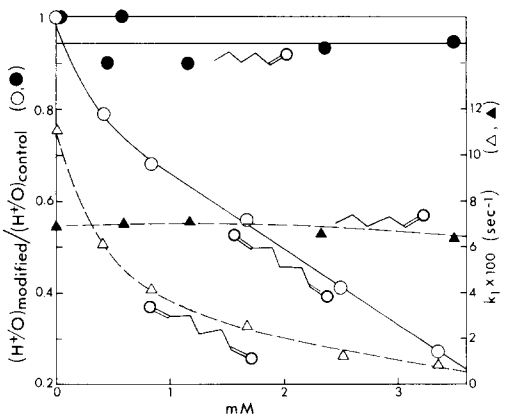

Fig. 7. Relative H^+/O ratio and leakage constant k_1 of mitochondria modified with glutaraldehyde and valeraldehyde. Composition of the incubation mixture: mitochondrial protein, 78 mg/ml; sucrose, 0.25 M; Hepes, 10 mM at pH 7.1. The assay procedure is the same as that for Fig. 2. (○, △) Glutaraldehyde-modified mitochondria; (●, ▲) for valeraldehyde-modified mitochondria.

TABLE I

DEPENDENCE OF CHEMICAL MODIFICATION ON THE OXIDATION STATE OF MITOCHONDRIA

The labeling and assay procedures were identical with those for the experiments in Figs. 1–6, as described in Experimental Procedures. δ_{ss}: number of nmol H^+ extruded/mg protein at the steady state. k_1: the first-order rate constant for the backward leakage of extruded protons during respiration. (a) Protein concentration in each incubation mixture, 64 mg/ml; 100-μl aliquots of the incubated mixtures were diluted to 1.67 ml with assay medium for the respiration and proton extrusion measurements. (b) Protein concentration in each incubation mixture, 75 mg/ml; 100 μl aliquots of the incubated mixtures were used for the assay.

Reagent and concentration	R_0 (nmol H^+/ mg protein per s)	Respiration rate (ng atom O/mg protein per s)	H^+/O (mol/g-atom)	δ_{ss} (nmol H^+/ mg protein)	k_1 (s^{-1})
(a) Mitochondria modified in reduced state					
Control	4.96	0.661	7.51	33.3	0.149
FDNB					
1.0 mM	2.64	0.445	5.92	51.7	0.051
2.0 mM	1.36	0.452	3.02	29.5	0.046
EEDQ					
2.0 mM	2.29	0.431	5.31	34.0	0.0673
4.0 mM	1.38	0.403	3.42	49.4	0.0279
(b) Mitochondria modified in oxidized state					
Control	2.39	0.556	4.30	35.5	0.0674
FDNB					
1.0 mM	1.32	0.319	4.14	29.7	0.0445
2.0 mM	0.96	0.249	3.83	23.5	0.0407
EEDQ					
2.0 mM	1.28	0.415	3.09	42.8	0.0299
4.0 mM	0.84	0.280	2.98	56.2	0.0149

state, prepared by dialysis as described in Experimental Procedures, were allowed to react with limited amounts of EEDQ and FDNB, respectively. The resulting samples of chemically modified mitochondria were assayed for respiration and proton extrusion and compared with the results on similar samples of mitochondria which had been labeled with EEDQ and FDNB, respectively, under highly reduced state. The experimental data are summarized in Table I.

Compared to the respective control samples, both EEDQ and FDNB cause a smaller percentage decrease of H^+/O ratio in the oxidized than in the reduced mitochondria. These results indicate that the participating carboxyl and amino groups are more reactive in reduced mitochondria than in oxidized mitochondria.

Discussion

Because of their hydrophobic nature, the reagents EEDQ, phenylglyoxal, FDNB and $C(NO_2)_4$ can penetrate the inner mitochondrial membrane readily. The observed decrease in H^+/O ratio by several chemical reagents suggests that in addition to redox loops mitochondria may also have an indirect molecular mechanism for pumping protons. A possible indirect proton-pumping mechanism consistent with the experimental data reported in this work is illustrated symbolically in

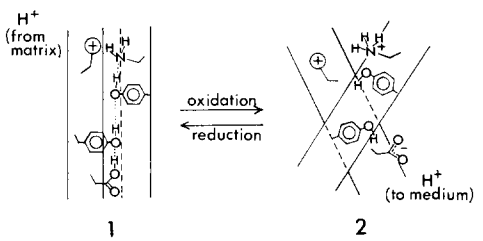

Fig. 8. A proposed conformational change mechanism for proton pumping coupled to electron transport as suggested by chemical modification studies. The unidentified positively charged group denotes the functional group of either a lysine or arginine residue.

Fig. 8. In this proposed model each energy-transducing electron carrier is assumed to have one or more membrane-imbedded proton-pumping assemblies, and that each proton-pumping assembly consists of two adjacent helical polypeptide segments represented by the cylinders in Fig. 8. One polypeptide segment has the functional amino group, which can pick up proton from the matrix, as well as one or more functional tyrosine residues. An adjacent segment has a positively charged functional group of either arginine or another lysine residue as well as one or more functional tyrosine residues. One of the segments also has the carboxyl group which is responsible for releasing proton at the cytoplasm or medium side of the membrane.

In the reduces state of the electron carrier, these polypeptide segments in each assembly are assumed to be in intimate contact as illustrated by structure 1 in Fig. 8, such that the functional amino group is unprotonated because of the nearby positively charged group, the functional carboxyl group is protonated because it is buried in a hydrophobic environment, and the participating tyrosine phenolic groups of the adjacent helical segments form a hydrogen-bonded proton-relay chain which is stabilized by the protein conformation. Upon oxidation, the assembly undergoes conformational change leading to a state represented by structure 2 in which the hydrogen-bonded proton-relay chain is disrupted, the amino group recovers its normal basic strength because of its separation from the neighboring positively charged group and thereby regains a proton from the matrix side, and the carboxylic acid group regains its normal acid strength because of its exposure to water and thereby dissociates a proton at the cytoplasm or medium side. When the electron carrier is again reduced, the assembly changes back to the conformation illustrated by structure 1 with rapid proton transfer from the protonated amino group to the carboxyl group along the hydrogen-bonded proton-relay chain [14–18]. In this way, the system will have completed a cyclic process with the net translocation of one proton per assembly from the matrix side to the cytoplasm side of the inner membrane. Recent studies indicate that there may be a sufficient number of properly located tyrosine residues to form such a hydrogen-bonded proton-relay chain in both subunit III of cytochrome *c* oxidase [19] and bacteriorhodopsin [20].

The rationale for assigning structure 1 to the reduced state and structure 2 to the oxidized state may be summarized as follows. Both FDNB and EEDQ are sufficiently hydrophobic to penetrate the inner mitochondrial membrane readily. But, since FDNB reacts more rapidly with the basic form of the amino group and EEDQ reacts faster with the protonated form of the carboxyl group [21], the observed higher reactivity of these functional groups in reduced mitochondria suggests that structure 2 represents the proton-pumping assembly when the electron carrier is in the oxidized state, and that structure 1 represents the assembly when the electron carrier is in the reduced state.

The nitration of anyone of the participating phenolic groups decreases the affinity of the corresponding phenolate ion for proton, and hence is expected to introduce a potential barrier in the proton-relay chain. Cross-linking with glutaraldehyde hinders the oxidation- or reduction-triggered conformational change which plays a crucial role in the active translocation of protons in the proposed model, and hence is also expected to decrease the H^+/O ratio as observed.

Acknowledgements

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References

1 Mitchell, P. (1961) Nature 191, 144–148
2 Mitchell, P. (1977) Annu. Rev. Biochem. 46, 996–1005
3 Reynafarje, B., Brand, M.D. and Lehninger, A.L. (1976) J. Biol. Chem. 251, 7442–7451
4 Papa, S., Capuano, C., Markert, M. and Altamura, N. (1980) FEBS Lett. 111, 243–248
5 Krab, K. and Wikström, M. (1978) Biochim. Biophys. Acta 504, 200–214
6 Tu, S.-I., Lam, E., Ramirez, F. and Marecek, J.F. (1981) Eur. J. Biochem. 113, 391–396
7 Ho, Y.-K. and Wang, J.H. (1981) J. Biol. Chem. 256, 2611–2614
8 Guerrieri, F., Izzo, G., Maida, I. and Papa, S. (1981) FEBS Lett. 125, 261–265

9 Chen, C.C., Yang, M.C., Durst, H.D., Saunders, D.R. and Wang, J.H. (1975) Biochemistry 14, 4122–4126
10 Ho, Y.-K., Liu, C.J., Saunders, D.R. and Wang, J.H. (1979) Biochim. Biophys. Acta 547, 149–160
11 Ho, Y.-K. and Wang, J.H. (1981) J. Biol. Chem. 256, 2611–2614
12 Casey, R.P., Thelen, M. and Azzi, A. (1979) Biochem. Biophys. Res. Commun. 87, 1044–1051
13 Casey, R.P., Thelen, M. and Azzi, A. (1980) J. Biol. Chem. 255, 39-4-4000
14 Eigen, M. and DeMayer, L. (1958) Proc. R. Soc. A247, 505–533
15 Wang, J.H. (1970) Proc. Natl. Acad. Sci. U.S.A. 66, 874–881
16 Nagle, J.F., Mille, M. and Morowitz, H.J. (1980) J. Chem. Phys. 72, 3959–3971
17 Nagle, J.F. and Mille, M. (1981) J. Chem. Phys. 74, 1367–1372
18 Papa, S. (1982) J. Bioenerg. Biomembranes 14, 69–86
19 Azzi, A. (1980) Biochim. Biophys. Acta 594, 231–252
20 Engelmann, D.M., Henderson, R., McLachlan, A.D. and Wallace, B.A. (1980) Proc. Natl. Acad. Sci. U.S.A. 77, 2023–2027
21 Pougeois, R., Satre, M. and Vignais, P.V. (1978) Biochemistry 17, 3018–3023