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EFFECTS OF MONOVALENT CATIONS ON THE ACTIVITIES ASSOCIATED WITH COUPLING SITE III OF RAT LIVER MITOCHONDRIA

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SUMMARY

The effects of substitution of K^+ by Li^+, Na^+, or Rb^+ in the assay medium on the processes of electron transfer and H^+ translocation associated with Site III are investigated. The replacement of K^+ with Rb^+ has little effect, if any, on the measured initial rates of H^+ extrusion and electron transfer. The substitution of K^+ by Li^+ increases the initial rate of both processes simultaneously while the replacement of K^+ by Na^+ causes an enhancement on the rate of electron transfer with concomitant inhibition of the observed acidification. The presence of either Na^+ or Li^+ decreases the proton-leak rate of the inner membrane. These results suggest that the link between electron transfer and H^+ translocation in Site III is weakened by the presence of Na^+.

Wikström and coworkers (1,2) first presented evidence for the existence of an intrinsic proton pump in cytochrome oxidase (coupling Site III) in mitochondria and sub-mitochondrial particles. Their findings were subsequently supported by the observations of Artzabanov, et al (3). Casey, et al (4) also showed that in the reconstituted cytochrome oxidase system, the observed acidification can be demonstrated repeatedly by using cytochrome c pulses. On the other hand, Moyle and Mitchell (5) and Lorusso, et al (6) contend that the observed transient proton efflux during active electron transfer at Coupling Site III is an artefact of the assay conditions rather than an intrinsic property of cytochrome oxidase. However, this criticism is unlikely to be valid as shown by the limited turnover studies on proton translocation in reconstituted cytochrome oxidase (4).

We have reported that the acidification process and the electron transfer process associated with Coupling Site III can be differentially inhibited by fluorescamine modification (7). This observation, correlated with the data of

Abbreviations used: EGTA, ethyleneglycol-bis-(β-aminoethyl ether) N,N'-tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonate; NEM, N-ethylmaleimide.

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Casey, et al (8), indicates that the proton translocation process is only linked indirectly to the electron transfer reactions associated with Coupling Site III. In the present report, the effects of monovalent cations in the assay medium on the coupled activities of Site III are described.

MATERIALS AND METHODS

Rat liver mitochondria were isolated from male rats weighing from 150 to 200 grams, as described previously (7), in 0.25 M sucrose containing 3 mM Hepes at pH 7.4. Mitochondria were incubated for about 1 minute at 22 °C with stirring before the additions of 40 nmol NEM/mg of protein, 1 μg oligomycin/mg, 100 ng valinomycin per mg of protein, 3 μM rotenone, 1 mM EGTA, and 0.45 μM antimycin A. The final volume of the suspension was about 2.2 mL. The suspension was further incubated for 2 minutes at the same temperature. The reaction was started by the addition of 8 μL of 0.21 M of potassium ferrocyanide. The oxygen consumption and the concomitant proton extrusion were measured simultaneously, as described previously by others (9), in a modified Gilson Medical oxygen chamber. The proton movement was monitored by a Markson J-445 combined electrode connected to a Corning 112 pH meter. The changes in proton concentration were recorded by a Varian A-25 recorder. The buffering capacity of the system was determined by the addition of a known quantity of standardized HCl. The concomitant oxygen consumption was followed by a Clarkson oxygen electrode connected to a Yellow Spring Model 53 Oxygen Monitor. The electron transfer rate measured by ferricyanide production was determined in a seperate but identically treated mitochondrial suspension, by an Aminco DW-2 spectrophotometer using the wavelength couple of 420-500 nm. The calibration procedure for the dual wavelength measurement was the same as that described by Lorusso, et al (6).

Valinomycin, oligomycin, Hepes, and EGTA were obtained from Sigma Co. NEM (Gold Label) was purchased from Aldrich Chemical. Antimycin A was from Calbiochem. All other reagents used were of analytical grade.

RESULTS

Effects of Substitution of K^+ by Other Monovalent Cations on Coupled Activities of Site III. Figure 1 shows the effects of substituting K^+ in the assay medium by Li^+, Rb^+, or Na^+ on the coupled activities of Site III under the conditions of constant ionic strength and nullified membrane potential. It is apparent that a) the replacement of K^+ by Rb^+ does not cause any significant change in the rates of coupled processes, b) the substitution of K^+ by Li^+ increases the rates of electron transfer and proton extrusion simultaneously, and c) the replacement of K^+ by Na^+ increases the rate of electron transfer but abolishes the coupled acidification.

Figure 2 shows the results of substitution of K^+ by Li^+ from a more detailed study. Clearly, the initial rates of both proton extrusion and electron transfer (measured either by ferrocyanide-oxidation or oxygen consumption) increase almost

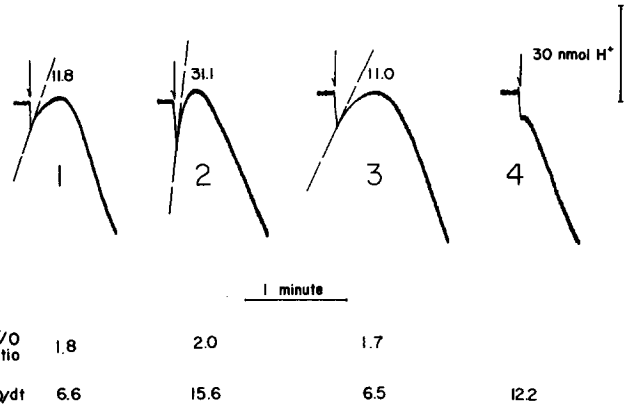

Figure 1. EFFECTS OF MONOVALENT CATIONS ON COUPLED ACTIVITIES ASSOCIATED WITH COUPLING SITE III. Mitochondria (5.5 mg) were added to assaying media containing 1 mM Hepes, pH 7.3, and various combinations of monovalent cations: 1 - 150 mM KCl; 2 - 10 mM KCl, 140 mM LiCl; 3 - 10 mM KCl, 140 mM RbCl; and 4 - 10 mM KCl, 140 mM NaCl. Coupled activities (e. g. medium acidification and the concomitant oxygen consumption) are measured as described in Materials and Methods. The numbers beside the broken lines correspond to the initial slope of the particular trace and represent the initial acidification rate in nmol H^+/(min. mg). The H^+/O ratios are obtained by dividing the corresponding initial oxygen consumption rate in ng atom O/(min.mg) into the particular initial acidification rate. The initial oxygen consumption rate of the traces (dO/dt) are in ng atom O/(min.mg). Arrows (↓) indicate the addition of ferrocyanide.

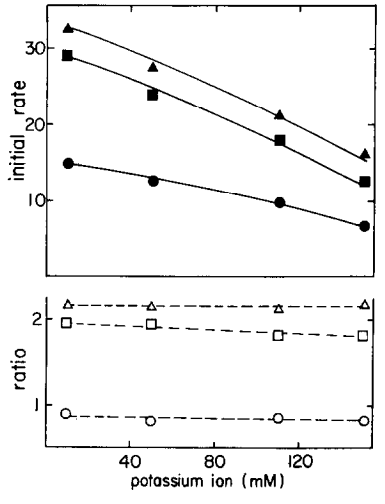

Figure 2. EFFECTS OF Li^+ SUBSTITUTION OF K^+ ON THE COUPLED EVENTS ASSOCIATED WITH COUPLING SITE III. Mitochondria (5.0 mg) were added to assaying media containing 1 mM Hepes, pH 7.3, and various combinations of KCl and LiCl (total K^+ and Li^+ concentration maintained as 150 mM). Assay conditions and procedures are as described in Materials and Methods. ▲▲▲, ■■■, and ●●● represent the initial rates of ferricyanide production (nmol ferricyanide/(min. mg)), proton extrusion (nmol H^+/(min.mg)), and oxygen consumption (ng atom O/(min.mg)), respectively. The ratios of ferricyanide/O, H^+/O, and H^+/ ferricyanide are shown by △△△, □□□, and ○○○, respectively.

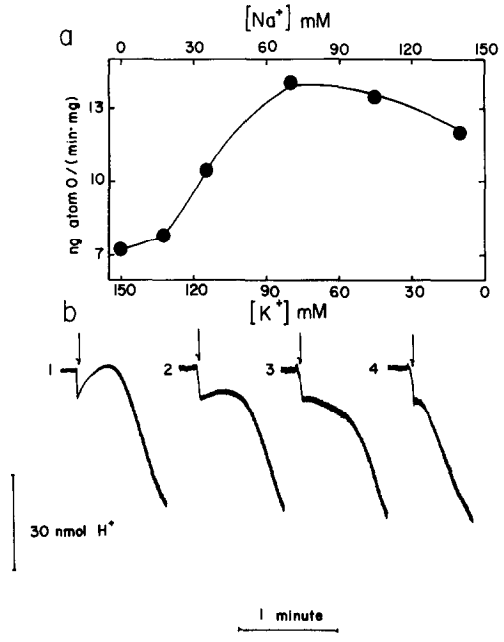

Figure 3. EFFECTS OF VARIOUS RATIOS OF Na^+ AND K^+ ON COUPLED ACTIVITIES OF COUPLING SITE III. Mitochondria (5.7 mg) were assayed essentially as in Figure 1. In a, the effect of varying the ratio of Na^+/K^+ in the medium on the rate of oxygen consumption is shown. In b, some representative traces of the concomitant proton movements are shown: 1 - 150 mM KCl; 2 - 132.5 mM KCl and 17.5 mM NaCl; 3 - 115 mM KCl and 35 mM NaCl; 4 - 10 mM KCl and 140 mM NaCl. Arrows (↓) indicate the addition of ferrocyanide.

linearly as the K^+ in the assay medium is replaced by Li^+. However, the H^+/e^- ratio determined from initial rates remains constant.

Figure 3 shows the effects of replacing K^+ by Na^+ on the coupled activities of Site III. As shown, the initial proton extrusion is completely abolished when about 35 mM of K^+ is substituted by Na^+ in the assay medium. The rate of electron transfer increases to a maximal level when about 70 mM K^+ is replaced by Na^+.

Effects of Substitution of K^+ by Monovalent Cations on the Proton-leak Rate of the Mitochondrial Inner Membrane. The inhibitory effects of Na^+ on proton efflux may be attributed to an increased membrane proton permeability. However, this possibility is eliminated by the study shown in Figure 4. Uncouplers are known to enhance the electron transfer rate by increasing the permeability of the membrane to protons (10). Using the oxygen pulse technique (7) we found that the proton-leak rate of mitochondrial membrane was decreased rather than increased by replacing K^+ with

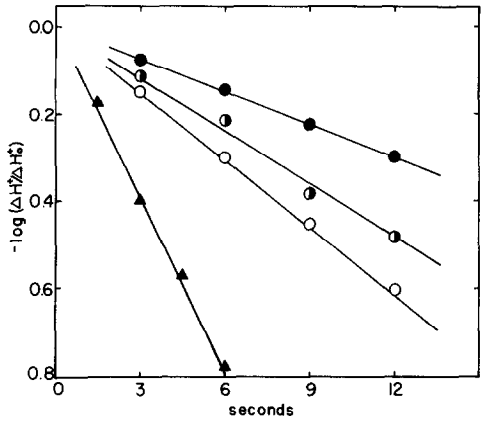

Figure 4. EFFECT OF MONOVALENT CATIONS ON PROTON-LEAKAGE OF THE MITOCHONDRIAL MEMBRANE. The proton-leakage of the mitochondrial membrane is determined as described previously in (7). The assay conditions are essentially the same as described in Figure 1 except that Antimycin A is omitted and succinate (1 mM final concentration) is used as substrate instead of ferrocyanide. The kinetics of H^+ uptake by mitochondria when the suspension becomes anaerobic again was analyzed by the first order rate law. $\Delta H^+/\Delta H^+_o$ represents the ratio of extruded protons remaining after a given time interval to that at the time when proton uptake was first noted. The monovalent cation(s) present in ▲▲▲, ○○○, ◐◐◐, and ●●● are 150 mM KCl; 10 mM KCl + 140 mM NaCl; 10 mM KCl + 70 mM LiCl + 70 mM NaCl; and 10 mM KCl + 140 mM LiCl; respectively. The apparent 1st-order rate constant for mitochondria suspended in these media are 0.13, 0.05, 0.04 and 0.027 sec^{-1}, respectively.

either Na^+ or Li^+. Figure 4 also shows that Li^+ is more effective than Na^+ in inhibiting the proton-leak of the membrane.

Effects of Substitution of Li^+ by Na^+ under A Constant Concentration of K^+.

Since Li^+ and Na^+ have opposite effects on the extrusion of protons, it is of interest to examine if the presence of Li^+ can reverse the effect of Na^+. The effects on the coupled activities by varing the ratio of Li^+ to Na^+ (total as 140 mM) in the medium also containing 10 mM K^+, were investigated. As shown in Figure 5, the initial acidification is preserved even when 105 mM of Li^+ is replaced by Na^+ in the medium. This observation is in a strong contrast to the case in which only K^+ and Na^+ are present in the assay medium (i.e. Figure 3). In that case, the proton extrusion is completely abolished by the presence of only about 35 mM of Na^+. As expected, since both Li^+ and Na^+ increase the electron transfer rate to about the same extent (see Figure 1), the substitution of Li^+ by Na^+ does not alter the oxygen consumption rate.

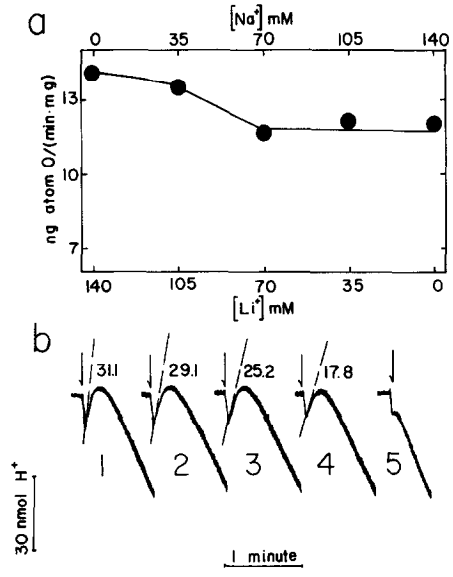

Figure 5. EFFECT OF VARYING THE RATIO OF Na^+/Li^+ IN THE PRESENCE OF A CONSTANT AMOUNT OF K^+ ON THE COUPLED ACTIVITIES. The assay conditions are essentially the same as in Figure 3. The concentration of KCl is held constant at 10 mM in the assay medium and then the ratio of Na^+ to Li^+ (total 140 mM) is varied. In a, the initial rates of oxygen consumption under various Na^+/Li^+ ratios are shown. In b, some representative traces of the concomitant proton movement are shown: 1 - 10 mM KCl + 140 mM LiCl; 2 - 10 mM KCl + 105 mM LiCl + 35 mM NaCl; 3 - 10 mM KCl + 70 mM LiCl + 70 mM NaCl; 4 - 10 mM KCl + 35 mM LiCl + 105 mM NaCl; and 5 - 140 mM NaCl + 10 mM KCl. Arrows (↓) indicate the addition of ferrocyanide.

DISCUSSION

The results presented in this study clearly demonstrate that the electron transfer rate of Site III depends on the ionic composition of the medium. Under the experimental conditions of constant ionic strength and nullified membrane potential (valinomycin plus K^+), the electron transfer rate follows the order of $Li^+ > Na^+ > K^+$, which qualitatively correlates well with the hydrated ionic radius of the principal monovalent cation. Since the proton permeability of the membrane follows the reverse order ($K^+ > Na^+ > Li^+$), the enhanced electron transfer rate caused by Li^+ or Na^+ cannot be attributed to the simple uncoupling action such as that caused by uncouplers which facilitate the influx of H^+ (10). However, it is well known that the inner membrane becomes negatively charged upon energization (11). Thus, the surface potential resulting from the absorption of positive ions with various sizes should be quantitatively different (i.e. different effective surface charge densities). This difference could then lead to the observed variations in electron transfer rate.

Unlike Li^+ or K^+, which does not change the coupling between proton movement and electron transfer of Site III (i.e. constant H^+/e^- ratio), the presence of Na^+ causes differential effects on the coupled activities. The observed inhibition on proton extrusion by Na^+ is not compatible with the possible enhancement of Na^+/H^+ antiporter activity (12) under our experimental conditions. As suggested by Azzone, et al (13), this enhancement would require an increased H^+-leak of the inner membrane.

According to the proton pump mechanism, a conformational link is required for the electron transfer and the vectorial proton movement (14). The fact that Na^+ inhibits the acidification while stimulating the electron transfer can be understood in terms of the following suggestion. We assume that the conformational link between electron transfer and proton movement in Site III is weakened, either directly or indirectly, by the binding of Na^+ to some specific site(s). This can be caused by the direct interaction of Na^+ with some critical negatively charged groups which are functionally important in the conformational link. Based on this hypothesis, the results shown in Figures 3 and 5 suggest that Li^+ is more effective than K^+ in either inhibiting the binding of Na^+ to or displacing Na^+ from the specific site(s), or both.

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