RESPIRATION-INDEPENDENT DISPLACEMENT OF PROTONS FROM EXTERNAL ANIONIC GROUPS OF MITOCHONDRIA BY ALKALI METAL CATIONS

A. R. L. Gear and A . L. Lehninger
Department of Physiological Chemistry, The Johns Hopkins University
School of Medicine, Baltimore, Maryland, U.S., 21205.

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This communication reports a hitherto undescribed respiration-independent interaction between alkali metal cations of the suspending medium and protonated groups of intact rat-liver mitochondria, presumably on the membrane surface. This interaction, which leads to the ejection of H+ into the medium and binding of alkali metal cation, was found in an investigation of the anomalous action of high concentrations of alkali metal salts in producing the "super-stoichiometry" effect during respiration-linked Ca++ uptake, i.e., the occurrence of Ca++:$\sim$ and H+:$\sim$ ratios greatly exceeding 2.0 in the absence of permeant anions (1, 2).

Experimental details. Acid-base changes in the suspending medium and mitochondria were measured as described earlier (3). Na+ binding was measured by emission spectroscopy. Deionized water was used to prepare the 0.25 M sucrose solutions, which were again deionized before use; atomic absorption and conductivity measurements showed that such solutions contained less than 1 μM total of Na, K, Ca, Mg, and Fe.

Results. Fig. 1 shows the pH trace obtained on mixing a stock suspension of rat-liver mitochondria in 0.25 M sucrose (50 mg protein per ml), having a measured pH of 7.00, with a medium of 80 mM NaCl - 5.0 mM Tris chloride buffer, also adjusted to pH 7.00. An immediate drop in the pH of the system occurred, which was almost complete within 5 sec. Following the rapid acidification, the pH remained stable for some minutes. The amount of acid formed was estimated by internal standardization with HCl. In a long series of such experiments between 18 and 25 nmoles H+ per mg protein were formed under the specific conditions shown in Fig. 1. The amount of H+ formed was found to be linear with the amount of mitochondrial protein added over the range 1.0 - 25.0 mg per ml.

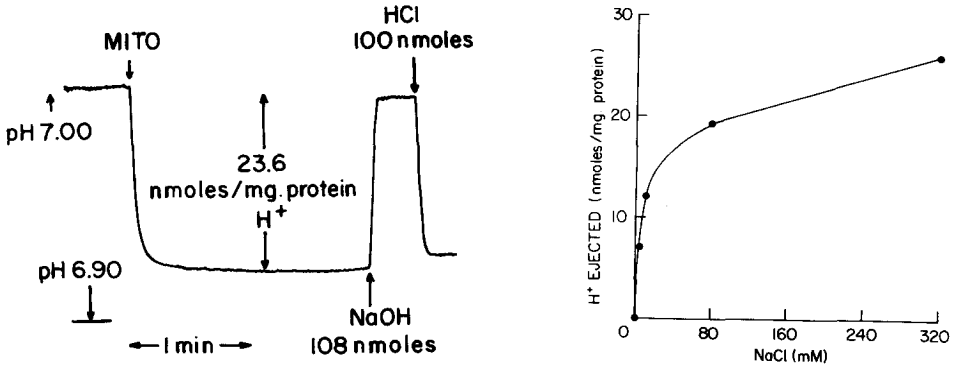

Fig. 1. The acid-base changes occurring on mixing mitochondria and medium. The medium (2.0 ml) contained 80 mM NaCl and 5 mM Tris chloride, adjusted to pH 7.00. At point shown, 0.1 ml. of a stock suspension of rat-liver mitochondria in 0.25 M sucrose (5.0 mg. protein) adjusted to pH 7.0 was rapidly mixed with the medium. Following the initial rapid H+ ejection, the pH remained relatively stable. After 2 min, NaOH was added to the system to bring the pH back to nearly 7.0 and a 100 n mole aliquot of HCl was added as internal standard. Temperature, 25°.

Fig. 2. Effect of NaCl concentration on the ejection of H^+. Details as in Fig. 1 except 0.5 mM histidylhistidine chloride, pH 6.60 was used as buffer. The pH of the mitochondrial suspension was 6.60. For construction of this curve, the H^+ ejected within 10 sec of mixing was measured. The data represent the mean determinations from seven mitochondrial preparations.

The rapid release of H^+ observed in such experiments is dependent on the presence of NaCl or other alkali metal salts in the medium, as is shown in Fig. 2. Mixing of the mitochondrial suspension in 0.25 M sucrose with salt-free 0.25 M sucrose buffered with 0.5 mM histidylhistidine chloride at the same pH yielded little if any H+ ejection. When increasing concentrations of NaCl were added to the buffered sucrose medium, the amount of H+ ejected on mixing with the mitochondria increased accordingly. Addition of 5.0 mM NaCl gave a large effect and 20 mM NaCl gave about half-maximal acidification. In such experiments the pH of the medium was always precisely adjusted to the pH of the mitochondrial suspension before mixing.

No respiratory substrates were added in the above experiments, nor were endogenous substrates required for the H+ ejection. The addition of rotenone, amytal, antimycin A, or cyanide to the salt medium prior to mixing with mitochondria produced no significant change in the amount of H+ ejected in the rapid phase. Furthermore, neither oligomycin nor atractyloside influenced the rapid salt-dependent H+ ejection. It is concluded that the H+ formation observed on mixing of mitochondria with a salt-containing medium is independent of respiration and energy-coupling mechanisms.

The uncoupling agents 2,4-dinitrophenol and m-chlorocarbonylcyanide phenylhydrazone also showed no intrinsic effects on the rapid salt-dependent acidification of the salt-containing medium on mixing with the mitochondria. Their addition produced only the known acceleration of H+ absorption from the medium reported by Mitchell (4), Chappell and Crofts (5), and others, which is much slower than the nearly instantaneous acidification produced on mixing mitochondria and medium. Similarly, neither gramicidin (NaCl medium)or valinomycin (KCl medium) showed any intrinsic effect on the rapid acidification occurring on mixing mitochondria and buffered salt medium at the same pH. As expected, they increased the total H+ formed because of their capacity to allow alkali metal cations to penetrate the membrane in exchange for H+ (6, 7), but this effect was additive to the rapid, salt-dependent acidification. The H+ produced during the mixing of mitochondria and salt-containing medium evidently does not involve those compartments of the mitochondria that are "opened" by dinitrophenol or the antibiotics. The simplest working hypothesis is that the observed respiration-independent acidification is caused by displacement of protons from dissociating groups on the mitochondrial membrane(s) by alkali metal cations, the amount of displacement increasing with cation concentration.

Table I

Binding of Na^+ and ejection of H^+

The medium (2.0 ml.) contained 0.5 mM histidylhistidine shloride, pH 6.60, and 320 mM NaCl. The control system contained 160 mM sucrose instead of NaCl. At zero time 0.1 ml mitochondria suspended in 0.25 M sucrose (5.0 mg. protein) was rapidly mixed. After 30 sec. at 25°, the suspensions were centrifuged at 0°. The resulting pellets were suspended in 2.0 ml. 0.25 M sucrose at 0°, centrifuged, and resuspended in 2.0 ml. deionised water for emission spectroscopy.

Exp.	H^+ ejected n moles per mg.	Endogenous Na^+ in control n moles per mg.	ΔNa^+ bound n moles per mg.
1.	23.0	2.0	22.8
2.	25.4	3.2	27.6
3.	24.2	1.2	29.6

Emission spectroscopy of the mitochondria recovered by centrifugation from the NaCl medium after mixing showed that Na+ was bound when H^+ was ejected. Table 1, Experiment 1, shows that 22.8 nmoles Na+ were taken up per mg mitochondrial protein accompanying the ejection of about 23.0 nmoles of H+ into the medium on mixing of salt-free mitochondria with NaCl medium, i.e. an approximate molar equivalence. That the

mitochondria increased in titratable alkalinity equivalent to the H^+ ejected was confirmed by direct micro-titration of the mitochondrial pellet by methods described earlier (3). These results will be reported elsewhere. The observed ejection of H^+, binding of Na^+, and increased alkalinization of the mitochondria are thus approximately stoichiometric.

Other experiments showed that H^+ ejection on mixing mitochondria with a NaCl medium increased with increase in the initial pH to which both mitochondria and medium were adjusted prior to mixing, that other alkali metal cations tested (in the form of their chlorides) promoted H^+ ejection, in the descending order $Li^+ > Na^+ > K^+ \cong Rb^+$, and that chloride could be replaced with bromide, iodide, nitrate, or fluoride.

Discussion. These data show that alkali metal cations can displace protons from dissociating groups of the mitochondria probably located on the outer surface of one or both of the membranes. The excellent agreement between the amount of H^+ so ejected, the increase in titratable alkalinity of the mitochondria, and the directly-measured binding of Na^+ by the mitochondria strongly supports this conclusion. The magnitude of the effect is rather large. Maximal H^+ ejection at the highest NaCl concentration (320 mM) was about 23 - 30 nmoles H^+ per mg protein at pH 6.6.

If Na^+ displaces protons by a mass-action effect it would be expected that the acid-base titration curve of whole intact mitochondria in 0.25 M sucrose would be displaced to the acid side by increasing NaCl concentration in the medium. This expectation was realized on direct experiment; the titration data will be reported elsewhere.

The fact that a large increment of H^+ ejection is produced on increasing the pH or on increasing the salt concentration, suggests that the effect described in this paper may be responsible for, or related to, the superstoichiometry phenomenon, which is also produced by increases in pH or salt concentration in this range (1, 2).

It is possible that the respiration-independent binding of univalent cations described here is related to respiration-independent binding of Ca^{++} (C. Rossi, et al. (8) and Mn^{++} (Chappell, et al. (9)); the former has been shown to be diminished in the presence of NaCl. However, Ca^{++} and Mn^{++} penetrate the membrane (review, Lehninger, et al. (10)) and some evidence has been reported that the energy-independent binding of Ca^{++} takes place in that compartment opened by valinomycin (8).

The molecular identity of the salt-sensitive anionic groups of the mitochondrial membrane is under investigation by titrimetric and chemical modification methods.

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Summary. When mitochondria suspended in salt-free 0.25 M sucrose are mixed with solutions of an alkali metal salt such as NaCl, previously adjusted to the same pH, there is an immediate release of H^+ to the medium, an equivalent binding of Na^+, and a corresponding increase in the apparent titratable alkalinity of the mitochondria. This interaction is also given by K^+ and other alkali metal cations. The acidification increases with cation concentration and initial pH of the medium. It is not directly influenced by electron transport inhibitors, by oligomycin or atractyloside, or by 2,4-dinitrophenol or gramicidin. The protonated groups involved are probably located on the surface of the membrane(s).

REFERENCES

1. Carafoli, E., Gamble, R. L., Rossi, C. S., and Lehninger, A. L., J. Biol. Chem., 242, 1199 (1967).
2. Rossi, C., and Azzone, G. F., Biochim. Biophys. Acta, 110, 434 (1965).
3. Gear, A. R. L., Reynafarje, B., Rossi, C. S., and Lehninger, A. L., J. Biol. Chem., In the press.
4. Mitchell, P., Symp. Biochem. Soc., 22, 142 (1962).
5. Chappell, J. B., and Crofts, A. R., Biochem. J., 95, 378 (1965).
6. Chappell, J. B., and Haarhoff, K. N., in E. C. Slater, Z. Kaniuga and L. Wojtczak (Editors), Biochemistry of Mitochondria, Academic Press, London and New York, 1967, p. 75.
7. Cockrell, R., Harris, E. J., and Pressman, B. C., Biochemistry, 5, 2326 (1966).
8. Rossi, C., Azzi, A., and Azzone, G. F., J. Biol. Chem., 242, 951 (1967).
9. Chappell, J. B., Cohn, M., and Greville, G. D., in Energy-Linked Functions of Mitochondria, B. Chance, Ed., Academic Press, New York, 1963, p. 232.
10. Lehninger, A. L., Carafoli, E., and Rossi, C. S., Adv. Enzymol., 29, 259 (1967).