Discussion Letter

H^+/SITE RATIO AND STEADY STATE DISTRIBUTION OF DIVALENT CATIONS IN MITOCHONDRIA

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1. Introduction

Mitochondria as well as bacteria are known to operate as proton pumps, i.e. the metabolic force is used to transport primarily protons across the membrane. The primary transport of protons is then coupled through various mechanisms to the movement of other species, cations, anions and water.

The mechanism of proton transport has been assumed to occur via either respiratory loops [1] or membrane Bohr effect [2,3] or H^+ carrier or channel [4]. Since the membrane Bohr effect and the proton channel may accomodate a stoicheometry of 4 H^+/site while the respiratory loops are compatible with a ratio only of 2 H^+/site, much interest has been concentrated over the exact determination of the H^+/site ratio. While Mitchell and Moyle [5] reported a ratio of 2 H^+/site, recently Lehninger and associates [6,7] reported that when the movement of endogenous phosphate is restricted a ratio of 3 or 4 H^+/site could be measured. This observation supports the earlier conclusions of Azzone and associates [8–10], who not only measured a ratio of 4 K^+/site but also indicated the opportunity of measuring the stoicheometry on cation rather than on H^+ charges [11]. This is due to the masking of the H^+ fluxes because of overlapping anion fluxes. A role of endogenous phosphate in affecting the H^+/site ratio is confirmed also by the appearance of a spin exchange signal in the ESR spectrum during Mn^{2+} uptake [12]; the signal is attributed to $(Mn)_3(PO_4)_2$ precipitate.

While the view of 4 H^+/site is gaining increasing consensus, Moyle and Mitchell [13] have brought a new argument in favour of 2 H^+/site, based on the properties of Ca^{2+} transport. The argument is based on two observations: (a) Ca^{2+} is generally transported with a stoicheometry of 2 Ca^{2+}/site [14] and of 1 H^+/Ca^{2+} [15]; this is in accord with a ratio of 2 H^+/site; (b) the steady state accumulation ratio of divalent cations is 10^3–10^4 [16], close to that of K^+. Both observations are taken in support of the view that the molecular mechanism of divalent cation transport involves only 1 charge, i.e., $\leftarrow Ca^+$. The calcium porter is then seen as catalyzing a $[(Ca_2)^{4+} - HPO_4^{2-}]^{2+}$ symport. In the present letter we discuss the implications of this hypothesis and their compatibility with available data. An alternative view is proposed to explain the steady state distribution of divalent cations of 10^3–10^4.

2. Thermodynamics

Suppose that the mitochondrial membrane is impermeable to divalent cations (C^{2+}) and anions (A^-), as such, but permeable to a cation–anion complex (CA^+) carrying a single positive charge. Two cases may be considered: (a) the anion A^- is the divalent cation carrier, i.e., a membrane component. (b) A^- is an external anion (HPO_4^{2-}, $H_2PO_4^-$, Cl^-, OH^-, acetate$^-$) which forms an anion–cation complex either in the bulk aqueous phase, or at the membrane–water interface, or directly at the site of interaction of the divalent cation carrier. In the first case the equilibrium distribution of the divalent cation is

$$\frac{RT}{2F} \ln \frac{[C^{2+}]_{in}}{[C^{2+}]_{out}} = \Delta\psi \qquad (1)$$

where $\Delta\psi$ is the electrical potential difference between outer and inner osmotic spaces. The stoicheometry H^+/C^{2+} is two, for reason of electroneutrality. In the second case the equilibrium condition is:

$$\Delta\tilde{\mu}_{C^{2+}} + \Delta\tilde{\mu}_{A^-} = 0 \qquad (2)$$

and the divalent cation distribution:

$$\frac{RT}{F}\left(\ln \frac{[C^{2+}]_{in}}{[C^{2+}]_{out}} + \ln \frac{[A^-]_{in}}{[A^-]_{out}}\right) = \Delta\psi \qquad (3)$$

In general, if the translocated species is:

$[(C^{2+})_n (A^{Z-})_m]^{(2n-Zm)+}$, eq. (3) becomes:

$$\frac{RT}{(2n-Zm)F}\left[n \ln \frac{[C^{2+}]_{in}}{[C^{2+}]_{out}} + m \ln \frac{[A^{Z-}]_{in}}{[A^{Z-}]_{out}}\right] = \Delta\psi \qquad (4)$$

and the stoicheometry is $(2n-Zm)/n$. If $n = Zm$ the stoicheometry is 1, and C^{2+} is distributed across the membrane as a monovalent cation. Choice between eq. (1) or eq. (4) can be made by studying: (a) the charge/site and the H^+/C^{2+} stoicheometries and (b) the parameters determining the steady state distributions.

3. Stoicheometries

3.1. *Anaerobic stoicheometry*

The hypothesis that C^{2+} is transported as CA^+ is, under anaerobic conditions, in contrast with the following observation: the K^+/Ca^{2+} ratio is 2 [17,18], whether in the presence of an excess of K^+ or Ca^{2+} [17], whether in the presence or in the absence of weak acids [17].

3.2. *Aerobic stoicheometry*

The charge/site ratio is 4 whether Ca^{2+} or K^+ is the permeant cation [6,11]. Furthermore the H^+/Mn^{2+} ratio rises from 1.2–1.8 if the movement of endogenous phosphate is minimized [12].

3.3. *Aerobic Ca^{2+}/P_i ratio*

The hypothesis that Ca^{2+} is transported via a porter as $[(Ca_2)^{4+}\,HPO_4^{2-}]^{2+}$ complex [3] implies: (a) a NEM insensitive P_i transport, and (b) a NEM insensitive constant Ca^{2+}/P_i ratio of 2. However: (a) addition of NEM completely inhibits the P_i re-uptake accompanying the uptake of Mn^{2+} [12]; (b) the Ca^{2+}/P_i ratio is NEM-dependent. Table 1 illustrates data which support this conclusion.

3.4. *Reconstituted systems*

A basic discrepancy still remains: the charge/site stoicheometry of the reconstituted cytochrome oxidase vesicles is 2 [19] while that of the third energy conserving site in the intact membrane is 4 [6–11]. This discrepancy implies that the charge separation accomplished by the electron transfer carriers is converted by the proton pump unit into H^+ transport according to an intrinsic stoicheometry of 2 H^+/e^-. Then, the charge separation performed by the reconstituted complex is not equivalent to the active H^+ transport carried out by the intact membrane. Probably, the conversion of the charge separation into real coupled H^+ transport is deleted in the reconstituted systems.

4. Steady state distribution

4.1. *Distribution of divalent cation in vitro*

The discovery that the steady state accumulation ratio of divalent cations is 10^3–10^4 [16,17] rather than 10^6, as predicted by a straightforward application of the Nernst equation, raises the question as to the reason for the discrepancy. Moyle and Mitchell [13] proposed that C^{2+} may be transported as univalent charged species in a complex with an anion. Equation (4) predicts that the C^{2+} distribution is affected also by the anion distribution. A steady state divalent cation distribution of either 10^4 or 10^3, in presence of a $\Delta\psi$ of 180 mV, implies that the concentration of the anion in the matrix is either 10 times lower or equal to that of the outer space. However the distributions of OH^-, P_i and acetate are opposite [14,20,17]. That the anion is Cl^- cannot be excluded, although this would render C^{2+} uptake markedly dependent on $[Cl^-]_{out}$.

Our view is that: (a) C^{2+} are not at electrochemical equilibrium; (b) the steady state distribution is achieved when the rate of cation influx (V_i) equals that of cation efflux (V_e).

Table 1
The effect of *N*-ethylmaleimide on the Ca^{2+}/P_i ratio

	Ca^{2+} uptake nmol/mg protein	Pi uptake nmol/mg protein	Ca^{2+}/P_i
– NEM	19.84 ± 0.014	14.62 ± 0.88	1.36
+ NEM	19.58 ± 0.13	3.9 ± 0.23	5.02
+ Mg^{2+} – NEM	19.56 ± 0.14	15.21 ± 0.03	1.29
+ Mg^{2+} + NEM	19.57 ± 0.02	3.97 ± 0.53	4.93

Rat liver mitochondria were prepared according to standard procedures. The incubation medium contained 200 mM sucrose, 20 mM Hepes, pH 7.2, 2.5 mM succinate–Tris. $Ca(acetate)_2$ 20 nmol . mg $protein^{-1}$. The reaction was initiated with addition of mitochondria. After 2 min the samples were centrifuged in a Sorvall RC2B at 20 000 rev/min for 5 min. The supernatants were collected. Ca^{2+} uptake was determined with $^{45}Ca^{2+}$. The release of endogenous P_i from the mitochondria was determined, colorimetrically, in the supernatant after centrifugation of an EDTA supplemented sample. The uptake of endogenous P_i was then determined by adding $^{32}P_i$ in catalytic amounts. When indicated *N*-ethylmaleimide (NEM) 30 nmol . mg $protein^{-1}$, 2.5 mM $MgCl_2$ were added.

In this model the accumulation ratios of permeant cations do not correspond to $\Delta\psi$ as predicted by the Nernst equation. It is further predicted that any variation of the rate of V_i or V_e leads to a variation of the accumulation ratio. This model is supported by several observations: (a) in the case of the univalent cations the steady state accumulation ratio for K^+ is dependent on the concentration of valinomycin [21,22] as well as that of organic cations is dependent on the amount of tetraphenylboron [22]; (b) in the case of divalent cations the accumulation ratio decreases either after the addition of Mg^{2+} or Ruthenium Red [17,23], which restrict the rate of native carrier operation, or after increase of the rate of cation efflux ([24] and Pozzan et al., in preparation).

4.2. *Distribution of Ca^{2+} in vivo*

Most cells possess enzymatic reactions requiring a cytosol Ca^{2+} concentration between 10^{-5}–10^{-8} M [25]. In the mitochondrial matrix the Ca^{2+} concentration is kept very low, presumably around 10^{-4} M or less [26] through the low concentrations of anions forming soluble complexes with Ca^{2+} and the binding of Ca^{2+} to the phospholipids (K_d around 100 μM) [27]. If Ca^{2+} would be distributed at electrochemical equilibrium with a $\Delta\psi$ of 200 mV, the cytosol concentration would be lower than 10^{-10} M and thus incompatible with the enzyme requirements. The conclusion that the accumulation ratio depends on the relative rates of influx and efflux suggest a convenient mechanism to maintain a relatively high cytosol Ca^{2+} concentration. Vinogradov and Scarpa [28] and Scarpa and Graziotti [29] have shown that the kinetics of Ca^{2+} uptake is sigmoid in nature and the K_m may reach values around 50 μM in the presence of Mg^{2+}. Such properties may efficiently operate to reduce the rate of Ca^{2+} influx to 1–2 nmol . mg $protein^{-1}$. min^{-1} at external Ca^{2+} concentrations around 10^{-6}–10^{-7} M, i.e., near to the physiological conditions. This rate corresponds to the rate of Ca^{2+} efflux in steady state [30]. Evidence has been provided [30] that the rate of Ca^{2+} efflux is correlated with the rate of H^+ leak. The accumulation ratio, both in vivo and in vitro, then, would not be strictly related to the force generated by the proton pump but rather determined by two kinetics parameters, namely the affinity of C^{2+} for the carrier and the H^+ leak.

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