Vol. 93, No. 1, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
March 13, 1980 Pages 243-249

STEADY STATE MEASUREMENTS OF THE INTERNAL PHOSPHORYLATION POTENTIAL AND THE CROSS MEMBRANE ELECTROCHEMICAL POTENTIAL FOR PROTON IN RESPIRING MITOCHONDRIA

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Received January 21, 1980

Summary

A preliminary report is made on the stoichiometry (H^+/ATP) of the ATPase reaction at energetically steady state in respiring mitochondria. The internal phosphorylation potential and the ΔpH were measured in situ by ^{31}P NMR and the electrical potential gradient ($\Delta\psi$) was estimated from the uptake of a membrane penetrable cation (tetraphenylphosphonium ion) which was monitored with an ion selective electrode during NMR measurements. The stoichiometric number was around 2.5. There were non specific but extensive bindings of the cation inside mitochondria and therefore a large correction factor was required to the uptake of the cation in order to apply Nernst's equation. This reduced the reliability of the $\Delta\psi$ measurement substantially.

INTRODUCTION

According to the chemiosmotic theory by P. Mitchell[1], mitochondrial ATPase synthesizes ATP from ADP and inorganic phosphate (P_i) inside mitochondria by taking up protons from outside along the gradient of the electrochemical potential ($\Delta\mu_H$) generated by the respiration across the membrane. The stoichiometric number (n) of protons required for one ATP synthesis is an important parameter for understanding the mechanism of the energy coupling and it has been measured with various methods and the value varies between 2 and 4[2-5]. One way to measure the stoichiometry is to

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* On leave from the Department of Biochemistry, State University of New York at Stony Brook, New York and supported by NIH Grant #HL16474.

Abbreviations: NMR, nuclear magnetic resonance
TPP^+, tetraphenylphosphonium ion
$\Delta\mu_H$, the gradient of electrochemical potential for proton
$\Delta\psi$, the gradient of electrical potential

0006-291X/80/050243-07$01.00/0

determine the internal phosphorylation potential ΔG_p and $\Delta\mu_H$ under the condition where ATPase equilibrates the two.

$$n \cdot \Delta\mu_H = \Delta G^\circ + \Delta G_p = \Delta G^\circ + 59 \log \frac{ATP}{ADP} \cdot \frac{1}{P_i} \quad (1)$$

$$\Delta\mu_H = \Delta\psi + 59\ \Delta pH \quad (2)$$

In (1) and (2), $\Delta G^{\circ\prime}$ is the standard free energy of the reaction, ADP + $P_i \rightleftarrows$ ATP, and $\Delta\psi$ is the cross membrane electrical potential. A recent application of ^{31}P NMR[6] to the field of bioenergetics has shown that ΔG_p inside mitochondria can be determined in situ together with ΔpH. With the NMR method one can study at steady state the internal energetics under the least energy consuming (or more specifically, least ATP consuming) condition where no external ADP or ATP is present. Under this type of condition the assumption for the equation (1) should be allowed. The only term not determined by NMR in (1) and (2) is the value of $\Delta\psi$. In this report the uptake of a membrane penetrable cation (tetraphenylphosphonium ion) by energized mitochondria (internally negative) was used to estimate $\Delta\psi$. This type of $\Delta\psi$ probe has been used in bacterial systems[7]. In order to monitor the external cation concentration during NMR measurements, a small ion selective electrode was made and accomodiated in a NMR sample tube. Since the amount of cation taken up by mitochondria did not necessarily represent the activity of the cation inside, a calibration of the cation distribution with $\Delta\psi$ was required.

MATERIAL AND METHODS

Mitochondria from rat liver were prepared according to the procedure described previously[6]. The mitochondrial concentration in NMR experiments were in the range of 30 to 60 mg protein/ml.

An ion selective electrode for tetraphenylphosphonium ion (TPP^+) was made of polyvinylchloride membrane as described in the procedure by Kamo et al[8]. Micro reference electrodes (Ag/AgCl) were purchased from Microelectrodes, Inc., New Hampshire, U.S.A. The capability of the electrode to measure the external TPP^+ concentration in mitochondrial suspension was excellent in the range of 10^{-6}M to several mM. The electrode reading in the presence of uncoupled mitochondria was exactly the same as in the supernatant of the suspension after centrifugation. The extent of the apparent binding of TPP^+ to respiring mitochondria was estimated from the simultaneous measure-

ments of ^{86}Rb distribution in the presence of valinomycin[7] in dilute mitochondrial suspensions (2 to 10 mg protein/ml). Aliquots of 0.5 ml sample were centrifuged down to pellets within one minute by a Beckman microfuge B. Specific radioactivities of the suspension and the supernatant were measured by a liquid scintilation counter (Beckman Model LS-230). The value of $\Delta\psi$ was obtained from this ^{86}Rb distribution assuming no binding of Rb to mitochondria and Donnan equilibrium across the membrane. Variation of $\Delta\psi$ was made by adding small amounts of KCl.

An ion selective electrode (5mmϕ) was placed at the lower end of a 25mmϕ glass tube which was concentrically connected to a 10mmϕ NMR tube. It required 12ml of sample solution to fill to the level of the electrode. A special sample mixing device was built in order to oxygenate mitochondria and to ensure the homogenity of the sample throughout its volume. Using a pneumatic control through a 5mmϕ tube at the center of a NMR sample container, a portion of the sample solution (about 2ml) at the bottom where NMR measurements were made was transferred to the top of the sample where the electrode measurements were made. Additional mixing was done by the movement of the 5mmϕ tube during the sample cycling. This mixing operation which required 2 to 4 sec was followed by NMR measuring period of about 10 sec and the cycle repeated for 10 min for NMR signal accumulation. This operation provided a gentle mixing of a highly concentrated mitochondrial sample so that a steady state of highly energized mitochondria was maintained for more than an hour. During the mixing period hydrogen peroxide was added at an averaged rate of up to 0.5mM/min into the sample solution to provide oxygen by rapid enzymatic activity of catalase in sample solutions. As an energy source for respiration, sodium succinate was added at the time of H_2O_2 addition. All these operations described above were controlled by a Nicolet computer for NMR measurements.

NMR peak positions in spectra were expressed by parts per million (ppm) from the peak position of 85% phosphoric acid as the reference. In actual spectra endogenous or exogeneous glycerophosphoryl choline peak at 0.494 ppm was used as a marker.

The internal volume of mitochondria was assumed to be 1.2μl/mg protein. This value was measured only in anaerobic mitochondrial suspensions at high concentration by comparing ^1H NMR signals of H_2O and sucrose or other non penetrable compounds with those in the supernatant of the suspension. This method was quite similar in its principle to the radioisotope method usually used in biochemical analysis[7]. The value (1.2μl/mg protein) fell in the range of the reported values between 0.4 to 1.5μl/mg protein.

RESULTS AND DISCUSSION

Using the sample mixing system described above, aerobic mitochondria in a NMR sample tube were kept at energetically steady state at 17°C. The spectrum shown in Fig. 1a was obtained from the 1st NMR sampling pulses immediately following the mixing period[9] and the peak intensities (areas under peaks) represented the observable concentrations of these compounds without any spin saturation effects. From the two P_i peaks, internal and external to mitochondria, one could estimate the value of ΔpH taking the pK_2 values of P_i's at 17°C to be 6.8[6] for the internal and 6.9 for the external P_i. The

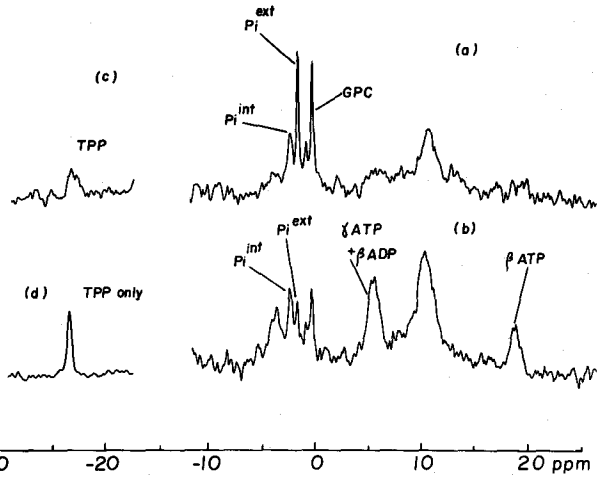

Figure 1. ^{31}P NMR spectra (6 min accumulation) of energized mitochondria at 60 mg protein/ml at 17°C.
The sucrose medium contained 15 mM KCl and 0.6 mM TPP^+.
Succinate and H_2O_2 were added during the sample mixing periods described in Material and Method.

(a) A spectrum obtained from the first 90° NMR sampling pulses immediately after the mixing periods. The total number of accumulations was 40.

(b) A spectrum obtained from 16 pulses after the first pulse at 0.25 sec repetition. Total number of accumulations was 640.

(c) The TPP^+ peak in the same mitochondrial suspension. The spectrum was obtained by 320 accumulations of signals from the first pulses after the mixing periods. The vertical gain was reduced by a factor of 4.

(d) The TPP^+ peak without mitochondria. The peak height was normalized to be equivalent to the peak in (c) as 0.6 mM concentration. Glycerophosphorylcholine (GPC) was added for a marker.

ΔpH in Fig. 1a was 0.4. The spectrum shown in Fig. 1b was obtained with a rapid repetition of NMR sampling pulses following the 1st pulse mentioned above. Those which had short T_1 like internal ATP or ADP (T_1's were ∿0.2 sec) appeared very strong in the spectrum. The chemical shifts of these internal ATP and ADP peaks showed that these phosphates were mostly in Mg^{+2} bound form[6]. The internal ATP did not decay at least 25 sec after mixing periods. The concentrations of internal P_i, ADP, and ATP observable in these NMR spectra were estimated to be 17 mM, 10 mM, and 6 mM respectively. The internal phosphorylation potential was estimated from the internal P_i concentration and ATP/ADP ratio.

In order to estimate $\Delta\psi$ from the uptake of TPP^+, the extent of TPP^+ binding to mitochondria was measured. In uncoupled mitochondrial suspen-

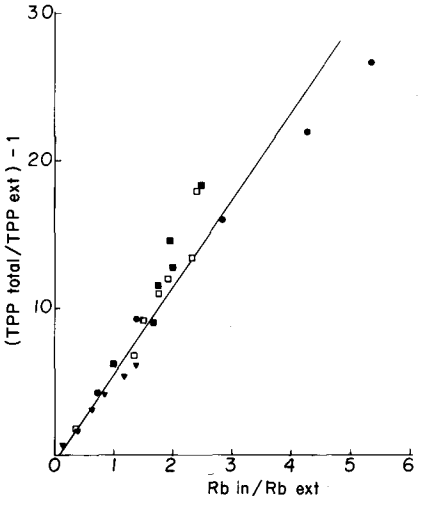

Figure 2. TPP^+ uptake and ^{86}Rb distribution in respiring mitochondria. The ratio of TPP^+ uptake ($TPP^+_{total} = TPP^+_{ext}$) over the external TPP^+ was plotted against the $^{Rb}in/^{Rb}ext$ ratio. The values of $^{86}Rb_{in}$ and TPP^+ uptake were not corrected for the mitochondrial concentration since the correction factor was common to both. Various symbols in the plot represent various experiments using mitochondrial suspensions at 6 to 10 mg protein/ml in 0.25^M sucrose with 5 mM NaCl, 0.3mM KCl, 50 μM cold Rb^+, 0.5μ Curie/ml of ^{86}Rb, 1 mM EDTA, 1 mM P_i, 2 mM succinate, 0.2 mM TPP^+ and 50 mM valinomycin. The Rb distribution was varied by adding KCl.

sion in 0.15^M KCl the ratio TPP^+ bound over TPP^+ external was 0.02m where m was mitochondrial concentration in mg protein/ml. Since in highly energized mitochondrial suspension most of TPP^+ were taken up inside, the internal binding was examined by comparing the distribution of ^{86}Rb in the presence of valinomycin. The uptake of TPP^+ by mitochondria ($TPP^+_{total} - TPP^+_{ext}$) over TPP^+_{ext} was plotted against $^{86}Rb_{int}/^{86}Rb_{ext}$ as shown in Fig. 2. The slope should be in principle independent of the mitochondrial concentration because it was related mostly to internal binding (detailed description of these experiments will be published elsewhere). It scattered among various samples of mitochondria and was 6.5 ± 2. The value of the slope in Fig. 2, which was far larger than 1, corresponded to an extensive binding inside mitochondria (∿85% apparent binding). The highly energized mitochondria in these experiments had $\Delta\psi$ of 130 to 150^{mV} as estimated from the ^{86}Rb distribution.

In spite of the extensive apparent binding of TPP^+ inside mitochondria, the ^{31}P NMR peak of this symmetric molecule had its intensity

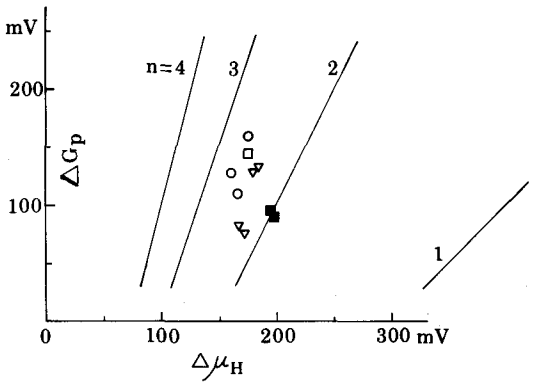

Figure 3. A plot of the internal phosphorylation potential ΔG_p versus the proton chemical potential gradient $\Delta\mu_H$. The lines (n = 1,2,3 and 4) were drawn taking $\Delta G°$ to be 300 mV. The values of ΔG_p and ΔpH were obtained from ^{31}P NMR spectra and $\Delta\psi$ was estimated from the TPP^+ uptake in the NMR samples. Various symbols in the plot represent different mitochondria preparations of which the concentrations ranged between 30 to 60 mg protein/ml. For the estimates of the internal volumes of mitochondria the value of 1.2µl/mg protein was used. For $\Delta\psi$ estimate the uptake of TPP^+ was corrected by a factor of 6.5 or -48^{mV}.

corresponding to more than 65% of the amount taken up into mitochondria (Fig. 1-c and -d). There could be a high degree of non specific bindings of TPP^+ either to proteins or membrane surfaces inside mitochondria, but these non specific bindings did not contribute much to the width of the NMR peak other than the extra broadening of about 50 Hz. These observations pose a basic question on the validity of using an observable ^{31}P NMR peak intensity as the activity of the molecule (or free concentration) in such a highly viscous space as mitochondrial matrix. In the case of P_i, however, the problem must be far less severe, since the P_i equilibrium between internal and external spaces measured by ^{31}P NMR peak intensities followed the simple scheme of $H_2PO_4^-/OH^-$ exchange equilibrium as shown in the previous report[6]. (Further detailed study will be published elsewhere). These basic questions in NMR measurements require further studies especially on the nature of the broad peak widths of those internal phosphate compounds.

The phosphorylation potential and ΔpH measured by ^{31}P NMR and $\Delta\psi$ estimated by the uptake of TPP^+ were combined in a plot, ΔG_p vs $\Delta\mu_H$, shown in Fig. 3. There were wide variations in ΔG_p among various experiments. We did

not positively control the value of ΔG_p except varying the total amount of P_i available to mitochondria. The cases which had lower ΔG_p values ($70 \sim 100^{mV}$) had relatively high internal P_i concentrations ($\gtrsim$15mM). The straight lines in Fig. 3 were drawn for n = 1,2,3 and 4 taking $\Delta G°$ to be 300^{mV} for the Mg^{+2} bound ATP and ADP in the system[10]. The points were scattered and did not give any systematic relation between ΔG_p and $\Delta\mu_H$ but they fell between n = 2 and 3 lines. Due to this scattering in experimental points, these results could not distinguish n to be 2 from 3, or be some non integer number between 2 and 3. Obviously we need more accuracy in those measurements. Especially the reliability of the measured value of $\Delta\psi$ should be improved. Similar experiments using K^+ electrode to monitor the external K^+ concentration are in progress.

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