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A ^{31}P-NMR STUDY OF THE CROSS-MEMBRANE pH GRADIENT INDUCED BY ATP HYDROLYSIS IN MITOCHONDRIA

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Summary

^{31}P-NMR has been used to study the increase of ΔpH in mitochondria by externally added ATP. Freshly prepared mitochondria was treated with *N*-ethylmaleimide to inhibit the exchange between internal and external P_i. Upon addition of ATP, phosphocreatine (30 mM) and creatine kinase to a NMR sample of mitochondria suspension (approx. 120 mg protein/ml) at 0°C, an increase of ΔpH by approx. 0.5 pH unit was observed. However the increased ΔpH could not be maintained, but slowly decayed along with the increase of external ADP/ATP ratio. Further addition of valinomycin to the suspension induced a larger ΔpH (approx. 1) which was maintained by the increased rate of internal ATP hydrolysis as seen in the growth of the internal P_i peak intensity in NMR spectra and the concomitant decrease of the external phosphocreatine peak. The external P_i and ATP peaks stayed virtually constant. When carboxyatractyloside was added to inhibit the ATP/ADP translocase, the internal P_i increase was stopped and the ΔpH decayed. These observations in conjunction with those made earlier in respiring mitochondria clearly show the reversible nature of the ATPase function in which the internal ATP hydrolysis is associated with outward pumping of protons.

Introduction

^{31}P-NMR has been used to study metabolic reactions in intact cells [1,2], cell organalles [3,4], tissues [5,7], and organs [8,9]. A study of bioenergetics

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in mitochondria by ^{31}P-NMR has been reported [4]. The NMR technique has an advantage in its capability of nondestructive measurement and it has been shown in the mitochondria study [4] that the internal phosphate compounds, especially inorganic phosphate (P_i), ADP, and ATP, can be distinguished from those in the external medium. The measurement can provide information on the pH gradient across membrane (ΔpH) and internal phosphorylation potential. The pH gradient can be measured because the chemical shift of P_i resonance is pH-sensitive around pH 7 and the P_i peaks from the internal and external media are distinct. It has also been shown that during respiration the pH gradient increased with the internal pH being more alkaline than the external pH [4].

In mitochondria under physiological conditions the contribution of ΔpH (approx. 0.5 pH unit or less) to the proton chemical potential gradient ($\Delta\mu_H$) is rather small [10,11] in contrast to the case of chloroplast [12]. There are many neutral exchange processes [13] across the mitochondrial membrane which utilize the ΔpH directly or indirectly and contribute to keep the ΔpH relatively small. When sufficient amounts of proton translocation occur, however, there should be a large enough change in ΔpH detectable by the P_i peaks in ^{31}P-NMR spectra. Inhibition of P_i/OH^- exchange carrier, which is the major neutral exchange transport system involving the ΔpH, should enhance the ΔpH change [14]. The purpose of the present study is to show by using ^{31}P-NMR that the mitochondrial ATPase functions as a proton pump [15] when it hydrolyses internal ATP and that ΔpH with the same sign as in respiration can be created by this ATP hydrolysis.

Materials and Methods

Liver mitochondria samples were prepared from 18-h starved male rats with the method [4] previously used. Respiratory control ratio of these samples tested with a Clark oxygen electrode was above 5. NMR samples contained 2 ml of mitochondrial suspension at about 120 mg protein/ml as measured by the biuret method [16]. The suspension medium was 0.25 M sucrose with 1 mM EDTA at pH 7.

Mitochondria were treated with *N*-ethylmaleimide prior to use in order to inhibit $H_2PO_4^-/OH^-$ exchange transport [17]. Freshly prepared mitochondria were first suspended in oxygenated sucrose medium at about 10 mg protein/ml concentration, then *N*-ethylmaleimide was added to a final concentration of 5 mM and incubated for 5 min. The mitochondria were then washed with and resuspended in the sucrose medium ready for measurements. In these samples the external P_i level was usually barely detectable in the NMR spectra, therefore extra P_i was added to make the external P_i at around 2 mM final concentration.

Rotenone, valinomycin and carboxyatractyloside were purchased from Boehringer-Mannheim. The concentrations of these compounds used in NMR samples were 1 nmol/mg protein for rotenone and valinomycin and 1.3 μg/mg protein for carboxyatractyloside. The added amounts scaled to the high mitochondrial concentration in NMR samples were adequate for our present experiments. Phosphocreatine, creatine kinase, ATP and adenosine monosulphate

were purchased from Sigma, and *N*-ethylmaleimide from Calbiochem. ATP was washed with Chelex 100 before use.

^{31}P-NMR measurements were carried out at 0°C with a Bruker HX 360 operated at 145.7 MHz. The following scheme of data acqusition was used. A sequence of 41 exciting pulses for NMR sampling with a repetition time of 0.4 s was applied, and this sequence was repeated 10 or 20 times after a delay of 7 s in between. The free induction decay signals from the first pulse (of 90° tilt angle) were stored separately from those from the rest of 40 pulses (of 60° tilt angles). This scheme allows one to have within the same time frame a fully recovered NMR spectrum from the first free induction decay, and also a partially saturated spectrum from the sum of the next 40 free induction decay signals which gives better signal-to-noise ratio especially for those compounds of short spin lattice relaxation times (T_1) such as internal P_i or internal ADP. The fully recovered NMR spectra were suitable for determining concentrations of phosphate compounds by measuring their peak intensities (integrated areas under peaks).

Resonance peak positions were expressed in parts per million (ppm) from external reference (85% phosphoric acid signal as 0 ppm) and the lower field region from this reference was given a negative sign. The actual calibration of our mitochondria spectra were made by using the endogenous or exogenously added glycerophosphorylcholine peak at —0.494 ppm as a marker.

Results

A typical ^{31}P-NMR spectrum taken from a suspension of *N*-ethylmaleimide treated mitochondria with rotenone was presented in Fig. 1a, and it was very similar to those of normal mitochondrial suspensions previously observed [4]. Assignments of the internal and external P_i peaks were made from their peak positions and also from the fact that the external P_i peak followed the external pH values determined by a pH meter. The presence of two distinct P_i peaks indicated the presence of ΔpH across the mitochondrial membrane since the chemical shift of P_i resonance is sensitive to the pH of its environment. This ΔpH in these *N*-ethylmaleimide-treated mitochondria was quite stable at 0°C in 0.25 M sucrose medium and the P_i peak positions did not change for at least 0.5 h. The two P_i peaks were collapsed to a single peak by H^+ conductors such as *p*-trifluoromethoxycarbonylcyanide phenylhydrazone.

Spectra in Fig. 1 were obtained with the rapid repetition (0.4 s) of the exciting pulses during signal accumulation, and therefore the slowly relaxing external P_i ($T_1 \approx 2.4$ s) was heavily saturated and its peak intensity appeared small. The broad peak at 5.5 ppm was assigned to ADP β-phosphate from its position. No internal ATP β peak was observed (usually at 18.6 ppm) indicating very low level of Mg^{2+} bound ATP in the matrix of these anaerobic mitochondria.

Adenosine monosulphate, phosphocreatine, creatine kinase and ATP were added in this sequence to a 2 ml suspension of *N*-ethylmaleimide treated mitochondria with rotenone. Adenosine monosulphate was used for retarding the adenylate kinase reaction. The final mitochondria concentration was 120 mg protein/ml. In the ^{31}P spectrum taken after ATP addition (Fig. 1(b)) the

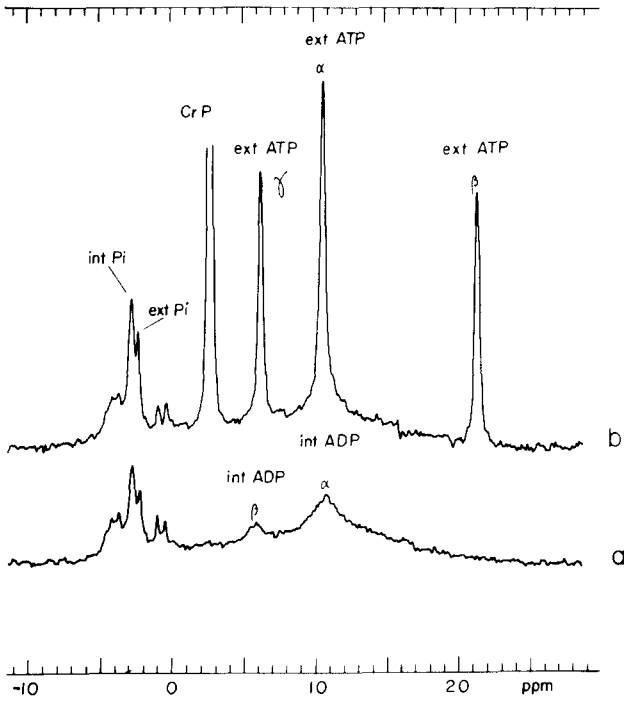


Fig. 1. ^{31}P-NMR spectra of *N*-ethylmaleimide treated mitochondria (120 mg protein/ml) with rotenone (1 nmol/mg protein) in 0.25 M sucrose at 0°C in the absence and the presence of externally added phosphocreatine (CrP) and ATP. (a) The spectrum was taken before the addition by 10 min signal averaging with 65° tilt angle exciting pulses and 0.4 s repetition time (see Materials and methods). (b) After addition of adenosine monosulphate (30 mM), phosphocreatine (29 mM), creatine kinase (1 mg/ml) and finally ATP (6.5 mM). The signal was time averaged for the period from 5 min to 10 min after the ATP addition. The condition for the NMR measurement was the same as in a. For the peak assignment, see text and reference [4].

strongest peak at 2.54 ppm was that of phosphocreatine and the external ATP added could be measured by the peak at 21.5 ppm which was characteristic to the β-phosphate of ATP free of divalent metal ion. The ATP γ-peak at 5.3 ppm overlapped with small amount of external ADP and with the internal ADP β-peaks (Fig. 1(a)) under this experimental condition.

^{31}P-NMR spectra were then taken of this mitochondria suspension after addition of valinomycin and finally after addition of carboxyatractyloside (an inhibitor of the ATP/ADP translocase).

Spectra emphasizing the P_i region at each stage of the experiment were presented in Fig. 2. These spectra were essentially free of nuclear spin saturation effects because before each 90° exciting pulse there was a recovery time of 7.5 s, which was about 3 T_1 of the external P_i. Changes in the pH values, ΔpH and the concentrations of the phosphate compounds, measured by NMR in this sequence of experiment were plotted in Figs. 3 I and 3 II. The apparent concentrations of the phosphate compounds were estimated from their respective peak intensities relative to that of phosphocreatine. The accuracy of this measurement was about ±0.5 mM. There was an uncertainty of several percent in calibrating the concentration of phosphocreatine. Addition of chemicals

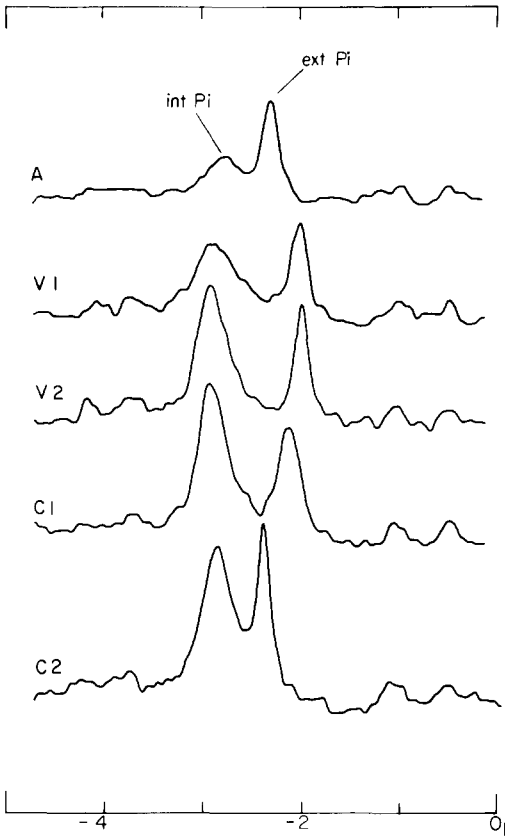


Fig. 2. The P_i resonance region of the spectra taken after addition of ATP, valinomycin and carboxyatractyloside. Spectrum A, 7.5 min after ATP addition (see also Fig. 1b); V_1, 3 min after valinomycin (1 nmol/mg protein) with 2.5 min signal averaging; V_2, 9 min after the valinomycin addition; C1, 3 min after carboxyatractyloside addition (1.33 μg/mg protein), 2.5 min signal averaging; C_2, 9 min after the carboxyatractyloside addition.

was done outside of the NMR instrument, and this led to a delay of approx. 0.5—1 min before data acqusition could proceed.

Upon addition of ATP (as marked by A in Fig. 3), there was an initial increase in the internal pH and ΔpH (Fig. 3 I). However the increased ΔpH could not be maintained as the level of external ATP, measured by the β-phosphate peak, decreased (Fig. 3 II). No internal ATP was observable as indicated by the absence of Mg^{2+} bound ATP β-phosphate peak around 18.6 ppm. The ATP γ-phosphate peak intensity included contributions from the external and internal ADP, the latter of which amounted to about 10% of the total intensity. The rates of the P_i increases were small, with that of the external P_i being smaller. The total P_i increase during this period of ATP hydrolysis did not match with the corresponding decrease in ATP plus phosphocreatine which was somewhat larger. The initial ΔpH jump upon the addition of ATP and phosphocreatine (at A in Fig. 3) was not always observed and differed in magnitude among several similar experiments. This variation was probably due to the variation of the energetic state of mitochondria in the starting

samples. For example, when mitochondria were stored anaerobically for 1 h on ice, the level of internal ADP signal diminished. With the initial ΔpH jump mentioned above a slight increase of total P_i (about 1 mM, data are not shown) was associated.

After valinomycin was added to the mitochondrial suspension, there appeared an increase of ΔpH resulted from a rise in the internal pH and fall in the external pH. This valinomycin effect was quite reproducible. Simple additions of valinomycin in the absence of ATP had no effect on the P_i peaks in either normal or *N*-ethylmaleimide treated mitochondria at anaerobic states. As seen in Figs. 2 and 3 I, a large value (ΔpH = 1 pH unit) was kept steady. The

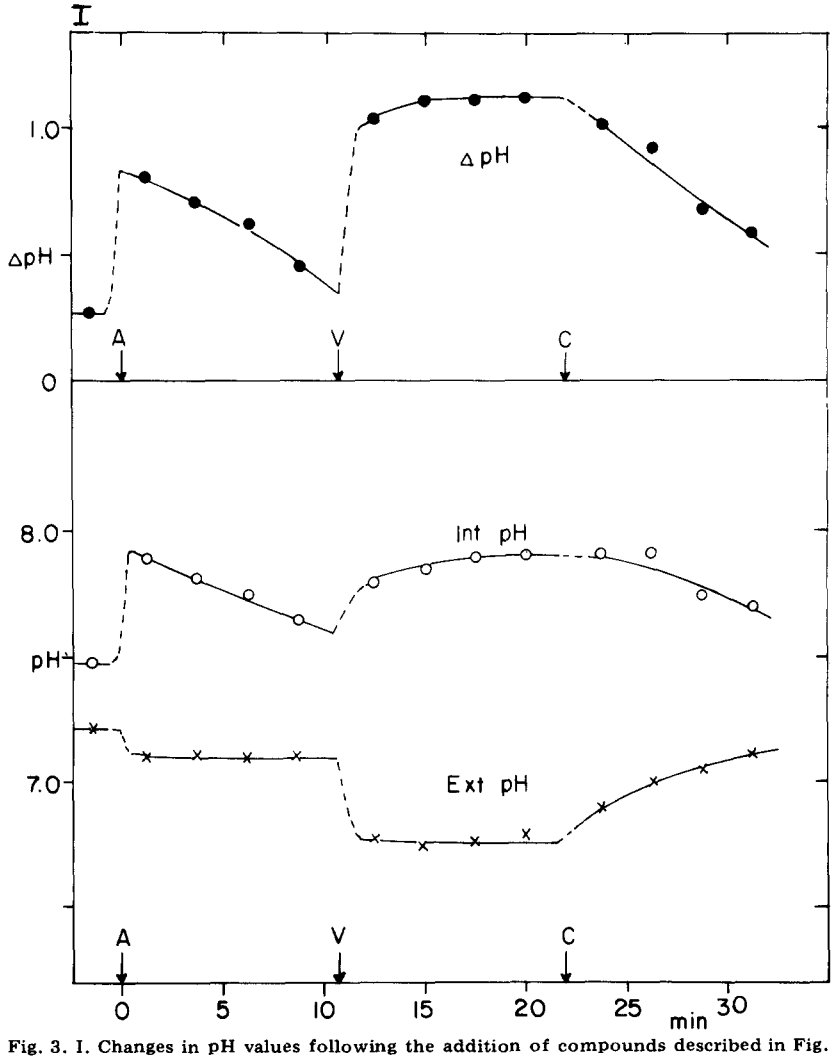
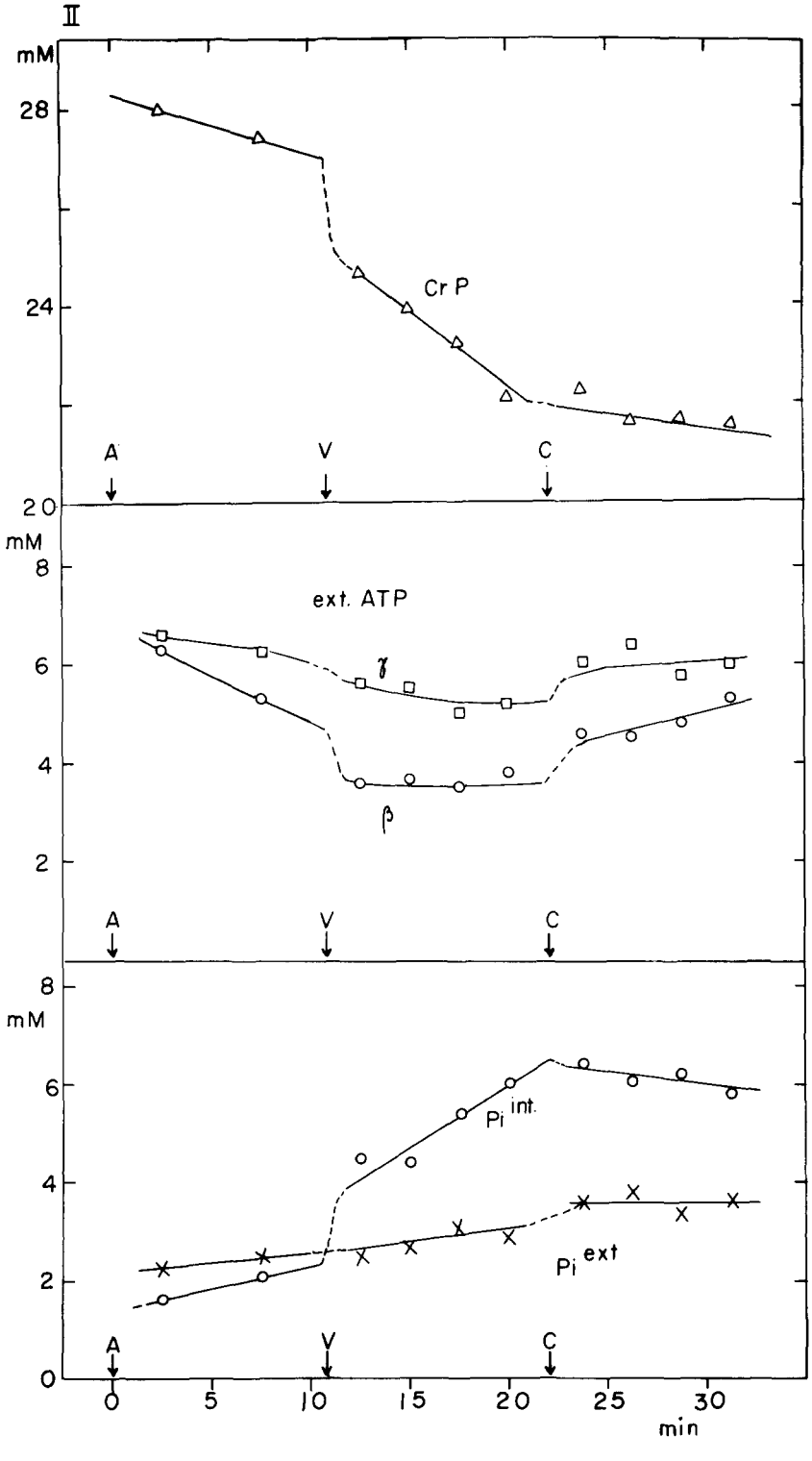


Fig. 3. I. Changes in pH values following the addition of compounds described in Fig. 2. The pK_2 values of the internal and external P_i were taken as 6.7 (see text). II. The changes of the concentrations of the phosphate compounds measured by NMR peak intensities. From the top of the figure, phosphocreatine (CrP), ATP (γ- and β-phosphate peak) and P_i (internal and external). The ATP γ peak intensity included the contribution of the overlapping external ADP β peak.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level of ATP was maintained relatively constant by the creatine kinase reaction, although it was slightly lower than before the valinomycin addition (Fig. 3 I). The γ phosphate peak of the external ATP shifted upfield because of the lowered external pH and was resolved from the β phosphate peak of the internal ADP [4], although the external ADP peak was still superimposing with the γ peak of the external ATP. There was no AMP production observed during this period. The increase in P_i was mostly confined to that of the internal P_i, and the rate of increase was much faster (2.6 nmol/min per mg protein) than that in the absence of valinomycin. The rate of total P_i increase was essentially the same as the rate of decrease in the phosphocreatine peak (Fig. 3 II). Since the ATP level was constant, the phosphate balance was well maintained.

Further addition of carboxyatractyloside to inhibit ATP/ADP exchange transport stopped the internal P_i increase (Fig. 3 II) and the ΔpH decayed slowly (Fig. 2 and Fig. 3 I), and the ATP level also recovered to some extent. Since there was no further increase of P_i observed, there must have been no external ATPase activity present, and the leakage of P_i from inside to outside was not appreciable during the decay of ΔpH.

In the pH measurements described above, the pK_2 value of 6.7 for the internal P_i was assumed to be the same as that previously measured in anaerobic mitochondria suspension [4]. In that previous experiment the pH titration curve of the internal P_i was measured using uncoupled and *N*-ethylmaleimide treated mitochondria so that the internal P_i was retained inside and ΔpH was very small. The titration curve thus obtained contained the effect of the internal ionic strength and also, if any, the effect of fast exchange binding of P_i to macromolecular components in the matrix of mitochondria. The pK_2 value for the external P_i was measured to be also 6.7 in a separate experiment where all compounds except creatine kinase and valinomycin were present at identical concentrations. Oligonycin was added (8 μg/mg protein) to prevent ATP hydrolysis. The external pH was varied by adding NaOH or HCl. Since there was no change in the chemical shift and intensity of the internal P_i peak, the titration curve of the external P_i chemical shift around pH 7 and also the external buffering capacity were obtained. The latter was calculated to be 15 mM of $[H^+]$/pH unit change. The values are in contrast to the $pK_2 = 6.9$ for external P_i and 3 mM $[H^+]$/pH unit change for external buffering capacity at 120 mg protein/ml previously determined for a suspension of *N*-ethylmaleimide treated mitochondria in the absence of added compounds.

When similar experiments to that shown in Fig. 1, 2 and 3 were done in 0.15 mM KCl solution instead of sucrose medium, the external P_i intensity increased in parallel to the internal P_i intensity especially after addition of valinomycin, in contrast to the constant level of the external P_i in the sucrose medium. After addition of carboxyatractyloside the total P_i intensity did not increase, but the relative intensity of the external P_i to the internal P_i increased, indicating the leak of P_i. When isotonic KCl solution was added to sucrose medium up to 30 mM in KCl concentration, the result was quite similar to those shown in Figs. 1—3.

Discussion

The present study demonstrates by means of ^{31}P-NMR that the mitochondrial ATPase pumps out protons by hydrolyzing internal ATP. The observation of the increased ΔpH especially in the presence of valinomycin as shown in Figs. 2 and 3 indicates the outward pumping of protons by the enzyme. In the present experiment the ATP hydrolysis occurred only internally since the internal P_i, not the external P_i, increased during the reaction in the *N*-ethylmaleimide treated mitochondria. Furthermore when the supply of ATP from outisde to inside was stopped by inhibiting the ATP/ADP translocation, the ATP hydrolysis ceased.

Before addition of valinomycin the rate of P_i increase was small as seen in the period between the arrow marks A and V in Fig. 3 II. This P_i increase rate was most likely representing the ATP hydrolysis rate, although there were extra changes in the concentrations of the external phosphocreatine and the external ATP. Since in the presence of valinomycin the ATP hydrolysis rate is much higher, the ATP supply was not rate limiting for the slow ATP hydrolysis. Instead it can be interpreted from the chemiosmotic theory [15] that the system was operating at near equilibrium between the internal phosphorylation potential and $\Delta\mu_H$ which were built up by using the externally added ATP [10] and also at near equilibrium between the internal and external ATP/ADP ratios [18]. Although we did not measure the value of $\Delta\mu_H$ in this present study, the observed effect of added valinomycin [10] is consistent with the above interpretation. Addition of valinomycin suddenly reduced the electrical potential, thereby $\Delta\mu_H$, by equilibrating the potassium concentration across the membrane (estimated external potassium concentration before valinomycin addition was about 10 mM). This sudden change of $\Delta\mu_H$ induced a jump in the rate of ATP hydrolysis (at the mark V in Fig. 3 II), and then a steady state period followed (between V and C in Fig. 3 II). The increased rate of ATP hydrolysis in this steady state as compared with the rate before the addition of valinomycin may indicate a larger deviation [19] from a new equilibrium among the parameters of the energetics as mentioned above. Most probably $\Delta\mu_H$ was too low for the equilibrium with the internal phosphorylation potential expected from the observed external ATP/ADP ratio [18] of about 1.5 and the internal P_i concentration. A large increase of ΔpH in the presence of valinomycin is simply due to the higher rate of ATP hydrolysis, therefore more outward proton pumping. This is quite analogous to the case where an increased rate of respiration in the presence of valinomycin induces a larger ΔpH [20].

NMR measurement is, in general, slow in acquiring reasonable spectra because of its intrinsic low sensitivity. It can follow slow reactions or can measure steady state kinetics, but it cannot measure rates of fast non-reversible reactions. This limitation is applicable to the present study. The jump of ΔpH or levels of phosphate compounds upon addition of valinomycin was too fast to allow any time resolved measurement, and only steady states with or without slow changes with time were measured. A steady state in terms of ΔpH was developed in the presence of valinomycin. This steady ΔpH must have been the result of a balance [21] between the outward proton pumping by the

ATPase and the inward proton leakage from the external medium to the matrix of the mitochondria. This proton leakage was indicated by the ΔpH decay after additions of carboxyatractyloside to prevent further influx of ATP through the translocase. If one knows the rate of this proton leakage, one can estimate the number of the vectorial protons per internal ATP hydrolyzed from the net rate of internal P_i increase. A rough estimate of the proton leakage was made from the decay of ΔpH after the carboxyatractyloside addition by converting the pH change to the amount of proton disappeared from the external medium, using the measured value of the external buffering capacity. From the proton leak rate ($0.5 \approx 1$ mM/min) and the number of the scalor proton of the reactions in the external medium (approx. 1), the number of vectorial protons per ATP hydrolyzed was estimated to be 2 to 4. Although this estimate falls in the range of the reported value [14,22] of the stoichiometry, the low accuracy of the estimate did not permit us to draw any specific conclusion.

As mentioned above, NMR is more suitable for measuring steady-state kinetics than for following relatively fast reactions in time. Simultaneous measurements of those phosphate compounds by NMR and $\Delta\mu_H$ at steady state should yield much clearer insight for those phenomena presented here. Estimates of the stoichiometry for ATP synthesis by respiration and for ATP hydrolysis by this simultaneous measurement are now being made in our laboratory in order to utilize the features of NMR measurements for bioenergetics.

In conclusion, the present study shows that the mitochondrial ATPase is a proton pump as described by the widely held view [15] of this membrane-bound enzyme.

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