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Proton Stoichiometry of Adenosine 5′-Triphosphate Synthesis in Rat Liver Mitochondria Studied by Phosphorus-31 Nuclear Magnetic Resonance†

Seiji Ogawa* and Tso-Ming Lee

ABSTRACT: The internal phosphorylation potential and the electrochemical potential for proton of respiring mitochondria at state 4 were measured simultaneously by using ^{31}P nuclear magnetic resonance and a K^+ distribution method with a K^+-specific electrode in the presence of valinomycin. From these measurements the apparent number for the proton stoichiometry for the synthesis of one ATP was estimated to be 2.2–2.4 for the scheme of the simple chemisomotic theory. It was not an integer number and showed some dependence on the metabolic conditions of mitochondria at steady states of no net phosphorylation.

According to the chemiosmotic theory of Mitchell (1966), the ATP synthesis by the ATPase inside mitochondria should be related to the electrochemical potential for proton ($\Delta\mu_H$),[1] generated across the mitochondrial membrane by respiration, in the chemical reaction

$$P_i + ADP + nH^+_{ext} \rightleftarrows ATP + nH^+_{int}$$

The free energy change, ΔG, for the ATP synthesis by this reaction is given by

$$\Delta G = \Delta G_p + n\Delta\mu_H \quad (1)$$

where

$$\Delta G_p = \Delta G_p^0 + (RT/F) \log [ATP]/([P_i][ADP])$$

and

$$\Delta\mu_H = \Delta\psi - (RT/F)\Delta pH$$

In the above equations, $\Delta\psi$ is the cross membrane electrical potential, which is negative in the internally negative case of mitochondria, and ΔG_p^0 is the standard free energy of ADP phosphorylation.

The parameter n is the stoichiometric number of protons pumped inward by the ATPase for synthesis of one ATP. In the simple chemiosmotic theory the value of n is expected to be a unique number, and for a molecular process it is likely to be an integer. Various measurements have been made to determine the value of n in mitochondria. Reported values range from 2 to 3 (Alexandre et al., 1978; Wiechmann et al., 1975; Azzone et al., 1978; Nicholls, 1974). These values were derived from measurements of the initial rates of phosphorylation and proton ejection after an oxygen pulse (Alexandre et al., 1978) or from measurements of ΔG_p and $\Delta\mu_H$ at energetically steady state, assuming the ATPase reaction is in equilibrium ($\Delta G = 0$) (Azzone et al., 1978; VanDam et al., 1980). In most of these studies the state of the external phosphorylation in the medium was monitored and the internal phosphorylation potential was estimated indirectly.

By the use of ^{31}P NMR, one can determine the internal phosphorylation potential of mitochondria directly (Ogawa et al., 1978; Shen et al., 1980) at energetically steady state. Therefore the complication due to ATP/ADP exchange transport or P_i transport can be avoided. ^{31}P NMR can also determine ΔpH at the same time, but $\Delta\psi$ has to be measured by other methods. In the present study the potassium ion distribution across the membrane in the presence of valinomycin has been measured to estimate $\Delta\psi$ (Rottenberg, 1979a). The external K^+ concentration can be monitored during NMR measurements by a K^+-specific electrode. This study is similar to our previous study where a lipophilic cation was used to estimate $\Delta\psi$ (Shen et al., 1980). In that study there was an extensive internal binding of the cation that required a large correction in estimating $\Delta\psi$. The potassium distribution should be more reliable for $\Delta\psi$ estimation because of the high concentration of K^+ inside mitochondria, so that any binding effect should be a small factor.

In the present study we have closely examined ^{31}P NMR spectra of respiring mitochondria in the presence of valinomycin in order to characterize the resonances of the internal phosphate compounds and to differentiate them from those of the external compounds. At state 4, energetical parameters in eq 1 have been measured to estimate the value of n. The apparent value of n ($n_a = \Delta G_p/-\Delta\mu_H$) is found to be 2.2–2.4

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[1] Abbreviations: $\Delta\mu_H$, electrochemical potential for proton across the mitochondrial membrane; $\Delta\psi$, cross membrane electrical potential; ΔG_p^0, standard free energy of ADP phosphorylation; ΔG_p, phosphorylation potential including ΔG_p^0; P_i, inorganic phosphate; $[P_i^{int}]$, internal concentration of P_i; NMR, nuclear magnetic resonance; ppm, parts per million for NMR peak positions; rf pulses, radio-frequency pulses for nuclear spin excitation; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)-phenylhydrazone; DNP, 2,4-dinitrophenol; Tris, tris(hydroxymethyl)-aminomethane; EDTA, ethylenediaminetetraacetic acid; NAD, nicotinamide adenine dinucleotide.

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and seems to have some dependence on metabolic conditions for respiration.

Materials and Methods

Mitochondria from rat livers were prepared according to the procedure described previously (Ogawa et al., 1978). Mitochondrial protein concentration was determined by the biuret method (Layne, 1952). Valinomycin and carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone (FCCP) were purchased from Boehringer-Mannheim, and 2,4-dinitrophenol (DNP) was from Sigma Chemical Co.

Potassium-specific electrodes (Moody & Thomas, 1979) were made to fit in NMR tubes of 15-mm diameter. The diameter of the tip of an electrode was several millimeters. The active membrane was prepared by casting poly(vinyl chloride) (PVC) film that contained valinomycin and dioctyl phthalate (0.4 g of PVC, 1.5 mL of dioctyl phthalate, and 10 mg of valinomycin in 15 mL of tetrahydrofuran for casting in a 60-mm diameter dish). The range of measurable potassium concentrations by those electrodes extended to 1 μM, and the sensitivity of 57–58 mV per a factor of 10 change in K^+ concentration at 25 °C was nearly the theoretical value. The selectivity against Na^+ was excellent. Sensitivity below 50 μM of K^+ in 20 mM NaCl solution was only slightly lower. The response time of those electrodes was about several seconds. Calibration of an electrode for K^+ measurements in a NMR sample was made in the NMR magnet under the same condition as the condition of NMR measurements.

Values of $\Delta\psi$ in mitochondria in the presence of valinomycin (0.5 nmol/mg of protein) were estimated from the external K^+ concentration monitored by a K^+ electrode and the internal K^+ concentration derived from the total K^+ in the sample. The Nernst equation was assumed to hold. The total K^+ concentration was measured by diluting the sample to about 1 mg of protein/mL and then by adding FCCP to uncouple the mitochondria. The amount of bound K^+ inside mitochondria was ignored ($\sim$10%). The amount of this extractable K^+ measured by a K^+ electrode was essentially identical with the amount measured by atomic analysis. The amounts of magnesium, calcium, and manganese measured by atomic analysis were 25, 2.8, and 0.4 ng-atoms/mg of protein, respectively. The internal volume of mitochondria was measured by the method described previously (Ogawa et al., 1981) in which ^1H NMR was used. The value of the internal volume was 1.2 μL/mg of protein in a mannitol suspension that contained 20 mM NaCl and 10 mM glutamate (with Tris) at 15 °C. The internal volume was measured only under anaerobic conditions (without valinomycin) immediately after preparation of fresh mitochondria in the cold. The increase of the internal volume upon respiration in the presence of valinomycin was ignored since the available K^+ in the medium for uptake by mitochondria was limited and only a 10% increase of the internal K^+ concentration was expected.

Respiration of mitochondria in a NMR sample (40–80 mg of protein/mL in mannitol with 20 mM NaCl medium at 14 ± 1 °C) was maintained by supplying, through capillary tubes, substrate and H_2O_2, the latter of which was converted to O_2 by a large activity of the endogenous or externally added catalase. The sample mixing was made by lowering and raising the K^+ electrode which also monitored K^+ concentration in the medium. The sample mixing period of 8–15 s was followed by a NMR signal acquisition period of 15–30 s.

The respiration rate was not monitored in these NMR samples. In dilute suspensions of mitochondria in the same medium as NMR measurements, the respiration rate at 15 °C with 10 mM glutamate at pH 7 was measured by a Clark-type oxygen electrode and was (10 ± 3) × 10^{-9} atoms of oxygen/(min·mg of protein). Addition of valinomycin increased the respiration rate by 10–15%. Addition of Mg^{2+} at 1 mM in excess over EDTA in the medium reduced the rate by a factor of 2. The maximum respiration rate with FCCP or dinitrophenol was around 40 × 10^{-9} atoms of oxygen/(min·mg of protein).

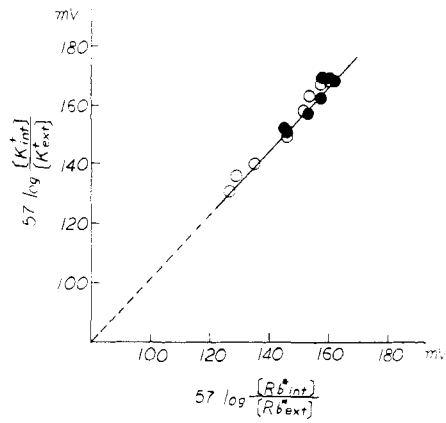

FIGURE 1: Comparison of K^+ electrode measurements with ^{86}Rb distribution measurements for $\Delta\psi$ estimation. Mitochondria were suspended (8 mg of protein/mL) in 210 mM mannitol with 20 mM NaCl at 15 °C. Before addition of valinomycin (0.5 nmol/mg of protein) the external K^+ concentration was around 0.5 mM. Cold RbCl at 20 μM and 0.5 μCi/mL ^{86}Rb were added. The glutamate concentration was varied up to 10 mM (●), and at 10 mM glutamate DNP was added at 0–10 μM (○). When the K^+ electrode reading was steady, ion distributions were measured and expressed in the potential term. Aliquots from a sample were taken to get the supernatant for ^{86}Rb counting by 1-min centrifugation (25 °C). The slope of the line was 1.05.

NMR measurements were made with a Bruker HX 360 instrument at 145.7 MHz by using a probe with a double-turn Helmholtz coil for a sample tube of 15-mm diameter. A Nicolet 1080 computer controlled NMR signal acquisition as well as sample mixing. Peak positions in ^{31}P NMR spectra were expressed in parts per million (ppm) from the peak position of the reference, 85% phosphoric acid. In actual spectra the resonance of endogenous or added glycerophosphocholine (GPC) at −0.494 ppm was used as a marker. The region at lower field than the phosphoric acid resonance position was marked by negative signs.

Results

Potassium Electrode Measurements and Tests for Energetically Steady States of Respiring Mitochondria. Prior to the NMR experiments various test experiments were performed with a K^+ electrode in mitochondrial suspensions at concentrations of about 10 mg of protein/mL. In the presence of valinomycin the Rb^+ distribution across the mitochondrial membrane was measured by using a trace ^{86}Rb radioisotope and was compared with the K^+ distribution simultaneously measured by a K^+ electrode. Figure 1 shows the comparison of the two types of measurements in terms of estimated $\Delta\psi$ values. The ion distributions (equivalent to $\Delta\psi$) were varied by adding various amounts of DNP. The K^+ electrode measurements always gave slightly higher values of $\Delta\psi$ than the Rb distribution. This was probably due to the way that the supernatants for $[Rb_{ext}]$ measurements were obtained. Aliquots of samples were simply spun down in 1 min at 25 °C instead of 15 °C for the incubation temperature where the K^+ electrode measurements were made. The correspondence between K^+ electrode measurements and ^{86}Rb radioactivity countings, therefore, was regarded quite good. In this test,

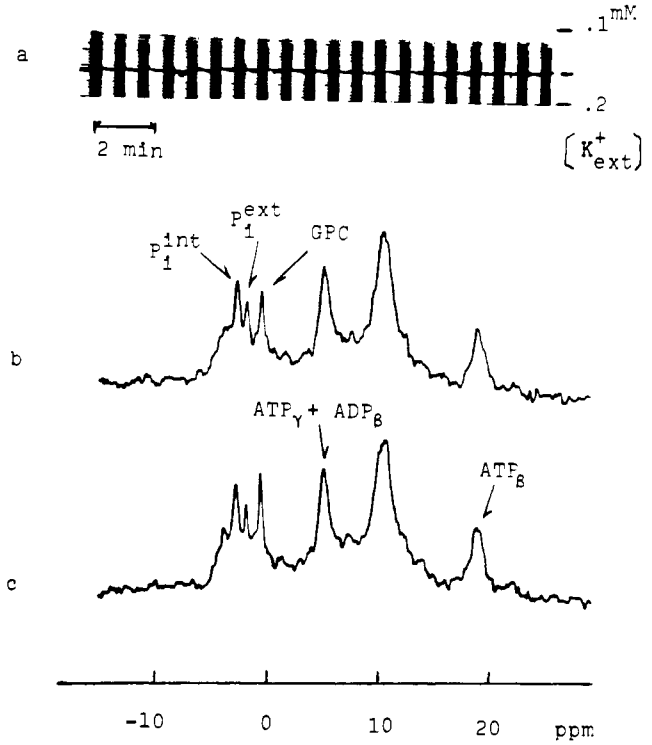

FIGURE 2: Steady-state measurements of external K^+ concentration and ^{31}P NMR spectra of a mitochondria suspension. (a) K^+ electrode output. Barlike spikes were due to microphonic disturbances during sample mixing. The external K^+ concentration measured during NMR signal acquisition periods was steady for 45 min. The sample contained 47 mg of protein/mL of mitochondria with valinomycin (~0.5 nmol/mg of protein) and 10 mM glutamate in mannitol–NaCl (20 mM) medium at 14 °C. (b) A ^{31}P NMR spectrum at 145.7 MHz obtained by a signal averaging over about 30 min during the first half of each NMR measuring period (see the text). (c) A spectrum obtained during the second half of each NMR measuring period. These spectra were accumulated 1600 times each with 60° rf pulses at a 4-s^{-1} repetition rate. Glycerophosphorylcholine (GPC) was added for a field marker.

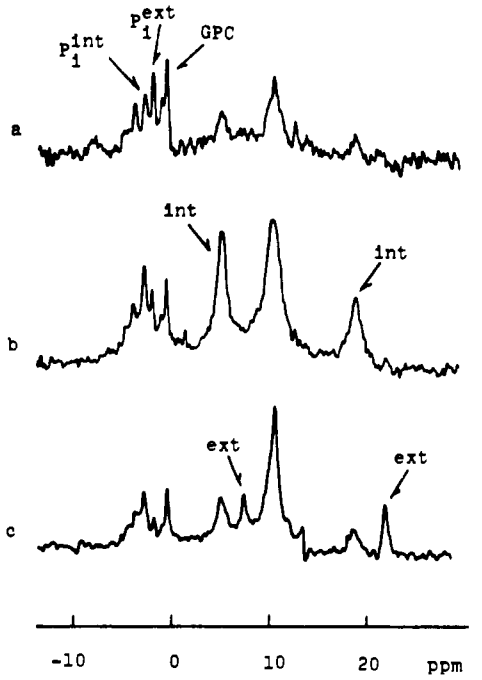

FIGURE 3: ^{31}P spectra of aerobic mitochondria with valinomycin. (a) 40 accumulations of signals with 90° rf pulses at a 0.33-s^{-1} repetition rate from mitochondria (61 mg of protein/mL) respiring with 10 mM glutamate. (b) In the same experiment as (a), 1280 accumulations of signals with 60° rf pulses at a 4-s^{-1} repetition rate. The saturation factors for P_i^{int} and γ ATP peaks in spectrum b were 0.48 and 0.8 as compared with those in spectrum a, and their estimated concentrations in the sample were 0.65 and 0.82 mM, respectively, with 15–20% uncertainties. (c) A spectrum with externally added ATP (0.9 mM) to a mitochondrial suspension (53 mg of protein/mL). Signals were accumulated 400 times with 90° rf pulses at a 1-s^{-1} repetition rate. The saturation factor for the external ATP β peak was 0.35.

glutamate was used as the substrate for respiration. The value of $\Delta\psi$ estimated from the K^+ distribution increased about 10 mV when the concentration of glutamate was increased from 1 to 10 mM. It took about 10 min to reach a steady reading of the K^+ electrode output when the glutamate concentration was varied. In the absence of valinomycin, glutamate addition or change in the glutamate concentration did not induce any significant changes in the K^+ electrode output. Therefore the K^+ uptake influenced by glutamate in the presence of valinomycin was due to the changes in $\Delta\psi$ (Tischler et al., 1976) rather than the K^+ involvement in glutamate transport. Under the conditions used in this study the changes in $[K^+_{ext}]$ in the suspension of respiring mitochondria without valinomycin were very slow, and it took nearly 1 h for the K^+ concentration to reach a certain steady level. In contrast the changes in $[K^+_{ext}]$ induced by addition of valinomycin occurred within the time for valinomycin to distribute among mitochondria in a sample suspension, and within 30 s the K^+ concentration reached a steady value.

So that energetical parameters at steady states could be measured by our sample mixing method for NMR measurements, the stability of a steady state was monitored by a K^+ electrode for the external K^+ concentration and also by spectral features of ^{31}P NMR of a mitochondrial suspension that contained valinomycin. A portion of the K^+ electrode output and two ^{31}P NMR spectra are shown in Figure 2. The barlike spikes in the K^+ electrode output (Figure 2a) were due to microphonic disturbances induced by mechanical movements of the electrode during sample mixing periods. Each mixing period (~15 s) was followed by a period of NMR signal acquisition (~25 s) during which the K^+ electrode was positioned away from the rf coil of the NMR probe. The electrode output during NMR signal acquisition was stable for 45 min or more. The stability of NMR spectral features was monitored by accumulating NMR signals in 10 of these measuring periods per one spectrum. Short-term stability within such a measuring period was checked by dividing each period into halves and storing the signals separately. The two spectra shown in Figure 2 were obtained by 30-min signal averaging for the first half (Figure 2b) and for the second half (Figure 2c) of each period. They were essentially identical, and there was no appreciable short-term oscillation or fluctuation of the energetic state as judged by the resonance peaks, such as the chemical shifts of two P_i peaks for ΔpH and the level of the ATP β-phosphate peak.

^{31}P NMR Spectra of Mitochondria. As reported in the previous studies of mitochondria by ^{31}P NMR, strong ATP resonances were observed at 18.7 ppm for the β-phosphate and at 5.1 ppm for the γ-phosphate. The ADP β-phosphate resonance of weak intensity was hidden in the ATP γ-phosphate peak. The α-phosphate peak around 10 ppm was superimposing on diphosphate peaks of dinucleotides such as NAD. For obtainment of a reasonable signal to noise ratio for these resonances that had short T_1 values, the repetition rate of rf sampling pulses was kept relatively high so that the slowly relaxing resonances such as the internal and external P_i resonances were heavily saturated and gave weak signals (Figure 2). A comparison of two spectra at a low repetition rate (0.33 s^{-1} with 90° rf pulses) and at a higher rate (4 s^{-1} with 60° rf

pulses) is made in Figure 3. The two spectra were obtained in a single experiment by dividing each NMR measuring period into two, one for each of the two repetition rates. The external P_i that had a T_1 value of about 3 s was still saturated in Figure 3a, but the internal P_i peak was essentially saturation free. The T_1 value of the internal P_i was estimated to be about 0.7 s in several experiments, but it appeared to vary to some extent and increased to 1 s when the internal P_i concentration was above 10 mM. At this high P_i concentration under anaerobic conditions the T_1 value of the internal P_i was also 1 s as estimated by a similar progressive saturation method. In order to measure the real P_i peak intensity to determine the internal P_i concentration, it was very useful to have saturation-free NMR signals. For each experiment NMR signals induced by the first rf pulse (90°) after each sample mixing period were stored separately from later NMR signals. One useful procedure to get the saturation-free intensity of the internal P_i was to make two linear combinations of two such spectra as shown in Figure 3a,b, so that in one linear combination the external P_i peak intensity was zero and in the other the internal P_i peak intensity was zero. From these linear combinations one could make a reasonable estimate of P_i concentration even when there was a strong overlap between two P_i peaks (Ogawa et al., 1981).

For the ATP resonances it was difficult to measure the T_1 values within the limited time available to observe these broad resonances ($\sim$100 Hz wide) at 0.5–1 mM concentration. If the shapes of the peaks at 5.1 and 18.7 ppm in the spectrum of Figure 3a were assumed to be the same as those seen in Figure 3b, the value of T_1 was estimated to be about 0.2 s for both resonances. When the rf repetition rates of 4 and 1.33 s^{-1} were alternately varied from one mixing measure period to another within one experiment, the ratios of the intensities of the two peaks (γ/β) were the same at a value of 1.3. Apparently the two resonances had very similar T_1 values. For measurements of the energetic parameters at steady state, a repetition rate of 60° rf pulses at 4 s^{-1} was used in most of the experiments done in this study. At mitochondrial concentrations of 70 mg of protein/mL or higher, a repetition rate of 90° rf pulses at 1.33 s^{-1} was also used.

In the previous studies we have assigned those ATP resonances described above to the internal ATP of mitochondria. This assignment was based upon the position of the peaks that corresponded to those of Mg^{2+}-bound ATP in spite of the presence of EDTA in the medium. When ATP (1 mM) was added to a mitochondrial suspension with 1 mM EDTA, the resonances of divalent metal free ATP (the T_1 value of the β peak at 21.8 ppm was about 2 s) were observable as reported previously (Figure 3c). The observation of metal-free ATP resonances as the external ATP, however, did not necessarily assure that the metal-bound ATP resonances assigned to the internal ATP or ADP were free from the contamination with resonances of some external metal-bound ATP. We found that under conditions similar to those described above the Mg^{2+} exchange rate between ATP–Mg and ATP in free solution without mitochondria was relatively slow in the NMR time scale of the chemical shift difference between the peaks of Mg^{2+}-bound and metal-free ATP β-phosphate (2.5×10^3 s^{-1}). In a solution of a 50/50 mixture of metal-free and -bound ATP, the two β-phosphate peaks appeared instead of a single peak at the weighted average position of the two peaks as expected for a fast exchange process. It is interesting to note that the intrinsic Mg^+ off rate from ATP–Mg under conditions similar to those of the experiments of Figure 3 was about 500 s^{-1}. Several attempts were made to find resonances associated with metal-bound ATP present externally in aerobic mitochondrial suspensions in the presence of 5 mM EDTA. When the external pH was lowered to 6.2 but the internal pH was 7.2, the γ peak did not shift upfield significantly nor was there an observable split of the γ peak. In another experiment similar to the one in Figure 3c, the supply of oxygen to the sample was reduced, driving mitochondria to a slightly lowered energy state (but not completely anaerobic). The metal-bound ATP β resonance assigned to the internal ATP decreased to a level where it was barely observable. However, the metal-free ATP β resonance was essentially the same as it was in the fully aerobic state. Since the external ATP/ADP ratio could be very high ($\gtrsim$ 10) even when the internal ATP/ADP ratio dropped substantially ($\lesssim$ 0.1), the presence of external metal-bound ATP or external ATP having resonances at the peak positions of metal-bound ATP was regarded to be undetectable by ^{31}P NMR under the conditions of the present study. This conclusion seemed obvious in the presence of more than enough EDTA to chelate all the divalent metal ions contained in mitochondria. However, these tests were necessary, since there were few cases of experiments where the internal γ peak seemed to have fairly longer T_1 values than the value mentioned above. As seen in Figure 2b,c or 3b, there was a small peak of metal-free ATP β-phosphate at 21.8 ppm that coincided with the peak position of added ATP (Figure 3c), although no extra ATP was added in the experiments of Figure 2 or 3b. The appearance of this small ATP peak might have been due to mechanical damage or leakage of ATP from inside, but its peak intensity did not increase with time. It could have been due to unwashable ATP or ADP bound to the outside of mitochondria in the cold and released to the medium at a high energy state of mitochondria. However, its intensity relative to the internal ATP β peak increased at lower mitochondrial concentration (20 mg of protein/mL). If it was due to some leakage of internal ATP, the external and internal ATP seemed to be in equilibrium. There was a small peak with noise near the internal ATP β peak in Figure 3a, but the center of this noisy peak was 0.6 ppm away from the small peak at 21.8 ppm in Figure 3b. Therefore, it was not reliable for the intensity estimate. If one assumes the same T_1 value for this small peak of Figure 3b as the externally added ATP peak in Figure 3c, although the T_1 value was likely to be shorter, the amount of this external ATP would be about one-fourth of the amount of the sum of the internal ATP and ADP. Without valinomycin this external ATP peak was, if any, a factor of 2 smaller.

Other types of experiments concerning the internal ADP and ATP were performed as shown in Figure 4. A spectrum (Figure 4a) of anaerobic mitochondria preincubated with succinate for respiration in the cold was compared with a spectrum (Figure 4c) of anaerobic mitochondria that were preincubated with succinate and acetate. The peak at 5.6 ppm (Figure 4a) was previously assigned to the internal ADP β-phosphate. This peak did not titrate with external pH. In the spectrum of Figure 4c, the intensity of this peak decreased to a substantial degree, and a broad resonance around −4.0 ppm increased its intensity. The difference between the two spectra is shown in Figure 4b. The α-phosphate peak of the ADP appeared in the difference spectrum, and its intensity was very similar to the β-phosphate peak. The presence of acetate seemed to have converted ATP to AMP through acetyl-CoA synthetase activity and reduced the content of ADP in anaerobic state. Although the acetyl-CoA synthetase activity and reduced the content of ADP in anaerobic state. Although the acetyl-CoA synthetase activity in rat liver mitochondria that

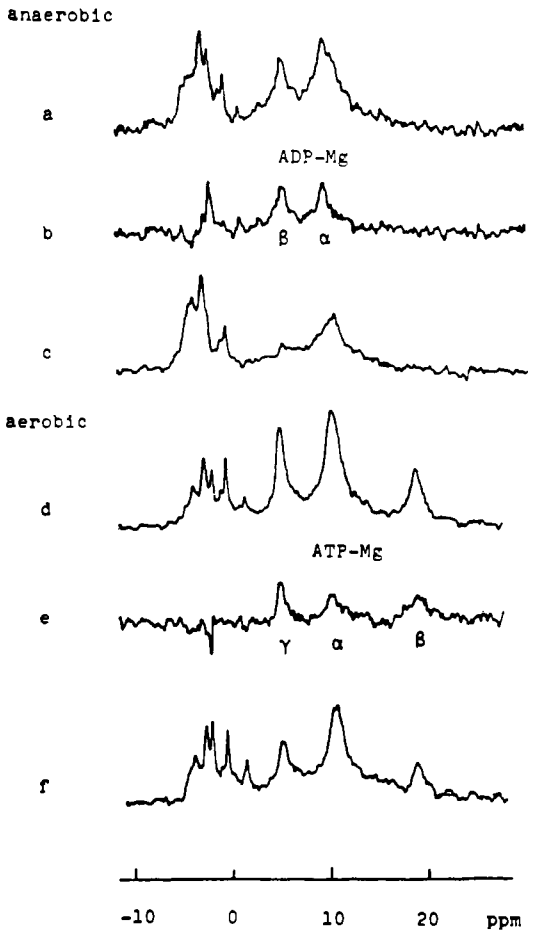

FIGURE 4: Mg-bound ADP and ATP resonances in anaerobic and aerobic mitochondria. (a) A spectrum from anaerobic mitochondria at 14 °C that were preincubated in the cold with 1 mM succinate for 20 min. (b) A difference spectrum between (a) and (c). (c) A spectrum of anaerobic mitochondria with 4 mM acetate that were preincubated in the cold with 1 mM succinate and 1 mM acetate. Spectrum c was accumulated 2 times longer with 12% higher mitochondria concentration than spectrum a. Contents of P_i were not controlled. The amount of added GPC was the same. (d) A spectrum from aerobic mitochondria similar to spectrum b of Figure 3. 960 accumulations of signals prior to acetate addition. (e) A difference spectrum between (d) and (f). (f) A spectrum after addition of 10 mM acetate. See the text for the details of the changes.

required ATP–Mg was reported to be distributed between inside and outside mitochondria (Aas & Bremen, 1968), the external activity must have been very low since the suspension medium contained EDTA. The broad peak intensity at −4.0 ppm was presumably due to the increased amount of the internal AMP. The T_1 value of this resonance was similar to or somewhat larger than the value of the internal P_i. When a difference spectrum was made between a spectrum of respiring mitochondria and the spectrum of Figure 4c, the internal AMP intensity was more clearly observed. In a ^{31}P NMR spectrum of a perchloric acid extract from respiring mitochondria, the content of AMP was very low as compared with anaerobic mitochondria. In the spectrum of Figure 4c (acetate-treated sample) there was some residual peak around 5.6 ppm. We have not characterized the nature of the peak. It looked somewhat heterogeneous.

An experiment similar to the one described above but with respiring mitochondria gave the spectra shown in Figure 4d–f. To mitochondria respiring with 10 mM glutamate (Figure 4d) was added 10 mM of acetate (pH 7). The K^+ electrode showed an transient increase of about 15 mV in $\Delta\psi$; then the increase was reduced to 6 mV to reach a steady state within several minutes. The spectrum (Figure 4f) was obtained after the K^+ electrode reading became steady. The ΔpH was reduced from 0.9 (Figure 4d) to 0.55 after the addition of acetate (Figure 4f) with the transient reduction of ΔpH being slightly larger. Therefore, $\Delta\mu_H$ became lower by a small amount. The level of ATP plus ADP decreased by a factor of nearly 2 and the γ/β peak ratio appeared to have slightly decreased. The difference spectrum between the two is shown in Figure 4e. The α,β,γ-phosphate resonances of ATP are visible in the spectrum. Between Figure 4d and Figure 4f there were some changes in the base line from 10 to 18 ppm that came from broad- and solid-state-like phospholipid resonances. Together with the high noise level, accurate comparison of the intensities of the ATP γ,β and γ resonances was not possible but they were very similar. We have not been able to isolated the ADP β resonances from the ATP γ resonances under aerobic condition where the ADP concentration was fairly low relative to ATP. In the present study we assumed that the ADP resonance at state 4 had the same characteristics as it had under anaerobic conditions.

The α-phosphate resonances of ATP and ADP were overlapping with dinucleotide resonances. The spectrum of Figure 4c where the ADP content was very low must have represented the resonance of NADH and NADPH at around 10 ppm. At the aerobic state one could generate NAD resonance from the two spectra of Figure 4d,f. The peak was slightly broader than the α-phosphate resonance of ATP but quite intense at 10.6 ppm. When the signal to noise ratio in those spectra is improved, this type of difference spectrum will be very useful. When mitochondria were returned to the anaerobic state from the aerobic state, a transient resonance appearing as a shoulder at around 14 ppm was always observed before the spectrum became the one shown in Figure 4a. We have not yet examined closely the relation of the resonance to the change in the base line in this field region seen in the difference spectrum (Figure 4e). A peak at 1.4 ppm seen in Figure 4f and also in Figure 4d has not been assigned. Their peak positions were very similar to that of carbamyl phosphate. Another small peak at 0.5 ppm seen in Figure 4a was also observed with stronger intensity in mitochondria respiring with succinate, and the peak intensity increased with time. It was found to be phosphoenolpyruvate resonance. The accumulation of phosphoenolpyruvate was more pronounced in the presence of valinomycin than in the absence.

Estimates of $\Delta\mu_H$ and ΔG_p. The energetic parameters for eq 1 were estimated from those NMR spectra and the K^+ electrode reading whenever the criteria were met for a steady state as mentioned earlier. Values of ΔpH were measured from the chemical shifts of the internal and external P_i resonances as reported previously (Ogawa et al., 1978, 1981). The pK_2 of 6.8 was used for the internal P_i at this temperature. For estimation of $\Delta\psi$, the Nernst equation was assumed. Internal concentrations of P_i and K^+ were calculated by using the value of 1.2 μL/mg of protein for the matrix space, which was measured in freshly prepared mitochondrial suspension at anaerobic state. No correction for swelling in the aerobic state was made, because of the limited availability of K^+ and P_i to mitochondria in a sample for uptake. The major question in estimating the internal phosphorylation potential[2] was whether the ATP/ADP ratio was measurable in the NMR spectra. As described earlier, the intensity ratio of the two

[2] The value of the standard free energy of ADP phosphorylation was taken from the report by Rosing & Slater (1972). The value is expressed in terms of the concentrations (not the chemical activities) of the phosphate compounds free in solution.

Table I

sample[a]	K^+_{int}[b]	P_i^{int} [b]	ATP + ADP[b]	ATP/ADP	pH_{int}	ΔpH	$-\Delta\psi$ (mV)	$-\Delta\mu_H$ (mV)	ph p. (mV)[c]
high glutamate, 47 mg of protein/mL	138	9 6	12	4.0	7.52	0.77	161	205	154
low glutamate, 66 mg of protein/mL	123	3.2	6	7	7.72	0.94	154	207	194
low glutamate, 47 mg of protein/mL	111	5.7	8.5	4.5	7.60	0.71	159	199	169
high succinate, 89 mg of protein/mL	191[d]	11	10	3	7.46	0.18	205	215	138
low succinate, 75 mg of protein/mL	151	7.6	8	7.5	7.53	0.62	172	207	174

[a] High substrate concentration was about 10 mM. Low substrate concentration was a few millimolar or less. Temperature was 14 ± 1 °C except in the high succinate case (11 °C). All samples contained valinomycin (~0.5 nmol/mg of protein). [b] nmol/mg of protein. [c] ph p.: (RT/F) log [ATP]/[P_i] [ADP]. [d] There was some unwashed potassium left in the sample suspension, and a volume correction might be needed for the estimates of the potentials.

peaks at 5.1 ppm for ATP plus ADP and at 18.7 ppm for ATP always appeared to be greater than 1, although there were cases where the ratio became close to 1 within the uncertainty of measurements (~15% at best). The external ATP/ADP ratio at state 4 was not measurable by NMR because of the low concentration of the external ADP.

When mitochondria were respiring with glutamate at high concentration (~10 mM), the results of these measurements were quite reproducible among several experiments with mitochondrial preparations at different times. Relevant parameters of a typical experiment were listed in Table I. The ATP/ADP ratio of about 4 was very similar to the reported value obtained by a chemical extraction method (Davis and Lumeng, 1975). The amount of ATP plus ADP was very high, and almost all the internal content of the ATP plus ADP plus AMP pool appeared to be observable as ATP plus ADP in the NMR spectra, although the uncertainty in estimating the absolute value of these contents could be 20%. When the level of glutamate concentration was low, $\Delta\mu_H$ was quite similar, but a slightly higher phosphorylation potential was observed. This phenomenon was first noticed in an experiment in which a limited amount of glutamate was present in a sample. The phosphorylation potential increased with time without changing $-\Delta\mu_H$ appreciably (a slight decrease in $-\Delta\psi$ was compensated by an increase of ΔpH). When the substrate ran out, both $-\Delta\mu_H$ and the phosphorylation potential dropped. In experiments with low glutamate concentration, we could not estimate glutamate concentrations in samples, since we did not monitor respiration rate in the NMR samples. With some glutamate supply rates lower than the consumption rate expected for respiration at high glutamate concentration, the system was allowed to become steady state, and then those parameters were measured. The results were not as reproducible as in the case of high glutamate concentration but seemed to give higher phosphorylation potential. The results from various experiments were plotted in terms of $\Delta\mu_H$ and the phosphorylation potential as shown in Figure 5, where closed and open circles represented the cases of high and low glutamate concentrations. When oligomycin was added to mitochondria respiring with high glutamate concentration in order to reduce the ATPase activity and to check ATP consumption by some reactions other than ATPase, the level of ATP did not decay much for at least 10 min under aerobic conditions and persisted for a while even when $-\Delta\psi$ was below 100 mV, where without oligomylin the level of the internal ATP decayed. Therefore there seemed to be no enzymatic reaction that could compete kinetically with ATPase even at the high glutamate concentration.

When succinate was used as the substrate for respiration (but without rotenone), there appeared to be similar phenomena. At a high succinate level, ΔpH was quite low as expected from the exchange process of succinate with P_i, but $-\Delta\psi$ was high (Table I). When the succinate level was low,

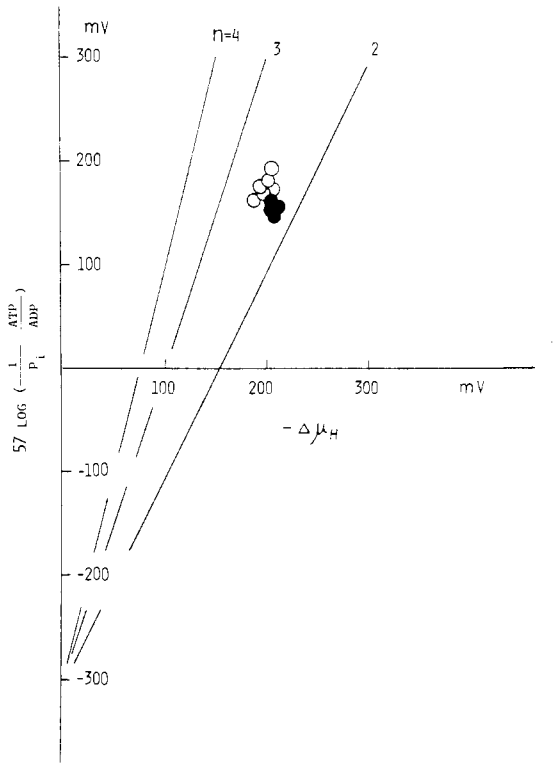

FIGURE 5: Plots of internal phosphorylation potential vs. $\Delta\mu_H$. Closed circles were from experiments with 10 mM glutamate. Open circles were with low glutamate concentrations. Straight lines for $n = 2$, 3, and 4 were drawn by using the value of 308 mV for ΔG_p^0 (standard free energy of ATP hydrolysis). Internval volume corrections were not made for those estimates of potentials although there was some variation in the amount of the internal K^+ (see Table I).

ΔpH was high but $\Delta\mu_H$ was similar to the case in high succinate concentration.

In the plot of Figure 5, the apparent value of n ($n_a = \Delta G_p/-\Delta\mu_H$) at a high glutamate level was 2.2, and at a low glutamate level n_a was 2.4. In the latter case, points scattered more but they were always at the side of higher phosphorylation potential than those in high-glutamate experiments. There seemed to be some dependence of n_a on the metabolic condition of mitochondria at state 4, although it was not clear what the factor was to control the phosphorylation potential. For measurement of the changes in ΔG_p by varying $\Delta\mu_H$, some experiments were carried out with H^+ ionophores such as DNP and FCCP. Data points showed some increases in the value of n_a toward 3 at lowered levels of $-\Delta\mu_H$. However, we could not maintain high glutamate concentration in those experiments. We need further study on this point.

Discussion

The main results of the present study of rat liver mitochondria are that the apparent number for the proton stoi-

chiometry ($n_a = \Delta G_p/-\Delta\mu_H$) of the ATPase as measured by the internal phosphorylation potential and $\Delta\mu_H$ is 2.2–2.4 and that it is somewhat dependent on the condition of state 4 respiration of mitochondria. In this thermodynamical approach to estimate the value of n, the apparent number n_a as defined above seems to be a noninteger and could vary.

When a fractional number instead of an integer number is indicated for the value of n, one has to question the absolute values of those estimated ΔG_p and $\Delta\mu_H$. Even if we were off by a factor of 2–3 in analyzing the NMR spectra, the uncertainty in the internal phosphorylation potential could be 17–27 mV. If the swelling of mitochondria at aerobic states were significant to increase the internal volume by a factor of 2, the correction would put the points in Figure 5 away from the line of $n = 2$ in the direction with a 17-mV decrease in $-\Delta\psi$ and a 17-mV increase in the phosphorylation potential. There are several millivolts of uncertainty in choosing the value of ΔG_p^0, although the chosen value (308 mV) in Figure 5 was rather a low value among reasonable choices for the solvent condition of the matrix space of mitochondria. The assumption of equilibrium distribution of K^+ across the mitochondrial membrane in the presence of valinomycin to estimate $\Delta\psi$ seemed to be reasonable. Without valinomycin the decrease in the external K^+ concentration (or uptake of K^+) in respiring mitochondria under the conditions used here occurred very slowly toward the level that was quickly reached by addition of valinomycin.

Heterogeneity in energetic characteristics of mitochondria in a sample is difficult to assess. The internal P_i peak had a comparable peak width at aerobic state to the one seen in the anaerobic condition, indicating the heterogeneous distribution of the internal pH value was not large (<0.2 pH unit) (Ogawa et al., 1981). There might be among mitochondria in a sample some distribution in energetic conditions caused by, for example, different degrees of proton leakage across the membrane. In the averaging of those relevant quantities to get the energetic parameters, however, the value of n_a does not depend much on the distribution, if n and ΔpH (and therefore $[P_i^{int}]$) are common among mitochondria. Even in a case of a 50/50 mixture of mitochondria with an ATP/ADP ratio of 5 and 1, n_a differs from n by only 0.1, assuming every mitochondrion is at steady state and has the same amount of ATP plus ADP content.

Although it is far from being established, the value of n_a seems to be a noninteger number between 2 and 3, and it could vary with the metabolic condition of mitochondria. We have not investigated the nature of the metabolic conditions that cause the variation of n_a. The present observations, however, are consistent to the report that n varried with $\Delta\mu_H$ upon additions of proton ionophores when the energetic state was monitored by the external phosphorylation potential (Azzone et al., 1978). The present results also supports the notion of Rottenberg (1978, 1979b) that there is some contribution of direct energy coupling between an energy producing site and the using site (ATPase) by local proton circulations in addition to the simple chemiosmotic process, in which protons go through the bulk solvent phases. If this is the case, those ATPase sites that operate in the scheme of the simple chemiosmotic mechanism must be hydrolyzing ATP at the steady state of no net ATP production. The sign of ΔG in eq 1 for those ATPases is therefore positive, not zero as in the case of equilibrium. The stoichiometric number n is then smaller than the apparent value n_a, and $n = 2$ could be the case.

It is interesting to note in the results shown in Figure 5 that there seems to be a maximum value in $-\Delta\mu_H$ at around 210 mV, although the internal phosphorylation potential varies at a near-maximum value of $-\Delta\mu_H$. Nicholls (1974) showed that at state 4 the proton leakage through the mitochondrial membrane increased nonlinearly with $-\Delta\mu_H$ beyond a certain level of $\Delta\mu_H$ so that there was a maximum in $-\Delta\mu_H$. The present results may well be explained by his observations. In that case at lower glutamate concentration (with slightly different lower respiration rates) mitochondria were converting energy more efficiently at a slightly lower level of $-\Delta\mu_H$.

The present study is still only a beginning of this type of measurements. We need to have well-defined metabolic conditions for the measurement of those energetic parameters. Improved sensitivity in recent NMR instrumentation by a factor of more than 2 from the instrument we have used here will allow us to have better control of the condition of these measurements, together with capabilities of monitoring more parameters such as respiration rates and concentrations of nonphosphorus metabolites simultaneously. It will be feasible to study effects of uncouplers and of transitions from state 4 to state 3 or from aerobic state to anaerobic state (ATP synthesis to ATP hydrolysis condition). Furthermore, detailed analysis of the characteristics of those NMR resonance of P_i, ATP, and ADP inside and outside mitochondria will give us good insight into what is happening to those compounds not only in their concentrations but also in their kinetical behaviors.

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