Kinetic Control of Mitochondrial ATP Synthesis†

K. F. LaNoue,* F. M. H. Jeffries, and G. K. Radda

Department of Biochemistry, University of Oxford, Oxford OX1 3QO, United Kingdom

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ABSTRACT: In order to gain a clearer understanding of the kinetic control of ATP synthesis, rat liver and rat heart mitochondria were incubated under conditions that resulted in various rates of net ATP synthesis or ATP hydrolysis. Radiolabeled phosphate was included in the incubation media, and exchange rates between phosphate and ATP were determined as a function of rates of net ATP synthesis. Since ATP synthase is a highly reversible enzyme, the catalyzed reaction was expected to approach equilibrium especially at low rates of respiration and net ATP synthesis. Thus ADP + $P_i \underset{V_2}{\overset{V_1}{\rightleftarrows}}$ ATP. If V_1 is the rate of incorporation of radiolabeled phosphate into ATP, then net ATP synthesis (or hydrolysis) is $V_1 - V_2$. Since V_1 and $V_1 - V_2$ could be measured, it was possible to calculate V_2. V_1 doubled in the transition from zero to maximal net ATP synthesis, whereas V_2 decreased by over 90% when the rate of ATP synthesis was high due to high-media ADP. In heart mitochondria at 37 °C when respiration increased from 104 ± 10 to 842 ± 51 nanoatoms of O_2/(min·mg), incorporation of [^{33}P]phosphate into ATP (V_1) increased from 1100 ± 60 to 1978 ± 121 and V_2 decreased from 1100 to near zero. These data demonstrate that mitochondrial ATP synthesis does not occur near equilibrium under physiological conditions and relatively high rates of ATP synthesis. A reaction with a high ratio of forward to reverse flux is obviously not near equilibrium. The important most sensitively controlled reaction appears to be V_2, ATP hydrolysis. Possible mechanisms of kinetic control of V_2 are discussed. Other observations made in connection with these studies showed a lack of correlation between exchange rates and $\Delta\mu H^+$. The possible contribution of the inhibitory subunit of the mitochondria ATPase to this lack of correlation is discussed.

In most cells oxygen consumption is linked stoichiometrically to rates of ATP[1] synthesis. The two processes are so tightly coupled that oxygen consumption is slow if ATP synthesis is blocked by lack of its substrates (ADP or inorganic phosphate) or by specific inhibitors of ATP synthesis such as oligomycin (Nicholls, 1982). Studies of isolated mitochondria show that respiration is a function of the ratios of ATP/ADP in the suspending media or of the media phosphorylation potential, ATP/(ADP)(P_i) when phosphate levels are lower than 2–3 mM (Davis & Davis-van Thienen, 1978; Kunz et al., 1981, 1983). Studies aimed toward defining the important rate-limiting steps in the pathways of net ATP synthesis have been carried out in recent years (Burat et al., 1984; Verhoeven et al., 1985; Jacobus et al., 1982; Erecinska & Wilson, 1982), while other studies have attempted to quantitate precise relationships between rates of ATP synthesis and its driving force $\delta\mu H^+$ (Rottenberg, 1983; Ferguson & Sorgato, 1982). Measurements of the relationship between $\Delta\mu H^+$ and rates of net ATP synthesis actually show rather poor correlation. This may be because of localized proton currents or because factors other than $\Delta\mu H^+$ have an important influence on the kinetics of ATP synthesis.

Studies designed to identify steps that are near equilibrium and rate-limiting step(s) in ATP synthesis are important because they define sites where hormones or other effectors may influence flux or where pathology may interfere with function. It is advantageous to identify metabolic steps that operate near equilibrium because moderate changes in the activity of enzymes which catalyze such steps do not influence flux in the pathways of which the enzymes are a part. Since their activity is in large excess, neither the ratio of substrates to products (mass action ratio) nor the net flux through such steps should be influenced by substantial decreases in enzymatic activity. If one could demonstrate that the adenine nucleotide translocase and the ATPase operate near equilibrium under most physiological conditions, one could conclude that their activities do not normally control or influence either flux or cytosolic ratios of ATP/ADP.

The "flux generating" step of ATP synthesis (Newsholme & Start, 1973) must be ATP utilization at least in viable cells, since ATP synthesis must equal ATP utilization. Therefore enzymes in the ATP synthetic pathway of viable cells should not be rate-controlling. If enzymes involved in synthesis such as the translocase and the ATP synthase are far from equilibrium, then their flux must be controlled by changes in the concentrations of substrates and/or products. Thus if the enzymes are far from equilibrium, substantial decreases in synthetic activity (V_{max}) would result either in a substantial decrease in flux near V_{max} or in a substantial change in the ratio of products/substrates (ATP/ADP) at lower net fluxes. A change in the cytosolic ratio of ATP/ADP at a fixed rate of ATP utilization by the cell would have dramatic influences on cytosolic functions. Data already reported (Burat et al., 1984; Verhoeven et al., 1985) for isolated mitochondria support the conclusion that if net flux is held constant a change in the activity of the translocase alters the steady-state ratio of ATP/ADP in the external media.

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* Address correspondence to this author at her present address: Department of Physiology, The Milton S. Hershey Medical Center, The Pennsylvania State University, Hershey, PA 17033.

[1] Abbreviations: EDTA, ethylenediaminetetraacetate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; ATP, adenosine 5′-triphosphate; ADP, adenosine 5′-diphosphate; P_i, inorganic phosphate; NADH, reduced nicotinamide adenine dinucleotide.

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It was initially proposed by Chance and Williams (1956) that respiration is kinetically controlled by ADP availability, a hypothesis which has received renewed support from the studies of Jacobus et al. (1982). However, a major controversy has centered on whether or not adenine nucleotide transport across the mitochondrial inner membrane is rate-limiting for respiration. As a consequence of this controversy, two hypotheses have emerged over the past several years. Erecinska and co-workers (Erecinska & Wilson, 1982; Erecinska et al., 1977) have proposed a "near-equilibrium hypothesis", whereas others have suggested that transport of ADP is rate-limiting.

According to the near-equilibrium hypothesis the mitochondrial electron-transport chain between NADH and cytochrome *c* is close to equilibrium with the external phosphorylation potential, (ATP)/(ADP)(P_i), through the enzymes of oxidative phosphorylation and the adenine nucleotides translocase. This implies that the redox state of cytochrome *c* is a function of the cytosolic phosphorylation potential. The cytochrome *c* redox state then sets the rate of O_2 consumption since it provides electrons to cytochrome oxidase. The reaction catalyzed by cytochrome oxidase, the reduction of molecular O_2, is demonstrably very far from equilibrium. The equilibrium theory of the control of respiration suggests that O_2 consumption is proportional to the concentration of the substrate of the oxidase, reduced cytochrome *c*, which is in turn set by the position of the adenine nucleotide and oxidation reduction state equilibria. Details of this theory of the control of O_2 consumption have been reviewed (Erecinska & Wilson, 1982).

When a reaction is in equilibrium, the forward and reverse reaction rates are equal; a near-equilibrium state is one in which they are nearly equal, or when the differrence is small with respect to total forward and reverse fluxes. When mitochondrial respiration is slow because of high external ratios of ATP/ADP, rapid exchange between radiolabeled phosphate and ATP can be demonstrated (Swanson, 1956; Cohn & Drysdale, 1955). The forward and reverse fluxes are equal when the steady state is achieved. This steady state is commonly called state 4, in accordance with the original terminology of Chance and Williams (1956), and it is clear that this is an equilibrium or at least a near-equilibrium state. Quantitation of isotopic ATP/P_i exchanges, as respiration rates increase, due to decreasing ratios of ATP/ADP has not been reported. However, measurements of the rate of the forward reaction, that is, the transfer of inorganic phosphate to ATP in perfused hearts (Matthews et al., 1981), in kidneys (Freeman et al., 1983), and in brain tissue (Shoubridge et al., 1982) in situ have been carried out by using the technique of nuclear magnetic saturation transfer. During the measurements, the organs were respiring (and thus synthesizing ATP) at normal but not maximal rates. These studies showed that the rate of transfer of P_i to ATP was approximately 6 times the rate of O_2 consumption on a mole-for-mole basis. Since the theoretical ratio of O_2 consumed to net ATP synthesized is 6, the results suggested that the reverse reaction was negligible in these organs, even when net ATP synthesis was less than maximal. Such a result was not anticipated in view of the data on isotope exchange rates (Swanson, 1956; Cohn & Drysdale, 1955).

In order to study the phenomenon in greater detail and determine whether the ATP synthase operates at near equilibrium during rapid net ATP synthesis, we have measured the rate of radiolabeled inorganic phosphate transfer to ATP as a function of net ATP synthesis and oxygen consumption, using conditions similar to those in the intact cell.

The results show that as respiration rates increase toward maximal, the forward reaction doubles and the backward reaction approaches zero. Additionally, when respiration was inhibited, by the respiratory poison rotenone, the exchange rate was significantly decreased. The results show that the mitochondrial ATP synthase reaction is not near equilibrium except at low rates of respiration. In addition they show that factors other than $\Delta\mu H^+$ and substrate levels may affect the activity of the synthase.

Experimental Procedures

Rat liver mitochondria were prepared by standard procedures of differential centrifugation (Schneider & Hogeboom, 1950). Rat heart mitochondria were prepared by a modification of the method of Chance and Hagihara (1960). As normally prepared these mitochondria contain high levels of a Mg^{2+}-stimulated ATPase activity, presumably due to broken pieces of mitochondrial membrane. A method of separating the Mg^{2+}-stimulated ATPase from intact mitochondria by density gradient centrifugation has been described (Saks et al., 1975). Since the high Mg^{2+}-ATPase activity interferes with precise measurement of rates of ATP synthesis, the method of Chance and Hagihara was modified to minimize that activity.

Rat hearts, with a small length of the aorta still attached, were quickly excised from anesthetized rats and placed in an ice-cold solution of mannitol (225 mM), sucrose (75 mM), EDTA (0.05 mM), and HEPES (5 mM) (MSE media) adjusted to pH 7.0. The aortas were cannulated, and hearts were flushed by retrograde Langendorff perfusion using 15 mL of the ice-cold MSE media. Then the hearts were perfused with 6 mL of MSE media containing 0.5 mg/mL of the protease Nagarse adjusted to pH 7.5. The Nagarse perfused hearts were minced and incubated at 0 °C in additional MSE containing 0.5 mg/mL Nagarse (~5 mL/heart). After 5 min, the Nagarse solution was diluted 5-fold and the minced heart was homogenized by hand with a Potter Elvehjem type homogenizer. Alternatively, the mince may be homogenized by very brief treatment (10 s) with a Polytron tissue disrupter set at very low speed. After the homogenization step, the normal procedures of differential centrifugation (Chance & Hagihara, 1960) were followed in order to isolate well-coupled heart mitochondria. The yield of mitochondria was 10–15 mg/heart. The respiratory control ratio of the heart mitochondria varied from 8 to 15. The respiration rate in the presence of substrate, 5 mM external ATP but no added ADP, was not stimulated by Mg^{2+} and was only slightly inhibited by carboxyatractyloside, an inhibitor of adenine nucleotide translocase (Vignais et al., 1973).

The rate of transfer of inorganic phosphate to ATP was measured isotopically as described by Rosing et al. (1977). Briefly, the mitochondria were incubated with radiolabeled inorganic phosphate and unlabeled ATP. At appropriate times the samples were quenched with 6% perchloric acid and neutralized, and aliquots were treated with ammonium molybdate. The ammonium molybdate inorganic phosphate complex was extracted from the aqueous media by vortexing with an equal volume of a 1:1 mixture of isobutyl alcohol and benzene. The aqueous fraction was counted to determine radioactivity of organic phosphate. Inorganic phosphate was assayed in the protein-free filtrate before extraction with isobutyl alcohol/benzene according to Baginsky et al. (1967). ATP, ADP, AMP, and glucose 6-phosphate were assayed spectrophotometrically by enzymatic procedures (Williamson & Corkey, 1969). Oxygen consumption was determined polarographically with a Clark electrode. Mitochondrial incu-

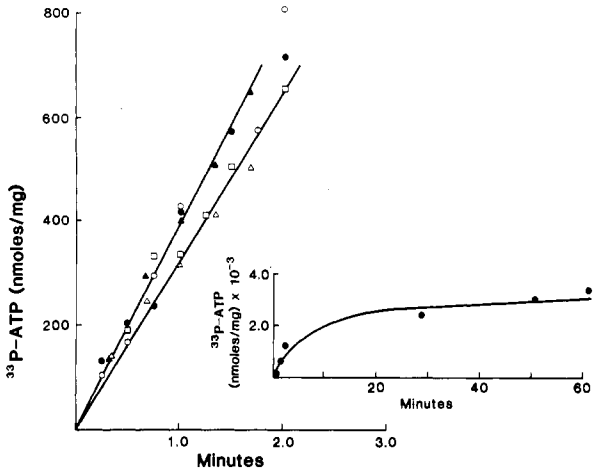

FIGURE 1: Effect of external ATP on the rate of incorporation of [^{33}P]phosphate into ATP under conditions of net ATP synthesis (state 3) in rat liver mitochondria. Rat liver mitochondria (1.0 mg/mL) were added to an incubation media containing 150 mM KCl, 20 mM HEPES, 5 mM $MgCl_2$, 5 mM KH_2PO_j (0.05 μCi/mL), and 10 mM glutamate (pH 7.2). In some cases (●, ▲) 5 mM ADP was present, in others (○, △) 5 mM ADP plus 5 mM ATP was present, and in still others (□) 1.0 mM ADP and 5 mM ATP was present. Samples (0.4 mL) were taken at the times shown, quenched with perchloric acid, and neutralized. The total sample radioactivity and initial phosphate concentrations were determined. The incorporation of ^{33}P into ATP was determined as described in Experimental Procedures, and radioactivity was converted to nanomoles per milligram of protein by dividing by the specific activity of phosphate and the milligrams of mitochondrial protein per milliliter.

bations were carried out in a buffer composed of 150 mM KCl, 20 mM HEPES, 5 mM KH_2PO_4, 0.05 mM EDTA, and 5 mM $MgCl_2$.

RESULTS

Since the mitochondrial ATP synthase is a readily reversible enzyme (Boyer et al., 1954), net ATP synthesis may be viewed as the difference between the forward (V_1) and the reverse (V_2) rates. Thus

$$ADP + P_i \underset{V_2}{\overset{V_1}{\rightleftharpoons}} ATP$$

The net ATP synthesized is available to enzymes in the cytosol which consume ATP, and the rate of net cellular ATP utilization is of course equal to the rate of net synthesis (i.e., $V_1 - V_2$). Oxygen consumption should be approximately equal to $6 \times (V_1 - V_2)$ on a mole-for-mole basis. In order to determine whether the mitochondrial ATP synthase is near equilibrium, attempts were made to measure V_1, the rate of the transfer of radiolabeled P_i to ATP, and its relationship to net ATP synthesis ($V_1 - V_2$) and O_2 consumption, using both rat liver and rat heart mitochondria.

Liver Mitochondria. Initial studies were carried out with rat liver mitochondria at 30 °C. The mean ± standard error of the mean of mitochondrial respiration rates in the presence of 5 mM ADP, 5 mM phosphate, 10 mM glutamate, and 1 mM malate was 169.3 ± 9.5 nanoatoms of O_2/(min·mg) (state 3). In the absence of ADP but in the presence of 5 mM ATP, rates were 27.3 ± 3.2 nanoatoms of O_2/(min·mg) (state 4). Multiplying the increase in respiration due to ADP by the theoretical P/O rate of 3 makes it possible to estimate maximal rates of net ATP synthesis to be 427 nmol/(min·mg) in state 3.

Respiration rates in the presence of 1 mM ADP were not changed by the addition of 1–5 mM ATP. In order to measure V_1, the rate of transfer of radiolabeled inorganic phosphate to ATP, liver mitochondria were incubated in dilute suspension

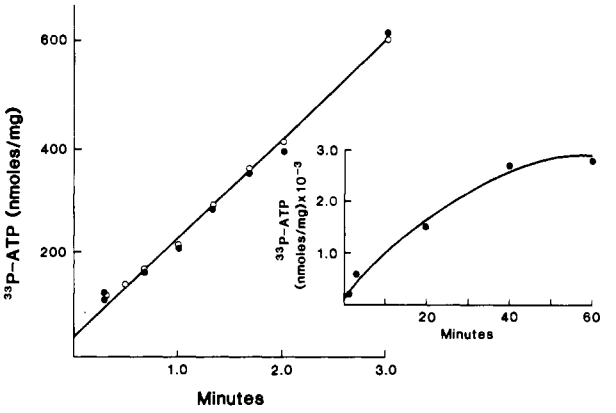

FIGURE 2: Incorporation of [^{33}P]phosphate into ATP under steady-state conditions with glutamate and ATP present (state 4). Conditions were the same as those described in Figure 1 except that 5 mM ATP was added instead of 5 mM ADP. In some cases (●) the incubation was begun by addition of mitochondria to the media, while in others (○) mitochondria were added 30 s prior to the addition of tracer 0.05 μCi [^{33}P]phosphate.

with 5 mM ADP and 5 mM [^{33}P]PO_4. The rate of transfer of radioactivity to the nucleotide was measured and is shown in Figure 1. Since $MgCl_2$ was present, it is likely that some adenylate kinase catalyzed conversion of the [γ-^{33}P]ATP formed to [β-^{33}P]ADP occurred during the course of the experiment. However, this does not affect the interpretation of the results. Since no ATP is present, initially one would expect V_2, the reverse reaction, to be negligible and V_1 to be similar to the maximum net rate of ATP synthesis, estimated from respiration rates. As shown, V_1 is 380 ± 25 nmol/(min·mg) when the ATP/ADP ratio is 0. This compares well with the value of 427 nanoatoms/(min·mg) estimated from O_2 consumption. The value of 380 was taken to be the correct rate of net ATP synthesis and compared to the rates obtained under other conditions in which the initial ATP level was in the millimolar range, equal to or higher than the initial concentration of ADP. In all these conditions (state 3) O_2 consumption was maximal at ~170 nanoatoms/(min·mg). As shown, the presence of ATP in 5-fold excess of ADP did not significantly alter the observed rate of transfer of phosphate to ATP. Thus, the reverse reaction, transfer of phosphate from ATP to inorganic phosphate, is negligible when ATP synthesis is maximal.

The inset of Figure 1 shows the incorporation of radiolabeled phosphate into ATP over a longer time period. The data were obtained from the same experimental reaction mixture in which the initial ratio of ATP/ADP was 5/1. The data show that [^{33}P]phosphate and ATP reach isotopic equilibrium relatively slowly and that during the initial early time points the rate of the back reaction is very slow relative to the forward reaction.

This experiment was repeated under three other metabolic conditions. In two of these conditions no ADP was added to the mitochondrial incubation media which contained 5 mM ATP and 5 mM [^{33}P]phosphate. In one of these conditions, glutamate and malate were also present (state 4). The results of those experiments are shown in Figure 2. In this state the ratio of ATP/ADP is maintained above 100 (Wanders et al., 1984; Gyulai et al., 1985), and no net ATP is synthesized. Figure 2 shows that the initial rate of ATP–phosphate exchange is 174 ± 14 nmol/(min·mg) and that equilibration requires about 40 min. Since net ATP synthesis is zero, the forward (V_1) and reverse (V_2) reaction rates are equal. In the third condition studied, respiratory substrate was omitted but the value of $\Delta\mu H^+$ was maintained high due to the net hy-

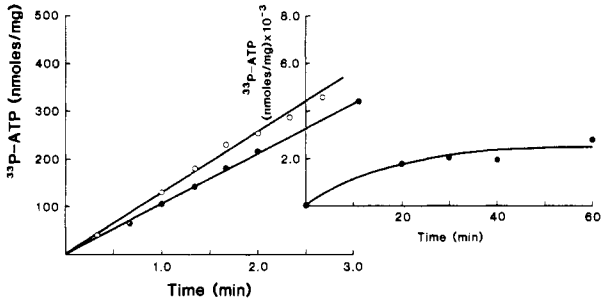

FIGURE 3: Effect of rotenone on the rate of incorporation of phosphate into ATP. The conditions were the same as those described for Figure 1 except that no ADP was added to the buffer and 5 μM rotenone plus 5 mM ATP were added. In some cases (●), the incubation was begun by addition of mitochondria to the buffer. In other cases (○), mitochondria were added 30 s before simultaneous addition of rotenone (5 μM) and 0.05 μCi [^{33}P]phosphate.

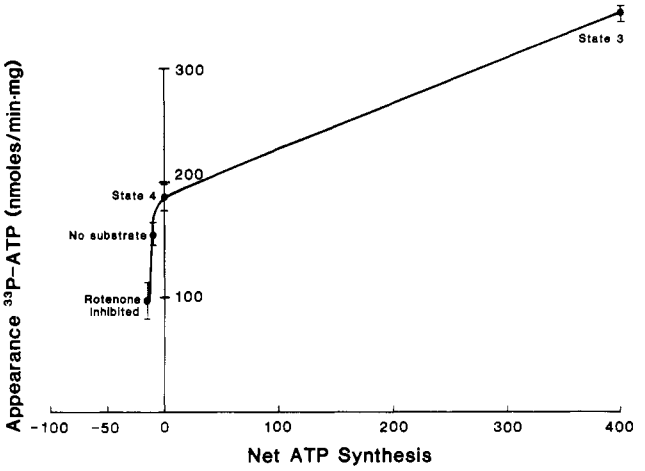

FIGURE 4: Relationship between net ATP synthesis and the rate of incorporation of radiolabeled phosphate into ATP. Conditions were the same as those described in Figures 1, 2, and 3, for the state 3, state 4, and rotenone-inhibited conditions. The "no substrate" condition shown here was obtained under the same conditions as those described for state 4 except that glutamate was omitted from the buffer. The values shown on the graph are the average of 3–4 separate experiments, and the error bars indicate SEM.

drolysis of ATP. The net rate of ATP hydrolysis was measured in separate experiments and found to be 12.1 ± 2 nmol of ATP/(min·mg). The rate of ATP/P_i exchange was 136 ± 11 nmol/(min·mg), only slightly less than the state 4 exchange rate. In the fourth metabolic state, glutamate and malate were present with ATP and phosphate, but rotenone was also added to inhibit the electron-transport chain. In this situation, ATP was hydrolyzed at about the same rate as in the absence of substrate and rotenone (11.5 nmol/(min·mg)), but the rate of the ATP/phosphate exchange was significantly slower (87 ± 12 nmol/(min·mg), cf. Figure 3). The possibility was considered that the exchange might be slow due to high mitochondrial AMP in the rotenone-inhibited state. If the AMP level in the presence of rotenone were much higher than in other conditions, it is possible that the initial transport of ATP into the mitochondria might be slow due to depletion of internal substrate for the adenine nucleotide exchange transporter (LaNoue et al., 1981). In order to avoid this potential artifact mitochondria were preincubated in state 4 for 1 min to convert most of the AMP to ADP and ATP. At 1 min, rotenone and ^{33}P were added to some incubations and ^{33}P alone to other incubations. The results of these studies are shown in Figures 2 and 3. The preincubation had very little effect on the results. Therefore, it appeared that the inhibition by rotenone was not due to substrate limitation of the transporter.

In order to determine whether rotenone had a direct effect on the ATPase or the translocase, other experiments were carried out in which the effect of succinate plus rotenone on the exchange rate was measured. Succinate bypasses the rotenone block of the electron-transport chain, so that in this condition, with rotenone present, the electron-transport chain is active, O_2 is consumed, and no net ATP is hydrolyzed. In this experiment, the exchange rate in state 4 with glutamate and no succinate was 176 ± 4 nmol/(min·mg), and rotenone lowered the rate to 93 ± 5. When succinate was also included, without rotenone the exchange was 164 ± 7 nmol/(min·mg) and with rotenone, 150 ± 10 nmol/(min·mg). Thus, in the presence of succinate, rotenone had no significant effect on the exchange rate. The average rates of exchange, obtained from four experiments with four different preparations of mitochondria, are shown in Figure 4, plotted as a function of net rates of ATP synthesis.

The data suggest that although the reverse reaction (V_2) of ATP synthesis is negligible when the forward reaction is maximal, at intermediate levels of ATP synthesis V_2 is appreciable. This is in contrast to the NMR data obtained with perfused hearts (Matthews et al., 1981), kidneys (Freeman et al., 1983) and brain (Shoubridge et al., 1982). However, no saturation transfer nuclear magnetic resonance data are available for liver. Therefore, isotopic exchange rates between [^{33}P]phosphate and ATP were measured in heart mitochondria.

Heart Mitochondria. Particular care was taken in these experiments to mimic the physiological situation in the heart as carefully as possible. The temperature was maintained at 37 °C, and experiments were performed not only in state 3 and state 4 but also at intermediate levels of ATP synthesis.

It also seemed important to assess the rate of net ATP synthesis directly rather than calculating it from oxygen consumption. This would not have been possibly initially in the presence of Mg^{2+} because of an external Mg^{2+} ATPase activity in the standard heart mitochondrial preparation. The rate of ATP hydrolysis by rotenone-inhibited heart mitochondria (at 37 °C) was increased from 149.2 to 354.0 nmol/(min·mg) by the presence of Mg^{2+} as assessed by rates of generation of inorganic phosphate. Mg^{2+} also decreased respiratory control rates from 8 to 3 or less. When an improved procedure for preparing heart mitochondria was developed (described in Experimental Procedures) the respiratory control ratios remained high even in 5 mM Mg^{2+}. In the presence of 5 mM Mg^{2+} and 5 mM phosphate the respiration rate in the absence of added ADP was 104 ± 21 nanoatoms of O_2/(min·mg) and this increased to 842 ± 51 nanoatoms/(min·mg) in the presence of excess ADP. Thus the rate of ATP synthesis assessed from the increase in O_2 consumption was 2214 nmol/(min·mg). ATP synthesis was measured directly by the increase in the amount of ATP in the media when mitochondria were incubated in the presence of 5 mM phosphate and ADP, and this was found be 1721 ± 150 nmol/(min·mg). The rate of synthesis of ATP and the rate of O_2 consumption were not changed by the addition of 5 mM ATP, in addition to 5 mM ADP, in the incubation media. The rate of transfer of radiolabeled phosphate to ATP was measured as in the experiments with liver mitochondria. The results are shown in Figure 5. Data shown as filled circles were obtained in the absence of added ATP, i.e., an initial ATP/ADP ratio of O, whereas the data shown as open circles were obtained when the initial ATP/ADP ratio was 1. In another experiment (shown as open squares, in Figure 5) the experiment was begun by simultaneous addition of mitochondria and hexokinase to

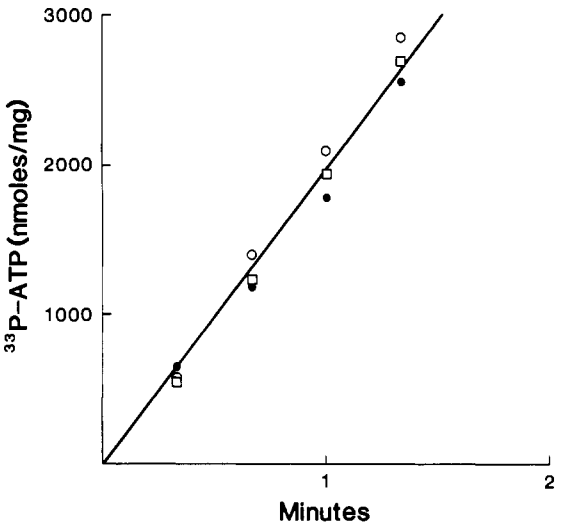

FIGURE 5: Effect of external ATP on the rate of incorporation of [^{33}P]phosphate into ATP under conditions of net ATP synthesis (state 3) in rat heart mitochondria. Rat heart mitochondria (0.4–0.5 mg/mL) were added to an incubation medium containing 150 mM KCl, 20 mM HEPES, 5 mM $MgCl_2$, 5 mM phosphate (0.05 μCi/mL), and 20 mM glutamate. In one case (□) 5 mM ADP was also present; in another case (○) 5 mM ATP and 5 mM ADP were present. In still another case (●), 5 mM ATP and 20 mM glucose were present, and hexokinase (1.5 units Sigma F300) was added 15 s prior to the addition of mitochondria. Assay of ATP and ADP indicated that the ratio of ATP/ADP in this condition was 5–7 during the first 40 s after addition of mitochondria and declined thereafter. The rate of incorporation of [^{33}P]phosphate into ATP was determined as in Figure 1.

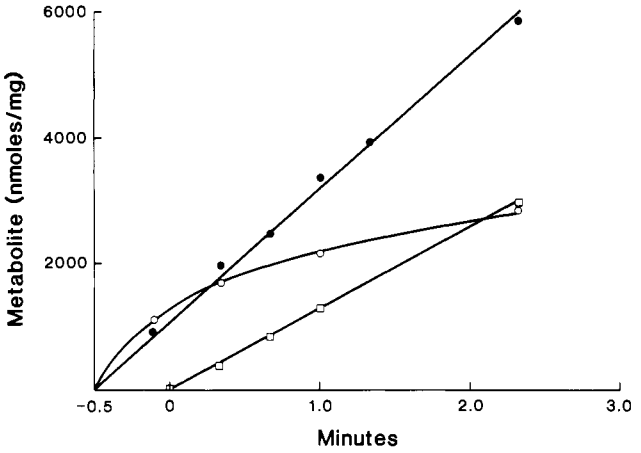

FIGURE 6: Changes in external nucleotides and in glucose 6-phosphate during incubation of heart mitochondria with a low concentration of a hexokinase. Mitochondria (~0.5 mg/mL) were added to the buffer described in Figure 5 plus 5 mM ATP. Glucose (20 mM) was included in the buffer, and hexokinase (0.82 units/mL) was added within 10 s after the mitochondria (–0.5 min). A t = 0 the tracer, 0.05 μCi/mL [^{33}P]phosphate, was added to the media. Samples were taken for determinations of ATP, ADP glucose 6-phosphate, and ^{33}P incorporation into ATP at the times shown. Net ATP synthesis (open squares) was calculated as the difference between the rate of glucose 6-phosphate formation (filled circles) and the rate of ADP formation (open circles).

a media containing 5 mM ATP and 5 mM phosphate. The hexokinase was sufficiently active to maintain the ratio of ATP/ADP = 4 during the first 20–40 s of the experiment. The rate of ATP synthesis was estimated by subtracting the rate of increase of ADP from the increase in glucose 6-phosphate. As indicated in Figure 5 the rate of transfer of phosphate to ATP was the same in all three conditions, and the initial ratio ATP/ADP did not alter the results.

However, when the mitochondria were incubated with much

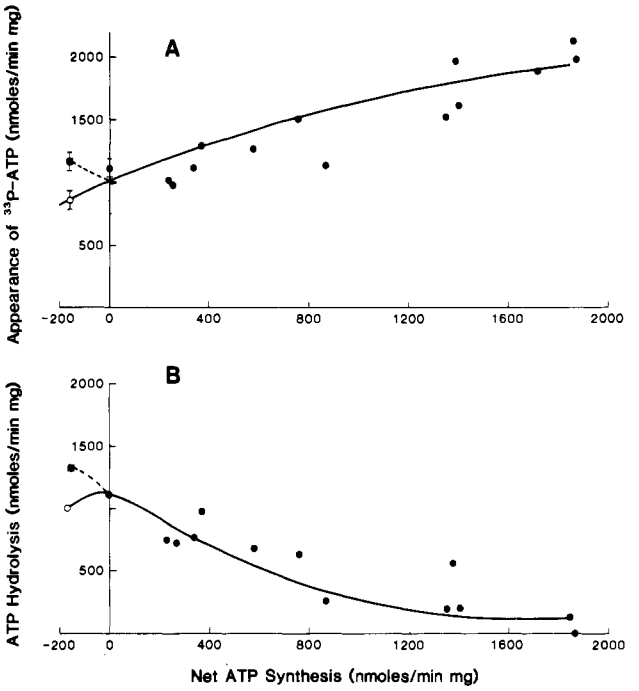

FIGURE 7: Relationship between net ATP synthesis and the incorporation of [^{33}P]phosphate into ATP in rat heart mitochondria. Rat heart mitochondria were added to the buffer described in Figure 5 which included 20 mM glutamate, 20 mM glucose, and 5 mM ATP. In most experiments (●) hexokinase (0.1–2 units/mL) was added to the media within 10 s after the mitochondria in order to vary rates of net ATP synthesis, which was determined as shown in Figure 6. At t = 0, tracer 0.05 μCi [^{33}P]phosphate was added. Samples were taken as shown in Figure 6 and assayed for ATP, ADP, glucose 6-phosphate, and [^{33}P]phosphate incorporation into ATP. In some incubations, no hexokinase was added, and glutamate was omitted from the media (■). In other incubations (○) no hexokinase was added, but rotenone (5 mM) was included in the buffer. In these incubations, [^{33}P]phosphate incorporation into ATP was measured. However, in both these conditions net rates of ATP hydrolysis were determined in separate incubations which included 5 mM ATP but no inorganic or tracer ^{33}P. Production of phosphate was used to monitor ATP hydrolysis. Thus panel A represents the relationship of V_1 to net ATP synthesis, and panel B represents the relationship of V_2 to net ATP synthesis.

higher ratios of ATP/ADP, different results were obtained. Respiration rates between states 3 and 4 were obtained by incubating mitochondria with suboptimal amounts of a hexokinase trapping system. Mitochondria were added to a buffer containing unlabeled phosphate (5 mM) and ATP (5 mM). Figure 6 is an example of how the data for net ATP synthesis were obtained for one of the data points shown in Figure 7. Suboptimal amounts of hexokinase insufficient to maximally stimulate respiration were added immediately after the mitochondria. Hexokinase generated ADP, which in turn stimulated respiration and the rate of ATP synthesis. Synthesis of ATP slowed down the apparent rate of ADP generation until a steady-state ratio of ATP/ADP was approached, and the rate of glucose 6-phosphate formation was equal to net synthesis. ATP, ADP, and AMP were measured enzymatically after perchloric acid extraction in these experiments. AMP levels remained below (~0.05 mM), demonstrating low activity of adenylate kinase under these conditions. The measured value of ADP at steady state varied between 0.1 mM in state 4 and 0.7 mM in conditions approaching state 3. Special precautions were not taken to prevent ATP hydrolysis in the 0.35 M perchloric acid mitochondrial extracts, and therefore these values are probably much higher than those present during the actual incubation. A recent study of conditions required for an accurate estimation of the extramito-

chondrial ATP/ADP ratios in isolated mitochondria showed that the conditions used in our study for extraction would have resulted in significant hydrolysis of ATP. Wanders et al. (1984) have shown that perchloric acid extraction results in artifactually low ATP/ADP values even when samples are neutralized immediately. When mitochondria were incubated in state 4 conditions and the incubation mixture quenched with an organic solvent mixture, the ratio ATP/ADP in state 4 was ~1500. When the rate of respiration was set at 70% of maximal with a hexokinase trap, the measured ratio ATP/ADP was 70. Moreover, in another study of the relationship of the phosphorylation potential to respiration rates in heart mitochondria the ATP/ADP ratio in state 4 was over 3000 even in the absence of added phosphate. These measurements were made without any chemical quenching, using NMR techniques. Our assays were used to estimate fluxes from changes in ADP and glucose 6-phosphate as a function of incubation time, and the absolute measured values of ATP/ADP are undoubtedly artifactually low.

In the experiment of Figure 6 the rate of mitochondrial ATP synthesis was estimated by subtracting the small rate of increase in ADP from the rate of synthesis of glucose 6-phosphate. This provided the abscissa values for the data points of Figure 7. In order to obtain the ordinant values tracer [^{33}P]phosphate was added and the rate of transfer of phosphate to ATP determined. A series of such experiments were carried out with different quantities of hexokinase from 0.05 to 0.75 units/mL. The rate of transfer of phosphate to ATP was also measured when net ATP synthesis was zero in state 4 and when it was negative (i.e., in the absence of substrate and also in the presence of substrate plus rotenone). The measured rate of ATP hydrolysis in the absence of respiratory substrate but in the presence of 5 mM Mg^{2+} and 5 mM ATP was 92 nmol/(min·mg). In the presence of glutamate and malate plus rotenone the rate was 114 nmol/(min·mg). Figure 7 summarizes the data obtained and illustrates the relationships between the net rate of ATP synthesis and the forward (V_1) and reverse (V_2) rates of the ATP synthase reaction. Rates of respiration were also measured, and P/O ratios were approximately constant and equal to 2.3. The inhibition of exchange by rotenone is not as great in heart as in liver but is nevertheless significant.

The qualitative relationship between net flux and the forward and reverse fluxes measured isotopically is similar in heart and liver. The cause of the inhibition of the exchange rates by rotenone is particularly intriguing. Also, the observation of little or no backward reaction in state 3 was unexpected. In this situation the back reaction must be inhibited by the product, ADP, either at the level of the translocase or at the ATPase.

Measurements of $\Delta\psi_m$ and ΔpH in Heart and Liver Mitochondria. Since the mitochondrial ATP synthase is a proton pump and the rate of unidirectional ATP synthesis or hydrolysis is likely to be controlled not only by the level of substrates and products (ADP, phosphate, and ATP) but also by the value of the electrochemical potential gradient of protons across the mitochondrial inner membrane, it seemed important to measure the electrical chemical potential gradient of protons ($\Delta\mu H^+$) across the inner mitochondrial membrane of liver and heart mitochondria under the conditions in which we had measured ATP/P_i exchange rates. Since $\Delta\mu H^+$ includes electrical ($\Delta\psi$) and chemical (ΔpH) components, both were measured. The definition of $\Delta\mu H^+$ is

$$\Delta\mu H^+ = \Delta\psi_m + \frac{RT}{F}\Delta pH$$

Determinations of $\Delta\psi_m$ were carried out under conditions very similar to those in which the exchanges were measured, except that the lipophilic cation tetraphenylphosphonium was included in the media. The use of the tetraphenylphosphonium ion (TPP^+) (Wilson & Forman, 1982; Lichtshtein et al., 1979; Brand & Felber, 1984; Rottenberg, 1984) to estimate $\Delta\psi_m$ has been extensively described. Using TPP^+, we estimated the value of $\Delta\psi_m$ from the Nernst equation

$$\Delta\psi_m = \frac{RT}{F}\log\frac{(TPP^+)_m}{(TPP^+)_e}$$

where R, T, and F have their usual meaning, $(TPP^+)_m$ is the concentration of TPP^+ free inside the mitochondria, and $(TPP^+)_e$ is the media TPP^+ concentration. Unfortunately, the method is complicated by the fact that TPP^+ binds to the mitochondrial membranes, and the measured accumulation of TPP^+ must be corrected for binding before the appropriate calculation is carried out.

We have used Rottenberg's method (1984) in order to correct for binding. It produces the best agreement between $\Delta\psi_m$ measured by using valinomycin plus K^+ and $\Delta\psi_m$ measured by using TPP^+. The method involves independent determinations of internal and external binding constants for TPP^+ and use of these constants to correct the observed gradients of TPP^+. The data obtained for liver are shown in Table I. The values obtained for the binding constants were determined independently by us but are the same as those obtained by Rottenberg (1984) for liver, and values of $\Delta\psi_m$ in state 4 are similar to published values. Our adaptation of Rottenberg's method has been described recently (LaNoue et al., 1985). The value of $\Delta\psi_m$ in state 3 obtained by this procedure is lower than values published previously. Values in the presence of ATP and without respiratory substrate or with substrate plus rotenone have not been published previously but are similar in magnitude to those measured for state 3. The method of Addanki (1968) was used to determine ΔpH. Briefly, mitochondria were incubated under the four standard conditions and 5,5′-dimethyloxazolidine-2,4-dione (DMO) was included in the media. This weak acid is permeable to the mitochondrial membrane in the protonated form, but impermeable as the anion. Thus the distribution of the radiolabeled compound can be used to calculate the H^+ distribution across the mitochondrial membrane. In order to allow correlations to be made between V_2 and $\Delta\mu H^+$, measured values for V_2 are also shown in the table.

Table II shows the same experiments carried out for heart mitochondria. The values obtained for the TPP^+ binding constants are not the same as for liver, and the difference is probably due to the larger area of inner mitochondrial membrane per milligram of mitochondrial protein in heart mitochondria as compared to liver. The absolute values of $\Delta\psi_m$ are significantly lower in heart than in liver, but the relative differences between metabolic states are the same. Thus, in both tissues $\Delta\psi_m$ is highest in state 4, as one would have expected, and lowest or insignificantly different from the lowest in the rotenone-inhibited state, with ATP. The value of $\Delta\psi_m$ in state 3 is somewhat lower than that measured with ATP and no substrate. For comparative purposes, values of V_2 for heart are also shown in Table II.

From these data it is possible to conclude that the low value of $\Delta\mu H^+$ in the rotenone-inhibited state does not limit the rate of phosphate incorporation into ATP (V_1). Likewise, as clearly demonstrated by Tables I and II, V_2 does not exhibit a negative correlation with $\Delta\mu H^+$. $\Delta\mu H^+$ is almost as low in state 3 as in the rotenone-inhibited state, and P_i incorporation into ATP

Table I: Influence of Metabolic State on Electrochemical Potential Gradient of H^+ across the Inner Membrane ($\Delta\psi_m$) of Rat Liver Mitochondria[a]

	$\{[TPP^+]_m \cdot mg\}/(TPP^+)_e$ (μL/mg)	$(TPP^+)_m/(TPP^+)_e$	$\Delta\psi_m$ (mV)	ΔpH	$\Delta\mu H$ (mV)	V_2 [nmol/(min·mg)]
state 4	1844 ± 129	194 ± 12.4	142.8 ± 1.5	0.85 ± 0.15	195.1	174 ± 14
state 3	417 ± 44	38.3 ± 4.7	96.6 ± 3.2	0.79 ± 0.10	145.2	0 ± 27
no substrate	942 ± 35.7	102 ± 4.4	122.5 ± 2.2	0.87 ± 0.16	176.0	148 ± 13
rotenone inhibited	423 ± 22.7	41.2 ± 2.5	98.5 ± 1.6	0.80 ± 0.09	147.7	98.5 ± 12

[a] Rat liver mitochondria were incubated at 30 °C in a buffer (pH 7.2) containing 130 mM KCl, 20 mM choline chloride, 7% dextran, 20 mM HEPES, 5 mM KH_2PO_4, 5 mM $MgCl_2$, 5 mM ATP, and 20 mM glutamate. In order to measure $\Delta\psi_m$ the protein concentration was 0.8 mg/mL, and 0.2 μM [^{3}H]TPP$^+$ (1.0 μCi/mL) and [^{14}C]sucrose (0.1 μCi/mL) were also included in the media. When ΔpH was measured, protein concentration was 1.5 mg/mL and 5,5′-dimethyloxazolidine-2,4-dione (DMO) (0.1 μCi/mL) and ^3H_2O (1 μCi/mL) were included in the media. In *state 4* there were no other additions; in *state 3*, 5 mM ADP was also present. *No substrate* signifies the omission of glutamate, and the *rotenone-inhibited* condition was the same as state 4, but 5 μM rotenone was also present. Samples were then taken at 3, 3.5, and 4.0 min, and mitochondria were rapidly separated from their media by using centrifugal filtration through silicone oil (LaNoue et al., 1973). The mitochondrial and supernatant fractions were counted in order to determine the radioactivity of (TPP$^+$) or (DMO) accumulated into the matrix space. The radioactivity of [^{14}C]sucrose was used to correct the mitochondrial fraction for TPP$^+$ in adhering external media. The term $\{[TPP^+]_m \cdot mg\}/(TPP^+)_e$ is cpm of TPP$^+$ in the mitochondrial fraction per mg of mitochondria divided by the cpm of TPP$^+$ in the external media per μL of external media. In separate incubations mitochondria were incubated in the same media replacing [^{3}H]TPP$^+$ with ^3H_2O (1.0 μCi/mL) or [^{14}C]DMO with [^{14}C]sucrose in order to calculate the volume of the matrix aqueous space. Binding constants external *a* and internal *b* for TPP$^+$ were determined in separate experiments as described by Rottenberg (1984) and modified by LaNoue et al. (1985). The method of Addanki et al. (1968) was used to calculate ΔpH from the gradients of [^{14}C]DMO across the membrane. The equation for TPP$^+$ binding is $[TPP^+]\text{bound/mg} = a(TPP^+)_e + b(TPP^+)_m$, where $(TPP^+)_m$ is the concentration of free TPP$^+$ in mitochondrial matrix (cpm/μL), $(TPP^+)_e$ is the concentration of TPP$^+$ in the external media (cpm/μL), $(TPP^+)_m/(TPP^+)_e$ is the ratio of TPP$^+$ in/out used to calculate $\Delta\psi_m$ according to the Nernst equation, *a* (external binding constant) = 55, *b* (internal binding constant) = 7.45, and V_2 is the rate reversal of ATP synthesis measured as the difference between net ATP synthesis and the rate of isotopic labeling of ATP by [^{33}P]phosphate as described in Figure 4.

Table II: Influence of Metabolic State on $\Delta\psi_m$ and ΔpH in Rat Heart Mitochondria[a]

	$\{[TPP^+]_m \cdot mg\}/(TPP^+)_e$ (μL/mg)	$(TPP^+)_m/(TPP^+)_e$	$\Delta\psi_m$ (mV)	ΔpH	$\Delta\mu H^+$ (mV)	V_2 [nmol/(min·mg)]
state 4	1191 ± 31	39.9 ± 6.4	97.6 ± 9.9	0.91 ± 0.13	153.6	1101 ± 60
state 3	624.5 ± 26	20 ± 5	78.9 ± 9.0	0.94 ± 0.12	136.7	0 ± 120
no substrate	652 ± 66	21.8 ± 4.6	82.3 ± 9.0	0.86 ± 0.08	135.2	1262 ± 97
rotenone inhibited	427 ± 33	13.9 ± 3.6	69.8 ± 8.1	0.81 ± 0.04	119.6	964 ± 48

[a] Mitochondria were incubated under conditions identical with those described in Table I except that the temperature was maintained at 37 °C and heart rather than liver mitochondria were used. The protein concentration was 0.5 mg/mL in the incubations used to measure $\Delta\psi_m$ and 1.5 mg/mL in the incubations used to measure ΔpH. The calculations were the same as those described in Table I except that the external binding constant (*a*) of TPP$^+$ to heart mitochondria was 35, and *b*, the internal constant, was 27.7. Experimental conditions used to obtain V_2 are described in the legend of Figure 7. ΔpH was determined according to Addanki et al. (1968).

is highest in state 3. Similarly, the rate of net ATP hydrolysis ($V_1 - V_2$) is only slightly faster in the presence of substrate plus rotenone than in the absence of substrate. Calculation shows that V_2, ATP hydrolysis, is faster in the absence of substrate than in the presence of substrate plus rotenone, yet $\Delta\psi_m$ is higher in the absence of rotenone. Thus V_2 does not correlate negatively with $\Delta\mu H^+$. These observations argue against control of reaction rates by $\Delta\mu H^+$ or its components $\Delta\mu H^+$ and ΔpH under the conditions studied.

Discussion

The data suggest that the mitochondrial ATPase is not in equilibrium with the external adenine nucleotides and phosphate except when rates of ATP synthesis are relatively low. Figures 4 and 7 show that when net ATP synthesis is half-maximal the forward reaction is 3 times the back reaction.

Whether this can be termed near equilibrium is a semantic problem. The data do imply that a change in the activity of the enzymes involved in synthesis, the ATP synthase and the adenine nucleotide translocase, would have important metabolic consequences. For example, in order to maintain the rate of ATP synthesis at 1000 nmol/(min·mg) in the face of a 30% degrease in the amount of active enzyme, the ratio ATP/ADP would change from one that supported 50% state 3 flux (V_{max} = 2000) to one that supported 71.4% state 3 flux (V_{max} = 1400), reportedly a very significant change in the ratio ATP/ADP·P_i (Gyulai et al., 1985; Davis & Davis-van Thienen, 1978; Wanders et al., 1984; Kunz et al., 1981, 1983).

Modulation of the back reaction is important in the control of net ATP synthesis. The forward reaction (V_1) only doubles while the back reaction (V_2) varies from 0 to 400 in liver and from 0 to 2000 in heart. The question of what controls the rate of the backward reaction arises. The kinetic characteristics and activity of the adenine nucleotide carrier (LaNoue & Schoolwerth, 1984; Tager et al., 1983) may be responsible for the relative unidirectionality of ATP synthesis. This is based on the assumption that the ATPase itself is highly reversible and could support a much higher incorporation of P_i into ATP than the intact mitochondria. ADP does cause product inhibition of ATP hydrolysis in isolated F_1 and submitochondrial particles, but the reported K_i's and K_m's would not be likely to cause complete cessation of ATP hydrolysis at an ATP/ADP ratio of 5, as seen here. However, the higher values of $\Delta\psi_m$ achieved in intact mitochondria may modulate values of K_m and K_i for ATP and ADP. Also, the rates of isotope exchange between ATP and phosphate in beef heart submitochondrial particles (Rosing et al., 1977; Kayalar, 1977) suggest that the maximum velocity of the enzyme is not very high. In fact, the exchange rates measured at 37 °C in beef heart submitochondrial particles are lower (~280 nmol/(min·mg)) than those reported here (1000–2000 nmol/(min·mg)).

On the other hand, when exchange rates of phosphate and ATP were studied in cell membrane preparations of *Paracoccus denitrificans* (Ferguson et al., 1976), transfer of phosphate to ATP occurred only in the forward direction when the rate of ATP synthesis was highest. Since there is no adenine nucleotide translocase present in the bacterial system, the suppression of the backward reaction must be considered a property of the ATPase itself. Thus, although one cannot

say whether the controlling enzyme is the ATPase or the translocase in the mammalian system, it is apparent that the control is a kinetic one with product inhibition playing an important role. Kinetic control of the ATPase, independent of the concentrations of substrates or products, could be mediated by the inhibitory subunit discovered by Pullman (van de Stadt et al., 1973; Pullman & Monroy, 1968). This regulatory subunit may inhibit ATP hydrolysis in low-energy states in the heart (Rouslin, 1983).

The saturation transfer nuclear magnetic resonance data, which estimate the rate of transfer of phosphate to ATP in perfused rat hearts, do not agree with the estimates of transfer obtained in these isotopic studies using isolated rat heart mitochondria. The NMR study (Matthews et al., 1981) was carried out with rat hearts using oxygen at the rate of 20.4 μmol of O_2/(min·g of dry weight). Since there are 280 mg of mitochondrial protein/g of dry weight in perfused rat hearts, the rate is equal to 145.7 nanoatoms of O_2/(min·mg of mitochondrial protein). Since the NMR experiments were carried out with Langendorff perfused hearts, perfused at a pressure of 70 cm of H_2O, it is not surprising that O_2 consumption was relatively low. During a study of the relationship between substrate and O_2 consumption and heart work, with hearts perfused in the working heart mode (Kobayashi & Neely, 1979), O_2 consumption was 30 μmol/(min·g of dry weight) when the product of peak systolic pressure and heart rate was 15 mmHg/min $\times 10^3$, whereas O_2 consumption increased to 112 μmol/(min·g of dry weight) dry when the product was 50 mmHg/min $\times 10^3$. This higher rate of O_2 consumption corresponds to 800 nanoatoms of O_2/(min·mg of mitochondrial protein), very close to our state 3 rates at 37 °C. On the other hand, the rate measured in the NMR experiment was very close to our state 4 rate, and thus one would have predicted that the rate of conversion of phosphate to ATP would have been slightly over 1000 nmol/(min·mg of mitochondrial protein) or 4.6 μmol/(s·g of dry weight) as compared to the value reported, 2.8 μmol/(s·g of dry weight). Several assumptions were made in calculating the NMR data which may or may not have been valid. These included assumptions about the amount of the metabolically active phosphate in the heart cells and the value of the intrinsic spin–lattice relaxation time (T_1) of the phosphorus atom in inorganic phosphate in the perfused heart. Inaccuracies in these assumptions may have led to some inaccuracy in the final flux calculation. A more detailed study at different work loads would be of interest.

Registry No. ATP, 56-65-5; ATP synthase, 37205-63-3.

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Spin-Label Electron Paramagnetic Resonance and Differential Scanning Calorimetry Studies of the Interaction between Mitochondrial Succinate–Ubiquinone and Ubiquinol–Cytochrome *c* Reductases†

Sung-Hee Gwak, Linda Yu, and Chang-An Yu*
Department of Biochemistry, Oklahoma State University, Stillwater, Oklahoma 74078
Received April 15, 1986; Revised Manuscript Received July 14, 1986

ABSTRACT: The interaction between succinate–ubiquinone and ubiquinol–cytochrome *c* reductases in the purified, dispersed state and in embedded phospholipid vesicles was studied by differential scanning calorimetry and by electron paramagnetic resonance (EPR). When the purified, detergent-dispersed succinate–ubiquinone reductase, ubiquinol–cytochrome *c* reductase, and cytochrome *c* oxidase undergo thermodenaturation, they show an endothermic transition. However, when these isolated electron-transfer complexes are embedded in phospholipid vesicles, they undergo exothermodenaturation. The energy released could result from the collapse of the strained interaction between unsaturated fatty acyl groups of phospholipids and an exposed area of the complex formed by removal of interacting proteins. The exothermic enthalpy change of thermodenaturation of a protein–phospholipid vesicle containing both succinate–ubiquinone and ubiquinol–cytochrome *c* reductases was smaller than that of a mixture of protein–phospholipid vesicles formed from the individual electron-transfer complexes. This suggests specific interaction between succinate–ubiquinone reductase and ubiquinol–cytochrome *c* reductase in the membrane. This idea is supported by saturation transfer EPR studies showing that the rotational correlation time of spin-labeled ubiquinol–cytochrome *c* reductase is increased when mixed with succinate–ubiquinone reductase prior to embedding in phospholipid vesicles. These results indicate that succinate–ubiquinone reductase and ubiquinol–cytochrome *c* reductase are indeed present in the membrane as a supermacromolecular complex. No such supermacromolecular complex is detected between NADH–ubiquinone and ubiquinol–cytochrome *c* reductases or between succinate–ubiquinone and NADH–uniquinone reductases.

The electron-transfer system of the mitochondrial inner membrane, which catalyzes the oxidation of NADH and succinate and generates the membrane potential and proton gradient for ATP synthesis (Mitchell, 1968), has been fractionated into four functionally well-defined multisubunit lipoprotein complexes—NADH–ubiquinone, succinate–ubiquinone, and uniquinol–cytochrome *c* reductases and cytochrome *c* oxidase—by treatment with detergents and salts (Green, 1966). These electron-transfer complexes are commonly known as complexes I, II, III, and IV, respectively. Fragments containing two adjacent complexes, such as NADH–cytochrome *c* reductase (complexes I and III) (Hatefi et al., 1962a) or succinate–cytochrome *c* reductase (Complexes II and III) (Takemori & King, 1964), have also been obtained. Much information about the subunit structures (Smith et al., 1978; Earley & Ragan, 1980; Millett et al., 1982) and topological arrangements (Mendel-Hartvig & Nelson, 1978; Ludwig et al., 1979; Gutweniger et al., 1981) of these complexes in the mitochondrial inner membrane has been obtained by using isolated complexes (Trumpower & Katki, 1979; Wikstrom et al., 1981; Wainio, 1983).

The native functional arrangement of these electron-transfer complexes in the mitochondrial inner membrane is not yet established. Whether these complexes are present individually (Schneider et al., 1980; Kawato et al., 1981; Gupte et al., 1984) or as macromolecular assemblies (Hochman et al., 1982, 1983, 1985) in the mitochondrial inner membrane is a matter of controversy. Early electron microscopic observation of the mitochondrial inner membrane suggested an order array arrangement (Klingenberg, 1964; Sjostrand & Cassel, 1978) of these electron-transfer complexes. The lack of a specific stoichiometric relationship between these complexes, however, raises questions concerning a supermacromolecular assembly. The facts that cytochrome *c* is diffusible on the surface of the membrane and ubiquinone is diffusible in the membrane also argue against the existence of a supermacromolecular assembly. The recent estimation of the lateral diffusion coefficients (Gupte et al., 1984) of each individual electron-transfer complex, such as cytochrome b–c_1 complex and cytochrome *c* oxidase, and of electron-transfer components such as cytochrome *c* and ubiquinone by the fluorescence recovery after photobleaching (FRAP)[1] technique has further supported the

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