Rates of Various Reactions Catalyzed by ATP Synthase as Related to the Mechanism of ATP Synthesis*

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Deborah A. Berkich†, Gerald D. Williams‡, Peter T. Masiakos‡, Michael B. Smith§, Paul D. Boyer‖, and Kathryn F. LaNoue‡‡

From the †Departments of Cellular and Molecular Physiology and Radiology, ‡Division of Nuclear Magnetic Resonance Research, The Milton S. Hershey Medical Center, Pennsylvania State University, Hershey, Pennsylvania 17033 and the §Molecular Biology Institute, University of California, Los Angeles, California 90024

The forward and reverse rates of the overall reaction catalyzed by the ATP synthase in intact rat heart mitochondria, as measured with "35"P, were compared with the rates of two partial steps, as measured with "34"O. Such rates have been measured previously, but their relationship to one another has not been determined, nor have the partial reactions been measured in intact mitochondria. The partial steps measured were the rate of medium P\textsubscript{i} formation from bound ATP (in state 4 this also equals the rate of medium P\textsubscript{i} into bound ATP) and the rate of formation of bound ATP from bound P\textsubscript{i} within the catalytic site. The rates of both partial reactions can be measured by "31"P NMR analysis of the "34"O distribution in P\textsubscript{i}, and ATP released from the enzyme during incubation of intact mitochondria with highly labeled "31"OP\textsubscript{i}.

Data were obtained in state 3 and 4 conditions with variation in substrate concentrations, temperature, and mitochondrial membrane electrical potential gradient (\(\Delta \psi_m\)). Although neither binding nor release of ATP is necessary for phosphate/H\textsubscript{2}O exchange, in state 4 the rate of incorporation of at least one water oxygen atom into phosphate is approximately twice the rate of the overall reaction rate under a variety of conditions. This can be explained if the release of P\textsubscript{i} or ATP at one catalytic site does not occur, unless ATP or P\textsubscript{i} is bound at another catalytic site. Such coupling provides strong support for the previously proposed alternating site mechanism. In state 3 slow reversal of ATP synthesis occurs within the mitochondrial matrix and can be detected as incorporation of water oxygen atoms into medium P\textsubscript{i} even though medium ["32"P]ATP does not give rise to "32"P, in state 3. These data can be explained by lack of translocation of ATP from the medium to the mitochondrial matrix. The rate of bound ATP formation from bound P\textsubscript{i} at catalytic sites was over twice the rate of the overall reaction in both states 4 and 3. The rate of reaction at the catalytic site is considerably less sensitive to the decrease in membrane potential and the concentration of medium ADP than is the rate of medium ATP formation. This supports the view that the active catalytic site is occluded and proceeds at a rapid rate which is relatively independent of \(\Delta \psi_m\) and of media substrates.

In order to probe the reaction mechanism of ATP synthase and to assess rate-limiting steps in net ATP synthesis by rat heart mitochondria, the rates of several reactions catalyzed by the synthase have been compared. Thus, the rate of incorporation of P\textsubscript{i} into ATP and of the phosphoryl group of ATP into P\textsubscript{i} was measured by use of "32"P or "33"P labeling (1). The rate of catalytic site ATP formation from bound P\textsubscript{i} and the rate of release of phosphate from the catalytic site to the medium were measured using inorganic phosphate highly enriched in "32"O as a probe. The results are correlated with mitochondrial membrane potential (1, 3, 4), temperature, and concentrations of medium P\textsubscript{i}, ATP, and ADP.

Previous publications based on the use of intact mitochondria report only measurement of one or two of these reaction steps without systematic alterations of possible control parameters. Measurements with submitochondrial particles (5), because of lack of tight coupling and because of high levels of ATP hydrolysis relative to synthesis, do not give an adequate evaluation of the effect of membrane potential on the various reaction rates. Although previous studies have demonstrated that P\textsubscript{i} transport across the mitochondrial membrane is rapid relative to other processes involved in net synthesis of ATP (6), there has been a continuing controversy about rate limitation of ATP synthesis, centering on whether the translocase or the ATP synthase might limit flux (7, 8). Another school of thought (9) has promoted the idea that both the translocase and the synthase are in rapid thermodynamic near-equilibrium even when net synthesis is high. According to this equilibrium theory, rate limitation occurs through coupling with the electron transport chain's final step, reduction of molecular O\textsubscript{2} by cytochrome aa\textsubscript{3}, which is far from equilibrium.

In the present study we assume that translocation does not impose a limitation on the rate of entry of medium ATP into reactions catalyzed by the synthase under steady state conditions of state 4. Isotopic studies carried out some years ago (10), aimed at assessing translocase flux in oligomycin-inhibited heart mitochondria, suggest that the obligatory 1:1 exchange of intramitochondrial ATP for extramitochondrial ATP would not limit "32"P/ATP exchange. In that early study, the flux of the translocase at 37 °C in heart mitochondria was assessed on the basis of an extrapolated Arrhenius plot to be about 2 \mu mol/min\cdot mg, whereas the ATP/P\textsubscript{i} exchange is \approx 1.0 \mu mol/min\cdot mg at 37 °C (1). Moreover, a recent study (11) of translocase flux in liver mitochondria using "31"P NMR saturation transfer as a tool to estimate transport rates, indicates...
that the previous isotopic estimates of flux may significantly underestimate these values.

In the present investigation $^{32}P$ and $^{18}O$ exchanges were measured to obtain information about the synthase operating within the mitochondrial membrane permeability barrier. Moreover, since measurements of $^{18}O$ incorporation into $P_iQ$ are dependent on the synthase but independent of translocase, a means of monitoring synthase independently was at hand, even under conditions where limitation by the translocase was possible or even likely.

Evaluations of the rates are reported under conditions of rapid net synthesis of ATP (state 3) and under steady state (state 4) conditions with rapid $^{32}P$ exchange between ATP and $P_i$. The reactions are assessed as a function of the concentration of ATP, $P_i$, ADP, $\Delta G_m$, and temperature.

The results reveal that during rapid net synthesis of ATP the synthase (within the mitochondrial matrix) operates far from thermodynamic equilibrium and that the slow step in the reaction pathway is not the formation of high energy phosphate bonds at the catalytic site, but the entry and exit of substrates into the catalytic site. The results reveal interesting characteristics of the synthase mechanism and concur with the concept of required sequential participation of catalytic sites on the multisubunit ATP synthase.

**EXPERIMENTAL PROCEDURES**

**Mitochondrial Isolation and Incubation Conditions**—Mitochondria were isolated from freshly excised rat hearts by a modification (1) of the procedure of Chance and Hagggara (12). The modification involves retrograde Langendorff perfusion of each heart with 50 ml of ice-cold isolation medium A (225 mM mannitol, 75 mM sucrose, 0.1 mM EDTA, and 5 mM MOPS, pH 7.0) followed by perfusion with the same isolation medium containing 0.4 mg/ml of the protease Nagarse adjusted to pH 7.5. The hearts are then minced in medium B and minced in ice-cold isolation medium A, and mitochondria are isolated from homogenates of minced preperfused hearts by standard techniques of differential centrifugation. The modified procedure results in a preparation of mitochondria with low matrix Ca$^{2+}$ levels and low Mg$^{2+}$-stimulatable ATPase activity. The respiratory control ratios are routinely over 8 in media containing 5 mM Mg$^{2+}$. The high Mg$^{2+}$-stimulated ATPase activity observed in most preparations of heart mitochondria has been attributed to broken and uncoupled fragments of inner mitochondrial membranes (15). Therefore, it was important to minimize such activity in the present study. The various reactions catalyzed by ATP synthase in isolated mitochondria were measured during incubation of the mitochondrial in a buffer (pH 7.2) containing 150 mM KCl, 20 mM MOPS, 5 mM MgCl$_2$, and 0.05 mM EDTA (medium C). Various concentrations of ATP, ADP, $P_i$, dinitrophenol, and electron transport chain substrates were added to medium C as noted in the figure and table legends. In order to measure reactions involving the exchange of $P_i$ with $H_2O$, the $P_i$ used in the buffer was KH$_2$P"O$_4$, prepared as racemic acid.

The respiratory control ratios are routinely over 8 in media containing 150 mM KCl, 20 mM MOPS, 5 mM MgCl$_2$, and 0.05 mM EDTA (medium C). Various concentrations of ATP, ADP, $P_i$, dinitrophenol, and electron transport chain substrates were added to medium C as noted in the figure and table legends. In order to measure reactions involving the exchange of $P_i$ with $H_2O$, the $P_i$ used in the buffer was KH$_2$P"O$_4$, prepared as racemic acid.

The abbreviations used are: MOPS, N-(2-hydroxyethyl)piperazine-\(N'\)-ethanesulfonic acid; CDTA, trans-1,2-diaminocyclohexane-N,N,N',N"-tetraacetic acid.

...
are given under "Experimental Procedures." The rates are
defined in Fig. 1. Both \( V_1 \) and \( V_2 \) reveal only that a catalytic
capacity for the conversion of medium \( P_i \) to medium ATP (\( V_1 \)) and of medium ATP to \( P_i \) (\( V_2 \)) exists. The measurement
of \( V_b \) based on fractional disappearance of the \( P^{18}O_4 \) isotopomer,
provides a measure of \( P_i \) that (a) binds to the catalytic
site, (b) reversibly forms bound ATP one or more times, and
(c) is released from the catalytic site as \( ^{18}O \) isotopomers of \( P_i \),
with three or less \( ^{18}O \) atoms present. Any \( P^{18}O_4 \) released to the
medium following conversion of medium ATP to bound
ATP and \( P^{18}O_4 \) also reduces the fraction of \( P^{18}O_4 \) in the
medium \( P_i \). The amount of this reaction (\( V_2 \)) is included in
the value for \( V_s \).

The ratios of the \( V_s/V_z \) rates are of particular interest as
they could reveal whether there is a mechanistic requirement
for coupling between the rates of entry of \( P_i \) into the catalytic
site and the rate of release of bound ATP from the catalytic
site. In order to make the measurement of \( V_s \) simultaneously
with \( V_1 \) and \( V_2 \), rat heart mitochondria were incubated with tracer \( ^{33}P \), and phosphate labeled with \( ^{18}O \) in all four oxygen
positions.

Fig. 2 gives the \( ^{31}P \) NMR spectra of the starting \(^{18}O\)-labeled
\( P_i \) and the spectra of the \( P_i \) following a 4-min incubation
under the dynamic equilibrium conditions of state 4. The initial \( P_i \) shows only about 1–2% \( ^{18}O \) detectable isotopomer other than the \( P^{18}O_4 \) species. After 4-min incubation under
state 4 conditions a prominent \( P^{18}O_4 \) peak is clearly evident,
resulting from appearance of medium \( P_i \) originating from
unlabeled ATP and from any medium \( P_i \) that reformed bound
ATP a sufficient number of times to lose all \( ^{18}O \), and the ATP
was again hydrolyzed and \( P^{18}O_4 \) released. A plot of the log of the percentage of \( P_i \) as \( P^{18}O_4 \) at 1-min intervals up to 4 min
(45% total loss) was linear as expected for constant rate of
loss (Fig. 3).

State 4 measurements of \( V_1, V_2, \) and \( V_3 \) are presented in
Table I. The assumption that \( V_1 \) equals \( V_2 \) was tested directly
by including \( ^{33}P \), and \( [\gamma-^{33}P] \) ATP in the same incubation
(those experiments with glutamate and malate as substrates).
The values for \( V_1 \) and \( V_2 \) are shown and as expected are the
same, within the experimental error.

With 5 mM \( P_i \) and 5 mM ATP present, the rates of \( V_1 \) and
\( V_2 \) are considerably higher with pyruvate and malate as substrates
than with glutamate and malate, as expected for the greater capacity for net ATP synthesis with the pyruvate-
malate system. The rate at which protonmotive force can be generated, not the capacity of the ATP synthase, limits the
rate of ATP synthesis.

With pyruvate and malate as substrates, lowering the medium
\( P_i \) concentration from 5 to 0.1 mM, with 5 mM ATP
present, cuts the \( V_1 \) and \( V_2 \) values about in half. A \( P_i \) concentra-
tion of 0.5 mM suffices for near maximal \( V_1 \) and \( V_2 \) rates.
When the ATP is decreased from 5 to 0.5 mM, with 5 mM \( P_i \)
present, the rates are decreased even more. Lowering the
temperature to 15°C cuts the \( V_1 \) and \( V_2 \) rates nearly 9-fold.

In striking contrast to the 8–10-fold change in the \( V_1 \) and
\( V_2 \) rates resulting from the changes in conditions as reported
in Table I, the ratio of \( V_1/V_2 \) remains between 1.8 and 2.3.
This maintenance of the \( V_1/V_2 \) ratio near 2.0 over these wide
variations in the individual rates, as considered under "Dis-
cussion," is consistent with a mechanism that would require
a 2:1 ratio of these rates and equality of the rate of release of
phosphate from the catalytic site and the rate of entry of
ATP.

The Rate of Bound ATP Formation from Bound \( P_i \) in State
The Mechanism of ATP Synthesis

Effect of substrate concentration and temperature on release of products from the ATP synthase catalytic site in state 4

Mitochondria (0.5 mg/ml at 37 °C and 2.0 mg/ml at 15 °C) were incubated in medium C. Samples were taken at 1, 2, 3, and 4 min. When present, glutamate and malate were 20 and 1 mM, respectively, whereas pyruvate was 5 mM. Incubations with glutamate included 5 mM P1 (0.5 μCi/ml 32P, ~98% P31) and 5 mM γ-32P [ATP (0.5 μCi/ml)] so that V1 and V2 could be assessed independently. When pyruvate was substrate, P1 was radiolabeled (0.5 μCi/ml 32P, ~98% P31) and the concentrations of P1 and ATP were varied as shown. Values shown are means ± S.E. (number of separate incubations).

<table>
<thead>
<tr>
<th>Temp. °C</th>
<th>Substrates</th>
<th>ATP mM</th>
<th>P1 (μmol/min·mg)</th>
<th>V1 (n) = V3 (μmol/min·mg)</th>
<th>Vi/Vi (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>37</td>
<td>Glutamate + malate</td>
<td>5.0</td>
<td>5.0</td>
<td>0.83 ± 0.08 (8)</td>
<td>1.90 ± 0.11 (3)</td>
</tr>
<tr>
<td>37</td>
<td>Pyruvate + malate</td>
<td>5.0</td>
<td>0.1</td>
<td>0.65 (1)</td>
<td>(1)</td>
</tr>
<tr>
<td>37</td>
<td>Pyruvate + malate</td>
<td>5.0</td>
<td>0.5</td>
<td>1.23 (1)</td>
<td>(1)</td>
</tr>
<tr>
<td>37</td>
<td>Pyruvate + malate</td>
<td>5.0</td>
<td>1.0</td>
<td>1.30 ± 0.01 (3)</td>
<td>(1)</td>
</tr>
<tr>
<td>37</td>
<td>Pyruvate + malate</td>
<td>5.0</td>
<td>2.5</td>
<td>1.20 ± 0.04 (4)</td>
<td>2.13 (1)</td>
</tr>
<tr>
<td>37</td>
<td>Pyruvate + malate</td>
<td>5.0</td>
<td>5.0</td>
<td>1.21 ± 0.08 (8)</td>
<td>2.19 ± 0.04 (3)</td>
</tr>
<tr>
<td>37</td>
<td>Pyruvate + malate</td>
<td>5.0</td>
<td>10.0</td>
<td>1.11 ± 0.09 (2)</td>
<td>2.17 (1)</td>
</tr>
<tr>
<td>37</td>
<td>Pyruvate + malate</td>
<td>0.5</td>
<td>5.0</td>
<td>0.50 ± 0.04 (3)</td>
<td>0.93 ± 0.04 (3)</td>
</tr>
<tr>
<td>37</td>
<td>Pyruvate + malate</td>
<td>1.0</td>
<td>5.0</td>
<td>0.73 (1)</td>
<td>(1)</td>
</tr>
<tr>
<td>15</td>
<td>Pyruvate + malate</td>
<td>5.0</td>
<td>5.0</td>
<td>0.15 ± 0.01 (5)</td>
<td>0.26 ± 0.03 (3)</td>
</tr>
</tbody>
</table>

* The value for V1 measured separately with γ-32P [ATP and 32P] in the reaction mixture was 0.88 ± 0.10 (4).

Influence of phosphate concentration and membrane potential on the formation of bound ATP from bound phosphate in state 4

The conditions of the experiments were the same as those described in the legend of Table I for the experiments which included 5 mM pyruvate and 1 mM malate. In an additional experiment, 50 μM dinitrophenol (DNP) was added as well as the other components described in the legend of Table I. Each condition represents a single incubation but R1 was determined separately in samples taken at different time points (2, 3, and 4 min). The value (n) shown for R1 is the number of time points used for the determinations. V1 and V2 were taken from Table I. In each case values shown are the mean ± S.E. (number of determinations).

<table>
<thead>
<tr>
<th>Pi mM</th>
<th>DNP μM</th>
<th>R1 (n)</th>
<th>V1 (n)</th>
<th>V2</th>
<th>V4</th>
<th>ΔVm (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5</td>
<td>0</td>
<td>3.96 ± 0.69 (3)</td>
<td>1.20 ± 0.05 (4)</td>
<td>2.13</td>
<td>10.55</td>
<td></td>
</tr>
<tr>
<td>5.0</td>
<td>0</td>
<td>4.27 ± 0.80 (4)</td>
<td>1.21 ± 0.08 (8)</td>
<td>2.26</td>
<td>11.92</td>
<td>129 ± 2 (8)</td>
</tr>
<tr>
<td>10.0</td>
<td>0</td>
<td>3.87 ± 0.37 (3)</td>
<td>1.11 ± 0.09 (2)</td>
<td>2.17</td>
<td>10.59</td>
<td></td>
</tr>
<tr>
<td>5.0</td>
<td>50</td>
<td>5.34 ± 1.93 (4)</td>
<td>0.09</td>
<td>1.78</td>
<td>11.25</td>
<td>95 ± 2 (3)</td>
</tr>
</tbody>
</table>

Reversal of the synthase inside the mitochondria.

The results of measurements under state 3 conditions with and without a hexokinase/ADP regenerating system present and with different substrates are given in Table III. Only with hexokinase present and with added medium ATP, could a value for V2 be assessed. With the initial conditions as given in Table III about half of the medium ATP was converted to the medium ATP during the incubation. But with γ-32P ATP added initially, no 32P, was detected in the medium. The sensitivity of the measurements was such that the rate was zero within experimental error (<0.003 μmol/min·mg).

Values for V1, were high, as anticipated. They were increased slightly by the presence of the hexokinase/ADP regenerating system and increased markedly when pyruvate replaced glutamate as a substrate. Values for V2 showed the opposite trend, decreasing from about one-third of the V1 rate with pyruvate and hexokinase present. Importantly, under all conditions V2 was appreciable although slow with respect to V1, demonstrating that the rate of formation of P1 from ATP in the mitochondrial matrix was greater than the formation of P1 from extramitochondrial ATP. The result is as anticipated if the adenine nucleotide translocase-catalyzed exchange of ATPout for ATPin is blocked by the presence of medium ADP.

The Effect of Uncoupler on V1 and on Reversals of Bound ATP Formation in State 3 Conditions—The effect of membrane potential (ΔVm) on the rate of appearance of 32P from P1 into ATP in state 3 and 4 conditions is shown in Fig. 4.
Effect of external ATP and ADP on the release of products from the ATP synthase catalytic site in state 3

Mitochondria (0.5 mg/ml) were incubated at 37°C in medium C containing 5 mM Pi (98% P32O4). In experiment 1 the medium initially contained 5 mM [γ-32P]ATP, 5 mM ADP, and 5 mM 33Pi (0.5 μCi/ml, ~98% P32O4) plus 20 mM glutamate and 1 mM malate. In experiment 2 the conditions were similar to experiment 1, but no ATP was added to the media and a hexokinase/ADP regenerating system was present (cf. Fig. 4 and “Experimental Procedures”). In experiment 3 conditions were the same as in experiment 2, but 5 mM pyruvate replaced 20 mM glutamate. Samples were taken at 1, 2, 4, and 6 min and analyzed as described under “Experimental Procedures.”

<table>
<thead>
<tr>
<th>Substrates</th>
<th>HK</th>
<th>Initial ATP</th>
<th>Initial ADP</th>
<th>Initial Pi</th>
<th>V1 (n)</th>
<th>V2 (n)</th>
<th>V3 (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutamate + malate</td>
<td>-</td>
<td>1</td>
<td>5</td>
<td>5</td>
<td>2.09 ± 0.08 (4)</td>
<td>0 (4)</td>
<td>0.72 ± 0.03 (3)</td>
</tr>
<tr>
<td>Glutamate + malate</td>
<td>+</td>
<td>0</td>
<td>1</td>
<td>5</td>
<td>2.22 ± 0.12 (4)</td>
<td>0.53 ± 0.09 (3)</td>
<td></td>
</tr>
<tr>
<td>Pyruvate + malate</td>
<td>+</td>
<td>0</td>
<td>1</td>
<td>5</td>
<td>3.39 ± 0.20 (5)</td>
<td>0.31 ± 0.04 (2)</td>
<td></td>
</tr>
</tbody>
</table>

HK, hexokinase/ADP regenerating system. +, present; −, absent.

The Mechanism of ATP Synthesis

The magnitude of ΔVm was decreased by including 20–120 μM dinitrophenol in the incubation medium. The well established sharp dependence of net synthesis on membrane potential is shown by the state 3 data. Whether the rate has reached a plateau at the highest membrane potential, with no uncoupler present, is not established by the data. They do suggest that an even higher V1 might be obtained if the membrane potential could be increased further.

Under the state 4 conditions, V1 is always lower than in state 3, as expected, because the synthase must bind ATP rapidly for potential hydrolysis as well as binding ADP and Pi for potential synthesis. In contrast to the state 3 result the V1 reaches a maximum when the potential is about 100 mV. At this stage, the availability of matrix ADP for V1 might become rate-limiting.

It was of particular interest to us to measure the rate of formation of bound ATP from bound Pi as membrane potential was decreased in state 3. Shown in Fig. 5 is the distribution of the 18O isotopomers in the glucose 6-phosphate formed with no uncoupler and with 40 μM dinitrophenol which reduced the steady state membrane potential by 35%. As noted in Fig. 4, this caused a decrease in the rate of net ATP synthesis of about 50%. The results in Fig. 5 give information about three reaction characteristics as follows. First, without uncoupler present, and with the tightly coupled mitochondrion capable of high ATP synthesis rates, an average of 1.03 water oxygen atoms is incorporated into each ATP molecule made from P1. (From the data of Fig. 5, total incorporation of 1.25 oxygen atoms was calculated. This was corrected for about 0.26 water oxygen atom arising from the average replacement of water oxygen atoms in medium Pi, that occurs in state 3 as noted in Table III.) As outlined previously (5), this result gave an average of 2.2 reversals (Ri) of bound ATP formation for each ATP molecule released to the medium. Second, the data show that upon uncoupler addition the average number of reaction reversals was about doubled. The rate of ATP released from the enzyme was only about half that without uncoupler, meaning that the rate of bound ATP formation (V2) was relatively constant with and without uncoupler present. The third characteristic derivable from the data of Fig. 5 is whether the distribution of 18O isotopomers in the glucose 6-phosphate formed can be accounted for by a single reaction pathway. The method for carrying out this determination has been described (19). Such evaluation shows that, within an experimental error of 1–2% in determination of the amount of the
TABLE IV

Effect of membrane potential on release of products and formation of ATP in the catalytic site (V4) of the ATP synthase in state 3

Mitochondria (0.5 mg/ml) were incubated at 37°C in medium C which included 5 mM 32P, (0.5 μCi/ml, ~98% P32O4-), 1 mM ADP, 5 mM pyruvate, 1 mM malate, the hexokinase/ADP regenerating system (cf. Fig. 4 and "Experimental Procedures"), and various concentrations of dinitrophenol (DNP) (0–120 μM). Samples were taken at 2 and 4 min and analyzed as described under "Experimental Procedures" for determination of Rm, V1, V2, and V4. The values of ∆Vm (means ± S.E. of three separate determinations) were determined in separate parallel incubations as described in the legend of Fig. 4 and Ref. 1. Rm is calculated from the corrected average water oxygen atom content of the glucose 6-phosphate formed.

<table>
<thead>
<tr>
<th>DNP (μM)</th>
<th>∆Vm (mV)</th>
<th>Rm</th>
<th>V1</th>
<th>V2</th>
<th>V4</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (2)</td>
<td>113 ± 3</td>
<td>2.0</td>
<td>3.46</td>
<td>0.31</td>
<td>11.0</td>
</tr>
<tr>
<td>0 (2)</td>
<td>118 ± 5</td>
<td>2.2</td>
<td>3.74</td>
<td>0.59</td>
<td>13.3</td>
</tr>
<tr>
<td>30 (2)</td>
<td>75 ± 4</td>
<td>4.8</td>
<td>1.51</td>
<td>0.42</td>
<td>10.8</td>
</tr>
<tr>
<td>40 (2)</td>
<td>75 ± 3</td>
<td>4.3</td>
<td>1.48</td>
<td>0.38</td>
<td>9.5</td>
</tr>
<tr>
<td>50 (2)</td>
<td>65 ± 2</td>
<td>4.3</td>
<td>0.86</td>
<td>0.48</td>
<td>6.6</td>
</tr>
<tr>
<td>50 (2)</td>
<td>57 ± 3</td>
<td>9.1</td>
<td>0.54</td>
<td>0.47</td>
<td>9.7</td>
</tr>
</tbody>
</table>

DISCUSSION

Several facets of the results provide increased understanding of how the ATP synthase catalysis proceeds in intact mitochondria. This includes the relative rates of reaction steps when maximal rates of net ATP synthesis are achieved (state 3), when the rate is limited by a decrease in the membrane potential, and when medium ADP is low and ATP high and dynamic reversal of the overall reaction is occurring (state 4). As discussed below, the relative values of two measured rates, V1 and V2, and the effect of membrane potential on V4 are of particular interest.

Rates in State 4 Conditions—Effects of concentration of ATP and phosphate on the P1/ATP exchange (Table I) show that V1 is decreased with a decrease in medium P1 concentration but not until the concentration is well below 0.5 mM. In contrast, about 1.0 mM P1 is required for half-maximal velocity of net ATP synthesis when excess ADP is present in agreement with the observation that the Km for phosphate transport is in the mM range. Thus, when phosphate is greater than 0.5 mM, it appears that some factor other than P1 concentration provides an important limitation on V1 and V2 in state 4. This could be the ability of ATP to bind, cleave, and form bound P1 accompanied by proton translocation against the high potential present. When the ATP concentration is decreased to 0.5 mM considerable decline in the rates of V1 and V2 occurs. This is probably the result of a decline of the level of free ATP in the mitochondrial matrix.
tion of energy-linked binding changes is considered. Only in scheme C is the energy input coupled to promotion of competent binding of ADP and P, and release of tightly bound ATP in a single step.

During net oxidative phosphorylation or photophosphorylation, the extent of water oxygen atom incorporation into each ATP made is sharply increased if either the P, ADP concentrations are lowered (2, 17). A similar behavior is shown with lowering of ATP concentration during net ATP hydrolysis by F1-ATPases or the ATP synthase preparations (22, 23). In a related experiment, the reversible formation of bound ATP from medium P, (V2) during net synthesis by submitochondrial particles is blocked by removal of medium ATP (2, 5). The results are all readily explainable by required alternating site participation. However, they could also be explained if the ATP, ADP, or P, acted through binding at some type of regulatory sites. This adds to the importance of the observation of a fixed ratio of V2/V1 reported here. The retention of the fixed ratio as ATP or P, concentrations or temperature are varied is readily explained by alternating site mechanisms but not by regulatory site occupancy.

**Flux through the Translocase and P/ATP Exchange during State 3 Conditions**—The rates observed when net oxidative phosphorylation is occurring (state 3 conditions) show that even when medium ATP is present, no formation of medium P, from medium ATP occurs (V2 is immeasurably small). The result is explainable by a strong preference of the translocase for ADP rather than ATP (20). That the translocase is indeed shutting down the V2 rate is demonstrated by the finding (Table III) that medium P, is still reversibly forming bound ATP even with a hexokinase/ADP regeneration system present. The coupling demonstrated in state 4 makes it highly probable that in state 3 the release of P, formed by hydrolysis and bound ATP is accompanied by binding of matrix ATP to the ATP synthase and such bound ATP can form bound P, which may be released in the next binding step. It follows that some P/ATP exchange is occurring.

**The Extent of Reaction Reversal during ATP Synthesis**—The extent of water oxygen atom incorporation observed during net ATP synthesis in the present experiments (an average of 1.03 oxygen atoms per ATP) is similar to that observed in net photophosphorylation (about 0.8 water oxygen atom per ATP released). In both oxidative phosphorylation and photophosphorylation some dynamic reversal of ATP formation always seems to occur. The extent of water oxygen atom incorporation reported here for mitochondrial ATP synthesis is considerably less than the incorporation of nearly two water oxygen atoms per ATP made during net ATP synthesis by submitochondrial particles (5). Submitochondrial particles appear to behave like partially uncoupled mitochondria.

As the membrane potential was lowered in state 3 by uncoupler addition (Table IV), the extent of water oxygen atom incorporation into the released ATP increased. A similar result was noted earlier with submitochondrial particles (5). The rate of formation of bound ATP (V2) remained relatively constant as shown in Table IV even though the rate of ATP release from the catalytic site is considerably decreased by the drop in membrane potential. The release of P, from the catalytic site under these conditions may be limited by low levels of matrix ATP. Although an increase in [P] occurs with decreasing A9m in state 3, a similar increase in R, does not occur in state 4. This could be because matrix ATP levels remain high in state 4 as A9m decreases (25). In state 3 the components of the reaction pathway must remain in the catalytic site for a longer period of time prior to release, as A9m decreases. The catalytic capacity of the synthase is still expressed but the net reaction is decreased because continued generation of proton motive force is necessary to drive the binding changes in the direction of net synthesis. The fact that V2 is the same in state 3 and in state 4 lends support to the notion that the catalytic site is occluded from the media and independent of the external environment.

**Evaluation of Catalytic Pathways**—It is also important to emphasize that the observed distribution of the 18O isotopomers of P, is consistent with a single reaction pathway for ATP synthesis. This result with intact mitochondria is similar to that observed earlier with ATP formation by submitochondrial particles (24) and by chloroplast thylakoids (18). A heterogeneity of isotopomer distribution has been observed by Sines and Hackney (24) with submitochondrial particles when net synthesis is inhibited by low ADP concentration. This heterogeneity could not be explained by multiple reaction pathways, but was explainable by an alternating site model in which reversals of bound ATP formation occurred readily but with limitation on the rate of cycling between reaction steps imposed by the lowered rate of ADP binding at one of the alternating sites. Measurements of the rates of 18O exchange as in this and earlier papers thus provide some of the strongest support for alternating site participation.

**REFERENCES**