Energy-dependent Dissociation of ATP from High Affinity Catalytic Sites of Beef Heart Mitochondrial Adenosine Triphosphatase*

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Incubation of [$\gamma$-$^{32}$P]ATP with a molar excess of the membrane-bound form of mitochondrial ATPase (F$_1$) results in binding of the bulk of the radioactive nucleotide in high affinity catalytic sites ($K_a = 10^{12} \text{ M}^{-1}$). Subsequent initiation of respiration by addition of succinate or NADH is accompanied by a profound decrease in the affinity for ATP. About one-third of the bound radioactive ATP appears to dissociate, that is, the [$\gamma$-$^{32}$P]ATP becomes accessible to hexokinase. The NADH-stimulated dissociation of [$\gamma$-$^{32}$P]ATP is energy-dependent since the stimulation is inhibited by uncouplers of oxidative phosphorylation and is prevented by respiratory chain inhibitors. The rate of the energy-dependent dissociation of ATP that occurs in the presence of NADH, ADP, and F$_1$ is commensurate with the measured initial rate of ATP synthesis in NADH-supported oxidative phosphorylation catalyzed by the same submitochondrial particles. Thus, the rate of dissociation of ATP from the high affinity catalytic site of submitochondrial particles meets the criterion of kinetic competency under the conditions of oxidative phosphorylation. These experiments provide evidence in support of the argument that energy conserved during the oxidation of substrates by the respiratory chain can be utilized to reduce the very tight binding of product ATP in high affinity catalytic sites and to promote dissociation of the nucleotide.

Studies with the soluble (1, 2) as well as the membrane-bound (3, 4) ATPase (F$_1$) from beef heart mitochondria have provided evidence that supports a four-step reaction mechanism for ATP hydrolysis by both forms of the enzyme. Two key features of the reaction mechanism are a high affinity catalytic site, $K_a = 10^{12} \text{ M}^{-1}$, and an equilibrium constant for the catalytic step that is near unity (1, 4). These observations support a model for ATP synthesis in oxidative phosphorylation which proposes that ATP forms from ADP and Pi bound in high affinity catalytic sites with little or no change in free energy and that the major requirement for energy in oxidative phosphorylation is the release of product ATP from high affinity catalytic sites (1-5). In terms of this model, the coupling mechanism linking the energy store to ATP synthesis in oxidative phosphorylation can be considered a device that lowers the affinity of the high affinity catalytic sites for ATP and facilitates dissociation (5). It was proposed that such a device could be expressed via changes in the state of ionization of the glutamate carboxyl group of subunit c (the carboxyl group of beef heart Fo (the membrane-embedded portion of the ATPase complex) that reacts with dicyclohexylcarbodiimide (6, 7) or by changes in the state of ionization of other charged amino acid residues in Fo. Conformational changes in Fo, resulting from changes in the state of ionization of charged groups of the submitochondrial proteins, could, when transmitted to F$_1$, bring about changes in the conformation of F$_1$ that reversibly alter the catalytic sites and thus the desired decrease in affinity (9). If the high affinity catalytic sites of membrane-bound F$_1$ participate in ATP synthesis in oxidative phosphorylation, it may be expected that these sites will exhibit energy-dependent changes in affinity for bound ATP. This paper examines the fate of [$\gamma$-$^{32}$P]ATP bound in high affinity catalytic sites of submitochondrial particles. It is shown, during respiration initiated by NADH or NADH plus ADP, that an energy-dependent dissociation of about one-third of the bound radioactive ATP takes place.

EXPERIMENTAL PROCEDURES

Materials—$^{32}$P, (enzyme grade) was purchased from ICN and used without further purification. [$\gamma$-$^{32}$P]ATP was prepared as described (8) and stored at $-20^\circ\text{C}$ in small volumes. The specific radioactivity of most preparations was about $10^7$ cpm/nmol. The preparations of radioactive ATP were used until the amount of free $^{32}$P, present reached about 5% of the total radioactivity. Polyethyleneimine-imregnated cellulose sheets (CEL PEI-UV) were purchased from Brinkmann Instruments. Myxothiazol was purchased from Boehringer Mannheim and antimycin, crystalline yeast hexokinase, and NADH were purchased from Sigma. Hexokinase was dialyzed before use to remove ammonium sulfate. FCCP was a gift from Dr. P. G. Heytler, E. I. du Pont de Nemours & Co., Wilmington, DE. S-13 was a gift from Dr. P. C. Herrmann, Monsanto, St. Louis, MO.

Methods—Submitochondrial particles prepared in the presence of ATP and magnesium were prepared as described (9) and activated by washing with buffered solutions of KC1 (3). The specific activity of the resulting submitochondrial particles ranged between 6 and 10 units/mg. The steady state rate of respiration catalyzed by the activated submitochondrial particles was 0.25 $\mu$A of O/min/mg of protein with succinate as substrate and 0.4-0.5 $\mu$A of O/min/mg of protein with NADH. The P/O ratio with succinate as substrate was 0.2-0.3. When NADH was incubated with 1 mg of activated submitochondrial particles in 1 ml of reaction mixture, at least 60 s of respiration were possible before anaerobiosis occurred. The rate of ATP synthesis during the initial 3 s following the addition of 1 mM NADH, 0.1 mM ADP, and 10 mM Pi to activated submitochondrial particles was 1.2 nmol of ATP formed per nmol of membrane-bound F$_1$/s.

When the formation of $^{32}$P was measured in ATPase experiments, reactions were stopped by adding 50 $\mu$l of a solution containing 60% perchloric acid and 5 mM ATP. The deproteinized reaction mixture was extracted with isobutyl alcohol/benzene (10) and the amount of $^{32}$P, formed was determined by counting the organic phase. The net

1 The abbreviations used are: FCCP, carbonyl cyanide p-trifluoromethoxyphenylhydrazone; S-13, S-chloro-3-tert-butyl-2-chloro-4'-nitrosoacyanilide; TNP-ATP and TNP-ADP, the 2',3'-O-(2,4,6-trinitrophenyl) derivatives of ATP and ADP, respectively; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.
amount of $^{32}$P, formed was calculated by subtracting any $^{32}$P present in controls to which perichloric acid was added before the submitochondrial particles. The amount of $[^{\gamma-32}P]$ATP remaining was determined by counting the aqueous phase.

When the amount of glucose-6-$^{32}$P was determined in reaction mixtures containing glucose and hexokinase, reactions were stopped by adding 1.0 ml of 2 N HCl that also contained 0.1 nmol of glucose-6-P. Samples of the deproteinized reaction mixtures were heated for 7 min in a boiling water bath to hydrolyze any $[^{\gamma-32}P]$ATP, esterified $^{32}$P was separated from $^{32}$P by extraction with isobutyl alcohol/benzene (10), and the amount of glucose-6-$^{32}$P was determined by counting the aqueous phase. Controls, minus hexokinase and particles, were routinely carried through the extraction procedures in order to obtain a correction factor for radioactivity appearing in the aqueous phase that was not glucose-6-$^{32}$P. This number was less than 0.5% of the total radioactivity in the reaction mixture.

Radioactivity was quantitated by adding 3.5 ml of the organic phase or 1 ml of the aqueous phase to 10 ml of a Triton/tohene-based scintillation mixture (11). Alternatively, Liquissint (National Diagnostics) was used in the same proportions. Radioactivity was counted in a Beckman LS-355 liquid scintillation counter.

Adenine nucleotides (ATP, ADP, and AMP) were separated by chromatography on plastic sheets layered with polyethelene-im-regnated cellulose (Brinkmann Instruments). The sheets were washed before use by ascending chromatography with water and air-dried. The samples containing radioactive and carrier nucleotides were applied to the sheets, and the chromatograms were developed with a solvent consisting of 2 N formic acid and 0.5 N LiCl. The sheets were dried, and the resolved nucleotides were identified under ultraviolet light and scraped into scintillation vials. The samples were extracted for 45 min with 0.5 ml of 1 N HCl, 5 ml of scintillant (Liquissint) was added, and radioactivity was determined in separate channels for $^3H$ and $^{32}P$.

The amounts of hexokinase used in these experiments (200 units/ml) were sufficient to convert 0.1 ml $[^{\gamma-32}P]$ATP to glucose-6-$^{32}$P in 200 ms. Addition of 200 units of hexokinase to 1 ml of buffer containing 0.1 nmol of $[^{\gamma-32}P]$ATP converted 98% of the radioactive ATP to glucose-6-$^{32}$P. If 1 mg or more of submitochondrial particles were added before the hexokinase, only about 3-4% of the radioactive ATP was available to the hexokinase. If 200 units of hexokinase were added to 1 mg of submitochondrial particles before adding 0.1 nmol of $[^{\gamma-32}P]$ATP, 95% or more of the added $[^{\gamma-32}P]$ATP was converted to glucose-6-$^{32}$P.

**RESULTS**

Incubation of $[^{\gamma-32}P]$ATP with activated submitochondrial particles (specific activity = 6 units/mg or higher) results in binding of the radioactive ATP in high affinity catalytic sites and the establishment of an equilibrium in the catalytic sites between bound substrate and bound hydrolysis products (4). Simultaneously, an equilibrium constant is approximately 0.5 (4), it is expected, as found in Table I, that a short-term incubation of radioactive substrate and a molar excess of membrane-bound F$_1$ results in 20–40% hydrolysis of the bound substrate. Although the net rate of ATP hydrolysis during single site catalysis is limited by the rate at which products dissociate from the catalytic site (1, 4), a considerable increase in rate is observed when an oxidizable substrate such as succinate or NADH is made available to the submitochondrial particles (Table I). The increased hydrolysis is energy-dependent, since the stimulation is reduced by rotenone (Experiment 3) or myxothiazol plus antimycin (Experiment 2) as well as the uncouplers FCCP and S-13 (Experiments 1, 3, and 4). It is noteworthy that only the reduced form of the pyridine nucleotide is effective in stimulating hydrolysis (Experiment 5) and that ethanol, the solvent for the various inhibitors, was without effect on the extent of hydrolysis (Experiment 2).

Fig. 1 shows the rate and extent of hydrolysis of $[^{\gamma-32}P]$ATP in the absence (curve A) and presence (curve B) of 1 mM NADH. It is clear that both the rate and the extent of hydrolysis is increased by the oxidizable substrate.

However, the stimulation of hydrolysis by NADH of $[^{\gamma-32}P]$ATP bound in high affinity catalytic sites is strongly dependent on the presence of P, and in fact the stimulation decreased with increasing P, concentration. In the absence of NADH, hydrolysis was not influenced by P, up to 8 mM (Fig. 2). Addition of NADH to a reaction mixture containing 0.8 added P, was accompanied by a small but detectable increase in hydrolysis. However, at higher concentrations of P, a considerable enhancement in the hydrolysis of bound radioactive ATP occurs and the levels observed, which reach

| Additions | $^{32}$P | $[^{\gamma-32}P]$ATP Hydrolysis | % |
|-----------|--------|-----------------------------|---|
| **Experiment 1** | | | |
| None | 0.033 | 0.056 | 37.1 |
| Succinate | 0.056 | 0.037 | 60.2 |
| FCCP | 0.035 | 0.054 | 39.3 |
| Succinate, FCCP | 0.026 | 0.056 | 51.7 |
| **Experiment 2** | | | |
| None | 0.029 | 0.062 | 31.9 |
| NADH | 0.081 | 0.010 | 89.0 |
| NADH, myxothiazol, antimycin | 0.040 | 0.052 | 43.5 |
| NADH, ethanol | 0.082 | 0.010 | 89.1 |
| Myxothiazol, antimycin | 0.028 | 0.062 | 30.8 |
| **Experiment 3** | | | |
| None | 0.025 | 0.026 | 27.3 |
| NADH | 0.082 | 0.026 | 89.3 |
| Rotenone | 0.026 | 0.028 | 28.8 |
| NADH, rotenone | 0.032 | 0.026 | 35.2 |
| **Experiment 4** | | | |
| None | 0.018 | 0.063 | 19.2 |
| NADH | 0.046 | 0.044 | 49.8 |
| NADH, FCCP | 0.021 | 0.062 | 22.9 |
| NADH, S-13 | 0.022 | 0.063 | 23.8 |
| FCCP, S-13 | 0.018 | 0.064 | 21.9 |
| **Experiment 5** | | | |
| None | 0.033 | 0.026 | 35.9 |
| NADH | 0.089 | 0.026 | 96.7 |
| NAD | 0.034 | 0.026 | 96.9 |
Energy-dependent Dissociation of ATP

The results are shown in Table 11. It may be seen that the reaction is hydrolysis. It may also be noted that relatively little (about 2%) of the 3H-nucleotide added is converted to [3H]AMP in this experiment.

Further investigation of the NADH-dependent hydrolysis of [γ-32P]ATP indicated that the mechanism of the stimulation included dissociation and rebinding of radioactive ATP, since the presence of hexokinase in the reaction mixture effectively prevented any stimulation. It may be seen in Fig. 2A that, while the NADH-stimulated hydrolysis (curve 1) was similar to that observed in Fig. 1, in the presence of hexokinase the rate of hydrolysis was similar to that observed in the absence of NADH (compare curve 2 of Fig. 3A and curve A of Fig. 1). In the absence of glucose, hexokinase failed to inhibit ATP hydrolysis (not shown). Additional evidence supporting the conclusion that the mechanism of the stimulation of hydrolysis by NADH includes dissociation and rebinding of [γ-32P]ATP is seen in Fig. 3B. In this experiment, a constant amount (0.092 nmol) of [γ-32P]ATP was added to increasing amounts of activated submitochondrial particles. At low concentrations of submitochondrial particles, a marked NADH-dependent stimulation of hydrolysis is observed. However, as the concentration of submitochondrial particles increases, and thus the availability of empty catalytic sites, the extent of the stimulation by NADH is reduced to a point approximating the per cent hydrolysis observed in the absence of NADH. It should be noted that at 6 mg of submitochondrial particles, respiration would continue for about 10 s before exhaustion of the oxygen in the reaction mixture (see "Experimental Procedures") and, in the absence of other considerations, should have caused a 50% increase in hydrolysis (Fig. 3A).

Direct evidence for the participation of NADH in an energy-dependent dissociation of [γ-32P]ATP from high affinity catalytic sites is shown in Table III. In Experiment 1, line a, it may be seen that: addition of 0.087 nmol of [γ-32P]ATP to 1 mg of submitochondrial particles results in the binding of 97% of the added nucleotide, since only about 3% is subsequently available to hexokinase. Addition of 1 mM NADH (line b) results in the dissociation of about 27% of the bound nucleotide in an energy-dependent fashion, since in the presence of either FCCP or S-13, the extent of dissociation is considerably reduced. Inhibitors of respiration also reduce the extent of NADH-dependent dissociation of [γ-32P]ATP (Table III, Experiment 2). Whereas 30% of the bound [γ-32P]ATP dissociates following addition of NADH (line b) if the submitochondrial particles are pretreated with myxothiazol and antimycin (line c), only 12% of the bound nucleotide dissociates. As in other experiments with water-insoluble inhibitors described in this paper, ethanol alone (line d) was without effect on NADH-dependent dissociation of [γ-32P]ATP. Table III, Experiment 3, also shows that the NADH-dependent dissociation of [γ-32P]ATP is not influenced by Pi.

In order to rule out the possibility that the NADH-stimulated hydrolysis of [γ-32P]ATP might represent an energy-dependent exchange reaction between the radioactive γ-phosphoryl of ATP bound in the high affinity catalytic site and Pi in the medium, an experiment was carried out with [3H,γ-32P]ATP. The results are shown in Table II. It may be seen that addition of NADH, in the presence of Pi, results in a net conversion of [3H]ATP to [3H]ADP and that the per cent hydrolysis measured in terms of the amount of [3H]Pi formed is the same as the conversion of [3H]ATP to [3H]ADP. Thus, the reaction is hydrolysis. It may also be noted that relatively small (about 2%) of the 3H-nucleotide added is converted to [3H]AMP in this experiment.

Further investigation of the NADH-dependent hydrolysis of [γ-32P]ATP indicated that the mechanism of the stimulation included dissociation and rebinding of radioactive ATP, since the presence of hexokinase in the reaction mixture effectively prevented any stimulation. It may be seen in Fig. 2A that, while the NADH-stimulated hydrolysis (curve 1) was similar to that observed in Fig. 1, in the presence of hexokinase the rate of hydrolysis was similar to that observed in the absence of NADH (compare curve 2 of Fig. 3A and curve A of Fig. 1). In the absence of glucose, hexokinase failed to inhibit ATP hydrolysis (not shown). Additional evidence supporting the conclusion that the mechanism of the stimulation of hydrolysis by NADH includes dissociation and rebinding of [γ-32P]ATP is seen in Fig. 3B. In this experiment, a constant amount (0.092 nmol) of [γ-32P]ATP was added to increasing amounts of activated submitochondrial particles. At low concentrations of submitochondrial particles, a marked NADH-dependent stimulation of hydrolysis is observed. However, as the concentration of submitochondrial particles increases, and thus the availability of empty catalytic sites, the extent of the stimulation by NADH is reduced to a point approximating the per cent hydrolysis observed in the absence of NADH. It should be noted that at 6 mg of submitochondrial particles, respiration would continue for about 10 s before exhaustion of the oxygen in the reaction mixture (see "Experimental Procedures") and, in the absence of other considerations, should have caused a 50% increase in hydrolysis (Fig. 3A).
Energy-dependent Dissociation of ATP

The reaction mixtures contained, in a final volume of 1 ml, 20 mM Tris-SO₄, pH 8, 5 mM MgSO₄, 1 mg of activated submitochondrial particles (specific activity = 9 units/mg), 0.12 nmol of [γ-3²P]ATP and, where shown, 4 mM Pi and 1 mM NADH. The specific activity of the [³²P]ATP was 1.4 × 10⁶ cpm/nmol and of the [γ-³²P]ATP was 1.3 × 10⁶ cpm/nmol. The particles were incubated with all additions except for radioactive ATP and NADH for 1 min at room temperature (20 °C). After ATP addition, the incubation was continued for 45 s (Experiments 1 and 3). For Experiments 2 and 4, the particles were incubated with radioactive ATP for 5 s, NADH was added, and incubations were continued for 40 s. Reactions were stopped by adding 50 μl of 60% perchloric acid followed by 25 μl of a solution containing 1 mM each nonradioactive ATP, ADP, and AMP. Experiments 3 and 4 received, in addition, 5 μl of 0.2 mM NaPi. Denatured protein was removed by centrifugation. The supernatants were cooled on ice and 90 μl of 6 N KOH were added. The neutralized extracts were cooled on ice for 10 min and centrifuged to sediment the precipitates. Aliquots of the supernatants (0.5 ml) were withdrawn and extracted with isobutyl alcohol/benzene as described under “Experimental Procedures” for analysis of ³²Pi formed. Individual amounts of the radioactive forms of ATP, ADP, and AMP present in the neutralized extracts were determined by chromatographing 10-μl aliquots on polyethyleneimine-impregnated cellulose sheets as described under “Experimental Procedures.” The values for [³²P]ATP bound and [³²P]AMP also were corrected for the small amounts of these compounds that were present in the original stock solutions: 0.002 and 0.001 nmol, respectively. Per cent hydrolysis was calculated as the ratio of [³²P]ADP to total ³²P-nucleotides present or as the ratio of ³²Pi formed to the total [γ-³²P]ATP added. Both ratios were multiplied by 100.

| Additions | [³²P]ATP | [³²P]ADP | [³²P]AMP | Hydrolysis [%] | [γ-³²P]ATP | ³²Pi | Hydrolysis [%] |
|-----------|---------|---------|---------|---------------|------------|------|---------------|
| Experiment 1 | 0.076 | 0.028 | 0.002 | 26.9 | 0.077 | 0.053 | 30.0 |
| Experiment 2 | 0.030 | 0.082 | 0.003 | 73.2 | 0.026 | 0.084 | 76.4 |
| Experiment 3 | 0.063 | 0.048 | 0.002 | 43.2 | 0.059 | 0.051 | 46.4 |
| Experiment 4 | 0.056 | 0.033 | 0.046 | 48.6 | 0.054 | 0.056 | 51.0 |

Fig. 3. Role of dissociation in the energy-dependent stimulation of the hydrolysis of [γ-³²P]ATP bound in high affinity catalytic sites. A, inhibition of hydrolysis by hexokinase. The reaction mixtures contained 20 mM Tris-SO₄, pH 8, 5 mM MgSO₄, 1 mg glucose, and 1 mg of activated submitochondrial particles (specific activity = 6.1 units/mg). The final volume was 1.0 ml. The submitochondrial particles were incubated for 1 min at room temperature, followed by the addition of 0.062 μM [γ-³²P]ATP and a further incubation of 5 s. Curve 1, 1 mM NADH was added and the incubation was continued for the times shown; curve 2, 200 units of hexokinase were added, followed immediately by 1 mM NADH. Reactions were allowed to continue for the times shown on the abscissa. B, reduction in the extent of hydrolysis at increased concentrations of submitochondrial particles. The reaction mixtures contained 20 mM Tris-SO₄, pH 8, 5 mM MgSO₄, 4 mM P, 0.062 μM [γ-³²P]ATP, and activated submitochondrial particles (specific activity = 6.1 units/mg) in the amounts shown on the abscissa. The final volume was 1.0 ml. Sub mitochondrial particles were added to reaction mixtures, minus [γ-³²P]ATP and NADH, and incubated for 1 min at room temperature. [γ-³²P]ATP was added and incubated for 20 s (lower curve). Upper curve, [γ-³²P]ATP was incubated with particles for 5 s, followed by the addition of 1 mM NADH and 15 s of further incubation. The symbols O—O and O—O in B represent data from two independent sets of experiments. All reactions were stopped by adding 50 μl of a solution containing 60% perchloric acid and 5 mM ATP. ³²Pi was separated from esterified ³²P as described under “Experimental Procedures.” All of the data are corrected for ³²Pi found in reaction mixtures when perchloric acid was added before the radioactive ATP.

**Discussion**

The most striking observation made in this paper is the energy-dependent dissociation of ATP bound in high affinity catalytic sites of submitochondrial particles. Dissociation requires an oxidizable substrate such as succinate or NADH, is dependent upon respiration, and is inhibited by uncouplers of oxidative phosphorylation. Of equal importance is the fact that the rate of the energy-dependent dissociation is at least as fast as the initial rate of ATP synthesis in oxidative phosphorylation. Thus, two criteria of kinetic competence suggest that the high affinity catalytic sites are normal catalytic sites on the enzyme. First, the maximum rate of hydrolysis of [γ-³²P]ATP bound in high affinity catalytic sites of the soluble (2, 12) or membrane-bound (4) forms is the same as the normally observed turnover number (600–700) for the enzyme. Second, as shown in Fig. 4, [γ-³²P]ATP bound in
where indicated, p1 of ethanol, and and MgS04, 50 mM glucose, and hexokinase were incubated at room temperature for indicated except [y-32P]ATP, NADH, and hexokinase. Experiment volumes of addition of NADH. The reaction was allowed to continue for an

\[ \text{nmol} \]

\[ \text{mg} \]

\[ \% \]

1 0.092 0.084 4.7 30.2
2 0.043 0.047 4.0 30.2
3 0.043 0.094 3.2 27.9
4 0.043 1.88 3.1 27.9
5 0.092 0.94 3.5 28.7

TABLE III
Energy-dependent dissociation of [\( \gamma \)-32P]ATP bound in high affinity catalytic sites on submitochondrial particles

The reaction mixture contained 20 mM Tris-SO1, pH 8, 5 mM MgSO4, 50 mM glucose, 0.092 \( \mu \)M [\( \gamma \)-32P]ATP (specific activity \( \approx 4.2 \times 10^5 \) cpm/nmol), 1 mg of activated submitochondrial particles and, where indicated, 1 mM NADH, 5 \( \mu \)M FCCP, 2.7 \( \mu \)M S-13, 4 mM Pi, 4 \( \mu \)l of ethanol, and 250 units of crystalline yeast hexokinase. FCCP and S-13 were dissolved in ethanol and added to reaction mixtures in volumes of 4 \( \mu \)l. The final volume was 1.0 ml. The specific activities of the submitochondrial particles were 6.1 units/mg (Experiments 1 and 3) and 9 units/mg (Experiment 2). The submitochondrial particles were incubated at room temperature for 1 min with all additions indicated except [\( \gamma \)-32P]ATP, NADH, and hexokinase. Experiment 1, line a, [\( \gamma \)-32P]ATP was added and the reaction continued for 25 s before addition of 1.3 ml of 2 N HCl; lines b–d, incubation with [\( \gamma \)-32P]ATP was continued for 5 s to form the enzyme-substrate complex, hexokinase was added, and an additional 5 s was allowed before addition of NADH. The reaction was allowed to continue for an additional 15 s before it was terminated with 1.0 ml of 2 N HCl. Experiment 2, lines a and b, the submitochondrial particles were incubated with [\( \gamma \)-32P]ATP for 5 s, hexokinase was added, and the incubation was continued for 40 s before addition of 1.0 ml of 2 N HCl; lines c and d, NADH was added immediately after the hexokinase, and incubation was continued for 40 s and stopped with 1 ml of 2 N HCl. Experiment 3, the submitochondrial particles were incubated with all additions above but Pi, NADH, and hexokinase for 1 min. When indicated, Pi was included in the incubations. [\( \gamma \)-32P]ATP was added, followed 5 s later by hexokinase. Lines a and b, the incubations were continued for 20 s; lines c and d, NADH was added 5 s after hexokinase and incubations were continued for 15 s. Reactions were stopped with 1 ml of 2 N HCl. The deproteinized reaction mixtures were treated as described under "Experimental Procedures" to determine the amounts of free [\( \gamma \)-32P]ATP. The amount of glucose-6-32P found in the aqueous phase is equated with "free" [\( \gamma \)-32P]ATP. Per cent [\( \gamma \)-32P]ATP dissociated is calculated as the nanomoles of glucose-6-32P divided by the nanomoles of [\( \gamma \)-32P]ATP added, multiplied by 100.

| Additions | Glucose-6-32P formed nmol | % |
|-----------|--------------------------|---|
| Experiment 1 | | |
| a. None | 0.003 | 3.3 |
| b. NADH | 0.025 | 27.1 |
| c. NADH, FCCP | 0.008 | 8.7 |
| d. NADH, S-13 | 0.008 | 8.7 |
| e. FCCP | 0.003 | 3.3 |
| Experiment 2 | | |
| a. None | 0.003 | 3.3 |
| b. NADH | 0.028 | 30.4 |
| c. NADH, myo3thiozal, antimycin | 0.011 | 12.0 |
| d. NADH, ethanol | 0.030 | 32.6 |
| e. Myo3thiozal, antimycin | 0.005 | 3.3 |
| Experiment 3 | | |
| a. None | 0.006 | 6.5 |
| b. Pi | 0.003 | 3.3 |
| c. NADH | 0.030 | 32.6 |
| d. NADH, Pi | 0.030 | 32.6 |

TABLE IV
Extent of the energy-dependent dissociation of [\( \gamma \)-32P]ATP from high affinity catalytic sites on submitochondrial particles

The reaction mixture contained 20 mM Tris-SO1, pH 8, 3 mM MgSO4, 4 mM Pi, 1 mM NADH, [\( \gamma \)-32P]ATP, and activated submitochondrial particles in the amounts shown. The final volume was 1.0 ml. The particles were incubated for 1 min at room temperature in the reaction mixture minus NADH, [\( \gamma \)-32P]ATP, and hexokinase. [\( \gamma \)-32P]ATP was added and incubated for 5 s, followed by hexokinase and an additional 5 s of incubation, and, finally, NADH was added and incubated for 40 s. The total incubation time for all tubes was the same. The reaction was stopped by adding 1 ml of 2 N HCl, and samples of the deproteinized reaction mixtures were treated as described under "Experimental Procedures" in order to separate [\( \gamma \)-32P]ATP free from esterified [\( \gamma \)-32P]ATP. The per cent [\( \gamma \)-32P]ATP free is equated with the amount of glucose-6-32P found in the aqueous phase and is calculated relative to the total [\( \gamma \)-32P]ATP added.

| Experiment | [\( \gamma \)-32P]ATP added | Submitochondrial particles added | Glucose-6-32P formed |
|-----------|--------------|-----------------|----------------|---|
| | Without | With | NADH | NADH | |
| 1 | 0.092 | 0.094 | 4.7 | 30.2 |
| 2 | 0.043 | 0.047 | 4.0 | 30.2 |
| 3 | 0.043 | 0.094 | 3.2 | 27.9 |
| 4 | 0.043 | 1.88 | 3.1 | 27.9 |
| 5 | 0.092 | 0.94 | 3.5 | 28.7 |

High affinity catalytic sites of submitochondrial particles dissociates at rates expected for the turnover of the membrane-bound enzyme in oxidative phosphorylation. These observations are directly relevant to the molecular mechanism of ATP synthesis in oxidative phosphorylation. The observations support a model for ATP synthesis which proposes that ATP is formed from bound ADP and Pi, with little or no change in free energy and that the major requirement for energy in oxidative phosphorylation is for the dissociation of product ATP from catalytic sites (1, 4, 5). Since the \( K_a \) for ATP binding in high affinity catalytic sites is \( 10^{-2} \) M\(^{-1} \) (1), release of product ATP formed in the same sites would require a decrease in binding affinity of many orders of magnitude. It is clear from experiments described in this paper that energy released during oxidation of substrates by the respiratory chain is capable of causing such a decrease in binding affinity.

The outline of a coupling device that might link the energy store to reversible alterations in the affinity of the catalytic site for ATP was presented recently. Treatment of submitochondrial particles with dicyclohexylcarbodiimide under conditions such that only the single carboxyl group of subunit c in F\(_0\) should be altered was followed by impaired binding of ATP in high affinity catalytic sites (9). Since the glutamate carboxyl group is approximately 20 \( A^\circ \) from a presumed catalytic site on F\(_{1}\) (13), it was proposed, in keeping with previous suggestions (6, 7), that chemical modification caused a conformational change in F\(_0\) which, transmitted to F\(_{1}\), caused a conformational change in the ATPase. The resulting reduced affinity of the catalytic sites for ATP was considered to arise from these changes in F\(_{1}\) (3). The possibility is thus raised that, during oxidative phosphorylation, a change in the state of ionization of charged amino acid residues in F\(_{0}\), in response to electrochemical proton gradients, also leads to a conformational change in F\(_{0}\) that is transmitted to F\(_{1}\) and gives rise to reversible changes in affinity for product ATP (3).

There is precedent for the suggestion that catalytic sites on F\(_{1}\) can undergo very large changes in binding affinity. Beef heart F\(_{1}\) binds TNP-ATP and TNP-ADP with equal high affinity. The affinity constant is 1 or more orders of magnitude greater than \( 10^6 \) M\(^{-1}\) (12). Since TNP-ATP is a substrate for F\(_{1}\) and product TNP-ADP must be released, it is clear that during turnover large, reversible shifts in binding affinity take place.

The energy-dependent stimulation of the hydrolysis of [\( \gamma \)-32P]ATP bound in the high affinity catalytic site is of some interest. The stimulation requires oxidation of substrates such as succinate or NADH and is inhibited by uncouplers (Table I). The stimulation of hydrolysis is preceded by dissociation and rebinding of the bound nucleotide since, if sufficient hexokinase is present in the medium to prevent rebinding, no
stimulation of hydrolysis is observed (Fig. 3). The order of addition of hexokinase is important. If there is a delay of more than 3 s between additions of NADH and hexokinase, very little glucose-6-\(^3\)P is formed. Rapid rebinding of dissociated \([\gamma-\text{32P}]\text{ATP}\) is expected from the bimolecular rate constant describing ATP binding in either the high affinity catalytic site or additional catalytic sites on the membrane-bound enzyme (4). Rapid rebinding is independent of the presence or absence of \(P\), and serves to explain why large amounts of hexokinase are required to demonstrate the energy-dependent dissociation of ATP. NADH-stimulated hydrolysis also is not observed in the presence of an excess of submitochondrial particles (condition favoring single site catalysis for the rebound ATP) (Fig. 3). While the NADH-stimulated hydrolysis requires \(P\), (Fig. 2), there was no effect of \(P\) on the NADH-dependent dissociation of ATP (Table III). The requirement for \(P\), cannot be explained in terms of a \(P\)-ATP exchange reaction that would cause \(\text{32P}\), to appear in the medium, since this is ruled out by the experiment of Table II. In addition, the rate of the \(\text{32P}\)-ATP exchange reaction continues to increase even at 40 mM \(P\), whereas maximum NADH-dependent hydrolysis occurred at about 4 mM \(P\) (Fig. 2).

The NADH-dependent stimulation of hydrolysis should be distinguished from the stimulated hydrolysis observed under the conditions of oxidative phosphorylation, that is, in the presence of NADH, \(\text{ADP}\), and hexokinase (Fig. 4). In the latter experiment, hexokinase prevented rebinding of dissociated ATP. The observed stimulation of hydrolysis is due to \(\text{ADP}\) alone, since \(\text{ADP}\) serves as a promoter molecule in the acceleration of the hydrolysis of \([\gamma-\text{32P}]\text{ATP}\) in high affinity catalytic sites (4). \(\text{ADP}\) alone, in the absence of NADH, also gives rise to a small, but reproducible dissociation of bound \([\gamma-\text{32P}]\text{ATP}\). Approximately 4% of the total ATP bound dissociates following addition of 0.1 mM \(\text{ADP}\).

The finding that only about one-third of the ATP bound in high affinity catalytic sites participates in an energy-dependent dissociation could be suggestive of heterogeneity in binding sites. However, there appears to be little heterogeneity in binding sites when cold chase experiments are carried out with submitochondrial particles (4). Alternatively, the extent of the observed dissociation might be influenced by the presence of nonfunctional phosphorylating assemblies which nevertheless bind ATP in high affinity catalytic sites. The fact that the extent of dissociation was the same for a number of different preparations of activated submitochondrial particles with differing specific ATPase activities (4.6-10 units/mg) argues against such an interpretation. The proposal of Boyer and co-workers (15) of alternation between three catalytic sites in the mechanism of action of \(P\) could help to explain the different dissociation of ATP (Table III). The requirement for \(P\) cannot be caused by the presence of an excess of \(P\), whereas maximum NADH-dependent hydrolysis occurred at about 4 mM \(P\) (Fig. 2).

Diagrams and figures cannot be represented here. The text includes references and a discussion of the methodology and results. The authors acknowledge help from Dr. H. S. Penefsky, unpublished experiments.

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