Partial Resolution of the Enzymes Catalyzing Oxidative Phosphorylation

XVI. CHEMICAL MODIFICATION OF MITOCHONDRIAL ADENOSINE TRIPHOSPHATASE*

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SUMMARY

1. The reaction of mitochondrial adenosine triphosphatase (coupling factor 1) with iodine resulted in rapid loss of ATPase activity, disappearance of sulfhydryl groups, binding of iodine to the protein, and partial dissociation of the molecule. The extent of the changes observed depended on the molar ratio of iodine to protein in the reaction mixture. At a molar ratio of 50, more than 99% of the ATPase activity was inhibited, about 20 atoms of iodine were bound per mole of enzyme and about 40% of the original protein (S20,w = 11.9 S) was converted to a subunit with a sedimentation coefficient of 3.5 S.

2. The iodinated enzyme, which contained less than 0.5% of the original ATPase activity, stimulated oxidative phosphorylation, the 32P-ATP exchange reaction and the ATP-dependent reduction of DPN catalyzed by submitochondrial particles which were partially but not completely devoid of coupling factor 1.

3. Reaction of coupling factor 1 with dicyclohexylcarbodiimide also resulted in virtually complete inhibition of ATPase activity. The inhibition was not accompanied by dissociation of the molecule or change in sulfhydryl content. Similar to the iodine-treated enzyme, dicyclohexylcarbodiimide-treated coupling factor 1 stimulated oxidative phosphorylation only in submitochondrial particles which contained endogenous coupling factor 1.

4. The failure of chemically modified ATPase to stimulate coupled phosphorylation in submitochondrial particles completely resolved with respect to coupling factor 1 supports the suggestion of a dual role of the coupling factor in oxidative phosphorylation.

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Coupling factor 1, a cold-labile adenosine triphosphatase from beef heart mitochondria, stimulates oxidative phosphorylation catalyzed by deficient submitochondrial particles (1, 2). The enzyme has been prepared in a highly purified form, homogeneous in the ultracentrifuge, and a number of its physical and chemical properties have been described (3).

Chemical modification of F1 was undertaken in an attempt to gain additional insight into the structure of the molecule as well as to further elucidate the role of F1 in oxidative phosphorylation. It was shown in a preliminary report (4) that treatment of F1 with iodine led to complete loss of ATPase activity with little or no loss in the ability of the preparation to stimulate oxidative phosphorylation catalyzed by N-particles.

The stimulation of oxidative phosphorylation by preparations of F1 in which ATPase was no longer manifest had already been demonstrated with a specific protein inhibitor of mitochondrial ATPase (5). An inhibitor-F1 complex was prepared which exhibited virtually no ATPase activity but stimulated coupled phosphorylation. The two forms of inhibited F1 differed fundamentally in that the effects of chemical modification were apparently irreversible whereas dissociation of the inhibitor-F1 complex was accompanied by "unmasking" of the ATPase (5).

Experiments described in this paper have bearing on recent findings that oligomycin stimulated the energy-dependent transhydrogenase reaction (6, 7) and coupled phosphorylation (8) in submitochondrial particles partially deficient with respect to coupling factors. It was proposed that oligomycin stimulated...
energy transfer reactions by preventing the hydrolysis of a non phosphorilated high energy intermediate (6, 8). A further suggestion by Lee and Ernster (8) that the stimulation by coupling factors was similar to that by oligomycin did not apply to submitochondrial particles completely resolved with respect to F1 (9). Stimulation by oligomycin of oxidative phosphorylation catalyzed by ASU-particles occurred in the presence but not in the absence of F1 (9). The purpose of this paper is to show that the stimulation of oxidative phosphorylation by chemically modified F1, like that by oligomycin and by a cold-labile ATPase from bakers' yeast mitochondria (10), occurred only in the presence of endogenous F1. These results support the concept of a dual role of F1, structural (10) as well as catalytic (2), in the stimulation of oxidative phosphorylation.

Experimental Procedure

Mitochondrial ATPase (1), N-particles (2), and SU-particles (9) were prepared as described earlier. F1 was equilibrated before use with selected buffers on columns (1 x 9 cm) of Sephadex G-50 (cf. Reference 3). 14C-ATP was synthesized from 8 14C ADP (Schwarz BioResearch) and P1 with glyceraldehyde-3-P dehydrogenase and P-glycerate kinase (2). ATP labeled in the γ position with 32P was made by the same method from ADP and 32Pi. A solution containing 14C,32P-ATP was obtained by mixing aliquots of the two solutions. Nucleotides labeled with 14C and 32P were counted in a liquid scintillation spectrometer as described by Wu (11). Potassium iodide and resublimed iodine were obtained from Merck. N,N'-Dicyclohexylcarbodiimide was obtained from Schwarz BioResearch. 5,5'-Dithiobis(2-nitrobenzoic acid) was purchased from Aldrich Chemicals. Arsenious oxide was purchased from the National Bureau of Standards. Stock solutions of approximately 20 mM iodine in 0.2 M KI were prepared fresh for each experiment. The exact iodine concentration was determined by titration with standard solutions of arsenious oxide. The iodinating mixture was added to solutions of F1, as indicated in the tables from a microburette or a Hamilton syringe in aliquots of about 5 μl with rapid stirring. Subsequent additions were made only after the color due to the previous addition had disappeared. In general, about 15 min were required (including decolorization) for iodination of 2 mg of protein at a ratio of 50 moles of iodine per mole of enzyme. When the last trace of color had disappeared, 0.5 μmole of cysteine was added to remove any unreacted iodine. Following the reaction of F1 with iodine, the enzyme was equilibrated with fresh buffer on a column of Sephadex G-50.

The procedure for reaction of F1 with 125I-labeled iodine was identical with that described above. 125I-Labeled iodine solution was prepared by allowing carrier free K125I to equilibrate with appropriate aliquots of the stock iodinating mixture for 6 hours. Samples of the column effluent were collected, dried, and counted in a Nuclear-Chicago low background counter in order to establish a clear separation of radioactivity in the protein-containing fractions from the unreacted iodide ion retardcd by the column. The number of atoms of iodine bound per mole of F1 was calculated on the basis of complete equilibration of added 125I with all the iodine and iodide of the iodinating mixture.

The reaction of F1 with dicyclohexylcarbodiimide was carried out in a buffer containing 0.25 M sucrose, 10 mM Tris-sulfate, pH 7.4, 2 mM EDTA and 4 mM ATP. Aliquots of a stock solution of the reagent (10 mM in 95% ethanol) were added in small increments with rapid stirring. The reaction was allowed to proceed 18 hours. Unreacted DCCD was removed by precipita
tion of the enzyme two times with 2 M (NH4)2SO4 followed by equilibration with STV-ATP on a column of Sephadex G-50.

ATPase activity (1), oxidative phosphorylation (2), the exchange between 32P and ATP (12), and the ATP-supported reduction of DPN by succinate (13) were measured as described previously. In order to obtain accurate measurements of the ATPase activity of chemically modified F1, about 20 μg of protein were used in the ATPase assay. The sulfhydryl group content of F1 was determined by a method similar to the one described earlier (3). The method depends upon the reaction of —SH groups with DTNB (14). A medium sufficient for 50 assays was prepared containing in a final volume of 25 ml, 40 mM imidazole sulfate, pH 7.0, 20 mM EDTA, and 0.2 mM DTNB. In order to eliminate stirring artifacts which gave rise to unpredictable increases in absorption of the blank during the assay, the medium was stirred vigorously for 3 hours on a magnetic stirrer before use. To each cuvette of 1-cm path length was added 0.5 ml of the medium, 0.1 ml of 10% sodium dodecyl sulfate, enzyme, and water to a final volume of 1.0 ml. The reaction was started by the addition of 0.5 to 5 μmoles of F1 and the change in absorbance at 412 nm was recorded in the Cary model 14 recording spectrophotometer with the 0 to 0.1 slide-wire. Approximately 5 μmoles of —SH (corresponding to a recorder pen deflection of about 50% of full scale) were measured in each experiment.

Sedimentation velocity experiments were carried out at 25° as described earlier (3) in a buffer containing 20 mM P1, pH 7.4, 2 mM EDTA, 4 mM ATP, and 0.1 M KCl.

ATPase protein concentration was measured with a differential refractometer (3). Particulate protein was measured by the biuret procedure after solubilization in 0.33% deoxycholate (15).

Definition of Unit and Specific Activity—One unit of ATPase activity is defined as that amount of enzyme which catalyzes the hydrolysis of 1 μmole of ATP per min. A unit of coupling activity is defined as the amount of protein required to stimulate the P:O ratio to one-half of the maximal ratio. In the 32Pi-ATP exchange reaction, a unit is defined as the amount of protein required to increase the specific activity of the ATP (counts per min per μmole) to one-half the maximal value. Specific activity is expressed as units per mg of protein.

Results

Previous studies have shown (3) that F1 contains about 10 —SH groups per mole of enzyme (mol wt 284,000). A study of the reaction of F1 with iodine revealed that loss of ATPase activity was accompanied by disappearance of sulfhydryl groups. As shown in Fig. 1, addition of iodine in mole ratios of iodine to protein up to 10, resulted in marked loss of ATPase activity, as well as —SH groups. Further addition of iodine was, however, less effective and in order to attain 90% inhibition of ATPase activity it was necessary to carry out the reaction at a ratio of 50 moles of iodine per mole of F1. Even at this large excess of iodine over protein, approximately 1 —SH per mole of F1 remained. Fig. 1 also shows that as much as 20 atoms of iodine were attached per mole of F1.

The effect of iodine on the physical properties of F1 was examined in the analytical ultracentrifuge. Virtually no change was observed in the sedimentation pattern following reaction of the enzyme with mole ratios of iodine to protein up to 4 (Fig. 2,
FIG. 1. Reaction of $^{131}$I-labeled iodine with $F_1$. $F_1$ was iodinated in a buffer containing 20 mM $P_i$, pH 7.4, 2 mM EDTA, and 4 mM ATP. Iodine was added at the molar ratios shown to 7.65 mmoles of enzyme in a final volume of 0.5 ml. Other conditions of iodination, as well as the measurement of $-\text{SH}$ groups and determination of iodine bound to protein are discussed under “Experimental Procedure.” The specific activity of the iodinating mixture was 486 cpm per mg atom of iodine.

Curve A). Thus, although ATPase activity was inhibited 50%, $F_1$ sedimented as a single component with a sedimentation coefficient of 11.9 S, identical with native $F_1$ (3). Reaction of $F_1$ with larger amounts of iodine led to the appearance in the sedimentation patterns of a second, slower moving component with a sedimentation coefficient of 3.5 S. The amount of 3.5 S material was found to be proportional to the mole ratio of iodine to protein in the original reaction mixture. In Experiment D (Fig. 2), ATPase activity was 99% inhibited and a considerable amount of 3.5 S material appeared. Measurement of the relative area under the two peaks in Fig. 2, Curve D, and correction for radial dilution revealed that 60% of the original material was in the 11.9 S component and 40% was in the 3.5 S component.

Additional information on changes which occurred when $F_1$ was treated with iodine was obtained from binding studies with radioactive adenosine di- and triphosphate. It has already been shown that native $F_1$ did bind both $^{14}$C-ADP and ATP and that the labeled nucleotides remained on the enzyme even after precipitation of the protein in 2 M ammonium sulfate. The label did, however, slowly dissociate from the protein (16). As shown in Table I, the iodinated enzyme had lost almost entirely the ability to bind adenine nucleotides with or without added Mg$^{++}$. Since Experiment 1 in Table I was carried out with $^{32}$P-ATP, it would have been possible to detect the formation of phosphorylated enzyme from labeled ATP and $F_1$. In agreement with earlier findings (15), native $F_1$ did not form such a compound. Not shown in Table I was the finding that iodinated $F_1$ also failed to form a $^{32}$P compound.

It was of considerable interest to determine whether the iodinated enzyme which had lost more than 99% of the original ATPase activity would stimulate oxidative phosphorylation. In Fig. 3A, it may be seen that iodine-treated $F_1$ did indeed restore coupled phosphorylation when added to N-particles. The specific activity of the native enzyme in the phosphorylation assay was 100 as compared with 67 for the iodinated material. In this experiment, $F_1$ was reacted with iodine at a mole ratio of iodine to protein of 50. The resulting protein, after separation from other components of the reaction mixture, contained only 0.4% of the original ATPase activity. The inhibition of ATPase activity by iodine was not affected by subsequent treatment with sulphydryl compounds such as mercaptoethanol or dithio-
threitol and, as shown in Fig. 3, treatment of the iodinated enzyme with 0.2 M mercaptoethanol also did not effect its specific activity in the coupling assay. Iodinated F₁ also stimulated the $^{32}$P-ATP exchange reaction catalyzed by N-particles. A, stimulation of oxidative phosphorylation. Iodine was added to 16.2 µmoles of F₁ in STV-ATP. The final volume was 0.5 ml. After incubation for 10 min at 25°, the entire reaction mixture was passed through a column (1 × 9 cm) of Sephadex G-50 equilibrated with buffer containing 0.25 M sucrose, 10 mM Tris-sulfate, pH 7.4, 2 mM EDTA, and 4 mM ATP (STV-ATP) was divided into three aliquots of 0.8 ml each. The protein concentration was 1.9 mg per ml. The second and third aliquots only were iodinated at a molar ratio of 50 as described under "Experimental Procedure." Aliquots 1 and 2 were then equilibrated with STV-ATP on fresh columns of Sephadex G-50. Aliquot 3 was equilibrated on a similar column in which 4 mM ADP and 4 mM MgSO₄ were substituted for ATP. The three protein preparations were assayed for the ability to stimulate the $^{32}$P-ATP exchange reaction catalyzed by N-particles on the days shown. ATPase activity of the iodinated enzyme was inhibited 99.4%. On Day 3, a portion of the iodinated enzyme stored in STV ADP-Mg$^{2+}$ was removed and clarified by centrifugation for 5 min in the Clay-Adams clinical centrifuge. The clear supernatant solution was assayed.

### Table I

Binding of $^{14}$C-labeled adenine nucleotide by native and iodinated F₁

<table>
<thead>
<tr>
<th>Enzyme and additions</th>
<th>Nucleotide bound</th>
<th>Molecules/mole F₁</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ADP</td>
<td>ATP</td>
</tr>
<tr>
<td><strong>Experiment 1</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F₁</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mg$^{2+}$, $^{14}$C-$^{32}$P-ATP</td>
<td>0.95</td>
<td>0.02</td>
</tr>
<tr>
<td>Iodinated F₁$^a$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mg$^{2+}$, $^{14}$C-$^{32}$P-ATP</td>
<td>1.02</td>
<td>0.08</td>
</tr>
<tr>
<td></td>
<td>$^{14}$C-ATP</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.11</td>
<td>0.11</td>
</tr>
<tr>
<td></td>
<td>0.05</td>
<td>0.05</td>
</tr>
<tr>
<td><strong>Experiment 2</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F₁</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mg$^{2+}$, $^{14}$C-ADP</td>
<td>0.39</td>
<td></td>
</tr>
<tr>
<td>Iodinated F₁$^a$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mg$^{2+}$, $^{14}$C-ADP</td>
<td>0.00</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ ATPase activity was inhibited 99.4%.

![Fig. 3](http://example.com/fig3.png)

Fig. 3. The stimulation by iodinated F₁ of oxidative phosphorylation and the $^{32}$P-ATP exchange reaction catalyzed by N-particles. A, stimulation of oxidative phosphorylation. Iodine was added to 10.2 µmoles of F₁ in STV-ATP. The final volume was 0.75 ml and the mole ratio of iodine to protein was 50. ATPase activity was inhibited 99.3%. ○, native F₁; O, iodinated F₁; □, iodinated F₁ incubated for 1 hour with 0.2 M mercaptoethanol before the assay was begun. The specific activity in the assay was: native F₁, 100 units per mg; iodinated F₁, 97 units per mg; iodinated F₁ treated with mercaptoethanol, 90 units per mg. B, stimulation of the $^{32}$P-ATP exchange. A sample of F₁ (10 µmoles in a final volume of 1.0 ml) was iodinated in STV-ATP at a molar ratio of 50. The resulting percentage of inhibition of ATPase activity was 90.7.

### Table II

Stability of iodinated F₁

<table>
<thead>
<tr>
<th>Sample</th>
<th>Storage at room temperature</th>
<th>Specific Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>days</td>
<td></td>
</tr>
<tr>
<td>1. Native F₁</td>
<td>0</td>
<td>358</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>450</td>
</tr>
<tr>
<td>2. Iodinated F₁ in STV-ATP</td>
<td>0</td>
<td>75</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>43</td>
</tr>
<tr>
<td>3. Iodinated F₁ in STV-ADP-Mg$^{2+}$</td>
<td>0</td>
<td>106</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>135</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>153</td>
</tr>
</tbody>
</table>

$^a$ Assayed after centrifugation.

(Fig. 3B) as well as the ATP-dependent reduction of DPN by succinate (not shown here). The specific activity of iodinated F₁ in the coupling assay, following reaction with iodine at a molar ratio of 50, ranged between 50 and 70% of the value obtained with the starting material. If the molar ratio were increased to between 70 and 100, the ability to stimulate oxidative phosphorylation was lost.

**Stability of iodine-treated ATPase**—The buffer in which the reaction of F₁ with iodine was carried out did not influence either the ATPase activity or the resulting physical and chemical properties of the enzyme such as —SH content or sedimentation behavior. However, the subsequent stability of the iodine-treated enzyme in the phosphorylation assay was influenced by the buffer composition.

In Table II it may be seen that native F₁ was stable when stored for 3 days in STV-ATP. However, when the iodinated enzyme was stored for 3 days in the same buffer, a decrease in specific activity of about 50% was observed. When the buffer contained ADP and Mg$^{2+}$, on the other hand, specific activity either remained constant or increased somewhat. Upon standing for 3 days at room temperature, the iodinated enzyme solutions, in contrast with native F₁, became quite turbid. Following centrifugation at low speed for 5 min, the clear supernatant solution was found to contain about one-half of the original protein concentration, and all of the coupling activity. Thus, the specific activity of iodinated F₁ in the coupling assay almost doubled and approached the value of native F₁ in the control (Table II).
The enzyme was iodinated in STV-ATP at a mole ratio of iodine to protein of 50 as described under "Experimental Procedure." The protein concentration was 2.8 mg per ml and the final volume was 0.9 ml. F1 was separated from the reaction products on a column of Sephadex G-50 equilibrated with a buffer containing STV-ATP. The iodinated enzyme solution as well as a similar control enzyme was then made 0.2 m in KCl and incubated at 0° or 25° for 2 hours. At the end of the incubation period, aliquots were removed and assayed for ATPase activity or for stimulation of the 32P-ATP exchange reaction catalyzed by N-particles.

**Table III**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Temperature</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>32P-ATP exchange reaction</td>
</tr>
<tr>
<td>F1</td>
<td>30°</td>
<td>264</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>154</td>
</tr>
<tr>
<td>Iodinated F1</td>
<td>30°</td>
<td>160</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

Further attempts at chemical modification of F1 were carried out with N,N'-dicyclohexylcarbodiimide. Recently it was reported that low concentrations of this reagent rapidly inhibited oxidative phosphorylation as well as ATPase activity in beef heart mitochondria (17). DCCD like iodine, inhibited ATPase activity of F1, virtually completely (Table IV). However, the effect of DCCD on F1 differed from its effect on mitochondrial ATPase where the inhibitor was shown to interact with CF1 (18). As shown in Table V, DCCD-treated F1, which was devoid of ATPase activity, stimulated oxidative phosphorylation in N-particles as well as native F1.

Two differences were observed between DCCD-treated and iodine-treated preparations of F1. The sulfhydryl group content as well as the sedimentation behavior of the DCCD-treated enzyme was identical with native F1.

As shown in Table VI, chemically treated F1, unlike native F1, but like oligomycin failed to stimulate oxidative phosphorylation in succinate-supported system. DCCD-F1, like iodinated F1, stimulated the 32P-ATP exchange reaction in a ratio of 50 and with DCCD at a mole ratio of 30. The final volume was 0.5 ml and the protein concentration was 2.5 mg per ml.

**Table V**

<table>
<thead>
<tr>
<th>Additions</th>
<th>Ratio of DCCD to F1</th>
<th>32P uptake</th>
<th>P:O</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-particles (500 µg)</td>
<td>0.5</td>
<td>3.9</td>
<td>0.2</td>
</tr>
<tr>
<td>Plus F1 (6 µg)</td>
<td>1</td>
<td>3.4</td>
<td>1.0</td>
</tr>
<tr>
<td>Plus F1 (12 µg)</td>
<td>2</td>
<td>3.0</td>
<td>1.3</td>
</tr>
<tr>
<td>Plus DCCD-F1 (6 µg)</td>
<td>3</td>
<td>18.7</td>
<td>3.5</td>
</tr>
<tr>
<td>Plus DCCD-F1 (12 µg)</td>
<td>4</td>
<td>18.7</td>
<td>4.0</td>
</tr>
<tr>
<td>Plus DCCD-F1 (6 µg)</td>
<td>5</td>
<td>37.4</td>
<td>3.1</td>
</tr>
<tr>
<td>Plus DCCD-F1 (12 µg)</td>
<td>6</td>
<td>37.4</td>
<td>3.6</td>
</tr>
</tbody>
</table>

**Table VI**

Failure of chemically modified F1 to stimulate oxidative phosphorylation catalyzed by SU-particles

A sample of F1 was equilibrated with STV-ATP on a column of Sephadex G-50. The enzyme was treated with iodine at a mole ratio of 50 and with DCCD at a mole ratio of 30. The final volume was 0.5 ml and the protein concentration was 2.5 mg per ml. Inhibition of ATPase activity by iodine and DCCD was 90.6% and 99.2%, respectively. The measurement of oxidative phosphorylation catalyzed by F1 supplemented with F1 has been described (9).
oxidative phosphorylation in N-particles. The failure of chemically modified F₁ to stimulate oxidative phosphorylation catalyzed by SU-particles was not due to failure of the particles to bind modified F₁. In separate experiments, not shown here, it was found that 14H-acetyl F₁ (19), treated with iodine or DCCD, was bound by SU-particles as well as N-particles. However, in agreement with the results shown in Table VI, oxidative phosphorylation catalyzed by N-particles only was stimulated by the chemically modified F₁.

DISCUSSION

Participation of F₁ in Oxidative Phosphorylation—One of the most interesting findings of this study was that more than 99% of the ATPase activity of F₁ could be destroyed by chemical modification without substantially affecting the ability of the preparation to stimulate the catalysis by N-particles of oxidative phosphorylation, the 32P-ATP exchange reaction, and the ATP dependent reduction of DPN by succinate. It was found, however, that chemically modified F₁ failed to stimulate oxidative phosphorylation in SU-particles under conditions which gave rise to a marked stimulation with native F₁. The effects of chemically modified F₁ on oxidative phosphorylation are remarkably similar to those of oligomycin (8) and a cold-labile ATPase, purified from baker’s yeast mitochondria (10), on coupled phosphorylation catalyzed by submitochondrial particles. It was shown that a stimulation of oxidative phosphorylation by low levels of oligomycin was observed in the presence, but not in the absence, of F₁ (9). It was suggested that oligomycin stimulated oxidative phosphorylation by preventing the hydrolysis of a high energy intermediate (6, 8).

Similarly, recent experiments with yeast F₁ have demonstrated that this enzyme stimulates coupled phosphorylation in N-particles but not in SU-particles which were completely resolved with respect to F₁. In addition, the stimulation with N-particles was inhibited by an antiserum to beef F₁ but not by an antiserum to yeast F₁ (10). On the basis of these experiments, it was suggested that the mechanism of stimulation of N-particles by the yeast enzyme was similar to that of oligomycin but that beef heart F₁, which is the only form of F₁ which stimulates oxidative phosphorylation in N-particles under conditions which gave rise to a marked stimulation with native F₁, has a dual role in that it stimulates oxidative phosphorylation by direct participation as a catalyst in the coupling process as reported earlier (2) as well as by an oligomycin-like mechanism. It seems reasonable to suppose that chemically modified F₁, like yeast F₁, changes the structure of the mitochondrial membrane and thereby prevents the hydrolysis of a nonphosphorylated high energy intermediate.

The observations with chemically modified F₁, described here, have bearing on recent reports of coupling factor preparations obtained from beef heart mitochondria which contained little or no ATPase activity (20) or preparations in which ATPase was inactivated (21, 22). In view of the finding that F₁ without ATPase activity stimulated oxidative phosphorylation by an oligomycin-like mechanism, consideration should be given to the possibility that these coupling factor preparations may be contaminated with inactivated F₁. Submitochondrial particles completely free of F₁ (9) constitute a convenient test system for the inactivated enzyme.

Alterations in F₁ following Chemical Modification—During the titration of F₁ with iodine it was seen (Fig. 1) that SH disappearance was stoichiometric with iodine added up to a molar ratio of about 4. Since under these conditions no iodine was attached to the protein and considerable inhibition of ATPase activity occurred, it is possible that —SH groups are intimately involved in the hydrolytic activity of the enzyme. The results were less clear at higher molar ratios since, in addition to the further disappearance of —SH groups, considerable amounts of iodine were bound to the protein (as much as 20 atoms per mole of F₁ at a molar ratio of 50). Although the reaction of iodine with cysteine would not be expected to lead to a stable iodine-containing product, covalent bonds with other amino acids such as tyrosine and histidine may have formed. Since DCCD inhibited ATPase activity of F₁ without affecting —SH, other groups on the enzyme must have been affected.

The possibility that tyrosine also may participate in the hydrolytic activity of F₁ has been strengthened by preliminary experiments with tetranitromethane, a reagent with considerable specificity for tyrosine (23). Treatment of F₁ with tetranitromethane under mild conditions led to complete loss of ATPase activity without affecting its capacity to stimulate oxidative phosphorylation with N-particles.

Since 1 mole of —SH remained per mole of enzyme even at a large excess of iodine over protein it may be interesting to explore whether this group participates in the stimulation of oxidative phosphorylation by iodinated F₁. It is not known, however, whether this last —SH is a discrete group or randomly distributed in the molecule.

The sedimentation properties of F₁ were considerably altered by iodination. It was seen (Fig. 2) that the enzyme not only appeared to dissociate partially at molar ratios higher than about 4, but that the extent of dissociation was proportional to the amount of iodine added. Of the two components observed in the sedimentation velocity experiments, the sedimentation coefficient of the more rapidly moving fraction was 11.9 S, the value found for the native enzyme (3), whereas the value for the more slowly sedimenting component was 3.5 S. A 3.5 S component also was observed when F₁ was incubated in the cold (3). It is thus possible that iodination and cold treatment gave rise to a subunit of similar size. A third component (s₂₀,₅ = 9.1 S) which was observed in cold inactivation studies (3) was not found in iodine-treated preparations of F₁.

Analysis by starch gel electrophoresis of F₁ reacted with iodine at molar ratios between 8 and 60 were in qualitative agreement with the sedimentation velocity experiment. Two fractions were observed on the gels, a slowly moving component with an s₅₀,₅ identical with that of native F₁ and a more rapidly moving component which appeared in relatively larger amounts as the molar ratio was increased.

In contrast to iodination, treatment of F₁ with DCCD under conditions which resulted in 99% inhibition of ATPase activity, led neither to dissociation of the molecule nor to loss of —SH groups. The nature of the chemical reaction between F₁ and DCCD is probably complex although it is likely that the e-amino group of lysine would be attacked at alkaline pH (24). This possibility is currently under investigation.

Acknowledgment—I would like to thank Mrs. J. Saltzgaber for her excellent technical assistance.

* H. S. Penefsky, unpublished experiments.
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