SEQUENCE OF REACTIONS IN THE PHOSPHORYLATION COUPLED TO THE OXIDATION OF REDUCED CYTOCHROME c

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The mitochondrial system catalyzing ATP synthesis coupled to the oxidation of reduced cytochrome c has been separated into a submitochondrial particle and a soluble protein factor (Webster, 1962). The soluble protein factor appears to be specifically required for the phosphorylation accompanying the oxidation of reduced cytochrome c, and has been designated the reduced cytochrome c coupling factor or RCCF (Green, et al., 1963). When the particles are incubated with reduced cytochrome c (Cyt c^{++}), RCCF, and magnesium ions, there is formed a soluble high-energy compound which reacts readily with ADP and P_i (in the absence of the particles) to form ATP (Webster, et al., 1963). The reaction is similar to that previously described by Pinchot (1960) during DPNH oxidation by particles from Alcaligenes faecalis. The high-energy compound has been purified by chromatography and by starch gel electrophoresis, and appears to contain both cytochrome c and RCCF (Webster, 1963).

It has now been found that the phosphorylation process coupled to the oxidation of reduced cytochrome c can be separated into four recognizable reactions:

Particle + n Cyt c^{++} ---> Particle ~ Cyt c + n Cyt c^{+++}

Particle ~ Cyt c + RCCF ---> RCCF ~ Cyt c + Particle

RCCF ~ Cyt c + P_i ---> RCCF ~ P + Cyt c^{+++}

RCCF ~ P + ADP ---> RCCF + ATP

Evidence for the occurrence of each of the reactions is presented in the following paragraphs.

Formation of a Particle-Bound High Energy Compound (Particle ~ Cytochrome c). Sub-mitochondrial particles are incubated with reduced cytochrome c for 1-5 min. The reaction mixture is quickly cooled, centrifuged, and the particles and supernatant fluid are incubated separately with KCN (to prevent further oxidation), RCCF, ADP, P_i^{32}, glucose, and crystalline yeast hexokinase as described in the legend of Table I. There is no detectable formation of glucose-6-phosphate by the supernatant fluid. In contrast, the particles produce 1.2 - 1.5 mμmoles of glucose-6-phosphate per mg of particle protein. The total amount of ATP formed by the system is directly proportional to the amount of particles present. If reduced cytochrome c is replaced by oxidized cytochrome c, or if 0.001 M KCN is present in the initial reaction, no ATP is subsequently formed. Likewise, omission of RCCF from the second reaction completely abolishes the formation of ATP. The particle catalyzes an exchange of P_i^{32} into ATP (probably formed from ADP as a result of adenyl kinase activity), but ATP formation due to oxidation of reduced cytochrome c is clearly evident in excess of the exchange.

In contrast to the catalytic function of the particles in the formation of the soluble high energy compound reported previously (Webster, et al., 1963), the particle-bound high energy compound appears to function as a reactant in the system. The amount of ATP formed in the second reaction is directly proportional to the amount of "activated" particles added to the reaction system. Furthermore, if particles are removed from the second reaction system after the formation of ATP, and are placed in a fresh mixture of KCN, RCCF, ADP, P_i^{32}, glucose, and hexokinase, there is no net formation of ATP unless the particles are first allowed to oxidize reduced cytochrome c.

Conversion of the Particle-Bound High Energy Compound to a Soluble High Energy Compound. When the "activated" particles are incubated with RCCF and Mg^{++}, the ability to form ATP is no longer associated with

TABLE I

FORMATION AND REACTIONS OF A PARTICLE-BOUND HIGH ENERGY COMPOUND

System	mμmoles ATP formed
Complete	53.
Minus reduced cytochrome c	25.
Reduced cytochrome c replaced by oxidized cytochrome c	24.
Plus 0.001 M KCN in 1st incubation	27.
Minus RCCF in 2nd incubation	23.
Plus 10^{-5} M dinitrophenol in 2nd incubation	0

Reaction system for the first reaction consisted of: 0.25 M sucrose, 0.03 M Tris-acetate (pH 7.5), 0.02 M $MgCl_2$, 2.25 x 10^{-4} M reduced cytochrome c, 1 x 10^{-6} M phenazine methosulfate, and 20 mg of ETP_H in a total volume of 3 ml. The ETP_H was prepared as described by Linnane and Ziegler (1958) from heavy beef heart mitochondria processed as described by Hansen and Smith (1963). Centrifugation at 0° was for 15 min at 105,000 x g.

The second reaction system consisted of: 0.01 M glucose, 0.01 M $MgCl_2$, 0.002 M KCN (pH 7.5), 0.002 M sodium ADP, 0.002 M potassium phosphate-P^{32} (pH 7.5) with a total activity of 2 x 10^7 cts/min, 0.15 mg of crystalline yeast hexokinase (Boehringer), 3 mg of RCCF, 0.25 M sucrose, 0.01 M Tris-acetate (pH 7.5), and 20 mg of the sedimented particles from the first reaction in a total volume of 5 ml. Incubation time was 5 min at 30°. ATP formation was assayed by the measurement of phosphate-P^{32} incorporation into glucose-6-phosphate.

the particles, but resides instead in the supernatant solution after sedimentation of the particles. This process exhibits an absolute requirement for both RCCF and Mg^{++}. The soluble activated material behaves upon chromatography on CM-cellulose in a manner identical with the soluble high energy compound reported previously (Webster, et al., 1963), and likewise contains cytochrome c and RCCF in approximately a 1:1 ratio. Because the cytochrome c in the soluble compound must have come from the particle, these results indicate the transfer of cytochrome c from the particle to the soluble high energy compound.

TABLE II

FORMATION OF THE PHOSPHORYLATED COMPOUND

System	mμmoles ATP formed
(1) Soluble high energy compound + P_i^{32}	55.
(2) "Activated" particles + RCCF + P_i^{32}	53.
Minus RCCF	0
(3) Particles + reduced cytochrome c + RCCF + P_i^{32}	65.
Minus RCCF	0
Minus particles	0
Minus reduced cytochrome c	0

The reaction mixture for system (1) above contained: 0.03 M Tris-acetate (pH 7.5), 0.02 M $MgCl_2$, 10 μmoles potassium phosphate-P^{32} (2 x 10^7 cts/min), and 3 mg of the soluble high energy compound, prepared and isolated as described previously (Webster, et al., 1963) in a total volume of 3 ml. The reaction mixture for system (2) was the same except 40 mg of "activated" particles (prepared as described with Table I) and 3 mg of RCCF were substituted for the high energy compound, and 0.25 M sucrose was present. The reaction mixture for system (3) was the same as for system (2) except 5 mg of particles, 10 mg of reduced cytochrome c, and 1 x 10^{-6} M phenazine methosulfate were substituted for the "activated" particles. After 5 min incubation at 30°, particles, if present, were removed by centrifugation, and excess P^{32} was removed from the supernatant solution by passage through Sephadex G-25. The phosphorylated substance was purified on a column of CM-cellulose and incubated with the second reaction system, consisting of: 0.01 M glucose, 0.01 M $MgCl_2$, 0.002 M KCN, 0.002 M ADP, 0.01 M Tris-acetate (pH 7.5), and 0.15 mg crystalline yeast hexokinase in a total volume of 5 ml. ATP formation was measured by the incorporation of P^{32} into glucose-6-phosphate.

Formation of a Phosphorylated Protein from the Soluble High Energy Compound. Incubation of the soluble high energy compound with P_i^{32} results in the formation of a new compound which requires only ADP to form ATP. The new compound has been purified by passage through a column of Sephadex G-25 (to remove unreacted P_i), followed by chromatography on a column of CM-cellulose. In contrast to the non-phosphorylated high energy compound described previously (Webster, et al., 1963), which

is held strongly on CM-cellulose, the new compound can be eluted from CM-cellulose by 0.1 M Tris-acetate (pH 7.5). Furthermore, in contrast to the red color of the nonphosphorylated compound, the new compound appears to be colorless. The purified compound contains approximately 0.04 μmoles of phosphate per mg protein. Only phosphate and protein have as yet been detected in the purified compound. Incubation of the phosphorylated protein (labeled with P^{32}) with ADP, glucose, and hexokinase in the presence of 0.01 M non-radioactive P_i causes no dilution of radioactivity incorporated into glucose-6-phosphate. As is shown in Table II, the phosphorylated compound can be formed: (1) from the non-phosphorylated high energy compound by incubation with P_i; (2) from the "activated" particles by incubation with P_i and RCCF; or (3) from incubation of P_i with the particles, reduced cytochrome c, and RCCF. Formation of the phosphorylated compound from the soluble high energy compound does not require the presence of RCCF, but formation of the phosphorylated compound from "activated" particles exhibits an absolute requirement for RCCF. Formation of the phosphorylated compound from particles which have not been oxidizing reduced cytochrome c requires not only the presence of RCCF, but also the concurrent oxidation of reduced cytochrome c. These observations are all consistent with the reaction sequence presented on the first page of this communication.

Although further investigation is necessary before it can be concluded that the reactions presented here depict the mechanism of phosphorylation coupled to the mitochondrial oxidation of reduced cytochrome c, the postulated reactions do explain the results which have been observed thus far with the submitochondrial system. Of especial interest is the nature of the particle-bound high energy compound, and the relationship of the phosphorylated compound to the phosphorylated compound observed by Boyer, et al. (1962). These questions are currently being investigated.

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