FORMATION AND ISOLATION OF A HIGH ENERGY PHOSPHORYLATED FORM OF COUPLING FACTOR II

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Studies in our laboratory (cf. Green et al., 1963) have led to the following hypothesis for the terminal sequence of reactions involved in the coupling of electron flow to the synthesis of ATP. At each of the three phosphorylation sites a high energy intermediate is generated by electron flow; this intermediate may be represented by the notation [particle ~ O.R. component] where O.R. component represents one of the oxidation-reduction components of the mitochondrial electron transfer chain. A specific coupling protein (or coupling factor, CF) then displaces the O.R. component and another high energy intermediate is formed [CF ~ O.R. component]. Two displacement reactions then ensue, first by inorganic orthophosphate leading to the formation of [CF ~ P] and then by ADP leading to [ADP ~ P] or ATP. In the present communication evidence will be presented that CF II, the coupling protein specific for phosphorylation in the segment of the electron transfer chain between reduced coenzyme Q and cytochrome c (Beyer, 1964), can be converted to a phosphorylated form and that about half of the protein-bound phosphorus can be transferred to glucose in the presence of ADP and hexokinase.

The isolation in purified form of CF II from beef heart mitochondria has been described in a previous communication (Beyer, 1964). When beef heart mitochondria, following disruption by 20 kc irradiation, were incubated with $^{32}P_i$, succinate, and Rotenone (cf. legend of Table I for full experimental details) the coupling factor was isolated from the mixture and shown to con-

tain radiophosphate (Table I). If p-triflouromethoxycarbonylcyanide phenylhydrazone (Heytler and Prichard, 1962) or 2,4-dinitrophenol were present during the incubation of the particle with $^{32}P_i$ and succinate, the isolated coupling factor contained little radioactivity. The addition of antimycin to block electron flow, or the deletion of substrate, resulted in a diminished incorporation of radioactivity into CF II. The latter incorporation may be due to exchange activity. Thus, the coupled oxidation of succinate is a prerequisite for effective incorporation of $^{32}P_i$ into the coupling factor.

Table I

FORMATION OF PHOSPHORYLATED CF II

System	Protein-bound ^{32}P, cts x min^{-1} x mg^{-1}
Complete	5583
plus 2,4-DNP	337
plus p-CF_3O-CCP	222
plus Antimycin	1246
minus succinate	1877

Heavy beef heart mitochondria were suspended in a solution which was 0.15 M in KCl and 10 mM in Tris-acetate, pH 7.5, and exposed to 20 kc irradiation. Rotenone, succinate, $MgCl_2$, and $^{32}P_i$ were added and the mixture incubated at 30° for 5 min. This is referred to as the complete system. The system was cooled, EDTA was added to 10 mM and the suspension centrifuged. The fraction precipitating from the clear supernatant solution between 45 and 70% saturation with ammonium sulfate (containing CF II; Beyer, 1964) was collected and passed through a column of Sephadex G-25M. Protein appearing immediately after the void volume was chromatographed on DEAE-substituted cellulose and the peak corresponding to CF II (Beyer, 1964) was collected. The final concentrations of additives were: 2,4-DNP, 10^{-4} M; p-CF_3O-CCP, 5 x 10^{-7} M; Antimycin, 0.5 µg/mg protein. These were added immediately after irradiation.

The same phosphorylated intermediate was formed by incubating CF II with ATP in the presence of Mg^{++} (Table III). The presence of particles was not needed for phosphorylation of CF II by ATP. This phosphorylation was not inhibited by any of the reagents which inhibit the formation of the phosphorylated intermediate during coupled electron flow. The entire ATP molecule was not introduced into CF II. When ATP labeled with ^{14}C in the purine ring was used instead of $\gamma(^{32}P)$ATP, no incorporation of label in CF II was observed.

Of the total radioactive phosphate introduced into the isolated CF II,

either by coupled phosphorylation or by ATP, about 50% was transferred to glucose (providing ADP, Mg^{++}, and hexokinase were present) (Tables II and III); 26-29% was nontransferable and was not released from the protein as inorganic phosphate under acid conditions; 17-23% was nontransferable and was released as inorganic phosphate under acid conditions. When the phosphorylated product, formed by way of coupled electron transfer, was incubated with hexokinase, glucose, Mg^{++}, and ADP prior to chromatography on DEAE-substituted cellulose, a phosphorylated product was eluted in the CF II peak which, in contrast to the normal preparation, transferred a negligible amount of its bound phosphate to form glucose-6-phosphate. The formation of radioactive glucose-6-phosphate by transfer of the protein-bound phosphate was verified in the paper chromatographic system of Bandurski and Axelrod (1951). For this transfer reaction, ADP, Mg^{++}, and hexokinase were all needed. Oligomycin did not inhibit the transphosphorylation reaction leading to glucose-6-phosphate formation from CF II$\sim$P when the phosphorylated derivative had been formed from labeled ATP.

Table II

TRANSFER OF ^{32}P FROM CF II$\sim^{32}P$ TO ADP

System	Protein-bound ^{32}P, cts x min^{-1} x mg^{-1}	% ^{32}P transferred to Acid protein	G-6-P	P_i
(1) Complete	2401	26	57	17
(2) Minus ADP	5466	6	1	93

Phosphorylated CF II prepared as in the legend under Table I was used as starting material. This was incubated for 5 min at 30° with hexokinase, Mg^{++}, glucose, and ADP and either chromatographed on Sephadex G-25M and the specific radioactivity of the protein assayed (protein-bound ^{32}P) or boiled for 10 min in 1 N acid and the distribution of radioactivity determined according to the method recommended for the determination of phosphate uptake in isolated systems by Lindberg and Ernster (1956).

Thus, CF II can be rapidly converted to a phosphorylated intermediate under conditions similar to those in which its capacity to restore deficient coupling at phosphorylation site II has been documented (Beyer, 1964). The formation of the intermediate, either by way of coupled electron flow or by way of ATP,

and the arrest of this reaction by the classical uncouplers provide strong evidence that the formation of the intermediate is not a coincidental property of the coupling protein. The high ATP-ADP exchange activity of CF II is in accord with the formation of a phosphorylated intermediate. It is noteworthy that other enzymic activities which might be associated with the transphosphorylation function (e.g. ATP-P_i exchange, pyrophosphatase, alkaline phosphatase) either were absent or were present only in trace amounts in CF II.

Table III

PHOSPHATE TRANSFER REACTIONS INVOLVING CF II

CF II + ATP ---> CF II~P + ADP	CF II~P formed, mμmoles	CF II~P + ADP + glucose + hexokinase ---> CF II + ADP + G-6-P	G-6-P formed, mμmoles
Complete	10.2	Complete	4.6
minus Mg^{++}	0.7	minus ADP	0.8
plus oligomycin	10.0	minus glucose	0.7
		plus oligomycin	4.3

CF II was prepared as previously described (Beyer, 1964). Phosphorylated CF II was prepared by incubating 40 mμmoles of CF II, 500 mμmoles of $\gamma(^{32}P)$ATP (ca. 10^6 cts/min), 17 μmoles of Tris-H_2SO_4, pH 7.5, and 5 μmoles of $MgCl_2$ in a volume of 1 ml, for 5 min at 30°. When present, the oligomycin concentration was 2×10^{-5} M. After cooling and passing through Sephadex G-25M, an aliquot was used to determine specific radioactivity. The mμmoles of CF II~P were calculated on the basis of an assumed stoichiometry of 1:1 between phosphorus and CF II. This value was taken as a measure of the formation of CF II~P.

The transfer of phosphate from CF II~P to glucose via ATP was measured as follows. A mixture of CF II~P, $MgCl_2$ (5 μmoles), glucose (20 μmoles), hexokinase (50 units), and ADP (5 μmoles), in a volume of 1 ml, was incubated at 30° for 10 min. The reaction was terminated by the addition of 4 ml of silicotungstic acid followed by boiling for 10 min. The formation of glucose-6-^{32}P was determined by the method recommended by Lindberg and Ernster (1956). Periodically glucose-6-P formation was also determined directly by the method of Bandurski and Axelrod (1951). $\gamma(^{32}P)$ATP was synthesized according to an unpublished modification (Drs. A. Worcel and L. Hokin, personal communication) of the method of Metzenberg et al. (1960) and was isolated from the reaction mixture by chromatography on DEAE-substituted cellulose, with gradient elution with triethylammonium bicarbonate according to Smith and Khorana (1963). Identification and purity of the product was established by paper chromatography according to System I of the Pabst Laboratories Circular OR-10.

The phosphorylated derivative of CF II is relatively stable at pH 7.5 in Tris-H_2SO_4 at 0°; 42% of the total bound phosphate was transferred to glucose after storage for 21 hours under these conditions (48% transferred prior

to storage). When solutions of the phosphorylated derivative of CF II were exposed to pH 2 or 11 for 30 min at 0° essentially all of the bound phosphate that had been previously transferable, was released as inorganic phosphate.

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