A PROTEIN FACTOR REQUIRED FOR PHOSPHORYLATION COUPLED TO ELECTRON FLOW BETWEEN REDUCED COENZYME Q AND CYTOCHROME c IN THE ELECTRON TRANSFER CHAIN

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Received June 11, 1964

Resolution of oxidative phosphorylation was first reported by Pinchot (1953) who demonstrated that a system of A. faecalis could be separated into particulate and soluble fractions, both being required for ATP synthesis. Reports on the isolation of soluble protein coupling factors from mammalian mitochondria first appeared in 1958 (Pullman et al., 1958; Linnane, 1958). The concept of three discrete coupling factors, each operating specifically at one of the three energy conservation sites of the mammalian electron transfer chain, was reported in 1962 (Smith and Hansen, 1962). Since that time coupling factors I and III (CF I, CF III) have been purified and shown to act specifically at sites I and III respectively [(Smith and Hansen, 1962; Webster, 1962; Green, Beyer, Hansen, Smith and Webster, 1963) (see latter reference for terminology of coupling factors and phosphorylation sites)]. In addition, CF III has been reported to be a component of intermediates which can give rise to ATP (Webster, Smith, and Hansen, 1963; Webster, 1963). Coupling factor II (CF II) has now been purified (Beyer, 1964); we report herein its specific action at phosphorylation site II of the electron transfer chain as well as some of its physical and enzymic properties.

CF II has been prepared from heavy beef heart mitochondria (HBHM) by sonic treatment, ammonium sulfate precipitation, DEAE chromatography, and Sephadex treatment. CF II is stable as a dry powder or when stored in 0.01 M Tris-SO_4, pH 7.5, at -20°. The coupling factor is destroyed by heat, is unstable at room temperature, is nondialyzable, is excluded from Sephadex

G-25M, and gives a positive biuret reaction. The absorption spectrum shows a maximum at 278 mμ, a minimum at 250 mμ and a shoulder at 290 mμ. The purified protein migrates as a single component toward the anode during electrophoresis on Sepraphore III between pH values 6 and 9, the isoelectric point appearing to be in the vicinity of pH 5.7. Ultracentrifugation data from approach to sedimentation equilibrium analysis indicate a mean molecular weight of 1.24×10^5 for the isolated product. The $S_{20,w}$ value is 6.1.

Table I

PURIFICATION OF COUPLING FACTOR II

Stage	Volume (ml)	Total Protein (mg)	Activity*
BHM	200	24,000	
HBHM	232	9,274	
S-1	380	1,370	
45-70% SAS	22	341	0.5
CF II	3.1	9.6	167

* Activity is defined as the reciprocal of the number of mgs of factor protein required to saturate one mg of particle protein in the phosphorylation assay described under Table II.

The various stages of purification of CF II from beef heart mitochondria (BHM) are mentioned in Table I. The initial step from BHM to HBHM represents a loss of 61.5% of the original protein but is not considered in the calculation of the yield of CF II since the "light" beef heart mitochondria are discarded. HBHM are subjected to sonic treatment and the soluble protein obtained (14.9%), termed S-1, is fractionated by the addition of solid ammonium sulfate. It has not been possible to demonstrate restoration of phosphorylative activity with the S-1 fraction because the S-1 fraction inhibits the residual phosphorylation of the modified electron transfer particle ($METP_H$) and thus precludes the calculation of the purification on an activity basis from the initial soluble fraction. The 45-70% saturated ammonium sulfate

fraction also appears to contain some inhibitory activity as evidenced by the apparent increase in total units from the stage where the coupling protein is precipitated by ammonium sulfate to the final preparation. As is implied in Table I the yield of CF II from the original HBHM is of the order of 0.1% on a protein basis.

Table II

EFFECT OF CF II ON P/O RATIOS OF $METP_H$

Substrate	Additions	ΔP_i^*	P/O Ratio	$\Delta P/O$
DPNH		3.3	0.32	
DPNH	100 μg CF II	5.0	0.47	+ .15
Succinate		1.7	0.17	
Succinate	100 μg CF II	3.5	0.34	+ .17
Reduced cyt. c		0.05	0.09	
Reduced cyt. c	100 μg CF II	0.05	0.07	- .02
Succinate	2,4-DNP, 100 μg CF II	0	0	
Succinate	Oligomycin, 100 μg CF II	0.2	0.02	

The reaction system was 0.33 M in sucrose, 0.033 M in glucose, 6.6 mM in $MgCl_2$, 5.2 mM in KPO_4 of pH 7.5, and 1.67 mM in ATP; it also contained, in a final volume of 3 ml, 0.1 mg of crystalline hexokinase and 1 mg of $METP_H$. DPNH was generated by the combination of 0.5 μmole of DPN, 0.3 mg of crystalline yeast ethanol dehydrogenase (100 U.), and 60 μmoles of ETOH. The initial concentration of succinate was 6.67 mM. Cytochrome c was maintained in the reduced state by the addition of 10 μmoles of ascorbate, 3 mμmoles of phenazine methosulfate, and 2 μg of antimycin A. The concentration of DNP was 5×10^{-5} M; 1 μg of oligomycin was added per mg of particle. Oxygen consumption was measured by conventional manometry for 30 min at 30°. After temperature equilibration for 5 min, the reaction was initiated by the addition of substrate. Phosphorylation was assayed by measuring the disappearance of inorganic orthophosphate as determined by the colorimetric method of Martin and Doty (1949) as described by Lindberg and Ernster (1956).

*ΔP_i refers to the μmoles of inorganic orthophosphate disappearing from the experimental flask corrected for a blank in which substrate was omitted.

Table II records the results of a representative experiment showing the effect of CF II on oxidative phosphorylation catalyzed by $METP_H$ prepared essentially according to Linnane and Titchener (1960) with DPNH, succinate, or

reduced cytochrome c as substrate. No technical difficulties were encountered in estimating changes in the P/O ratios because the amounts of both quantities were sufficiently large; approximately 10 μatoms of oxygen were utilized when DPNH or succinate was used as substrate and, in the presence of CF II, the increase in the P/O ratio varied between 0.15 and 0.24 with different preparations of factor and particle. For example, when succinate was used as substrate there was little change in oxygen consumption attributable to the factor; however, the amount of inorganic phosphate that disappeared in the control (Table II) flask in the absence of added CF II was 1.7 μmoles as compared with 3.5 μmoles in the experimental flask in the presence of 100 μg of CF II. An equal or greater effect has been noted with approximately thirty preparations of CF II during the past twelve months.

The presence of coupling factor rarely affected the rate of electron transfer which, with DPNH or succinate as substrate, was in the vicinity of 0.3 μatoms of oxygen per min per mg of particle. CF II (100 μg) increased the P/O ratio to the same extent whether succinate or DPNH served as substrate but was without effect when reduced cytochrome c was employed as substrate. The response of the particle to CF II was not affected by Rotenone (succinate as substrate). That all three phosphorylation sites of $METP_H$ respond to the addition of coupling factors is indicated by the demonstration that the crude ammonium sulfate fraction obtained during the preparation of CF II increased phosphorylative capacity at sites I and II and that purified CF III restored phosphorylation at site III (Webster, 1962). The data of Table II indicate that CF II specifically restores the ability to synthesize ATP at phosphorylation site II. All phosphorylation by $METP_H$, including that increased by the presence of CF II, was uncoupled by DNP and inhibited by oligomycin. Although 100 μg of CF II was employed in the experiment described in Table II, saturation of the particle was achieved at 60 μg per mg of particle. Other proteins, such as bovine serum albumin, did not substitute for CF II.

During purification of CF II both the capacity to restore phosphorylation and ATP-ADP exchange activity were enriched. ATPase, ATP-$^{32}P_i$ exchange, and adenylate kinase activities were diminished considerably. The initial rates of these reactions as catalyzed by CF II are listed in Table III. The enrichment of ATP-ADP exchange activity with purification of CF II is in agreement with the data of Webster (1962) on purification of CF III and also with the data of Glaze and Wadkins (1964) on the purification of an ATP-ADP exchange enzyme capable of restoring phosphorylation at site III between cytochrome c and O_2. Unlike the factor reported by Pullman et al. (1960), CF II was not labile to cold and did not exhibit significant ATPase activity.

Table III

ENZYMIC PROPERTIES OF CF II

Reaction	Rate, mμmoles per min per mg
ATPase	0.50
ATP-$^{32}P_i$ exchange	0.31
Adenylate kinase	180
ATP-ADP exchange	770

All reactions were carried out at 30°. ATPase, ATP-$^{32}P_i$ exchange, and adenylate kinase activities were assayed according to Wadkins and Lehninger (1958) with minor variations. ATP-ADP exchange was assayed according to Glaze and Wadkins (1964) with minor variations.

The ability of CF II to catalyze an active exchange between ATP and ADP, in addition to its ability to restore the phosphorylative capacity specifically associated with site II of the electron transfer chain, indicates that the factor may catalyze the substitution reaction by which the high energy intermediate generated at site II of the chain is transferred to ATP. Since site-specific coupling factors have previously been established for phosphorylation sites I and III (Smith and Hansen, 1962; Webster, 1962) we now consider the existence of three site-specific coupling factors in oxidative phosphorylation to be experimentally established.

ACKNOWLEDGEMENTS

The author thanks Dr. D. E. Green for advice and encouragement during this investigation, colleagues too numerous to mention for valuable suggestions and discussions, Dr. Pauline Yang for performing the experiments in the analytical ultracentrifuge, and Mr. Earl Fronk for skilled technical assistance. The author is the recipient of a Research Career Development Award from the Division of General Medical Sciences of the National Institutes of Health. This research was supported by Grant AM06751-03 MET from the National Institute of Arthritis and Metabolic Diseases of the National Institutes of Health. Meat by-products were generously supplied by Oscar Mayer and Co., Madison.

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