EFFECT OF COUPLING FACTOR 3 ON OXIDATIVE PHOSPHORYLATION*

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Submitochondrial particles prepared by exposing mitochondria to sonic oscillation either in the presence of phosphatides (Conover et al., 1963) or at pH 9.2 (Fessenden and Racker, 1966) require addition of F_1[1] and F_4 for oxidative phosphorylation. After prolonged dialysis of the F_4 preparation at an alkaline pH, a partial dependency on F_2 also became apparent (Fessenden and Racker, 1966). F_3 which stimulates the $^{32}P_i$-ATP exchange (Racker, 1962) as well as the ATP-dependent reduction of DPN by succinate (Conover et al., 1963) has no apparent effect on oxidative phosphorylation in the presence of F_1 and F_4.

It is the purpose of this communication to show that F_3 substitutes for F_4 in stimulating oxidative phosphorylation in A-particles. With succinate as substrate P:O ratios in excess of 1.0 can be obtained with A-particles in the presence of F_1, F_2 and F_3. These experiments support the earlier suggestion (Conover et al., 1963) that F_4 which has the properties of a structural protein (Zalkin and Racker, 1965) may stimulate oxidative phosphorylation by supplying firmly attached coupling factors. It will be shown that F_2 in contrast to F_1, F_3 and F_4 does not stimulate the ATP-dependent reduction of DPN by succinate.

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1 The abbreviations used are: F_1, F_2, F_3, and F_4: coupling factor 1 (ATPase), coupling factor 2, coupling factor 3, and coupling factor 4, respectively; A-particles: particles exposed to sonic oscillations in the presence of ammonia at pH 9.2.

RESULTS

A-particles and F_2 (Fessenden and Racker, 1966), F_1 (Pullman et al., 1960), F_3 (Fessenden and Racker, in press), F_4 (Conover et al., 1963) were prepared as described in the references. Measurements of oxidative phosphorylation and of related reactions were carried out as described (Fessenden and Racker, 1966) with the modifications specified in the legends of the tables.

It can be seen from Table I that in the presence of F_1 addition of F_3 almost completely replaced the requirement of A-particles for F_4. Addition of F_2 without F_3 had little or no effect on the P:O ratio, but in the presence of F_3 there was a small but consistent stimulation by F_2. In many experiments the P:O ratio was doubled by addition of F_2. Addition of F_4 to particles supplemented with F_1, F_2 and F_3 gave no further increase in the P:O ratio. After extensive dialysis of F_4 (over 70 hours at pH 10.5) most of its coupling factor activity was lost without apparent damage to the structural protein as

TABLE I

Effect of F_3 on oxidative phosphorylation

A-particles (540 μg) were incubated in the presence of 30 μmoles succinate, 2 μmoles $MgSO_4$, 2 μmoles ATP, and 40 μg F_1. Where indicated 200 μg F_2, 307 μg F_3 and 400 μg F_4 were added. Incubation conditions and assay procedures were as described previously (Fessenden and Racker, 1966).

Addition of factors to A-particles	μatoms O	μmoles Glucose-6-^{32}P	P:O
F_1	3.4	0.835	0.24
$F_1 + F_4$	3.4	3.50	1.03
$F_1 + F_2$	4.1	1.24	0.3
$F_1 + F_3$	4.2	3.75	0.9
$F_1 + F_2 + F_3$	3.1	4.23	1.36

measured by a reconstitution of the oligomycin-sensitive ATPase.[2] It thus appears likely that the stimulation of oxidative phosphorylation in A-particles by F_4 is due to the presence of at least two coupling factors (F_2 and F_3).

As shown in Table II, F_3 stimulated oxidative phosphorylation at site 1, site 3 and the phosphorylation associated with succinate oxidation (site 2 + site 3). These results suggest that F_3 is required at each of the three phosphorylation sites. Small but significant stimulations by F_2 were also noted.

TABLE II

Effect of F_2 and F_3 on the three phosphorylation sites

A-particles (430 µg) were incubated with 39 µmoles P_i, pH 7.4, 2 µmoles ATP, 2 µmoles $MgSO_4$ and 40 µg F_1. Where indicated 200 µg F_2 and 300 µg F_3 were added. Incubation conditions and assay procedures were as described previously (Fessenden and Racker, 1966, Schatz and Racker, 1966).

Factor additions to A-particles	DPNH	CoQ_1	Succinate	O_2	Ascorbate	O_2
	µmoles DPNH oxidized	P:O*	µatoms O	P:O	µatoms O	P:O
F_1	95	0.03	3.3	0.11	4.1	0.04
$F_1 + F_2$	97	0.04	3.9	0.18	3.7	0.02
$F_1 + F_3$	93	0.24	4.1	0.80	4.3	0.39
$F_1 + F_2 + F_3$	95	0.36	4.1	1.10	3.8	0.53

* Determined as glucose-6-^{32}P : DPNH ratio.

The experiment in Table III shows that F_2 stimulates the $^{32}P_i$-ATP exchange reaction even in the presence of large amounts of F_3. This indicated that a partial separation of these two factors has been obtained.

2 Unpublished experiments by B. Bulos showed that extensively dialyzed F_4 preparations still restored to F_0 preparations depleted in F_4 (Kagawa and Racker, 1966) the ability to confer oligomycin sensitivity to added ATPase. Neither preparations of F_3 or F_2 nor combinations of both substituted for dialyzed F_4.

TABLE III

Effect of F_3 and F_2 on the $^{32}P_i$-ATP exchange

A-particles (410 µg) were incubated as described in Table II except that 180 µg F_2 and 240 µg F_3 were used. The exchange reaction was measured as described previously (Fessenden and Racker, 1966).

Factor additions to A-particles	µmoles $AT^{32}P$ formed/ 10 min/mg protein
F_1	0.06
$F_1 + F_2$	0.30
$F_1 + F_3$	1.05
$F_1 + F_2 + F_3$	2.20

TABLE IV

Effect of coupling factors on the ATP-dependent reduction of DPN by succinate

A-particles (275 µg) were incubated in a quartz cuvette at room temperature in a final volume of 0.6 ml containing the following reagents: 150 µmoles Tris-sulfate, pH 7.5, 10 µmoles $MgSO_4$, 1.5 mg dialyzed bovine serum albumin, 15 µmoles Tris-succinate, pH 7.5, and where indicated 10 µg F_1, 100 µg F_2 and 200 µg F_3. After 10 min, 2.33 ml of distilled water, 0.02 ml of 0.3 M Na_2S and 0.02 ml of 0.03 M DPN were added. The reaction was started 1-2 min after the DPN addition with 0.02 ml of 0.2 M ATP. The appearance of fluorescence due to DPNH formation was measured in an Eppendorf fluorometer as described previously (Prairie et al., 1963).

Factor additions to A-particles	mµmoles DPNH formed/ min/mg protein
None	2.9
F_1	5.5
F_3	3.6
F_2	13.0
$F_1 + F_2$	11.9
$F_1 + F_3$	81.1
$F_1 + F_3 + F_2$	80.8

In Table IV the effect of F_2 and F_3 on the ATP-dependent reduction of DPN by succinate is shown. It can be seen that A-particles exhibit high rates of DPN reduction in the presence of both F_1 and F_3. Omission of either factor resulted in over 90% loss of activity. Addition of F_2 together with F_1 and F_3 had no effect on the rate of DPN reduction.

F_2 had no effect on the DPN reduction by succinate in the presence of F_1 and F_3. The partial stimulation by F_2 alone suggests this factor may be either involved in the formation of a non-phosphorylated high-energy intermediate or may perhaps help to preserve it as proposed for oligomycin (Racker and Monroy, 1964, Lee et al., 1964).

DISCUSSION

The stimulation of oxidative phosphorylation in submitochondrial particles by addition of rather insoluble preparations of F_4 was always difficult to understand. Possible explanations, e.g., that the structural protein supplies a soluble coupling factor (Conover et al., 1963) or that it may counteract an inhibitor (Zalkin and Racker, 1965) were presented without direct evidence. The demonstration of the replacement of F_4 by two soluble coupling factors, F_2 and F_3, not only provides the necessary evidence for the first alternative but helps to explain numerous examples of experimental variabilities which were due to varying contaminations by the two factors.

The difficulties encountered in removing F_2 and F_3 activity from F_4 indicate that these proteins are firmly bound to F_4. Binding of succinate dehydrogenase to F_4 was also observed[3] whereas several other proteins such as F_1, glucose-6-P dehydrogenase and lactate dehydrogenase were not bound. Contamination of F_4 with F_2 and F_3 may account entirely for its stimulatory effect on oxidative phosphorylation. On the other hand experimental evidence[2] points to an additional function of F_4 (Kagawa and Racker, 1966). The appar-

[3] A. Bruni, unpublished experiments.

ently specific binding of certain mitochondrial proteins to F_4 would be in line with such a view. Yet another possibility is that F_4 is a polymerized product of F_3, but loses the ability to depolymerize after prolonged dialysis. In line with this notion is the experimental observation of a progressive loss of solubility during purification of F_3 (Fessenden and Racker, in press).

The properties of factor B described by Lam et al., (1966) suggest that it may be identical with F_3.

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