*STUDIES OF FACTORS INVOLVED IN OXIDATIVE PHOSPHORYLATION**

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The oxidation of substrates of the citric acid cycle in mitochondria is coupled with the phosphorylation of ADP to ATP.[1] Considerable progress has been made in our understanding of the three electron transport reactions between DPNH and oxygen (DPNH → cytochrome b → cytochrome c → O_2), which are associated with the phosphorylation mechanism.[2] In several laboratories[3-7] actively phosphorylating submitochondrial particles were prepared by either physical or chemical fragmentation of mitochondria. Moreover, "coupling factors," soluble components which are required for the phosphorylation process, have been obtained from bacteria[8, 9] and animal tissues.[7, 10-12] The study of a multi-enzyme system such as the one involved in oxidative phosphorylation, which is embedded within the mitochondrial structure, presents considerably greater difficulties than that of soluble multi-enzyme systems. First, physical separation of the individual components is more difficult and rarely complete. Secondly, the individual components, after solubilization and purification, often have properties quite different from those they have within the mitochondria.[2] It is the purpose of this communication to describe some new procedures involving treatment of submitochondrial particles with urea and trypsin that have facilitated resolution and analysis of the oxidative phosphorylation system; and to report on a new coupling factor (F_3) which is required for the incorporation of P^{32}-labeled phosphate into ATP (P_i^{32}-ATP exchange) in submitochondrial particles. This exchange appears to be the expression of a partial reaction of oxidative phosphorylation.[2]

Results.—Coupling factor 1: It was previously shown that submitochondrial particles obtained by mechanical disintegration, although capable of oxidizing succinate, produced ATP from ADP only when a soluble protein was added.[7] This coupling factor 1 (F_1) was isolated from beef-heart mitochondria as a homogenous protein and was also shown to catalyze the hydrolysis of ATP to P_i and ADP.[13] It will therefore be referred to either as ATPase or as F_1, depending on whether hydrolytic activity or coupling in oxidative phosphorylation was measured. When a solution of this protein was exposed to 0°, both activities were lost. More recent studies of this curious cold-lability revealed that a number of factors influence the rate of inactivation. Among them are ionic environment, protein concentration and, particularly, prior exposure of the enzyme to ATP. For example, a solution of the enzyme was completely inactivated at 0° in 10 min in Tris-HCl buffer, whereas several hours were required in Tris-H_2SO_4 buffer.[14] Stored as a suspension in 2 *M* ammonium sulfate at 0°, the enzyme lost little activity even after several months. A fresh solution in 4 mM ATP was over 70 per cent inactivated after 15 min at 0°, but was fully active after 2 days at 25°. On aging at 25° with ATP, the enzyme acquired increasing stability to cold exposure, so that after 48 hr at 25° it was only 15 per cent inactivated after 15 min at 0°.

Effect of urea on ATPase and on the P_i^{32}-ATP exchange reaction: The enzyme,

which was stabilized by exposure to ATP, was very labile in the cold in the presence of relatively low concentrations of urea (0.8 *M*). Without urea, about 2 hr at 0° were required for 50 per cent inactivation; in the presence of urea the half-life was about 10 min at 0° and about 2 hr at 25°.

In contrast to soluble ATPase, the ATPase activity of submitochondrial particles was very stable at 0°. It was readily inactivated, however, at 0° in the presence of 1.6 *M* urea, as shown in Table 1. At higher urea concentration (4 *M*), exposure for 5 min sufficed to eliminate most of the ATPase activity. Submitochondrial particles treated in this manner did not catalyze a P_i^{32}-ATP exchange reaction unless ATPase (F_1) was added (Table 2). Addition of coupling factor 3 (F_3), which will be discussed later, resulted in a relatively small stimulation. The exchange stimulated by F_1 was eliminated in the presence of dinitrophenol. These experiments with urea-treated particles re-emphasize the role of F_1 (ATPase) in the phosphorylation process.

TABLE 1

INACTIVATION OF ATPASE IN SUBMITOCHONDRIAL PARTICLES BY UREA*

Time of exposure at 0°	ATPase activity, μmoles P_i/min/mg
None	0.48
40 min	0.19
80 min	0.12
120 min	0.07
210 min	0.02

* Submitochondrial particles prepared as described under *Preparations and Methods* were suspended at 10 mg protein per ml in 1.6 *M* urea at 0° for the time periods indicated and then assayed for ATPase activity as follows. In a final volume of 0.5 ml were added: Tris-H_2SO_4 buffer, pH 7.5 (50 mM), ATP (6 mM), $MgSO_4$ (3 mM), and 200 μg of submitochondrial particles. After 10 min at 30° the mixture was deproteinized with 5 per cent trichloroacetic acid and analyzed for P_i.

TABLE 2

EFFECT OF UREA ON THE P_i^{32}-ATP EXCHANGE REACTION IN SUBMITOCHONDRIAL PARTICLES*

	μmoles ATP^{32}/mg
Treated particles	0.021
Treated particles + F_1 (13.5 μg)	0.140
Treated particles + F_3 (800 μg)†	0.042
Treated particles + F_1 + F_3	0.268
Untreated particles	0.30
Untreated particles + F_1	0.32

* To submitochondrial particles (60 mg protein/ml) an equal volume of 8 *M* urea was added at 0°. After 5 min the particles were diluted with 8 volumes of 0.25 *M* sucrose, exposed to sonic oscillation for 1 min, and isolated by centrifugation for 30 min at 105,000 × *g*. The measurements of the exchange rate were carried out in a final volume of 0.5 ml containing ATP (15 mM), $MgSO_4$ (15 mM), Tris buffer, pH 7.4 (25 mM), 400 μg of particles, and 10^4 cpm P_i^{32} (40 mM). After 10 min at 30° the reaction was stopped by addition of 0.5 ml of 10 per cent trichloroacetic acid containing 50 mg of charcoal. After filtration and washing with 30 ml of 5 per cent trichloroacetic acid the radioactivity was determined in the entire charcoal sample.

† Although a rather crude preparation of F_3 was used in this experiment, no P_i^{32}-ATP exchange was catalyzed by this fraction in the absence of mitochondria.

Effect of oligomycin on ATPase: Among the many altered properties of solubilized ATPase was its resistance to oligomycin, which was in contrast to the high sensitivity of ATPase in mitochondria.[15, 16] Attempts to confer oligomycin sensitivity to the soluble enzyme were partly successful with particles[16] but were thus far unsuccessful with soluble fractions from mitochondria. However, it was found that urea-treated submitochondrial particles conferred essentially complete oligomycin sensitivity to added ATPase, as shown in Table 3.

Effect of trypsin on submitochondrial particles: Exposure of submitochondrial particles to trypsin resulted in a rapid loss of the P_i^{32}-ATP exchange reaction. As shown in Table 4, addition of a new factor (F_3) restored the exchange activity.

Addition of F_1 to these particles gave rise to relatively small stimulations. The exchange activity in the presence of F_3 and F_1 was sensitive to 2,4-dinitrophenol.

Since recent investigations[17, 18] have implicated mitochondrial pyrophosphatase in phosphate transfer reactions, various soluble protein fractions obtained from mitochondria were examined for pyrophosphatase activity. It was noted that during the purification of F_3 the pyrophosphatase activity was purified to approximately the same extent as the stimulatory activity in the P_i^{32}-ATP exchange reaction. However, purification of either component has not advanced far enough to suggest the identity of F_3 with pyrophosphatase. What makes the pyrophosphatase a rather intriguing mitochondrial component is its interaction with ATPase. As shown in Table 5, pyrophosphate hydrolyzing activity was markedly stimulated by the addition of ATPase, which by itself did not cleave pyrophosphate under these conditions. The pyrophosphatase activity was also activated by SH compounds, e.g., α-monothioglycerol. However, with some preparations of solubilized pyrophosphatase, hydrolysis of pyrophosphate was considerably more rapid in the presence of ATPase than with the SH compound. Moreover, remarkably small concentrations of ATPase (2×10^{-7} M) were effective in this system.

TABLE 3

CONFERRAL OF OLIGOMYCIN SENSITIVITY ON SOLUBLE ATPASE BY UREA-TREATED PARTICLES*

	μmoles P_i formed in 10 min
Urea-treated particles (1 mg)	0.13
Urea-treated particles (1 mg) + oligomycin (3.3 μg)	0.04
Untreated particles (200 μg)	1.00
Untreated particles (200 μg) + oligomycin	0.07
ATPase (3 μg)	1.63
ATPase (3 μg) + oligomycin	1.68
ATPase (3 μg) + urea-treated particles	1.04
ATPase (3 μg) + urea-treated particles + oligomycin	0.14

* Submitochondrial particles were treated at 10 mg protein/ml with 1.6 M urea for 20 hr at 0°, then centrifuged for 30 min at 105,000 × g and suspended in 0.25 M sucrose. ATPase was added to the particles with or without oligomycin and the mixture was immediately tested for ATPase activity, as described in Table 1.

TABLE 4

EFFECT OF TRYPSIN ON P_i^{32}-ATP EXCHANGE IN SUBMITOCHONDRIAL PARTICLES*

	μmoles ATP^{32}/mg
Treated particles	0.012
Treated particles + F_1 (6 μg)	0.018
Treated particles + F_3 (650 μg)	0.280
Treated particles + F_1 + F_3	0.320
Treated particles + F_3 + F_1 + DNP (5×10^{-4} M)	0.025
Untreated particles	0.305
Untreated particles + F_3	0.388

* Submitochondrial particles were diluted to 10 mg protein/ml in a mixture containing: Tris buffer, pH 8.0 (50 mM), EDTA (2 mM), and 32 μg/ml of a freshly prepared trypsin solution. After 30 min at 30° the reaction was stopped by addition of 120 μg/ml of trypsin inhibitor and the mixture was centrifuged for 45 min at 105,000 × g. The particles were collected in 0.25 M sucrose in a final suspension of approximately 10 mg protein/ml. The exchange reaction was measured as described in Table 2.

Discussion.—There are at least three soluble protein factors that stimulate phosphorylation processes in submitochondrial particles. Coupling factor 1 (ATPase) and coupling factor 2 (perhaps identical with the factor described by Linnane and Titchener[10]) are required for the phosphorylation of ADP associated with the oxidation of succinate.[20] Coupling factor 3, described in this paper, is required for the P_i^{32}-ATP exchange reaction in particles treated with trypsin, but a direct participation in the net formation of ATP has not as yet been demonstrated. It

TABLE 5

EFFECT OF ATPASE ON PYROPHOSPHATASE*

		μmoles Pi formed in 10 min
Exp. 1:	BHM-pyrophosphatase (60 μg)	0.61
	ATPase (50 μg)	0.0
	Both	2.78
Exp. 2:	RLM-pyrophosphatase (140 μg)	0.66
	ATPase (30 μg)	0.0
	Both	1.42

* Beef-heart mitochondria were used as source of the pyrophosphatase in Exp. 1, and rat-liver mitochondria in Exp. 2. Both preparations were carried through the purification procedure exclusive of the DEAE-cellulose step (cf. *Preparations and Methods*). Pyrophosphatase activity was measured in a final volume of 0.5 ml containing Tris buffer (120 mM), pH 7.4, $MgSO_4$ (12 mM), pyrophosphate (8 mM), pH 7.4, and the reagents listed in the table.

is conceivable that this factor is involved in the phosphorylation step between DPNH and cytochrome b, which has been considered to be the major contributor to the P_i^{32}-ATP exchange observed in mitochondria.[21]

The most probable assignment for the specific function of coupling factor 1 is the last transphorylation step between a phosphorylated intermediate X $\sim$ P and ADP. Assuming that X $\sim$ P is identical for the three electron transfer steps in oxidative phosphorylation, then F_1 should be required at each site. One might postulate at least three enzymes associated with the formation of X $\sim$ P at the three phosphorylating sites of the electron transport chain. Coupling factors 2 and 3 are visualized to operate as catalysts at the sites where cytochrome b and DPNH respectively are oxidized. It should be emphasized, however, that direct evidence for catalysis of a specific reaction is thus far available only for F_1 (ATPase).

Preparations and Methods.—The preparation of beef-heart mitochondria and coupling factor 1 (ATPase), and the analytical procedures, were essentially as described previously.[7, 13] Measurements of the P_i^{32}-ATP exchange were carried out according to Boyer *et al.*[19] It was found essential to recrystallize commercial preparations of P_i^{32} as the magnesium ammonium salt in order to eliminate high blank values due to adsorption of radioactivity to charcoal. Moreover, the specific activity of phosphate used in these experiments was kept at about 500 cpm per μmole.

Submitochondrial particles: Beef-heart mitochondria (heavy layer) stored frozen at −55° in 0.25 *M* sucrose (at about 40 mg protein/ml) were diluted with water and 0.1 *M* pyrophosphate buffer, pH 7.4, to a final protein concentration of 20 mg per ml and a final pyrophosphate concentration of 0.01 *M*. 30-ml batches of these suspensions were exposed for 2 min to sonic oscillation in a 10 kc Raytheon sonic oscillator. A heavy pellet was centrifuged off at 26,000 × *g* in a preparative Spinco centrifuge for 15 min and discarded. The supernatant solution was carefully decanted and centrifuged at 105,000 × *g* for 45 min. The pellet thus obtained was washed and resuspended in 0.25 *M* sucrose to yield a heavy suspension containing about 60 mg protein per ml. These particles, kept frozen at −55° in a Revco deep freeze, were stable for several months.

Preparation of F_3 and pyrophosphatase: The supernatant solution obtained after sonic oscillation of the mitochondria was adjusted to pH 5.4 with 1 *N* acetic acid and centrifuged at 0°. To the clear supernatant solution were added 29.1 gm of ammonium sulfate per 100 ml solution, and the precipitate was discarded after centrifugation for 10 min at 18,000 × *g*. The yellow supernatant solution was treated with 15.9 gm ammonium sulfate per 100 ml. The precipitate was collected by centrifugation for 15 min at 18,000 × *g* and dissolved in $^1/_{20}$ the original extract volume. After dialysis against 0.01 *M* pyrophosphate, pH 7.4, for 1 hr and against 0.005 *M* Tris-H_2SO_4 buffer, pH 7.4, for two more hr, 3 ml of the solution (containing 20 mg protein/ml) was applied to a DEAE-cellulose column (5.0 cm × 1.1 cm) previously equilibrated with 0.005 *M* Tris buffer, washed with 0.005 *M* ammonium sulfate, and eluted with 0.04 *M* ammonium sulfate. A rather sharp yellow band was eluted and collected in a small volume. This fraction contained both F_3 and pyrophosphatase activity, whereas the colorless fractions collected before and after this fraction were virtually inactive.

Summary.—Several soluble factors (coupling factors) have been shown to be required for phosphorylation associated with electron transport in submitochondrial particles. Requirement for coupling factor 1 in the P_i^{32}-ATP exchange reaction has been demonstrated after brief exposure of submitochondrial particles to 4 *M* urea. Such urea-treated particles were devoid of ATPase activity but conferred oligomycin sensitivity on added ATPase. Treatment of submitochondrial particles with trypsin also resulted in a loss of the P_i^{32}-ATP exchange reaction. Restoration of activity was achieved by the addition of a new factor (F_3). Partially purified preparations of this factor contained pyrophosphatase activity, which was stimulated by addition of ATPase. The possible role of the soluble coupling factors was discussed.

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[1] Abbreviations: ATP and ADP for adenosine triphosphate and adenosine diphosphate; P_i for inorganic phosphate; Tris for tris(hydroxymethyl) aminomethane; EDTA for ethylenediaminetetraacetate; DNP for 2,4-dinitrophenol; DPNH for reduced diphosphopyridine nucleotide; DEAE for diethylaminoethyl.

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