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ON COUPLING FACTORS OF OXIDATIVE PHOSPHORYLATION

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SUMMARY

1. The isolation of three ATPase preparations from beef-heart mitochondria is described. One of these (F_1-X) is soluble, oligomycin insensitive, cold labile and has a high coupling activity. The second is insoluble, oligomycin sensitive, cold stable and without coupling activity. From both of these preparations a soluble ATPase (F_1) can be obtained, which is identical with Coupling Factor 1 of PULLMAN, PENEFSKY AND RACKER.

2. The high coupling activity of F_1-X seems to be due to the fact that it is a complex between F_1 and another factor (or other factors) that is cold stable but labile at temperatures over 30°.

3. A factor that confers oligomycin sensitivity to F_1 was obtained from the insoluble ATPase by treatment with 2 M urea.

4. A factor (F_2) present in a crude succinate dehydrogenase preparation doubled the stimulation induced by F_1-X of the ATP-dependent NAD^+ reduction by succinate.

5. Cold-treated F_1-X inhibited the ATPase activity of F_1 and of EDTA particles.

INTRODUCTION

The intensive work that has been carried out since 1958 in RACKER's laboratory[1–16] and elsewhere[17–27] in an attempt to resolve the mammalian mitochondrial energy-transfer system into discrete components has resulted in the description of a large number of soluble coupling factors that are able to increase oxidative phosphorylation and related processes in deficient submitochondrial particles. The most thoroughly studied is the soluble ATPase (or F_1) purified from beef-heart mitochondria by PULLMAN *et al.*[2]. It has been proposed that F_1 catalyses the transfer of phosphate from a high-energy intermediate to ADP, and that its ATPase activity is the result of structural damage to the mitochondria and to the enzyme during purification[3]. The ATPase activity of F_1, in contrast to that of mitochondria, is oligomycin insensitive and cold labile, but insoluble factors have been described (F_0 and CF_0) that restore the oligomycin sensitivity to F_1 (refs. 12, 13).

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In addition to F_1, other factors (F_2, F_{3A}, F_{3B}, F_4) have been reported[2,5–10] to be necessary for maximal stimulation of the energy-transfer process in different types of submitochondrial particles. A very considerable stimulation of the P/O ratio was obtained when several factors were used together[10,11]. However, the coupling activity of F_4 has recently been attributed to a contamination with F_2 and F_3 (ref. 11). There was also some contamination of F_3 with F_2 (ref. 15).

The present paper reports the purification and properties of a soluble ATPase (F_1-X) with a high coupling activity in the absence of other factors. It is probably a complex of F_1 with another factor or other factors. The paper also reports the preparation, from an insoluble oligomycin-sensitive and cold-stable ATPase, of a soluble oligomycin-insensitive and cold-labile ATPase (F_1) and an insoluble factor that restores oligomycin sensitivity to F_1.

A preliminary account of this investigation has been presented[28].

METHODS

Preparation of beef-heart mitochondria and submitochondrial particles

Heavy and light beef-heart mitochondria were prepared according to CRANE, GLENN AND GREEN[29] and HATEFI AND LESTER[30] with slight modifications.

Ammonia particles (A particles) and EDTA particles were prepared from heavy beef-heart mitochondria as described by FESSENDEN AND RACKER[10] and LEE, AZZONE AND ERNSTER[31], respectively, with slight modifications.

Measurement of ATPase activity

ATPase activity was assayed at 30° in the presence of the ATP-regenerating system described by PULLMAN *et al.*[2] with 50 mM Tris–sulphate buffer (pH 7.4), 6 mM ATP, 3 mM $MgCl_2$, 5 mM phosphoenolpyruvate and 32 μg of pyruvate kinase (EC 2.7.1.40) in a final volume of 1 ml.

Measurement of oxidative phosphorylation

Submitochondrial particles (0.5 mg protein) were preincubated alone or with factors for 5 min at room temperature in 0.3 ml of a solution containing 2 μmoles $MgSO_4$, 2 μmoles ATP and 30 μmoles potassium phosphate (pH 7.4) in a small manometer flask. The reaction was started by adding 0.2 ml of a solution containing 1 μmole $MgSO_4$, 0.5 μmole ATP, 16 μmoles glucose, 2.5 μmoles Tris–sulphate buffer (pH 7.4), 0.25 μmole EDTA, 10 μmoles sodium succinate, 0.5 mg bovine serum albumin, 5 units (μmole/min) of hexokinase (EC 2.7.1.2) and about 300000 counts/min of carrier-free $^{32}P_i$. The reaction was carried out at 25° for 20–30 min and stopped with 0.5 ml of 10% trichloroacetic acid.

Oxygen consumption was determined with differential manometers according to SLATER AND HOLTON[32]. The ^{32}P incorporated into glucose 6-phosphate was determined in an aliquot of the supernatant by extraction of the $^{32}P_i$ with molybdate and isobutanol–benzene according to NIELSEN AND LEHNINGER[33] and counting the aqueous layer in a Nuclear Chicago gas-flow counter.

Measurement of the P_i–ATP exchange reaction in submitochondrial particles

Particles and factors were preincubated at 25° in a test tube under the conditions described above for measuring oxidative phosphorylation. The reaction was

started by adding 0.2 ml of a solution containing 8 μmoles $MgSO_4$, 8 μmoles ATP and about 300000 counts/min carrier-free $^{32}P_i$. After 15 min the reaction was stopped with 0.05 ml of 50% trichloroacetic acid. The incorporation of ^{32}P into ATP was determined as described above.

Measurement of the ATP-dependent reduction of NAD^+ by succinate

The ATP-dependent reduction of NAD^+ by succinate was measured in the Aminco-Chance double-beam spectrophotometer at 350 *minus* 375 nm. Submitochondrial particles (0.5 mg protein) were preincubated alone or with factors for 5 min at room temperature in a glass cuvette in 0.5 ml of a solution containing 150 μmoles Tris–sulphate (pH 7.4), 10 μmoles $MgSO_4$, 1.5 mg bovine serum albumin and 30 μmoles sodium succinate. 3 μmoles KCN, 3 μmoles NAD^+ and water to 2.9 ml were then added and the reaction was started 1 or 2 min later by adding 0.1 ml of 40 mM ATP.

Analytical methods

P_i was determined by the method of FISKE AND SUBBAROW as modified by SUMNER[34]. Soluble protein was measured spectrophotometrically[35] and insoluble protein by the biuret method[36]. Occasionally the method of LOWRY *et al.*[37] was used. The phospholipid composition was determined, after extraction with methanol–chloroform[38], by thin-layer chromatography according to HABERMANN, BANDTLOW AND KRUSCHE[39], and total P according to BÖTTCHER, VAN GENT AND PRIES[40].

Materials

Carrier-free $H_3{}^{32}PO_4$ was purchased from Philips-Duphar. It was boiled with 1 M HCl for 1 h and then neutralized with 4 M KOH.

ATP, hexokinase, pyruvate kinase and phosphoenolpyruvate were obtained from Boehringer and Söhne. Oligomycin was kindly provided by the Upjohn Chemical Co.

All other chemicals were from the British Drug Houses Ltd.

RESULTS

Preparation of factors

Acetone powder of light beef-heart mitochondria. An acetone powder of light beef-heart mitochondria was prepared by extracting twice the mitochondrial suspension (40–50 mg/ml) with 20 vol. of cold acetone (—20°) and once with anhydrous ether. Finally the preparation was dried under vacuum and stored in the cold.

Extraction of the acetone powder. Aliquots of about 800 mg of the acetone powder (dry wt.) were homogenized with 18 ml of water and treated with sonic oscillations (Mullard 60 W, maximum output) for 2 min at room temperature. The powder was recovered by centrifugation at 38000 × *g* for 10 min and the extraction repeated three times with 12 ml of water (*cf.* ref. 41). In this way four extracts with ATPase activity were obtained (Fig. 1). The specific activity increased from the first to the fourth extract while the oligomycin sensitivity diminished (Table I).

Ammonium sulphate fractionation. The aqueous extracts obtained from the acetone powder were clarified by centrifugation at 105000 × *g* for 30 min and then

precipitated with $(NH_4)_2SO_4$ (pH 7.4) to 50% satn. At this stage the preparation may be stored in the cold. The precipitates were recovered by centrifugation (supernatant solutions were discarded), suspended in 0.25 M sucrose, 10 mM Tris–sulphate (pH 7.4) and 1 mM EDTA, and centrifuged at 38000 × *g* for 20 min at room temperature. The supernatant solutions contained F_1-X. The precipitates (insoluble ATPase)

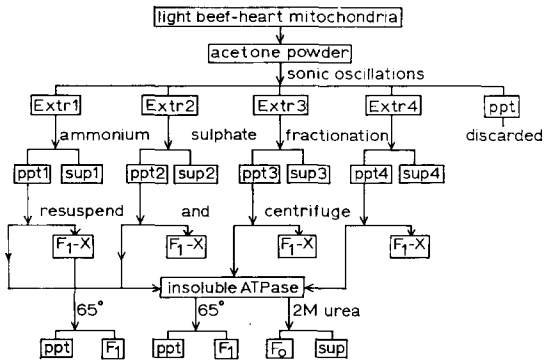


Fig. 1. Preparation procedure. See text and Table I for explanation.

TABLE I

ATPase ACTIVITIES AT VARIOUS STAGES OF PURIFICATION OF F_1-X AND F_1

The isolation procedure is described in the text and in Fig. 1. 1.2 g (protein) of light beef-heart mitochondria were used to obtain 2.2 g (dry wt.) of acetone powder which was extracted as described. The ATPase activity is expressed as μmoles P_i/min.

	Total protein (mg)	*Total activity*		*Specific activity***
		— Oligomycin	*+ Oligomycin**	
Extract 1	130	27	19	0.2
Extract 2	83	75	49	0.9
Extract 3	57	117	102	2.0
Extract 4	28	77	68	2.8
F_1-X from Extract 1	27	38	33	1.4
F_1-X from Extract 2	12	66	58	5.6
F_1-X from Extract 3	6	93	91	15.3
F_1-X from Extract 4	1.5	39	39	26.0
Insoluble ATPase	51	124	55	2.4
(Assayed after 24 h)		168	38	3.3
F_1 from insoluble ATPase	1.5	47	47	31.4
(Precipitate	48	25	25	0.5)
F_1 from F_1-X from Extract 2	0.7	23	23	33.0
(Precipitate	10.2	5	4	0.5)

* 2.5 μg.
** μmoles/min per mg protein.

were pooled and suspended in 0.25 M sucrose and stored in liquid nitrogen. The F_1-X preparations were stored at 4° after adding 1 vol. of satd. $(NH_4)_2SO_4$ (pH 7.4).

This procedure yielded a 6- to 10-fold increase in the specific activity of the extracts (Table I). F_1-X obtained from Extract 4 always had the highest specific activity (usually about 30 μmoles P_i/min per mg). The activity of the insoluble oligo-

mycin-sensitive ATPase increased after storage. The total activity found in the insoluble ATPase and the four preparations of F_1-X exceeded the total activity in the four initial extracts, indicating the presence of a latent ATPase (*cf.* ref. 2) in the extracts.

Purification of F_1. A soluble ATPase with the properties of F_1 (*cf.* refs. 2, 3) was obtained from either the insoluble ATPase or F_1-X (at a concentration of about 15 mg/ml) by warming to 65° for 2 min in 0.25 M sucrose, 10 mM Tris–sulphate (pH 7.4), 1 mM EDTA and 4 mM ATP (*cf.* refs. 2, 42). The precipitate was discarded by centrifugation and F_1 was recovered from the supernatant solution by precipitation with $(NH_4)_2SO_4$ (pH 7.4) at 50% satn. The yield of this step was low (30–50%) but F_1 was purified up to 10 times. The oligomycin sensitivity of the insoluble ATPase was destroyed by this treatment (Table I).

The ATPase activity of F_1-X, the insoluble ATPase and F_1 was dependent on Mg^{2+} and was stimulated by 2,4-dinitrophenol.

The effects of cold treatment and of oligomycin on the three ATPase preparations are shown in Table II. The ATPase activities of both F_1 and F_1-X are cold labile and oligomycin insensitive. On the other hand, the insoluble ATPase was cold stable and oligomycin sensitive.

TABLE II

COLD LABILITY AND OLIGOMYCIN SENSITIVITY OF ATPase PREPARATIONS

F_1 (18 μg), F_1-X (11 μg from Extract 3) or insoluble ATPase (420 μg) was incubated in 0.5 ml 25 mM Tris–sulphate (pH 7.4) at 0° or 25° for 1 h. The assay was started by adding 0.5 ml of reaction mixture and the ATPase activity determined at 30° as described in METHODS. Reaction period, 10 min.

Incubation temperature (°C)	*Oligomycin* (μg)	*ATPase activity* (μmoles P_i *liberated*)		
		F_1	F_1-X	*Insoluble ATPase*
25	0	0.77	1.66	2.82
0	0	0.12	0.18	2.62
25	2.5	0.77	1.66	0.49
0	2.5	0.06	0.20	0.48

The specific ATPase activity of our F_1 preparation (30–40 μmoles/min per mg protein) was about one-half that of the highest specific activity reported for F_1 (*cf.* ref. 2).

Preparation and properties of F_0. A factor, similar to F_0 of KAGAWA AND RACKER[12] that confers oligomycin sensitivity on the ATPase activity of F_1, was obtained from the insoluble ATPase by incubation with 2 M urea at 0° for 45 min. The supernatant solution was discarded after centrifugation at 26000 × *g* for 10 min and the precipitate was washed twice with 0.25 M sucrose and stored in liquid nitrogen. The treatment with urea destroyed more than 99% of the ATPase activity of the insoluble ATPase. Little protein and no activity was found in the discarded supernatant solution.

The succinate dehydrogenase activity of both our preparation of F_0 and of the insoluble ATPase was very low (18–39 nmoles $Fe_3(CN)_6$ reduced/min per mg protein)

and the NADH dehydrogenase was not detectable. Both preparations contained about 0.45 μmole of total phospholipid P per mg protein. Lecithin comprised about 30–40 % of the phospholipids and cephalin 26 %*.

Neither albumin nor phospholipids had any effect on the activity of our F_0 preparation (Table III; *cf.* refs. 12, 13). It was stable for months when stored in liquid nitrogen but when exposed to more than 30° was quickly inactivated (*cf.* ref. 8).

TABLE III

EFFECT OF F_0 ON F_1

F_1 (10 μg in Expt. 1, 14 μg in Expt. 2) was preincubated for 5 min at 30° in 25 mM Tris–sulphate (pH 7.4) with the additions stated in a final volume of 0.5 ml. The ATPase assay was started by adding 0.5 ml of the reaction mixture (at 30°) and carried out as described in METHODS. Reaction time, 10 min. Soybean phospholipids were prepared from Asolectin[5].

Expt.	*Additions*	*ATPase activity (μmoles P_i liberated)*	
		– Oligomycin	*+ Oligomycin (2.5 μg)*
1	None	0.68	0.68
	F_0 (150 μg)	0.70	0.25
	F_0 (300 μg)	0.65	0.19
	F_0 (450 μg)	0.67	0.17
2	None	1.04	0.98
	F_0 (300 μg)	0.94	0.26
	F_0 (300 μg), albumin (10 mg)	0.88	0.25
	F_0 (300 μg), phospholipids (500 μg)	1.02	0.28

Coupling activity of F_1 and F_1-X

Table IV, Expts. 1 and 2, shows that F_1 prepared from F_1-X, like the F_1 of RACKER and co-workers[3,10], stimulated the P/O ratio of A particles and that this effect was cold labile and additive with the stimulation by oligomycin. F_1 also stimulates the ATP-dependent NAD^+ reduction by succinate (Table V) and the P_i–ATP exchange reaction[28]. F_1 prepared from the insoluble ATPase, which itself had no coupling activity, was also active. Preincubation of F_1 with F_0 strongly inhibited its coupling activity.

The coupling activity of F_1-X was greater than that of F_1 as measured by its effect on P/O ratio (Table IV, Expt. 3), the P_i–ATP exchange reaction[28] and the ATP-dependent NAD^+ reduction by succinate (Table V). The coupling activity of F_1-X was not additive with the effect of oligomycin (Table IV, Expt. 3).

The coupling and ATPase activities of F_1-X were closely associated as can be seen in Fig. 2, where two F_1-X preparations with different specific ATPase activities show the same titration curve when the effect on P/O ratio is plotted against the ATPase activity. Under the conditions of the experiment illustrated in Fig. 2, maximal stimulation was obtained with 42 μg of the more purified F_1-X preparation (or 25 μg, when the protein concentration was determined by the method of LOWRY *et al.*[37]). FESSENDEN, DANNENBERG AND RACKER[11] reported a comparable stimulation with a mixture of 40 μg F_1, 200 μg F_2 and 307 μg F_3 under similar conditions.

* These determinations were kindly done by Dr. B. ROELOFSEN.

TABLE IV

EFFECT OF F_1, F_1-X AND OLIGOMYCIN ON P/O RATIOS OF SUBMITOCHONDRIAL PARTICLES

The substrate was succinate. F_1-X used in Expt. 3 was obtained from Extract 3 and in Expt. 4 from Extract 2 (see Table I). F_1 was prepared from F_1-X. A particles were used in Expts. 1–3 and EDTA particles in Expt. 4.

Expt.	*Additions*	*Glucose 6-phosphate (μmoles)*	*P/O*
1	None	0.37	0.09
	F_1 (37 μg)	0.85	0.18
	Oligomycin (0.05 μg)	0.84	0.17
	Oligomycin (0.05 μg) + F_1 (37 μg)	1.42	0.31
2	None	0.10	0.05
	F_1 (120 μg)	0.26	0.14
	F_1 (120 μg), cold treated*	0.08	0.05
3	None	0.18	0.04
	F_1 (88 μg)	0.45	0.10
	F_1-X (120 μg)	3.31	0.68
	Oligomycin (0.05 μg)	1.17	0.24
	Oligomycin (0.1 μg)	1.25	0.26
	F_1-X (120 μg) + oligomycin (0.05 μg)	3.31	0.66
	F_1-X (120 μg) + oligomycin (0.1 μg)	3.38	0.66
4	None	0.16	0.04
	F_1 (18 μg)	0.35	0.08
	F_1 (72 μg)	0.41	0.09
	F_1-X (500 μg), cold treated*	0.28	0.06
	F_1 (72 μg) + F_1-X (500 μg), cold treated*	1.91	0.47

* 2 h at 0°.

TABLE V

EFFECT OF F_1, F_1-X AND F_2 ON THE ATP-DEPENDENT NAD^+ REDUCTION BY SUCCINATE IN A PARTICLES

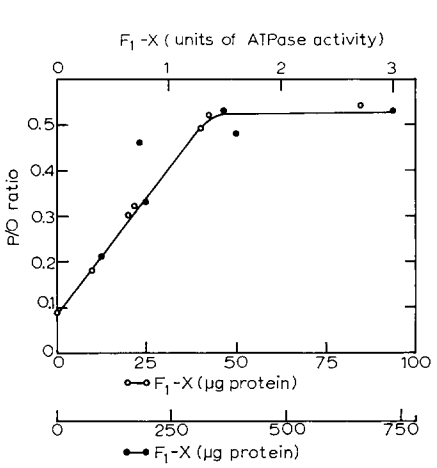
F_1-X was obtained from Extract 3 (see Table I). F_1 was prepared from F_1-X. A particles (0.5 mg protein) were used. F_2 was a crude preparation of succinate dehydrogenase (see text).

Additions	*NADH formation (nmoles/min)*
None	0.6
F_1 (37 μg)	1.8
F_2 (236 μg)	1.3
F_1 (37 μg) + F_2 (236 μg)	2.4
F_1-X (96 μg**)	10.7
F_1-X (96 μg) + F_2 (236 μg)	22.5
F_1-X (96 μg) + F_2 (236 μg); without A particles	0.3
F_1-X (96 μg), cold treated*	1.3
F_1-X (96 μg), cold treated* + F_1 (37 μg)	10.5

* 150 min at 0°.

** In a separate experiment (not shown here) it was found that this amount of F_1-X was saturating.

It was found that the response of submitochondrial particles to F_1-X varied with the type of particle and with different preparations of the same type of particle, as is illustrated in Fig. 3. With A particles the maximal P/O ratio was 0.5; with EDTA particles P/O ratios of 0.7 and 0.9 could be reached. This variability may be explained by different degrees of inactivation or extraction of the endogenous coupling factors during treatment of mitochondria by sonic oscillation.



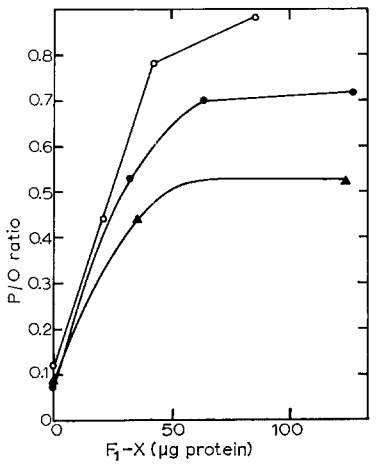


Fig. 2. Effect of F_1-X on P/O ratio. P/O ratios were determined as described in METHODS. A particles were used. The specific ATPase activity of F_1-X was: ○—○, 32 µmoles P_i/min per mg protein; ●—●, 4.1 µmoles P_i/min per mg protein.

Fig. 3. Variability of response of submitochondrial particles to F_1-X. The effect of F_1-X (obtained from Extract 3; specific ATPase activity, 21 µmoles P_i/min per mg protein) on the P/O ratio was determined as described in METHODS. ▲—▲, A particles; ●—● and ○—○, two different preparations of EDTA particles.

The coupling activity of F_1-X is also cold labile (Table IV, Expt. 4, Table V and Fig. 4) but it can be completely restored after cold treatment by addition of F_1 (Fig. 4, Curve C). This suggests the presence in F_1-X of another factor (or factors) differing from F_1 with respect to its stability at low temperatures. Also in contrast to F_1, the extra factor is labile at 37°, particularly when F_1-X is previously cold-treated (half inactivation after 40 min with untreated F_1-X, 20 min with cold-treated). This suggests that the extra factor (X) is, to some extent, protected by native F_1 against inactivation at 37°. This is supported by the fact that added F_1 partially protects cold-treated F_1-X against inactivation at 37° (half inactivation after 30 min).

Following ZALKIN AND RACKER[8] we used a crude preparation of succinate dehydrogenase* (eluate after adsorption on calcium phosphate gel) as a source of F_2. The eluate was concentrated by precipitation with $(NH_4)_2SO_4$ and suspended in 10 mM Tris–sulphate (pH 7.4) and 4 mM ATP. Table VI shows that this preparation of F_2 somewhat stimulated both the P_i–ATP exchange and the P/O ratios of A particles, but the stimulation was much less than that given by F_1-X. A small (15–35 %) but significant stimulation of the P_i–ATP exchange but not that of the P/O ratios

* Kindly supplied by Mr. W. P. ZEYLEMAKER.

was observed in the presence of saturating amounts of F_1-X. The small P_i–ATP exchange activity in F_2 could not account for the stimulation.

F_2 alone or with F_1 had a rather small effect on the ATP-dependent NAD^+ reduction by succinate in A particles (Table V) in agreement with FESSENDEN, DANNENBERG AND RACKER[11] but it doubled the stimulation induced by saturating amounts of F_1-X.

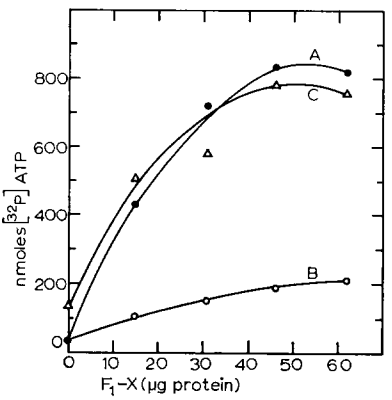


Fig. 4. Effect of cold treatment on F_1-X. *Curve A* (●—●). The effect of F_1-X (obtained from Extract 4; specific ATPase activity, 32 µmoles P_i/min per mg protein) on the P_i–ATP exchange reaction of EDTA particles was determined as described in METHODS. Reaction period, 15 min. *Curve B* (○—○). As in Curve A, but F_1-X was preincubated at 4° for 24 h in 0.25 M sucrose, 10 mM Tris–sulphate (pH 7.4) and 1 mM EDTA. *Curve C* (△—△). As in Curve B, but F_1 (30 µg) was present during the assay.

Although a more purified succinate dehydrogenase (precipitated between 30 and 45 % satd. $(NH_4)_2SO_4$) had the same effects on the P_i–ATP exchange reaction and the ATP-linked reduction of NAD^+ by succinate as the crude preparation, it is unlikely that F_2 and succinate dehydrogenase are identical, since a 3- to 5-fold increase in succinate dehydrogenase specific activity was not associated with any in-

TABLE VI

EFFECT OF F_2 AND F_1-X ON THE P_i–ATP EXCHANGE AND ON THE P/O RATIO OF A PARTICLES

F_2 was a crude preparation of succinate dehydrogenase (see text). F_1-X was obtained from Extract 3 (see Table I). 0.5 mg A particles were used.

Additions	*P_i–ATP exchange (nmoles [^{32}P]ATP per 15 min)*	*P/O**
None	33	0.09
F_2 (128 µg)	79	
F_2 (236 µg)	99	0.17
F_1-X (81 µg)	763	0.73
F_1-X (81 µg) + F_2 (128 µg)	858	
F_1-X (81 µg) + F_2 (236 µg)	825	0.69
F_2 (236 µg); without A particles	20	

* With succinate.

crease in coupling activity. Moreover, the coupling activity of succinate dehydrogenase preparations is more stable than the dehydrogenase activity.

It is clear from Table V that, in the presence of F_1, cold-treated F_1-X (*i.e.* X) is much more effective than the preparation of F_2. Thus X is unlikely to be F_2.

TABLE VII

EFFECT OF F_1-X (COLD TREATED) ON THE ATPase ACTIVITY OF F_1 AND EDTA PARTICLES

F_1-X (obtained from Extract 4) was preincubated at 4° for 24 h. 90% or more of the ATPase activity was lost. The ATPase activity of 9 μg F_1 and 114 μg EDTA particles, alone or *plus* F_1-X (cold treated), was determined as described in METHODS. Reaction time, 10 min. The values in the column *Expected* resulted from adding up the values found for F_1 or EDTA particles alone *plus* those corresponding to the amount of F_1-X (cold treated) added. Numerals in parentheses refer to % inhibition.

Expt.		*ATPase activity (μmoles P_i)*	
		Found	*Expected*
1	F_1-X (31 μg)	0.10	
	F_1	1.79	
	F_1 + F_1-X (15 μg)	1.63	1.84 (11)
	F_1 + F_1-X (31 μg)	1.41	1.89 (25)
	EDTA particles	3.82	
	EDTA particles + F_1 (15 μg)	2.45	3.87 (37)
	EDTA particles + F_1-X (31 μg)	1.74	3.92 (56)
2	F_1-X (38 μg)	0.73	
	EDTA particles	4.29	
	EDTA particles + F_1-X (10 μg)	3.33	4.47 (26)
	EDTA particles + F_1-X (19 μg)	2.89	4.65 (38)
	EDTA particles + F_1-X (29 μg)	2.54	4.84 (48)
	EDTA particles + F_1-X (38 μg)	2.39	5.02 (52)
	EDTA particles + oligomycin (0.01 μg)	3.16	(26)
	EDTA particles + oligomycin (0.02 μg)	2.67	(38)

Effect of cold-treated F_1-X on the ATPase activity of F_1 and EDTA particles

Table VII shows that cold-treated F_1-X, containing less than 10% of its original ATPase activity, was able to inhibit the ATPase activity of F_1 and EDTA particles. It can be calculated from other data that 80–160 μg of untreated F_1-X per mg of EDTA particles would give the maximum coupling effect. This amount of cold-treated F_1-X caused 26–38% inhibition of the ATPase activity of EDTA particles. A similar inhibition was brought about by a concentration of oligomycin (0.09–0.18 μg/mg) that was in the range of concentration where stimulation of the energy-transfer process occurred.

DISCUSSION

The multiplicity of coupling factors obtained from beef-heart mitochondria reported in the literature is at first sight bewildering. However, some of the factors described in different laboratories may result from alterations occurring to a single native factor during isolation. Factor A (ref. 18) and SONE AND HAGIHARA's factor[20] are probably functionally identical with F_1, LINNANE AND TITCHENER's factor[17] with F_2 and Factor B (ref. 19) with F_3 (*cf.* ref. 11). Coupling factors specific for all three

sites have been reported[23–27], but the claim with respect to the Site-III factor has been retracted[43]. The role of the ADP–ATP exchange enzyme[21–22] in oxidative phosphorylation has now been questioned[44]. FESSENDEN, DANNENBERG AND RACKER[11] have found that the stimulation of oxidative phosphorylation by F_4 was due to contamination with F_2 and F_3.

The experimental evidence reported in this paper supports the existence of at least three different factors (F_1, F_2 and X), two of which may be obtained in a closely associated form (F_1-X). The two components of F_1-X have the opposite temperature lability. The coupling activity of F_1-X is destroyed by cold treatment but is completely restored by F_1, indicating that the cold-labile component of F_1-X is F_1 and that X is cold stable. The other component is, however, quite labile at higher temperatures and is rapidly destroyed at 65°, the temperature used by PULLMAN *et al.*[2] for the purification of F_1. As would be expected, the coupling activity of heat-treated F_1-X cannot be restored by F_1.

The nature of X has not been established by this investigation. Like F_2 and F_3, X requires the simultaneous presence of F_1. However, as already discussed, it cannot be F_2. Not only is F_2 unable to replace cold-treated F_1-X as a coupling factor in the presence of F_1, the addition of F_2 had a marked effect on the ATP-linked reduction of NAD^+ by succinate, measured in the presence of F_1-X. This stimulatory effect of F_2 is in contrast to the lack of effect reported for F_2 in the presence of F_1 and F_3 (ref. 11). Perhaps the F_3 used in the experiments of FESSENDEN, DANNENBERG AND RACKER[11] contained some F_2 (*cf.* ref. 15). The striking effect of F_2 in our experiments suggests that this factor might have a special role in Site-I phosphorylation.

It is quite possible that X is a purified form of F_3 or Factor B. Like F_3 (ref. 7) X inhibits the ATPase activity of F_1 and it resembles Factor B (ref. 19) in its instability at 37°. The nature of the interaction between F_1 and X is under further study.

During this investigation a factor has been isolated that confers oligomycin sensitivity to F_1. In this respect, the factor resembles F_0 of KAGAWA AND RACKER[12]. In contrast to the latter, our preparation of F_0 has a very low succinate dehydrogenase activity and no detectable NADH dehydrogenase activity. Moreover the phospholipid content of our F_0 preparation is much lower. In some of our preparations the phospholipid content was as low as 0.2 μmole of phospholipid phosphorus per mg of protein, as compared with 0.94 μmole in KAGAWA AND RACKER's preparation[12]. Incubation with active phospholipase A did not affect the activity of our F_0 preparation (contrast ref. 12). Also the preparation procedure is very different. RACKER[7] was unable to extract F_0 from mitochondria with acetone, whereas our preparation of F_0 was obtained from an acetone-powder extract.

In some respects, such as the low content of phospholipids and respiratory enzymes, our preparation of F_0 also resembles the factor CF_0 of KAGAWA AND RACKER[13], but in contrast to the latter it does not inhibit the ATPase activity of F_1.

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REFERENCES

1 M. E. PULLMAN, H. S. PENEFSKY AND E. RACKER, *Arch. Biochem. Biophys.*, 76 (1958) 227.
2 M. E. PULLMAN, H. S. PENEFSKY, A. DATTA AND E. RACKER, *J. Biol. Chem.*, 235 (1960) 3322.
3 H. S. PENEFSKY, M. E. PULLMAN, A. DATTA AND E. RACKER, *J. Biol. Chem.*, 235 (1960) 3330.
4 E. RACKER, *Proc. Natl. Acad. Sci. U.S.*, 48 (1962) 1659.
5 T. E. CONOVER, R. L. PRAIRIE AND E. RACKER, *J. Biol. Chem.*, 238 (1963) 2831.
6 R. L. PRAIRIE, T. E. CONOVER AND E. RACKER, *Biochem. Biophys. Res. Commun.*, 10 (1963) 422.
7 E. RACKER, *Biochem. Biophys. Res. Commun.*, 10 (1963) 435.
8 H. ZALKIN AND E. RACKER, *J. Biol. Chem.*, 240 (1965) 4017.
9 H. S. PENEFSKY AND R. C. WARNER, *J. Biol. Chem.*, 240 (1965) 4694.
10 J. M. FESSENDEN AND E. RACKER, *J. Biol. Chem.*, 241 (1966) 2483.
11 J. M. FESSENDEN, M. A. DANNENBERG AND E. RACKER, *Biochem. Biophys. Res. Commun.*, 25 (1966) 54.
12 Y. KAGAWA AND E. RACKER, *J. Biol. Chem.*, 241 (1966) 2461.
13 Y. KAGAWA AND E. RACKER, *J. Biol. Chem.*, 241 (1966) 2467.
14 Y. KAGAWA AND E. RACKER, *J. Biol. Chem.*, 241 (1966) 2475.
15 E. RACKER AND L. L. HORSTMAN, *J. Biol. Chem.*, 242 (1967) 2547.
16 G. SCHATZ, H. S. PENEFSKY AND E. RACKER, *J. Biol. Chem.*, 242 (1967) 2552.
17 A. W. LINNANE AND E. B. TITCHENER, *Biochim. Biophys. Acta*, 39 (1960) 469.
18 T. E. ANDREOLI, K. W. LAM AND D. R. SANADI, *J. Biol. Chem.*, 240 (1965) 2644.
19 K. W. LAM, J. B. WARSHAW AND D. R. SANADI, *Arch. Biochem. Biophys.*, 119 (1967) 477.
20 N. SONE AND B. HAGIHARA, *J. Biochem. Tokyo*, 60 (1966) 622.
21 C. L. WADKINS AND A. L. LEHNINGER, *J. Biol. Chem.*, 233 (1958) 1589.
22 C. L. WADKINS AND A. L. LEHNINGER, *J. Biol. Chem.*, 238 (1963) 2555.
23 A. L. SMITH AND M. HANSEN, *Biochem. Biophys. Res. Commun.*, 8 (1962) 136.
24 G. WEBSTER, *Biochem. Biophys. Res. Commun.*, 7 (1962) 245.
25 D. E. GREEN, R. E. BEYER, M. HANSEN, A. L. SMITH AND G. WEBSTER, *Federation Proc.*, 22 (1963) 1460.
26 R. E. BEYER, *Biochem. Biophys. Res. Commun.*, 16 (1964) 460.
27 R. E. BEYER, *Biochem. Biophys. Res. Commun.*, 17 (1964) 764.
28 R. H. VALLEJOS AND E. C. SLATER, *Biochim. Biophys. Acta*, 143 (1967) 441.
29 F. L. CRANE, G. L. GLENN AND D. E. GREEN, *Biochim. Biophys. Acta*, 22 (1956) 475.
30 Y. HATEFI AND R. L. LESTER, *Biochim. Biophys. Acta*, 27 (1958) 83.
31 C.-P. LEE, G. F. AZZONE AND L. ERNSTER, *Nature*, 201 (1964) 152.
32 E. C. SLATER AND F. A. HOLTON, *Biochem. J.*, 55 (1953) 530.
33 S. O. NIELSEN AND A. L. LEHNINGER, *J. Biol. Chem.*, 215 (1955) 555.
34 J. B. SUMNER, *Science*, 100 (1944) 413.
35 O. WARBURG AND W. CHRISTIAN, *Biochem. Z.*, 310 (1941) 384.
36 L. SZARKOWSKA AND M. KLINGENBERG, *Biochem. Z.*, 338 (1963) 674.
37 O. H. LOWRY, N. I. ROSENBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
38 C. F. REED, S. N. SWISHER, G. V. MARINETTI AND E. G. EDEN, *J. Lab. Clin. Med.*, 56 (1960) 281.
39 E. HABERMANN, G. BANDTLOW AND B. KRUSCHE, *Klin. Wochschr.*, 39 (1961) 816.
40 C. J. F. BÖTTCHER, C. M. VAN GENT AND C. PRIES, *Anal. Chim. Acta*, 24 (1961) 203.
41 J. KETTMAN AND T. E. KING, *Biochem. Biophys. Res. Commun.*, 11 (1963) 255.
42 K. H. BRYINGTON AND A. TZAGALOFF, *Federation Proc.*, 25 (1966) 413.
43 D. R. SANADI, *Ann. Rev. Biochem.*, 34 (1965) 21.
44 G. S. P. GROOT AND S. G. VAN DEN BERGH, *Biochim. Biophys. Acta*, 153 (1968) 22.