A PROTEIN FACTOR REQUIRED FOR THE PHOSPHORYLATION ACCOMPANYING OXIDATION OF REDUCED CYTOCHROME c

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When beef heart mitochondria are disrupted by sonic treatment in the presence of EDTA, the resulting sub-mitochondrial particle (modified ETP_H) requires a soluble protein fraction for maximal phosphorylation (Linnane, 1958). This factor was purified 8-15 fold by Linnane and Titchener (1960), who showed that the factor restored phosphorylation coupled to the oxidation of both succinate and DPNH. Furthermore, evidence was obtained that the restoration of phosphorylation depended upon a recombination of the soluble factor and the particle in the presence of magnesium.

This factor has now been purified about 250-fold by fractionation with CM-cellulose, DEAE-cellulose, and Sephadex. The factor appears to be a protein. Activity is destroyed by exposure to 100° C for three minutes, or by treatment with crystalline trypsin for two hours at 30° C. The absorption spectrum of the factor exhibits a maximum at 277 mμ and a minimum at 252 mμ. It can be precipitated from solution by ammonium sulfate, by ethanol, or by lowering the pH of the solution to 5.4.

In Table I is shown the effect of the factor on the P/O ratios of modified ETP_H in the presence of either succinate or DPNH. It can be seen that the factor increases the P/O ratio by approximately 1.0 in the presence of each of the substrates. Maximal increase is obtained when approximately 5 μg of factor protein are added to each mg of modified ETP_H. As the concentration of factor is lowered, the increase in P/O ratio is lowered proportionately. Increase in factor concentration above the optimal value, however, produces no further increase in P/O ratio.

TABLE I

EFFECT OF PHOSPHORYLATION FACTOR ON P/O RATIOS OF MODIFIED ETP_H

Substrate	Additions	P/O Ratio	
		No Factor	Plus Factor
1 μmole DPNH	-	0.1	1.0
0.01 μmole DPNH	-	0.8	1.8
1 μmole Succinate	-	0.1	0.8
0.01 μmole DPNH	0.1 mg. Phenazine Methosulfate plus 0.001 M Cyanide	1.1	1.0
1 μmole Succinate	0.1 mg. Phenazine Methosulfate plus 0.001 M Cyanide	0.1	0.1
30 μmoles Ascorbate	0.02 mg. Antimycin	0.1	0.9

The reaction system consisted of: 0.25 M sucrose, 0.002 M ATP, 0.002 M ADP, 0.006 M $MgCl_2$, 0.024 M glucose, 0.01 M Tris-HCl (pH 7.5), 0.003 M potassium phosphate-P^{32} (20,000,000 cts/min), 10,000 units of crystalline hexokinase, approximately 2 mg of modified ETP_H, prepared as described by Linnane and Titchener (1960), the additions listed in the table, and, where indicated, approximately 9 μg of the purified factor in a total volume of 2 ml. In the case of DPNH oxidation, the system also contained 0.04 M ethanol and approximately 0.03 μg of crystalline alcohol dehydrogenase. Reaction rates were measured during the initial 1-5 min. following temperature equilibration at 37° C. Oxygen consumption was measured with a recording GME Oxygraph, and phosphorylation was measured by determination of the phosphate-P^{32} incorporated into glucose-6-phosphate.

This observed increase in P/O ratio by a value not exceeding 1.0 is apparently due to the fact that this factor is necessary only for the phosphorylation step accompanying the oxidation of cytochrome c by cytochrome oxidase. Table I shows that the factor, even in high concentrations, has no effect on the phosphorylation accompanying the oxidation of DPNH or succinate by phenazine methosulfate (A. Smith, unpublished results) in a system in which the cytochrome oxidase step has been blocked by cyanide. In contrast, the phosphorylation accompanying the oxidation of cytochrome c (kept reduced by ascorbate) by cytochrome oxidase in an antimycin-blocked system is almost entirely dependent upon the presence of the factor.

Although the original soluble fraction obtained by sonic disruption of mitochondria contains considerable ATPase activity, this activity is eliminated during the purification procedure (Table II).

TABLE II

EFFECT OF PURIFICATION OF THE PHOSPHORYLATION FACTOR ON ASSOCIATED ENZYMATIC ACTIVITIES

Activity	Original Supernatant Solution	250-fold Purified Fraction
ATPase activity	3.6	0
ATP-P_i exchange	0.4	0
ATP-ADP exchange	0.2	12.5

Reaction system for ATPase assay: 0.05 M Tris-HCl (pH 7.5), 0.01 M ATP, 0.01 M $MgCl_2$, and 0.1 mg of protein in a total volume of 1 ml. Activity is expressed as μmoles P_i formed in 10 min/mg protein at 37° C.

Reaction system for ATP-P_i exchange: 0.05 M Tris-HCl (pH 7.5), 0.005 M potassium phosphate-P^{32} (20,000,000 cts/min), 0.005 M $MgCl_2$, 0.15 M sucrose, and 0.1 mg of protein in a total volume of 1 ml. Activity is expressed as μmoles P_i incorporated into ATP in 10 min/mg protein at 37° C.

Reaction system for ATP-ADP exchange: 0.05 M Tris-HCl (pH 7.5), 0.001 M $MgCl_2$, 0.003 M ATP, 0.003 M ADP-C^{14} (120,000 cts/min), and 0.1 mg of protein in a total volume of 1 ml. Activity is expressed as μmoles ADP incorporated into ATP in 10 min/mg protein at 37° C.

The purified factor fails to exhibit any detectable ATPase activity, either alone, or in the presence of dinitrophenol, calcium ion or magnesium ion. The factor, therefore, would appear to be different from the cold-labile ATPase-containing phosphorylation factor which has been purified by Pullman, et al. (1960) from disrupted beef heart mitochondria. The soluble fraction from disrupted mitochondria also contains a detectable ATP-P_i exchange activity which is likewise completely eliminated by the purification procedure (Table II). In this connection, it should be noted that modified ETP_H possesses an unusually low ATP-P_i exchange activity (about 0.1 that of ETP_H or of intact beef heart mitochondria). This ATP-P_i exchange is not increased to a measurable extent by the addition of ten times the amount

of factor necessary to increase the P/O ratio of the particles by approximately 1.0. In contrast to the complete disappearance of the ATPase and ATP-P_i exchange activities during the purification of the factor, the ability to catalyze an ADP-ATP exchange increases with the increase in ability to promote oxidative phosphorylation (Table II).

The ability of the purified factor to catalyze an ADP-ATP exchange, together with the specificity of the factor for the phosphorylation accompanying the oxidation of reduced cytochrome c, suggest that the factor may be a transphosphorylase specific for one phosphorylation site. Evidence has been obtained in this laboratory (A. Smith and M. Hansen, unpublished results) indicating that additional factors occur which are specific for the other phosphorylation sites. In the light of these results, one would expect that the enzyme preparation with ADP-ATP exchange activity, which Lehninger (1960) has found to increase the P/O of particles from digitonin-treated mitochondria, might likewise be specific for only one phosphorylation site.

The evidence indicates that the factor loses both ATPase and ATP-P_i exchange activity during purification. Furthermore, the lack of restoration of the already-low ATP-P_i exchange activity of modified ETP_H when phosphorylation is restored, suggests that ATPase and ATP-P_i exchange may not be reliable criteria for the study of oxidative phosphorylation. Although ATPase and ATP-P_i exchange have been studied extensively in connection with oxidative phosphorylation, the present results suggest that considerable caution should be exercised in the interpretation of the results so obtained. The recent finding of Vignais, et al. (1961) that potassium atractylate strongly inhibits mitochondrial ATP-P_i exchange and dinitrophenol-promoted ATPase, while hardly affecting oxidative phosphorylation, is in line with this suggestion.

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