THREE DISCRETE COUPLING PROTEINS IN OXIDATIVE PHOSPHORYLATION

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Studies of oxidative phosphorylation from our laboratory (Linnane and Titchner, 1960) and elsewhere (Pullman et al., 1960) have demonstrated that the submitochondrial system for coupled oxidation can be resolved into a soluble fraction and a particle containing the electron transfer system. Phosphorylation can be reconstituted by combining the particle and the soluble fraction. The coupling activity of the soluble fraction has now been resolved into three discrete coupling factors. The present communication provides evidence that these coupling factors intervene at different sites of the electron transfer system.

When beef heart mitochondria are fragmented (by sonication) in the presence of EDTA, a particle is obtained (modified ETPH) which has a very low phosphorylative capacity (Linnane and Titchner, 1960). The phosphorylation accompanying the oxidation of succinate and DPNH by this particle can be increased by the addition of the supernatant solution from which the particle was isolated. The coupling activity of the supernatant solution has been resolved into factors which promote phosphorylation accompanying the oxidation of: 1) DPNH; 2) reduced coenzyme Q; 3) reduced cytochrome c. These factors have been called the DPNH (I), reduced coenzyme Q (II), and reduced cytochrome c (III) coupling factors. The present communication deals with I and II while the properties of III have been described in a previous communication (Webster, 1962).

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In order to localize the site of action of each of the coupling factors, assays were devised to measure phosphorylation accompanying oxidation in a restricted segment of the electron transfer system. It was found that phenazine methosulfate (PMS) could be used as an electron acceptor for coupled oxidation of DPNH and succinate. In ETPH preparations, the PMS-mediated oxidation of succinate and DPNH is insensitive to antimycin, and the P/O ratios observed in the presence of this inhibitor are 0.9 and 2.0 respectively (cf. Table I)*. These findings indicate that two sites involved in

Table I

Oxidative Phosphorylation Mediated by Phenazine Methosulfate (PMS)

Substrate	Electron Acceptor	Electron Transfer Inhibitor	Oxygen Uptake S.A.[a]	P/O
DPNH	O_2	-	0.15	1.7
	PMS	-	0.10	1.6
	PMS	Antimycin	0.10	2.0
	PMS	Amytal	0.18	0.9
Succinate	O_2	-	0.27	0.7
	PMS	-	0.06	0.7
	PMS	Antimycin	0.06	0.9

The reaction mixture (3 ml) contained: 10 mM KPO_4 (pH 7.5), 1.6 mM $MgCl_2$ 0.25 M sucrose, 20 mM glucose, 0.5 mg hexokinase, 2 mg ETPH, 15 mM succinate or 0.3 mM DPN, 0.02 ml ethanol, 50 µg alcohol dehydrogenase. Where indicated the following additions were made: 0.1 mg PMS, 2 µg antimycin, 6 µmoles Amytal. Oxygen consumption was measured manometrically at 30° and the decrease in inorganic phosphate determined. The oxidation of reduced PMS by oxygen results in the formation of hydrogen peroxide; therefore,a four electron change is involved in the reduction of oxygen,and the calculations have been made on that basis.

[a] S.A. = µatoms O_2/min/mg protein

*Although PMS mediates a non-enzymic oxidation of DPNH, under conditions of low DPNH concentrations employed in the assay,almost all of the DPNH is oxidized via the flavoprotein as evidenced by the theoretical P/O ratio obtained. The PMS mediated coupled oxidation is sensitive to uncoupling agents such as DNP and oligomycin.

the coupled oxidation of DPNH must precede the antimycin site whereas in the coupled oxidation of succinate only one site is operative in the same segment of the electron transfer system. According to the present formulation of the electron transfer sequence, the oxidation of DPNH and of succinate proceed through a common pathway at a point in the chain including and beyond coenzyme Q (Green, 1961). It seems most probable that one of the two phosphorylation sites shared in the coupled oxidation of DPNH and succinate must lie between coenzyme Q and the antimycin site. This would necessarily involve the complex which catalyzes the oxidation of $CoQH_2$ by cytochrome c and which contains cytochromes b and c_1 as well as non-heme iron. In a previous communication we have pointed out that ETPH may be devoid of the terminal phosphorylation site (Smith and Hansen, 1962). The present findings that the P/O ratios obtained with PMS and oxygen are nearly equal, further confirm that ETPH preparations are deficient in the factor (III) required for phosphorylation at the terminal site.

PMS can also be used to study the first phosphorylation site associated with DPNH oxidation. When the electron flow from DPNH is interrupted by Amytal, PMS mediates DPNH oxidation with P/O ratio of 0.9 (cf. Table I). The Amytal block suggests that only the segment of the DPNH-CoQ reductase that includes the flavin and non-heme iron is operative in the coupled oxidation. Phenazine may thus be used to study coupling in two segments of the chain - one involved in the oxidation of DPNH by flavin-non-heme iron, and the other in the oxidation of reduced coenzyme Q by cytochrome b - non-heme iron. Amytal brackets the first segment and antimycin the second.

The first coupling factor (I) promotes phosphorylation associated with DPNH oxidation and is called the DPNH coupling factor (cf. Table II). In an Amytal-blocked system, this coupling factor increases the P/O ratio for DPNH oxidation about 0.5 when PMS is used as the electron acceptor. The fact that there was no further increase in the P/O ratio when electron flow was extended through cytochrome b (in presence of antimycin rather than Amytal)

Table II

Restoration of Phosphorylation by the DPNH Coupling Factor (I)

Substrate	Electron Acceptor	Electron Transfer Inhibitor	Increase in P/O
DPNH	PMS	Amytal	0.5
DPNH	PMS	Antimycin	0.5
Succinate	O_2	-	0.0
Cytochrome c (reduced)	O_2	-	0.0

The assays were performed as described in the legend for Table I. The reduced cytochrome c assay was performed as described by Webster, (1962). Modified ETPH (2 mg) was used in place of ETPH and 5 µg coupling factor I was added in the assay. The increase in P/O represents the Δ P/O ratio of the particle assayed in the presence and absence of coupling factor I.

indicates that this coupling factor operates solely in the flavin-non-heme iron region of the electron transfer system. In line with this assumption, the fact that the P/O ratio with either succinate or reduced cytochrome c is not affected by the factor provides further evidence that I is specific for the coupling that is operative in the DPNH-flavin-non-heme iron region. Factor I, on occasions, increases the P/O ratio for the coupled oxidation of DPNH by 1.0.

The second coupling factor (II) increases phosphorylation accompanying oxidation of both DPNH and succinate, but not of reduced cytochrome c (cf. Table III). In presence of antimycin, II increases the P/O ratio for the oxidation of succinate and DPNH about 0.4 (PMS was used as the electron acceptor). The lack of effect of this coupling factor on phosphorylation associated with the oxidation of either cytochrome c or DPNH in an Amytal blocked system, strongly suggests that phosphorylation is promoted solely by the $CoQH_2$-cytochrome c reductase complex in the segment of the complex between $CoQH_2$ and the antimycin site - a segment which includes cytochrome b and non-heme iron.

Table III

Restoration of Phosphorylation by the Reduced Coenzyme Q Coupling Factor (II)

Substrate	Electron Acceptor	Electron Transfer Inhibitor	Increase in P/O
Succinate	PMS	Antimycin	0.4
DPNH	PMS	Antimycin	0.4
DPNH	PMS	Amytal	0.0
Cytochrome c (reduced)	O_2	-	0.0

The assays were performed as described in the legend for Table I. Oxygen consumption was measured with a recording GME Oxygraph and phosphorylation was measured by determination of the phosphate-P^{32} incorporated into glucose-6-PO4. Modified ETPH (2 mg) was used in place of ETPH and 30 μg coupling factor II was added to the assay. The increase in P/O represents the Δ P/O ratio of the particle assayed in the presence and absence of coupling factor II.

Both the DPNH (I) and the reduced coenzyme Q (II) coupling factors have been purified 100 fold by fractionation with DEAE cellulose and CM cellulose, and they appear to be proteins. The coupling activity of I and II is destroyed by heating at 100°. The two factors are precipitated from solution by ammonium sulfate. The coupling activity of I and II can not be replaced with serum albumin, coenzyme A, ribonucleic acid or carbon dioxide.

I and II do not have either ADP-ATP or ATP-Pi exchange activities. Furthermore, when I and II are added back to the particle, the ATP-Pi exchange activity of the particle is not enhanced even under conditions which lead to restoration of phosphorylation. It is interesting to note that the ATP-Pi exchange activity of modified ETPH is only 2% of that of intact mitochondria, even after the coupling factors have been added back to the particle.

Factor II does have some ATPase activity (1.4 μmoles ATP hydrolyzed/min/mg protein) while I is devoid of this activity. The ATPase activity of II is three times higher than that of the original supernatant solution whereas the coupling activity is 100 times higher. This difference in the

degree of purification of the two activities suggests that ATPase activity is unrelated to the coupling activity. During the purification of II, a fraction rich in ATPase activity (40 μmoles ATP hydrolyzed/min/mg protein) has been separated from the fraction containing the coupling factor (II). Occasionally this fraction with high ATPase activity also shows some coupling activity and in this respect resembles the ATPase preparation of Pullman et al. (1960). The coupling factors (I, II, III) do not have "latent" ATPase activity as evidenced by the fact that exposure to 60° for two minutes in the presence of ATP (M. Pullman, personal communication) does not lead to increase of ATPase activity. The coupling factor described by Pullman et al. (1960) probably represents a modified form of the reduced coenzyme Q coupling factor. Factor II is inactivated by incubation at zero degrees for an extended period of time.

In the present study it has been demonstrated that a coupling protein can be removed from each of the phosphorylation sites and that under appropriate conditions maximal phosphorylation can be restored at each specific site by adding back the appropriate factor (also see Webster, 1962). Furthermore, these studies demonstrate that the coupling activity of each of the factors is specific for a different site in the electron transfer chain.

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