Preservation of Coupling Capacity in Submitochondrial Particles

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The stepwise fragmentation of the mitochondrion into submitochondrial particles has proved to be decisive in the identification of components of the electron transfer system and in defining their sequence and their arrangement within four discrete complexes (Green, 1961). Precise information about the electron transfer process was essential for success in the next phase in the study--that of determining how phosphate esterification is coupled to electron transfer. The difficulty we have experienced in applying the tactic of stepwise fragmentation of the mitochondrion to the study of oxidative phosphorylation has been the apparent instability of the coupling apparatus during the fragmentation process. Therefore, it became mandatory to determine experimentally the conditions which must be used and the additions which must be made in order to preserve or restore fully the coupling capacity in the submitochondrial particles.

We have observed three phenomena which underlie the loss or preservation of coupling capacity. The first is the uncoupling action of the substrate itself (succinate and DPNH); the second is the requirement for Mg and Mn ions; and the last is the requirement for coupling factors which are involved in the synthesis of ATP. The present communication deals with these phenomena, and a submitochondrial particle is described which retains maximal phosphorylative capacity.

Suspensions of heavy beef heart mitochondria (HBHM) show maximal phosphorylative efficiency (P/O ratio of 2 for succinate and 3 for the DPN-

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linked substrates). When these suspensions are frozen and thawed, the P/O ratio for succinate declines from 2.0 to 1.2 whereas the P/O ratio for the DPN-linked substrates only declines from 3.0 to 2.8. A similar discrepancy in coupling efficiency has been found to apply to suspensions of light beef heart mitochondria (LBHM). In LBHM, the P/O ratio for succinate oxidation is 0.3 whereas DPN-linked substrates exhibit P/O ratios greater than 2.5. The available evidence points to a common electron transfer sequence in the oxidation of succinate and DPNH. Therefore, it was difficult to see how the coupling efficiency of succinate oxidation could decline so sharply while the coupling efficiency of DPNH oxidation was essentially unaffected.

The dilemma has been largely resolved by the finding that the concentration of succinate employed in the assay system has a profound influence on the coupling efficiency. Whereas maximal coupling efficiency can be achieved in intact unfrozen mitochondria over a wide range of succinate concentrations (5-50 mM) this is definitely not the case for mitochondrial suspensions which have been frozen. (cf. Table I). Succinate acts as an uncoupling agent at concentrations greater than 5 mM. The explanation of this unexpected observation is probably straightforward. The organization of the intact mitochondrion provides a permeability barrier to succinate. Regardless of the external concentration of succinate, the level of internal succinate (ie. the actual concentration of succinate which prevails at the coupling site) is below the critical range in which uncoupling effects are exerted. Less effective barriers exist in frozen HBHM and LBHM, and thus the internal concentration of succinate may approach that of the external concentration. When the permeability barriers are non-operative, theoretical phosphorylative efficiency can be achieved by maintaining the succinate concentration at less than 5 mM. In LBHM the succinate linked P/O ratio can only be raised to 1.0 by maintenance of succinate at low concentration levels.

The uncoupling action of succinate and DPNH has been examined in the submitochondrial particle, ETPH. At concentrations greater than 5 mM,

Table I

Influence of Succinate Concentration on Oxidative Phosphorylation

Particle (After freezing and thawing)	Succinate Concentration mM	ΔO_2 S.A*	P/O
HBHM	5	0.04	2.0
	20	0.12	1.4
	40	0.19	1.2
LBHM	5	0.06	0.8
	40	0.25	0.3

The reaction mixture (3 ml) contained: 10 mM KPO_4 (pH7.5), 1.6 mM $MgCl_2$, 0.25M sucrose, 20 mM glucose, 0.5 mg hexokinase, 1-3 mg particle protein and substrate. Oxygen consumption was measured manometrically at 30° and the decrease in inorganic phosphate was determined. Particles were maintained at -20° for one day.

*S.A. = μatoms O_2/min/mg protein.

succinate has an uncoupling action but the degree of uncoupling is less than that noted in frozen mitochondria. A pronounced uncoupling action by DPNH in concentrations greater than 1 mM is readily demonstrable. Webster (1962) in our laboratory has also noted the uncoupling action of DPNH in modified ETPH.

The mechanism of the uncoupling action of succinate and DPNH is under investigation in our laboratory and will be the subject of another communication. Metal chelation undoubtedly underlies the effect.

Even if due regard is given to the uncoupling action of substrate, the coupling efficiency of ETPH, when prepared according to the method of Linnane and Ziegler (1958), is still less than theoretical (P/O of 1 for succinate and 2 for DPNH). Linnane and Ziegler stressed the need for the presence of Mg ions in the suspending medium used during the preparation of ETPH. It occurred to us that some metal other than Mg might also be essential for preservation of coupling efficiency. Indeed, Mn has proved to be the second metal required. (cf. Table II).

Table II

Influence of Manganese and Magnesium on Oxidative Phosphorylation

Particle	Metal ion used in the preparation	Substrate	Additions to Reaction Mixture	P/O
I	Mg	Succinate	-	0.8
		DPNH	-	1.5
II	Mn + Mg	Succinate	-	1.9
		DPNH		2.8
IIa	Mn + Mg	Succinate	-	0.9
		Succinate	Reduced cyt c coupling factor	1.5

The assays were performed as described in the legend for Table I. DPNH was generated by alcohol dehydrogenase (50γ), 0.02 ml ethanol and 0.3 mM DPN. Succinate was used at 8 mM. Particle I was prepared according to the procedure of Linnane and Ziegler (1958). Particles II and IIa were prepared in the same manner except that the $MgCl_2$ concentration was lowered to 5 mM and $MnCl_2$ was added with a final concentration of 10 mM. Where indicated, (50γ) reduced cytochrome c coupling factor was added.

When Mg and Mn ions are both present in the medium used during sonic fragmentation of mitochondria, preparations of ETPH have been obtained which have theoretical P/O ratios (2 for succinate and 3 for DPNH). It is not yet possible to say whether the two metals are essential components of the coupling mechanism or whether they prevent the loss of other factors that are essential for the coupling process. Whatever the correct interpretation may be, the fact of their essentiality for preservation of coupling efficiency is apparent.

Some preparations of ETPH, which have been prepared and assayed under optimal conditions, still fail to show theoretical P/O ratios (cf. Particle IIa in Table II). In such particles the phosphorylative efficiency can in large measure be restored by the addition of one of the three highly purified coupling factors isolated in our laboratory. As shown in Table II the addition of the coupling factor essential for phosphorylation associated with the electron flow from reduced cytochrome c to oxygen (Webster, 1962) restores the phosphorylative capacity of particle IIa. The fact that complete restoration of phosphorylative efficiency of particle IIa was not

observed, even upon addition of the reduced cytochrome c coupling factor, would suggest that the coupling factor at the reduced coenzyme Q phosphorylation site was also partially deficient. In order to restore fully the phosphorylative capacity of various ETPH preparations, one or more of the three coupling factors would have to be added back to the particle.

In a communication to follow, the evidence for the existence of three different coupling factors and some information about their properties will be provided. Crude preparations of the coupling factors are contaminated with uncoupling agents and the full restoration of the coupling capacity of any given submitochondrial particle may require the addition of the three factors in a highly purified state, free of contaminating uncoupling agents.

In most cases the loss of the coupling factors can be avoided by the addition of Mg and Mn ions to the fragmentation medium. Particles prepared in this way show theoretical phosphorylative capacity. There is no need to postulate any feature of the coupling process which cannot survive the fragmentation of the mitochondria into subunits. Providing due care is paid to the conditions for fragmentation (eg. by having metal ions present in the medium) and providing a deficiency in any of the three coupling factors is rectified, the way is now open for the systematic study of the coupling process in submitochondrial particles of decreasing complexity.

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