*THE ENZYMES AND THE ENZYME-CATALYZED REACTIONS OF MITOCHONDRIAL OXIDATIVE PHOSPHORYLATION**

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The process of oxidative phosphorylation in mitochondria involves the formation of ATP coupled to the oxidation of DPNH or succinate. Considerable evidence exists[1] that the ATP is produced at three separate and discrete sites along the electron transfer chain (Fig. 1). Experiments in this laboratory have been directed toward an understanding of the chemical steps involved in mitochondrial oxidative phosphorylation, and have led to the separation of the enzyme system that catalyzes oxidative phosphorylation into four components: a submitochondrial particle, ETP_H, which is deficient in the ability to phosphorylate, and three discrete soluble proteins, each of which restores a portion of the phosphorylative ability to the functionally deficient particle.[2-5] The soluble proteins were collectively termed "coupling factors" until more could be learned concerning their individual functions. Each coupling factor has been found to be specifically required for phosphorylation at only one of the three phosphorylation sites. Thus, coupling factor I (the DPNH coupling factor[6]) is required only for phosphorylation coupled to that sector of the electron transfer chain in which electrons are transferred from DPNH to coenzyme Q; coupling factor II (the $CoQH_2$ coupling factor[6]) is required only for phosphorylation coupled to that sector in which electrons are transferred from reduced coenzyme Q to cytochrome *c*; and coupling factor III (the reduced cytochrome *c* coupling factor[6]) is required only for phosphorylation coupled to that sector in which electrons are transferred from reduced cytochrome *c* to oxygen. Each of the three coupling factors has been purified considerably.[3-5] Several properties of the factors are listed in Table 1. It is noteworthy that the purified factor preparations have negligible ATPase, ATP-P_i exchange, and adenyl kinase activities, but that each has a measurable ADP-ATP exchange activity.

TABLE 1

Some Properties of the Site-Specific Coupling Factors

Property	DPNH factor[a]	$CoQH_2$ factor[b]	Cyt^{++} factor[c]
Molecular weight (at neutrality)[d]	85,000	124,000	35,000
Isoelectric point (pH)	9.5	5.7	7.8
Absorption maximum (mμ)	279	278	277
Absorption minimum (mμ)	251	250	252
ATPase activity[e]	15	0.5	<0.1
ATP-P_i exchange activity[e]	50	0.3	<0.1
ATP-ADP exchange activity[e]	220	1500	1410
Adenyl kinase activity[e]	<0.1	<0.1	<0.1

[a] Data of A. L. Smith and M. Hansen.
[b] Data of R. E. Beyer.
[c] Data of G. Webster.
[d] Data of P. Yang.
[e] mμMoles per min per mg of protein.

The relationship of the three site-specific proteins to various coupling factors which have been reported by other laboratories is slowly becoming clarified. Wadkins and Lehninger[7] have now found that their ADP-ATP exchange enzyme is re-

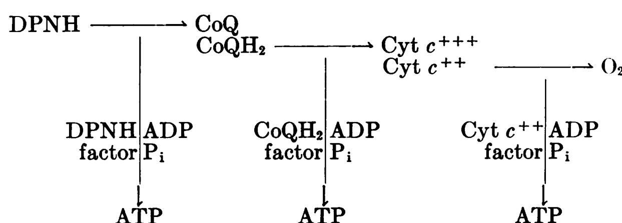


FIG. 1.—Sites of ATP formation along the electron transfer chain.

quired for phosphorylation at the cytochrome oxidase site, and appears to be quite similar to our reduced cytochrome *c* coupling factor. Experiments in this laboratory, which will be presented *in extenso* elsewhere, have shown that most of the other reported coupling factors are either site-specific or exhibit no evidence of being an integral part of the enzyme system catalyzing oxidative phosphorylation.

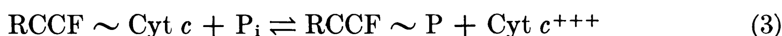
Our discovery of three site-specific proteins required for oxidative phosphorylation has posed a question concerning the mechanism of their participation as enzymes in the phosphorylation process. Recent experiments have provided new insight into the mode of action of the coupling factors and into the chemical nature of the enzymic reactions comprising oxidative phosphorylation. These experiments are summarized and discussed in this communication.

Synthesis of High-Energy Compounds during Electron Transfer.—Following the pioneering experiments of Pinchot,[8, 9] we were able to demonstrate[10] that a soluble compound was formed during the oxidation of reduced cytochrome *c* by submitochondrial particles from beef heart, which could then react very rapidly with ADP and P_i (in the absence of the particles and in the presence of KCN to block oxidation by any "solubilized" particles) to form ATP. The formation of this reactive compound entailed an absolute requirement for the reduced cytochrome *c* coupling factor, and subsequent experiments provided evidence that the reactive compound contained both the coupling factor and cytochrome *c*. A similar formation of a high-energy compound during DPNH oxidation was observed.[10] The DPNH coupling factor was specifically required for this formation.

Further experiments[11] established the fact that oxidation of reduced cytochrome *c* in the presence of the reduced cytochrome *c* coupling factor and orthophosphate-P^{32} resulted in the formation of a coupling factor $\sim P^{32}$ compound which could react readily with ADP to form ATP. The coupling factor $\sim$P compound could also be formed by reaction of the cytochrome *c* $\sim$ coupling factor compound, discussed above, with orthophosphate. Finally, a particle-bound high energy compound has been detected, which appears also to involve cytochrome *c*.[11] The formation of this compound precedes the formation of the soluble cytochrome *c* coupling factor compound, and is the only high-energy compound which is formed in the absence of the coupling factor. The sequence—particle-bound high-energy compound $\rightarrow$ soluble high-energy compound $\rightarrow$ phosphorylated high-energy compound $\rightarrow$ ATP—has been studied in detail, and all of the observations to date are best explained by the following series of four reactions (where RCCF represents the reduced cytochrome *c* coupling factor):

$$\text{Particle} + \text{n Cyt } c^{++} \rightleftharpoons \text{Particle} \sim \text{Cyt } c + (\text{n} - 1) \text{ Cyt } c^{+++} \quad (1)$$

$$\text{Particle} \sim \text{Cyt } c + \text{RCCF} \rightleftharpoons \text{Particle} + \text{RCCF} \sim \text{Cyt } c \quad (2)$$



$$\text{RCCF} \sim \text{Cyt } c + \text{P}_i \rightleftharpoons \text{RCCF} \sim \text{P} + \text{Cyt } c^{+++} \quad (3)$$

$$\text{RCCF} \sim \text{P} + \text{ADP} \rightleftharpoons \text{RCCF} + \text{ATP}. \quad (4)$$

The formation of high-energy compounds similar to those observed at the cytochrome *c*–cytochrome oxidase site has now been observed at the other two phosphorylation sites. Thus, at each site, the formation, during electron transfer, of a phosphorylated form of the respective coupling factor has been demonstrated,[12, 13] and at the DPNH-CoQ site, a soluble, nonphosphorylated, high-energy compound has been isolated.[10, 14] The various high-energy compounds which have been detected thus far at each of the three phosphorylation sites are specified in Figure 2. Especially noteworthy is the parallel formation, during electron transfer in the presence of orthophosphate and in the absence of ADP, of phosphorylated forms of the three coupling factors.

Synthesis of Coupling Factor $\sim$ *P^{32} from ATP^{32}.*—The formation of coupling factor $\sim$P compounds during electron transfer, together with the ADP-ATP exchange catalyzed by each coupling factor, suggests that the terminal reaction of oxidative phosphorylation is reversible at each site. That this is indeed the case has now been demonstrated by the finding[12–14] that each coupling factor can react with ATP^{32} to form coupling factor $\sim P^{32}$. The isolated coupling factor $\sim P^{32}$ can subsequently react with ADP to form ATP^{32}.

$$\text{Coupling factor} \sim \text{P} + \text{ADP} \rightleftharpoons \text{Coupling factor} + \text{ATP}. \quad (5)$$

The three coupling factor $\sim$P compounds, formed by the reaction of each coupling factor with ATP, exhibit the same physical and chemical characteristics as do the three respective coupling factor $\sim$P compounds formed during substrate oxidation in the presence of coupling factor and orthophosphate; it must be concluded that they are identical.

Information on the reaction of the $CoQH_2$ coupling factor with ATP^{32}, and on the nature of the $CoQH_2$ coupling factor $\sim$P compound has been presented by Beyer.[12] The reduced cytochrome *c* coupling factor likewise reacts with ATP^{32} to form a coupling factor $\sim$P compound (Table 2). Approximately one molecule of P^{32} is bound to each molecule of protein. This bound P^{32} is rapidly converted into glu-

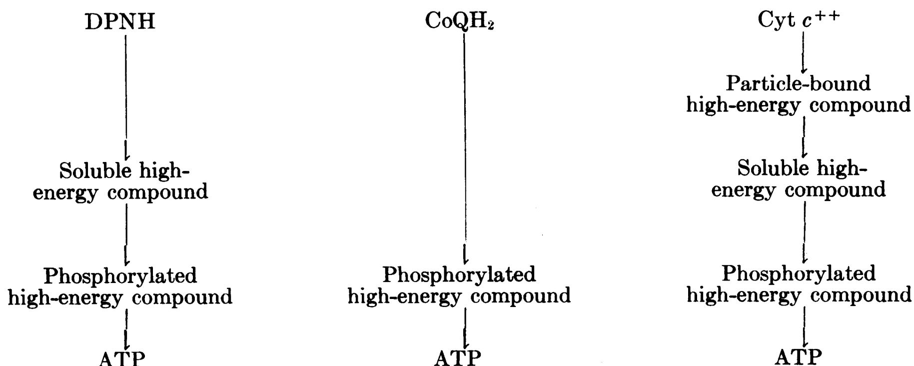


FIG. 2.—High-energy compounds detected at the three phosphorylation sites.

TABLE 2

FORMATION AND STABILITY OF A PHOSPHORYLATED FORM OF THE REDUCED CYTOCHROME *c* COUPLING FACTOR

Reaction system*	Total μmoles P^{32} bound to protein	μmoles P^{32} per μmole RCCF
RCCF (3 mg) + ATP^{32}	0.10	1.17
RCCF (5 mg) + ATP^{32}	0.17	1.19
RCCF (10 mg) + ATP^{32}	0.38	1.33
RCCF (5 mg, boiled 3 min) + ATP^{32}	0.01	0.07
RCCF$\sim P^{32}$ (5 mg, incubated at pH 3 for 30 min at 0°)	0.03	0.21
RCCF$\sim P^{32}$ (5 mg, incubated at pH 11 for 30 min at 0°)	0.03	0.21

* The reaction system for RCCF$\sim P^{32}$ formation was 0.01 *M* in Tris-acetate (pH 7.5), 0.001 *M* in $MgCl_2$, 0.001 *M* in ATP^{32} (with a total activity varying, in different experiments, between 110,000 and 180,000 cpm). The total volume, 5 ml, contained the indicated amount of reduced cytochrome *c* coupling factor (RCCF). The RCCF$\sim$P was isolated as described previously.[11] Incubation time was 10 min at 30°.

cose-6-P^{32} when the coupling factor $\sim P^{32}$ is incubated with ADP, glucose, and hexokinase.

It is noteworthy that each of the coupling factor $\sim$P compounds is split by exposure either to pH 2 or to pH 11 for 30 min at 0°. In this respect, the stability of the coupling factor phosphates differs from the stability characteristic of protein-bound histidine phosphate.[15] Our findings, therefore, are not in accord with the view that histidine phosphate serves as a phosphorylated intermediate in mitochondrial oxidative phosphorylation. Our view is in agreement with the findings of other investigators,[16, 17] and with the recent report[18] that the principal locus of histidine phosphate is succinyl-CoA synthetase. In view of these results, the following question may well be raised. If the phosphorylated coupling factor proteins are indeed intermediates in oxidative phosphorylation, how could the methods employed by Boyer and his colleagues (which were sensitive enough to detect the formation of histidine phosphate in succinyl-CoA synthetase in mitochondria) fail to detect the formation of the phosphorylated coupling factors? The answer apparently lies in the techniques employed by Boyer *et al.*[19] for mitochondrial disruption. We have observed that the phosphorylated forms of the reduced cytochrome *c* coupling factor and the DPNH coupling factor are destroyed when these techniques are applied; quite possibly the same holds true for the $CoQH_2$ factor $\sim$P compound. It is not surprising, therefore, that Boyer failed to see the phosphoproteins which may well be the intermediates in oxidative phosphorylation, and found instead the phosphoprotein of succinyl-CoA synthetase. Thus, one cannot argue that the phosphorylated coupling factors are not involved in oxidative phosphorylation in intact mitochondria merely because they are not detected in extracts of mitochondria disrupted in an arbitrary manner. For studies on the formation of the phosphorylated coupling factors in intact mitochondria, it will be necessary to employ a special method for mitochondrial disruption that will not degrade the labile phosphorylated compounds.

The significance of the finding that different coupling factor $\sim$P compounds are formed at the three phosphorylation sites should not be overlooked. The view has sometimes been advanced that the energy released at the three phosphorylation sites is channeled through a single set of ATP-forming enzymes. Our finding that the three site-specific coupling factors can act as direct precursors of ATP does not support this view, and suggests instead that each phosphorylation site has its own enzyme system for ATP formation.

Catalysis of ATP-P_i Exchange by the Coupling Factor and Synthesis of Coupling

Factor ~ Substrate Compound.—Although none of the three coupling factors alone has been found to catalyze an exchange of P_i with ATP,[3-5] the reduced cytochrome *c* coupling factor has now been found to catalyze an ATP-P_i exchange in the presence of cytochrome *c* (Table 3). The rate of the reaction is very sensitive to the

TABLE 3

ATP-P_i EXCHANGE CATALYZED BY THE REDUCED CYTOCHROME *c* COUPLING FACTOR

Reaction system*	μMoles P_i^{32} incorporated into ATP/min/mg protein
Complete	0.31
Minus cytochrome *c*	0.05
Minus magnesium ion	0.03
Minus ADP	0.02

* The reaction system was 0.05 *M* in Tris-acetate (pH 7.5), 0.02 *M* in ATP, 0.01 *M* in ADP, 0.01 *M* in potassium phosphate-P^{32} (2×10^7 cpm), 0.01 *M* in $MgCl_2$. The total volume, 1 ml, contained 1 mg of reduced cytochrome *c* coupling factor and 4 mg of cytochrome *c*. Incubation time was 5 min at 30°. Exchange activity was assayed by partition of ATP and P_i between silicotungstate and isobutanol-benzene.

ratios among the components P_i, ADP, ATP, and cytochrome *c* in the reaction system; further work is required to determine the conditions required for maximal rates of exchange. However, the discovery that the coupling factor will catalyze the exchange under specified conditions indicates that the last two reactions in the reaction scheme shown above [eqs. (3) and (4)] are measurably reversible. The occurrence of this exchange reaction also suggests that the nonphosphorylated high-energy compound, RCCF ~ Cyt *c*, which we first discovered during the oxidation of reduced cytochrome *c* by submitochondrial particles, can also be formed from ATP. We have now observed that incubation of RCCF, cytochrome *c*, and ATP results in the formation of small, but detectable, quantities of a high-energy compound which behaves in a fashion identical with that of RCCF ~ Cyt *c*.

Synthetase-like Activity of the Coupling Factors.—In view of the above results, the direct participation of the coupling factors in the synthesis of ATP from the primary high-energy bond formed during substrate oxidation is apparent. For example, at the cytochrome oxidase site, the first detectable reaction is that defined by equation (1) (see above). This reaction is catalyzed either by cytochrome oxidase, or by some, as yet undetected, enzyme working in conjunction with cytochrome oxidase. The coupling factor then catalyzes the reaction [sum of the reaction defined by equations (3) and (4)]:

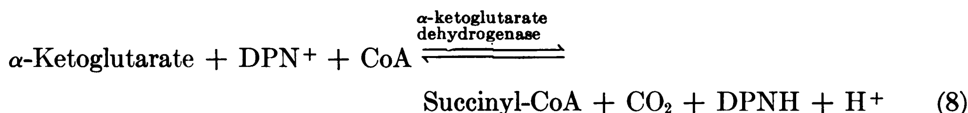
$$\text{Particle} \sim \text{Cyt } c + \text{ADP} + P_i \rightleftharpoons \text{Particle} + \text{Cyt } c^{+++} + \text{ATP}. \quad (6)$$

Measurements of the stoichiometry of the reaction have demonstrated that it proceeds as depicted here. Our conclusion that the coupling factors function in the formation of ATP from a high-energy bond initially formed during substrate oxidation is in agreement with the conclusion of Ernster and Lee,[20] based on the observation that particles devoid of coupling factors catalyze certain energy-linked reactions,[21] that the coupling factors function only in ATP formation, "rather than energy coupling in the true sense."

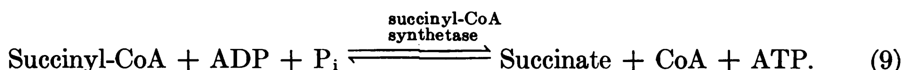
The reaction catalyzed by the coupling factor closely resembles a synthetase-catalyzed reaction:

$$\text{A} + \text{B} + \text{ATP} \rightleftharpoons \text{A—B} + \text{ADP} + P_i. \quad (7)$$

Depending upon the equilibrium point of the reaction, a synthetase may catalyze ATP formation from ADP and P_i when linked to the splitting of some bond, A—B. The action of the coupling factors resembles most closely the action of succinyl-CoA synthetase (succinic thiokinase) which catalyzes the "substrate-level" phosphorylation associated with α-ketoglutarate oxidation:[22, 23]



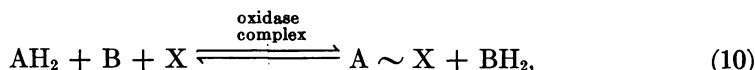
$$\alpha\text{-Ketoglutarate} + DPN^+ + CoA \xrightleftharpoons{\alpha\text{-ketoglutarate dehydrogenase}} \text{Succinyl-CoA} + CO_2 + DPNH + H^+ \quad (8)$$



$$\text{Succinyl-CoA} + ADP + P_i \xrightleftharpoons{\text{succinyl-CoA synthetase}} \text{Succinate} + CoA + ATP. \quad (9)$$

The first reaction represents the oxidative formation of the high-energy bond of succinyl-CoA, catalyzed by an oxidative enzyme, α-ketoglutarate dehydrogenase. The second reaction results in the formation of ATP from succinyl-CoA, catalyzed by succinyl-CoA synthetase. It is noteworthy that succinyl-CoA synthetase, like the coupling factors, catalyzes an ADP-ATP exchange.[24]

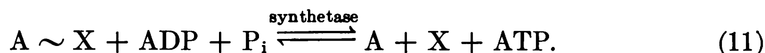
Similarly, oxidative phosphorylation now appears to proceed by two recognizable reactions. The first, catalyzed by the oxidative enzyme complex results in the oxidation of the substrate and the generation of a high-energy bond:



$$AH_2 + B + X \xrightleftharpoons{\text{oxidase complex}} A \sim X + BH_2, \quad (10)$$

where A is DPN, CoQ (yet to be shown), or cytochrome c, and X is some, as yet unknown, functional group with which the substrate forms a high-energy bond as a result of coupled oxidation.

The second reaction, catalyzed by the synthetase (coupling factor) results in the formation of ATP:



$$A \sim X + ADP + P_i \xrightleftharpoons{\text{synthetase}} A + X + ATP. \quad (11)$$

We realize that the term, "synthetase," is a trivial name. However, in view of the fact that there is an entire class of enzymes, the synthetases (or kinases), whose general characteristics encompass the enzymic activities we have found for the coupling factors, the term, "synthetase," seems considerably more definitive than does the term, "coupling factor."

We propose, therefore, that more accurate terms for the three site-specific proteins which restore phosphorylation in submitochondrial particles are: site I—linked ATP synthetase, site II—linked ATP synthetase, and site III—linked ATP synthetase for the DPNH coupling factor, the $CoQH_2$ coupling factor, and the reduced cytochrome c coupling factor, respectively.

The evidence for ATP formation by a synthetase mechanism has important implications for the claim[25] that multiple coupling factors are operative at each site in oxidative phosphorylation. Although a number of synthetases have been studied by various investigators, in no case has the synthetase been separated into multiple protein components. Instead, a single protein has always been found to catalyze the synthetase reaction. Our observations that only a single protein is required at each phosphorylation site to produce ATP from the high-energy bond formed

during electron transfer are in agreement with a large amount of biochemical information concerning synthetase action. Not only is there no need to invoke a requirement for multiple coupling factors for ATP production from the initial high-energy compound, but also there is no definitive evidence to support the claim that multiple coupling factors are required at each phosphorylation site.

In conclusion, we wish to emphasize the point that the soluble proteins which we called "coupling factors" catalyze the displacement of the two groups which are joined by the high-energy bond formed initially during electron transfer—displacement first by P_i and then by ADP. The coupling factors appear not to participate in the formation of the initial high-energy bond. Our steadily increasing knowledge of the coupling factor catalyzed conversion of the initial high-energy bond into ATP by a synthetase reaction indicates that this portion of the oxidative phosphorylation mechanism will soon be largely understood. It is evident that presently the central problem in oxidative phosphorylation concerns the nature of the high-energy bond initially formed during oxidation.

Abbreviations used: ATP, adenosine triphosphate; ADP, adenosine diphosphate; P_i, orthophosphate; DPN^+ and DPNH, the oxidized and reduced forms of diphosphopyridine nucleotide; CoQ and $CoQH_2$, the oxidized and reduced forms of coenzyme Q (ubiquinone); RCCF, reduced cytochrome *c* coupling factor; Tris, tris (hydroxymethyl) aminomethane; CoA, coenzyme A.

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