THE ROLE OF FACTOR B IN THE ENERGY TRANSFER REACTIONS OF OXIDATIVE PHOSPHORYLATION*

BY D. R. SANADI,[†] K. W. LAM,[‡] AND C. K. RAMAKRISHNA KURUP

INSTITUTE OF BIOLOGICAL AND MEDICAL SCIENCES, RETINA FOUNDATION, BOSTON, MASSACHUSETTS

Communicated by Britton Chance, June 14, 1968

One approach to the study of the mechanism of multienzyme systems is resolution of the constituent parts and reconstitution of the system from the purified components. This has proved to be difficult in the case of oxidative phosphorylation because the catalytic units also form an integral part of the mitochondrial structure and thus appear to have a dual function. Resolution is further complicated by the association of the enzymes with the insoluble structural protein and phospholipid. The resolved components often show alterations in properties due either to changes produced by the isolation methods or to a change in environment from a particle-bound state to a free soluble protein. A major step in this direction was made in 1958^{1, 2} when it was found that a soluble protein fraction from mitochondria stimulated phosphorylation in submitochondrial particles (see Table 1). Since these soluble proteins were concerned with reactions leading to ATP synthesis, they were given the operational name of "coupling" factors. A more appropriate name would be "energy transfer" factors since they appear to be acting in the steps subsequent to the coupling event. A factor that has been studied in detail is F_1 which has high ATPase activity.³ Another preparation, Factor A, which shows similar stimulatory activity in several energy-linked reactions has been isolated in our laboratory.^{4, 5} This protein has no ATPase activity but can be converted to as active an ATPase as F_1 by exposure to elevated temperatures. It has been suggested that the factor, as it occurs in mitochondria, has no inherent ATPase activity,^{3, 5} but the hydrolytic property is induced as a result of changes in environmental conditions during the isolation.⁵ At least three other factors closely related to F_1 have been reported (Table 1).

The claims for additional factors have proliferated during the last few years. However, the only other extensively purified protein is Factor B. The role of the purified ADP-specific nucleotide diphosphokinase (ADP-ATP exchange enzyme) and of the phosphoryl-transferase in oxidative phosphorylation needs further exploration. The status of the remaining factors is uncertain since they have not been adequately purified, and milligram quantities are needed for significant effect on similar amounts of particle protein. The stimulation of coupled phosphorylation under these conditions may be caused by contaminants present in the preparation. The activity of F_4 , for example, is now attributed to contaminating F_2 and F_3 ,⁶ and McLennon and Tzagoloff have been able to prepare OSCP (Table 1) from F_4 . It is often difficult to see the relationship between the different factor preparations since several types of deficient particles are in use, most of them having a considerable complement of bound factors resulting in rather high basal activity. The significance of small changes in P/O above the basal level or above the level after partial supplementation becomes difficult to evaluate. Sometimes the superficial analogies drawn between different factors

TABLE 1. *Energy-transfer and related factors.*

Trivial name	Properties and function	Reference
Coupling factor	Not purified	Linnane (1958) ^a
F ₁	ATPase, cold labile, mol wt 284,000 Terminal phosphorylation of ADP to ATP	Pullman <i>et al.</i> (1958) ^b
Factor A	Latent ATPase, cold stable, otherwise similar to F ₁	Sanadi <i>et al.</i> (1962) ^c
Factor	No ATPase, functionally similar to F ₁	Sone and Hagihara (1966) ^a
Factor	ATPase, similar to F ₁	Ling and Wu (1964) ^e
F ₁ -X	Complex of F ₁ and other factor(s)	Vallejos and Slater (1967) ^f
ADP-ATP exchange enzyme	Subunit of approx. 30,000 mol wt Gains dinitrophenol sensitivity when added to particles.	Lehninger <i>et al.</i> (1958) ^{g, h}
Phosphoryl-transferase	Mol wt 124,000	Beyer (1964) ⁱ
Factor B	Mol wt about 32,000. Functional-SH	Sanadi <i>et al.</i> (1963) ^j
F ₂	Not purified	Racker (1964) ^k
F ₃ (F _{3A} , F _{3B})	Not purified	Racker (1962) ^{l, m}
F ₄	Structural protein with contaminating factors	Prarie <i>et al.</i> (1963) ^{n, n}
Factor	Partially purified	Hommes (1963) ^o
F ₀	Confers oligomycin sensitivity to F ₁ -ATPase	Racker (1963) ^p
OSCP	Oligomycin sensitivity conferring protein	McLennon ^q

^a A. W. Linnane, *Biochim. Biophys. Acta*, **30**, 221 (1958).

^b M. E. Pullman, H. Penefsky, and E. Racker, *Arch. Biochem. Biophys.*, **76**, 227 (1958).

^c D. R. Sanadi, A. L. Fluharty, and T. E. Andreoli, *Biochem. Biophys. Res. Commun.*, **8**, 200 (1962).

^d N. Sone and B. Hagihara, *J. Biochem.*, **60**, 622 (1966).

^e C.-S. Ling and C.-Y. Wu, quoted: *Chem. Abstr.*, **62**, 14956 (1965).

^f R. H. Vallejos and E. C. Slater, *Biochim. Biophys. Acta*, **143**, 441 (1967).

^g C. L. Wadkins and A. L. Lehninger, *J. Biol. Chem.*, **233**, 1589 (1958).

^h C. L. Wadkins and R. P. Glaze, in *Oxidases and Related Redox Systems*, ed. T. E. King, H. S.

Mason, and M. Morrison (New York: John Wiley, 1965), vol. 21, p. 1018.

ⁱ R. E. Beyer, *Biochem. Biophys. Res. Commun.*, **16**, 460 (1964).

^j D. R. Sanadi, T. E. Andreoli, R. L. Pharo, and S. R. Vyas, in *Energy-Linked Functions of Mitochondria*, ed. B. Chance (New York: Academic Press, 1963), p. 26.

^k E. Racker, *Biochem. Biophys. Res. Commun.*, **14**, 75 (1964). (See also statements in Penefsky *et al.*, *J. Biol. Chem.*, **235**, 3330 (1960) and in Racker and Conover, *Federation Proc.*, **22**, 1088 (1963).

^l E. Racker, these PROCEEDINGS, **48**, 1659 (1962).

^m R. L. Prarie, T. E. Conover, and E. Racker, *Biochem. Biophys. Res. Commun.*, **10**, 422 (1963).

ⁿ J. M. Fessenden, M. A. Dannenberg, and E. Racker, *Biochem. Biophys. Res. Commun.*, **25**, 54 (1966).

^o F. A. Hommes, *Biochem. Biophys. Res. Commun.*, **8**, 248 (1962); *Biochim. Biophys. Acta*, **71**, 595 (1963).

^p E. Racker, *Biochem. Biophys. Res. Commun.*, **10**, 435 (1963). F₀ cannot be regarded as an energy transfer factor since its effect on P/O has not been reported. It is a membranous protein which confers oligomycin sensitivity to the ATPase activity of F₁.

^q D. H. McLennon and A. Tzagoloff, *Biochemistry*, **7**, 1603 (1968).

are premature. For example, it has been suggested that Factor B "may be identical with F₃".⁶ However, the tendency of F₃ (still relatively crude) to undergo polymerization⁶ and its stability to iodine treatment⁷ are properties that are not shared by Factor B.⁹

Implicit in the approach to the study of oxidative phosphorylation by resolution of the mitochondrial components is the concept of high-energy intermediates and stepwise transfer of energy for ADP phosphorylation. The intermediates could actually be activated forms of energy transfer factors in which the activa-

tion occurs by interaction of the active sites with each other or with the respiratory carrier. Thus, the isolation and characterization of the energy transfer factors would be prerequisite for the identification of the chemical steps in oxidative phosphorylation.

The factors with ATPase activity, overt or latent, are evidently concerned with the terminal reaction leading to the synthesis of ATP from ADP. In the case of Factor B,⁸ no individual enzyme activity has been observed. It is assayed by its ability to stimulate energy-linked reactions when added to deficient particles. It could be acting as an energy transfer enzyme, as a stabilizer, as a structural component, or by removing an inhibitor. By taking advantage of its mercurial sensitivity and of the activation of several types of energy-linked reactions, it is now possible to localize the site of action of Factor B in the sequence of energy transfer steps.

Materials and Methods.—The preparation of ammonia and EDTA-treated particles and of Factor B have been described.⁸ The Factor B used in many of these experiments was purified in the later steps in the presence of DTT⁹ and was also subjected to purification by sucrose density gradient centrifugation. The purified protein migrated as a single band in polyacrylamide disc electrophoresis comprising over 95% of the protein and gave a symmetrical peak in sedimentation experiments (unpublished data by K. W. Lam and D. Swann). Preparation of ETPH¹⁰ and assay of ATP-driven NAD reduction by succinate¹¹ have been described.

Results.—Mercurial inhibition of ATP-driven reactions: Factor B increases P/O and stimulates energy-linked reactions in the ammonia particle.⁸ It has been shown recently that the activity of Factor B is inhibited by CMPS and restored by DTT, indicating that the factor has functional -SH group.⁹ It is thus expected that energy-linked reactions involving bound Factor B in nondepleted particles should be sensitive to mercurials. In fact, the ATP-P_i exchange is inhibited 85 per cent by 150 μ M CMPS while similar levels of the mercurial have no effect on the ATPase activity of ETPH (see also ref. 12). The inhibition is reversed by 5 mM DTT. These results clearly show that -SH groups may not be essential for the segment of the energy transfer reactions concerned with the ATPase (see Fig. 1) but are essential for the segment concerned with the ATP-P_i exchange reaction. The mercurial also inhibits the ATP-driven nicotinamide nucleotide transhydrogenase (60% at 67 μ M), while in the energy-independent direction the reaction is only 23 per cent inhibited.

Stimulation of ATP-driven reactions by Factor B: Factor B stimulates oxidative phosphorylation and ATP-driven NAD reduction by succinate in the ammonia particle, but it has only slight inhibitory effect, if any, on the ATPase activity.⁸ As shown in Table 2, Factor B also stimulates ATP-P_i exchange activity fivefold and the ATP-driven transhydrogenase activity threefold.

Effect of mercurials on reactions driven by oxidative energy: The energy-dependent transhydrogenase when driven by coupling to the oxidation of ascorbate-TMPD (via the cytochrome oxidase) is inhibited 89 per cent by 10 μ M CMPS. The reduction of NAD by succinate, when similarly coupled, is inhibited almost completely by 10 μ M CMPS or mersalyl (data are not shown). The inhibitions are relieved by excess DTT.

TABLE 2. Stimulation of ATP-driven reactions by Factor B.

	Nmoles $\times$ min ⁻¹ $\times$ mg protein ⁻¹
<i>Transhydrogenase</i>	
Particle, 0.5 mg	36
Particle + 40 μ g Factor B	100
<i>ATP-P_i exchange^a</i>	
Particle 1 mg	16
Particle + 20 μ g Factor B	47
Particle + 60 μ g Factor B	78
Particle + 80 μ g Factor B	82

Transhydrogenase: Ammonia particle (0.5 mg protein) was mixed with the factor as indicated and then suspended in the reaction medium consisting of 150 μ moles Tris-sulfate, 3 μ moles KCN, 0.3 μ mole NAD, 5 μ moles MgCl₂, 750 μ moles sucrose, 6 μ moles ATP, 80 μ moles ethanol, 2 mg BSA, and 100 μ g alcohol dehydrogenase in 2.95 ml at pH 7.8. After incubation for 2 min at 38°, the reaction was initiated with 0.05 ml of 0.03 M NADP. Since a stored preparation of Factor B which generally shows stimulation by DTT^a was used in this experiment, 0.17 mM DTT was also present in the assay medium.

ATP-P_i exchange: Ammonia particles (1 mg protein) was incubated with 10 μ moles of phosphate, 4.4×10^4 cpm of ³²P, 5 μ moles MgCl₂, and 50 μ moles Tris-sulfate in 0.9 ml, pH 7.5 at 30° for 2 min. The exchange reaction was initiated by the addition of 5 μ moles ATP in 0.1 ml. The reaction was stopped with 0.5 ml of 20% TCA 5 minutes after the addition of ATP. A fresh preparation of Factor B, which does not require DTT for activity, was used in this experiment.

^a The stimulation of the reaction by Factor B was first observed by Dr. Joseph B. Warshaw in this laboratory.

assays mentioned previously, which essentially rules out a role of BSA in assisting the accessibility of the mercurial to the -SH site in the particle.

Discussion.—The participation of -SH in oxidative phosphorylation reactions has been documented by several workers.^{12, 19–24} Evidence has been presented that the -SH functions in an energy-transfer reaction occurring between the respiratory chain and the site of oligomycin inhibition.^{23, 24} The results presented here are consistent with previous data and conclusions. The only reaction that is insensitive to thiol inhibitors is the oligomycin-sensitive ATPase. Reactions utilizing the energy from ATP are all inhibited by mercurials, and the inhibition is reversed in each case by DTT. These reactions are also stimulated by Factor B (Table 2 and ref. 8) which has a functional -SH group.⁹ The simplest hypothesis would assume that the site of mercurial inhibition in the above reactions is the endogenous Factor B in the particles. However, involvement of other -SH groups in the multistep energy transfer reactions must be recognized as a distinct possibility.

The results with reactions driven by oxidative energy, particularly those coupled to the mercurial-resistant cytochrome *c* oxidation, also point to -SH involvement in the energy transfer reactions. These reactions are best carried out in the presence of a high level of oligomycin to prevent the breakdown of the energized intermediates. The utilization of the oxidative energy for the energy-requiring transhydrogenase reaction and the pH gradient across the membrane is inhibited by mercurial (Fig. 2), and the inhibition is reversed by DTT.

In all these experiments, it has been observed that reactions driven by coupling to aerobic oxidation appear to be more sensitive to CMPS inhibition than the ATP-driven reactions. For example, ATP-driven transhydrogenase is inhibited 60 per cent by 67 μ M CMPS while 10 μ M CMPS shows 88 per cent inhibition of the same reaction driven by ascorbate-TMPD oxidation. The discrep-

Fig. 2.—Sensitivity of the BTB color change to mercurial. The reaction medium contained 20 mM Tris-Cl buffer, pH 7.4, 0.25 M sucrose, 1 mM EDTA, 0.5 mg ammonia particle protein, 20 nmoles BTB, 1 μ g antimycin, 1 μ g rotenone, and 0.5 μ g oligomycin in 3 ml. The additions are shown on the 618–700-m μ absorbance trace. TB was pre-reduced with ascorbate to avoid large absorbance changes. A downward deflection indicates decrease in absorbance. The medium contained 2 mg BSA for the experiment on the left. The measurements were carried out in an Aminco-Chance dual wavelength spectrophotometer with a cutoff filter below 430 m μ .

ancy may be attributed either to unrecognized minor differences in experimental conditions, or to the involvement of another -SH group which is functional only when energy is provided by oxidation. In most of these experiments, the bovine serum albumin present in the medium was approximately 10 μ M. It has one -SH per molecule which may provide competition for the mercurial.

Two of the simpler mitochondrial reactions related to oxidative phosphorylation are ATPase and ATP- P_i exchange, since they appear to be independent of red-ox reactions. The exchange reaction requires the preservation of an energized intermediate ($X \sim C$) and liberation of P_i in the medium so that when the reaction is reversed, $^{32}P_i$ can be incorporated into ATP. Breakdown of an intermediate in the energy transfer sequence leads to ATPase activity. Both reactions are inhibited by oligomycin while only the exchange is inhibited by mercurials. Thus, the oligomycin-sensitive ATPase site (or, more correctly, the site of energy discharge) must lie between $X \sim C$ and ATP, as represented by the empty block in Figure 1.

It is seen from Figure 1 that the intermediate common to all the mercurial-sensitive reactions is the nonphosphorylated $X \sim C$ intermediate. The results on inhibition of partial reactions by mercurial suggest that an -SH group may be associated with X or C , or involved in the formation of $X \sim C$ both from ATP and from respiration. It is also apparent from Figure 1 that the same intermediate is common to all of the ATP-driven reactions that are stimulated by Factor B (NAD reduction by succinate, transhydrogenase, and ATP- P_i exchange—ref. 8 and Table 2). Therefore, the data are consistent with the conclusion that the

-SH-containing Factor B is X or C , or a component concerned with the synthesis of $X \sim C$.

Summary.—Several energy-linked reactions driven by ATP or by coupling to cytochrome c oxidation have been examined for -SH involvement, with inhibition by mercurials and reversal by thiols as the criteria. The same reactions have been tested for activation by the highly purified -SH-containing mitochondrial energy transfer (coupling) factor, Factor B. Mercurials inhibit ATP- P_i exchange and ATP-driven transhydrogenase (NADP reduction by NADH), but do not inhibit the ATPase reaction in heart mitochondrial particles. The mercurials also inhibit the transhydrogenase reaction and the establishment of a proton gradient when the energy supply is coupled to ascorbate oxidation. The inhibition is reversed in all instances by dithiothreitol. Factor B, which has a functional thiol group, stimulates all reactions driven by ATP, except the ATPase, in the phosphorylation-deficient ammonia-EDTA particle. In terms of the present concepts of the mechanism of mitochondrial oxidative phosphorylation, Factor B has the properties expected of X or C in the hypothetical $X \sim C$ intermediate or of some component involved in the synthesis of $X \sim C$.

Abbreviations: CMPS for p -chloromercuriphenylsulfonate, DTT for dithiothreitol, FCCP for trifluoromethoxycarbonylcyanide phenylhydrazide, BSA for bovine serum albumin, TMPD for N,N,N',N' -tetramethyl- p -phenylenediamine, TB for toluidene blue, and BTB for bromthymol blue.

* This work was supported by grants from the U.S. Public Health Service (1-RO1-GM13641-02 and GRS 5-S01-FR-05527-05), the Life Insurance Medical Fund (G-67-20), and the American Heart Association (67-749).

† Also at the Department of Biological Chemistry, Harvard Medical School, Boston, Massachusetts.

‡ Recipient of a Public Health Service Career Development Award (1-K3-HE-24, 202-01).

¹ Pullman, M. E., H. Penefsky, and E. Racker, *Arch. Biochem. Biophys.*, **76**, 227 (1958).

² Linnane, A. W., *Biochim. Biophys. Acta*, **30**, 211 (1958).

³ Penefsky, H. S., M. E. Pullman, A. Datta, and E. Racker, *J. Biol. Chem.*, **235**, 3330 (1960).

⁴ Andreoli, T. E., K. W. Lam, and D. R. Sanadi, *J. Biol. Chem.*, **240**, 2644 (1965).

⁵ Warshaw, J. B., K. W. Lam, B. Nagy, and D. R. Sanadi, *Arch. Biochem. Biophys.*, **123**, 385 (1968).

⁶ Fessenden, J. M., M. A. Dannenberg, and E. Racker, *Biochem. Biophys. Res. Commun.*, **25**, 54 (1966).

⁷ Racker, E., and L. L. Horstman, *J. Biol. Chem.*, **242**, 2547 (1966).

⁸ Lam, K. W., J. B. Warshaw, and D. R. Sanadi, *Arch. Biochem. Biophys.*, **119**, 477 (1967).

⁹ Lam, K. W., *Arch. Biochem. Biophys.*, **123**, 642 (1968).

¹⁰ Linnane, A. W., and D. M. Ziegler, *Biochim. Biophys. Acta*, **29**, 630 (1958).

¹¹ Lam, K. W., J. B. Warshaw, and D. R. Sanadi, *Arch. Biochem. Biophys.*, **117**, 594 (1966).

¹² Boyer, P. D., L. L. Bieber, R. A. Mitchell, and G. Szabolcsi, *J. Biol. Chem.*, **241**, 5384 (1966).

¹³ Mitchell, P., *Biol. Rev. Cambridge Phil. Soc.*, **41**, 445 (1966).

¹⁴ Lehninger, A. L., *The Mitochondrion* (New York: Benjamin Press, 1964).

¹⁵ Penniston, J. T., R. A. Harris, J. Asai, and D. E. Green, these PROCEEDINGS, **59**, 624 (1968).

¹⁶ Chance, B., and L. Mela, *J. Biol. Chem.*, **241**, 4588 (1966).

¹⁷ *Ibid.*, **242**, 830 (1967).

¹⁸ Slater, E. C., *Biochem. J.*, **49**, 130 (1949).

¹⁹ Cooper, C., and A. L. Lehninger, *J. Biol. Chem.*, **219**, 519 (1956).

²⁰ *Ibid.*, **224**, 561 (1957).

²¹ Fluharty, A., and D. R. Sanadi, these PROCEEDINGS, **46**, 608 (1960).

²² Kielley, W. W., in *Proceedings, Fifth International Congress of Biochemistry*, Moscow, 1961 (New York: The Macmillan Co., 1963), vol. 5, p. 378.

²³ Fluharty, A., and D. R. Sanadi, *Biochemistry*, **2**, 519 (1963).

²⁴ Fonyo, A., and S. Bessman, *Biochem. Biophys. Res. Commun.*, **24**, 61 (1966).