REQUIREMENT FOR MULTIPLE FACTORS FOR THE ATP-LINKED REDUCTION OF DPN BY SUCCINATE*

R. L. Prairie,** T. E. Conover,*** and E. Racker

Department of Biochemistry, The Public Health Research Institute of The City of New York, Inc., New York 9, New York

Received February 6, 1963

The reversal of oxidative phosphorylation was discovered by Chance and his collaborators (Chance and Hollunger, 1957, 1960; Klingenberg et al., 1959). The reduction of DPN by succinate via cytochrome b and flavoprotein requires energy which can be provided by ATP or by the oxidation of either succinate or reduced cytochrome c (Löw et al., 1961; Ernster, 1963; Snoswell, 1962; Packer, 1962). The reaction with ATP in the presence of an inhibitor of respiration presents an opportunity to study electron flow between succinate and DPNH, independently of other steps of respiration.

In view of the requirements for multiple soluble protein factors in oxidative phosphorylation and in the P_i^{32}-ATP exchange reaction of submitochondrial particles from beef heart (Penefsky et al., 1960; Linnane and Titchener, 1960; Smith and Hansen, 1962; Racker, 1962), a study of the specific requirements at a single site was desirable. It is the purpose of this communication to report on the absolute requirement for two soluble protein factors (F_1 and F_4) for the ATP-linked reduction of DPN by succinate, and on a marked stimulation by at least two others.

---

* Supported by Grant C-3463 from the U.S. Public Health Service.

** Postdoctoral Fellow of the National Institutes of Health.

*** Postdoctoral Fellow of the National Institutes of Health. Present address: The Eldridge Reeves Johnson Foundation for Medical Physics, The University of Pennsylvania.

Treatment of mitochondria or submitochondrial particles by various physical or chemical procedures (Penefsky et al., 1960; Linnane and Titchener, 1960; Lehninger, 1960; Smith and Hansen, 1962; Racker, 1962) has yielded derivative particles with marked dependencies on soluble mitochondrial proteins for oxidative phosphorylation. A recent addition to this series are submitochondrial particles prepared by sonication of mitochondria in the presence of phosphatides (P-particles). These particles show a dependency on F_1 (ATPase) as well as on a new factor (F_4)† for the P_i^{32}-ATP exchange as well as for oxidative phosphorylation (Conover et al., 1963). As shown in Table I, P-particles exhibit the same requirements for the ATP-linked reduction of DPN by succinate. Solutions of F_1 kept at 0° instead of 25° were inactive in this reaction. The cold-lability of F_1 has been shown previously for oxidative phosphorylation and ATP hydrolysis (Pullman et al., 1960; Penefsky et al., 1960). Bovine serum albumin was included in all assays because it gave a 2- to 3-fold stimulation of DPN reduction in the presence of all other components. This action was presumably due to the binding of fatty acid uncouplers (Pressman and Lardy, 1956). The rate of DPN reduction was further stimulated (about 2-fold) by addition of F_3, which also stimulated the P_i^{32}-ATP exchange (Racker, 1962; Conover et al., 1963).

The time course of the reaction is shown in Fig. 1. Addition of oligomycin resulted in an abrupt cessation of DPN reduction. That the increased fluorescence was due to DPNH was shown by the addition of pyruvate and lactate dehydrogenase, which led to a rapid disappearance of the fluorescence. Both these tests were used frequently to check on the validity of the measurements. The test for oligomycin sensitivity was most valuable for ruling out DPN reduction by malate formed from succinate (Krebs, 1961). Partially purified preparations of F_3 contained soluble enzymes, presumably malate dehydrogenase and phosphopyruvate carboxykinase, which catalyzed an ATP-dependent reduction of DPN by malate. This latter reaction was not affected by oligomycin.

† The preparation of the P-particles and F_4 will be described elsewhere, but full details of the procedure are available on request.

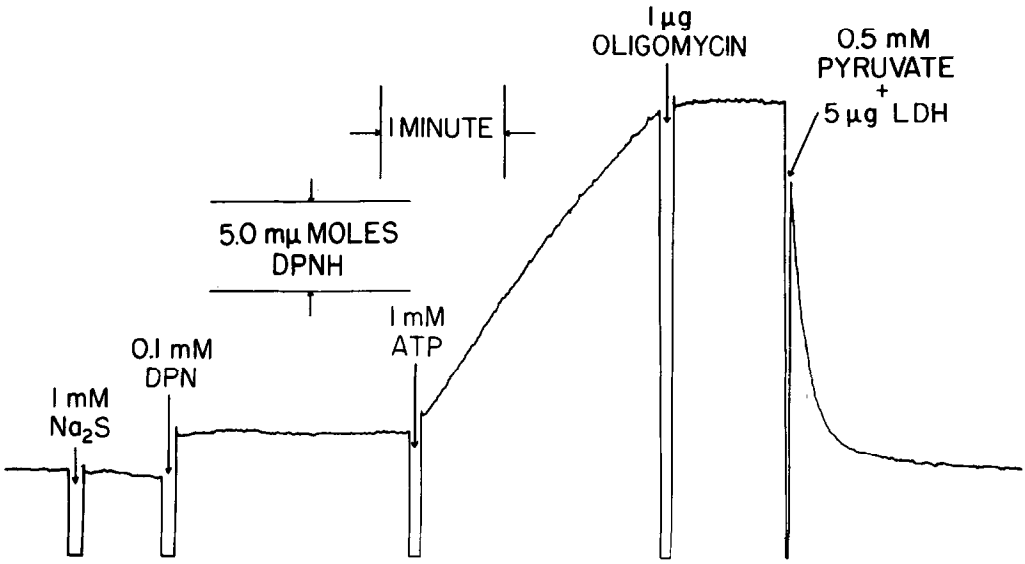

Figure 1. Time Course of the Reduction of DPN by Succinate. After preincubation of the P-particles for 5 minutes as described in Table I, various reagents were added as indicated, in a final volume of 2 ml. The recording was made from an Eppendorf Photometer equipped with fluorimetric attachments: primary filter 313 + 366 nm, secondary filter 420-3000 nm, and a device for cancelling background fluorescence (Estabrook, 1962).

The dependency of P-particles on F_3 was only partial, as seen in Table I. Particles that were treated with trypsin (Racker, 1962) exhibited a variable but often more pronounced dependency on this factor. As shown in Table II, these T-particles exhibited a marked dependency on F_1 and partially purified F_3 (Experiment 1). Further purification of the F_3 preparation on a DEAE-cellulose column gave a faintly brown fraction (F_{3A}) that was not retained by DEAE-cellulose, and a yellow fraction (F_{3B}) that was eluted with 0.1 M ammonium sulfate. Titration of these two factors revealed that both F_{3A} and F_{3B} were required for maximal stimulation (Table II, Experiment 1). P-particles showed similar dependencies (Table II, Experiment 2). The yellow fraction (F_{3B}) had been reported previously (Racker, 1962) to stimulate the $P_i{}^{32}$-ATP exchange reaction in T-particles.

Two soluble factors have previously been reported to stimulate the ATP-linked reduction of DPN by succinate. Sanadi et al. (1962) reported that repeated washing of submitochondrial particles yielded inactive particles

that were stimulated by a soluble factor. Hommes (1963) has reported the partial purification of a soluble factor that stimulated DPN reduction by succinate and eliminated a lag period observed under his experimental conditions. The relation of these two factors to those reported in this paper was explored. In our hands DPN reduction by succinate in particles prepared as

TABLE I

DEPENDENCY OF DPN REDUCTION BY SUCCINATE ON SOLUBLE PROTEIN FACTORS

The formation of DPNH was measured fluorimetrically (Estabrook, 1962). The following components were incubated in a final volume of 0.4 ml for 5 minutes at room temperature: 100 μmoles Tris-H_2SO_4, pH 7.4; 6 μmoles $MgSO_4$; 10 μmoles sodium succinate; 1 mg dialyzed bovine serum albumin; 250 μg P-particles (25 mg/ml of 0.25 M sucrose); 9.2 μg F_1 (ATPase, specific activity 54.5, kept at room temperature at a concentration of 920 μg/ml in a solution containing 250 mM sucrose, 10 mM Tris, pH 7.4, 1 mM EDTA, and 2 mM ATP); 500 μg F_3 (25 mg/ml of a fraction precipitated between 50% and 75% ammonium sulfate saturation, dissolved in H_2O, pH 5.6); and 170 μg F_4 (17 mg/ml of a fraction precipitated between 12% and 30% ammonium sulfate saturation, dissolved in 20 mM Tris, pH 7.4, containing 1 mM EDTA). After the addition of 1.57 ml H_2O, the cuvette was placed in a fluorimeter (Figure 1) at room temperature. Two μmoles of Na_2S, 0.2 μmoles of DPN, and 2.0 μmoles of ATP were sequentially added (total volume 2.0 ml) and the maximal rate of the formation of DPNH recorded. Cold-inactivated F_1 was prepared by storing a solution of F_1 for 48 hours at 2°. No increase in fluorescence with time was noted upon DPN addition, except in the control without submitochondrial particles, in which experiment ATP was without effect on the slow rate of DPNH formation. The final pH of the reaction mixture was 7.3 to 7.35.

Additions	mμmoles DPNH/minute
Complete system	11.2
- submitochondrial particles	0.2
- F_1	0.3
- F_1 + cold-inactivated F_1	0.5
- F_4	0.7
- F_3	5.5
- Bovine serum albumin	4.3
- $MgSO_4$	0.0
- Na_2 succinate	0.0
+ 0.33 μg oligomycin	0.2

TABLE II

STIMULATION OF THE ATP-LINKED REDUCTION OF DPN BY SUCCINATE WITH FRACTIONS OF FACTOR 3

The reaction was assayed as described in Table I. In Experiment 1, 150 μg T-particles, 4.6 μg F_1, 500 μg F_3, 290 μg F_{3A}, 170 μg F_{3B}, and 0.66 μg oligomycin were used. In Experiment 2, 600 μg P-particles, 9.2 μg F_1, 170 μg F_4, and the same amounts of F_3 preparations as in Experiment 1 were used. F_3 (42 mg) was passed through a 1.5 x 12 cm column of Sephadex G-25, equilibrated and eluted with 2 mM Tris, pH 7.8. The eluate was placed on a 1.1 x 5 cm column of DEAE-cellulose equilibrated with 2 mM Tris, pH 7.8 (temperature 0°, bed volume 3.0 ml). One bed volume of buffer was added and the F_{3A} fraction (8.1 mg) collected. The column was washed with 2 bed volumes of 0.005 M ammonium sulfate, eluted with 0.1 M ammonium sulfate, and the yellow fraction F_{3B} collected (12.4 mg).

Experiment 1	mμmoles DPNH/minute	Experiment 2	mμmoles DPNH/minute
T-particles	0.8	P-particles + F_1 + F_4	5.8
+ F_1	1.3	+ F_3	10.8
+ F_3	1.6	+ F_{3A}	7.0
+ F_1 + F_3	5.4	+ F_{3B}	6.7
+ F_1 + F_3 + oligomycin	0.0	+ F_{3A} + F_{3B}	9.3
+ F_1 + F_{3A}	2.2		
+ F_1 + F_{3B}	2.0		
+ F_1 + F_{3A} + F_{3B}	4.7		

described by Sanadi et al. (1962) and by Hommes (1963) were stimulated by addition of ATPase. The purified fractions prepared as described by Hommes (1963) contained ATPase activity. The lag period in DPNH formation was eliminated by F_3.

The multiplicity of coupling factors shown in this paper to be required at a single site may appear bewildering but is not unexpected. Experimental evidence from several laboratories indicates the formation of a non-phosphorylated high-energy intermediate during oxidative phosphorylation (Pinchot and Hormanski, 1962; Webster, 1962; Danielson and Ernster, 1963; Snoswell, 1962).

Thus the formation of this intermediate, its cleavage by P_i, and P-transfer to ADP, may well require separable protein catalysts. It is suggested that F_1 acts at the transfer step to ADP, and that F_3 and F_4 may be involved in the formation of high-energy intermediates prior to this step.

ACKNOWLEDGMENTS

We wish to thank Drs. Chance, Estabrook, and Hommes for their helpful assistance with the fluorimetric measurements, and for sending us manuscripts prior to publication.

REFERENCES

Chance, B., and Hollunger, G. (1957), Federation Proc. 16, 163.
Chance, B., and Hollunger, G. (1960), Nature 185, 666.
Conover, T. E., Prairie, R. L., and Racker, E. (1963), J. Biol. Chem. (submitted for publication).
Danielson, L., and Ernster, L. (1963), Biochem. Biophys. Res. Commun. 10, 91.
Ernster, L. (1963), in "Symposium on Intracellular Respiration," Proc. Vth Intern. Congr. Biochem. Moscow 1961, Pergamon Press, Oxford, 5, 115.
Estabrook, R. W. (1962), Anal. Biochem. 4, 231.
Hommes, F. A. (1963), Biochim. Biophys. Acta (in press).
Klingenberg, M., Slenczka, W., and Ritt, E. (1959), Biochem. Z. 332, 47.
Krebs, H. A. (1961), Biochem. J. 80, 225.
Lehninger, A. L. (1960), Federation Proc. 19, 952.
Linnane, A. W., and Titchener, E. B. (1960), Biochim. Biophys. Acta 39, 469.
Löw, H., Krueger, H., and Ziegler, D. M. (1961), Biochem. Biophys. Res. Commun. 5, 231.
Packer, L. (1962), J. Biol. Chem. 237, 1327.
Penefsky, H. S., Pullman, M. E., Datta, A., and Racker, E. (1960), J. Biol. Chem. 235, 3330.
Pinchot, G. B., and Hormanski, M. (1962), Proc. Nat. Acad. Sci. (Wash.) 48, 1970.
Pressman, B. C., and Lardy, H. A. (1956), Biochim. Biophys. Acta 21, 458.
Pullman, M. E., Penefsky, H. S., Datta, A., and Racker, E. (1960), J. Biol. Chem. 235, 3322.
Racker, E. (1962), Proc. Nat. Acad. Sci. (Wash.) 48, 1659.
Sanadi, D. R., Fluharty, A. L., and Andreoli, T. E. (1962), Biochem. Biophys. Res. Commun. 8, 200.
Smith, A. L., and Hansen, M. (1962), Biochem. Biophys. Res. Commun. 8, 136.
Snoswell, A. M. (1962), Biochim. Biophys. Acta 60, 143.
Webster, G. (1962), Biochem. Biophys. Res. Commun. 7, 245.