The Isolation of a Basic Coupling Factor from Preparations of Factor F₃

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A large number of mitochondrial protein fractions that increase the efficiency of energy conservation in depleted mitochondrial-membrane preparations have been described over the past decade (Vallejos, Van den Berg & Slater, 1968). Precise comparison of the properties of those coupling factors that do not have ATPase* activity is very difficult. However, there are sufficient similarities both in the methods of extraction of the coupling factors from mitochondria and in the functional properties of these heterogeneous preparations to suggest that they may contain a common active component, which is positively charged in the pH range 7–8.

We therefore decided to examine various preparations of coupling factors for the presence of a basic fraction that, in the presence of coupling factor F₁ (mitochondrial ATPase), would stimulate in A-particles (Lam, Warshaw & Sanadi, 1967) the ATP-driven reduction of NAD⁺ by succinate and both the aerobic and ATP-driven energy-linked nicotinamide nucleotide transhydrogenase reactions and also confer oligomycin-sensitivity on factor F₁ ATPase activity in the presence of TUA-particles (Bulos & Racker, 1968). The basicity of the protein fraction was established by its ability to adhere to CM-cellulose and the subsequent elution with 200mM-KCl–20mM-tris sulphate buffer, pH 8. These are among the properties of the oligomycin-sensitivity-conferring protein (MacLennan & Tzagoloff, 1968), but we believe that a title describing a physical property rather than a putative function is more apt. We have termed our extract the basic coupling factor. The results presented here show the isolation of basic coupling factor from preparations of factor F₃ (Fessenden & Racker, 1967).

Methods. Ox heart mitochondria were prepared by the method of Sanadi & Fluharty (1963) and stored frozen at –25° in 250mM-sucrose–10mM-tris sulphate buffer, pH 7.7. A mitochondrial extract was prepared by sonicating the mitochondria at pH 9.2. The particulate material was removed by centrifuging for 1 hr. at 105 000g. Factor F₃ was prepared from this extract by the method of Fessenden & Racker (1967). Protein was measured spectrophotometrically (Layne, 1957). A-particles were prepared by the method of Lam *et al.* (1967).

The coupling factors were assayed initially by the

* Abbreviation: ATPase, adenosine triphosphatase.

stimulation of the ATP-driven reduction of NAD⁺ by succinate in A-particles. A-particles (288 µg. of protein) and factor F₁ (5 µg. of protein) were added to a mixture containing 150 µmoles of tris sulphate, pH 8, 10 µmoles of MgSO₄, 15 µmoles of sodium succinate and 1.5 mg. of crystalline bovine plasma albumin. To this was added the coupling-factor preparation and the reaction mixture was incubated in a final volume of 0.5 ml. for 5 min. at 30°. Then 2.36 ml. of water, 0.6 µmole of NAD⁺ and 6 µmoles of KCN were added and incubation was continued for a further 2 min. The reaction was started by adding 6 µmoles of ATP. The total reaction volume was 3 ml. The change in extinction at 340 nm. was measured on a Unicam SP. 800 spectrophotometer. ATPase activity was measured by the spectrophotometric ATP-regenerating method of Pullman, Penefsky, Datta & Racker (1960) by using TUA-particles (210 µg. of protein) and factor F₁ (5 µg. of protein).

A unit of coupling-factor activity is defined as that amount which will enhance the rate of the ATP-driven reduction of NAD⁺ by succinate catalysed by A-particles by 1 µmole/min. over the rate obtained in the absence of the coupling factor. Specific activity is obtained by titrating A-particles with coupling factor and calculating the units/mg. of protein from the initial slope of the curve.

Results and discussion. In the experiment described here at each stage in the preparation of factor F₃ the behaviour of coupling-factor activity on CM-cellulose was examined.

(a) Preparation of factor F₃. Mitochondrial extract (containing 783 mg. of protein, specific activity 0.026 unit/mg., total units of coupling-factor activity 20.3) was put on to a column (8.9 cm. long × 2.5 cm. diam.) of DEAE-cellulose (Whatman DE 52) that had been equilibrated with 20mM-tris sulphate buffer, pH 8. The column was washed with 150 ml. of this buffer. This removed 204 mg. of protein with a specific activity of 0.011 unit/mg. (total units 2.2). The column was then eluted with 100mM-(NH₄)₂SO₄–20mM-tris sulphate buffer, pH 8. The coupling-factor activity was eluted with the protein band, which had a specific activity of 0.06 unit/mg. (total units 9.8).

A portion of this DEAE-cellulose eluate (103 mg. of protein) was fractionated with (NH₄)₂SO₄ as described by Fessenden & Racker (1967). The

Fig. 1. Chromatography on CM-cellulose of the basic coupling factor eluted from a DEAE-cellulose column. ○, Protein content; ●, coupling-factor activity.

0–33%-saturated- $(\text{NH}_4)_2\text{SO}_4$ fraction had a specific activity of 0.11 unit/mg.

(b) Behaviour of the factor F_3 fractions on CM-cellulose. Mitochondrial extract (containing 283 mg. of protein, specific activity 0.026 unit/mg., total units 7.45) was put on to a column (5 cm. long $\times$ 1.5 cm. diam.) of CM-cellulose (Whatman CM 52) that had been equilibrated with 2 mM-tris sulphate buffer, pH 8. The column was washed with 20 ml. of this buffer. Most of the protein (261 mg., specific activity 0.017 unit/mg., total units 4.4) was removed by this washing procedure. The column was then eluted successively with 20 ml. portions of 50 mM-, 100 mM- and 200 mM-KCl solutions in 20 mM-tris sulphate buffer, pH 8. The coupling-factor activity was eluted with 50 mM-KCl–20 mM-tris sulphate buffer (3.8 mg. of protein, specific activity 0.57 unit/mg., total units 2.18).

This procedure was repeated on the coupling factor eluted from DEAE-cellulose (61.6 mg. of protein, total units 3.7). Again much of the protein (56.9 mg., specific activity 0.02 unit/mg., total units 1.14) was not adsorbed by the column. Fig. 1 shows that with each concentration of KCl protein was eluted from the column. The basic coupling factor was eluted with 200 mM-KCl–20 mM-tris sulphate buffer, pH 8 (specific activity 0.4 unit/mg., total units 0.3).

When the behaviour of the 0–33%-saturated- $(\text{NH}_4)_2\text{SO}_4$ fraction on CM-cellulose was examined,

the basic-coupling-factor activity was eluted by 200 mM-KCl–20 mM-tris sulphate buffer, pH 8.

The basic coupling factor prepared from the mitochondrial extract that had been chromatographed first through DEAE-cellulose and then through CM-cellulose stimulated both the aerobic and the ATP-driven energy-linked nicotinamide nucleotide transhydrogenases with specific activities relative to the transhydrogenase of 0.88 and 1.0 respectively. A 2 μg . sample of oligomycin B, in the presence of 3 μg . of protein of this fraction, gave 33% inhibition of factor F_1 ATPase activity in the presence of TUA-particles.

We have also been able to isolate a basic-coupling-factor preparation from the mitochondrial extract by the acid precipitation method used by Bulos & Racker (1968) to isolate factor F_c from factor F_4 . This protein was eluted from CM-cellulose by 200 mM-KCl–20 mM-tris sulphate buffer, pH 8. This procedure raised the specific activity from 0.01 to 0.12 unit/mg.

Our interpretation of these results is that in the mitochondrial extract there are complexes of anionic proteins with the cationic basic-coupling-factor molecules. The ratio of the anionic to cationic proteins is changed on passing through DEAE-cellulose, such that the subsequent elution pattern from CM-cellulose is different from that obtained before DEAE-cellulose chromatography. Whether this complex of anionic and cationic

proteins is an artifact of isolation or is related to the situation in the mitochondrion is not known. Our most active fractions are heterogeneous, as shown by polyacrylamide-gel electrophoresis at pH 9.5 and 2.3.

However, these results show unequivocally that factor F_3 contains a basic coupling factor that has many functional properties in common with the oligomycin-sensitivity-conferring protein and behaves exactly like it on chromatography on CM-cellulose. In similar experiments we have also shown that factor F_4 (Conover, Prairie & Racker, 1963) and factor F_c (Bulos & Racker, 1968) also contain basic coupling factor.

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- Bulos, B. & Racker, E. (1968). *J. biol. Chem.* **243**, 3891.
Conover, T. E., Prairie, R. L. & Racker, E. (1963). *J. biol. Chem.* **238**, 2831.
Fessenden, J. & Racker, E. (1967). In *Methods in Enzymology*, vol. 10, p. 530. Ed. by Estabrook, R. W. & Pullman, M. E. New York: Academic Press Inc.
Lam, K. W., Warshaw, J. B. & Sanadi, D. R. (1967). *Arch. Biochem. Biophys.* **119**, 477.
Layne, E. (1957). In *Methods in Enzymology*, vol. 3, p. 451. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
MacLennan, D. H. & Tzagoloff, A. (1968). *Biochemistry*, **7**, 1603.
Pullman, M. E., Penefsky, H. S., Datta, A. & Racker, E. (1960). *J. biol. Chem.* **235**, 3322.
Sanadi, D. R. & Fluharty, A. L. (1963). *Biochemistry*, **2**, 523.
Vallejos, R. H., Van den Berg, S. G. & Slater, E. C. (1968). *Biochim. biophys. Acta*, **153**, 509.