Journal of Supramolecular Structure 8:111–117 (1978)

Purification and Characteristics of Hydrophobic Membrane Protein(s) Required for DCCD Sensitivity of ATPase in Mycobacterium Phlei

Natalie S. Cohen, Soon-Ho Lee, and Arnold F. Brodie

Department of Biochemistry, University of Southern California School of Medicine, Los Angeles, California 90033

The energy-transducing N,N′-dicyclohexylcarbodiimide-sensitive (DCCD-sensitive) ATPase complex consists of two parts, a soluble catalytic protein (F_1), and an intrinsic membrane protein (F_0). The bacterial coupling factor complex, BCF_0-BCF_1, has recently been purified from Mycobacterium phlei, and used to reconstitute oxidative phosphorylation in detergent-extracted membranes. The BCF_0 moiety has been purified by being recovered from the purified BCF_0-BCF_1 complex by affinity chromatography. BCF_0 is a lipoprotein or lipoprotein complex with an approximate molecular weight of 60,000. The preparation contained 0.15 mg of phospholipid per milligram protein. There appear to be three polypeptides, with approximate molecular weights of 24,000, 18,000, and 8,000 as determined by sodium dodecylsulfate acrylamide gel electrophoresis. Purified BCF_0 conferred DCCD sensitivity on a purified BCF_1 preparation. Reconstitution of oxidative phosphorylation was achieved after incubation of detergent-extracted membranes with purified BCF_0 and purified BCF_1.

Key Words: hydrophobic membrane proteins(s), DCCD-sensitive ATPase, oxidative phosphorylation, affinity chromatography

The membrane-bound adenosine triphosphatase (EC 3.6.1.3) from bacteria and mitochondria plays an essential role in energy transduction. This ATPase is actually a complex of an integral (hydrophobic) membrane protein and a peripheral (soluble) protein [1–7]. The soluble portion exhibits ATPase activity (F_1), while the hydrophobic

Abbreviations used: DCCD, N,N′-dicyclohexylcarbodiimide; BCF_1, bacterial coupling factor-latent ATPase from Mycobacterium phlei (equivalent to BCF_4 of previous publications); BCF_0-BCF_1 complex, DCCD-sensitive latent ATPase; BCF_0, intrinsic membrane portion of BCF_0-BCF_1 complex; ETP, electron transport particles; DETP, depleted ETP, ie, ETP with BCF_1 removed; TxETP or TxDETP, detergent-extracted ETP or DETP.

This paper was presented at the ICN-UCLA Symposium on Molecular Aspects of Membrane Transport held at Keystone, Colorado, March 13–18, 1977, but was inadvertently omitted from the published proceedings of that symposium.

Received April 15, 1977; accepted October 30, 1977.

0091-7419/78/0801-0111$01.70

protein (F_0) is presumably the membrane component across which protons can be translocated [3, 5, 8, 9]. The ATPase complex is inhibited by N,N'-dicyclohexylcarbodiimide (DCCD) [4–8]. The DCCD affects the F_0 portion of the complex; the F_1 alone is unaffected by the inhibitor.

The DCCD-sensitive complex has been purified from yeast [3], from a thermophilic bacterium [5], and from M phlei [6]. Recently we reported the reconstitution of oxidative phosphorylation by the purified complex from M phlei [6]. The F_1 portion of the complex has been purified from many sources [10–16] and is rather well characterized. Recently Sone et al have reported the purification of the F_0 protein [5]. Some of the several polypeptides of F_0, such as the DCCD-binding protein, the oligomycin sensitivity-conferring protein, and a binding protein have also been isolated and purified [17–22].

This communication describes the solubilization and purification of the F_0 moiety (BCF_0) from M phlei and the reconstitution of oxidative phosphorylation in detergent-extracted membranes with purified preparations of BCF_0 and BCF_1.

METHODS

Solubilization and Purification of BCF_0

M phlei (ATCC 354) was grown as previously described [23] and the electron transport particles (ETP) were prepared by sonication as described by Brodie [24]. The bacterial coupling factor-latent ATPase (BCF_1) was removed from ETP by washing with 0.25 M sucrose in the absence of cations [25]. These washed ETP are referred to as depleted ETP (DETP).

The DCCD-sensitive ATPase (BCF_0-BCF_1) was solubilized from ETP and the BCF_0 portion was solubilized from DETP by treatment of the membranes first with sodium cholate and then with Triton X-100 essentially according to the method of Sone and associates as previously described [5, 6]. The crude extract was then fractionated on a 0.7–1.0 M sucrose density gradient containing 0.15 M KCl [6]. The gradients were centrifuged in a Spinco SW 50.1 rotor at 175,000g for 16.5 h in a Spinco L-2 centrifuge.

The sucrose fraction containing the BCF_0-BCF_1 complex from ETP was applied to columns containing CH-Sepharose 4B (Pharmacia Fine Chemicals) bound to ADP. The BCF_1 and the BCF_0-BCF_1 complex have been purified by affinity chromatography on this medium [6, 16]. In the present investigation, the buffer employed was 50 mM Tris-acetate containing 0.25 M sucrose, 0.25% sodium cholate, and 0.15 M KCl, pH 8.0. The KCl was included in order to keep the BCF_0 and BCF_1 moieties bound together. After thorough washing of the column with this buffer, the BCF_0 was eluted with the same buffer lacking KCl. Under these conditions the BCF_1 remained on the column.

ANALYTICAL PROCEDURES

Latent ATPase activity was assayed by a procedure previously described [16]. When DCCD was used, the samples were incubated with the inhibitor (0.6 mM) for 10 min at 30° before trypsin treatment. Oxygen consumption was measured in a Gilson differential respirometer at 30°, and phosphorylation was estimated as previously described [23]. Protein was measured by Lowry's method [26] with BSA as a standard. Phospholipids were extracted by the method of Folch, Lees, and Stanley [27] and phospholipid phosphorus was assayed by King's method [28]. Disc gel electrophoresis was performed by

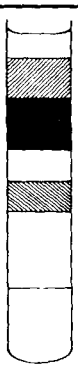
the method of Davis [29] with 5.5%, 6.5%, 7.0% and 7.5% gels (acrylamide: bis, 30:1). SDS gel electrophoresis was performed by the method of Laemmli [30], with 12.5% or 15% gels (acrylamide: bis, 30:1). Samples were prepared by boiling for 3 min in a solution containing 2% SDS, 1% β-mercaptoethanol, and 10% glycerol.

RESULTS

Solubilization and Purification

The solubilization procedure employed resulted in the release of many membrane-bound components in addition to the BCF_0-BCF_1 complex or BCF_0. This was especially so when DETP served as the starting material. A typical sucrose density gradient fractionation of the crude Triton X-100 extract from DETP is depicted in Figure 1. Striking color variations were noted along the length of the tube, and these formed the basis for the fractionation. The BCF_0-BCF_1 complex was found in fraction V when the extract was from ETP rather than from DETP [6]. The BCF_0 solubilized from DETP appeared in fraction III, along with the carrier protein for proline [31]. Also solubilized, and present in varying amounts in the different fractions, were large quantities of the cytochromes. Especially interesting was the separation of the two different cytochromes b [32] into fractions III and V. Preliminary results indicate that while succinate reduced some of the cytochrome b of all fractions, NADH was capable of reducing only the cytochrome b in fraction III. Much lower quantities of the membrane-bound cytochromes (b_{559}, b_{563}, and $a+a_3$) were solubilized from ETP than from DETP.

Although the reconstitution of DCCD sensitivity to a purified BCF_1 preparation and of oxidative phosphorylation to detergent-extracted membranes could be demonstrated

COLOR	GRADIENT	FRACTION	ACTIVITIES	TOTAL CYTOCHROMES (nmole)			
				b_{559}	b_{563}	$c+c_1$	$a+a_3$
Yellow-orange		VI		0.2	< 0.1	2.7	< 0.1
Green-yellow		V	[BCF_0-BCF_1]*	0.8	< 0.1	1.7	1.6
Pale yellow		IV		0.2	0.5	0.7	0.6
Dark yellow		III	BCF_0 Proline carrier	< 0.1	2.1	2.8	1.3
Clear		II		–†	–	–	–
Clear		I		–	–	–	–

*The BCF_0-BCF_1 complex was found in fraction V when the extract was made from ETP. This complex was not found in DETP, since the BCF_1 had already been removed from these membranes.
†No cytochromes were detectable in fractions I and II.

Fig 1. Components solubilized from DETP by detergent extraction. The detergent extraction and sucrose density gradient centrifugation were performed as described in Methods. Fractions were removed by puncturing the bottom of the tube. These data represent a typical sucrose density gradient fractionation of the crude detergent extract from DETP. When the extract was made from ETP instead, the quantities of cytochromes b_{559}, b_{563}, and $a+a_3$ removed were much lower, and the cytochrome c removed was all in fraction VI.

with the partially purified BCF_0 from DETP fraction III [6], attempts at further purification from this fraction were unsuccessful. For this reason, the BCF_0 was purified from the BCF_0-BCF_1 complex as outlined under Methods. It had already been established that the BCF_0-BCF_1 complex could be purified to homogeneity by affinity chromatography on Sepharose-ADP [6]. Therefore, after binding of the complex to the Sepharose-ADP, and thorough washing of the column, a fraction containing purified BCF_0 was eluted with the same buffer minus KCl.

Characterization

The hydrophobic nature of BCF_0 produces anomalous behavior on gel electrophoresis, as well as on gel filtration. Therefore, the molecular weight could not be established directly. The MW of BCF_0 has been estimated to be approximately 60,000 by subtracting the MW of BCF_1, which has been rigorously established at 404,000 [33] by polyacrylamide gel electrophoresis, from that of the BCF_0-BCF_1, which is approximately 460,000. SDS gel electrophoresis, using γ-globulin, glyceraldehyde-3-phosphate dehydrogenase, myoglobin, and cytochrome c as standards, provided polypeptide molecular weights of approximately 24,000, 18,000, and 8,000. BCF_0 is difficult to denature with SDS, and some apparently undenatured material remained, which ran at a MW of approximately 58,000. These values are therefore preliminary. The presence of phospholipid may also affect the polypeptide MW analysis. Purified BCF_0 contained 0.15 mg of phospholipid per milligram protein.

Reconstitution of DCCD Sensitivity

Purified BCF_0 was assayed by determining its ability to confer DCCD sensitivity on BCF_1, which in the solubilized state is not inhibited by DCCD (Table I). The addition of 15 μg of BCF_0 protein to 25 μg of BCF_1 resulted in a 22% inhibition of ATPase activity by DCCD. An inhibition of 33% was achieved with 30 μg of BCF_0, while 45 μg produced no further increase in the inhibition observed. The concentration of DCCD used, 0.6 mM,

TABLE I. Reconstitution of DCCD Sensitivity by Purified BCF_0

	ATPase activity (μmoles Pi)	% Inhibition by DCCD
BCF_1	0.74	
BCF_1 + DCCD	1.00	0
BCF_1 + 15 μg BCF_0	1.57	
BCF_1 + 15 μg BCF_0 + DCCD	1.22	22
BCF_1 + 30 μg BCF_0	1.65	
BCF_1 + 30 μg BCF_0 + DCCD	1.10	33
BCF_1 + 45 μg BCF_0	1.65	
BCF_1 + 45 μg BCF_0 + DCCD	1.15	30

Latent ATPase activity was measured after trypsin activation as described previously [16]. After BCF_0 was added, the reaction mixture, containing 50 mM Hepes, 4(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.5), 5 mM $MgCl_2$, and 25 μg of BCF_1 protein, was incubated 10 min at 30°. DCCD, dissolved in 60% methanol was then added (0.6 mM) and incubation was continued for 10 min more. Samples without DCCD received an equivalant amount of 60% methanol. The final methanol concentration was 6%. ATP was used at a concentration of 10 mM, and the final volume was 0.5 ml. The ATPase reaction was allowed to proceed for 15 min at 30°.

was found to be the optimal concentration for the inhibition of ATPase activity and oxidative phosphorylation in M phlei ETP [34]. The addition of sonicated soybean phospholipid did not increase either the ATPase activity or the DCCD inhibition.

Reconstitution of Oxidative Phosphorylation

Since the primary function of BCF_0 would appear to involve energy transduction, the reconstitution of oxidative phosphorylation in detergent-treated ETP (TxETP) was attempted with purified BCF_0 and BCF_1 preparation. Neither TxETP alone, TxETP plus BCF_0, nor TxETP plus BCF_1 showed significant levels of phosphorylation (Table II). Oxidative phosphorylation was reconstituted, however, by the addition of both BCF_0 and BCF_1 to TxETP, and this was inhibited by DCCD. The addition of larger amounts of BCF_0 produced a higher level of phosphorylation.

DISCUSSION

Previous work from this laboratory has employed the term BCF_4 for the bacterial coupling factor-latent ATPase [6, 16, 25, 33] from M phlei. The corresponding enzyme from mitochondrial and other bacterial systems has come to be denoted by the subscript 1. Therefore, in accordance with this accepted common usage, we have adopted this terminology. What has previously been called BCF_4 will be called BCF_1 in this and all future publications.

The study of the functional aspects of purified BCF_0 preparations should provide some insights as to the mechanism of energy transduction across the membrane. Some of the components of F_0 proteins from various sources have been isolated and purified. These include the DCCD-binding protein [17, 19, 22] and a protein responsible for the binding of F_1 to the membrane [20, 21]. It is not known whether all of the polypeptides are required for energy transduction, although there is some evidence that the DCCD-reactive protein alone can serve as a proton translocator [22, 35].

The objective of the studies described here was to purify the intact BCF_0 moiety, ascertain its functional capabilities, and begin a characterization of this essential membrane

TABLE II. Reconstitution of Oxidative Phosphorylation in Detergent-Extracted Membranes by Purified BCF_0

Preparation	P/O
TxETP	0.02
TxETP + 20 μg BCF_0	0.00
TxETP + BCF_1	0.04
TxETP + 16.5 μg BCF_0 + BCF_1	0.15
TxETP + 16.5 μg BCF_0 + BCF_1 + DCCD	0.03
TxETP + 28 μg BCF_0 + BCF_1	0.24

The reaction mixture contained detergent-extracted membranes (TxETP) (7.5 mg of protein), the stated amount of BCF_0, and 100 μg of BCF_1 where indicated. The final concentration of DCCD was 0.6 mM (methanol, 6%). Also present were 15 μmoles of $MgCl_2$, 100μmoles of Hepes-KOH buffer (pH 7.5), 50-μmoles of glucose, 10 μmoles inorganic phosphate, 6 mg of yeast hexokinase, 25 μmoles of KF, 10 μmoles of semicarbazide, 2.5 μmoles of ADP, 0.5 mg of yeast alcohol dehydrogenase, 40 μmoles of ethanol, and 1 μmole of NAD, in a final volume of 1.5 ml. The reaction was allowed to proceed for 30 min.

protein. The purified material has been shown to confer DCCD sensitivity on a BCF_1 preparation. Optimal DCCD inhibition (at 0.6 mM DCCD) was observed when BCF_0 and BCF_1 were present in approximately equivalent quantities, although complete inhibition did not occur. In contrast, the purified BCF_0-BCF_1 from M phlei was inhibited 84% by 0.6 mM DCCD [6]. It is possible that the separated components do not always recombine in the proper orientation. The functional capabilities of BCF_0 were also ascertained by the reconstitution of oxidative phosphorylation in detergent-extracted ETP by the purified BCF_0 plus BCF_1. As expected, this activity was inhibited by DCCD.

Attempts to characterize the purified BCF_0 have presented some problems due to the extreme hydrophobicity of the protein, necessitating the presence of detergent throughout all procedures. The MW of BCF_0 as such could not be established by polyacrylamide gel electrophoresis. Therefore, the MW was estimated from the difference between the MW of the BCF_0-BCF_1 complex ($\sim$ 460,000) and that of BCF_1, which has been well established at 404,000 [33] by statistical analysis of polyacrylamide gel electrophoresis. The BCF_0 MW is estimated to be 60,000 by this method. The MW of the F_0 from yeast mitochondria has been reported as approximately 100,000 [3], while one estimate of Sone et al [5] for the TF_0 from a thermophilic bacterium was about 170,000. Both of these values were derived from analysis of SDS gel electrophoresis bands of F_0-F_1 complex and based on previously calculated MW values for F_1. The reasons for these MW discrepancies are probably to be found in the hydrophobic nature of the protein, variations in conditions employed that might affect aggregation, and phospholipid and detergent contents. It should be emphasized that there is as yet no unequivocal evidence that the F_0 moiety is a single protein rather than a complex of proteins.

The polypeptide MW values reported here (24,000, 18,000, and 8,000) are somewhat higher than those reported Yoshida et al [8] for TF_0 (19,000, 13,500, and 5,400). The DCCD-binding protein from E coli has been assigned a molecular weight of 8,000–9,000 by Fillingame [19] and by Altendorf [22]. A similar MW has been reported for a protein involved in the binding of mitochondrial F_1 to the membrane [20, 21]. Peptides of 29,000 and 9,000 MW were found to be a part of the F_0 portion of the E coli DCCD-sensitive ATPase [36], while the oligomycin sensitivity conferring protein of mammalian mitochondria has a MW of 22,500 [21]. The M phlei BCF_0 was incompletely dissociated by boiling for 3 min in 2% SDS; it displayed a major band at 58,000. The presence of 8 M urea during the sample preparation or in the gels did not improve the dissociation. The difficulties of denaturing hydrophobic proteins with SDS have been discussed in a recent review by Tanford and Reynolds [37] in which it was noted that in some cases more SDS can be bound by the protein in the native state than in the denatured state. In such cases the protein will tend to remain in the native state. New techniques will be required to handle proteins of this nature for analytical purposes.

The purified BCF_0 preparation contained 0.15 mg of phospholipid per milligram protein. The further addition of phospholipid was not required for the reconstitution of DCCD sensitivity by BCF_0. The TF_0 of Sone et al [5], which contained virtually no phospholipid (< 0.005 mg/mg protein) did require phospholipid for activity.

The work described here represents our initial attempts to characterize the chemical and functional aspects of BCF_0, and work along these lines is continuing in our laboratory. It is hoped that a better understanding of the role of this component in bioenergetic mechanisms will result.

ACKNOWLEDGMENTS

The authors wish to acknowledge the technical assistance of Miss Esperanza Gonzalez. The work was supported by grants from the National Science Foundation (GB 32351, X 2), the National Institutes of Health (AI05637), and the Hastings Foundation of the University of Southern California School of Medicine.

REFERENCES

1. Kagawa Y, Racker E: J Biol Chem 241:2467, 1966.
2. Tzagoloff A, Byington KH, MacLennan D: J Biol Chem 243:2405, 1968.
3. Tzagoloff A, Meagher P: J Biol Chem 246:7328, 1971.
4. Ryrie I: J Supramol Struct 3:242, 1975.
5. Sone N, Yoshida M, Hirata H, Kagawa Y: J Biol Chem 250:7917, 1975.
6. Lee SH, Cohen NS, Brodie AF: Proc Natl Acad Sci USA 73:3050, 1976.
7. Soper JW, Pedersen PL: Biochemistry 15:2682, 1976.
8. Yoshida M, Sone N, Hirata H, Kagawa Y: J Biol Chem 250:7910, 1975.
9. Senior AE: Biochim Biophys Acta 301:249, 1973.
10. Catterall WA, Pedersen PL: J Biol Chem 246:4987, 1971.
11. Lambeth DO, Lardy HA: Eur J Biochem 22:355, 1971.
12. Schatz G, Penefsky HS, Racker E: J Biol Chem 242:2552, 1967.
13. Munoz E, Salton MRJ, Ng MH, Schor MT: Eur J Biochem 7:490, 1969.
14. Hanson RL, Kennedy EP: J Bacteriol 114:772, 1973.
15. Abrams A, Smith JB: In Boyer PD (ed): "The Enzymes." New York: Academic, 1974, vol 10, p 396.
16. Higashi T, Kalra VK, Lee SH, Bogin E, Brodie AF: J Biol Chem 250:6541, 1975.
17. Cattell KJ, Lindop CR, Knight LG, Beechey RB:Biochem J 125:169, 1971.
18. MacLennan DH, Tzagoloff A: Biochemistry 7:1603, 1968.
19. Fillingame RH: J Biol Chem 251:6630, 1976.
20. Kanner BI, Serrano R, Kandrach MA, Racker E: Biochem Biophys Res Commun 69:1050, 1976.
21. Russell LK, Kirkley SA, Kleyman RR, Chan SHP: Biochem Biophys Res Commun 73:434, 1976.
22. Altendorf K: FEBS Lett 73:271, 1977.
23. Brodie AF, Gray CT: J Biol Chem 219:853, 1956.
24. Brodie AF: J Biol Chem 234:398, 1959.
25. Higashi T, Bogin E, Brodie AF: J Biol Chem 244:500, 1969.
26. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ: J Biol Chem 193:265, 1951.
27. Folch J, Lees M, Stanley GHS: J Biol Chem 191:833, 1951.
28. King EJ: Biochem J 26:292, 1932.
29. Davis BJ: Ann NY Acad Sci 121: 404, 1964.
30. Laemmli VK: Nature (London) 227:680, 1970.
31. Lee SH, Cohen NS, Jacobs AJ, Brodie AF: J Supramol Struct Supplement 1:135, 1977.
32. Cohen NS, Brodie AF: J Bacteriol 123:162, 1975.
33. Lee SH Kalra VK, Ritz CJ, Brodie AF: J Biol Chem 252:1084, 1977.
34. Kalra VK, Brodie AF: Arch Biochem Biophys 147:653, 1971.
35. Patel L, Schuldiner S, Kaback HR: Proc Natl Acad Sci USA 72:3387, 1975.
36. Hare JF: Biochem Biophys Res Commun 66:1329, 1975.
37. Tanford C, Reynolds JA: Biochim Biophys Acta 457:133, 1976.