THE CONVERSION OF CARBON DIOXIDE TO ACETATE. III. DEMONSTRATION OF FERREDOXIN IN THE SYSTEM CONVERTING Co-$^{14}CH_3$-COBALAMIN TO ACETATE.

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The obligate anaerobe, Clostridium thermoaceticum, catalyzes the net synthesis of acetate from CO_2 according to the overall reaction (Barker and Kamen, 1945; Wood, 1952).

$$2\,CO_2 + 8\,H \dashrightarrow CH_3COOH + 2\,H_2O$$

Our earlier studies (Poston, et al., 1966) have shown that crude cell-free extracts of this organism are able to utilize the methyl group of Co-methyl cobalamin as a source of the methyl group of acetate. By chromatography on DEAE cellulose, two protein fractions were obtained from the crude bacterial extracts which, when recombined and supplemented with ferredoxin from another bacterial source, could catalyze the synthesis of acetate from Co-methyl cobalamin and CO_2. The obligatory requirement for ferredoxin by the fractionated enzyme system suggested that this non-heme iron protein might be a normal component of the acetate synthesizing system in untreated extracts. However, our efforts to demonstrate the presence of ferredoxin in extracts of C. thermoaceticum by the conventional procedures (Mortenson, 1964; Valentine, 1964; Lovenberg and Sobel, 1965) were unsuccessful. After equally unsuccessful efforts, Li, Ljungdahl, and Wood (1966) concluded that C. thermoaceticum probably does not contain ferredoxin.

It is the purpose of the present report to show that, with appropriate modifications of the conventional procedures, a protein fraction can be obtained from extracts of C. thermoaceticum that can replace ferredoxin in the reconstituted acetate synthesizing system of this organism and that also exhibits ferredoxin activity in the resolved hydrogenase system from Clostridium kluyveri. These results and the spectral characteristics of a partially purified preparation of the active material indicate that it is a member of the family of bacterial ferredoxins.

Materials and Methods --- C. thermoaceticum were grown and harvested as described previously (Poston, et al., 1966). Extracts were prepared as described with the ex-

ception that the streptomycin treatment was omitted. Dried cells of C. kluyveri were obtained by the method of Stadtman and Barker (1949) and the ferredoxin-dependent hydrogenase was prepared from these cells by the method of Fredricks and Stadtman (1965). Ferredoxin was prepared from cells of Clostridium pasteurianum after the method of Lovenberg and Sobel (1965). Clostridium acidi-urici ferredoxin was a gift from Dr. Jesse Rabinowitz. Co-methyl cobalamin was prepared by the method of Smith, et al. (1962). All reactions and operations involving cobalamin were performed in dim light under an atmosphere of helium. The assay system for ability to convert Co-$^{14}CH_3$-cobalamin to acetate has been described (Poston, et al., 1966).

Experimental and Results --- In a typical experiment, cell-free extract (1.9 g protein) of C. thermoaceticum was passed over a DEAE cellulose column (25 x 160 mm) equilibrated with 0.02 M potassium phosphate buffer, pH 6.6. The effluent (Fraction A), containing 1.0 g protein, was collected in 45 ml. The column was washed with 1 liter of 0.02 M pH 6.6 phosphate buffer and then with 500 ml 0.1 M buffer of the same pH. Elution of the column with 0.5 M pH 6.6 phosphate buffer (53 ml) removed a protein band (Fraction B) which contained 0.9 g protein. This elution was then followed by successive washes with 800 ml 0.5 M buffer, 500 ml 0.02 M buffer, and finally with 1.0 N NaCl in 0.02 M potassium phosphate buffer, pH 6.6. A brown band eluted from the top of the column with the NaCl was collected in 50 ml and the proteins in this solution were precipitated with $(NH_4)_2SO_4$ (0.9 sat.). The precipitate was taken up in 30 ml 0.02 M buffer and passed over a 19 x 47 mm DEAE cellulose column. This column was then washed successively with pH 6.6 potassium phosphate buffers as follows: 200 ml of 0.02 M, 100 ml of 0.5 M, and 100 ml of 0.02 M. Finally the brown protein band was eluted with 1.0 N NaCl in 0.02 M buffer. The protein precipitating from the eluate between 60 and 90% saturation with respect to $(NH_4)_2SO_4$ (Fraction C) was taken up in 2 ml 0.02 M buffer (3.5 mg protein per ml).

Fraction C was examined for its ability to restore activity to the reconstituted acetate synthesizing system, i.e., to supplement fraction A plus fraction B. It is evident from the data of Table I that fraction C can replace ferredoxin.

To see if fraction C exhibited ferredoxin activity in another reaction, the hydrogenase system of C. kluyveri which was shown to be ferredoxin dependent by Fredricks and Stadtman (1965) was utilized as an assay system. Among the various fractions obtained from C. thermoaceticum (Table II) it is evident that fraction C is the only one exhibiting significant ferredoxin activity. Fig. 1 shows the linear response of the hydrogenase system to increasing concentration of fraction C. The ability of fraction C to replace

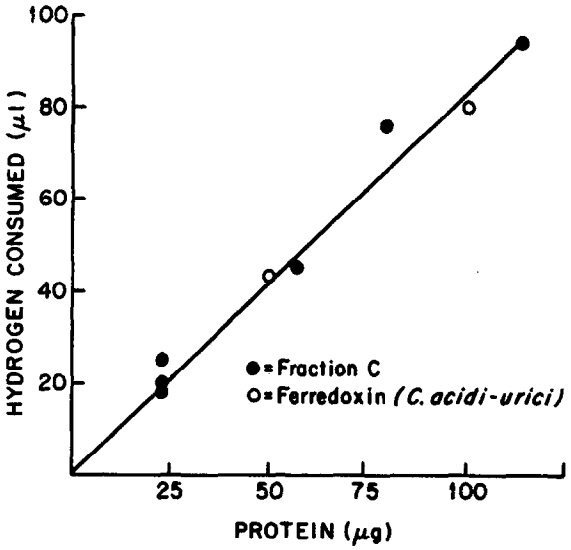

Fig. 1. Hydrogen consumption as a function of the addition of increasing amounts of fraction C and ferredoxin protein to the resolved hydrogenase system. Conditions of the assay are described in Table II.

ferredoxin in the hydrogenase system is further illustrated by the data of table III.

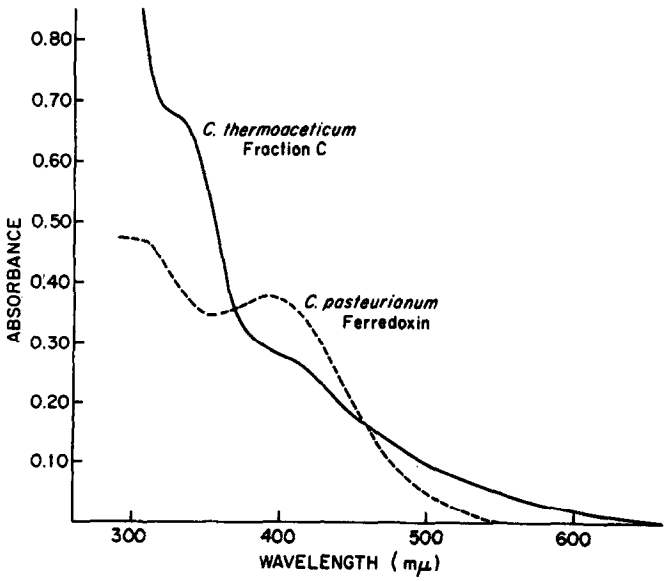

Fig. 2. Absorption spectrum of fraction C and authentic ferredoxin.

Table 1. Ability of Fraction C to replace ferredoxin in acetate synthesis

Expt.	Additions	Co-$^{14}CH_3$-cobalamin converted to acetate	
		With no ferredoxin added	With added ferredoxin
		mμmoles	mμmoles
1	None	7	-
	Crude extract	208	-
	Fraction A	10	-
	Fraction B	13	-
	Fraction C	13	-
	Fractions A + B	11	121
	Fractions A + B + C	93	-
2	None	7	3
	Crude extract	127	142
	Fractions A + B	13	81
	Fraction C	3	3
	Fractions A + B + C	169	157
3	None	11	-
	Crude extract	146	167
	Fraction A	7	18
	Fraction B	12	14
	Fraction C	8	4
	Fractions A + B	35	171
	Fractions A + C	19	-
	Fractions B + C	6	16
	Fractions A + B + C	110	172

The reaction mixture (1.0 ml) contained 100 μmoles triethanolamine buffer, pH 8.5, 1 μmole CoASH, 10 μmoles $MgCl_2$, 1 μmole $FeSO_4$, 10 μmoles $KHCO_3$, 1 μmole neutralized GSH, 30 μmoles Na pyruvate, and, where indicated, protein from crude extracts, 7.5 mg; fraction A, 4.5 mg; fraction B, 1.9 mg; fraction C, 0.6 mg; or ferredoxin, 0.1 mg. Co-$^{14}CH_3$-cobalamin was present in experiment 1. Co-$^{14}CH_3$-cobalamin was 0.465 μmoles (113400 cpm/μmole) in expts. 1 and 3 and 0.393 μmoles (127500 cpm/μmole) in expt. 2. Gas phase, helium; incubation temperature 57.5° C. for 60 minutes.

These results indicate the fraction C contains ferredoxin or a component with ferredoxin-like activity.

Disc gel electrophoresis of the various protein solutions obtained in the purification procedures revealed that fraction C is enriched in a rapidly migrating material that has the same mobility and color as does C. pasteurianum ferredoxin. The absorption spectrum of fraction C in which the rapidly migrating material represents about 50% of the protein (as estimated by visual inspection following disc gel electrophoresis) is shown in Fig. 2. This spectrum is similar to those of other clostridial ferredoxins of comparable purity.

Table II. Ability of Fraction C to replace ferredoxin in the C. kluyveri hydrogenase assay system

Additions	Hydrogen consumed	
	With no ferredoxin added	With added ferredoxin
	µliters	µliters
None	3	123
Crude extract	13	72
Fraction A	6	79
Fraction B	0	119
Fraction C	53	100

Incubations were carried out in small Warburg vessels (5 ml) under an atmosphere of hydrogen at 30° C for 300 minutes. The center well contained 0.2 ml 20% KOH. The 1.0 ml reaction mixture contained 60 µmoles TRIS buffer, pH 7.8, 5 µmoles DPN, 20 µmoles GSH, 20 µmoles Na pyruvate, 8 units lactate dehydrogenase 8 mg C. kluyveri hydrogenase, and, where indicated protein from C. thermoaceticum crude extracts, 5.6 mg; fraction A, 1.3 mg; fraction B, 0.5 mg; fraction C, 0.16 mg; or C. acidi-urici ferredoxin, 0.1 mg. The reaction was begun by tipping the hydrogenase from the side arm into the reaction mixture.

Table III. Effect of increasing ferredoxin concentration on the response of the C. kluyveri hydrogenase assay system to various levels of fraction C.

Fraction C. added	Hydrogen consumed, µliters			
	C. pasteurianum ferredoxin added			
	None	0.091 mg	0.182 mg	0.455 mg
mg protein				
None	10	41	66	102
0.085	25	--	--	--
0.425	54	84	94	109
0.850	77	--	106	107

Conditions of incubation were the same as those shown in Table II. Fraction C was dialyzed against 0.02 M potassium phosphate buffer (pH 6.6) for 4 hours to lower the $(NH_4)_2SO_4$ concentration.

Ferredoxin prepared from C. pasteurianum is eluted from DEAE cellulose with phosphate buffer at pH 6.5 or with Tris buffer at pH 8.0 at molarities greater than 0.3 (Mortenson, 1964). Attempts to elute the active component of fraction C either with 1 M potassium phosphate, pH 6.6, or with 1 M K_2HPO_4 were unsuccessful. A brown band could be eluted with dilute KOH but this had no activity. When 1 M NaCl was used, however, a ferredoxin with similar characteristics has been isolated from extracts of Methanosarcina barkeri (B. Blaylock, personal communication). It may be significant that the metabolism

of both C. thermoaceticum and M. barkeri involves the reduction of CO_2 to methyl derivatives (Blaylock and Stadtman, 1966) and that methyl cobalamin can serve as a source of methyl groups in both organisms.

In their studies of formate dehydrogenase in C. thermoaceticum Li, Ljungdahl, and Wood (1966) showed that there is no ferredoxin involvement in that system. Using techniques standard for the isolation of ferredoxin, they failed to find any protein with ferredoxin activity and concluded that either ferredoxin is not present or, if present, differs from those of C. pasteurianum or C. thermosaccharolyticum. It is now apparent that their second conclusion was correct since C. thermoaceticum ferredoxin differs from other ferredoxins in its binding to DEAE cellulose.

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