THE PARTICIPATION OF A FERREDOXIN OF CLOSTRIDIUM NIGRIFICANS IN SULFITE REDUCTION[1]

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Clostridium nigrificans, a thermophilic sulfate-reducing bacterium (Campbell et al., 1957), reduces inorganic sulfate to hydrogen sulfide by a dissimilatory pathway involving adenosine-5'-phosphosulfate as the active intermediate compound (Peck, 1959; Ishimoto and Fujimoto, 1959).

The electron transport system operating in this reductive process was not established for C. nigrificans. Previous attempts, in this laboratory, to isolate a ferredoxin from this organism were unsuccessful (Akagi, 1964). The failure to detect ferredoxin-activity was not due to the absence of the carrier but rather, was primarily due to the method of assay employed.

In this communication evidence for the presence of a ferredoxin in C. nigrificans and a brief description of some of its properties are presented.

Methods: C. nigrificans, strain 8351, was grown at 55 C in a medium containing yeast extract, 5.0 g; K_2SO_4, 1.5 g; $MgSO_4$ (anhydrous), 0.75 g; and sodium lactate, 6.0 g per 1 liter of distilled water. After sterilizing at 121 C for 20 minutes the medium was cooled to 55 C. Just prior to inoc-

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ulation, 1.0 ml of a 1.2 per cent solution of filter-sterilized $Fe(NH_4)_2(SO_4)_2$ was added to the medium. Because the organism is a strict anaerobe, it was grown in the presence of a potassium carbonate-pyrogallol seal.

The cultivation of the organism on a large-scale basis and the preparation of cell-free extracts were previously described (Akagi and Campbell, 1962).

The ferredoxins of Clostridium pasteurianum and Clostridium thermosaccharolyticum were isolated as previously described (Wilder et al., 1963). The ferredoxin and ferredoxin-free extract of C. nigrificans were obtained from a crude extract passed through a DEAE-cellulose column (chloride) by the method of Mortenson et al. (1962). The column was washed with a solution of 0.1 M KCl in 0.15 M Tris-HCl buffer, pH 7.0 until a clear eluate resulted. A dark amber-colored ferredoxin remained adsorbed at the top of the DEAE-cellulose column. This was eluted with a solution of 0.5 M KCl in Tris-HCl buffer, pH 7.0. The ferredoxin fraction was desalted by passing through sephadex, G-25 column and lyophilized.

Hydrogen evolution studies were conducted in a Gilson Respirometer (Gilson Medical Electronics). Acetyl phosphate was determined by the method of Lipmann and Tuttle (1945). Hydrogen sulfide was estimated by the method of Fogo and Popowski (1949). Protein was measured according to Lowry et al. (1951). Coenzyme A (Co A) was removed from the extracts by the procedure of Stadtman et al. (1951).

Results and Discussion: Acetyl phosphate formation from pyruvate by crude extracts of C. nigrificans was not observed to any great extent using the procedure of Mortenson et al. (1962). However, when a crude extract was centrifuged at 105,000 x g for 2 hours, the supernatant fraction (USS*) formed significant amounts of acetyl phosphate when sulfite was added to

*USS and DSS represents Untreated Spinco Supernate and DEAE-treated Spinco Supernate respectively.

the reaction mixture (Table 1). Passing the USS through a DEAE-cellulose column, to remove ferredoxin, yielded an extract (DSS) that had lost the ability to form acetyl phosphate; the activity was restored by the addition of ferredoxin. The results suggest that an inhibitor(s) is removed from crude extracts by high-speed centrifugation and also, that the ferredoxin is closely associated with a sulfite reducing system. To eliminate the possibility that sulfite was exerting a reducing effect, the addition of B-mercaptoethanol, glutathione or cysteine did not substitute for sulfite under identical conditions. Other inorganic sulfur compounds tested, in equimolar amounts, showed that metabisulfite was equally as effective as sulfite, thiosulfate was 50 per cent as effective and sulfate was inactive.

Table 1: Effect of Sulfite on Acetyl Phosphate Formation by Extracts of Clostridium nigrificans.

System	Acetyl Phosphate (umoles)
Crude extract	0.9
Crude extract + $SO_3^=$	1.4
USS	1.7
USS + $SO_3^=$	8.4
DSS	0.5
DSS + $SO_3^=$	1.8
DSS + ferredoxin	0.3
DSS + ferredoxin + $SO_3^=$	6.2

Reaction mixtures contained in umoles: K phosphate buffer, pH 6.0, 100; Na pyruvate, 50; Co A, 0.13; Na_2SO_3, where added, 10; ferredoxin, where added, 0.17 mg and extract in a total volume of 1.0 ml. Extract concentration in all mixtures was 3.0 mg. Mixtures were incubated at 55 C for 10 minutes and terminated by the addition of 1.0 ml of 2 M neutral hydroxylamine solution. Acetyl phosphate was determined as the hydroxamic acid.

The ferredoxin of C. nigrificans participated as an electron carrier in the pyruvic phosphoroclastic reaction catalyzed by a ferredoxin-free C. pasteurianum extract. Although it was not possible to demonstrate this reaction by the test tube method (Mortenson et al., 1962), under manometric

conditions the reaction proceeded smoothly. Table 2 shows a comparison of this activity with the ferredoxins of C. pasteurianum and C. thermosaccharolyticum. In addition, the formation of molecular hydrogen and acetyl phosphate was proportional to ferredoxin concentration (Fig. 1).

The absorption spectrum for this carrier is shown in Fig. 2. Unlike

Table 2: Comparison of Clostridium nigrificans Ferredoxin with the Ferredoxins of Clostridium pasteurianum and Clostridium thermosaccharolyticum.

Ferredoxin Added	H_2 Evolved (umoles)	Acetyl Phosphate (umoles)
None	0.4	0.8
C. pasteurianum, 0.13 mg	3.0	3.6
C. thermosaccharolyticum, 0.18 mg	4.9	6.0
C. nigrificans, 0.19 mg	2.4	2.9

Reaction mixtures contained in umoles: K phosphate buffer, pH 6.0, 100; Na pyruvate, 50; Co A, 0.13; ferredoxin, as indicated; and ferredoxin-free C. pasteurianum extract, 3.0 mg in a total volume of 1.1 ml. Center well contained 0.1 ml of 20 per cent KOH. Incubated at 30 C under a helium atmosphere for 15 minutes. Flasks were analyzed for acetyl phosphate after the final readings were taken.

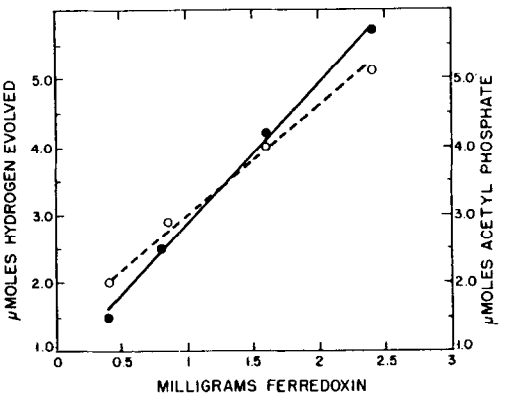

Fig. 1 Effect of ferredoxin concentration on hydrogen and acetyl phosphate production by a ferredoxin-free C. pasteurianum extract. Solid line denotes hydrogen evolved, broken line denotes acetyl phosphate formation.

The protocol for this experiment was identical to that described for Table 2.

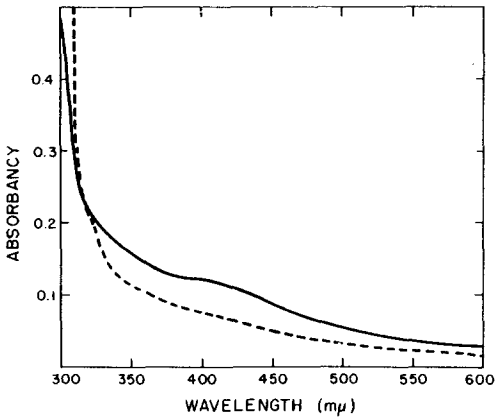

Fig. 2 Absorption spectrum of C. nigrificans ferredoxin. Solid line, oxidized form; broken line, reduced with dithionite.

Table 3: Participation of Ferredoxin in Sulfite Reduction by Cell-Free Extracts of Clostridium nigrificans.

System	uMoles H_2S Formed, Experiment Number				
	1	2	3	4	5
Complete	3.0	4.4	1.2	3.0	2.0
- pyruvate	0.0	0.0	0.0	0.0	0.0
- Co A	0.6	1.6	0.1	0.9	0.5
- ferredoxin	0.4	1.6	0.1	1.1	0.6
- extract	0.0	0.0	0.0	0.0	0.0

The complete mixture contained in umoles: K phosphate buffer, pH 6.0, 100; Na pyruvate, 50; Co A, 0.13; Na_2SO_3, 10; ferredoxin, 0.2 mg and extract (ranged from 10 to 12 mg) in a total volume of 1.1 ml. Center well contained 0.1 ml of 20 per cent $CdCl_2$ solution. After 30 minutes incubation under a helium atmosphere at 55 C, 0.1 ml of 20 N H_3PO_4 was tipped into the main compartment to release dissolved H_2S. The $CdCl_2$-impregnated filter papers were analyzed for H_2S after 10 additional minutes of incubation.

The extracts used in these experiments were passed through a DEAE-cellulose column, to remove ferredoxin, and subsequently treated with Dowex-1 resin to remove Co A.

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the ferredoxins from other bacterial systems a definite 390 mu peak was not observed. Instead, a small peak in the region of 390 to 400 mu was evident, which disappeared upon reduction with dithionite.

The ability of this ferredoxin to transport electrons between pyruvic dehydrogenase and the hydrogenase of C. nigrificans was extremely poor. In contrast, the coupling of the phosphoroclastic reaction to a sulfite-reductase system was interposed by this carrier (Table 3). Other known electron carriers tested were NAD, NADP, FMN and FAD. None of these, either alone or in various combinations, enhance the formation of hydrogen sulfide by the complete system shown in Table 3.

The data presented show that C. nigrificans contains a ferredoxin which appears to be closely associated with a sulfite-reductase system. The electrons released during pyruvate oxidation are transported by ferredoxin to furnish the reducing potential for sulfide formation. It was not possible to demonstrate an increase in sulfide production concomitant with an increase concentration of ferredoxin. Presumably other electron carriers, not detected during these studies, are participating together with ferredoxin in the reduction of sulfite to sulfide.

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