AN ELECTRON TRANSPORT FACTOR FROM CLOSTRIDIUM PASTEURIANUM

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An electron-transferring protein that links hydrogenase with a variety of electron donors or acceptors has been obtained from Clostridium pasteurianum. One of its functions is to couple pyruvate dehydrogenase with hydrogenase in the formation of H_2 from pyruvic acid by the clostridial phosphoroclastic reaction.

$$CH_3\overset{O}{\overset{\|}{C}}\text{-}COOH \xrightarrow[\text{enzymes}]{H_3PO_4} CH_3\overset{O}{\overset{\|}{C}}\text{-}OPO_3H_2 + CO_2 + H_2$$

The new factor, which at the present stage contains iron but no detectable heme or flavin, has been named "ferredoxin".

Experimental results by several investigators have pointed to the possibility of an unknown electron carrier in the pyruvate metabolism of saccharolytic clostridia. Thus, reaction rate was strongly increased by furacin in preparations of C. butyricum (Wolfe and O'Kane, 1953) and by methyl viologen in preparations of C. pasteurianum (Shug and Wilson, 1956). Also, C. butyricum preparations that had lost activity for pyruvate oxidation as a result of treatment with 2-propanol were reactivated by adding methyl viologen (Mortlock et al., 1959). Partially purified preparations of hydrogenase from C. butylicum did not catalyze H_2 formation in aqueous $Na_2S_2O_4$ unless methyl viologen was added (Peck and Gest, 1957). Natural electron carriers were not found to substitute for methyl viologen in these systems.

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In the course of investigating nitrogen fixation in cell extracts of C. pasteurianum, it was observed in this laboratory that a centrifugal supernatant fraction from heated extracts stimulated the phosphoroclastic reaction. The effect was in excess of any stimulation by known coenzymes. Furthermore, this fraction substituted for methyl viologen in restoring pyruvate oxidation to preparations that had been subjected to a 2-propanol precipitation procedure (0-38%) adapted from that of Mortlock et al. (1959). Accordingly, the supernatant fraction contained a phosphoroclastic factor apparently serving in electron transport. This factor, ferredoxin, has been purified 60-fold from C. pasteurianum extracts by a three-step fractionation procedure employing DEAE-cellulose, ammonium sulfate, and 2-propanol as summarized in Table I.

An extract of 500 g. of dried cells of C. pasteurianum, strain W-5, was prepared as described previously (Carnahan, et al., 1960) with the exception that distilled H_2O was used in place of phosphate buffer to effect autolysis. All subsequent operations were performed at 4-5° without exclusion of air. The aqueous extract was applied to the top of a column of DEAE-cellulose (Peterson and Sober, 1958), measuring 7 cm. high and 14.3 cm. in cross-section. Prior to being packed into the column, the DEAE-cellulose was stored overnight in 2M phosphate buffer, pH 6.5, freed of fine particles by decantation, and after being packed in the column, was washed with about 10 volumes of distilled H_2O. The major portion of the protein (about 95%) representing the phosphoroclastic system minus ferredoxin passed directly through the column and was collected for use in assaying ferredoxin. A dark band containing ferredoxin remained adsorbed at the top of the column, which was washed with 10 volumes of distilled H_2O followed by 10 volumes of 0.05M phosphate buffer, pH 6.5, to remove unwanted proteins. The ferredoxin was eluted with 1M phosphate buffer, pH 6.5, and the solution freed from salts by dialysis against distilled H_2O. The dialyzed solution of ferredoxin was purified further by adding solid $(NH_4)_2SO_4$, and the fraction precipitating between 0.62 and 0.89 $(NH_4)_2SO_4$ saturation was collected by centrifugation, dissolved in 80 ml. H_2O, and dialyzed 24 hours against 4 l. H_2O. Cold 2-propanol (-20°) was added to a final concentration of 60% by volume, and after centrifugation the supernatant solution was dialyzed 12 hours

TABLE I

PURIFICATION OF FERREDOXIN

Fraction	mg. Total Protein	Specific* Activity	Total Activity (units)	% Recovery
Crude extract	41,000	2.05	84,000	100
DEAE-cellulose, 1M phosphate eluent	1,400	46.0	64,500	77
Ammonium sulfate fraction, 0.62-0.89 saturation	430	89.0	38,500	46
2-Propanol 60% supernatant solution dialyzed	310	120	37,000	44

* Specific activity is expressed as μmoles of acetyl phosphate formed per mg. of protein in 15 minutes in the standard assay; 1 unit of ferredoxin catalyzes formation of 1 μmole acetyl phosphate in 15 minutes in the standard assay.

Ferredoxin activity was assayed in terms of acetyl phosphate formation from pyruvate in the reconstituted phosphoroclastic system. Assays were conducted in test tubes without excluding air. Standard reaction mixture was: 10 mg. phosphoroclastic enzymes minus ferredoxin from procedure in text, 110 μmoles pyruvate, 0.13 μmoles coenzyme A, 50 μmoles potassium phosphate buffer, pH 6.5, up to 0.2 mg. ferredoxin fraction depending upon its specific activity (Figure 1), H_2O to 1 ml. Mixture was incubated 15 minutes at 30° and terminated by addition of 1 ml. 2M neutral NH_2OH. Acetyl phosphate was measured as acethydroxamic acid (Lipmann and Tuttle, 1945). The amount of ferredoxin fraction used was chosen to give an optical density of 0.1-0.3 (530 mμ) in a "Lumetron" colorimeter in the acetyl phosphate determination. The same batch of phosphoroclastic enzymes minus ferredoxin, dried and stored at -10° for stability, was used throughout.

against 4 l. H_2O and finally concentrated by dialysis against "Carbowax". This procedure resulted in the recovery of 310 mg. (44%) of ferredoxin with a total purification of 60-fold (Table I).

Neither the protein fraction issuing from the DEAE-cellulose column initially nor those eluted with 0.05M phosphate buffer or 1M phosphate buffer were alone capable of catalyzing pyruvate oxidation. However, combination of the initial protein fraction with the ferredoxin fraction (1M phosphate eluent, dialyzed) resulted in rapid oxidation of pyruvate as determined either colorimetrically by the production of acetyl phosphate (Figure 1) or manometrically by the evolution of H_2.

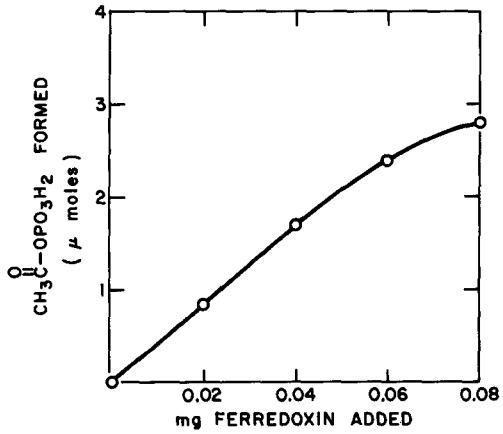

Figure 1. Catalysis of pyruvate oxidation by ferredoxin as measured by acetyl phosphate formation. Experimental conditions as described in Table I.

In addition to its role in pyruvate oxidation, ferredoxin participates in the reduction of NO_2^- to NH_3 with H_2 and in the evolution of H_2 from aqueous $Na_2S_2O_4$ (Figure 2) in preparations of C. pasteurianum. Methyl viologen could substitute for ferredoxin in the dithionite reaction but was less effective in nitrite reduction.

Ferredoxin is a relatively stable protein and does not lose significant activity during storage for several days at room temperature exposed to air. After 60-fold purification it is brown in color, contains about 0.5 μmole Fe/mg. protein, and has absorption maxima near 400 mμ and 280 mμ. Absorption spectra characteristic of a heme or flavin have not been observed. More detailed reports on the chemistry and biochemistry of ferredoxin and its occurrence in other organisms will be presented in subsequent publications.

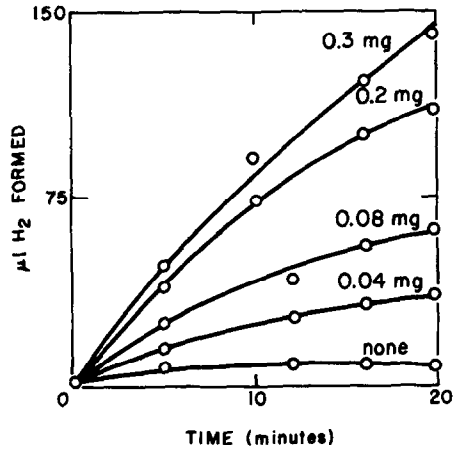

Figure 2. Catalysis of H_2 evolution from $Na_2S_2O_4$ by ferredoxin. H_2 was measured by standard manometric techniques (Umbreit, 1957). Reaction vessel contained 1 mg. phosphoroclastic enzymes minus ferredoxin (see text), 100 µmoles phosphate buffer, pH 6.5, 20 µmoles $Na_2S_2O_4$ in 1M phosphate buffer, pH 6.5, ferredoxin preparation as indicated, and H_2O to 3 ml. The center well contained 0.2 ml. 20% KOH. Argon was the gas phase. Temperature was 30°.

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