FERREDOXIN-DEPENDENT REACTIONS IN MICROCOCCUS LACTILYTICUS[1]

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Evidence had been presented earlier (Whiteley and Ordal, 1956) that an iron-containing protein functions as an electron carrier in the dehydrogenation of hypoxanthine to xanthine and hydrogen by extracts of Micrococcus lactilyticus. An iron-containing protein, named ferredoxin, has recently been isolated and purified from Clostridium pasteurianum (Mortenson et al., 1962) and M. lactilyticus (Valentine et al., 1962) and has been found to participate in several hydrogenase-coupled reactions. The evolution of hydrogen from hypoxanthine by M. lactilyticus, the hydrogenase-linked reduction of nitrite and hydroxylamine by C. pasteurianum, and the production of hydrogen from pyruvate and dithionite by both anaerobes have been shown to require ferredoxin (Mortenson et al., 1962; Valentine et al., 1962). Ferredoxin also mediates the photoevolution of hydrogen by Chromatium and the photosynthetic reduction of TPN by chloroplasts (Tagawa and Arnon, 1962); ferredoxin isolated from the latter source differs slightly in properties from the bacterial ferredoxin.

Extracts of M. lactilyticus catalyze the evolution of hydrogen from several organic compounds and are able to utilize hydrogen to reduce a variety of dyes, inorganic anions, organic acids, purines and coenzymes. These reactions have now been examined with respect to ferredoxin dependency.

Methods: The evolution or utilization of hydrogen was measured by conventional manometric techniques before and after extracts were depleted of ferredoxin by passage through a column of DEAE-cellulose following the procedure of Valentine et al. (1962) and Mortenson et al. (1962). The preparation

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of extracts and conditions used for the reduction of pyruvate have been described previously (McCormick et al., 1962); the reduction of fumarate was measured under the same conditions. The reductions of dyes, vitamins, coenzymes and uric acid were assayed under the conditions used for uric acid reduction (Whiteley and Ordal, 1956) except that the concentration of phosphate buffer was increased to 0.22 M and the reducing agent was omitted. The reduction of cyanocobalamin (vitamin B_{12}) was measured at pH 8.0. Reactions with inorganic anions were determined according to Woolfolk and Whiteley (1962). The oxidative decarboxylation of pyruvate to carbon dioxide, hydrogen and acetyl phosphate was measured in the presence of coenzyme A, thiamin pyrophosphate and Co^{++} (Whiteley and McCormick, 1962).

Ferredoxin was isolated and purified from extracts of M. lactilyticus by gradient elution on a column of DEAE-cellulose as described by Valentine et al. (1962) and Mortenson et al. (1962). These preparations were dialyzed and diluted to a known absorbancy at 385 mμ; one to two times the amount of ferredoxin originally present in the untreated extracts was added to the treated extract.

Cobalamins resulting from the reduction of cyanocobalamin were separated by extraction with phenol and ether (Veer et al., 1950) and their spectra determined with a Beckman DK-2 recording spectrophotometer. Cyanide was measured by the method of Fisher and Brown (1952). Aquocobalamin was prepared by photolysis of cyanocobalamin at an acid pH. We wish to thank Dr. D. Perlman and Dr. G. Scrimgeour for samples of hydroxocobalamin and aquocobalamin, respectively.

Results and Discussion: Of the dyes tested, only the reduction of methylene blue was affected by removal of ferredoxin from the extract (Table 1). The rate of this reaction was restored to the level of the untreated extract by addition of ferredoxin. The hydrogenase-coupled reduction of pyruvate to lactate (McCormick et al., 1962), of fumarate to succinate (Peck et al., 1957) and of uric acid to hypoxanthine (Whiteley and Ordal, 1956) was decreased significantly by the removal of ferredoxin. Addition of the carrier restored the pyruvate and uric acid reductions to the original level but was not as

effective a carrier for these reactions as trace amounts of methyl viologen. Contrary to the results reported by Valentine et al. (1962), ferredoxin was found to stimulate the reduction of fumarate and could replace methyl viologen at higher concentrations (i.e., at three to four times the level found in untreated extracts).

Table 1: Effect of Ferredoxin Depletion on the Reduction of Dyes and Organic Acids and on the Evolution of Hydrogen from Pyruvate.

Substrate	Product	Untreated extract	DEAE-treated extract		
			No addition	+ MV*	+ Ferredoxin
Benzyl viologen	**	920***	920	---	930
Methyl viologen	**	43.5	46.6	---	46.6
Saffranin	**	56.1	59.5	---	57.5
Methylene blue	**	60.0	22.9	---	57.4
Pyruvate	Lactate	2.12	0.0	37.3	2.09
Fumarate	Succinate	3.01	1.03	50.1	8.50
Uric acid	Hypoxanthine	3.94	0.0	17.6	3.81
Pyruvate	CO_2, H_2, acetyl~P	30.4	6.90	25.6	37.1

* = 2 x 10^{-4} M final concentration; ** = reduced dye; *** = specific activity = μmoles H_2 x 10^3/min/mg extract protein; MV = methyl viologen.

As previously reported (Valentine et al., 1962; Mortenson et al., 1962; Whiteley and McCormick, 1962), the rate of evolution of hydrogen from pyruvate, and also from α-ketobutyrate and α-ketoglutarate, was decreased if ferredoxin was removed from the extract and restored by the subsequent addition of this carrier (Table 1). The rate of evolution (as well as the rates of reduction in the ferredoxin-dependent reactions shown in Tables 1-3) was directly proportional to the amount of ferredoxin added and the activity of untreated extracts was also stimulated markedly by ferredoxin. As reported elsewhere (Whiteley and McCormick, 1962), the addition of ferredoxin stimulated the exchange of carbon dioxide into pyruvate and stabilized treated extracts to storage in the

cold with respect to their ability to carry out the oxidative decarboxylation of pyruvate. The effect of ferredoxin on the exchange of formate into pyruvate is the subject of a separate communication.

Flavins readily serve as substrates for the hydrogenase of M. lactilyticus (Table 2) and the reactions require ferredoxin. The reduction of TPN was also found to be ferredoxin-dependent although this coenzyme was reduced at a much lower rate than are the flavins. DPN did not serve as a substrate for reduction by hydrogenase whether ferredoxin was added or not. In the course of other experiments (Whiteley, unpublished), the reduction of cyanocobalamin was noted. This reaction was carried out at a low rate and did not appear to be linked to ferredoxin. The uptake of hydrogen by extracts in the presence of cyanocobalamin was accompanied by the release of cyanide and the appearance of hydroxocobalamin and trace amounts of aquocobalamin in the reaction mixture. This reaction appears to be identical to the reduction of cyanocobalamin under hydrogen with a platinum catalyst (Kaczka, et al., 1951).

Table 2: Effect of Ferredoxin Depletion on the Hydrogenase-Coupled Reduction of Vitamins and Coenzymes.

Substrate	Product	Untreated extract	DEAE-treated extract	
			No addition	+ Ferredoxin
Riboflavin	Reduced riboflavin	203*	63.0	215
FMN	$FMNH_2$	221	86.2	302
FAD	$FADH_2$	157	42.3	173
TPN	$TPNH^2$	6.85	0.0	6.35
Cyanocobalamin	Hydroxocobalamin + CN^-	1.71	1.50	0.89

* = Specific activity = μmoles H_2 x 10^3/min/mg extract protein.

As seen from Table 3, the reaction with nitroprusside was independent of ferredoxin, as were the reductions of benzyl and methyl viologen, saffranin and cyanocobalamin. The reductions of other anions and 2,4-dinitrophenol, however, were decreased to varying degrees by treatment of the extract and restored by the subsequent addition of ferredoxin. Ferredoxin was either nearly as effec-

tive or more effective than methyl viologen in the reduction of sulfite to dithionite, selenite to selenium, tellurite to tellurium, vanadate to vanadyl, molybdate to molybdenum blue and arsenate to arsenite (Woolfolk and Whiteley, 1962).

Table 3: Effect of Ferredoxin Depletion on the Hydrogenase-Coupled Reduction of Inorganic Compounds and 2,4-Dinitrophenol.

Substrate	Product	Untreated extract	DEAE-treated extract		
			No addition	+ MV*	+ Ferredoxin
Nitroprusside	---	173**	168	161	169
Nitrate	Ammonia	0.83	0.56	22.2	1.0
Hydroxylamine	Ammonia	8.8	0.71	24.0	8.5
2,4-Dinitrophenol	2-Amino,4-nitrophenol	102	25.8	71.0	106
Sulfite	Dithionite	37.6	2.7	10.7	33.6
Selenite	Selenium	46.5	8.8	72.0	51.0
Tellurite	Tellurium	36.0	10.1	82.0	51.5
Vanadate	Vanadyl	132	10.9	48.0	139
Molybdate	Molybdenum blue	45.0	0.0	25.2	50.3
Arsenate	Arsenite	2.92	0.31	2.50	2.82

* = 2 x 10^{-4} M final concentration, ** = specific activity = μmoles H_2 x 10^3/min/mg extract protein; MV = methyl viologen.

It is of interest that methyl viologen was considerably more effective than ferredoxin in mediating the reduction of nitrate and hydroxylamine to ammonia, whereas ferredoxin was slightly better than the dye as an intermediate carrier in the reduction of 2,4-dinitrophenol to 2-amino,4-nitrophenol. Methyl viologen also mediated the reduction of pyruvate, fumarate and uric acid more effectively than ferredoxin (Table 1). The differences in effectiveness of the two carriers may be related to differences in structure or redox potential (E'_0 for ferredoxin = -0.418 V, Tagawa and Arnon [1962]; and E'_0 for methyl viologen = -0.440 V) or to the properties of the specific reductases which accept electrons from reduced ferredoxin. It is known that specific reductases (nitrate, nitrite and sulfite reductases, lactic, succinic and xanthine dehydrogenases, etc.) participate in certain of the reductions, but non-enzymatic reactions between reduced ferredoxin and certain of the anions are possible.

A non-enzymatic reaction between nitrite and reduced benzyl viologen or reduced cytochrome c_3 (E'_0 = -0.205 V), for example, has been shown (Senez and Pichinoty, 1958). The mechanisms of some of the ferredoxin-mediated reductions are now under investigation using purified hydrogenase.

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