THE ISOLATION OF A NEW, LOW-POTENTIAL, IRON-CONTAINING ELECTRON-TRANSFER PROTEIN FROM RUMEN BACTERIA.

Roy S. Bush and Frank D. Sauer
Animal Research Institute, Agriculture Canada
Ottawa, Ontario, Canada, K1A 0C6

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SUMMARY: A new low-potential iron-containing electron-transfer protein has been isolated from cell free extracts of rumen bacteria. This protein, like some bacterial ferredoxins, can 1) transfer electrons from illuminated spinach chloroplasts to NADP; 2) catalyze ferredoxin dependent reductive carboxylation reactions; 3) act as electron acceptor in the pyruvate lyase catalyzed reaction in which pyruvate is oxidized to acetyl-CoA and CO_2. Unlike ferredoxin, this low potential iron protein contains no acid-labile sulfide; contains tightly bound iron that is released only after prolonged hydrolysis with 6 N HCl at 105°C and with an optical spectrum different from that associated with the bacterial ferredoxins.

INTRODUCTION: Cell free extracts of some anaerobic bacteria carboxylate acetate to pyruvate in the presence of a spinach chloroplast photoreducing system, an electron donor, a strongly electronegative electron carrier such as ferredoxin, and an ATP generating system (1, 2). The ability to carboxylate acetate is lost if the cell free extract is passed over DEAE cellulose but full activity can be restored by the addition of either bacterial or plant ferredoxins (1). We have shown that rumen bacteria can synthesize 2-oxo-acids by reductive carboxylation of the appropriate precursor acid by a similar mechanism (3, 4).

This report deals with the isolation of a non-ferredoxin, low potential iron protein (LPIP) from cell free extracts of rumen microorganisms which can replace ferredoxin in the pyruvate synthase assay or in the pyruvate lyase assay (Pyruvate: ferredoxin oxidoreductase, CoA acetylating, EC 1.2.7.1), but lacks the acid-labile sulfide and iron that is characteristic of the ferredoxins (5).

MATERIALS AND METHODS: Rumen bacteria were collected from fistulated Holstein cows, sedimented and washed as described previously (3). Bacterial pellets (50 g) were suspended in 150 ml of 50 mM potassium phosphate buffer

Abbreviations: LPIP, low-potential iron protein, DPIP, dichlorophenolindophenol.

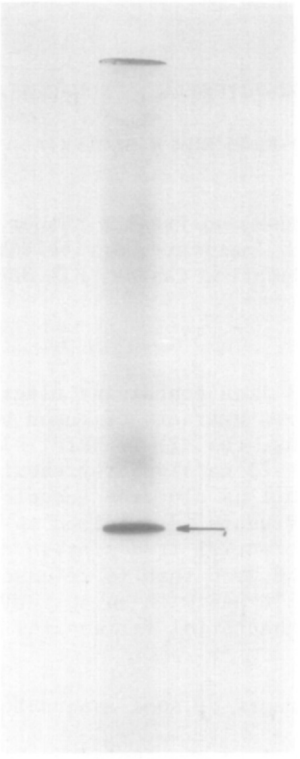

Fig. 1. Electrophoresis of LPIP in 15% polyacrylamide gel. Electrophoresis was continued for 1.5 h at 4 mA in 0.38 M Tris-glycine buffer, pH 8.9 (4). Gels were stained with 1% (w/v) Amido Black in 7% (v/v) acetic acid. Arrow points to LPIP band.

(pH 7.5) containing 50 mM 2-mercaptoethanol and disrupted in a Ribi Cell Fractionator (Model R7-1) Norwalk, Conn., at 27,000 p.s.i. while cooled to 2-4°C. The suspension was centrifuged first at 27,000 g for 20 min to remove heavier particles, then at 166,000 g for 1 h in a Spinco Ultracentrifuge (Model L-2 65B). The supernatant contained LPIP, pyruvate lyase and pyruvate synthase. LPIP was adsorbed on a DEAE cellulose column (2.4 x 4.5 cm) prepared as described by Mortenson et al. (6), and washed with 10 column vol. of 0.01 M Tris buffer (pH 7.5). The active protein was eluted with a linear gradient of Tris buffer, pH 8.0 (500 ml, 0.1 to 0.7 M) and collected in 10 ml fractions. The active fractions were pooled. The pooled sample was diluted with 2 vol. of water and concentrated by passage through a DEAE cellulose column (1.0 x 7.0 cm) and eluted with 0.7 M Tris buffer, pH 8.0. LPIP was used either directly or purified further by passage through Sephadex G-75. LPIP showed as a single major band after electrophoresis in 15% polyacrylamide gel at pH 8.9 (Fig. 1). Traces of contaminating protein, present as two diffuse bands, were not removed by this procedure. Throughout the purification procedure, fractions were tested for LPIP activity in the pyruvate lyase assay described in Fig. 2.

Ferredoxin was isolated from Clostridium pasteurianum grown in 20 L flasks as described in Carnahan and Castle (7). Ferredoxin was isolated essentially as described by Buchanan et al. (8) to step 5 in their purification procedure and had O.D. 390/280 ratios of approximately 0.40.

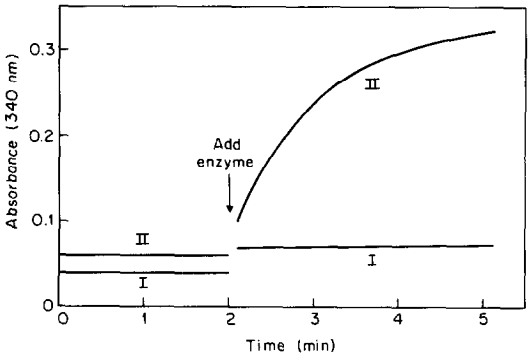

Fig. 2. Acetyl-CoA formation from pyruvate by pyruvate lyase in the presence and absence of a suitable electron acceptor. Each cuvette contained the following (mM) in a final vol. of 1.0 ml: Tris buffer pH 8.0, 50; coenzyme A, 0.18; 2-mercaptoethanol, 25; sodium malate, 7.5; sodium pyruvate, 5; NAD, 0.38; malate dehydrogenase, 15 µg; citrate synthase, 15 µg; LPIP as indicated. After 2 min, the reaction was started by the addition of 100 µg of ferredoxin-free (6) pyruvate lyase isolated from C. pasteurianum. Curve I, reaction in the absence of LPIP; Curve II, reaction with 70 µg LPIP.

The iron content of LPIP or ferredoxin was measured by the o-phenanthroline method, exactly as described by Lovenberg et al. (9). Alternately the samples were deproteinized at 80°C in 0.1 N HCl (or hydrolyzed as indicated) and the free iron measured by atomic absorption spectrophotometry.

Acid-labile sulfide was measured exactly as described by Brumby et al. (10). Protein was measured by the Lowry phenol reagent method (11) with crystalline bovine serum albumin as standard. No correction factor was used for the protein determination of ferredoxin (9).

RESULTS AND DISCUSSION: The reductive carboxylation of acetate to pyruvate requires, in addition to an acetate-activating system and the appropriate enzymes, an electron donor and an electron carrier of low oxidation-reduction potential. The reducing system commonly used consists of DPIP, illuminated spinach chloroplasts and any one of a number of ferredoxins. The requirement is not specific for ferredoxin which can be replaced by methyl viologen (E_o' = -0.432 V at pH 7) (2). LPIP, which, when photoreduced by illuminated spinach chloroplasts drives the reductive carboxylation of acetate to pyruvate (Table 1), therefore must have a low oxidation reduction potential.

LPIP replaces ferredoxin in the pyruvate synthase assay (Table 1) which measures the reductive carboxylation of acetate to pyruvate. The system is completely dependent on enzyme and illuminated chloroplasts but retains some activity in the absence of added LPIP or ferredoxin because DEAE cellulose

Table 1. The requirement of reduced ferredoxins or LPIP in the synthesis of pyruvate from [1-^{14}C]acetate and CO_2.

Incubation system	Pyruvate hydrazone (dpm)
Complete + *C. pasteurianum* ferredoxin	53,070
Complete + Spinach ferredoxin*	62,060
Complete + LPIP	25,020
Complete - ferredoxin - LPIP	3,660
- chloroplasts + LPIP	290
- enzyme + LPIP	200

The incubation medium, assay procedure and hydrazone isolation were done exactly as described previously (4). The pyruvate synthase activity was from cell-free rumen bacterial extract freed of ferredoxin by passage through DEAE cellulose (6) immediately before use. The reaction mixture contained 1 mg of this protein. Other additions: *C. pasteurianum* ferredoxin, 170 µg; Spinach ferredoxin, 140 µg; LPIP, 170 µg. Each incubation contained 10 µmoles of [1-^{14}C]acetate (2.92 x 10^5 d.p.m. per µmole) in a final volume of 3.0 ml.

* Spinach ferredoxin, Type III was a commercial product obtained from Sigma Chemical Co. and used without further purification.

chromatography does not completely remove the endogenous ferredoxin.

LPIP substitutes for ferredoxin in the reduction of NADP (E_o' = -0.320 V at pH 7) by illuminated spinach chloroplasts. LPIP was less active than *C. pasteurianum* ferredoxin but the rate of NADP reduction was dependent on, and responded linearly to, increasing LPIP concentrations (results not shown).

Ferredoxin or FAD serve as electron acceptors in the pyruvate lyase assay which measures the cleavage of pyruvate to acetyl-CoA and CO_2 (4, 12). LPIP is also an electron acceptor in this assay (Fig. 1). In this assay, there is no NAD reduction in the absence of a suitable electron acceptor. The assay described in Fig. 1 was routinely used in the purification of LPIP from the crude, cell free extract.

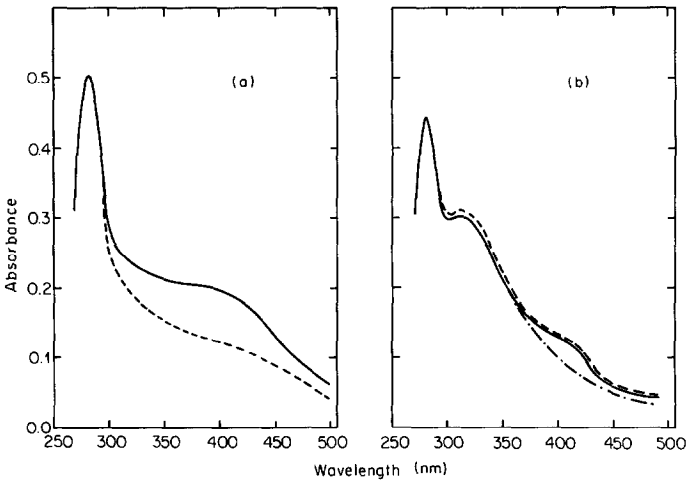

Fig. 3a. C. pasteurianum ferredoxin before acidification(——), after acidification to pH 1.5 for 15 min then readjusting with 1N alkali to pH 7.5 (---).

Fig. 3b. LPIP before acidification (——) at acid pH (—·—) after readjusting to pH 7.5 (---).

Table 2. Iron and acid-labile sulfide content of ferredoxin and LPIP.

Sample	Iron * (μmoles/mg protein)	Acid-labile sulfide
Spinach ferredoxin	0.18	0.29
C. pasteurianum ferredoxin	0.53	0.47
LPIP	0.06	0.06
LPIP hydrolyzed in 6 N HCl for 24 h at 105°C	0.37	----

* Iron measurements were done by both the o-phenanthroline technique and by atomic absorption spectrophotometry. Values obtained by the two methods were in excellent agreement.

Clostridial ferredoxins, when acidified, evolve H_2S and lose acid-labile sulfide and iron (13). This results in a decrease in absorbancy at 390 nm (Fig. 3a) with an irreversible loss of the shoulder characteristic of the

spectra for bacterial ferredoxins. By contrast, the peak at 412 nm observed with LPIP (Fig. 3b) disappears at acid pH but reappears on neutralization. This shows that, unlike the ferredoxins, no irreversible spectral alterations are noted on acidification of LPIP.

The same result is expressed quantitatively in Table 2. Mild acid treatment (0.12 N HCl for 10 min at 80°C) releases the ferredoxin iron when measured by either atomic absorption spectrophotometry or by the o-phenanthroline method. The iron of LPIP cannot be measured by either method unless LPIP is first hydrolyzed 24 h in 6 N HCl at 105°C. No significant amounts of acid-labile sulfide were detected in LPIP (Table 2).

In summary, LPIP has the electron transferring properties generally associated with the bacterial and plant ferredoxins. LPIP differs from ferredoxin in its optical spectrum, absence of acid-labile sulfide and with tightly bound iron that requires acid hydrolysis of the protein for release. Studies on EPR spectra and the nature of iron binding in LPIP are currently in progress.

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