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Formate Dehydrogenase from *Clostridium acidiurici*

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Partial purification of formate dehydrogenase from *Clostridium acidiurici* has been accomplished, and some properties of the enzyme have been determined. The molecular weight of the protein is at least 200,000 daltons. The enzyme showed marked instability to freezing and thawing and was inhibited strongly by oxygen and by light. Such inhibition was not reversed by incubation in the presence of thiol compounds. Cyanide inhibited the enzyme 90% at 0.1 mM concentrations, but ethylenediaminetetraacetate produced only slight inhibition at concentrations as high as 50 mM. The purified enzyme showed no ferredoxin activity in the *Clostridium pasteurianum* clastic system during pyruvate oxidation. Crude preparations of the enzyme could be coupled through ferredoxin to the reduction of nicotinamide adenine dinucleotide during formate oxidation, but the purified enzyme could not catalyze the reduction of pyridine nucleotides by formate in the presence of ferredoxin. Formate oxidation with the purified enzyme was readily coupled to benzyl viologen reduction, in which case ferredoxin was not required. An exchange between formate and bicarbonate was catalyzed by both crude and purified preparations of the enzyme, but the net synthesis of formate from CO_2 was not accomplished.

Formate dehydrogenase is widely distributed throughout nature in bacteria, plants, and animals. The enzyme may be associated with the soluble cytoplasmic fraction of some bacterial and plant cells, whereas in others the enzyme is bound to the mitochondria or cell membranes of the insoluble particulate fractions of these cells.

Extracts from pea and bean seeds have yielded soluble formate dehydrogenases which may be linked to nicotinamide adenine dinucleotide (NAD) (6, 16). Similar enzymes have also been isolated from the mitochondria and particulate preparations of pea epicotyls and tissues and leaves of higher plants (5, 17).

The oxidation of formate in rat liver tissue and the larvae of the black blowfly, *Phormia regina*, appears to be coupled with catalase activity (16, 33). Formate dehydrogenase activity has also been found in four species of trichomonads (14).

Formate dehydrogenase has been found in a wide variety of bacterial species. A soluble enzyme which is linked to the reduction of NAD has been isolated from two different *Pseudomonas* species grown on methanol (11). The enzyme is an integral part of the formate hydrogenlyase systems of *Escherichia coli* (20), *Rhodopseudomonas palustrus* (22), and *Enterobacter (Aerobacter) aerogenes* (12). The *E. coli* and *E. aerogenes* enzymes were found to be associated with the particulate fraction of the cell, whereas the enzyme from *R. palustrus* is a soluble protein. None of these three enzymes in soluble form reduced NAD, but they were effective in reducing several artificial electron acceptors such as the viologen dyes, ferricyanide, and dichloroindophenol.

Soluble formate dehydrogenase preparations have been obtained from aerobically and anaerobically grown *E. coli* by treating the particulate fractions of these cells with deoxycholate and snake venoms (10, 35). In these preparations the enzyme was found to be associated with cytochrome b_1.

The existence of formate dehydrogenase in strictly anaerobic bacteria has also been reported. In crude preparations of *Methanobacillus omelianskii* (1) and *Clostridium pasteurianum* (28, 31, 32), formate oxidation was shown to be coupled to NAD reduction by way of ferredoxin. A formate dehydrogenase specific for NADP during formate oxidation was not dependent on ferredoxin (13).

Clostridium acidiurici contains a very active formate dehydrogenase, but neither the role of

this enzyme in the fermentation of purines to acetate nor the physical and kinetic properties of the protein has been defined.

The purpose of this investigation was to purify the enzyme and examine its properties.

MATERIALS AND METHODS

Cultivation of C. acidiurici. Cells were grown in bottles containing 13 liters of medium as described by Sagers and Carter (26).

Cultivation of C. pasteurianum. *C. pasteurianum*, strain W-5, ATCC 6013 was grown in the medium described by Carnahan and Castle (3).

Preparation of cell-free extracts. Wet, packed cells of *C. acidiurici* or *C. pasteurianum* weighing 50 g were suspended in 150 ml of 0.05 M potassium phosphate, *p*H 7.1, containing 2 mM ethylenediaminetetraacetate (EDTA) and 4 mM mercaptoethanol. The cells were ruptured in batches of 20 ml of suspension by shaking with 25 g of 0.1-mm glass beads in a Bronwill MSK homogenizer for 2 min. The homogenate was first centrifuged at 16,000 × *g* for 10 min to remove large cell debris and glass beads and then subjected to a second centrifugation at 27,000 × *g* for 20 min. Where possible, extracts were kept under a nitrogen atmosphere during the purification procedures.

Preparation of "clastic enzymes" from C. pasteurianum. Ferredoxin-free "clastic enzymes" were prepared by the method described by Mortenson, Valentine, and Carnahan (18). A 22-g amount of frozen cells of *C. pasteurianum* suspended in 0.02 M potassium phosphate, *p*H 6.5, was ruptured as previously described. Centrifuged extracts were placed over a diethylaminoethyl (DEAE) cellulose column (1.5 by 8 cm) to remove ferredoxin. The ferredoxin-free enzymes were divided into 6-ml portions containing 15 mg of protein per ml and lyophilized. The lyophilized enzymes retained activity for several months and were dissolved in 3 ml of 0.05 M potassium phosphate buffer, *p*H 6.5, when needed.

Preparation of ferredoxin from C. acidiurici. Ferredoxin was prepared by the method of Buchanan, Lovenberg, and Rabinowitz (2). The purified material had a 280 to 390 nm absorbancy ratio of 0.76.

Assay for ferredoxin activity. Activity of the ferredoxin was assayed by measuring its stimulation of acetyl phosphate formation from pyruvate with the ferredoxin-free *C. pasteurianum* "clastic systems" as described above. The standard assay mixture (total volume, 1.0 ml) contained in μmoles: potassium phosphate buffer (*p*H 6.5), 50; coenzyme A (CoA), 1; sodium pyruvate, 50. DEAE cellulose-treated "clastic enzymes" from *C. pasteurianum* (7 mg) and ferredoxin from *C. acidiurici* were added as required. After a 10-min incubation at 37 C, 1 ml of neutralized hydroxylamine, prepared by the method of Lipmann and Tuttle (15), and 1 ml of 0.1 M acetate buffer, *p*H 5.4, was added to the mixture. After ten more min, 1 ml of 4 N HCl was added, and the mixture was centrifuged for 5 min at 3,000 rev/min in a clinical centrifuge to remove the precipitated proteins. The colored hydroxamic acid-ferric iron complex formed in the supernatant solution was measured at 540 nm in a Beckman DU spectrophotometer. Succinic anhydride was used as a standard for constructing a standard curve for acylhydroxamates (15).

Protein determinations. Protein content of extracts (except ferredoxin) was determined by the ratio of absorbancies at 280 and 260 nm (34). Ferredoxin was measured by the trichloroacetic acid precipitation method of Stadtman, Novelli, and Lipmann (29). Crystalline bovine serum albumin was used as a standard.

Spectrophotometry. Spectrophotometric determinations were made with a Beckman DU or Cary model 15 spectrophotometer.

Partial purification of formate dehydrogenase from C. acidiurici. Cell-free extracts (500 ml) containing 4.7 g of protein and 150,000 units of formate dehydrogenase activity were obtained from rupturing 50 g of cells as described above. Because of the instability of the enzyme at this stage, the extract was quickly treated with protamine to remove nucleic acid materials and then fractionated with ammonium sulfate.

Protamine treatment. Precipitation of nucleic acid material was accomplished by slowly adding 10 ml of freshly prepared 1% protamine sulfate (Sigma Chemical Co., St. Louis, Mo.) per 100 ml of the above extract. After being stirred for 20 min, the extract was centrifuged at 16,000 × *g* for 20 min, and the precipitate was discarded. The 280 to 260 nm ratio of the supernatant extract was 0.9 to 1.0. This supernatant solution (558 ml containing 4.1 g of protein and 140,000 units) contained 87% of the original protein and 95% of the original activity.

Ammonium sulfate precipitation. Precipitation of proteins by addition of ammonium sulfate was carried out according to conventional methods with solid ammonium sulfate. Precipitates were dissolved in a minimal volume of 0.05 M potassium phosphate, *p*H 7.0, containing 2 mM EDTA and 4 mM mercaptoethanol and then stored in an ice bath under nitrogen. The fraction precipitating between 0.50 and 0.55 saturation was recovered in 10.6 ml of solution and contained 254 mg of protein and 107,000 units. Although recovery of 98% of the units was possible in the ammonium sulfate fraction precipitating between 0.35 and 0.65 saturation, the 0.50 to 0.55 fraction consistently gave 60 to 70% recovery of units and a 10- to 12-fold purification of the enzyme over the crude extracts. This fraction was used for column chromatography.

Sephadex G-200 chromatography. Sephadex G-200 (10 to 40 μm bead size) was soaked in double distilled water for at least 3 days to allow for adequate swelling. Fine particles were removed by repeated washing and decantation until particles sedimented evenly. Excess water was removed on a Buchner funnel. The gel thus obtained was suspended at a ratio of two parts buffer to one part gel in 0.05 M potassium phosphate, *p*H 7.0, containing 50 mM EDTA. The gel suspension was heated to near boiling and then degassed under negative pressure. After cooling under a nitrogen atmosphere to 5 C, the gel was poured into a glass column, giving

final bed dimensions of 2.0 by 90 cm of gel. The gel was allowed to settle under a head pressure of not more than 10 cm of H_2O. The column was then equilibrated for 2 days with boiled and degassed 0.05 M potassium phosphate buffer, *p*H 7.0, containing 2 mM EDTA. The flow rate of the column was 12 ml/hr. A few hours before application of the protein sample, mercaptoethanol was added to the buffer to a concentration of 3 mM, and at least two void volumes of buffer were allowed to pass through the column. Protein was layered on the column above a stainless-steel (100 mesh) disc that had been placed on top of the column to prevent disturbances of the gel. Ten milliliters of the 0.50 to 0.55 fraction representing 102,000 units of activity was placed on the column and eluted in the equilibrating buffer in 5-ml fractions. Although large losses of total units were observed during gel filtration, a two- to threefold increase in specific activity was achieved by this step.

Measurement of formate dehydrogenase activity. Three methods were employed to measure enzyme activity: (a) benzyl viologen reduction by formate; (b) the exchange of ^{14}C-formate with bicarbonate; and (c) the reduction of NAD by formate. In method (a), the reduced benzyl viologen was measured at its absorption maximum of 555 nm. The assay was performed in Thunberg cuvettes kept anaerobic by alternatively evacuating and flushing with pure nitrogen which had been bubbled through a pyrogallol train. The standard reaction mixture (total volume, 3 ml) contained in μmoles: tris(hydroxymethyl)aminomethane (Tris) buffer (*p*H 8.0), 250; EDTA, 2.5; benzyl viologen, 25; $Na_2S \cdot 9H_2O$, 9; sodium formate, 200. Crystalline benzyl viologen was added to the buffer-EDTA solution, this mixture was boiled to remove oxygen and quickly cooled, and then sodium sulfide was added. This mixture was added to the main compartment of the cuvette. The formate solution was boiled, and 0.2 ml was added to the side arm. After addition of enzyme to the main compartment, the cuvettes were closed, quickly evacuated, and flushed with nitrogen. After a 3-min equilibration at 36 C, the contents of the cuvette were mixed together, and the rate of the dye reduction was measured in a Cary model 15 spectrophotometer. A nonformate blank was used as a control. One unit of activity was defined as the amount of protein required to produce an absorbancy change at 555 nm of 0.1 per min as benzyl viologen was reduced.

In method (b), the formate-bicarbonate exchange reaction $HCO_3^- + H^{14}COOH \rightleftharpoons H^{14}CO_3^- + HCOOH$ was measured by recovering radioactive $^{14}CO_2$ after the exchange with ^{14}C-formate had occurred and counting the $^{14}CO_2$ in a liquid scintillation counter. The standard reaction mixture (total volume, 1.0 ml) contained in μmoles: Tris-hydrochloride buffer (*p*H 8.0), 70; sodium ^{14}C-formate, 100; sodium bicarbonate, 100; dithiothreitol, 5; and enzyme. Reactions, carried out in test tubes (13 by 100 mm), were initiated by addition of either formate or enzyme. The tubes were flushed with nitrogen gas, stoppered, and incubated for 20 min at 37 C. At the end of the incubation period, a fluted filter paper (2.5 by 10 cm) saturated with 0.2 ml of 1 M hydroxide of hyamine was inserted into the neck of each reaction tube to absorb the CO_2. The reaction was stopped, and CO_2 was liberated from solution by addition of 0.5 ml of 5 N sulfuric acid inserted below the hyamine absorbent over a 30-min period. During this time the tubes were shaken frequently. (It had been determined experimentally that quantitative recovery from solution of 200 μmoles of CO_2 could be achieved within 20 min by this method.) The carbon dioxide-laden filter paper was then transferred to a scintillation vial containing 15 ml of fluid consisting of 0.5% 2,5-diphenyloxazole and 0.03% 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene in toluene. Scintillation solutes and hyamine solution were obtained from New England Nuclear Corp., Boston, Mass.

In method (c), NAD reduction by formate was coupled through formate dehydrogenase to the reduction of ferredoxin, thence NAD, as follows:

$$\text{formate} + \text{ferredoxin (ox)} \rightleftharpoons CO_2 + \text{ferredoxin (red)}$$

$$\text{ferredoxin (red)} + NAD^+ \rightleftharpoons NADH + \text{ferredoxin (ox)}$$

This reaction could be found in crude extracts but not in the purified fractions and was measured by the increase in absorption at 340 nm of NADH. The assay was performed the same way as the benzyl viologen assay except that 3 μmoles of NAD replaced benzyl viologen as the electron acceptor. The NAD was added directly to the main compartment of the cuvette and not subjected to boiling. NADH was found to be stable for at least 30 min at *p*H 8.0 under the conditions of these experiments.

RESULTS

A summary of the purification procedure is given in Table 1, and Fig. 1 shows the elution pattern of the proteins placed on the Sephadex G-200 column, the proteins having been obtained as the 50 to 55% ammonium sulfate fraction of the extract described earlier. Fractions 16 through 27 contained the formate dehydrogenase activity. Fractions 17, 18, 19, and 20 contained 11.4% of the total units, with a specific activity 30-fold greater than the cell-free extract. The formate dehydrogenase apparently is a large entity, having a molecular weight of 200,000 or greater, inasmuch as it was eluted in the first void volume. A sample of blue dextran placed on the column was eluted at the front of the first protein peak.

Stability to freezing, thawing, and storage. The purified formate dehydrogenase showed a marked instability towards storage under certain conditions and toward freezing and thawing. Increased ionic strengths (30% ammonium sulfate) and reducing agents (1% mercaptoethanol) did not protect the enzyme

during freezing and thawing. In fact, under such conditions 65% of the activity commonly was lost after a single freezing. Stability was observed for at least 3 weeks, however, if the enzyme was stored at 5 C in the dark in 0.05 M potassium phosphate buffer (*p*H 7) containing 1 mM EDTA, 5 mM mercaptoethanol, and 10% ammonium sulfate and was under a nitrogen atmosphere. Stability was enhanced if the protein concentration was at least 3 mg/ml.

Sensitivity to light. The enzyme was sensitive to light, as shown in Fig. 2. Two samples, each containing 8 mg of protein in 2 ml, were kept at 5 C in 0.05 M potassium phosphate, *p*H 7.1, containing 1 mM EDTA and 10% ammonium sulfate. Both fractions contained 5 mM mercaptoethanol and were flushed with nitrogen. The fraction exposed to light was contained in a clear Pyrex tube (13 by 100 mm) and placed 20 cm from a 6-watt fluorescent lamp. The other sample was contained in a completely blackened tube and placed also at a distance of 20 cm from the light. Samples were removed at the intervals shown and assayed for residual activity. After 48 hr, the fraction exposed to light retained only 12% of the initial activity, while the fraction kept in the dark showed 81% of its initial activity.

Sensitivity to oxygen. The enzyme was also sensitive to oxygen, as shown in Fig. 3. Three samples of enzyme, each containing 8 mg of protein in 2 ml, were kept at 5 C in 0.1 M potassium phosphate buffer, *p*H 7.1, containing 1 mM EDTA and 1 mM mercaptoethanol. These were placed in 10-ml Erlenmeyer flasks which had been blackened to prevent inactivation by light. Flask 1 was flushed with nitrogen and stoppered but not stirred. Flask 2 was flushed with nitrogen, stoppered and stirred slowly by use of a magnetic stirrer. Flask 3 was not flushed, but it was stoppered and stirred at the same rate as flask 2. After 16 hr, flasks 1 and 2 retained 80 and 65% of their initial activity, respectively, whereas flask 3 retained only 4% of its initial activity. When air was introduced to flasks 1 and 2 for 1 hr, each preparation lost 50% of the activity remaining at 16 hr. Attempts to recover lost activity by incubation in the presence of reducing agents and under a nitrogen atmosphere failed.

Amelioration of enzyme activity by reducing agents. Employment of benzyl viologen as the electron acceptor for formate oxidation required that the reaction vessels be entirely free of oxygen to prevent autooxida-

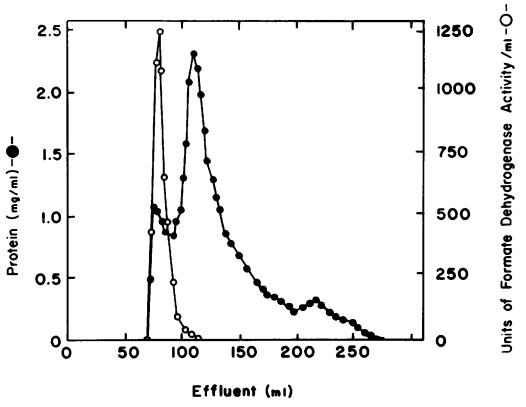

FIG. 1. *Elution of formate dehydrogenase from Sephadex G-200.*

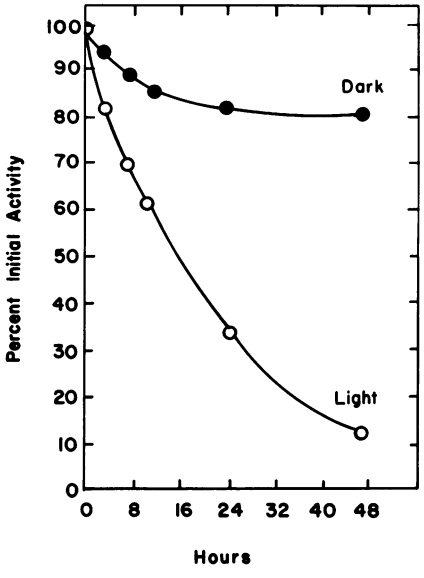

FIG. 2. *Inactivation of formate dehydrogenase activity by light.*

TABLE 1. *Partial purification of formate dehydrogenase*

Step	Protein (mg)	Units	Recovery (%)	Specific activity (units/mg)
1. Cell-free extract	4,720	150,000	100	31.8
2. Treatment with protamine sulfate	4,100	140,000	93.5	35.0
3. 50–55% $(NH_4)_2SO_4$ precipitate	254	107,000	71.0	412.0
4. Sephadex G-200 chromatography	18	17,500	11.4	901.0

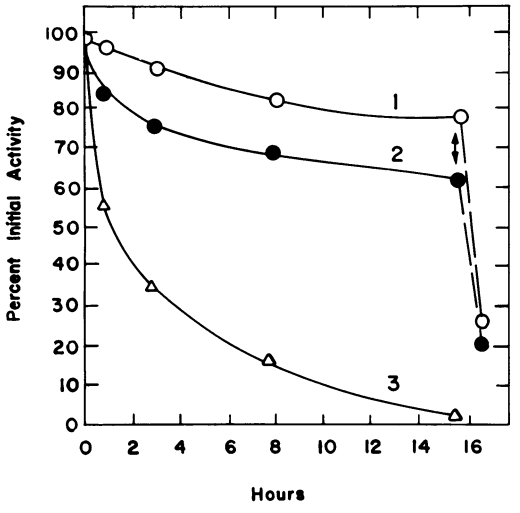

FIG. 3. *Inactivation of formate dehydrogenase activity by oxygen. Flask 1* (○) *was flushed with* N_2 *and not stirred; flask 2* (●) *was flushed with* N_2 *and stirred; flask 3* (△) *was not flushed with* N_2 *but was stirred. Air was introduced into flasks 1 and 2 at arrow.*

tion of the dye. Addition of certain levels of reducing agents were beneficial in this regard as well as in enhancing the rate of electron transfer. Precautions were necessary, however, to assure that the levels of reducing agents added were not sufficient to cause nonenzymatic reduction of the dye. Control cuvettes with formate omitted from the reaction mixture (but with enzyme present) assured the substrate and enzyme dependency of the benzyl viologen reduction. The effect on enzyme activity by reducing agents during enzyme assay is shown in Fig. 4. Dithionite (0.25 mM), $Na_2S \cdot 9H_2O$ (2.5 mM) and dithiothreitol (5 mM) gave a linear rate with no lag period, whereas 5 mM cysteine, mercaptoethanol, and glutathione exhibited lesser degrees of enhancement. It should be noted that, although each reaction mixture, including the one minus reducing agent, reached linearity of rate after lag periods of up to 15 min, none of the rates reached the maximum attained by those reaction mixtures containing Na_2S, dithionite, or dithiothreitol. Of the reducing agents tested, sodium sulfide was chosen for routine use during assays because of the nonspecific reduction of benzyl viologen by enzyme preparations when the organic thiol reagents were used. Concentrations of 2 through 5 mM Na_2S showed optimal protection, while 10 mM Na_2S inhibited the enzyme activity by approximately 20%.

Inhibition by NaCN and EDTA. The sensitivity of formate dehydrogenase activity to NaCN and EDTA is shown in Fig. 5. NaCN showed 30% inhibition at 10^{-5} M and 90% inhibition at 10^{-4} M concentration. EDTA showed only slight inhibition at concentrations as high as 10^{-2} M but did produce 50% inhibition at 0.1 M.

Kinetics of benzyl viologen reduction. The reduction of benzyl viologen during formate oxidation, with increasing amounts of purified enzyme, was linear from 8 to 64 μg of protein in the standard assay procedure.

The saturation curve and Lineweaver-Burk plot for formate is shown in Fig. 6. The approximate K_M for formate is 3.1×10^{-3} M. The K_M for benzyl viologen was determined to be approximately 10^{-4} M, although the curve is not shown.

Any one of the three buffers, Tris, Tricine, or potassium phosphate, at a concentration of 0.1 M, could be used for enzyme assay without any effect on enzyme activity. The enzyme showed maximal activity between *p*H 7.8 and 8.2. Attempts to couple formate oxidation to other electron acceptors such as tetrazolium dyes, ferricyanide, dichloroindophenol, and methylene blue failed.

Ferredoxin coupling to formate oxidation in crude preparations of formate dehydrogenase. The involvement of ferredoxin in the

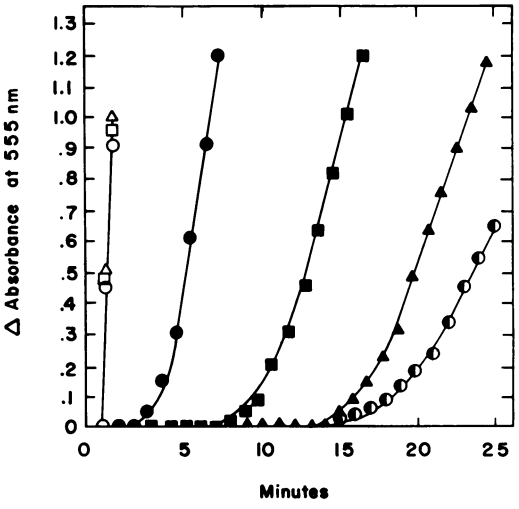

FIG. 4. *Effect of reducing agents on formate dehydrogenase activity. Standard reaction mixtures containing* (△) *0.25 mM dithionite,* (□) *2.5 mM* $Na_2S \cdot 9H_2O$, (○) *5 mM dithiothreitol,* (●) *5 mM cysteine,* (■) *5 mM mercaptoethanol,* (▲) *5 mM glutathione, and* (◐) *no reducing agent were supplied with 53 μg of protein.*

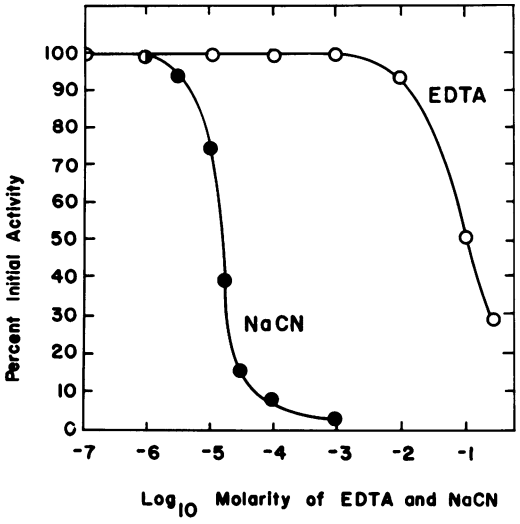

FIG. 5. *Comparative inhibition of formate dehydrogenase by (○) ethylenediaminetetraacetate (EDTA) and (●) NaCN. Standard reaction mixtures contained 27 μg of protein.*

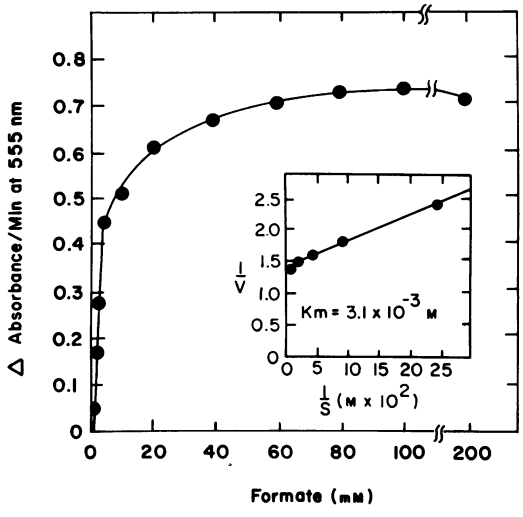

FIG. 6. *Saturation curve for formate. Standard reaction mixtures contained 16 μg of protein.*

couple between formate oxidation and benzyl viologen or NAD reduction and the relative efficiency of benzyl viologen and NAD as electron acceptors are shown in Table 2. Ferredoxin-free crude extracts of *C. acidiurici* containing formate dehydrogenase activity reduced benzyl viologen but not NAD or NADP. The ability to transfer electrons to NAD was restored by addition of ferredoxin, but it should be noted that the rate of transfer, on the basis of protein concentration, was approximately 30-fold greater to benzyl viologen than to NAD. Calculations of the amount of acceptor reduced were done with the aid of the molar extinction coefficients of NADH (6.22×10^3) and benzyl viologen (6.3×10^3). The crude extracts used here contained appreciable amounts of NADH oxidase, but, under the strictly anaerobic conditions employed, the oxidase showed little or no activity. Thus it was possible to make a fair approximation of the rate of NAD reduction.

Ferredoxin and formate dehydrogenase activity in the pyruvate clastic system from C. pasteurianum. Ferredoxin was purified from *C. acidiurici* and assayed by the methods described above. The activity of purified ferredoxin and 30-fold-purified formate dehydrogenase (both from *C. acidiurici*) in the clastic system of *C. pasteurianum* is shown in Table 3. A 100-μg amount of purified ferredoxin showed a ninefold stimulation of the clastic system, while 150 μg of formate dehydrogenase showed no ferredoxin activity and gave no stimulation.

TABLE 2. *Electron transfer to benzyl viologen and pyridine nucleotides by crude extracts*

C. acidiurici crude extract (ferredoxin-free) (mg/ml)	Formate (mM)	*C. acidiurici* ferredoxin (mg/ml)	Electron acceptor[a] (mM)	Acceptor reduced (μmoles per 10 min per mg of protein)
0.12	30	0	BV 10	11.6
0.12	30	0.9	BV 10	11.3
2.6	30	0.0	NAD 1	0.03
2.6	30	0.9	NAD 1	0.4
2.6	0	0.9	NAD 1	0
0	30	0.9	NAD 1	0
2.6	30	0.9	NADP 1	0

[a] Abbreviations: BV, benzyl viologen; NAD, nicotinamide adenine dinucleotide; NADP, NAD phosphate.

TABLE 3. *Comparison of ferredoxin and formate dehydrogenase activation of C. pasteurianum clastic system*

C. pasteurianum clastic enzymes (mg)	Pyruvate (μmoles)	*C. acidiurici* ferredoxin (mg)	*C. acidiurici* formate dehydrogenase (mg)	Acetyl-P formed[a] (μmoles)
7.0	50	0	0	0.3
7.0	50	0.1	0	2.6
7.0	0	0.1	0	0
7.0	50	0	0.15	0.3

[a] Total volume, 1.0 ml; 20-min incubation.

Possible involvement of ferredoxin in purified preparations of formate dehydrogenase. In contrast to the ability of crude extracts to couple formate oxidation to NAD by way of ferredoxin, the partially purified formate dehydrogenase, as shown in Table 4, was incapable of reducing NAD or NADP. The reduction of benzyl viologen by formate proceeded at a maximal rate in the absence of ferredoxin, and ferredoxin itself showed no formate dehydrogenase activity, again showing the noninvolvement of ferredoxin in this couple. Attempts to couple formate oxidation with NAD and NADP reduction in the presence of ferredoxin were negative, even with increased levels of protein and longer incubation times. NADH oxidase activity was measured in both the ferredoxin and formate dehydrogenase preparations and found to be negligible. Less than 0.01 μmole of NADH was oxidized per mg of protein in 10 min under the conditions of the assay, whereas the rate of benzyl viologen reduction (and potentially the rate of NAD reduction) was at least 0.6 μmoles per 10 min per 14 μg of protein, or a rate approximately 400-fold greater than the potential rate of NADH oxidation.

Formate-bicarbonate exchange. Sagers and Beck (25) showed that whole cells of *C. acidiurici* catalyzed the exchange of bicarbonate with ^{14}C-formate. This phenomenon was also observed in DEAE cellulose-treated crude extracts of *C. acidiurici* and is shown in Table 5. The reaction was not stimulated by ferredoxin, and ferredoxin itself showed no CO_2 exchange activity. The formate-bicarbonate exchange reaction was also catalyzed by the 30-fold-purified preparations of formate dehydrogenase, and, under conditions employed in Table 5, 60 μmoles of CO_2 was exchanged in 10 min per mg of protein.

Failure of formate dehydrogenase to reduce carbon dioxide to formate. It seemed possible that the formate-bicarbonate exchange capability of the enzyme might indicate a route for net CO_2 fixation by *C. acidiurici*. In an attempt to demonstrate the direct reduction of CO_2 and the net synthesis of formate, 160 μg of purified formate dehydrogenase was added to 200 mM ^{14}C-bicarbonate and 10 mM dithiothreitol either with or without 10 mM reduced benzyl viologen. But under these conditions there was no decrease in the labeled CO_2 after 2 hr of incubation. The same amount of enzyme catalyzed the formate-bicarbonate exchange reaction at the rate of 10 μmoles exchanged per 10 min.

DISCUSSION

Since 1901, when Pakes and Jollyman (19) described the bacterial oxidation of sodium formate to carbon dioxide and hydrogen, attempts to purify formate dehydrogenase have met with only limited success.

A two- to threefold purification of the soluble enzyme from *Rhodopseudomonas palustris* was achieved by fractional precipitation with ammonium sulfate, but poor recovery of the enzyme was common and rarely exceeded 50% of its initial activity (22). The formate dehydrogenase from this source lost activity when stored at −10 C. Storage in the presence of formate under anaerobic conditions improved the stability of the enzyme. The NADP-specific enzyme from *C. thermoace-*

TABLE 4. *Electron transfer to benzyl viologen and pyridine nucleotides by purified formate dehydrogenase*

C. acidiurici formate dehydrogenase (mg/ml)	Formate (mM)	*C. acidiurici* ferredoxin (mg/ml)	Electron acceptor[a] (mM)	Acceptor reduced (mM)
0.014	30	0	BV 10	0.67[b]
0.014	30	0.03	BV 10	0.64
0.014	0	0.03	BV 10	0
0	30	0.03	BV 10	0
0.014	30	0.03	NAD 0.1	0[c]
0.045	30	0.1	NAD 0.1	0
0.014	30	0.03	NADP 0.1	0

[a] Abbreviations: BV, benzyl viologen; NAD, nicotinamide adenine dinucleotide; NADP, NAD phosphate.

[b] Amount reduced in 10 min.

[c] Amount reduced in 30 min.

TABLE 5. *Formate-bicarbonate exchange reaction in cell-free extracts of C. acidiurici*

C. acidiurici crude extracts (mg/ml)	*C. acidiurici* DEAE-treated extracts (mg/ml)	Ferredoxin (mg/ml)	Na $^{14}COOH$[a] (mM)	HCO_3 (mM)	CO_2 exchanged (μmoles per 10 min per mg of protein[b]
1.4	0	0	100	100	1.62
1.4	0	0	100	0	0.46
0	1.8	0	100	100	1.66
0	1.8	0.9	100	100	1.70
0	0	0.9	100	100	0.02
0	1.8	0	0	100	0
0	1.8	0	100	0	0
0	0	0	100	100	0
0	1.8	0.9	100	0	0.25

[a] 1,720 disintegrations per min per μmole.

[b] Counts were corrected for quenching by the hydroxide of hyamine and filter paper by use of internal standards.

ticum was purified sevenfold by absorption and elution from DEAE cellulose and Amberlite CG-50 (batch method) with concurrent losses of activity (13). Stability of the purified enzyme was maintained up to 5 days when the protein was stored as a precipitate after ammonium sulfate fractionation. A 20-fold-purified soluble formate dehydrogenase was obtained from the particulate fractions of *E. coli* by the use of deoxycholate in the presence of ammonium sulfate, but loss of activity accompanied any further attempts to purify the enzyme on column chromotography (10). A 30% recovery of activity and a threefold purification of NAD-specific soluble formate dehydrogenase from *Pseudomonas Sp. AM1* was achieved by heat treatment and two consecutive ammonium sulfate precipitations (11). All extracts were kept in the presence of mercaptoethanol. The enzyme lost 50% of its activity in one month when stored at −15 C.

The instability of the enzyme from *C. acidiurici* is not easily explained but may be a function of any one or all of the following factors: large size of the enzyme (Fig. 1); light sensitivity (Fig. 2); and oxygen sensitivity (Fig. 3). The NADP-specific enzyme from *C. thermoaceticum* was also inhibited by the introduction of air to the system (13), and the inhibition due to oxygen of the formate dehydrogenase from *R. palustris* could not be reversed by the addition of catalase. Although activity was restored when this system was subsequently made anaerobic, the renewed rate was lower than the rate attained before inhibition (22).

Description of the light sensitivity of formate dehydrogenase is unique to this report. This sensitivity may result from the presence of flavines or vitamin B_{12}-like substances in the enzyme. The formate dehydrogenase solubilized from particulate preparations of *E. coli* has shown a characteristic lumiflavine peak at 530 nm when excited at 365 nm (10). It would be tempting to equate light sensitivity to the presence of particular prosthetic groups, but until more highly purified preparations of the enzyme are obtained this must remain as speculation.

Inhibition of formate dehydrogenases by EDTA, 8-hydroxyquinoline, or orthophenanthroline has been commonly associated with the binding of free divalent metal cations or with the formation of protein-metal-chelate complexes (9, 17, 22, 23, 27). Reasons for the variance in stimulation and inhibition by chelators may not be clear at present, but it would appear in the case of *C. acidiurici* that free divalent metal ions are not involved in the catalytic activity of the formate dehydrogenase.

Cyanide inhibition can proceed by any one of three ways: complexing with metals on metallo proteins; cyanohydrin formation with carbonyl groups of aldehydes and ketones; or the irreversible reaction with disulfide bonds to give thio salts and thiocyanates (9). The enzyme from *C. acidiurici* appears to be the most sensitive to cyanide inhibition of all the formate dehydrogenases described (10, 11, 16, 22). The observation that bacteria of the coli-aerogenes group (normally containing high levels of the enzyme) show no formate dehydrogenase activity when grown in the absence of iron and molybdenum (7, 8, 21) may indicate the presence of these metals on the enzyme. It is interesting to note that cyanide binds irreversibly to molybdenum which is present in high-molecular-weight metalloflavoenzymes—milk xanthine oxidase, rabbit liver aldehyde oxidase, and chicken liver xanthine dehydrogenase (4). To draw any parallels between these enzymes and formate dehydrogenase from *C. acidiurici* at this point may be unwarranted but should be considered in future studies.

The failure of formate oxidation to couple to NAD reduction, when purified preparations of formate dehydrogenase and ferredoxin were used (Table 4), could be explained simply by the removal during purification of some factor (either an enzyme or a coenzyme) which is essential in this couple. Earlier reports (1, 31) indicated, as demonstrated in this report, that crude preparations of formate dehydrogenase from *C. acidiurici* could couple to NAD but not NADP when supplemented with ferredoxin.

The reversal of the formate dehydrogenase system has been investigated in a variety of bacterial and plant systems, but such studies have not reported a net synthesis of formate (6, 13, 16). It is possible that one function of the enzyme in *C. acidiurici* might be the reduction of CO_2 to formate, with the formate thus derived being converted to *N*-5,10-methenyltetrahydrofolate in the presence of adenosine triphosphate and an active tetrahydrofolate formylase, which is present in this organism. This methenyl derivative could then be reduced to *N*-5,10-methylenetetrahydrofolate through the NADP-specific dehydrogenase also known to be present in the cells and thus find its way into the metabolic pathways of the organism.

Another possible method of CO_2 fixation into acetate has been described for *Clostridium thermoaceticum*. In the total synthesis of

3 moles of acetate from 1 mole of glucose by this organism, 2 moles of acetate are derived from pyruvate, while the third apparently is synthesized from CO_2. It has been proposed that CO_2 is fixed into the carbonyl carbon of acetate by its condensation with the methyl carbon of an active B_{12} derivative. The details of this route are not clear, but indications are that formate may be an intermediate (30). Both *C. acidiurici* and *C. thermoaceticum* catalyze the exchange of bicarbonate with formate (13, 25), but adequate experiments to show the net synthesis of formate from carbon dioxide have not been performed. Raeburn and Rabinowitz (24), using extracts of *C. acidiurici*, showed that the synthesis of pyruvate from CO_2 and acetyl-CoA was dependent on reduced ferredoxin. Reduced low-potential dyes such as methyl viologen and benzyl viologen could replace ferredoxin as sources of electrons in this system. Since it has been shown that the formate dehydrogenase from *C. acidiurici* couples readily with ferredoxin and with benzyl viologen, these two sources of low-potential electrons might serve in the reduction of CO_2 to formate, provided the system is coupled to an appropriate formate-trapping mechanism. However, the appropriate experiments in this regard have not yet been performed.

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