JOURNAL OF BACTERIOLOGY, Apr. 1978, p. 139-146
0021-9193/78/0134-0139$02.00/0

Vol. 134, No. 1
Printed in U.S.A.

Identity of Proline Dehydrogenase and Δ^1-Pyrroline-5-Carboxylic Acid Reductase in *Clostridium sporogenes*†

RALPH N. COSTILOW* AND DALE COOPER

Department of Microbiology and Public Health, Michigan State University, East Lansing, Michigan 48824

Received for publication 20 October 1977

Proline dehydrogenase and Δ^1-pyrroline-5-carboxylic acid (PCA) reductase activities were copurified 60- and 130-fold, respectively, from extracts of *Clostridium sporogenes*. The primary change in the ratio of activities was the result of a loss of proline dehydrogenase activity during dialysis. Both activities were eluted in single peaks from diethylaminoethyl-cellulose, hydroxylapatite, and Sephadex G-200 columns. They had identical sedimentation coefficients (10.3S), as determined in linear sucrose gradients, and identical isoelectric points (4.95 to 5.12) based on isoelectric focusing. The proline dehydrogenase activity was dependent on nicotinamide adenine dinucleotide and L-proline, and the PCA reductase required L-PCA and reduced nicotinamide adenine dinucleotide. The optimum pH for the assay of proline dehydrogenase was ~10.2, whereas that for PCA reductase was 6.5 to 7.5. An increase in pH from 8.0 to 10.2 greatly decreased the apparent Michaelis constant observed for L-proline, and an increase from pH 8.3 to 8.6 resulted in a large shift in the reaction equilibrium toward PCA. Both the dehydrogenase and reductase activities were stabilized to heating at 65°C for 5 min by solutes of high ionic strength and were inactivated in a similar fashion when dissolved in low-ionic-strength buffer. The specific activities for both were reduced by about 50% when glucose was added to the growth medium. The data support the conclusion that L-proline and L-PCA are interconverted by either a single enzyme or an enzyme complex in extracts of *C. sporogenes* cells.

Earlier studies in this laboratory (1) demonstrated that *Clostridium sporogenes* and *Clostridium botulinum* type A contained a nicotinamide adenine dinucleotide (NAD)-dependent enzyme(s) that catalyzes the interconversion of proline and Δ^1-pyrroline-5-carboxylic acid (PCA). In crude extracts, the reaction appeared reversible, with the equilibrium far in the direction of proline. The conversion of PCA to proline by PCA reductase [L-proline:NAD(P)$^+$ 5-oxidoreductase, EC 1.5.1.2] has been demonstrated in a number of tissues (8, 11, 16, 18, 23-25). However, this activity is believed to be irreversible and either reduced NAD (NADH) or reduced NAD phosphate (NADPH) can serve as reductants (11, 16).

In animal tissues and some microorganisms, proline is oxidized to PCA by proline oxidase (3, 19). However, NAD-dependent proline dehydrogenase activities that produce PCA have been described in a number of plant tissues (7, 9, 14) and in *Chlorella* (5). These same tissues contain high levels of PCA reductase activities. There is some evidence that both activities are catalyzed by the same protein in extracts of pumpkin cotyledons (14). Evidence presented herein indicates that *C. sporogenes* contains a single NAD-dependent proline dehydrogenase which catalyzes a reversible reaction between proline and PCA.

† Journal article no. 8267, Michigan Agricultural Experiment Station.

MATERIALS AND METHODS

Culture, cultural methods, and preparation of cell extracts. *C. sporogenes* (ATCC 7955, National Canners Association PA 3679) was used for all experiments. Cultural media and procedures and methods for harvesting and breaking cells were as described previously (2). Crude cell extracts were in 0.25 M potassium phosphate buffer (pH 7.5).

Enzyme assays. Routine assays for PCA reductase were conducted by monitoring the loss of absorbancy at 340 nm (A_{340}). Reaction mixtures of 1 ml contained 0.125 M potassium phosphate (pH 7.5), 0.1 mM NADH, 1 to 1.3 mM DL-PCA, and enzyme. Corrections were made for endogenous rates of NADH oxidation where necessary. For routine assays, the DL-PCA was synthesized by the procedure of Williams and Frank (21). L-PCA used in kinetic studies was synthesized by the biological method of Strecker (19). The concentration of PCA in routine stock solutions was estimated by the reaction with *o*-aminobenzaldehyde, using an extinction coefficient of 2.94 mM^{-1} cm^{-1} at 444 nm (21). For kinetic studies, the concentration was also checked by determining the amount of NADH oxi-

dized by the PCA reductase at pH 6.5. The results of both assays were very close with L-PCA preparations.

Unless otherwise stated, proline dehydrogenase was assayed by measuring NADH formation at 340 nm. Reaction mixtures of 1 ml contained 20 mM L-proline, 20 mM NAD, and enzyme in 0.125 M sodium carbonate buffer, pH 10.2. In a study of pH optima, the proline dehydrogenase was also assayed by measuring the formation of a colored complex between PCA and *o*-aminobenzaldehyde. Reaction mixtures of 1 ml contained 0.4 ml of 0.25 M buffer, which had been saturated with *o*-aminobenzaldehyde, 0.3 M L-proline, 20 mM NAD, and enzyme. These were incubated for 10 min, and the reaction was stopped by the addition of 1 ml of 10% trichloroacetic acid in ethanol. After centrifugation, the A_{444} was determined, and the amount of PCA formed was calculated.

Glutamate dehydrogenase assays were conducted by measuring the α-ketoglutarate-dependent oxidation of NADH by the procedure of Winnacker and Barker (22). Aldolase was determined by the method of Sibley and Lehninger (17), acetokinase was determined by that of Rose et al. (15), and alcohol dehydrogenase was determined according to Racker (13).

All enzyme activities were determined at 37°C. All light absorbancy measurements were made with a Gilford model 2000 recording spectrophotometer equipped with a Haake constant-temperature circulator. One unit of enzyme is defined as that amount which converts 1 μmol of substrate per min, and the specific activity is defined in units per milligram of protein. Protein was routinely estimated by the procedure of Lowry et al. (4), but in highly purified preparations the spectrophotometric method of Warburg and Christian (20) was used.

Purification procedures. All procedures were conducted in the cold (0 to 7°C). Three different lots of extract were purified through all of the steps outlined, and other extracts were purified through only some of them with minor variations. A summary of one complete procedure is presented in Results (Table 1). For heating, the crude extract was divided into 5- to 6-ml lots and sealed in 16-mm screw-cap vials. These vials were immersed in a 65°C water bath for 5 min and then cooled in an ice bath. The procedures used for the treatment with streptomycin sulfate and ammonium sulfate were fundamentally the same as described previously to prepare ornithine cyclase (deaminating) (2). The remaining purification steps were performed as follows.

(i) Step 5: DEAE-cellulose chromatography. The 60 to 80% $(NH_4)_2SO_4$ fraction was dialyzed overnight against 1 liter of 0.15 M tris(hydroxymethyl)aminomethane (Tris)-chloride buffer, pH 7.5. This was added to a 1.5- by 17-cm column of diethylaminoethyl (DEAE)-cellulose (DE 52) which had been equilibrated with 0.15 M Tris-chloride, pH 7.5. The column was washed with this buffer until the A_{280} was <0.1. The enzyme activities were eluted with a linear gradient from 0.15 to 0.3 M Tris-chloride, pH 7.5 (250 ml of each). The elution rate was about 30 ml/h, and fractions of 4.5 ml were collected. Both the reductase and dehydrogenase activities were eluted in the trailing edge of an A_{280} peak, which was slightly less than one-half way through the elution gradient. Fractions 36 through 46 were combined and concentrated by ultrafiltration through a Diaflo UM-10 (Amicon Corp.) membrane, using nitrogen as the pressuring gas.

(ii) Step 6: Dialysis. The concentrated extract was dialyzed against 500 ml of 0.02 M potassium phosphate, pH 7.4.

(iii) Step 7: Hydroxylapatite column chromatography. The dialyzed extract was added to a hydroxylapatite column (2.0 by 24 cm) which had been equilibrated with the same buffer. The column was washed with 50 ml of this buffer and then eluted with a linear gradient from 0.02 to 0.4 M potassium phosphate buffer, pH 7.4 (250 ml of each). Fractions of about 4.5 ml were collected at a flow rate of 25 ml/h. Most of the enzyme activities were eluted between fractions 42 and 50. These fractions were combined and concentrated by ultrafiltration as described above.

Sucrose gradient centrifugation. Linear sucrose gradients (5 to 20%) of 4.55-ml total volume were prepared by the procedure of Martin and Ames (6) in cellulose nitrate tubes. After the tubes were held at 5°C for 3 h, 0.15 ml of a mixture of extract from step 7, Table 1 (0.05 mg of protein), and 2 μg of lactic acid dehydrogenase (beef heart) were layered on duplicate tubes. The gradients were centrifuged in an SW39L rotor in a Spinco model L ultracentrifuge (Beckman-Spinco) at 37,000 rpm for 15 h. Five-drop fractions (23 from one gradient and 24 from another) were collected and analyzed.

Isoelectric focusing. Two milliliters of extract from step 4, Table 1, was dialyzed for 24 h versus 1% glycine (pH 7.4; 100 ml) with one change of the glycine solution. The dialysis bag was then transferred to 500 ml of 0.001 M Tris-chloride, pH 7.5, and dialysis continued for 48 h. A sample (0.3 ml with 6.6 mg of protein) of this extract was isoelectrically focused in a 110-ml-capacity glass column (model 1801, LKB Instruments Inc.) according to the manufacturer's directions, with the anode at the bottom, modified only in that the light and dense solutions were 50 ml each and the light solution contained 5 g of sucrose. The linear sucrose gradient contained 1.2% ampholine (pH range, 4 to 6). Constant voltage was applied at 300 to 400 V and increased gradually to 700 or 800 V. After 46 h, 1.5-ml fractions were collected and assayed for enzyme activities and pH.

Disc gel electrophoresis. Samples of the most highly purified preparation were subjected to electrophoresis on three polyacrylamide gels, using a Buchler apparatus with 6-mm-ID glass tubing. The procedure used was as described previously (2). One of the gels was stained with amido black protein stain and destained in 7% acetic acid. One gel was used to detect glutamate dehydrogenase, and the other was used to detect proline dehydrogenase based on the reduction of tetrazolium by NADH produced during the reactions. For glutamate dehydrogenase, the gel was incubated at 37°C in 23 ml of 25 mM Tris-chloride (pH 7.5) containing 10 mM L-glutamate, 2 mM NAD^+, 12 mg of phenazine methosulfate, and 2.5 mg of nitroblue tetrazolium. The glutamate in this mixture was replaced with 300 mM L-proline for detection of proline dehydrogenase on the other gel.

Chemicals. Nicotinamide adenine dinucleotides, *o*-aminobenzaldehyde, and the δ-hydroxylysine mono-

hydrochloride (a mixture of hydroxy-DL-lysine and allohydroxy-DL-lysine) used for preparation of DL-PCA were from Sigma Chemical Co. These and all other chemicals used were of the highest standards of purity available.

RESULTS

Copurification of PCA reductase and proline dehydrogenase activities. A summary of the purification of one representative lot of extract is presented in Table 1. The relative activities of the proline dehydrogenase and PCA reductase remained reasonably constant through the early purification steps (through step 5). However, dialysis of the preparation from step 5 resulted in about a 78% loss in the specific activity of the dehydrogenase, whereas only about a 44% loss of reductase activity occurred. The resulting high PCA reductase/proline dehydrogenase ratio was also observed in the concentrate from the hydroxylapatite column. This is not a consistent observation. During purification of a separate extract, a similar change in the ratio of the two activities was observed during dialysis of the $(NH_4)_2SO_4$ fraction, but the ratio returned to that routinely observed in crude extracts after concentration of the active fractions from DEAE-cellulose chromatography.

While glutamate dehydrogenase activity was never eliminated from the preparations, the relative activities changed dramatically. In this particular preparation (Table 1), the $(NH_4)_2SO_4$ fractionation was most effective in removing the glutamate dehydrogenase, but this was not consistent. The DEAE-cellulose and hydroxylapatite steps were usually more effective.

Passage of the most highly purified preparation through a Sephadex G-200 column (1.5 by 36 cm) equilibrated with 0.025 M potassium phosphate buffer, pH 7.4, resulted in about a 50% loss in the specific activities of both proline dehydrogenase and PCA reductase. They both eluted in the trailing edge of the void volume of the column. The fractions with the highest enzyme activities were pooled and concentrated by ultrafiltration. After disc gel electrophoresis of this concentrate, two protein bands were detected on staining with amido black. A major protein band with an R_f of 0.3 also stained with enzyme stains for both proline-dependent and glutamate-dependent NAD reduction. A minor protein band at R_f 0.18 did not stain with the enzyme stain. The gel stained for glutamate dehydrogenase was diffusely stained from the origin to the major band.

Elution patterns of PCA reductase and proline dehydrogenase activities from three very different columns were similar. Peak activities of both enzymes were found in the same fractions from DEAE-cellulose, hydroxylapatite, and Sephadex G-200 columns (Fig. 1). Also, the relative magnitudes of the two activities were similar across the peaks. Glutamate dehydrogenase activity was consistently present in the same peaks from all three columns, but the peak glutamate dehydrogenase fraction was always different from the peak fraction for the other two activities.

Both the PCA reductase and proline dehydrogenase activities in the preparations from step 7 were completely stable during 2 months of storage at −18°C. This concentrated fraction has a relatively high ionic strength (0.15 to 0.2 M potassium phosphate buffer), and this is believed to contribute to stability. However, freezing and thawing resulted in considerable losses in activity.

Sedimentation pattern and isoelectric points. Peak activities of both PCA reductase and proline dehydrogenase were found in the same fraction from a linear sucrose gradient (Fig. 2). These peak activities were separated by one fraction from the high endogenous glutamate dehydrogenase peak. Using a value of 7.6 for the sedimentation coefficient of lactic acid dehydrogenase, beef heart (10), the calculated coefficient for both the PCA reductase and proline dehydrogenase is 10.3S, and that for glutamate dehydrogenase is 11.4S. Costilow and Laycock (2)

TABLE 1. *Purification of proline dehydrogenase (PDH), PCA reductase, and glutamate dehydrogenase (GDH)*[a]

Step	Prepn	Vol (ml)	Protein (mg)	Sp act (U/mg)			Activity ratio		
				PDH	PCA reductase	GDH	PDH	PCA reductase	GDH
1	Crude	16	590	0.04	0.54	34.0	1	13.5	850
2	Heated, 65°C, 5 min	13	281	0.10	1.15	86.3	1	11.5	863
3	Streptomycin sulfate	26	283	0.09	0.96	55.3	1	10.7	614
4	$(NH_4)_2SO_4$ (60–80%)	5	110	0.14	1.61	5.1	1	11.5	36
5	DEAE-cellulose	6.4	6.5	1.29	12.08	19.7	1	9.4	15
6	Dialyzed	6.3	6.4	0.28	6.8	18.9	1	24.3	67
7	Hydroxylapatite	5.1	0.34	2.4	70.1	197.6	1	29.2	82

[a] See Materials and Methods for experimental details.

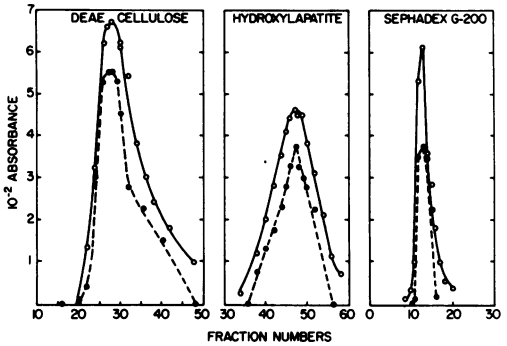

FIG. 1. *Elution patterns of PCA reductase and proline dehydrogenase activities from DEAE-cellulose, hydroxylapatite, and Sephadex G-200 columns. See text for experimental details and Table 1 for recoveries. Symbols: ○, PCA reductase ($-\Delta A_{340}$ per minute with 10 μl of each fraction); ●, proline dehydrogenase (ΔA_{340} per minute with 0.1 ml of each fraction).*

previously observed a value of 10.7S for the PCA reductase from this organism.

The protein(s) or protein aggregate(s) responsible for the two enzymatic activities not only appears to be of one size and conformation, but also has a single isoelectric point. Three separate determinations by isoelectric focusing demonstrated that peak activities of the PCA reductase and proline dehydrogenase coincided in the elution patterns. The pH of the peak fractions in the different runs varied from 4.95 to 5.12. The endogenous glutamate dehydrogenase consistently eluted earlier in the gradient; the pH of peak fractions varied from 4.70 to 4.85. The results of one of the isoelectric focusing runs are presented in Fig. 2. The reason for the asymmetry of the glutamate dehydrogenase peak in this run is not known, and it was not observed consistently.

pH optima. The apparent optimal pH for proline dehydrogenase activity was dependent on the assay method (Fig. 3). When activity was measured by monitoring NADH formation, the optimal pH was about 10.2. In contrast, when the activity was measured by measuring the formation of the colored complex of PCA and *o*-aminobenzaldehyde, the optimal pH was about 7.5. There was very little dehydrogenase activity detectable by this assay at an intermediate pH of 9.0 at the substrate concentrations used. The PCA reductase exhibited maximal activity over a pH range of 6.2 to 7.5, but activity declined rapidly at higher pH values. Negligible reductase activity was detected at pH 10.2.

Kinetic studies. The concentration of L-proline necessary for half-maximal reaction rates (apparent K_m) with proline dehydrogenase was very dependent upon pH (Fig. 4). In the presence of 20 mM NAD, the apparent K_m for L-proline at pH 8.0 was 31 mM, whereas at pH 10.2 it was 1.25 mM. In contrast, K_m values for NAD determined under the same conditions were the same at both pH values (K_m, ~1.0 to 1.2 mM). No activity was detectable when NADP (21 mM) was substituted for NAD or when D-proline was used as substrate.

The apparent K_m for L-PCA at pH 8.0 as determined by the PCA reductase assay in the presence of 0.1 mM NADH was 0.33 mM (Fig. 5). This is about 1/100 that observed with proline at the same pH. At pH 6.5, the apparent K_m for L-PCA was 0.2 mM. The reductase is specific for L-PCA. In the presence of excess NADH, L-

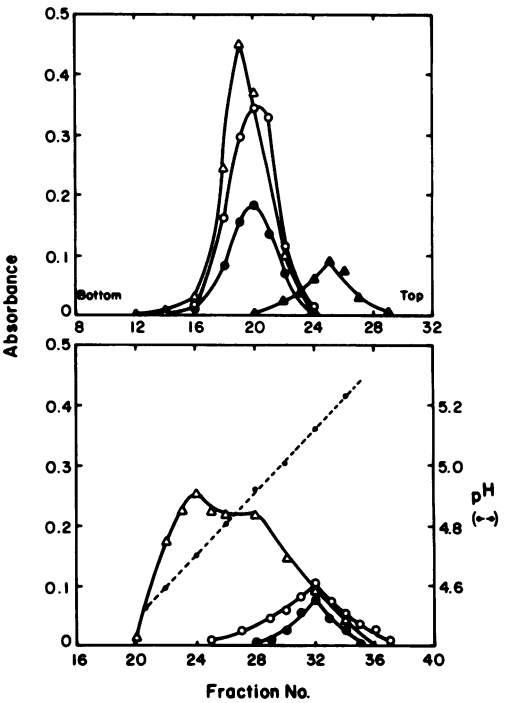

FIG. 2. *Fractionation of glutamate dehydrogenase, PCA reductase, and proline dehydrogenase activities on a linear sucrose gradient (top) and by isoelectric focusing (bottom). Protein, 0.05 mg (step 7, Table 1), was used for the sucrose gradient, and 6.6 mg of protein from step 4, Table 1, was used for the isoelectric focusing runs. Lactic acid dehydrogenase, 2 μg, was included as a marker in the sucrose gradient. Symbols: Δ, glutamate dehydrogenase ($-\Delta A_{340}$ per minute with 1 μl [top] and 5 μl [bottom] of each fraction); ○, PCA reductase ($-\Delta A_{340}$ per minute with 5 μl [top] and 25 μl [bottom] of each fraction); ●, proline dehydrogenase (ΔA_{340} per minute with 50 μl [top] and 200 μl [bottom] of each fraction); ▲, lactic acid dehydrogenase ($-\Delta A_{340}$ per minute with 10 μl of each fraction).*

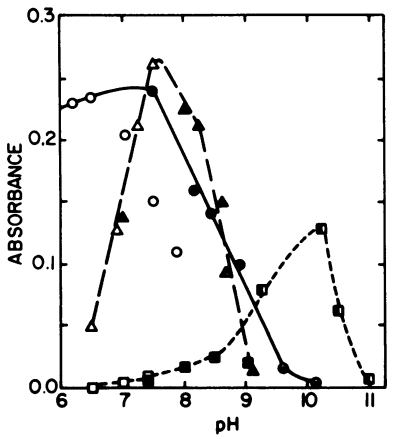

FIG. 3. *Effect of pH on the activities of PCA reductase and proline dehydrogenase. Extract from step 4, Table 1, was used for PCA reductase (0.4 mg of protein) assays and for assays of proline dehydrogenase by the o-aminobenzaldehyde reaction (2 mg of protein). Extract (9 μg of protein) from step 7, Table 1, was used for assays of proline dehydrogenase based on NAD reduction. See text for experimental details. Symbols: Circles and solid line, PCA reductase ($-\Delta A_{340}$ per minute); triangles with long dashes, proline dehydrogenase assayed with o-aminobenzaldehyde (ΔA_{444} per 10 min); squares with short dashes, proline dehydrogenase as measured by following NADH formation (ΔA_{340} per minute); open points, phosphate buffer; closed points, Tris-chloride buffer; half-closed points, bicarbonate buffer.*

PCA was completely reduced at pH 6.5, whereas only 50% reduction occurred when DL-PCA was added as substrate. When high concentrations of L-proline were added to reaction mixtures at pH 8.0, the PCA reductase activity was strongly inhibited (Fig. 5). This inhibition appeared to be competitive in nature. As reported previously (2), NADPH was not oxidized by the PCA reductase.

The equilibria for both the PCA reductase and proline dehydrogenase activities were strongly affected by pH (Table 2). A decrease in pH from 8.6 to 8.3 resulted in an average four- to fivefold increase in the average equilibrium constant (K_{eq}) as measured by the PCA reductase assay, whereas the opposite effect was observed when K_{eq} values were estimated from proline dehydrogenase assays. As expected, the K_{eq} values at each pH for the two activities were essentially reciprocals.

Thermostability. Initial rates of thermal inactivation at 60 and 65°C of both reductase and dehydrogenase activities were identical (Fig. 6). However, the inactivation curves for both activities became concave downward, and proline dehydrogenase activity then decreased more rapidly than the reductase.

The results in Fig. 6 were obtained with an extract in a relatively low-ionic-strength buffer (0.01 M). In one experiment, with an extract eluted with hydroxylapatite at relatively high buffer concentration (~0.20 to 0.25 M), little inactivation of either activity was observed even at 65°C. This led to the finding that both activities were stabilized by high ionic strength (Table 3). Maximal stabilization was achieved with 0.25 M potassium phosphate buffer, but 0.25 M Tris-chloride and 0.25 M KCl were also very effective. There was consistently more loss of proline dehydrogenase than of reductase activity. The endogenous glutamate dehydrogenase was quite heat stable even in the 0.01 M Tris-

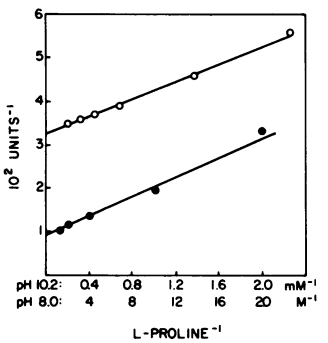

FIG. 4. *Influence of pH on Lineweaver-Burk plots of proline dehydrogenase as a function of L-proline concentrations. Reaction mixtures at pH 8.0 of 1 ml contained 9 μg of extract protein from step 7 (Table 1), 20 mM NAD, and varying L-proline concentrations in 0.1 M Tris-chloride buffer. Reaction mixtures at pH 10.2 contained 0.4 mg of extract protein from step 4 (Table 1), and 20 mM NAD, in 0.125 M carbonate buffer. Reaction rates were all measured by the increase in A_{340}. Symbols: ○, pH 8.0; ●, pH 10.2.*

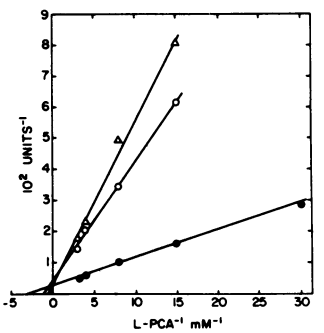

FIG. 5. *Lineweaver-Burk plots of PCA reductase activities as a function of L-PCA concentrations in the presence and absence of L-proline. Reaction mixtures of 1 ml contained 0.1 mM NADH, enzyme (4 μg of protein) from step 7, Table 1, and the indicated concentrations of L-PCA and L-proline in 0.1 M Tris-chloride buffer, pH 8.0. Symbols: ●, no added proline; ○, 225 mM L-proline; Δ, 300 mM L-proline.*

chloride buffer, but it was completely stable for 5 min at 65°C in 0.25 M phosphate buffer. The specific activities of PCA reductase, proline dehydrogenase, and glutamate dehydrogenase were all increased by a factor of 2.0 to 2.5 by heating extract in 0.25 M phosphate buffer at 65°C for 5 min followed by centrifugation (see Table 1).

Influence of growth media on specific activities. The specific activities of both PCA reductase and proline dehydrogenase in cell extracts were reduced by about 50% when glucose was added to the growth medium (Table 4). In contrast, the specific activities of both fructose 1,6-diphosphate aldolase and acetokinase were about two times higher in cells grown in the

TABLE 2. *Effect of pH on equilibria observed with PCA reductase and proline dehydrogenase (PDH) activities*[a]

pH	L-proline (mM)	K_{eq} calculated	
		PCA reductase	PDH
8.6	5	3.9×10^{4}	2.7×10^{-5}
	10	4.0×10^{4}	2.6×10^{-5}
	20	4.5×10^{4}	2.5×10^{-5}
	50		2.5×10^{-5}
8.3	10	1.9×10^{5}	4.5×10^{-6}
	20	1.6×10^{5}	5.5×10^{-6}
	50	1.7×10^{5}	5.2×10^{-6}

[a] All reaction mixtures contained 0.2 M Tris-chloride buffer, 20 mM NAD, purified enzyme from step 7 (Table 1), and the indicated levels of L-proline. DL-PCA, 0.2 mM, and 0.09 mM NADH were used in PCA reductase assays. The total changes in optical density at 340 nm at 37°C were measured, and the final concentrations of reactants were calculated. The final pH of each reaction mixture was measured.

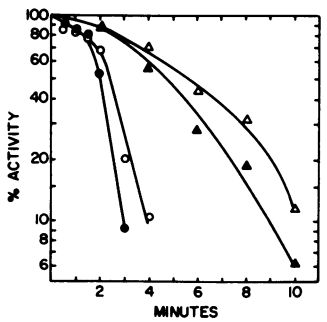

FIG. 6. *Heat inactivation of PCA reductase and proline dehydrogenase activities. Crude extract, 0.2 ml (0.6 mg of protein in 0.01 M Tris-chloride buffer, pH 7.4), was heated for the times indicated and cooled in an ice bath. Activities were estimated by standard assay procedures, using 10 μl of extract for PCA reductase and 0.1 ml for proline dehydrogenase. Symbols: Open, PCA reductase; closed, proline dehydrogenase; triangles, 60°C; circles, 65°C.*

TABLE 3. *Effect of buffers and ionic strength on heat stability of enzymes*[a]

Solvent (pH 7.5)	% Activity remaining[b]		
	PCA reductase	PDH	GDH
0.01 M Tris-chloride	1	0	62
0.25 M Tris-chloride	61	45	91
0.25 M potassium phosphate	92	81	105
0.25 M KCl	67	50	90

[a] Dialyzed enzyme from step 4 of the purification procedure (Table 1), 28 mg of protein per ml, was diluted 10^{-1} in the solvents indicated. For heated samples, the dilution was made after the solvent was equilibrated in a water bath. PDH, Proline dehydrogenase; GDH, glutamate dehydrogenase.

[b] (ΔA_{340} per min after heating at 65°C for 5 min/initial ΔA_{340} per min) × 100.

TABLE 4. *Effect of growth medium on the activity of various enzymes*[a]

Medium	Sp act (μmol/min per mg of protein)			ΔA/min per mg of protein	
	PCA reductase	PDH	ADH	FDP aldolase	Acetokinase
Trypticase	0.80	0.15	0.07	0.02	0.04
+ Glucose	0.40	0.08	0.07	0.04	0.08

[a] Cells were produced in the routine Trypticase medium and in the same medium plus 1% glucose. Dialyzed crude extracts were prepared in 0.25 M phosphate buffer, pH 7.3, and used for the enzyme assays. See Materials and Methods for assay procedures. PDH, Proline dehydrogenase; ADH, alcohol dehydrogenase; FDP, fructose 1,6-diphosphate.

presence of glucose. Alcohol dehydrogenase activity was not affected by the presence of glucose.

The presence of glucose in the growth medium had no significant influence on the stability of any of these enzymes to heating in 0.25 M phosphate buffer. Of the enzymes tested in this experiment, only the PCA reductase and proline dehydrogenase activities were stable (75 to 100% recoveries) to heating at 65°C for 5 min.

DISCUSSION

With few exceptions, the data indicate that the interconversion of L-proline and L-PCA by *C. sporogenes* is catalyzed by a single protein or a protein complex. Thus, both proline dehydrogenase and PCA reductase activities (i) copurify extensively (60 to 130 times), (ii) elute in single peaks from DEAE-cellulose, hydroxylapatite, and Sephadex G-200 columns, (iii) cosediment in a sucrose gradient, (iv) have identical isoelectric points as determined by isoelectric focusing, (v) are specific for the oxidized or reduced forms of NAD, (vi) are stabilized to heating by high ionic strengths, and (vii) are repressed by about

50% when glucose is present in the growth medium. Similar evidence of identity of the protein(s) catalyzing these reactions was obtained with extracts of pumpkin cotyledons (14).

Some changes in ratios of the two activities were observed during purification and heating of the clostridial extracts, but not with the pumpkin (14). However, during storage of pumpkin extract at low ionic strength, the PCA reductase activity was lost more rapidly than proline dehydrogenase activity. This resulted in a decrease in the reductase/dehydrogenase ratio. In contrast, with the clostridial extracts, when a change in ratio was observed, a loss in the relative activity of the dehydrogenase was consistently indicated. The ratios of the two activities in extracts from pumpkin and *C. sporogenes* are also quite different. The reductase/dehydrogenase ratio was about 1.5 in crude or purified preparations from pumpkin (14), whereas the ratio in the crude clostridial extract was about 10 to 13 and increased to 29 during purification. Therefore, if the two activities are catalyzed by the same protein(s), the enzymes from these two different sources are quite different with respect to relative activities.

Some questions have been raised regarding the identity of proline dehydrogenase and PCA reductase from plants because of differences in pyridine nucleotide specificity. In extracts of peanut plants (7, 8), the reductase utilized NADPH more effectively than NADH, whereas NAD was preferred over NADP as oxidant for the proline dehydrogenase. In pumpkin (14) the dehydrogenase was specific for NAD, whereas PCA was reduced with either NADH or NADPH, although the latter was preferred. In fact, there was some evidence that there are two reductases in pumpkin tissues, one specific for NADH and the other for NADPH. This is not indicated in *C. sporogenes*, since both of the activities are dependent on the same coenzyme, NAD or NADH.

The protein(s) catalyzing the proline dehydrogenase and PCA reductase activities is of a relatively high molecular weight. Data presented herein and those of Costilow and Laycock (2) demonstrate that the PCA reductase from *C. sporogenes* has a sedimentation coefficient of 10.3 to 10.7, which would correspond to a molecular weight for a globular protein of about 200,000. The molecular weight of the purified PCA reductase from *E. coli* was estimated at 320,000 (16). Proline dehydrogenases from plants also have molecular weights in excess of 100,000 (5, 7). Therefore, there is a distinct possibility that the two activities depend on an aggregate of two or more enzymes.

The effects of pH and the kinetic data are consistent with the proposal that the reductase and dehydrogenase reactions are both catalyzed by a reversible enzyme. Racker (12) demonstrated that the observed equilibrium constants for the NAD-linked alcohol and lactic acid dehydrogenases were increased 10 times with each increase in 1 pH unit. This results from ignoring the H^+ concentration in calculating the constants. Therefore, the equilibrium constant for a reversible proline dehydrogenase would be predicted to be 1,000 times higher at pH 10.2 than at pH 7.2. Equilibrium data indicated that a pH change of only 0.3 resulted in a four- to sixfold change in the K_{eq} values for both the proline dehydrogenase and PCA reductase. Based on H^+ concentration alone, one would expect only a twofold change in the equilibria with this pH difference. Although the reason(s) for the four- to sixfold change observed have not been experimentally established, the data indicate that proline becomes an increasingly better substrate as the pH increases. Increasing the pH from 8.0 to 10.2 resulted in a decrease in the apparent K_m for L-proline from 31 to 1.25 mM and a threefold increase in the maximum velocity of the dehydrogenase reaction. In contrast, the apparent K_m for L-PCA increased from 0.2 mM at pH 6.5 to 0.33 mM at pH 8.0. These data indicate that the relative affinity of the enzyme for proline as compared to PCA may increase rapidly with pH, which might also be expected to shift the equilibrium.

The reason for the difference in pH optima obtained for the proline dehydrogenase activity in the presence and absence of *o*-aminobenzaldehyde (pH 7.5 and 10.2, respectively) is not known. However, it may be related to the stability and reactivity of *o*-aminobenzaldehyde at high pH. It was demonstrated previously that the presence of *o*-aminobenzaldehyde in reaction mixtures at pH 7.5 increased the conversion of proline to PCA (2). The presence of pyruvate and lactic acid dehydrogenase along with *o*-aminobenzaldehyde dramatically increased the amount of PCA formed at this pH. These reagents would be expected to pull the reaction toward PCA.

The role(s) of proline dehydrogenase and PCA reductase activity in *C. sporogenes* has not been determined. In some organisms, the reductase is important in the biosynthesis of proline from either glutamic acid or ornithine (23). *C. sporogenes* can convert ornithine to proline by ornithine cyclase (deaminating), and we have been unable to demonstrate the conversion of [^{14}C]glutamate to proline. The NAD-dependent proline dehydrogenase in plants is believed to be involved in the catabolism of proline to glutamate (14). This may also be true with *C. sporo-*

genes. Preliminary experiments (Costilow, unpublished data) indicate that growing cells can convert proline to glutamate.

ACKNOWLEDGMENTS

The technical assistance of Barbara Goelling and Susanne Keller is gratefully acknowledged.

This research was partially supported by a Public Health Service grant (AI 10951) from the National Institute of Allergy and Infectious Diseases.

LITERATURE CITED

1. **Costilow, R. N., and L. Laycock.** 1969. Reactions involved in the conversion of ornithine to proline in clostridia. J. Bacteriol. **100**:662–667.
2. **Costilow, R. N., and L. Laycock.** 1971. Ornithine cyclase (deaminating). Purification of a protein that converts ornithine to proline and definition of the optimal assay conditions. J. Biol. Chem. **246**:6655–6660.
3. **Frank, L., and B. Ranhand.** 1964. Proline metabolism in *Escherichia coli.* III. The proline catabolic pathway. Arch. Biochem. Biophys. **107**:325–331.
4. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
5. **McNamer, A. D., and C. R. Stewart.** 1974. Nicotinamide adenine dinucleotide-dependent proline dehydrogenase in *Chlorella.* Plant Physiol. **53**:440–444.
6. **Martin, R. G., and B. N. Ames.** 1961. A method for determining the sedimentation behavior of enzymes: application to protein mixtures. J. Biol. Chem. **236**:1372–1379.
7. **Mazelis, M., and R. K. Creveling.** 1974. L-Proline dehydrogenase of *Triticum vulgare* germ: purification, properties and cofactor interactions. Phytochemistry **13**:599.
8. **Mazelis, M., and L. Fowden.** 1969. Conversion of ornithine to proline by enzymes from germinating peanut cotyledons. Phytochemistry **8**:801–809.
9. **Mazelis, M., and L. Fowden.** 1971. The metabolism of proline in higher plants. II. L-Proline dehydrogenase from cotyledons of germinating peanut (*Arachis hypogaea* L.) seedlings. J. Exp. Bot. **22**:137–145.
10. **Millar, D. B. S.** 1962. Physico-chemical properties of lactic dehydrogenase. J. Biol. Chem. **237**:2135–2139.
11. **Peisach, J., and H. J. Strecker.** 1962. The interconversion of glutamic acid and proline. V. The reduction of Δ^1-pyrroline-5-carboxylic acid to proline. J. Biol. Chem. **237**:2255–2260.
12. **Racker, E.** 1950. Crystalline alcohol dehydrogenase from bakers' yeast. J. Biol. Chem. **184**:313–319.
13. **Racker, E.** 1955. Alcohol dehydrogenase from baker's yeast. Methods Enzymol. **1**:500–503.
14. **Rena, A. B., and W. E. Splittstoesser.** 1975. Proline dehydrogenase and pyrroline-5-carboxylate reductase from pumpkin cotyledons. Phytochemistry **14**:657–661.
15. **Rose, I. A., M. Grunberg-Manago, S. R. Korey, and S. Ochoa.** 1954. Enzymatic phosphorylation of acetate. J. Biol. Chem. **211**:737–756.
16. **Rossi, J. J., J. Vender, C. M. Berg, and W. H. Coleman.** 1977. Partial purification and some properties of Δ^1-pyrroline-5-carboxylate reductase from *Escherichia coli.* J. Bacteriol. **129**:108–114.
17. **Sibley, J. A., and A. L. Lehninger.** 1949. Determination of aldolase in animal tissues. J. Biol. Chem. **177**:859–872.
18. **Smith, M. E., and D. M. Greenberg.** 1957. Preparation and properties of partially purified glutamic semialdehyde reductase. J. Biol. Chem. **226**:317–327.
19. **Strecker, H. J.** 1971. The preparation of animal proline oxidase (rat liver), and its use for the preparation of Δ^1-pyrroline-5-carboxylate. Methods Enzymol. **17B**: 251–254.
20. **Warburg, O., and W. Christian.** 1942. Isolation and crystallization of enolase. Biochem. Z. **310**:384–421.
21. **Williams, I., and L. Frank.** 1975. Improved chemical synthesis and enzymatic assay of Δ^1-pyrroline-5-carboxylic acid. Anal. Biochem. **64**:85–97.
22. **Winnacker, E. L., and H. A. Barker.** 1970. Purification and properties of a NAD^+-dependent glutamate dehydrogenase from *Clostridium* SB_4. Biochim. Biophys. Acta **212**:225–242.
23. **Vogel, H. J.** 1955. On the glutamate-proline-ornithine interrelation in various microorganisms, p. 335–346. *In* W. D. McElroy and H. B. Glass (ed.), A symposium on amino acid metabolism. Johns Hopkins Press, Baltimore.
24. **Vogel, H. J., and B. D. Davis.** 1952. Glutamic γ-semialdehyde and Δ^1-pyrroline-5-carboxylic acid, intermediates in the biosynthesis of proline. J. Am. Chem. Soc. **74**:109–112.
25. **Yura, T., and H. J. Vogel.** 1959. Pyrroline-5-carboxylate reductase of *Neurospora crassa*: partial purification and some properties. J. Biol. Chem. **234**:335–338.