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Partial Purification and Some Properties of Δ^1-Pyrroline-5-Carboxylate Reductase from *Escherichia coli*

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Δ^1-Pyrroline-5-carboxylate (PCA) reductase [L-proline:NAD(P)$^+$5-oxidoreductase, EC 1.5.1.2] has been purified over 200-fold from *Escherichia coli* K-12. It has a molecular weight of approximately 320,000. PCA reductase mediates the pyridine nucleotide-linked reduction of PCA to proline but not the reverse reaction (even at high substrate concentrations). The partially purified preparation is free of competing pyridine nucleotide oxidase, PCA dehydrogenase, and proline oxidase activities. The Michaelis constant (K_m) values for the substrate, PCA, with reduced nicotinamide adenine dinucleotide phosphate (NADPH) or NADH as cofactor are 0.15 and 0.14 mM, respectively. The K_m values determined for NADPH and NADH are 0.03 and 0.23 mM, respectively. Although either NADPH or NADH can function as cofactor, the activity observed with NADPH is severalfold greater. PCA reductase is not repressed by growth in the presence of proline, but it is inhibited by the reaction end products, proline and NADP.

In *Escherichia coli*, the path of proline biosynthesis is: glutamate $\rightarrow\rightarrow$ glutamate γ-semialdehye $\rightleftharpoons$ Δ^1-pyrroline-5-carboxylate (PCA) $\rightarrow$ proline (21). The first enzyme, γ-glutamyl kinase, mediates the adenosine 5'-triphosphate-dependent conversion of glutamate to a glutamyl-enzyme intermediate, most likely a glutamyl-phosphate-enzyme complex (1). The second enzyme, γ-glutamyl phosphate reductase, which probably functions in a complex with γ-glutamyl kinase, converts glutamyl phosphate to glutamate γ-semidaldehyde (2, 8). The conversion of glutamate γ-semialdehyde to PCA, the substrate for the final step in proline biosynthesis, occurs spontaneously (21). PCA reductase [L-proline:NAD(P)$^+$5-oxidoreductase, EC 1.5.1.2], the third enzyme, mediates the reduction of PCA to proline (21). Overall control of proline biosynthesis in bacteria is accomplished at the first step by feedback inhibition (3) and repression (18). Synthesis of PCA reductase, however, is not subject to repression by proline (3, 5).

It has been assumed that PCA reductase is not regulated in bacteria because of a report that *E. coli* W excreted proline when fed PCA (3). We have found, however, that most isolates of *E. coli* (including our laboratory strains of K-12, B, C, and W) do not excrete proline when fed PCA (4). This regulation is bypassed in two classes of proline-excreting mutants: *proB* (3, 5) and *argD* (4).

Proline is synthesized from glutamate and broken down to glutamate by two different, irreversible pathways. PCA, the intermediate common to both pathways, can be reduced by PCA reductase to proline or oxidized by PCA dehydrogenase to glutamate. In mammals, PCA is channeled into the appropriate pathway by compartmentation: PCA dehydrogenase in mitochondria (10) and PCA reductase in the non-mitochondrial portion of the cell (11). It is not clear, however, how, or to what extent, PCA is channeled in prokaryotes. As a first step in elucidating the mechanism by which PCA metabolism is regulated in bacteria, we have undertaken a partial purification and characterization of PCA reductase from *E. coli* K-12. A comparison of the properties of PCA reductase from a non-excreting strain of *E. coli* K-12 and a proline-excreting derivative will be reported elsewhere (J. J. Rossi, J. Vender, and C. M. Berg, Biochem. Genet., in press).

(This work was taken in part from a thesis submitted by J.J.R. in partial fulfillment of the Ph.D. degree at the University of Connecticut, 1976.)

MATERIALS AND METHODS

Bacterium employed. Strain CB0401 is a *proB*$^-$ derivative of *E. coli* K-12 W3110 (*thy*$^-$) (12). It is defective in the synthesis of Δ^1-pyrroline-5-carboxylate (PCA). (This permits manipulation of the lev-

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els of PCA and proline available to the cell.) *proB* is in a different operon from *proC*, which codes for PCA reductase.

Chemicals and substrates. Ethylenediaminetetraacetic acid tetrapotassium salt was purchased from K & K Laboratories, Inc., Plainview, N.Y. Nicotinamide adenine dinucleotide (NAD), nicotinamide adenine dinucleotide phosphate (NADP), their respective reduced forms (NADH and NADPH) and *o*-aminobenzaldehyde were purchased from Sigma Chemical Co., St. Louis, Mo.

DL-Δ^1-Pyrroline-5-carboxylate (PCA) was synthesized by periodate oxidation of DL-δ-hydroxylysine as described by Williams and Frank (24). For use in enzyme assays the PCA was neutralized with 5 N potassium hydroxide to a final pH of 6.9. Quantitation of the neutralized PCA was accomplished by reaction with *o*-aminobenzaldehyde (24).

Media and buffers. The complete medium used was L broth supplemented with 10 μg of thymine per ml, and the minimal medium was medium E plus glucose (0.5%), thymine (10 μg/ml), thiamine (10 μg/ml), and proline (30 μg/ml, unless otherwise indicated) (12).

Potassium phosphate buffer (0.05 M) was used for enzyme assays at pH 6.9, except where noted. All purification steps were carried out in buffer at the indicated molarities and pH. The buffer contained 10^{-3} M dithiothreitol and 10^{-4} M ethylenediaminetetraacetic acid unless otherwise noted.

Polyacrylamide gel electrophoresis. After the last purification step the peak PCA reductase fraction was assayed for homogeneity by disc-gel electrophoresis in glass tubes (5 by 50 mm) at 2 mA per tube. Portions of 40 to 60 μg of the enzyme preparation were applied to stacking gels. Acrylamide separating gels (5 and 7.5%) were run at pH 9.5. Protein bands were detected by staining with 1% Coomassie brilliant blue.

Protein determination. Protein was determined by the ultraviolet spectrophotometric method of Waddell (23). Blanks were identical to the sample being measured except for the absence of protein.

Enzyme assays. All assays were carried out at 22°C in 0.05 M buffer, pH 6.9. Reactions were initiated by the addition of enzyme (diluted to yield a linear reaction for 5 min). Enzymatic activity was monitored by following absorbance at 340 nm.

PCA reductase was assayed during the purification by monitoring PCA-dependent oxidation of NADPH in buffer containing 0.5 mM PCA and 0.12 mM NADPH.

Glutamate dehydrogenase and *N*-acetylornithine transaminase were assayed by the methods of Vender et al. (19) and Vogel and Jones (22), respectively.

Preparation of extracts. Two liters of minimal medium was inoculated with 1 ml of a broth-grown culture and incubated, with aeration, at 37°C. When the cells reached late log phase (optical density at 420 nm, 0.8), they were chilled and harvested by centrifugation at 4°C for 20 min at 8,000 × *g*. The cells were washed once in buffer, resuspended in 10 to 20 ml of buffer, and disrupted with a Bronwill Biosonic IV ultrasonic generator at 4°C until the suspension was clarified. The extract was then centrifuged at 4°C for 20 min at 27,000 × *g*, and the supernatant was retained.

Molecular weight determination. Molecular weight was estimated by gel filtration on Bio-Gel A-1.5m, 100 to 200 mesh. The elution volumes of the pure protein standards were determined by monitoring the column at 280 nm. The elution volumes of endogenous PCA reductase and glutamate dehydrogenase were determined by applying a sample of the partially purified preparation (30 to 50% ammonium sulfate-saturated fraction) to the column and assaying for activity.

RESULTS

Specific activity of PCA reductase in crude extracts. PCA reductase from *E. coli* has been reported to be insensitive to repression by proline (3, 5). We examined the specific activity of PCA reductase from cultures of W3110 (*pro*$^+$) grown without supplementation or with proline (30 μg/ml) and from cultures of CB0401 (*proB*$^-$) grown with proline (6 to 150 μg/ml) or PCA (60 μg/ml). No differences in specific activity were found.

Purification of PCA reductase from *E. coli* K-12. The partial purification of PCA reductase from *E. coli* K-12 was accomplished by the steps described below and in Table 1.

(i) Step 1. $(NH_4)_2SO_4$ precipitation. The crude cell-free extract was brought to 30% saturation using solid $(NH_4)_2SO_4$ with stirring at

TABLE 1. *Enzyme purification*

Fraction	Vol (ml)	Activity		Recovery (%)	Purification (fold)
		Total units[a]	Units per mg of protein		
Cell-free extract	20.0	56.8	0.19	100	-
$(NH_4)_2SO_4$ (30 to 50%)	4.0	47.2	0.67	83	3.5
Sephadex G-200	13.5	36.4	2.33	64	12
DEAE[b]-Sephadex	23.8	29.3	4.82	51	25
Hydroxylapatite					
Active fractions (pooled)	41.0	11.0	24.45	19	129
Peak fraction	8.0	3.3	41.25	6	217

[a] Micromoles of NADPH oxidized per minute.
[b] DEAE, Diethylaminoethyl.

4°C. After 20 min the suspension was centrifuged at 16,000 × *g* for 15 min, and the pellet was discarded. Additional $(NH_4)_2SO_4$ was added to the supernatant fluid to a final concentration of 50% saturation. After centrifugation, the pellet obtained from this precipitation was dissolved in 2 to 5 ml of 0.02 M buffer (pH 7.0).

(ii) Step 2. Sephadex G-200 gel filtration. The enzyme solution from step 1 was applied directly to a Sephadex G-200 column (1.6 by 36 cm) previously equilibrated with 0.02 M buffer (pH 7.0) and eluted with this same buffer at a flow rate of 2.0 ml/h. The enzyme eluted as a single peak of activity. Fractions containing the bulk of the PCA reductase activity were pooled for further purification.

(iii) Step 3. Diethylaminoethyl-Sephadex A-50 column chromatography. The pooled fractions from step 2 were applied directly to a diethylaminoethyl-Sephadex A-50 column (1.5 by 23 cm) equilibrated with 0.033 M buffer (pH 6.9), and the enzyme was eluted with a 200-ml linear gradient of NaCl (0 to 0.8 M). Fractions of 4.0 to 8.0 ml were collected and assayed for PCA reductase activity. A single peak of activity was obtained between 0.43 and 0.49 M NaCl (not shown). The fractions containing PCA reductase activity were pooled and diluted to a final phosphate concentration of 0.02 M.

(iv) Step 4. Hydroxylapatite column chromatography. The enzyme solution was applied to a hydroxylapatite column (1.5 by 20 cm) previously equilibrated with 0.02 M buffer (pH 6.9, lacking ethylenediaminetetraacetic acid). The column was eluted with a 400-ml linear gradient of this buffer (0.02 to 0.4 M). Fractions of 4.0 to 8.0 ml were collected and assayed for PCA reductase activity. The highest enzyme activity was eluted from the column between 0.15 and 0.22 M phosphate (Fig. 1).

Criteria for purity. No proline oxidase, NADP-dependent or NAD-dependent PCA dehydrogenase activity could be detected in enzyme solutions purified through step 3. The enzyme solution purified through step 4 is free of contaminating, nonspecific pyridine nucleotide oxidase activity.

Fractions of the hydroxylapatite eluate containing high enzyme activity were analyzed by polyacrylamide gel electrophoresis. Eight protein bands were found. Attempts to develop an in-gel activity stain using modifications of the procedure for *E. coli* lactate dehydrogenase (17) were unsuccessful. Although the PCA reductase purified through step 4 is not homogeneous, it is sufficiently free of competing enzyme activities for use in kinetic studies.

Optimal conditions and stability. Maximal activity of both crude and purified PCA reductase was observed using 0.05 M buffer at pH 6.7 to 6.9 with NADPH as the cofactor. Only 25 to 50% of the maximal specific activity was found when NADH was used as the cofactor. PCA reductase did not catalyze the reverse reaction, the oxidation of proline to PCA, in the pH range 6.0 to 8.0, with cofactor (NADP or NAD) concentrations up to 1.5 mM and substrate (proline) concentrations up to 5 mM.

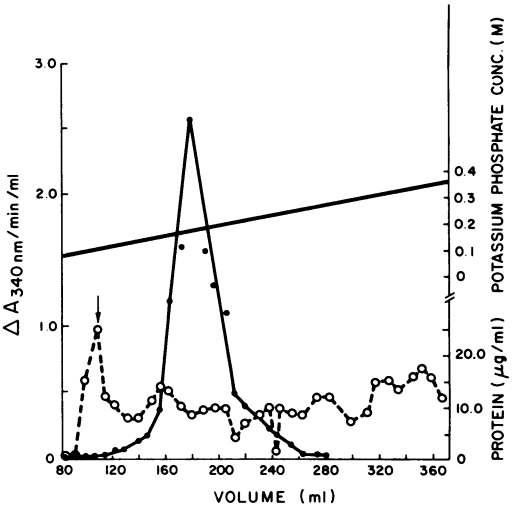

FIG. 1. *Elution profile from hydroxylapatite column chromatography. Symbols:* ●, *PCA reductase activity;* ○, *absorbance at 280 nm. The arrow* (↓) *indicates the elution peak of endogenous glutamate dehydrogenase.*

The purified enzyme is stable between 4 and 40°C, but is rapidly inactivated above 60°C with 50% inactivation occurring after 5 min of incubation at 67°C. While NADPH affords some protection against heat inactivation, NADH and PCA had no significant protective effect.

Dialysis of crude or ammonium sulfate-fractionated extracts against buffer containing dithiothreitol and ethylenediaminetetraacetic acid usually resulted in some loss of activity.

PCA reductase is inactivated by freezing and thawing. Therefore, the enzyme preparations are routinely maintained at 4°C (with no appreciable loss in activity over a period of several weeks). Stability during purification is dependent on the presence of a thiol reagent such as dithiothreitol.

Kinetic studies. Typical Lineweaver-Burk plots for NADPH and NADH utilization are shown in Fig. 2. NADPH at concentrations greater than 0.13 mM and NADH at concentrations greater than 0.16 mM inhibit PCA reductase activity (Fig. 3). At lower cofactor concen-

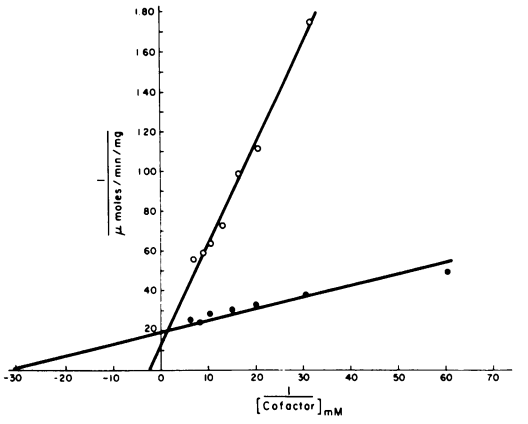

FIG. 2. *Lineweaver-Burk plot of PCA reductase activity as a function of varying cofactor concentration.* DL-*PCA was used at 0.5 mM. Assays were performed as described in the text. Symbols:* ●, *NADPH oxidation;* ○, *NADH oxidation.*

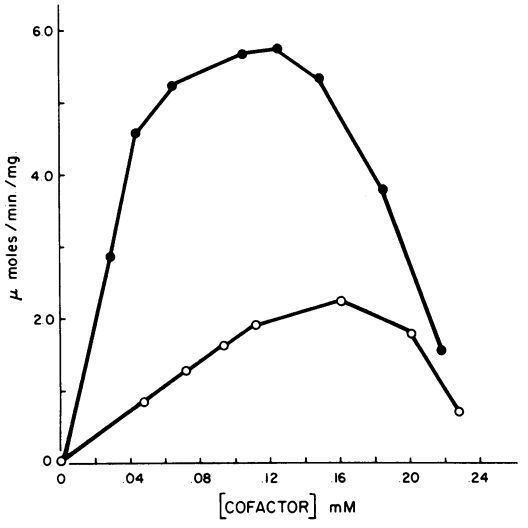

FIG. 3. *Inhibition of PCA reductase activity by NADPH and NADH.* DL-*PCA was used at 0.5 mM. Symbols:* ●, *Varying NADPH concentrations;* ○, *varying NADH concentrations.*

trations, there is a marked difference between the activity observed with the two cofactors. The half-saturation (K_m) values calculated for NADPH and NADH within the range of 0.3 to 0.8 mM PCA, at pH 6.9, are approximately 0.03 and at least 0.23 mM, respectively (Table 2). (The maximum useable concentration is lower than the K_m because commercially available preparations of NADH contain an inhibitor [6].)

Lineweaver-Burk plots for activity with varying PCA concentrations are presented in Fig. 4. It can be seen from these plots, as well as from the Michaelis-Menten plot insert, that PCA at high concentrations also inhibits PCA reductase, but that this inhibition is less severe with NADPH, than with NADH, as the cofactor. Kinetic studies using NADPH concentrations between 0.09 and 0.14 mM and NADH concentrations between 0.15 and 0.20 mM revealed no significant differences in K_m values for PCA obtained with either cofactor. These data (Fig. 4) also show that NADPH is the preferred cofactor under these conditions. The apparent K_m values for DL-PCA obtained from extrapolation of Lineweaver-Burk plots, utilizing either NADPH or NADH as cofactor are approximately the same, 0.15 and 0.14 mM, respectively (Table 2).

Williams and Frank (24) have demonstrated that PCA prepared by their methods is a racemic mixture of the D and L isomers, and that *E. coli* PCA reductase is specific for the L isomer. Therefore, the true K_m values for PCA may differ from the observed values.

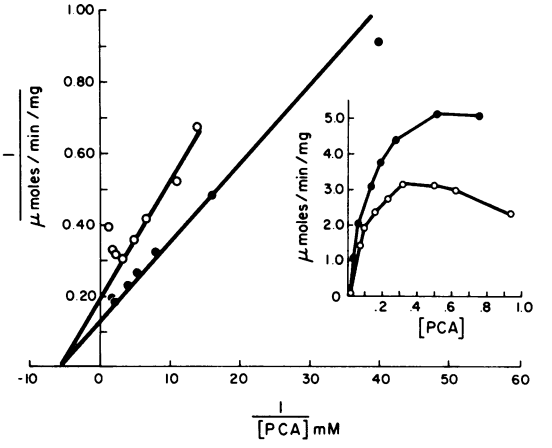

FIG. 4. *Lineweaver-Burk plot of PCA reductase activity as a function of varying* DL-*PCA concentrations. Insert depicts Michaelis-Menten plot for these data. NADPH was used at 0.12 mM, and NADH was used at 0.15 mM. Symbols:* ●, *NADPH as cofactor;* ○, *NADH as cofactor.*

TABLE 2. *Kinetic constants of substrate and cofactors for PCA reductase*[a]

Ligand	Cofactor (mM)	Ligand K_m (mM)[b]	No. of determinations
NADPH		0.030 ± 0.003[c]	6
NADH		0.229 ± 0.067[c]	5
DL-PCA	NADPH (0.12)	0.154 ± 0.056	8
DL-PCA	NADH (0.15)	0.139	2

[a] All kinetic experiments were performed with partially purified extract at pH 6.9 at 22°C.
[b] Means and standard deviations.
[c] DL-PCA was used at 0.5 mM.

Inhibitor studies. The reaction end products proline, NADP, and NAD were tested for their inhibitory activity on PCA reductase (Table 3). Proline inhibited competitively, with an approximate K_i of 15.0 mM (Fig. 5). NADP inhibition of NADPH utilization was competitive, with an approximate K_i of 0.6 mM (Fig. 6). NADP inhibition of NADH utilization was uncompetitive at low concentrations (Fig. 7A), but at 1.9 mM or higher the inhibition was mixed (Fig. 7B). NAD was not found to be an inhibitor of either NADPH or NADH utilization over a wide range of NAD concentrations (0.26 to 4.17 mM).

Incubation of the enzyme with the sulfhydryl blocking agent *p*-chloromercuribenzoate resulted in marked inhibition of enzyme activity, which is largely prevented by the addition of excess dithiothreitol (Table 3). Preincubation of the enzyme with NADPH, NADH, PCA, or proline does not protect against *p*-chloromercuribenzoate inhibition.

Estimation of molecular weight. The molecular weight of PCA reductase was estimated to be 320,000, based upon the elution of PCA reductase activity from a Bio-Gel A-1.5 column calibrated with purified enzymes of known molecular weights. PCA reductase activity elutes just before endogenous glutamate dehydrogenase (Fig. 8). Our elution volume for glutamate dehydrogenase is consistent with the molecular weight of 300,000 reported by Sakamoto et al. (13) but not with the molecular weight of 250,000 reported by Veronese et al. (20).

TABLE 3. *Inhibitors of PCA reductase activity*[a]

Inhibitor	Cofactor	Inhibitor concn (mM)	Inhibition (%)
Proline	NADPH (0.10 mM)	1.0	7
		5.0	17
		15.0	31
		20.0	45
NADP	NADPH (0.10 mM)	0.24	19
		0.47	26
		0.93	49
		1.87	54
		7.50	69
	NADH (0.14 mM)	0.47	60
		0.93	65
		1.87	75
		3.75	97
NAD	NADPH (0.10 mM)	4.17	0
	NADH (0.14 mM)	4.17	0
PCMB[b]	NADPH (0.12 mM)	0.0125	2
		0.025	16
		0.075	36
		0.100	95
		0.125	100
PCMB[b,c] (plus DTT)	NADPH (0.12 mM)	0.125	19

[a] DL-PCA was used at 0.5 mM.

[b] Final protein concentration was 76 μg/ml. Enzyme was incubated for 2 min in the presence of *p*-chloromercuribenzoate (PCMB) at room temperature before the addition of substrate and cofactor.

[c] A 0.25 mM concentration of dithiothreitol (DTT) was added before PCMB.

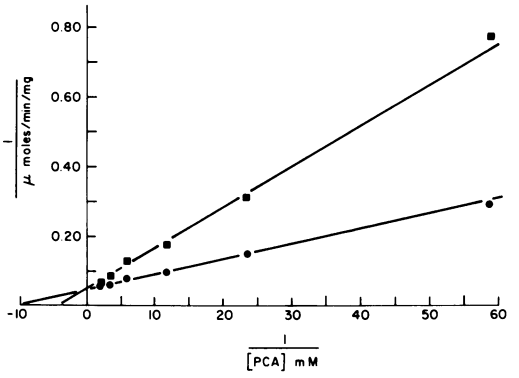

FIG. 5. *Lineweaver-Burk plot of proline inhibition of PCA reductase activity as a function of varying* DL-*PCA concentrations. NADPH was used at 0.12 mM. Symbols:* ●, *Without added proline;* ■, *with 20 mM proline.*

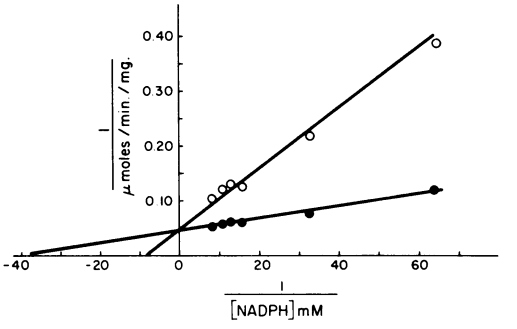

FIG. 6. *Lineweaver-Burk plots of NADP inhibition of PCA reductase as a function of varying NADPH concentrations.* DL-*PCA was used at 0.5 mM. Symbols:* ●, *Without added inhibitor;* ○, *with 1.9 mM NADP.*

DISCUSSION

PCA reductase has previously been partially purified from *Neurospora* (25), rat liver (14), and calf liver (16). In addition, an eightfold purification of the enzyme from *E. coli* B has been reported recently (24).

As in eukaryotes, PCA reductase can utilize either NADPH or NADH as a cofactor. Like the *Neurospora* enzyme (25), but unlike the mammalian enzyme (16), it shows a marked preference for NADPH in vitro (Fig. 3 and 4) and has similar K_m values for PCA with either NADPH or NADH as cofactor (Table 2; Fig. 4).

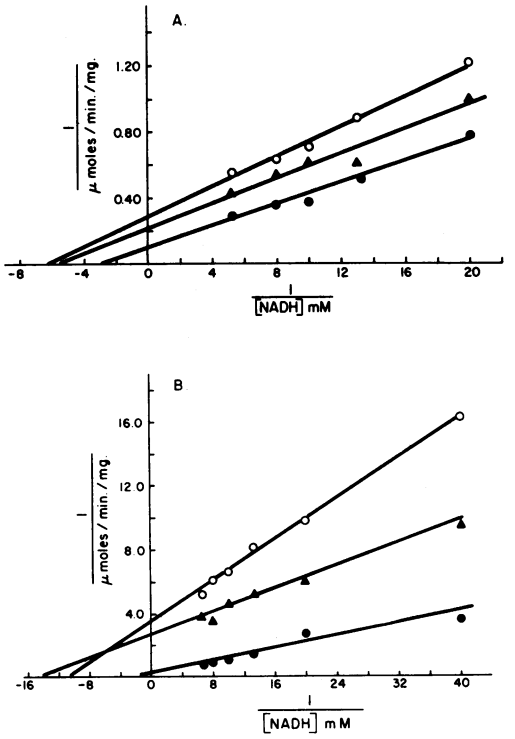

FIG. 7. *Lineweaver-Burk plots of NADP inhibition of PCA reductase activity as a function of varying NADH concentrations.* DL-*PCA was used at 0.5 mM. (A) Symbols: ●, Without added NADP; ▲, 0.095 mM NADP; ○, 0.19 mM NADP. (B) Symbols: ●, Without added NADP; ▲, 0.95 mM NADP; ○, 1.9 mM NADP.*

Two of the three end products of the PCA reductase-catalyzed reaction, proline and NADP, are competitive inhibitors of PCA reductase, while the third, NAD, does not inhibit the reaction (Table 3; Fig. 5 and 6). In spite of its high K_i (15 mM), inhibition of PCA reductase by proline (Fig. 5) may be physiologically significant when proline is present in excess (such as when the cells are growing on proline as a nitrogen source). NADP is a particularly potent inhibitor of PCA reductase activity when NADH serves as the cofactor. At low NADP concentrations the inhibition is uncompetitive (Fig. 7A), but at high NADP concentrations the inhibition appears mixed (Fig. 7B), with an observed increase in the slope of the inhibition curve, suggesting more than one binding site for NADP (15). (NADP is also a noncompetitive inhibitor of NADH-linked PCA reductase activity from rat liver [14].) Although PCA reductase isolated from eukaryotic and from prokaryotic sources is capable of utilizing either NADPH or NADH as cofactor, we do not know which is most available to the enzyme in vivo or whether inhibition by NADP is physiologically significant.

Although proline represses synthesis of the first biosynthetic enzyme (18) and induces the catabolic pathway, it does not appear to repress PCA reductase (5; this paper). What, then, prevents PCA reductase from competing with PCA dehydrogenase for PCA generated catabolically? Frank and Ranhand (7) have suggested that the two catabolic enzymes may function only as a complex. If so, PCA generated by proline oxidase would not enter a free pool and, hence, would not be available as a substrate for PCA reductase. Such channeling of a common intermediate has been described for aromatic amino acid metabolism in *Neurospora* (9). Physical compartmentation may also play a role in separating proline catabolism and anabolism, since both degradative enzymes are found in the membrane (7), whereas the biosynthetic enzymes are found in the cytoplasm (8). Finally, a high proline concentration, which induces the catabolic pathway, also inhibits PCA reductase activity. The relative importance of channeling, compartmentation, and end product inhibition in avoiding cycling between proline and PCA is yet to be determined.

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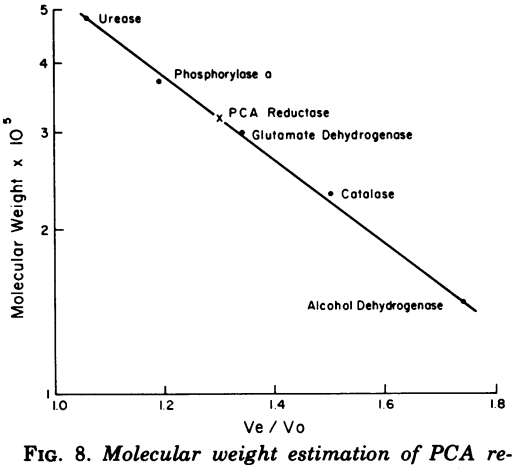

FIG. 8. *Molecular weight estimation of PCA reductase by gel filtration on a Bio-Gel A-1.5m column. Standard proteins (and molecular weights) are: jack bean urease (483,000), rabbit muscle phosphorylase a (370,000), endogenous glutamate dehydrogenase (300,000), bovine liver catalase (232,000), and yeast alcohol dehydrogenase (148,000).*

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