Phytochemistry, Vol. 22, No. 6, pp. 1345–1355, 1983.
Printed in Great Britain.

0031-9422/83/061345-11$03.00/0

OCTOPINE DEHYDROGENASE OF A *VINCA ROSEA* CROWN GALL TUMOR*

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(*Received* 16 *September* 1982)

Key Word Index—*Vinca rosea*; Apocynaceae; crown gall; opines; octopine; lysopine; octopine dehydrogenase; lysopine dehydrogenase.

Abstract—Octopine dehydrogenase (ODH) (EC 1.5.1.11) from crown gall tumor tissue induced by *Agrobacterium tumefaciens* strain W1 on *Vinca rosea* was purified 300-fold, yielding a single active enzyme with a MW of 38 000. The enzyme shows activity with four α-ketoacids and 16 amino acids. Both NADP(H) and NAD(H) serve as coenzymes; the enzyme has lower K_ms with NADPH and $NADP^+$, but higher V_{max}s with NADH and NAD^+. Pyruvate, NADPH and NADH at higher concentrations show substrate inhibition. The kinetics of the enzyme suggest that it may act *in vivo* to synthesize lysopine, octopinic acid, octopine and the corresponding N^{α}-derivatives of glutamine. The apoenzyme is protected against inactivation by $NADP^+$ and binds the coenzyme as the first substrate in the reaction; pyruvate potentiates binding of arginine to the holoenzyme and vice versa. The *Vinca* enzyme is similar to other crown gall ODHs in many respects, but differs in having relatively high affinities for ornithine and glutamine and a relatively low affinity for histidine. It also differs in showing inhibition by NADPH and NADH. These differences suggest that the primary structure of the *Vinca* W1 ODH differs from that of the crown gall ODHs previously described.

INTRODUCTION

Crown gall tumors induced by *Agrobacterium tumefaciens* typically synthesize unique compounds called opines which fall into four classes: octopine-type opines [N^2-(D-1-carboxyethyl)-L-amino acid], nopaline-type opines [N^2-(D-1,3-dicarboxypropyl)-L-amino acid], agropine, and agrocinopine [1–5]. The opine induced by a given strain of *A. tumefaciens* can also be used by that bacterium as a sole carbon and nitrogen source. Genes controlling bacterial utilization of at least the first three types of opines have been located in the Ti-plasmid [5]. The genetic information controlling tumor production of octopine and nopaline-type opines is located at one end of the T-DNA region, that part of the Ti-plasmid DNA which is maintained in bacteria-free tumor cells [5].

Theoretically, the Ti-plasmid gene controlling production of any opine could be a structural gene for the enzyme involved in its production or a gene that affects the regulation of a normal host gene for this enzyme. Octopine dehydrogenase (ODH) is responsible for octopine synthesis in animals [6–8] and similar activity has been demonstrated in octopine-type tumors in plants [9–15]. A characterization of the ODH in crown gall tumors was undertaken since the results could help resolve the origin as well as define the properties of this unique enzyme in plants.

Enzyme extracts from crown gall tissues which catalyse the reaction

L-amino acid + pyruvate + NADH (or NADPH) → octopine-type opine + NAD^+ (or $NADP^+$)

have been described [9–15]. This manuscript describes the partial purification, substrate specificity, and kinetic characteristics, of the ODH from a cultured *Vinca rosea* tumor tissue induced by *A. tumefaciens* strain W1.

RESULTS

Fractionation of Vinca *ODH*

All of the ODH activity of initial tissue extracts was recovered in the cytosol fraction after centrifugation. Fractions enriched for either cell organelles or membranes showed no ODH activity. Most of the enzyme precipitated in a 1.95–2.83 M (50–70% saturated) ammonium sulfate fraction. When this fraction was chromatographed on a column of Sephadex G-100, little or no activity was detectable in the eluate unless $NADP^+$ was included in the buffer. Although only *ca* 1% of the ODH applied to these columns was recovered in the absence of $NADP^+$, this activity eluted at the same volume as ODH applied and eluted with $NADP^+$ (Fig. 1). The elution volume of ODH corresponded to a MW of 38 000 and was not significantly different from the scallop ODH. Since a similar peak of *Vinca* ODH activity was detected by both radiometric and spectrophotometric assays (Fig. 1), enzyme activities catalysing octopine oxidation, octopine-dependent $NADP^+$ reduction and arginine + pyruvate-dependent NADPH and NADH oxidation were present in this fraction.

Ammonium sulfate fractionated ODH was applied to affinity columns of NADP-Agarose and NAD-Agarose, washed and eluted with the appropriate coenzyme (Fig. 2). ODH activity was not bound by the NAD column as determined by tests for NADH- or NADPH-dependent activity. NADPH-dependent ODH activity was bound by the NADP-Agarose column, and from 35 to 80% could be recovered on elution with $NADP^+$.

The separation of *Vinca* W1 ODH activity on Cellex-D

*This paper is dedicated to David Shemin in honor of his 70th birthday.

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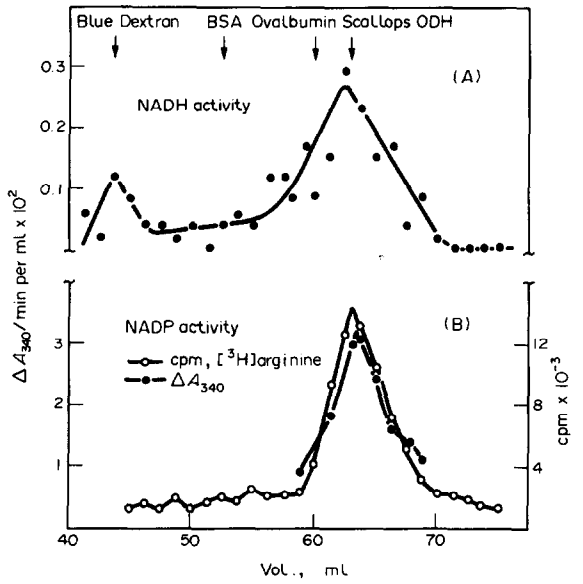

Fig. 1. Chromatography of the *Vinca* W1 ODH on Sephadex G-100. The 50–70% ammonium sulfate fraction of a crude *Vinca* W1 extract was chromatographed without added $NADP^+$ (A), and with 1 mg/ml $NADP^+$ added to the enzyme and to the eluant (B). Assays for NADH-dependent (A) or $NADP^+$-dependent (B) ODH activity were done spectrophotometrically (●) and radiometrically (○). The elution volumes of five protein standards were determined in a separate experiment and their elution volumes indicated by arrows; myoglobin elutes off-scale at 83 ml.

anion-exchange columns by elution with a linear sodium chloride gradient is shown in Fig. 3. The apparent recovery of NADH-dependent ODH activity in different experiments showed wide variation. In the major peak eluting at *ca* 0.12 M sodium chloride (fractions 62–78) (Fig. 3), the recovery of NADPH-dependent activity was 72% and that for NADH-dependent activity was 725%. The ratio of activity of NADPH- to NADH-dependent ODH activity in this peak varied in different experiments, but was always less than 0.6. However, this ratio was greater than 1.0 in all initial extracts and also for most enzyme extracts after ammonium sulfate fractionation. Recovery of NADPH-dependent ODH activity in the major peak was never greater than 75%, while that for NADH-dependent activity varied from 140 to 725% (average of 290%).

In all experiments, the bulk of the ODH activity detected with either NADPH or NADH as coenzyme eluted from Cellex-D at *ca* 0.12 M sodium chloride as a single peak. This fraction also catalysed the oxidation of octopine with either $NADP^+$ or NAD^+. A small peak of ODH activity (Fig. 3) eluting just after the void volume and prior to the start of the salt gradient was observed in most experiments; the relative position of the peak activity of NADPH- and NADH-dependent ODH activity in this fraction varied somewhat. A similar minor peak of NADH-dependent ODH activity appeared on rechromatography of the ODH fraction eluting at *ca* 0.12 M sodium chloride, suggesting that the minor peak may be an artifact of aggregation.

Vinca tumor ODH activity was not detectable after gel electrophoresis when stained by the phenazine methosulfate–nitroblue tetrazolium method. Pre-electrophoresis of the gel with β-mercaptoethanol to remove impurities allowed detection of a single band of $NADP^+$-dependent dehydrogenase that stained more strongly in the presence than in the absence of octopine. The band was faint in most experiments and the difference between gels stained with and without octopine was only clear after 2–4 hr. A faint NADP-ODH band was also detected at the same R_f using ODH chromatographically purified on both the Cellex-D and G-100 columns. The band was obtained by using 200 μl of a highly active *Vinca* ODH extract which was then concentrated 50-fold by ammonium sulfate precipitation prior to electrophoresis. No NAD^+-dependent ODH activity was detected on these gels.

To determine if the NADPH and NADH activities could be distinguished by slight differences in their temperature sensitivity samples from the major ODH

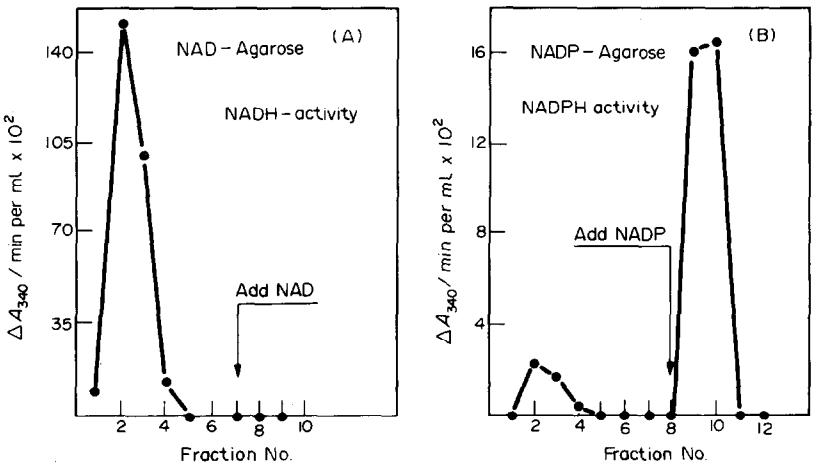

Fig. 2. Affinity chromatography of *Vinca* W1 ODH on NAD-Agarose (A) and NADP-Agarose (B). ODH, partially purified by ammonium sulfate fractionation, was applied to each column and eluted either with NAD^+ (A) or $NADP^+$ (B). Fractions (1 ml each) were assayed for either NADH-dependent ODH(A) or NADPH-dependent ODH(B). The void volume of each column was *ca* 2.5 ml.

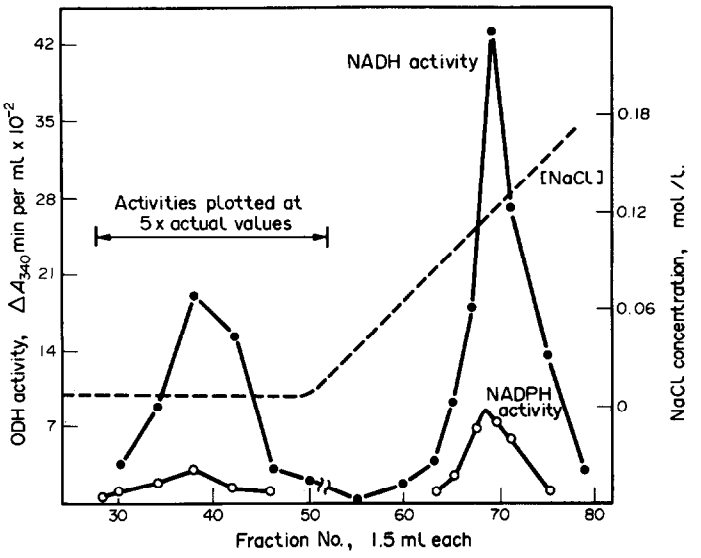

Fig. 3. Anion-exchange chromatography on Cellex-D of the 50–70% ammonium sulfate fraction of a *Vinca* W1 crude extract. After sample application, the column was eluted with 0.01 M sodium chloride and then with a linear 0.01 to 1.0 M sodium chloride gradient.

fraction obtained by anion-exchange chromatography were treated for 5 min at various elevated temperatures. The NADPH and NADH activities in the absence of $NADP^+$ are virtually identical in regard to their temperature sensitivity between 40 and 48°. The presence of $NADP^+$ during the heat treatment protected the NADPH-dependent ODH activity, as indicated by a small increase in the temperature required for inactivation, but made it impossible to measure NADH-dependent activity as described below.

Purification and properties of Vinca *W1 NADPH-ODH*

A protocol based on the above procedures was used to purify the *Vinca* tumor ODH activity. After ammonium sulfate fractionation, the enzyme was further fractionated by sequential chromatography on NADP-Agarose with $NADP^+$ as eluant, Cellex-D and Sephadex G-100. Results of a typical purification are given in Table 1. Final purifications of *ca* 300-fold were consistently obtained. However, a pressure-dialysis step, employed prior to application of the ODH to G-100, resulted in a loss of *ca* 75% of the ODH activity without apparent loss of protein, indicating that the ODH is inactivated by this procedure. Therefore, the final 300-fold purified ODH eluted from the G-100 column probably had a ratio of ODH-protein to total protein equivalent to *ca* 1200-fold purification.

The 300-fold purified ODH is relatively stable at pH 6.5–8.5, but at 4° loses most of its activity within 24 hr at lower or higher pHs. When this 'purified' enzyme was analysed by gel electrophoresis and stained for protein, several faint protein bands were obtained, none of which had the R_f of ODH. Since further purification did not seem practicable, the 300-fold purified preparations were used for further characterization of the enzyme.

Table 1. Purification of the *Vinca* W1 NADPH-dependent ODH

Purification step	Total activity (A_{340}/min)	Total protein (mg)	Specific activity (A_{340}/min · mg)	Yield (%)	Purification factor
Initial extract	77.0	280	0.27	100	1
$(NH_4)_2SO_4$	120.6	103	1.2	156	4.2
Affinity chromatography	40.5	1.09	37	53	137
Anion-exchange chromatography	42.0	0.62	68	55	251
Pressure dialysis	10.7	0.66	16	14	59
Gel permeation chromatography	9.9	0.12	82	13	304

Substrates

Many amino acids were effective substrates in lieu of arginine in the forward reaction (Table 2). Since the relative activities of these different amino acids as substrates depend greatly on the pH (see below), compounds supporting 10% of the activity observed with arginine may reasonably be assumed to be potential *in vivo* substrates. The characteristics of the 11 such substrates suggest that enzyme binds amino acids with side chains of 3–6 carbons in length having a carbon terminal group which is basic or capable of donating electrons to a hydrogen bond. There seems to be a strict requirement for both the α-carboxyl and the α-amino group, as well as the L-configuration at the α-carbon. The lack of activity with methyl octopine as substrate shows that, as expected for a dehydrogenase attacking an imino group, a free hydrogen at this position is essential for the oxido-reduction to take place. The high K_i for methyl octopine suggests that this hydrogen is involved in the binding of the enzyme to the imino acid.

Unlike arginine, which saturates but does not inhibit the enzyme at higher concentrations (e.g. above 100 mM when the pyruvate concentration is 3 mM), pyruvate inhibits the reaction at relatively low concentrations (e.g. 3 mM when the arginine concentration is 50 mM). For this reason, substrates tested in lieu of pyruvate were added at 3 mM. As this concentration is near the K_m for pyruvate, the relative activities obtained for these substrates (Table 3) may not represent a true V_{max}. The list of active compounds indicates that to substitute for pyruvate, a compound must be an α-ketoacid with a short (0–3 carbons) alkyl side chain.

pH optima

The *Vinca* ODH exhibits maximal activity with arginine and histidine between pH 6.0 and 6.6, while the optimum for lysine and ornithine occurs at *ca* pH 5.2. A shoulder or second peak of activity appears in the curves of lysine and ornithine just above the optima (at pH 5.5–6.0) and another, smaller shoulder or auxiliary peak at *ca* pH 7. The pH optimum for octopine oxidation is *ca* pH 9.2.

Affinity constants of several substrates

Because the activity vs pH curves for four basic amino

Table 2. Substrates in lieu of arginine or octopine for the *Vinca* W1 ODH and inhibition constants for certain inactive compounds

Substrate	Relative activity*	I_{50}†
L-Arginine	100	nd
L-Histidine	78	nd
L-Citrulline	73	nd
L-Methionine	67	nd
L-Homoarginine	62	nd
L-Canavanine	56	nd
L-Lysine	31	nd
L-Ornithine	30	nd
L-Cysteine	27	nd
L-Serine	25	nd
L-Glutamine	18	nd
L-Leucine	8	nd
L-Alanine	7	nd
L-Asparagine	7	nd
L-Threonine	5	nd
L-Valine	5	nd
L-Aspartate	0	nd
L-Glutamate	0	nd
L-Glycine	0	nd
L-Phenylalanine	0	nd
L-Tyrosine	0	nd
L-Argininic acid	0	not inhibitory
L-Agmatine	0	not inhibitory
L-Arginine, Me-ester	0	not inhibitory
D-Arginine	0	not inhibitory
D-Octopine	6	nd
D-Lysopine	2	nd
Methyl octopine	0	5 mM

*Forward reactions were measured at pH 6.4 with NADPH at 0.066 mg/ml, and pyruvate at 3 mM. Reverse reactions were measured at pH 9 with $NADP^+$ at 0.5 mg/ml. Concentrations of amino acids were 50 mM and of opine 10 mM.

†Concentration required to reduce activity by 50% when arginine is at 50 mM or octopine at 10 mM. Not inhibitory indicates no inhibition at 10 mM. nd, Not determined.

Table 3. Keto-compounds as substrates for *Vinca* W1 ODH and inhibition constants for certain inactive compounds

Substrate	Relative activity*	I_{50}†
Pyruvate	100	nd
α-Ketobutyrate	21	nd
Glyoxylate	5	nd
α-Ketovalerate	1	nd
α-Ketocaproate	0	nd
α-Ketopimelate	0	nd
Oxaloacetate	0	nd
α-Ketoglutarate	0	not inhibitory
α-Ketomalate	0	not inhibitory
Propionate	0	not inhibitory
Acetate	0	not inhibitory
Acetone	0	not inhibitory
Lactate	0	not inhibitory
Oxamate	0	20 mM

*Reactions were measured at pH 7.0 with NADPH at 0.2 mg/ml, arginine at 50 mM, and other substrates at 3 mM.

†Concentration required to reduce activity by 50% under these conditions with pyruvate as substrate. Not inhibitory indicates no inhibition at 18 mM. nd, Not determined.

acids suggested that they were most active at an acidic pH, the K_m for each was determined at both pH 7 and the pH for optimal activity (Table 4). The results indicate that the higher activity observed at the lower pH is due to a higher V_{max} rather than to a lower K_m. These amino acids have a greater apparent affinity for the enzyme at pH 7.0 than at the more acidic pH optima. For this reason, all other K_m determinations were made at pH 7.0.

Michaelis–Menten kinetics were observed for all substrates except at high concentrations of the following: (a) amino acids with $K_m > 10$ mM, where apparent inhibition of ODH activity occurred at concentrations above the K_m; and (b) α-ketoacid substrates, all of which inhibited the enzyme at concentrations greater than twice the K_m.

Table 4. K_m s of various substrates

Substrate	K_m(mM)	Assay conditions*	pH
L-Ornithine	0.70	(1)	7.0
L-Lysine	1.00	(1)	7.0
L-Glutamine	2.70	(1)	7.0
L-Arginine	5.1	(1)	7.0
L-Canavanine	3.9	(1)	7.0
L-Cysteine	5.4	(1)	7.0
L-Citrulline	22	(1)	7.0
L-Methionine	22	(1)	7.0
L-Histidine	23	(1)	7.0
L-Lysine	⩾300	(1)	5.2
L-Ornithine	⩾200	(1)	5.2
L-Arginine	66	(1)	6.4
L-Histidine	61	(1)	5.8
D-Octopine	0.095	(2)	9.0
D-Lysopine	0.050	(2)	9.0
Pyruvate	0.88	(3)	7.0
Glyoxylate	1.8	(3)	7.0
α-Ketobutyrate	4.3	(3)	7.0

*Concentrations: (1) Pyruvate = 3 mM, NADPH = 66 μg/ml; (2) $NADP^+$ = 500 μg/ml; (3) arginine = 50 mM, NADPH = 66 μg/ml.

The relative K_ms of the *Vinca* ODH for the basic amino acids lysine, ornithine, arginine and histidine (Table 4) suggest that the enzyme is more sensitive to the size of the side group than to its basicity. The intermediate K_ms of the non-basic amino acids are consistent with this observation. The K_ms of the three α-ketoacids tested fall in a similar range to those of the more active amino acids; pyruvate, the presumed natural substrate, had the lowest K_m (Table 4).

The reaction rates at high substrate concentrations for octopine and lysopine at pH 9.0 are *ca* 20-fold lower than those for arginine and lysine in the reverse reaction at pH 6.4 (Table 2). However, the apparent affinity constants for lysopine and octopine at pH 9.0 are at least 20-fold lower than those for arginine and lysine at pH 7.0 (Table 4). Therefore, *in vivo*, the enzyme probably is poised to function in either direction depending on the relative concentrations of substrates and the pH in the microenvironment of the enzyme.

It is noteworthy that for the two opines tested, lysopine bound more strongly to the enzyme than octopine, and lysine more strongly than arginine. However, the V_{max}s of arginine and octopine are *ca* three times that of lysine and lysopine, respectively (Table 2). This suggests that the products of *Vinca* ODH *in vivo* are strongly dependent on the relative size of the lysine and arginine pools. Also, given the kinetic characteristics of the enzyme with ornithine, cysteine and glutamine, one would anticipate that opines based on these amino acids may be detected in *Vinca*.

Interdependence of the K_ms for arginine and pyruvate

The K_m of the *Vinca* W1 ODH for arginine was determined at three different concentrations of pyruvate

Table 5. Dependence of the *Vinca* W1 ODH K_m s for arginine and pyruvate on the concentration of the other substrate

Pyruvate concentration (mM)	K_m of arginine (mM)
25	1.3
3.1	5.0
0.2	19.0
Arginine concentration (mM)	**K_m of pyruvate (mM)**
50	0.9
1.6	1.8
0.2	2.5

Assay conditions: pH 7; NADPH concentration = 0.066 mg/ml.

and vice versa (Table 5). Interdependence was observed, the K_m of arginine being especially sensitive to the pyruvate concentration, decreasing nearly 15-fold over a 125-fold increase in the concentration of pyruvate.

Coenzyme kinetics

Enzyme extracts purified *ca* 20-fold by ammonium sulfate fractionation and anion-exchange chromatography were used to determine coenzyme kinetics. This permitted determinations without interference from the $NADP^+$ required to maintain ODH activity in both the affinity and molecular sieve chromatography.

Velocity vs coenzyme concentration curves for the oxidized and reduced forms of both coenzymes were obtained and the K_m and V_{max} determined (Table 6). The K_m for $NADP^+$ was 0.15 ± 0.01 mg/ml with 6 mM octopine as substrate, as determined with three different enzyme preparations. The K_m for NADPH was sufficiently low that accurate measurements were not possible (initial rate measurements could only be made for short periods at concentrations of NADPH near the K_m) but it is of the order of 1 μg/ml.

The apparent K_m of *Vinca* W1 ODH for NADH and NAD^+ varied with the preparation, that for NADH varying from 0.07 ± 0.01 to 0.7 ± 0.1 mg/ml, and that for NAD^+ from 0.7 to 7.0 mg/ml. In each preparation, the K_ms obtained for NAD^+ or NADH were at least 10-fold higher than that for $NADP^+$ or NADPH, respectively.

$NADP^+$ and NAD^+ were also tested to determine if they would act as inhibitors of NADH-dependent activity. NAD^+ was non-inhibitory, even at 100 μg/ml, but $NADP^+$ was a very effective competitive inhibitor with a K_i of 1.0 μg/ml at pH 7.0.

The combination of NADH and NADPH at total coenzyme concentrations above 0.2 mg/ml reduces the apparent ODH acaivity (Figs. 4 and 5). At concentrations between 0.001 and 0.2 mg/ml, NADPH is virtually saturating for the enzyme (Fig. 4) and the addition of NADH at 0.2 mg/ml inhibits the activity at all concentrations of NADPH. NADH alone gives optimal ODH activity at *ca* 0.2 mg/ml (Fig. 5) and the addition of NADPH, even at very low concentrations, reduces ODH activity. This substrate inhibition observed with both coenzymes seems to be additive: the higher the NADPH concentration, the lower the concentration of NADH required to obtain inhibition and vice versa.

The complex behavior of the *Vinca* ODH in response to various concentrations of different coenzymes suggested a possible explanation for the unusual variations in apparent NADH-dependent ODH activity during purification. Retention of low levels of either $NADP^+$ or NADPH from the tissue when the ODH is extracted, and subsequent loss or partial loss of this coenzyme at the anion-exchange step, could account for the enhanced but variable recovery of NADH-dependent ODH activity. In fact, $NADP^+$ was found to elute from the Cellex-D column at *ca* 0.08 M sodium chloride and NADPH at *ca* 0.15 M sodium chloride, both peaks partially overlapping that of the ODH which elutes at *ca* 0.12 M sodium chloride. Also, while the NADPH-dependent ODH activity in ammonium sulfate fractionated ODH is proportional to the amount of enzyme used, NADH-dependent activity follows a convex curve as the amount of enzyme is increased, a response typical of the presence of an endogenous inhibitor. To further test this possibility, an ammonium sulfate fractionated preparation of ODH was treated with a crude preparation of NADase to remove the nicotinamide group from any $NADP^+$ or NAD^+ present. Although the NADase treatment reduced both activities, the ratio of NADH-dependent ODH to NADPH-dependent ODH changed from 2.2 to 3.5, consistent with the proposed presence of endogenous coenzyme.

DISCUSSION

Evidence presented in a previous paper [10] suggested the presence of an NADH-specific ODH and an NADPH-specific ODH in *Vinca* W1 tumor extracts. Efforts to separate these two enzymes point to some artifact of the preparations rather than separate enzymes being responsible for this result. Activities with both coenzymes were co-purified by fractional precipitation with ammonium sulfate and both co-eluted at the same

Table 6. Coenzyme kinetic parameters of the *Vinca* W1 ODH

Coenzyme	pH	Relative V_{max}	K_m(mg/ml)	K_i*
$NADP^+$	9.0	5	0.015	1 μg/ml
NADPH	7.0	100	0.001–0.01	nd
NAD^+	9.0	≤25	0.7–7.0	≥100 μg/ml
NADH	7.0	≤500	0.07–0.7	nd

*With NADH as coenzyme.

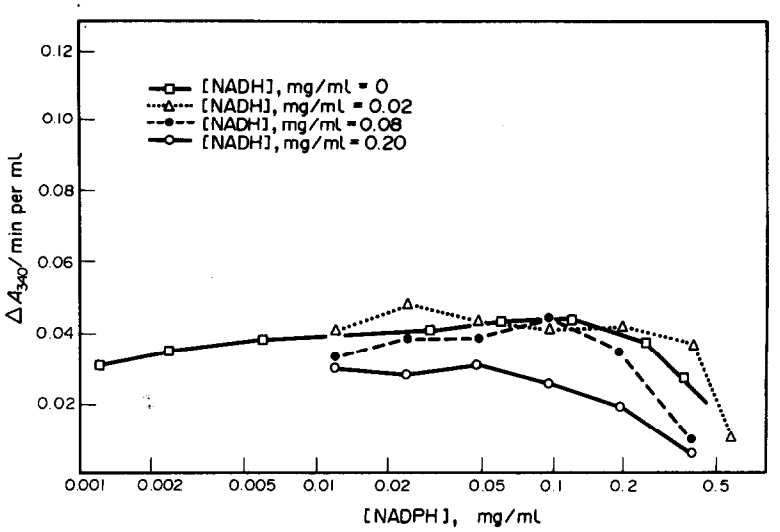

Fig. 4. Activity of *Vinca* W1 ODH as a function of NADPH concentration and in the presence of different concentrations of NADH. The four curves show results for NADPH alone (□——□); in the presence of 0.02 mg/ml NADH (△.....△); 0.08 mg/ml NADH (●---●); and 0.2 mg/ml NADH (○——○).

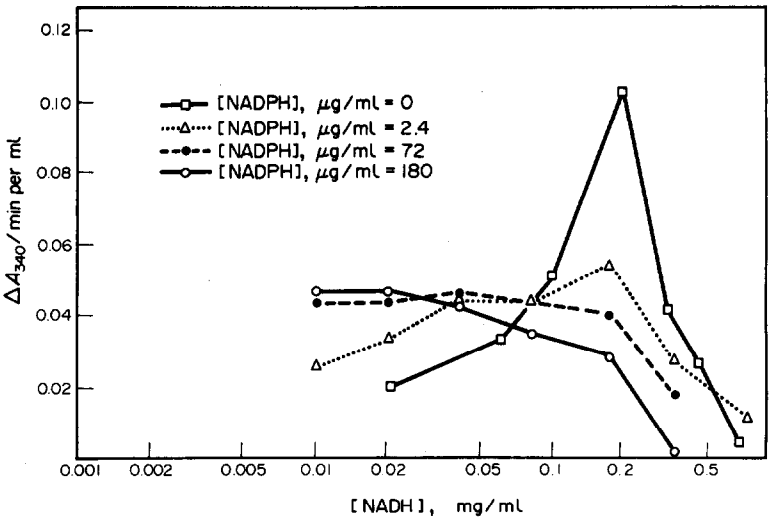

Fig. 5. Activity of *Vinca* W1 ODH as a function of NADH concentration and in the presence of different concentrations of NADPH. The four curves show results for NADH alone (■——■); in the presence of 2.4 μg/ml NADPH (△.....△); 72 μg/ml NADPH (●---●); and 180 μg/ml (NADPH (○——○).

volume from columns of molecular sieve (Fig. 1) and hydroxyapatite (data not shown). Only an $NADP^+$-dependent ODH could be detected on gels following polyacrylamide gel electrophoresis. Anion-exchange chromatography (Fig. 3) did yield two active fractions, but both contained NADH- and NADPH-dependent ODH activity in approximately the same ratio. Moreover, the smaller of these active fractions seems to be an artifact resulting from either degradation or aggregation of the ODH since rechromatography of the principal active fraction gives rise to both fractions.

Affinity chromatography on NAD- or NADP-Agarose (Fig. 2) also yielded evidence for only a single ODH, one which accepts both coenzymes but which has a much higher affinity for NADP(H). While the NADP-Agarose column typically bound more than half the ODH activity in crude extracts, none of the ODH bound to the NAD-Agarose column under otherwise identical conditions. Enzyme bound to NADP-Agarose and eluted with sodium chloride was active with both coenzymes and, like the major anion-exchange ODH fraction, showed greater activity with NADH than with NADPH (data not shown).

The ratio of NADPH-dependent activity to NADH-dependent activity for ODH purified by ammonium sulfate fractionation and elution from Cellex-D anion-exchange columns varied eight-fold. Different ODH preparations had apparent K_ms for both NADH and NAD^+ that varied by at least 10-fold while the K_ms for NADPH and $NADP^+$ were similar in all determinations. Regardless of oxidation state, K_ms for the triphospho-

pyridine nucleotides were consistently lower than for diphosphonucleotides. Recovery of NADPH activity was never greater than 72%, while that for NADH was at least 140% in all experiments. This implies that the *Vinca* W1 ODH in crude extracts has a several-fold greater V_{max} with NADH than with NADPH, but a considerably greater affinity for the latter.

The consistent recovery of more than 100% of the initial NADH-ODH activity after anion-exchange chromatography suggests the presence of an inhibitor that is largely removed by this fractionation. Unlike the straight line relationship of velocity vs NADPH concentration, the velocity vs NADH concentration curve was convex. This suggests a possible explanation for the apparent specificity of this inhibitor. Because the NADPH activity was not affected significantly by this inhibitor, it must be either: (a) a competitive inhibitor with greater affinity for the enzyme than NAD(H) but not NADP(H); or (b) an allosteric inhibitor which reduces activity of the enzyme with NADH. This second possibility is unlikely because, as discussed below, the *Vinca* W1 ODH has a MW of 38 000 and, therefore, appears to be a monomeric enzyme too small to exhibit an allosteric binding site. Also, even after the apparent removal of most of the inhibitor by anion-exchange chromatography, the ODH still shows a very large K_m for NADH and NAD^+ (typical coenzyme K_ms with dehydrogenases are 0.0001–0.05 mM for the reduced form and 0.01–0.1 mM for the oxidized form [16]). This strongly suggests that the inhibitor does not increase the K_m for NADH or NAD^+.

The simpler explanation, that a competitive inhibitor is present in the crude and partially purified enzyme preparations, seems probable. This inhibitor would need a K_i which is less than the K_m for NAD(H). Since $NADP^+$ and NADPH were shown to inhibit the NADH-supported reaction, it is possible that tumor-derived $NADP^+$ and/or NADPH is the inhibitor in these extracts. Both forms of the coenzyme are present in plant extracts at a concentration of *ca* 10 nM [17] and elute from Cellex-D near ODH. Thus, slight variations in the concentration of NADP(H) in the crude extract and in their relative elution volumes could account for the observed results. In fact, NADase, which degrades $NADP^+$, increased the apparent activity with NADH relative to NADPH. Therefore, competition by tissue $NADP^+$ and/or NADPH is the presumed basis for the somewhat anomalous recovery of NADH-ODH activity after anion-exchange chromatography.

Hack and Kemp [12, 15] found that the crude extract of cultured sunflower B6 galls showed ODH activity with either NADH or NADPH, as did the purified enzyme, and concluded that only one enzyme was present. The sunflower ODH had a much higher K_m for NAD(H) than for NADP(H) and also a higher V_{max} for NADH than for NADPH, similar to the *Vinca* ODH results. Goldmann [11] and Otten *et al.* [13, 14] also detected only a single ODH (utilizing either NADH or NADPH) in cultured *Scorzonera* A6 and tobacco B6 crown gall tissue, respectively. Interestingly, Goldmann [11] reported differences in the relative ODH activity with NADH or NADPH that varied with the substrate amino acid. Different pH optima for the reaction with different amino acids and different coenzymes, or the unusual coenzyme kinetics we observed with crude extracts might account for her [11] results.

Bomhoff's report [18] of an NADH-specific ODH was not confirmed by a later publication from the same laboratory [13] which describes an ODH preferentially using NADP(H). Lejeune and Jubier's [9] earlier paper describing an NADH-specific lysopine dehydrogenase may be a manifestation of the greater lysopine dehydrogenase activity with NADH than with NADPH reported by Goldmann [11]. The spectrophotometric assay used in Goldmann's work, as opposed to the radiometric one employed by Lejeune and Jubier, might also have contributed to this difference.

The MW of *Vinca* W1 ODH, estimated by gel filtration (Fig. 1) to be 38 000, agrees with the results of Goldmann [11] and Hack and Kemp [15] for tobacco and sunflower ODHs, respectively. Otten [14] reported an apparent MW of 31 000 for tobacco ODH from similar measurements. The very high ammonium sulfate concentration of 400 mM, which was used to elute the enzyme, may be responsible for this difference. The MW of Otten's ODH estimated by polyacrylamide gel electrophoresis was 38 000 [14].

The highly purified *Vinca* W1 ODH, obtained by the protocol described here, contained less than 0.1% of the protein in crude extracts but when examined by gel electrophoresis it appeared at most, 1% pure. Such preparations, however, were essentially free of non-specific dehydrogenase activity, and the reaction kinetics with arginine or histidine as substrates were the same as with less purified samples (data not shown). This constancy indicates that the kinetic constants obtained would not be altered by further purification and that the kinetic values determined with less pure preparations are valid.

Unlike most animal ODHs, which accept only arginine, homoarginine, or canavanine as amino acid substrates, the *Vinca* W1 enzyme (Tables 2 and 4) and other crown gall ODHs [11, 13–15] accept a broad range of amino acids. Only L-amino acids are substrates and these must have an R-group of 2–6 carbons containing a basic or an electron-donating group. In animals, only the sea anemone ODH has been reported to accept a similar broad range of amino acids as substrates [7, 8].

The *Vinca* W1 ODH does have somewhat different affinities for the amino acids than other ODHs reported (Table 4) [11, 13–15]. Histidine, a good substrate with the other plant tumor ODHs, is the poorest substrate for the *Vinca* W1 ODH; ornithine and glutamine are especially good substrates for the *Vinca* enzyme.

The animal and plant ODHs are all very similar in the range of ketones accepted as substrates, as shown here and by others [6–8, 11, 13, 15]. Only pyruvate, glyoxylate, α-ketobutyrate and α-ketovalerate are active, and the latter three are poor substrates relative to pyruvate. The K_m for pyruvate is the lowest and the V_{max} by far the highest with all the enzymes reported. It is also a more abundant plant metabolite and, therefore, should be considered the natural substrate for this enzyme. The absence of reports of octopine-type tumors containing reductive condensation products of amino acids with the other three α-ketoacids supports this conclusion.

It is not clear which amino acid(s) is the 'natural' substrate for the plant enzymes. Ornithine and lysine have the lowest K_ms with *Vinca* ODH, as was also found for the sunflower, tobacco and *Scorzonera* ODHs [11, 13–15]. However, the V_{max}s for lysine and ornithine are also relatively low. The K_ms of the amino acids vary considerably more than does the ratio of K_m to V_{max}, as noted by Hack and Kemp [15] for the sunflower ODH. This seems to indicate that, depending on abundance, any one

of these amino acids may be the preferred substrate. Lysopine, octopinic acid, octopine and histopine have all been reported in crown gall tissue [1, 19–21]. Other octopine-type opines may be present [Chang, C. C., personal communication].

The small size of the *Vinca* tumor ODH implies that it is monomeric, like the ODHs of animals [22]. The smallest known dehydrogenase, pea formaldehyde dehydrogenase, has a MW of 19 000, about the minimum required to form the 'NADH fold' (*ca* 12 000) plus a binding site for formaldehyde [23]. The NADPH-accepting *Vinca* W1 ODH needs a larger coenzyme fold and a much larger binding site to accommodate octopine as opposed to formaldehyde. At 38 000 its MW is probably near the minimum necessary to catalyse the ODH reaction and, by implication, contains only a single catalytic site and no allosteric site.

As with the scallop ODH, the K_m for arginine with the *Vinca* W1 ODH depends on the concentration of pyruvate and, similarly, the K_m of pyruvate varies with the concentration of arginine (Table 5) [6]. This implies that, as with the animal enzyme, the catalytic site includes a site for some sort of arginine–pyruvate adjunct. This does not mean that arginine and pyruvate are necessarily joined before binding, as suggested initially for scallop ODH by van Thoai *et al.* [6], but merely that the holoenzyme–arginine–pyruvate complex involves a coordinated binding of arginine and pyruvate. This coordinated binding of amino acid and α-ketoacid manifests itself in another way. The K_m of the amino acid can depend on which α-ketoacid is supplied as substrate and the K_m of the ketoacid on which amino acid is supplied [14, 15]. These reports indicated that amino acid K_ms were lower with pyruvate as the ketoacid than with the other ketoacids, and that ketoacid K_ms were lower with lysine and ornithine as substrate than with arginine and histidine. With more strongly binding amino acids more of the enzyme should be in the form that binds α-ketoacids strongly (holoenzyme–amino acid) than in the one that binds weakly (holoenzyme alone). Similarily, the K_m for the amino acid should depend on the binding strength of the ketoacid.

Like most dehydrogenases, the *Vinca* ODH binds to coenzyme first, as demonstrated by binding of the apoenzyme to NADP-Agarose (Fig. 2) in the absence of arginine or pyruvate. Unlike the scallop enzyme [24], the *Vinca* ODH is not mnemonic, as high concentrations of $NADP^+$ do not inhibit oxidation of octopine (data not shown).

In summary, the *Vinca* W1 ODH, similar to other crown gall ODHs [11, 13, 15], appears to be a monomeric protein capable of generating a wide range of octopine-type opines using either NADPH or NADH as coenzyme. Unlike other plant ODHs [14, 15], the *Vinca* W1 ODH demonstrates substrate inhibition at high concentrations of either NADH or NADPH and appears to be different in the relative affinities of amino acid substrates. This suggests that the ODHs of different crown galls vary in their primary structure.

EXPERIMENTAL

Tumor tissues and enzyme extracts. Crown gall tissue of *Vinca rosea* induced by *Agrobacterium tumefaciens* strain W1 was cultured on White's 10X medium and enzyme extracts prepared as described in ref. [10]. The enzyme extraction buffer (TDEG buffer) was 0.05 M Tris–HCl, pH 8, containing 10 μM dithiothreitol and 100 μM EDTA plus 5% glycerol. Tissues frozen in liquid N_2 and stored at −80° were also used and showed no apparent difference in the levels of enzyme activity. For enzyme preparations from tissue samples greater than 10 g, homogenization was in a Waring blender for three 1 min bursts with cooling between bursts.

Radiometric estimation of ODH activities. Octopine formation was assayed radiometrically as described previously [10]. For the radiometric assay of octopine oxidation, the reaction mixture was 25 μl of enzyme extract, 5 μl $NADP^+$ at 200 mg/ml, 10 μl 0.5 M glycine–NaOH, pH 9, and 10 μl [^{3}H]octopine (*ca* 200 000 dpm with sp. act. of 16.7 Ci/mmol). The reaction was stopped by adding 100 μl cold, redistilled EtOH and cooling at −22° for at least 15 min. The mixture was centrifuged at 12 000 *g* for 15 min and an aliquot of the supernatant applied to a small (5 × 20 mm) anion-exchange resin column (Biorad Ag-2 hydroxide form, in H_2O). Any [^{3}H]arginine formed was eluted with 3 ml H_2O, and the eluate collected directly in a 20 ml scintillation vial; at least 98% of the substrate octopine remained bound to the column under these conditions. Radioactivity was estimated by scintillation spectrometry. Typically, 2000–3000 cpm were obtained in the arginine fraction with active enzyme. The reaction was linear for at least 150 min.

Spectrophotometric assay of the synthetic reaction. The reaction mixture was 150 μl of enzyme extract, 10 μl of either NADPH at 3.3 mg/ml or of NADH at 10 mg/ml, 50 μl α-ketoacid (30 mM Na pyruvate, unless otherwise indicated), and 350 μl of reaction buffer (0.1 M NaPi, pH 7.0) in a 1-ml cuvette at room temp. Either 50 μl H_2O (control) or of amino acid (0.5 M arginine–HCl, pH 7, unless otherwise indicated) was added to initiate the reaction. Change in A at 340 nm was followed and enzyme activity estimated from the difference between the rate of decrease in A in the presence and in the absence of arginine. Reactions were linear to at least a change of 0.5 A. Typically, activity was sufficient to provide a change in A of 0.002 to 0.02 per min. For assays involving less activity, the reaction was recorded for at least 10 min before adding amino acid, and again for at least 10 min after addition to the same cuvette.

Spectrophotometric assay of the oxidative reaction. The reaction mixture was 200 μl of enzyme extract, 10 μl of either $NADP^+$ at 25 mg/ml or NAD^+ at 50 mg/ml and 250 μl of reaction buffer (0.2 M glycine–NaOH, pH 9). Either 50 μl of H_2O (control) or of 100 mM octopine or lysopine were added and the difference in rates of change of A_{340} used to estimate activity as above. Typically, activities were 0.001–0.01 A/min.

Determination of pH optimum. Enzyme activity as a function of pH was determined using the above assays with the following modifications in the reaction mixtures. The reaction mixture for the synthetic reaction contained 200 μl 0.5 M NaPi at the indicated pH, 100 μl 0.5 M amino acid, 50 μl Na pyruvate at 30 mM, 10 μl NADPH at 3.3 mg/ml, 100 μl H_2O and 50 μl enzyme. The pH optimum for the oxidative reaction was determined using the radioactive assay with 25 μl enzyme, 10 μl [^{3}H]octopine, 5 μl $NADP^+$ at 200 mg/ml and 10 μl 0.5 M glycine–NaOH adjusted to the desired pH. The pH did not change during the reaction.

Measurement of K_ms. The K_ms of the ODH for different substrates were measured using the standard spectrophotometric assays and varying the concn of the substrate in question. The data were plotted by the Lineweaver–Burk method and a line fitted by linear regression. K_is were determined similarily, with $(1/V)$ vs $(1/S)$ determined at three concns of inhibitor as well as without inhibitor.

Enzyme purification. All enzyme purification steps were carried out at 4°. Unless otherwise noted, TDEG buffer was used with

appropriate additions for CC. Enzyme preparations were stored at −22° at various stages of purification in 50% TDEG buffer–50% glycerol which contained 1 mg/ml $NADP^+$.

$(NH_4)_2SO_4$ fractionation. Partial pptn of proteins in the crude extracts was achieved by rapidly mixing appropriate amounts of solid $(NH_4)_2SO_4$ with the enzyme extract. In the purification protocol, the enzyme was brought to 1.95 M $(NH_4)_2SO_4$, incubated 4 hr at 4° and, after centrifugation at 20000 *g* for 1 hr, the supernatant was decanted. This supernatant was then brought to 2.83 M $(NH_4)_2SO_4$, incubated at 4° for at least 4 hr (usually overnight) and the ppt collected by centrifugation as above. The enzyme ppts were resuspended in 1–2 ml of buffer, dialysed against 1 or 2 changes of 100–200 vols. of TDEG buffer for 2–4 hr and clarified by centrifugation. ODH activity was found exclusively in the 1.95 M supernatant and the 2.83 M ppt (referred to as the 50–70% fraction).

Affinity chromatography. Columns of NADP-Agarose and NAD-Agarose (linkage at the position 8 of adenine, Type 3, P&B Biochemicals) were prepared in TDEG buffer and a 5 × 35 mm column bed prepared. Enzyme in a vol. of 1–10 ml and containing less than 60 mg protein was applied, the column washed with *ca* 16 ml TDEG buffer and the enzyme eluted with TDEG buffer containing 1 mg/ml $NADP^+$, 1 mg/ml NAD^+, or 0.5 M NaCl. Fractions (1–2 ml each) with less than 15% of the ODH activity of the peak fraction were discarded and the rest pooled and saved for further tests.

Gel permeation chromatography. A column of Sephadex G-100 (1 × 120 cm) was prepared according to the manufacturer's instructions and equilibrated with TDEG buffer containing 1 mg/ml $NADP^+$. Enzyme in a vol. of 0.5–2 ml (density adjusted to above 1.05 mg/ml with sucrose) and containing less than 100 mg of protein was applied to the column. The column was eluted with TDEG buffer containing 1 mg/ml $NADP^+$ and, after the first 30 ml, 1 ml fractions were collected. Fractions having at least 15% of the activity of the peak ODH fraction were pooled, an equal vol. of glycerol added, the $NADP^+$ concn restored to 1 mg/ml and the enzyme stored at −22°.

The Sephadex column was calibrated using a mixture of 8 mg of Fraction V bovine serum albumin, 8 mg of × 3-recrystallized ovalbumin, 1.6 mg myoglobin, and *ca* 3 μkat of scallop ODH dissolved in 0.5 ml 12% sucrose. The mixture was applied to and eluted from the column as above except $NADP^+$ was omitted from the buffer. The void vol. was 43 ml as determined with Blue Dextran.

Anion-exchange chromatography. Cellex-D (Biorad), a chromatographic matrix of cellulose with diethylaminoethanol groups covalently attached, was 'ultrapurified' according to the manufacturer's instructions and a column bed of 15 × 110 mm prepared from gel swollen in TDEG buffer. Up to 100 mg protein was applied in vol. of 1–100 ml and protein eluted, first with TDEG buffer containing 0.01 M NaCl and then with a 200 ml linear gradient of 0.01–1.0 M NaCl in TDEG buffer. Fractions of 1–2 ml were collected and the concn of NaCl in each fraction determined refractometrically. Fractions having at least 15% of the ODH activity of the peak fraction were pooled, an equal vol. of glycerol added, the $NADP^+$ concn restored to 1 mg/ml and the enzyme stored at −22°.

Hydroxyapatite chromatography. A 15 × 55 mm column bed of hydroxyapatite (Biorad) was equilibrated in a buffer of 0.01 M NaPi, pH 8.0, 100 μM EDTA, 10 μM dithiothreitol and 5% glycerol. Enzyme (2 ml, containing less than 20 mg protein) was applied to the column and eluted by washing the column with the equilibrating buffer.

Polyacrylamide gel electrophoresis. The electrophoretic separation of ODH was determined as described in ref. [25]. Briefly, 7% acrylamide gels (0.5 × 6 or 9 cm) and a pH 8.9 Tris–glycine buffer (0.19 M) were employed. Enzyme in 12% sucrose was applied to each gel in a vol. of 25–200 μl. A tracking dye, bromophenol blue (20 ppm) and β-mercaptoethanol (5 mM) were added to the upper reservoir and the gels electrophoresed at 1.25 mA/gel until the dye formed a thin band; the current was then doubled. Electrophoresis was terminated when the dye had migrated *ca* 90% of the length of the gel. In some expts, separation gels were prepared as described in ref. [25] and then pre-electrophoresed for 3 hr at 2.5 mA/gel with β-mercaptoethanol in 9.15% (w/v) Tris, 0.057% (v/v) *N,N,N',N'*-tetramethylethylenediamine and 0.12 M HCl in both the upper and lower reservoirs. The stacking gel was then polymerized on top of the pre-electrophoresed separation gel and used for ODH separation.

The gels were stained for ODH by incubation for 4–5 hr in the dark at room temp. in a soln containing 0.5 M glycine, pH 8.9, $NADP^+$ or NAD^+ at 3 mg/ml, nitroblue tetrazolium at 0.8 mg/ml and phenazine methosulfate at 0.15 mg/ml in the presence or absence of 25 mg/ml octopine. The reaction was stopped by transferring the gels to 7% HOAc.

Gels were stained for protein with Coomassie Brilliant Blue (0.2%) in MeOH–H_2O–HOAc (5:4:1) for at least 12 hr. The gels were destained in a soln of H_2O–MeOH–HOAc (13:5:2) containing nylon netting (60 cm^2 of commercial hosiery per l.). Destaining required 1–3 days at room temp. to achieve a constant pattern of bands. As little as 0.5 μg of bovine serum albumin could be detected after electrophoresis.

Protein assays. Total protein assays were performed according to ref. [26], or by using the Biorad Protein Reagent according to the manufacturer's instructions. High concns of glycerol interfered with both assays and for samples with little protein the protein was first isolated by pptn with 10% $HClO_4$.

Chemicals. L-[5-^{3}H]Arginine and D-[5-^{3}H]octopine were prepared as described previously [10]. Imino-*N*-methyl octopine (methyl octopine) was prepared by reductive condensation of formaldehyde and D-octopine with $Na(CN)BH_3$ as reducing agent as described in ref. [27]. $(NH_4)_2SO_4$ (enzyme grade) was from Sigma or Mallinckrodt. For most expts, coenzymes were from Sigma; the highest purity coenzymes from Boehringer gave similar results. D-Octopine was from Sigma. Lysopine was obtained from R. Manasse as the lysopine–isolysopine mixture [28] and results were corrected assuming the isolysopine to be unreactive.

Acknowledgement—This investigation was supported by Grant No. PCM 7809225 from the National Science Foundation.

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