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Oxidation of 3,4-dehydro-D-proline and other D-amino acid analogues by D-alanine dehydrogenase from *Escherichia coli*

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Abstract

3,4-Dehydro-DL-proline is a toxic analogue of L-proline which has been useful in studying the uptake and metabolism of this key amino acid. When membrane fractions from *Escherichia coli* strain UMM5 (*putA1*::Tn*5 proC24*) lacking both L-proline dehydrogenase and L-Δ^1-pyrroline-5-carboxylate reductase were incubated with 3,4-dehydro-DL-proline, pyrrole-2-carboxylate was formed. There was no enzyme activity with 3,4-dehydro-L-proline, but activity was restored after racemization of the substrate. Oxidation of 3,4-dehydro-DL-proline by membrane fractions from strain UMM5 was induced by growth in minimal medium containing D- or L-alanine, had a pH optimum of 9, and was competitively inhibited by D-alanine. An *E. coli* strain with no D-alanine dehydrogenase activity due to the *dadA237* mutation was unable to oxidize either 3,4-dehydro-D-proline or D-alanine, as were spontaneous Dad$^-$ mutants of *E. coli* strain UMM5. Membrane fractions containing D-alanine dehydrogenase also catalyzed the oxidation of D-2-aminobutyrate, D-norvaline, D-norleucine, *cis*-4-hydroxy-D-proline, and DL-ethionine. These results indicate that D-alanine dehydrogenase is responsible for the residual 3,4-dehydro-DL-proline oxidation activity in *putA proC* mutants of *E. coli* and provide further evidence that this enzyme plays a general role in the metabolism of D-amino acids and their analogues.

Keywords: D-alanine dehydrogenase; D-amino acid dehydrogenase; 3,4-dehydroproline; *Escherichia coli*; Proline analogue

1. Introduction

Escherichia coli is the major cause of urinary tract infections (UTIs) in adolescent and adult women and in older adults fitted with catheters [1]. While sulfamethoxazole/trimethoprim and fluoroquinolones can be used to treat UTIs, the incidence of antibiotic-resistant *E. coli* strains has been increasing [2]. The pathogenicity of *E. coli* is due in part to adhesive surface organelles that allow the bacteria to attach to and eventually invade epithelial cells [3]. It also may depend on the ability of the bacteria to accumulate osmotically compatible solutes such as L-proline and glycine betaine from the urine [4]. These solutes may counteract the dehydrating effect of increased osmolarity and protect against the toxicity of high concentrations of urea.

Most laboratory strains and clinical isolates of *E. coli* have three active transport systems that mediate the uptake of L-proline and glycine betaine [5,6]. The *putP* gene encodes a Na^+-dependent transporter whose synthesis is induced by L-proline. The *proP* gene codes for a H^+-dependent carrier whose formation and activity increase in hyperosmotic media; it also facilitates the uptake of glycine betaine. The *proU* operon, which consists of the *proV*, *proW*, and *proX* genes, encodes an ATP-dependent transporter for both proline and glycine betaine whose synthesis increases 100-fold during growth in hyperosmotic media. In addition, some pyelonephritis isolates such as *E. coli* strain HU734 contain

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an additional glycine betaine carrier called BetU [7]. The role of these transport systems in UTIs is still unclear. Deletion of *proP* and *proU* slowed the growth of strain HU734 in high osmolarity human urine but did not affect its colonization of the mouse urinary tract following transurethral inoculation [8]. Deletion of *proP* and *proU* from the clinical isolate CFT073 [9] had no effect on either its growth rate or experimental colonization activity [8].

The transport systems encoded by *putP*, *proP*, and *proU* also allow the uptake of a number of L-proline and glycine betaine analogues [5,10,11]. Accordingly, an alternative approach to treating UTIs may be to use toxic analogues of the normal solutes as antibiotics [12]. Among the L-proline analogues to which *E. coli* is most sensitive are L-azetidine-2-carboxylate, 3,4-dehydro-DL-proline, and L-thiazolidine-4-carboxylate. The sensitivity of *E. coli* to L-azetidine-2-carboxylate and L-thiazolidine-4-carboxylate increases with osmotic stress and is greater under aerobic conditions [13]. While the growth of *E. coli* can be inhibited by these analogues, the clinical effectiveness of these compounds may be limited as a result of their degradation by enzymes that normally act on proline. L-Proline is degraded by the *putA*-encoded membrane-associated enzyme L-proline dehydrogenase, which catalyzes both the FAD-dependent oxidation of proline to form Δ^1-pyrroline-5-carboxylate (P5C) and the NAD^+-dependent oxidation of P5C to form L-glutamate [14,15]. 3,4-Dehydro-DL-proline and L-thiazolidine-4-carboxylate are also substrates for L-proline dehydrogenase [16,17]. In addition, they can be degraded by the cytoplasmic enzyme Δ^1-pyrroline-5-carboxylate reductase, which normally catalyzes the last step in proline biosynthesis [18]. Wood [16] noted that membrane fractions from an *E. coli* mutant lacking L-proline dehydrogenase could still catalyze the oxidation of 3,4-dehydro-DL-proline using 2,6-dichlorophenolindophenol as an electron acceptor. I now report that this reaction reflects the oxidation of 3,4-dehydro-D-proline by another membrane-associated enzyme, D-alanine dehydrogenase [19–21]. This enzyme also catalyzes the degradation of several other D-amino acid analogues.

2. Materials and methods

2.1. Strains and culture conditions

E. coli strain UMM5 was constructed by transducing the *putA1*::Tn*5* mutation from strain JT31 [16] into strain X^{342}(*proC24 metB1 relA1 spoT1 bglF18*::IS*150*) with phage P1 [18]. *E. coli* strains EC972 (*araD139* Δ(*argF-lac*)*169flb-5301 trpB202 fruA25 relA1 rpsL150 metB185 deoC1*) and EC989 (*dadA237 araD139* Δ *(argF-lac)169flb-5301 trpB202 fruA25 relA1 rpsL150 metB185 deoC1*), which were originally described by Wild and Klopotowski [22], were obtained from the *E. coli* Genetic Stock Center. Spontaneous Dad^- derivatives of *E. coli* strain UMM5 were selected on the basis of resistance to β-chloro-D-alanine [22]. Strains EC972-1 and EC989-1 were constructed by transducing the *putA1*::Tn*5* mutation from strain JT31 [16] with phage P1 into strains EC972 and EC989. Bacteria were grown aerobically at 37 °C in Minimal Medium A with appropriate supplements as previously described [17].

2.2. Preparation of membrane fractions

Bacteria were growth to late exponential phase (A_{600} = 0.8–1.0) in minimal medium, harvested by centrifugation at 8200*g* in a Sorvall RC-5B refrigerated centrifuge, washed once with 0.85% NaCl, and stored as a pellet at −20 °C. The cells were thawed, suspended in Buffer B (10 mM MOPS, pH 7.5, 20 mM $MgCl_2$, 10% glycerol), and disrupted by three passages through a French Pressure Cell at 12,000 psi. Membrane fractions were isolated as previously described [23], stored in small aliquots at −80 °C, and used only once after thawing.

2.3. Assays

Oxidation of 3,4-dehydro-DL-proline was measured by the formation of pyrrole-2-carboxylate. Membrane fractions (5–20 μl) were combined with Buffer B to give a total volume of 900, 100 μl of 0.1 M 3,4-dehydro-DL-proline (Sigma) were added, and the absorbance at 255 nm was measured in a Shimadzu UV160U spectrophotometer for 5 min. The amount of pyrrole-2-carboxylate formed was determined using a molar extinction coefficient of 12,700 [24]. Oxidation of D-alanine and other D-amino acids was measured as described by Franklin and Venables [19] through the reduction of 2,6-dichlorophenolindophenol (DCPIP). Reactions contained 50 mM potassium phosphate buffer (pH 7.0), 10 mM KCN, 0.5 mM phenazine methosulfate, 0.1 mM DCPIP, and 30–50 mM amino acid substrate. The absorbance at 600 nm was measured in a Shimadzu 160-U spectrophotometer for 5 min, and the amount of DCPIP reduced was determined using a molar extinction coefficient of 21,500 [21]. L-lactate dehydrogenase activity was measured in the same way using 30 mM sodium L-lactate as the substrate. Activities with DCPIP were corrected for spontaneous reduction in the presence of Buffer B and the substrate. All enzyme assays were done in triplicate. Rates were normally determined from the first 2 min of activity and varied by <10%. Protein concentrations were determined by the bicinchoninic acid (BCA) method [25] using reagents from Pierce and bovine serum albumin as the standard, and specific activities were

expressed as nmol min^{-1} mg protein^{-1}. All experiments were done at least twice.

3. Results

3.1. Identification of a 3,4-dehydro-D-proline dehydrogenase activity in E. coli

When membrane fractions from *E. coli* strain UMM5 (*putA1*::Tn*5 proC24*) lacking both L-proline dehydrogenase and L-Δ^1-pyrroline-5-carboxylate reductase activities were incubated with 10 mM 3,4-dehydro-DL-proline, an oxidation product with the characteristics of pyrrole-2-carboxylate (P2C) was formed (Fig. 1). The product had an absorption spectrum identical to that of pure P2C with a λ_{max} of 255 nm [24], and clearly differed from 3,4-dehydro-DL-proline which had a λ_{max} of 220 nm. The product reacted with *p*-dimethylaminobenzaldehyde to form a pink complex [26] and gave the same R_f value (0.61) as P2C during paper chromatography in ethanol–water (77:23) [24]. P2C has been previously shown to be the product of 3,4-dehydroproline oxidation by a D-amino acid oxidase from hog kidney [27], by an L-amino acid oxidase from snake venom [28], and by proline oxidase (L-proline dehydrogenase) from rat liver mitochondria [29].

While the formation of P2C from 3,4-dehydro-DL-proline could be measured quantitatively in fixed-time assays by reaction with *p*-dimethylaminobenzaldehyde [29], it was more conveniently determined in continuous assays as an increase in absorbance at 255 nm [28]. In these assays, the formation of P2C was linear with time for at least 2 min and proportional to the volume of membranes. The 3,4-dehydro-DL-proline dehydrogenase activity was detectable in membrane fractions of *E. coli* strain UMM5 after growth in a minimal medium containing D-glucose, glycerol, or sodium succinate as carbon sources.

Surprisingly, there was no enzyme activity when membrane fractions from *E. coli* strain UMM5 were incubated with purified 10 mM 3,4-dehydro-L-proline as the substrate. However, when a solution of 3,4-dehydro-L-proline was adjusted to pH 11 with 0.1 M NaOH and heated at 90 °C for 2 h, conditions known to result in racemization and formation of 3,4-dehydro-D-proline [30], enzyme activity returned to 80% of that seen with 3,4-dehydro-DL-proline. Although 3,4-dehydro-D-proline could not be tested directly as a substrate because it is not commercially available, these results indicated that membrane fractions from *E. coli* strain UMM5 contain an enzyme that specifically oxidizes 3,4-dehydro-D-proline to pyrrole-2-carboxylate.

3.2. Biochemical analysis of 3,4-dehydro-D-proline dehydrogenase activity

E. coli contains a number of membrane-associated enzymes that oxidize organic substrates and transfer electrons to ubiquinone in the aerobic electron transport chain [31]. To identify the enzyme responsible for the oxidation of 3,4-dehydro-D-proline, membrane fractions were assayed for P2C formation in the presence of potentially competitive substrates (Table 1). Activity was not inhibited by sodium succinate, sodium

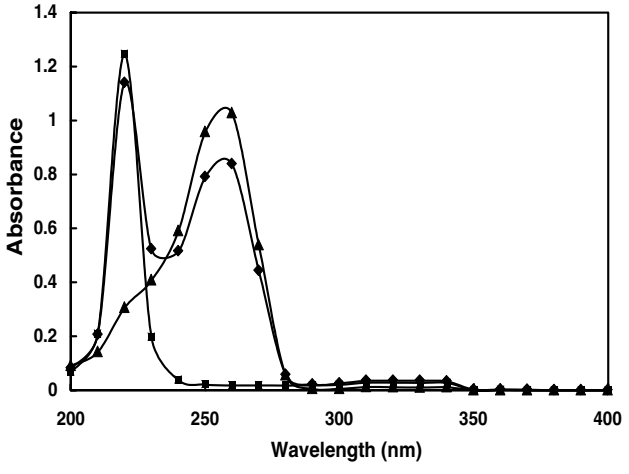

Fig. 1. Absorption spectra of 3,4-dehydro-DL-proline and pyrrole-2-carboxylate. Spectra were determined for a solution of Buffer B containing 10 μmoles of 3,4-dehydro-DL-proline (■), a solution of Buffer B containing 0.2 μmoles of pyrrole-2-carboxylate (▲), and a reaction mixture in Buffer B in which 23.3 μg of a membrane fraction from *E. coli* strain UMM5 were incubated with 10 mM 3,4-dehydro-DL-proline for 30 min (◆).

Table 1
Inhibition of 3,4-dehydro-D-proline dehydrogenase activity by potentially competitive substrates

Inhibitor	Concentration (mM)	Percent of control activity[a]
Na succinate	10	99.1
Na D,L-α-glycerophosphate	10	89.5
Na L-5,6-dihydroorotate	10	100
D-alanine	10	42.7
	3	85.2
L-alanine	10	96.7
Li D(−)-lactate	10	48.1
	3	66.7
	1	89.0
Na pyruvate	10	36.8
	3	78.7
	1	89.0
Li L(+)-lactate	10	4.9
	3	15.4
	1	37.2

[a] Membrane fractions were prepared from *E. coli* strain UMM5 after growth in minimal medium containing 50 mM sodium succinate. Oxidation activity was measured as an increase in A_{255} in the presence of 10 mM 3,4-dehydro-DL-proline. The control activity was 99.2 nmol of P2C formed min^{-1} mg protein^{-1}.

D,L-α-glycerophosphate, or sodium L-5,6-dihydroorotate. The oxidation of 3,4-dehydro-DL-proline was partially inhibited by D-alanine but not by L-alanine. It also was partially inhibited by lithium D(−)-lactate and sodium pyruvate and more strongly inhibited by lithium L(+)-lactate.

The oxidation of D-alanine and L-lactate in *E. coli* are catalyzed by the inducible enzymes D-alanine dehydrogenase and L-lactate dehydrogenase, respectively, while the oxidation of D-lactate is catalyzed by the constitutive enzyme D-lactate dehydrogenase [31]. Membrane fractions were prepared from *E. coli* UMM5 after growth in minimal media containing various substrates and tested for 3,4-dehydro-D-proline dehydrogenase, D-alanine dehydrogenase, and L-lactate dehydrogenase activities (Fig. 2). The specific activity of 3,4-dehydro-D-proline dehydrogenase increased after growth in media containing D-alanine or L-alanine and correlated with the specific activity of D-alanine dehydrogenase. It did not increase after growth in medium containing L-lactate, even through bacteria grown in this medium had much higher specific activity for L-lactate dehydrogenase. These results suggested that the oxidation of 3,4-dehydro-D-proline is mediated by D-alanine dehydrogenase.

D-alanine dehydrogenase (EC 1.4.99.1) in *E. coli* and the related bacterium *Salmonella typhimurium* (now called *S. enterica* serovar Typhi) is a relatively nonspecific enzyme that has also been called D-amino acid dehydrogenase because it catalyzes the oxidation of a number of D-amino acids including D-proline [19,21,32]. The pH optimum for D-alanine oxidation has been reported to be 8.9, with a K_m for this substrate of about 8 mM [32] or 30 mM [19] depending on the assay procedure used. The pH optimum for the oxidation of 3,4-dehydro-D-proline by membrane fractions of *E. coli* strain UMM5 also was found to be about 9. When the kinetics of the 3,4-dehydro-D-proline dehydrogenase reaction were examined in 100 mM Tris–HCl buffer, pH 9.0, the enzyme showed simple Michaelis–Menten kinetics with an apparent K_m for 3,4-dehydro-DL-proline of 6.4 mM (Fig. 3). D-alanine was a competitive inhibitor of the 3,4-dehydro-D-proline dehydrogenase reaction. Enzymes that act on D-alanine such as alanine racemase, D-alanyl-D-alanine ligase, and D-alanine dehydrogenase are particularly sensitive to the antibiotic D-cycloserine [33–35]. When membrane fractions from *E. coli* strain UMM5 were tested for the oxidation of 3,4-dehydro-DL-proline in the presence of varying concentrations of D-cycloserine, activity was strongly inhibited (complete inhibition at 10 mM, 90.3% inhibition at 3 mM, and 42.6% inhibition at 1 mM). These results were consistent with the hypothesis that oxidation of 3,4-dehydro-D-proline to form P2C is mediated by D-alanine dehydrogenase.

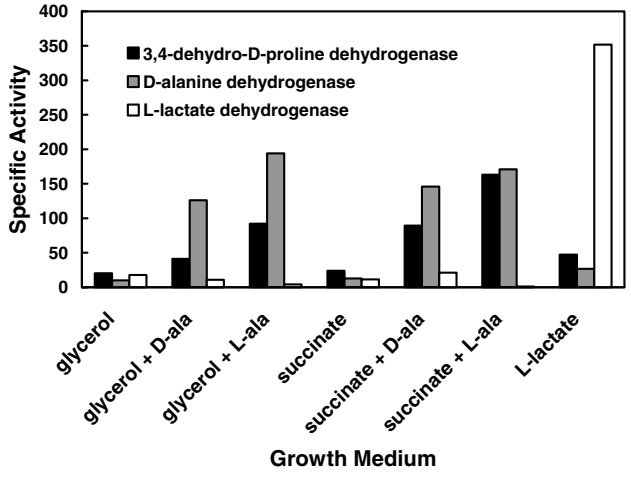

Fig. 2. Specific activities of 3,4-dehydro-D-proline dehydrogenase, D-alanine dehydrogenase, and L-lactate dehydrogenase in membrane fractions of *E. coli* strain UMM5 after growth in minimal medium with various substrates. Oxidation of 3,4-dehydro-D-proline was measured as a increase in A_{255} in the presence of 10 mM 3,4-dehydro-DL-proline. Oxidation of D-alanine or L-lactate was measured as a decrease in A_{600} in the presence of 10 mM KCN, 0.5 mM phenazine methosulfate, 0.1 mM dichlorophenolindophenol, and 30 mM of each substrate.

3.3. Genetic analysis of 3,4-dehydro-D-proline dehydrogenase activity

To confirm this hypothesis, the specific activities of 3,4-dehydro-D-proline dehydrogenase and D-alanine dehydrogenase were measured in membrane fractions from a well-characterized Dad$^-$ strain of *E. coli* (EC989, containing the *dadA237* mutation) and its wild-type parent (EC972). The mutant was originally selected on the basis of its resistance to β-chloro-D-alanine [22] and the formation of white colonies on 2,3,5-triphe-

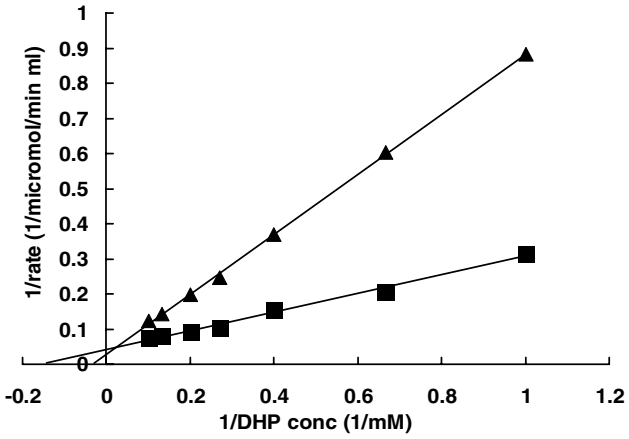

Fig. 3. Kinetics of 3,4-dehydro-D-proline oxidation by membrane fractions from *E. coli* strain UMM5. Oxidation of varying concentrations of 3,4-dehydro-DL-proline was measured as an increase in A_{255} in 100 mM Tris–HCl buffer, pH 9 in the absence (■) or the presence (▲) of 5 mM D-alanine.

nyltetrazolium chloride (TTC) indicator plates containing D- or L-alanine [36]. While the parental strain had high specific activities for both enzymes after growth in minimal medium containing succinate and L-alanine, the Dad$^-$ mutant was unable to oxidize either 3,4-dehydro-D-proline or D-alanine (Table 2). To confirm this result in the genetic background of *E. coli* strain UMM5, spontaneous Dad$^-$ mutants of strain UMM5 also were selected based on resistance to β-chloro-D-alanine. Membrane fractions were prepared from four isolates that formed white colonies on TTC indicator plates containing L-alanine but could still grow on minimal medium containing succinate. There was a significant reduction in the D-alanine dehydrogenase specific activity and a concurrent decrease in 3,4-dehydro-D-proline dehydrogenase activity in all four preparations. As might be expected for spontaneous mutants, two of the derivatives had partial but consistently low activity with both substrates. Combined with the biochemical data, these results indicated that both oxidation activities are due to the same enzyme.

3.4. Oxidation of other D-amino acid analogues by D-alanine dehydrogenase

To determine if D-alanine dehydrogenase also catalyzes the oxidation of other D-amino acid analogues, membrane fractions from EC972-1 (PutA$^-$ Dad$^+$) and EC989-1 (PutA$^-$ Dad$^-$) were tested for their ability to catalyze the oxidation of a series of related compounds using DCPIP as the electron acceptor. Among the substrates oxidized by D-alanine dehydrogenase were D-alanine, D-2-aminobutyrate, D-norvaline, D-valine, D-norleucine, D-leucine, D-isoleucine, D-*allo*-isoleucine, D-methionine, DL-ethionine, D-serine, D-theonine, and D-*allo*-threonine (Table 3). In addition to D-proline and 3,4-dehydro-D-proline, the enzyme also oxidized *cis*-4-hydroxy-D-proline. The specific activities for those substrates with linear R groups were higher than those with branched-chain R-groups. Introduction of a hydroxyl group into the side chain was tolerated but amino or carboxyl groups were not. In each case, activity was lost in the Dad$^-$ strain, indicating that oxidation was due to D-alanine dehydrogenase.

Table 2
Specific activities of 3,4-dehydro-D-proline dehydrogenase and D-alanine dehydrogenase in *E. coli* mutants

Strain	Relevant genotype	3,4-dehydro-D-proline	D-alanine
		Dehydrogenase[a]	Dehydrogenase[b]
EC972	*dad*$^+$	229	217
EC989	*dadA237*	<1	<1
UMM5	*dad*$^+$	163	171
UMM5-3	*dad*$^-$	5.5	7.1
UMM5-5	*dad*$^-$	12.9	15.9
UMM5-11	*dad*$^-$	<1	<1
UMM5-18	*dad*$^-$	<1	<1

[a] Membrane fractions were prepared after growth of the *E. coli* strains in minimal medium containing 50 mM sodium succinate and 20 mM L-alanine. Activity was measured as a increase in A_{255} in the presence of 10 mM 3,4-dehydro-DL-proline. Specific activity is expressed in nmoles of P2C formed min^{-1} mg protein^{-1}.

[b] Activity was measured using the same membrane fractions as a decrease in A_{600} in the presence of 10 mM KCN, 0.5 mM phenazine methosulfate, 0.1 mM dichlorophenolindophenol (DCPIP), and 30 mM D-alanine. Specific activity is expressed in nmoles of DCPIP reduced min^{-1} mg protein^{-1}.

Table 3
Oxidation of D-amino acids and their analogues by D-alanine dehydrogenase[a]

Substrate	EC 972-1 (Put$^-$Dad$^+$)	EC989-1 (Put$^-$Dad$^-$)
D-alanine	360.9	<5
D-2-aminobutyrate	211.1	<5
D-valine	15.5	<5
D-norvaline	156.5	<5
D-leucine	19.9	<5
D-norleucine	160.3	<5
D-isoleucine	7.6	<5
D-*allo*-isoleucine	8.1	<5
D-methionine	231.8	<5
DL-ethionine	33.2	<5
D-phenylalanine	234.3	<5
D-proline	47.4	<5
3,4-dehydro-DL-proline	403.5	<5
cis-4-hydroxy-D-proline	116.2	<5
D-serine	80.8	<5
D-threonine	32.9	<5
D-*allo*-threonine	13.0	<5
D-aspartate	<5	<5
D-ornithine	<5	<5
Cycloleucine	<5	<5

[a] Membrane fractions were prepared after growth of the *E. coli* strains in minimal medium containing 50 mM sodium succinate and 20 mM L-alanine. Activity was measured as a decrease in A_{600} in the presence of 10 mM KCN, 0.5 mM phenazine methosulfate, 0.1 mM dichlorophenolindophenol, and 50 mM of each substrate. Specific activities are expressed in nmoles of DCPIP reduced min^{-1} mg protein^{-1}.

4. Discussion

These results show that D-alanine dehydrogenase is responsible for the residual 3,4-dehydro-DL-proline oxidation activity in *putA proC* mutants of *E. coli*. This enzyme catalyzes the oxidation of the N–C_α bond in D-amino acids, which normally leads to deamination [21]. With 3,4-dehydro-D-proline, oxidation results in formation of pyrrole-2-carboxylate. Interestingly, this same product is formed during oxidation of 3,4-dehydro-L-proline by L-proline dehydrogenase, although oxidation in this case occurs at the N–C_δ bond [14,29]. The oxidation of 3,4-dehydro-D-proline by D-alanine dehydrogenase was partially inhibited by L-lactate and pyruvate,

as is the oxidation of L-proline and its analogues by L-proline dehydrogenase [37]. While no specific toxicity has been ascribed to 3,4-dehydro-D-proline, 3,4-dehydro-DL-proline inhibits the growth of *E. coli* because the L-isomer is incorporated into proteins in place of proline [38]. 3,4-Dehydro-L-proline inhibits prolyl hydroxylase activity and collagen formation in cultured mammalian cells [39] and cell wall assembly in plant cells [40]. Degradation of 3,4-dehydro-DL-proline by both membrane-associated and cytoplasmic enzymes thus may have an important protective effect both for the bacteria and their host.

D-alanine dehydrogenase appears to play a general role in the metabolism of D-amino acids and their structural analogues. *E. coli* contains D-alanine and D-glutamate in its peptidoglycan, and D-alanine dehydrogenase allows the use of these compounds as carbon or nitrogen sources [19,35]. D-amino acids commonly occur in foods as a result both of their natural occurrence in plants and various processing steps [41] and are inhibitory to *E. coli*, particularly under conditions of osmotic stress [42]. While they are not normally incorporated into proteins and can be removed from tRNAs by specific deacylases [43], D-amino acids can be incorporated into cell wall precursors and so can interfere with peptidoglycan formation [44]. Some D-amino acid analogues have specific inhibitory effects on bacteria: DL-ethionine and D-norleucine inhibit the synthesis of lipids used in the assembly of the envelope of *Mycobacterium avium* [45] and D-2-aminobutyrate and D-norvaline inhibit L-alanine-dependent germination of *Bacillus subtilis* spores [46]. Mutants of *E. coli* which have been selected on the basis of their ability to metabolize D-amino acids more efficiently have increased levels of D-alanine dehydrogenase activity [47]. The enzyme thus appears to have broad metabolic significance and further studies of its substrate specificity and biochemistry would be helpful.

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