Oxidation of L-thiazolidine-4-carboxylate by L-proline dehydrogenase in *Escherichia coli*

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L-Thiazolidine-4-carboxylate (T4C, γ -thioprolin) is a toxic analogue of L-proline. T4C can be oxidized by *Escherichia coli* to form N-formylcysteine, which is hydrolysed to yield formate and cysteine. To determine if L-proline dehydrogenase (EC 1.5.99.8) catalyses T4C degradation, membrane fractions from *E. coli* were tested for T4C and proline oxidation activity. The specific activity for T4C oxidation in membranes from bacteria grown with 10 mM-proline was similar to the specific activity for proline oxidation and about 100 times that in membranes from bacteria grown without proline. Both oxidation activities were inactivated at 45 °C at the same rate. Membranes from a strain with a deletion of the *putA* gene encoding L-proline dehydrogenase or a strain with a *putA*::Tn5 insertion mutation had no detectable activity with either substrate. Although T4C was a simple competitive inhibitor of proline oxidation, proline inhibited T4C oxidation in a way that gave competitive but sigmoidal kinetics. At low concentrations, T4C induced proline dehydrogenase synthesis. Cysteine auxotrophs containing the *putA*::Tn5 mutation could still use T4C as a cysteine source, and bacteria with this mutation consumed oxygen in the presence of T4C at half the control rate. These results indicate that T4C is a substrate and an inducer of L-proline dehydrogenase but suggest that *E. coli* also contains a second enzyme catalysing T4C degradation.

Introduction

Toxic analogues are often used in studies of amino acid uptake and metabolism in micro-organisms (Fowden *et al.*, 1967). L-Thiazolidine-4-carboxylate (T4C, γ -thioprolin) is a sulphur-containing proline analogue that is a therapeutic agent for some tumours (Brugarolas & Gosalvez, 1980; Hahn, 1980) and a scavenging agent for reactive nitrosamines (Tsuda *et al.*, 1988; Miwa *et al.*, 1989). Although rarely used for bacterial studies, T4C inhibits the growth of *Escherichia coli* (Unger & DeMoss, 1966a). It competes with proline for uptake by the major proline carrier (Roland & Tristram, 1975) and for aminoacylation by prolyl-tRNA synthetase (Papas & Mehler, 1970; Busiello *et al.*, 1979).

T4C is oxidized by whole cells of *E. coli* (Unger & DeMoss, 1966b) and by mitochondria from rat liver (Cavallini *et al.*, 1956; Mackenzie & Harris, 1957; Johnson & Strecker, 1962) and barley (Elthon & Stewart,

1984). The degradative pathway in mitochondria is similar to that for proline (Fig. 1). T4C is first converted to 2,3-thiazoline-4-carboxylate; ring opening yields N-formylcysteine, which then is cleaved to give formate and cysteine (Cavallini *et al.*, 1956; Mackenzie & Harris, 1957). The initial oxidation of T4C in mitochondria is catalysed by L-proline dehydrogenase [L-proline:(acceptor) oxidoreductase; EC 1.5.99.8]; hydrolysis of N-formylcysteine in liver is due to a second cytosolic enzyme (Boehler *et al.*, 1989).

Unger & DeMoss (1966b) concluded that L-proline dehydrogenase was not involved in T4C degradation in *E. coli* because oxidation was detected in cells grown without proline or T4C. Proline dehydrogenase activity in *E. coli* and *Salmonella typhimurium* is known now to be contained in a multifunctional protein encoded by the *putA* gene (Ratzkin & Roth, 1978; Menzel & Roth, 1981a). The PutA protein is a dimeric peripheral membrane protein that oxidizes proline and Δ^1 -pyrroline-5-carboxylate and transfers electrons to the aerobic electron transport chain (Scarpulla & Soffer, 1978; Menzel & Roth, 1981a; Abrahamson *et al.*, 1983). PutA also occurs in the cytoplasm where it binds to DNA and

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Abbreviation: T4C, L-thiazolidine-4-carboxylate (γ -thioprolin).

Fig. 1. Pathways of proline and T4C degradation.

regulates transcription of *putA* and the adjacent *putP* gene for the major proline carrier (Menzel & Roth, 1981*b*; Ostrovsky de Spicer *et al.*, 1991). Transcription of these genes is induced by proline (Dendinger & Brill, 1970; Maloy & Roth, 1983). I have re-examined the relationship between proline dehydrogenase and T4C oxidation and demonstrated that this enzyme is partly responsible for T4C degradation.

Methods

Bacterial strains. *E. coli* K12 strains CSH4 (*thi trp lacZ rpsL*), JT31 (*thi trp lacZ rpsL putA1::Tn5*), JT34 (*thi trp lacZ rpsL putP3::Tn5*) and RM2 (*thi trp lacZ rpsL ΔputA101*) were obtained from Dr Janet Wood of the University of Guelph, Canada. Strains JM15 (*cysE50 tfr-8*) and JM96 (*cysH56 thr-1 leuB6 trp-1 hisG1 argH1 thi-1 ara-13 lacY1 gal-6 malA1 xyl-1 mtl-1 strA9 tonA2 supE44 λ⁻*) were provided by Dr Barbara Bachmann of Yale University, CT, USA. The *putA1::Tn5* mutation in strain JT31 was moved into the cysteine auxotrophs by P1kc transduction (Curtiss, 1981). Kanamycin-resistant colonies were selected on LB agar (Miller, 1972) containing 25 μg kanamycin ml⁻¹, and tested on *put*-indicator medium (Menzel & Roth, 1981*b*).

Growth conditions. Bacteria were grown aerobically at 37 °C in minimal Medium A containing 0.5% (v/v) glycerol as previously described (Deutch *et al.*, 1985, 1989). L-Proline, L-thiazolidine-4-carboxylate (Sigma) and other supplements were added as specified. Radial streak tests for inhibition of growth by T4C were done as described by Wood (1981).

Preparation of membrane fractions. Membrane fractions were prepared from exponential-phase cells by the method of Abrahamson *et al.* (1983) and stored in small aliquots at -80 °C. Membranes were thawed prior to each experiment and used only once.

Identification of products. Membranes (50 μl) from strain CSH4 grown with 10 mM-L-proline were combined with 50 μl 0.1 M-T4C and 150 μl of Buffer B (10 mM-MOPS, pH 7.5, 20 mM-MgCl₂, 10% v/v,

glycerol). After incubation at 37 °C for various times, the reaction mixtures were heated in boiling water for 2 min and insoluble material was removed by centrifugation at 10000 r.p.m. for 10 min in a TOMY microcentrifuge. Supernatant fractions were spotted on 20 × 20 cm squares of Whatman 3MM paper along with standards, and the chromatograms developed in phenol/water (80:20, v/v). The chromatograms were dried overnight and dipped in acetone/ether (1:1, v/v). They were sprayed with 0.3% (w/v) ninhydrin in acetone and heated at 100 °C to reveal amino acids, or sprayed with 4,4'-bis(dimethylamino)-diphenylcarbinol (BDC-OH) reagent (Burt *et al.*, 1976) to detect compounds with thiol or carboxylic acid groups.

Thiazolidine-4-carboxylate oxidation assays. Aliquots of membranes (10–50 μl) were combined with Buffer B to a volume of 0.9 ml in 1.5 ml microcentrifuge tubes. After equilibration to 37 °C, 0.1 ml of 0.1 M-T4C was added to each tube. After various times, the reactions were stopped with 0.1 ml 50% (w/v) trichloroacetic acid; insoluble material was removed by centrifugation for 10 min at 10000 r.p.m. in a TOMY microcentrifuge, and the cysteine content of each supernatant fraction was determined by the method of Gaitonde (1967). Duplicate 0.1 ml aliquots were combined with water to a volume of 0.5 ml; 0.5 ml glacial acetic acid and 0.5 ml acidic ninhydrin reagent (250 mg ninhydrin in a mixture of 6 ml acetic acid and 4 ml HCl) were added to each tube. After heating the tubes at 100 °C for 10 min and cooling them in water, 3.5 ml 95% ethanol was added to each tube and the absorbances at 560 nm measured in a Shimadzu 160U spectrophotometer. Cysteine contents were determined from a L-cysteine standard curve. Assays of a particular preparation were reproducible ± 10%. An activity unit was defined as the formation of 1 nmol cysteine equivalents min⁻¹.

Oxygen uptake studies. Exponential-phase bacteria were harvested, suspended in Medium A without a carbon source, and incubated at 37 °C for 60 min to reduce endogenous substrates. A 2.7 ml volume of each suspension was combined with 0.3 ml of 0.1 M-substrate solution and equilibrated to 25 °C; oxygen uptake was measured with a Clark-type electrode connected to a monitor (Yellow Springs Instruments, model 5300) and a recorder (Kipp and Zonen, model B41). Assays were reproducible ± 10% for a particular culture. Substrate-dependent rates of oxygen uptake were calculated by subtracting the rate without added substrate.

Other assays. Proline dehydrogenase activity in toluene-treated whole cells was determined as described previously (Deutch *et al.*, 1985). Assays of a particular culture were reproducible $\pm 15\%$. Proline dehydrogenase activity in membrane fractions was measured at room temperature using *o*-aminobenzaldehyde (Abrahamson *et al.*, 1983). Assays of a particular preparation were reproducible $\pm 10\%$. Protein concentrations were determined by the Lowry method using bovine serum albumin as the standard.

Results

Spectrophotometric assay of T4C oxidation in membrane fractions

In previous studies (Mackenzie & Harris, 1957; Unger & DeMoss, 1966*b*; Elthon & Stewart, 1984), oxidation of T4C by intact bacteria or mitochondria was measured manometrically as oxygen uptake. To determine if T4C degradation in *E. coli* is mediated by the membrane-associated enzyme proline dehydrogenase, a spectrophotometric assay for T4C catabolism was developed. Attempts to measure T4C oxidation by reduction of 2,6-dichlorophenolindophenol (Abrahamson *et al.*, 1983) or *p*-iodonitrotetrazolium (Scarpulla & Soffer, 1978) were unsuccessful, so an assay based on product formation was used. The degradative pathway in Fig. 1 was confirmed for *E. coli* by combining T4C with membranes from strain CSH4 (*putA*⁺) grown with 10 mM-L-proline. When the reaction mixtures were analysed by paper chromatography, T4C (R_F 0.76), a small amount of cysteine (R_F 0.63), and a product with an R_F of 0.55 were observed. The latter spot reacted with the BDC-OH reagent for thiols but not with ninhydrin; it was absent in mixtures stopped at 0 min and easily distinguished from cysteine (R_F 0.32). When samples containing the product were acidified and heated, the compound disappeared and the cysteine spot intensified. Although efforts to synthesize *N*-formylcysteine (Mackenzie & Harris, 1957) were unsuccessful, the fact that the reaction product reacted with a thiol reagent but not with ninhydrin and was hydrolysed to give cysteine suggested it was this intermediate.

To measure T4C degradation quantitatively, T4C and membrane fractions were combined, incubated at 37 °C for various times, and the reactions stopped with acid. Aliquots of the acid-soluble material were mixed with the acidic ninhydrin reagent of Gaitonde (1967) and heated. This resulted simultaneously in the conversion of *N*-formylcysteine to cysteine and in the reaction of cysteine with ninhydrin to form a pink product with an absorbance maximum at 560 nm. T4C reacted with the acidic ninhydrin to form an orange product with a maximum absorbance at 430 nm and a small absorbance at 560 nm; T4C also showed some spontaneous hydrolysis to cysteine. However, using control mixtures lacking membranes and stopped at the same time-points, T4C oxidation could be measured quantitatively. Cysteine formation was linear for 20 min and proportional to the amount of membranes up to 500 µg protein.

Degradation of T4C by membrane fractions was similar to membrane-dependent proline oxidation (Abrahamson *et al.*, 1983). Oxidation required intact membranes containing a functional electron transport chain. Formation of cysteine from T4C was inhibited completely by detergents (0.1% Tween 20, Brij 58, Triton X-100 or SDS). KCN (10 mM) abolished T4C oxidation completely and sodium azide (100 mM) reduced it by 60%. NaCl (1.0 M) or KCl (1.0 M) completely inhibited T4C oxidation and 1.0 M-(NH₄)₂SO₄ reduced it by more than 60%. When membrane fractions from strain CSH4 were heated at 45 °C, there was a simultaneous and nearly linear inactivation of both activities.

Genetic analysis of T4C oxidation

Membrane fractions from wild-type bacteria and several *put* mutants were tested for T4C and proline oxidation (Table 1). Membranes from strain CSH4 (*putA*⁺) grown without proline contained low levels of both activities, while membranes from bacteria grown with 10 mM-proline contained about 100-fold higher activity with each substrate. The mean specific activity for T4C oxidation at 37 °C in five equivalent preparations was 88.2 ± 11.1 nmol min⁻¹ (mg protein)⁻¹. The mean

Table 1. Specific activities of T4C and proline oxidation in membrane fractions from *E. coli* CSH4 and derivatives

Strain	Relevant genotype	Growth conditions	No. of expts	T4C oxidation [nmol min ⁻¹ (mg protein) ⁻¹ , 37 °C]	Proline oxidation [nmol min ⁻¹ (mg protein) ⁻¹ , 25 °C]
CSH4	<i>purA</i> ⁺ <i>putP</i> ⁺	- Proline	3	0.6 $\pm$ 0.3	0.4 $\pm$ 0.4
		+ Proline	5	88.2 $\pm$ 11.1	35.2 $\pm$ 4.1
JT31	<i>putA1::Tn5</i>	+ Proline	2	<0.2	<0.2
RM2	Δ (<i>putPA101</i>)	+ Proline	2	<0.2	<0.2
JT34	<i>putP3::Tn5</i>	+ Proline	3	64.0 $\pm$ 17.6	25.1 $\pm$ 9.6

Fig. 2. Kinetics of proline and T4C oxidation in membrane fractions from *E. coli*. Membranes fractions prepared from strain CSH4 (*putA*⁺) after growth in the presence of 10 mM-L-proline were assayed as described in Methods using substrate concentrations ranging from 0.312–10 mM. (a) Eadie-Hofstee plot of the kinetics of proline oxidation without T4C present (●), or with 1 mM-T4C (○) or 3 mM-T4C (▲). (b) Eadie-Hofstee plot of the kinetics of T4C oxidation without proline present (●), or with 1 mM-proline (○) or 3 mM-proline (▲). Only the regression line for no proline present is shown in (b).

specific activity at room temperature (25 °C) was 29.8 ± 4.4 nmol min⁻¹ (mg protein)⁻¹, or 34% of the value at 37 °C. This was comparable to the specific activity of proline dehydrogenase in these membranes [35.2 ± 4.1 nmol min⁻¹ (mg protein)⁻¹ at room temperature]. Membranes from strains JT31 (*putA1*::Tn5) and RM2 (Δ *putA101*) exhibited no measurable T4C or proline oxidation. By contrast, membranes from strain JT34 (*putP3*::Tn5) had activities similar to the wild-type.

Kinetics of T4C oxidation

The kinetics of T4C and proline degradation in membranes from strain CSH4 (*putA*⁺) were analysed. Both T4C and proline oxidation activities showed simple Michaelis-Menten characteristics (Fig. 2). The mean apparent K_m value for T4C in seven similar preparations was 1.86 ± 0.23 mM; the mean apparent K_m value for proline in four of these preparations was 2.65 ± 0.98 mM. T4C was a competitive inhibitor of proline oxidation and vice versa. In each case, the apparent K_m increased while V_{max} was not affected. Although inhibition of proline oxidation by T4C showed simple competitive kinetics, the kinetics of T4C oxidation were altered in the presence of proline. At low substrate concentrations, a marked deviation from linearity was noted in Eadie-Hofstee or Lineweaver-Burk plots of the data. Sigmoidicity was observed in graphs of velocity as a function of substrate concentration.

Two possible explanations for these unusual kinetics were investigated. The possibility that the results were an artifact of the T4C oxidation assay was rejected because (1) the unusual kinetics were observed in four preparations made over a period of 2 years; (2) control reactions containing proline did not have significant absorbance at 560 nm that might distort the results; and (3) temporal linearity in the assay was maintained even in the presence of proline. The possibility that proline might be binding to the primary proline carrier encoded by the *putP* gene and exerting an allosteric effect on the reaction

(Wood & Zadworny, 1979; Maloy, 1987) was also rejected. Membrane fractions from strain JT34 (*putP3*::Tn5) exhibited simple Michaelis-Menten kinetics for T4C oxidation in the absence of proline; the K_m was the same as that in strain CSH4. In the presence of 1 or 3 mM proline, nonlinear but competitive kinetics again were noted.

Induction of T4C and proline oxidation activities

L-Proline is an inducer of proline dehydrogenase formation (Dendinger & Brill, 1970; Maloy & Roth, 1983), and membranes from strain CSH4 (*putA*⁺) grown without T4C or proline exhibited low levels of T4C and proline oxidation (Table 1). To determine if T4C also can induce synthesis of this enzyme, strain CSH4 was grown in minimal media containing different concentrations of either T4C or proline and proline dehydrogenase activities were measured in toluene-treated whole cells (Fig. 3). At low concentrations of T4C (up to 0.5 mM), the specific activities were somewhat higher than those in cultures with equivalent concentrations of proline. At higher concentrations of T4C, the specific activity was reduced as growth was inhibited. The highest activity was found in cultures with 1–2 mM-proline.

Identification of a second T4C oxidation activity

Although these results were consistent with the oxidation of T4C by proline dehydrogenase, further experiments suggested that another enzyme might be involved in T4C degradation. If proline dehydrogenase were the sole enzyme catalysing T4C catabolism, strains with the *putA1*::Tn5 mutation should be more sensitive than wild-type strains to inhibition by T4C and unable to use T4C as a cysteine source. To test the first prediction, the sensitivities of strains CSH4 (*putA*⁺) and JT31 (*putA1*::Tn5) to T4C inhibition were compared by radial-streak assays on plates and by growth in liquid

Fig. 3. Induction of proline dehydrogenase activity in *E. coli* by T4C and proline. *E. coli* strain CSH4 (*putA*⁺) was grown for at least 10 generations in a series of minimal media containing 0.5% glycerol as carbon source and increasing concentrations of T4C (●) or proline (○). Exponential-phase cells were assayed for proline dehydrogenase activity after treatment with toluene, and the specific activities plotted as a function of the initial concentration of the inducer.

Table 2. Oxygen uptake by whole cells of *E. coli*

Data shown are the means and standard deviations from three separate experiments.

Added substrate	Oxygen uptake [$\mu\text{l min}^{-1}$ (mg protein) $^{-1}$]	
	CSH4 (<i>putA</i> ⁺)	JT31 (<i>putA1::Tn5</i>)
None	19.3 $\pm$ 2.8	25.5 $\pm$ 0.3
Proline	115 $\pm$ 13	0
T4C	24.6 $\pm$ 6.4	14.0 $\pm$ 8.7

cultures with varying concentrations of T4C. When the cells were tested for T4C sensitivity after prior growth without proline, JT31 was somewhat more sensitive than strain CSH4. However, if the bacteria were tested after prior growth in 10 mM-proline, there was no difference between the strains. Because the PutA protein regulates transcription of *putP*, the greater sensitivity of JT31 in the first case appeared due to constitutive formation of the major proline carrier and increased T4C uptake. To test the second prediction, the *putA1::Tn5* mutation was moved by P1 transduction into two Cys⁻ auxotrophs. The resulting Cys⁻Put⁻ strains were compared to their Cys⁻Put⁺ parents in their use of different cysteine sources. Both Put⁺ and Put⁻ strains could use T4C as well as cysteine or cystine to satisfy the auxotrophic requirement.

The rates of oxygen uptake by strains CSH4 (*putA*⁺) and JT31 (*putA1::Tn5*) were determined in the presence of proline and T4C (Table 2). The strains showed similar

rates of consumption with endogenous substrates. With proline as the added substrate, strain CSH4 exhibited a high rate of oxygen uptake while strain JT31 showed no activity above the control level. With T4C as the substrate, strain CSH4 consumed oxygen at one-quarter the rate seen with proline. Strain JT31 showed a low but significant rate of uptake which was about half that seen in strain CSH4.

Discussion

These results indicate that T4C is degraded by membrane fractions from *E. coli* through a pathway similar to that in mitochondria. The initial oxidation reaction is catalysed by L-proline dehydrogenase. Whether an additional enzyme is required to convert *N*-formylcysteine to formate and cysteine is unclear. It has not been possible to synthesize 2,3-thiazoline-4-carboxylate or *N*-formylcysteine and to test them as substrates. However, the spontaneous formation of cysteine in the T4C reaction mixtures suggests the hydrolysis of *N*-formylcysteine is non-enzymic. T4C is also a good inducer of proline dehydrogenase synthesis. It is currently thought that addition of proline to uninduced cultures leads to reduction of the flavin cofactor in the cytoplasmic form of the PutA protein. This allows PutA to associate with the cell membrane, and as its cytoplasmic concentration drops, transcription of the *putA* gene increases (Maloy, 1987; Wood, 1987). The K_m for T4C is lower than that for proline, which may explain why T4C is a better inducer at low concentrations.

Although Michaelis-Menten kinetics were found in reactions with single substrates, the presence of proline as a competitive inhibitor of T4C oxidation led to sigmoidal kinetics. This was not a trivial property of the assay or a result of the presence of the PutP protein. In preliminary experiments with two other proline analogues, azetidine-2-carboxylate (3 mM) had no effect on T4C oxidation, but 3,4-dehydro-L-proline (3 mM) gave the same kinetics seen with proline. However, L-lactate (3 mM), a competitive inhibitor of purified proline dehydrogenase (Scarpulla & Soffer, 1978), inhibited T4C oxidation in a simple competitive fashion. It is possible that another membrane-associated protein with proline-binding sites may interact with the PutA protein and modulate its activity. Alternatively, the PutA protein may have multiple binding sites, some of which are catalytic and others regulatory. These possibilities might be distinguished by further kinetic analysis of T4C oxidation by both membrane-associated and solubilized forms of the PutA protein.

Although membrane fractions from strain JT31 containing the *putA1::Tn5* mutation could not convert

T4C to cysteine, these bacteria consumed oxygen in the presence of T4C at a low rate. It seems likely that there is a distinct cytoplasmic enzyme catalysing T4C oxidation. Although the assay method described in this paper would not be suitable for studying this enzyme, an alternative procedure could be developed to analyse this reaction.

These studies imply that the sensitivity of cells to T4C may be determined by the specific activity of proline dehydrogenase. The efficacy of T4C as an antitumour agent or as a scavenger of reactive nitrosamines thus might be reduced in cells with high proline dehydrogenase activity, but tumour cells with reduced activity might be more sensitive to this compound. Furthermore, T4C may be a useful selective agent for studying the regulation of proline dehydrogenase synthesis in *E. coli*. In a genetic background in which other enzymes degrading T4C have been eliminated, mutants with an increased resistance to T4C may have elevated concentrations of proline dehydrogenase. On the other hand, bacteria that are unable to use T4C as a cysteine source may have decreased amounts of this enzyme. By selecting and analysing such mutants, new insights into the control of proline dehydrogenase may be obtained.

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