Proline biosynthesis from L-ornithine in *Clostridium sticklandii*: purification of Δ^1 -pyrroline-5-carboxylate reductase, and sequence and expression of the encoding gene, *proC*

Janet Kenklies, Renate Ziehn, Kathrin Fritsche, Andreas Pich and Jan R. Andreessen

Author for correspondence: Andreas Pich. Tel: +49 345 5526360. Fax: +49 345 5527010.
e-mail: a.pich@mikrobiologie.uni-halle.de

Institut für Mikrobiologie,
Martin-Luther-Universität
Halle-Wittenberg, Kurt-
Mothes-Str. 3, D-06099
Halle, Germany

***Clostridium sticklandii* utilizes combinations of amino acids for growth by Stickland reactions. Proline is an efficient electron acceptor in these reactions and is reduced to 5-aminovalerate. Proline can be partly synthesized from ornithine by the action of ornithine aminotransferase and Δ^1 -pyrroline-5-carboxylate (PCA) reductase. Both enzymes were present in crude extracts of *C. sticklandii* in sufficient activity of 0.93 nkat (mg protein)⁻¹ and 4.3 nkat (mg protein)⁻¹, respectively, whereas enzymes involved in proline biosynthesis from glutamate were not detected. PCA reductase was purified to homogeneity in a three-step procedure involving ammonium sulfate precipitation, affinity chromatography with Procion Red and gel filtration on Sephadex GF200. The homogeneous enzyme was most likely an octamer of 230 kDa with a subunit size of 25 kDa as obtained by SDS-PAGE and 28.9 kDa as calculated from the sequence. Apparent K_m values for PCA and NADH were 0.19 mM and 0.025 mM, respectively. The enzyme also catalysed *in vitro* the reverse reaction, the oxidation of proline, at alkaline pH values above 8 and higher substrate concentrations (apparent K_m values: 1.55 mM for proline and 10.5 mM for NAD at pH 10.0). Studies with growing cells of *C. sticklandii* and [¹⁵N]proline revealed that proline is not oxidized *in vivo* because ¹⁵N was solely detected by HPLC-MS in 5-aminovalerate as the product of proline reduction. The *proC* gene encoding PCA reductase of *C. sticklandii* was cloned, sequenced and heterologously expressed in *Escherichia coli*. The enzyme exhibited high homologies to PCA reductases from different sources. Thus, *C. sticklandii* is able to synthesize the electron acceptor proline from ornithine (a degradation product of arginine) by action of ornithine aminotransferase and PCA reductase.**

Keywords: *Clostridium sticklandii*, Δ^1 -pyrroline-5-carboxylate reductase, proline, ornithine, Stickland reaction

INTRODUCTION

The Gram-positive anaerobe *Clostridium sticklandii* belongs to the proteolytic clostridia and requires com-

binations of amino acids for growth (Stadtman & McClung, 1957; Hippe *et al.*, 1991; Wagner & Andreessen, 1995; Zinecker *et al.*, 1998). The amino acids are degraded in a coupled oxidation–reduction reaction called the Stickland reaction whereby one amino acid is utilized as electron donor and another as electron acceptor (Seto, 1980; Andreessen *et al.*, 1989). In *C. sticklandii* proline and glycine are predominantly utilized as electron acceptors whereas amino acids like

Abbreviation: PCA, Δ^1 -pyrroline-5-carboxylate.

The EMBL accession number for the sequence described in this paper is AJ010739.

Fig. 1. Interconversion of arginine, ornithine, glutamate and proline by anaerobic bacteria. The numbers refer to enzymes: 1, γ -glutamyl kinase; 2, γ -glutamylphosphate reductase; 3, glutamate-semialdehyde dehydrogenase; 4, ornithine aminotransferase; 5, non-enzymic reaction; 6, PCA reductase; 7, L-proline dehydrogenase; 8, proline racemase; 9, D-proline reductase; 10, arginine deiminase; 11, ornithine carbamoyl-phosphate transferase; 12, ornithine racemase; 13, D-ornithine 4,5-aminomutase; 14, 2,4-diaminopentanoate dehydrogenase; 15, 2-amino-4-ketopentanoate thiolase; 16, glutamate dehydrogenase; 17, ornithine cyclase (deaminating).

serine, arginine and ornithine serve predominantly as electron donors. Ornithine is formed from arginine via citrulline and can also be reduced depending on the growth conditions used (Stadtman & White, 1954; Andreessen *et al.*, 1989).

Proline and glycine are reduced by the enzymes D-proline reductase and glycine reductase (Stadtman, 1956; Stadtman & Elliot, 1956; Tanaka & Stadtman, 1979; Seto & Stadtman, 1976; Andreessen *et al.*, 1989). Glycine is converted by glycine reductase to ammonia and acetyl phosphate, which can be used for ATP synthesis by acetate kinase (Arkowitz & Abeles, 1989). In contrast, no high-energy compound is produced during proline reduction to 5-aminovalerate (Arkowitz *et al.*, 1994), indicating that proline reduction is predominantly used as an electron sink in *C. sticklandii* (Seto & Stadtman, 1976). At least, proline is preferentially reduced by *C. sticklandii* and, thus, proline reductase might play a major role in redox-balancing in the cell (Seto, 1980). Therefore, enzymes involved in proline biosynthesis should be present in *C. sticklandii* to synthesize this important electron acceptor if it is not available from the medium.

Pathways of proline biosynthesis are well-established for several organisms (Adams & Frank, 1980; Leisinger, 1996; Brandriss & Falvey, 1992; Dougherty *et al.*, 1992; Delauney *et al.*, 1993): either glutamate is converted to proline by γ -glutamyl kinase (EC 2.7.2.11), glutamate- γ -semialdehyde dehydrogenase (EC 1.2.1.41) and Δ^1 -pyrroline-5-carboxylate (PCA) reductase (EC 1.5.1.2) or proline is formed from ornithine [which derives in most cases from arginine (Cunin *et al.*, 1986)] by the

enzymes ornithine aminotransferase (EC 2.6.1.13) and PCA reductase (EC 1.5.1.2) (Fig. 1). *Clostridium sporogenes*, physiologically related to *C. sticklandii*, possesses an ornithine cyclodeaminase (EC 4.3.1.12) that directly produces proline from ornithine (Muth & Costilow, 1974). An enzyme assigned as proline dehydrogenase (EC 1.5.99.8) was purified from *C. sporogenes* and shown to be identical to PCA reductase catalysing the interconversion of proline and PCA (Costilow & Cooper, 1978). The dehydrogenase activity was inhibited by glutamate, thus indicating that it might be involved in proline oxidation *in vivo* (Monticello & Costilow, 1981).

C. sticklandii utilizes arginine employing the arginine deiminase pathway to ornithine like *C. sporogenes* and the pathogen *Clostridium botulinum* (Mitruka & Costilow, 1967; Cunin *et al.*, 1986). In the latter organism arginine utilization repressed the production of neurotoxins (Patterson-Curtis & Johnson, 1992). An arginine deiminase – as indicated by its N-terminal sequence – was co-purified with L-serine dehydratase from *C. sticklandii* (Zinecker *et al.*, 1998).

In early tracer studies, Stadtman & White (1954) showed that 6% of ornithine added to the medium of *C. sticklandii* is converted to 5-aminovalerate; similar results were obtained by Dyer & Costilow (1968). So far, enzymes synthesizing the electron acceptor proline from ornithine have not been identified in *C. sticklandii*. We now report about the enzymes ornithine aminotransferase and PCA reductase which are responsible for this conversion in *C. sticklandii*. The purification of PCA reductase, and the DNA sequence and the expression of the corresponding gene are presented.

METHODS

Strains and growth conditions. *C. sticklandii* strain HF (DSM 519^T) was grown at 30 °C under a nitrogen atmosphere in a complex medium (Stadtman & McClung, 1957) or in a defined medium as described by Wagner & Andreessen (1995). *Escherichia coli* XL2-Blue (Stratagene) was used for genetic techniques. This strain was grown at 37 °C in LB medium (Miller, 1972) supplemented with ampicillin (0.1 g l⁻¹) for maintenance of the plasmid.

Preparation of cell-free extracts of *C. sticklandii*. Cells were harvested from the late-exponential growth phase by centrifugation at 7000 g (4 °C, 30 min). For the preparation of extracts, 1 g cells (wet weight) was dissolved in 1.5 ml 250 mM Tris/HCl buffer, pH 7.4, containing 1 mM dithioerythritol. After addition of 2.5 µl PMSF (0.2 M in 2-propanol), 2.5 mg lysozyme and 0.2 mg DNase I, the suspension was incubated for 30 min at 37 °C and subsequently sonicated at 0 °C at several intervals for 10 s with intermitting cooling periods of 60 s until nearly all cells were broken. Cell debris was removed by centrifugation at 25000 g (4 °C, 30 min) and the supernatant was designated the crude extract. To detect ornithine aminotransferase activity, a 50 mM Tris buffer (pH 8.0) was used instead.

Enzyme assays. PCA reductase activity was routinely measured at 40 °C in 200 mM Tris buffer, pH 7.4, containing 1 mM PCA and 0.3 mM NADH by following the oxidation of NADH at 365 nm as described by Costilow & Cooper (1978). PCA was synthesized by the procedure of Williams & Frank (1975), and its concentration in stock solutions was determined by reaction with *o*-aminobenzaldehyde, using an absorption coefficient of 2.94 mM⁻¹ cm⁻¹ at 444 nm. The L-proline-oxidizing activity of PCA reductase was measured at 40 °C in a 200 mM diethanolamine buffer, pH 10.0, containing 20 mM NAD and 20 mM L-proline by following the increase in A₃₄₀ (Monticello & Costilow, 1981).

The activity of ornithine aminotransferase was measured by determination of the PCA formed (Takechi *et al.*, 1994). In a total volume of 0.5 ml 50 mM Tris buffer, pH 8.5, 10 mM L-ornithine, 10 mM 2-oxoglutarate, 0.1 mM pyridoxal phosphate and enzyme solution were incubated at 30 °C for 15–30 min. The reaction was stopped by addition of 0.3 ml 6 M HCl and incubated at 95 °C for 20 min. The assay was neutralized with 1 ml 3.6 M sodium acetate, and 0.2 ml 33 mM *o*-aminobenzaldehyde was added. The A₄₄₄ was determined after 15 min.

The activities of γ -glutamyl kinase and glutamate- γ -semialdehyde dehydrogenase were assayed as described before (Hayzer & Leisinger, 1982; Smith *et al.*, 1984; Limauro *et al.*, 1996). The hydroxamate assay was used to detect γ -glutamyl kinase. The reaction mixture contained in a volume of 0.5 ml 20 mM ATP, 20 mM MgCl₂, 100 mM hydroxylamine, 50 mM Tris, pH 7.0, and enzyme. The reaction was carried out at 30 °C for 30–60 min and terminated by the addition of 1 ml stop mix. Stop mix contained in 100 ml: 5.5 g FeCl₂·6H₂O, 2 g trichloroacetic acid and 2.1 ml 10 M HCl. The amount of hydroxamate was determined from the A₅₃₅ by comparison to a standard curve prepared with γ -glutamyl hydroxamate (Sigma). The assay parameters were modified by using ATP and MgCl₂ concentrations of 0.1–100 mM and hydroxylamine concentrations between 0.1 and 500 mM; pH values were varied between pH 6 and 7 by using 50 mM MOPS buffer and between pH 7 and 9 by using Tris buffer. The reaction temperature was varied from room temperature to 50 °C and the incubation time was extended up to several hours.

Glutamate- γ -semialdehyde dehydrogenase was assayed in a final volume of 0.5 ml containing 5 mM PCA, 1 mM NAD(P), 100 mM KH₂PO₄, pH 7.0, and enzyme. The increase in A₃₄₀ was recorded at room temperature or 30 °C. PCA and NAD(P) concentrations were varied from 0.1 to 20 mM. Potassium phosphate or Tris buffer was used ranging from pH 5 to pH 8.

No activity was obtained for γ -glutamyl kinase and glutamate- γ -semialdehyde dehydrogenase in extracts of *C. sticklandii* despite these variations; however, activities were obtained in controls performed with extracts from *E. coli*.

Purification of PCA reductase. All procedures were performed at 4 °C or on ice and could be done under aerobic conditions. The crude extract was ultracentrifuged at 120000 g for 1 h. The resulting supernatant contained all enzyme activity and was subsequently fractionated with ammonium sulfate. Solid ammonium sulfate was added slowly to give 70 % saturation. After centrifugation at 25000 g for 30 min, the pellet was discarded and the ammonium sulfate concentration was increased to 80 % saturation. After centrifugation the resulting pellet contained most of the activity and was dissolved in a Tris buffer (100 mM, pH 7.4, 1 mM DTT) and dialysed against this buffer. The dialysed solution was further purified on a Procion Red affinity chromatography column equilibrated with the 100 mM Tris buffer. Elution of the column (10 × 2.6 cm) was performed with a linear KCl gradient from 0 to 0.7 M KCl in 700 ml. The PCA reductase eluted in a single peak at about 300 mM KCl. Active fractions were combined, dialysed against 250 mM Tris buffer (pH 7.4, 1 mM DTT) and concentrated by a Centriprep-30 concentrator (Amicon). The concentrated enzyme solution was applied to a Sephadex GF200 FPLC column (Amersham Pharmacia Biotech) equilibrated with the previously used 250 mM Tris buffer. Fractions containing enzyme activity were combined, concentrated by a Centricon concentrator (Amicon) and used for characterization.

Molecular mass determination. The molecular mass of native PCA reductase was estimated using a Sephadex GF200 FPLC column. The calibration standards were thyroglobulin (686000 Da), ferritin (440000 Da), catalase (232000 Da), aldolase (158000 Da), BSA (69000 Da) and α -chymotrypsinogen (25000 Da). The molecular mass of the subunit was determined by SDS-PAGE.

Electrophoresis. SDS-PAGE was performed as described by Laemmli (1970). For molecular mass determination the electrophoresis calibration kit Sigma 7 was used as standard. Proteins were stained with silver according to Blum *et al.* (1987).

Analytical methods. Protein concentration was determined by the method of Bradford (1976) with BSA as standard. N-terminal amino acid sequence determinations were done by automated Edman degradation in a 476A protein sequencer (Applied Biosystems).

Analysis of proline degradation by HPLC-MS. [¹⁵N]Proline (about 10 % enriched in nitrogen; Cambridge Isotope Laboratories) was added at 20 mM to the synthetic growth medium of *C. sticklandii* (Wagner & Andreessen, 1995). Samples were taken after 1, 3 and 7 h, centrifuged for 5 min at 10000 g, and 5-aminovaleate, glutamate, ornithine, alanine and ammonia were analysed for their isotope deviation by HPLC-MS. Amino acids were first derivatized with 9-fluorenylmethyl chloroformate as described by Bank *et al.* (1996). The amino acids were solubilized in DMSO, applied to an RP18 HPLC column using a HPLC system from Hewlett Packard, and

separated by an acetonitrile gradient from 25 to 75% in H₂O containing 0.1–0.09% trifluoroacetic acid. Detection was done using a prototype of a HPLC mass spectrometer detector developed by Hewlett Packard.

Molecular biology methods. DNA was isolated from *C. sticklandii* according to Bertram & Dürre (1989). Isolation of plasmid DNA, digestion by restriction enzymes, ligation and other standard DNA techniques were done as described by Sambrook *et al.* (1989). DNA–DNA hybridizations were done using the DIG system from Boehringer and carried out as recommended by the manufacturer. Both strands of plasmid pJK2 were sequenced (Sanger *et al.*, 1977) using the AutoRead Sequencing kit and the ALF automated sequencing system (Amersham Pharmacia). Plasmid DNA was used as template and sequencing was performed by the method of primer walking (Sambrook *et al.*, 1989).

Construction of a genomic library of *C. sticklandii*. Genomic DNA was partially digested with the restriction enzymes *Sau*3A and *Eco*RI. Fragments with a size of 2–3 kb and 4–6 kb, respectively, were ligated into pBluescript II SK (Stratagene) and transformed into *E. coli* XL2-Blue. Nearly 7500 recombinant clones were obtained and stored in pools of 100 clones. The plasmid mixtures from these pools were isolated and used to screen for a *proC* gene.

RESULTS

Biosynthesis of L-proline in *C. sticklandii*

High specific activities of L-ornithine aminotransferase [$0.93 \text{ nkat (mg protein)}^{-1}$] and PCA reductase [$4.3 \text{ nkat (mg protein)}^{-1}$] were detected in crude extracts of *C. sticklandii*. The specific activities of both enzymes varied only slightly in cells obtained from different growth conditions. The presence of L-proline in the growth medium had no influence on the enzyme activities obtained, indicating that these enzymes were constitutively expressed. γ -Glutamyl kinase and glutamate- γ -semialdehyde dehydrogenase activities could not be detected in these extracts, even after variation of different assay parameters as outlined in Methods (data not shown).

L-Proline is converted by an active proline racemase to the D-isomer (Yagasaki & Ozaki, 1998) and further converted by D-proline reductase to 5-aminovalerate (Seto & Stadtman, 1976), which was detectable in the growth medium shortly after inoculation (data not shown). However, an oxidation of proline was also catalysed *in vitro* by PCA reductase. Therefore, an

Fig. 2. Steps of purification of PCA reductase from *C. sticklandii*. Proteins from different purification steps were separated by SDS-PAGE (10%, w/v) and silver-stained. Molecular masses of standard proteins are given in kDa on the left side. Lanes: 1, standard proteins; 2, crude extract; 3, ammonium sulfate precipitation (70–80%); 4, chromatography on Procion Red; 5, purified PCA reductase.

analysis was performed using [^{15}N]proline and HPLC-MS to see whether proline might also be oxidized *in vivo* by *C. sticklandii*. The possible nitrogen-containing products of proline metabolism (Fig. 1), 5-aminovalerate, glutamate, ornithine, alanine and ammonia, were analysed. Only 5-aminovalerate was labelled with ^{15}N , indicating that proline was solely reduced to 5-aminovalerate under the conditions employed.

Purification of PCA reductase

The PCA reductase was purified from 30 g cells of *C. sticklandii* (Table 1). The enzyme was purified 672-fold with a recovery in activity of 2.7%. The unusually high ammonium sulfate concentration (80% saturation) necessary to precipitate the enzyme and the use of the affinity material Procion Red led to a three-step purification procedure that resulted in a homogeneous enzyme preparation as judged by SDS-PAGE (Fig. 2).

Table 1. Purification of PCA reductase from *C. sticklandii*

Preparation step	Total protein (mg)	Total activity (nkat)	Specific activity (nkat mg ⁻¹)	Purification (-fold)	Yield (%)
Crude extract	2270.00	9760	4.30	—	100.0
Ammonium sulfate	57.40	6310	110.00	26	65.0
Procion Red	3.80	4600	1210.00	281	47.0
Gel filtration	0.09	260	2890.00	672	2.7

Molecular mass and subunit analysis of PCA reductase

PCA reductase of *C. sticklandii* eluted from a Sephadex GF200 column corresponding to a molecular mass of 230 kDa (data not shown). The enzyme partly decomposed during purification into a still active 150 kDa species (data not shown). In both cases, only one subunit was detected after analysis by SDS-PAGE exhibiting a molecular mass of 25 kDa (Fig. 2). The sequence of the N-terminus of this protein was determined to be NH₂-MDKKIGFIGXGNMAQAMISALVSKSLIESNQI. This sequence revealed similarities to PCA reductases from several sources (see below).

Characteristics of PCA reductase

Homogeneous PCA reductase of *C. sticklandii* exhibited high activities with NADH as electron donor. Using NADPH, an approximately tenfold lower specific activity was obtained. PCA reduction exhibited a pH optimum of pH 7.4, and the apparent K_m values for PCA and NADH were determined to be 0.19 and 0.025 mM, respectively. PCA reductase activity was slightly stimulated by addition of Mg²⁺ (3 mM), whereas 2 mM ATP decreased the activity to about 70%. No effect on enzyme activity by D- or L-proline, L-glutamate or L-ornithine was observed, even in concentrations of up to 20 mM.

The L-proline-oxidizing activity of PCA reductase was 2.6 times lower than the PCA reductase activity employing optimized conditions. Proline oxidation by purified PCA reductase was strictly NAD-dependent and required pH values between 8.0 and 11.0, exhibiting an optimum at pH 10.0. No activity could be detected at physiological pH values even if 5 mM *o*-aminobenzaldehyde or semicarbazide was added to remove PCA or its hydrolysis product γ -glutamic semialdehyde, respectively. Also high substrate concentrations and long incubation periods did not allow proline oxidation at pH values below pH 8. The proline-oxidizing activity was specific for the L-isomer, and the apparent K_m values for L-proline and NAD were 1.55 and 10.5 mM, respectively.

Cloning and sequence analysis of the gene encoding PCA reductase

A 26-base oligonucleotide primer (5' ATG GAT/C AAA AAA ATT/A GGT/A TTT ATT/A/C GG 3') was designed on the basis of the N-terminal sequence of the PCA reductase and the codon usage of *C. sticklandii* (Garcia & Stadtman, 1992). This primer was employed in combination with commonly used sequencing primers (T3, T7) to amplify a DNA fragment from the genomic library of *C. sticklandii* by PCR. A diluted mixture of all 75 plasmid pools available from the genomic library was used as template. A specific DNA fragment of 1.4 kb was obtained which contained the gene for the PCA reductase, commonly called *proC*, and a small part of the vector pBluescript II SK. Subsequently, only the *proC*

Fig. 3. Structure of the *proC* gene region.

Cs	MDK-----KIGFIGCGNMAQAMISALVSKSLIESNQIIVSNRKNIL	42
Ec	MEK-----KIGFIGCGNMGKAILGGLIASGQVLPGQIWWYTPSPDKV	42
Cg	MEK-----KIGFIGCGNMGKAILGGLIASGQVLPGQIWWYTPSPDKV	42
Ps	MEILPILSDSYTLGFIGAGKMAESIAKASRGVLPSSRIVTAHSNP--S	48
Hs	MSVGFITAGAGQLAFALAKGFTAAAGVLAHKIMASSPMDMLA	40
	..****.*..	
Cs	EKMN-KEYGITIAANNIEVAEKCDIVFLAVKQNLVEMVKEIKDSVTDKD	91
Ec	AALH-DQPGINAESAQEAQIADIIFAAVKPGIMIKVLSEITSSLNKDS	91
Cg	AALH-DQPGINAESAQEAQIADIIFAAVKPGIMIKVLSEITSSLNKDS	91
Ps	RRAAFESIGITVLSNDDVVRASNVVFSVKPQLVKDVLKPLLTDKD	98
Hs	TVSALRKMVKLTPHNKETVQHSVDLFLAVKPHIIPFILDIEGADIEDRH	90
	..****.*..	
Cs	IFVSIAPGKTMEFLEAHLGA---YAKILRTPMNTPSMVSEGMTAICPNSN	138
Ec	LUVSIAAGVTLDQLARALGH---DRKIIRAMPNTPALVNAGMTSVTPNAL	138
Cg	LUVSIAAGVTLDQLARALGH---DRKIIRAMPNTPALVNAGMTSVTPNAL	138
Ps	LLVSVAAGIKL---KDLQEWAGHERFIRVMPNTPAVQQAASVMSLGA	144
Hs	IVVSCAAGVTISSIEKKLSAFRPAVRIRCMTNTPVVREGATVYATGTH	140
	..****.*..	
Cs	IASEEELLVVKLIESFGAVEIIEEKLFDVAVAVSGSSPAYVFMFIEMAD	188
Ec	VTPEDTADVLNIFRCFGEAEVIAEPMIHPVVGSGSSPAYVFMFIEMAD	188
Cg	VTPEDTADVLNIFRCFGEAEVIAEPMIHPVVGSGSSPAYVFMFIEMAD	188
Ps	ATEEDANLISQLFGSIGIKWKADDFDAITGLSGSGPAYIYLAIEALAD	194
Hs	AQVEDGRLMEQLLSTVGPFCTEVEEDLIDAVTGLSGSGPAYATDALAD	190
	..****.*..	
Cs	AAVIQGMPPRAQAYFAAQAVLGSAKMVLSEKSHPGELKDMVCSPGGTTIE	238
Ec	AAVLGGMPPRAQAYKFAAQAVMGSAKMVLSETEHPGALKDMVCSPGGTTIE	238
Cg	AAVLGGMPPRAQAYKFAAQAVMGSAKMVLSETEHPGALKDMVCSPGGTTIE	238
Ps	GGVAAGLPRDLASLSAQTVLGAASMATLSGKHPGQLKDDVTSPPGGTTIA	244
Hs	GGVKGMLPRRLAVRLGAQALLGAARKMLHSEHQHPGQLKDNVSSPGGATIH	240
	..****.*..	
Cs	AVAVLEERGMRSAVIEAMRKCAQKSKEM-----	266
Ec	AVRVLEEKGFRAAVIEAMTKCKEKSLSKS-----	266
Cg	AVRVLEEKGFRAAVIEAMTKCKEKSLSKS-----	266
Ps	GVHELEKGGFRGTLNNAVVAARSRSELS-----	272
Hs	ALHVLSEGGFRSLINAVEASCIRTELQSMADQEQVSPAIAKKTILDKV	290
	..****.*..	
Cs	-----	266
Ec	-----	269
Cg	-----	269
Ps	-----	273
Hs	KLDSFAGTALSPSGHTKLLPRSLAPAGKD	319

Fig. 4. Alignment of PCA reductase sequences from *C. sticklandii* and selected other organisms. Identical amino acids are indicated by an asterisk and the similar amino acids by a stop. The potential nucleotide-binding motif (Walker *et al.*, 1982) in the N-terminal region is printed in bold. Cs, *C. sticklandii* (this paper); Ec, *E. coli* (Deutch *et al.*, 1982); Cg, *Corynebacterium glutamicum* (Ankri *et al.*, 1996); Ps, *Pisum sativum* (EMBL accession no. Q04708); Hs, *Homo sapiens* (Dougherty *et al.*, 1992).

gene was used as a probe to screen the library by hybridization. One plasmid pool showed a positive reaction and was used to transform *E. coli*. The transformants obtained were screened again with the *proC* probe and four identical clones were obtained. Plasmid pJK2 was isolated from one of these clones and contained a 2.9 kb *EcoRI* fragment which encoded one complete and one truncated ORF (Fig. 3). The 3'-localized ORF encoded the complete gene for the PCA reductase (*proC*) of 798 bp, which corresponded to 266 amino acid residues with a deduced molecular mass of 28902 Da (Fig. 4). The derived N-terminal amino acid sequence encoded by the *proC* gene corresponded to the

sequence obtained from protein sequencing. The coding region of *proC* was preceded by the sequence GGAGGG, which might be a ribosome-binding site (Shine & Dalgarno, 1974) located eight bases upstream of the start codon, ATG. A consensus promoter sequence for Gram-positive bacteria (Moran, 1993) and a terminator structure could not be identified in the upstream and downstream regions, respectively. Expression of PCA reductase from *C. sticklandii* was demonstrated in *E. coli* containing the pJK2 plasmid. These clones exhibited a specific activity for PCA reductase of 0.75 nkat (mg protein)⁻¹; the control, vector-transformed cells of *E. coli* had a specific activity below 0.2 nkat (mg protein)⁻¹.

The ORF located in the 5' region of pJK2, *orfX*, was 1503 bp long, corresponding to 501 amino acid residues. *orfX* probably proceeds further into the upstream region which has not been isolated (Fig. 3). *orfX* only exhibited similarities to proteins with unknown functions according to the databases. *proC* and *orfX* were divergently oriented and separated by an AT-rich region of 262 bp exhibiting a GC content of 17 mol%, in contrast to the overall GC content of 34.6 mol% of *C. sticklandii* (Hippe *et al.*, 1991).

DISCUSSION

C. sticklandii can actually synthesize its important electron acceptor proline partly from ornithine as has already been indicated by tracer studies (Stadtman & White, 1954). Both enzymes involved, L-ornithine aminotransferase and PCA reductase, were now found to be sufficiently active in crude extracts of *C. sticklandii*. This organism is able to disproportionate L-ornithine (Dyer & Costilow, 1968): reducing equivalents are generated during ornithine oxidation via 2-amino-4-ketopentanoate to alanine and acetyl-CoA (Tsuda & Friedmann, 1970; Jeng *et al.*, 1974) and by regeneration of the co-substrate of ornithine transaminase (2-oxoglutarate) by glutamate dehydrogenase (Fig. 1). They can be consumed by reduction of PCA to proline (this study) and of proline to 5-aminovalerate (Stadtman, 1956). *C. sticklandii* contains an active glutamate dehydrogenase (Buckel & Barker, 1974). However, γ -glutamyl kinase and glutamate- γ -semi-aldehyde dehydrogenase activities were not detected in our studies, and, therefore, glutamate seems not be a precursor for proline in *C. sticklandii*. In addition, glutamate inhibited growth of *C. sticklandii* if added to the growth medium (A. Uhde & J. R. Andreesen, unpublished result).

PCA reductase of *C. sticklandii* was purified to homogeneity as judged by SDS-PAGE. On the basis of the molecular mass of 230 kDa for the native PCA reductase and the subunit size of 25 kDa as estimated from SDS-PAGE and 28.9 kDa as deduced from the DNA sequence, the enzyme is most likely an octamer. The subunit composition of other PCA reductases varies from four, e.g. for *Saccharomyces cerevisiae* (Brandriss & Falvey, 1992), to 16, e.g. for barley (Krüger *et al.*, 1986). PCA reductase of *C. sticklandii* could also catalyse the

oxidation of proline to PCA at a nonphysiological pH value of 10 and higher substrate concentrations. This ability seems to have no physiological function for *C. sticklandii* because proline was solely converted to 5-aminovalerate as indicated by the obtained ¹⁵N data.

Some properties of PCA reductase from *C. sticklandii* were similar to those of proline dehydrogenase from *C. sporogenes* (Costilow & Cooper, 1978; Monticello & Costilow, 1981). Both enzymes were more stable in a high-ionic-strength buffer, slightly thermotolerant, and precipitated by high ammonium sulfate concentrations. Both enzymes exhibited a proline-oxidizing activity around pH 10 with high *K_m* values for L-proline and NAD and a PCA-reducing activity at physiological pH values. A different property resides in the ability of the enzyme from *C. sporogenes* to oxidize proline at pH 7.5 if 450 mM L-proline (*K_m* 110 mM for proline) is present and to be inhibited by glutamate. Proline dehydrogenase of *C. sporogenes* is a dimer of 220 kDa (Monticello & Costilow, 1981) in contrast to the possible octameric structure of the PCA reductase from *C. sticklandii*. Both organisms also differ in the enzymes used for proline biosynthesis from ornithine: in *C. sticklandii* proline is synthesized from ornithine by the action of ornithine aminotransferase and PCA reductase, whereas in *C. sporogenes* proline is directly formed from ornithine by ornithine cyclodeaminase, a dimeric enzyme of 81 kDa (Muth & Costilow, 1974). Proline dehydrogenase/PCA reductase of *C. sporogenes* might be involved in the interconversion of proline and glutamate under special physiological conditions (Costilow & Cooper, 1978).

The PCA reductases of diverse bacterial species, yeast, plants and animals are quite conserved in their amino acid sequence (Fig. 4). The enzymes from *Glycine max* (Delauney & Verma, 1990) and human origin (Dougherty *et al.*, 1992) exhibit 35% and 33% similarity, respectively, in amino acid residues to the PCA reductase of *C. sticklandii*. PCA reductases vary in chain length from 251 amino acids, as described for *Methanobrevibacter smithii* (Hamilton & Reeve, 1985), to 319 amino acids for human sources (Dougherty *et al.*, 1992). The high degree of interspecies complementation reported (Delauney & Verma, 1990; Dougherty *et al.*, 1992) confirms the functional similarity of the PCA reductases. An alignment of the primary sequence of selected PCA reductases shows several conserved amino acids including the putative NAD-binding site (Walker *et al.*, 1982) localized in the N-terminal part of PCA reductase (Fig. 4).

The bacterial *proC* gene is generally not localized together with other genes of proline biosynthesis as is evident from sequence data deposited in databases. The adjacent genes known to be upstream and downstream of *proC* differ between species. The derived amino acid sequence of *orfX* of *C. sticklandii* exhibited no similarities to proteins known to be present in the neighbourhood of *proC* genes. However, the genes *proBA* encoding γ -glutamyl kinase and γ -glutamylphosphate reductase generally form an operon structure in bacteria

(Ankri *et al.*, 1996; Limauro *et al.*, 1996; Leisinger, 1996). So far, transcriptional analyses have not been performed with *proC* genes. The *proC* gene of *E. coli* has three σ^{70} -dependent promoters as is evident from sequence analyses (see accession no. P00373), indicating that this gene is constitutively expressed. The *proBA* gene products of *E. coli* and *Corynebacterium glutamicum* are regulated to some extent during osmotic stress (Dadekar & Uratsu, 1988; Ankri *et al.*, 1996). To accumulate proline during osmotic stress, the genes encoding proline uptake systems are highly induced in *E. coli* (Leisinger, 1996). In plants genes involved in proline biosynthesis and proline degradation are divergently regulated before and after applying osmotic stress (Peng *et al.*, 1996).

ACKNOWLEDGEMENTS

This study was supported by grants from the Deutsche Forschungsgemeinschaft to A.P. and by the Fonds der Chemischen Industrie to J.R.A. We thank Peter Rücknagel for the N-terminal sequencing and Angelika Schierhorn for the HPLC-MS analysis (MPG Forschungsstelle 'Enzymologie der Proteinfaltung', Halle).

REFERENCES

- Adams, E. & Frank, L. (1980). Metabolism of proline and the hydroxyprolines. *Annu Rev Biochem* **49**, 1005–1061.
- Andreesen, J. R., Bahl, H. & Gottschalk, G. (1989). Introduction to the physiology and biochemistry of the genus *Clostridium*. In *Clostridia*, pp. 27–62. Edited by N. P. Minton & D. P. Clarke. New York: Plenum.
- Ankri, S., Serebrijski, I., Reyes, O. & Leblon, G. (1996). Mutations in the *Corynebacterium glutamicum* proline biosynthetic pathway: a natural bypass of the *proA* step. *J Bacteriol* **178**, 4412–4419.
- Arkowitz, R. A. & Abeles, R. H. (1989). Identification of acetyl phosphate as the product of clostridial glycine reductase: evidence for an acyl enzyme intermediate. *Biochemistry* **28**, 4639–4644.
- Arkowitz, R. A., Dhe-Paganon, S. & Abeles, R. H. (1994). The fate of the carboxyl oxygens during D-proline reduction by clostridial proline reductase. *Arch Biochem Biophys* **311**, 457–459.
- Bank, R. A., Jansen, E. J., Beekmann, B. & te Koppele, J. M. (1996). Amino acid analysis by reversed phase high performance liquid chromatography: improved derivatization and detection conditions with 9-fluorenylmethyl chloroformate. *Anal Biochem* **240**, 167–176.
- Bertram, J. & Dürre, P. (1989). Conjugal transfer and expression of streptococcal transposons in *Clostridium acetobutylicum*. *Arch Microbiol* **152**, 551–557.
- Blum, H., Beier, H. & Gross, H. J. (1987). Improved silver staining of plant proteins, RNA, and DNA in polyacrylamide gels. *Electrophoresis* **8**, 93–99.
- Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Anal Biochem* **72**, 248–254.
- Brandriss, M. C. & Falvey, D. A. (1992). Proline biosynthesis in *Saccharomyces cerevisiae*: analysis of the PRO3 gene, which encodes Δ^1 -pyrroline-5-carboxylate reductase. *J Bacteriol* **174**, 3782–3788.
- Buckel, W. & Barker, H. A. (1974). Two pathways of glutamate fermentation by anaerobic bacteria. *J Bacteriol* **117**, 1248–1260.
- Costilow, R. N. & Cooper, D. (1978). Identity of proline dehydrogenase and Δ^1 -pyrroline-5-carboxylic acid reductase in *Clostridium sporogenes*. *J Bacteriol* **134**, 139–146.
- Cunin, R., Glansdorff, N., Pierard, A. & Stalon, V. (1986). Biosynthesis and metabolism of arginine in bacteria. *Microbiol Rev* **50**, 314–352.
- Dadekar, A. M. & Uratsu, S. L. (1988). A single base pair exchange in proline biosynthesis genes causes osmotic stress tolerance. *J Bacteriol* **170**, 5943–5945.
- Delauney, A. J. & Verma, D. P. (1990). A soybean gene encoding Δ^1 -pyrroline-5-carboxylate reductase was isolated by functional complementation in *Escherichia coli* and is found to be osmo-regulated. *Mol Gen Genet* **221**, 299–305.
- Delauney, A. J., Hu, C. A., Kishor, P. B. & Verma, D. P. (1993). Cloning of ornithine Δ^1 -aminotransferase cDNA from *Vigna aconitifolia* by trans-complementation in *Escherichia coli* and regulation of proline biosynthesis. *J Biol Chem* **268**, 18673–18678.
- Deutch, A. H., Smith, C. J., Rushlow, K. E. & Kretschner, P. J. (1982). *Escherichia coli* Δ^1 -pyrroline-5-carboxylate reductase: gene sequence, protein overproduction and purification. *Nucleic Acids Res* **10**, 7701–7714.
- Dougherty, K. M., Brandriss, M. C. & Valled, D. (1992). Cloning human Δ^1 -pyrroline-5-carboxylate reductase cDNA by complementation in *Saccharomyces cerevisiae*. *J Biol Chem* **267**, 871–875.
- Dyer, J. K. & Costilow, R. N. (1968). Fermentation of ornithine by *Clostridium sticklandii*. *J Bacteriol* **96**, 1617–1622.
- Garcia, G. E. & Stadtman, T. C. (1992). *Clostridium sticklandii* glycine reductase selenoprotein A gene: cloning, sequencing and expression in *Escherichia coli*. *J Bacteriol* **174**, 7080–7089.
- Hamilton, P. T. & Reeve, J. N. (1985). Structure of genes and an insertion element in the methane producing archaeobacterium *Methanobrevibacter smithii*. *Mol Gen Genet* **200**, 47–59.
- Hayzer, D. J. & Leisinger, T. (1982). Proline biosynthesis in *Escherichia coli*. Purification and characterization of glutamate-semialdehyde dehydrogenase. *Eur J Biochem* **121**, 561–565.
- Hippe, H., Andreesen, J. R. & Gottschalk, G. (1991). The genus *Clostridium* – nonmedical. In *The Prokaryotes*, 2nd edn, pp. 1800–1866. Edited by A. Balows, H. G. Trüper, M. Dworkin, W. Harder & K. H. Schleifer. New York: Springer.
- Jeng, I. M., Somack, R. & Barker, H. A. (1974). Ornithine degradation in *Clostridium sticklandii*; pyridoxal phosphate and coenzyme A dependent thiolytic cleavage of 2-amino-4-ketopentanoate to alanine and acetyl coenzyme A. *Biochemistry* **13**, 2898–2903.
- Krüger, K. A., Jäger, H. J., Hintz, M. & Pahlich, E. (1986). Purification to homogeneity of Δ^1 -pyrroline-5-carboxylate reductase of barley. *Plant Physiol* **80**, 142–144.
- Laemmli, U. K. (1970). Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.
- Leisinger, T. (1996). Biosynthesis of proline. In *Escherichia coli and Salmonella: Cellular and Molecular Biology*, pp. 434–441. Edited by F. C. Neidhardt and others. Washington, DC: American Society for Microbiology.
- Limauro, D., Falcioratore, A., Basso, A. L., Forlani, G. & DeFelice, M. (1996). Proline biosynthesis in *Streptococcus thermophilus*: characterization of the *proBA* operon and its products. *Microbiology* **142**, 3275–3282.
- Miller, J. H. (1972). *Experiments in Molecular Genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Mitruka, B. M. & Costilow, R. N. (1967). Arginine and ornithine catabolism by *Clostridium botulinum*. *J Bacteriol* **93**, 295–301.

- Monticello, D. J. & Costilow, R. N. (1981). Purification and partial characterization of proline dehydrogenase from *Clostridium sporogenes*. *Can J Microbiol* 27, 942–948.
- Moran, C. P. (1993). RNA polymerase and transcription factors. In *Bacillus subtilis and Other Gram-positive Bacteria: Biochemistry, Physiology, and Molecular Genetics*, pp. 653–667. Edited by A. L. Sonenshein, J. A. Hoch & R. Losick. Washington, DC: American Society for Microbiology.
- Muth, W. L. & Costilow, R. N. (1974). Ornithine cyclase (deaminating). Properties of the homogeneous enzyme. *J Biol Chem* 249, 7457–7462.
- Patterson-Curtis, S. I. & Johnson, E. A. (1992). Roles of arginine in growth of *Clostridium botulinum* OkraB. *Appl Environ Microbiol* 58, 2334–2337.
- Peng, Z., Lu, Q. & Verma, D. P. S. (1996). Reciprocal regulation of Δ^1 -pyrroline-5-carboxylate synthetase and proline dehydrogenase genes controls proline levels during and after osmotic stress in plants. *Mol Gen Genet* 253, 334–341.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Sanger, F., Nicklen, S. & Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci USA* 74, 5463–5467.
- Seto, B. (1980). The Stickland reaction. In *Diversity in Bacterial Respiratory Systems*, vol. 2, pp. 50–64. Edited by C. J. Knowles. Boca Raton, FL: CRC Press.
- Seto, B. & Stadtman, T. C. (1976). Purification and properties of proline reductase from *Clostridium sticklandii*. *J Biol Chem* 251, 2435–2439.
- Shine, J. & Dalgarno, L. (1974). The 3'-terminal sequence of *Escherichia coli* 16S ribosomal RNA: complementarity to non-sense triplets and ribosome binding site. *Proc Natl Acad Sci USA* 71, 1342–1346.
- Smith, C. J., Deutch, A. H. & Rushlow, K. E. (1984). Purification and characteristics of a γ -glutamyl kinase involved in *Escherichia coli* proline biosynthesis. *J Bacteriol* 157, 545–551.
- Stadtman, T. C. (1956). Studies on the enzymic reduction of amino acids: a proline reductase of an amino acid-fermenting *Clostridium*, strain HF. *Biochem J* 62, 614–619.
- Stadtman, T. C. & Elliot, P. (1956). A new ATP-forming reaction: the reductive deamination of glycine. *J Am Chem Soc* 78, 2020–2021.
- Stadtman, T. C. & McClung, L. S. (1957). *Clostridium sticklandii* nov. spec. *J Bacteriol* 73, 218–219.
- Stadtman, T. C. & White, F. H. (1954). Tracer studies on ornithine, lysine, and formate metabolism in an amino acid fermenting *Clostridium*. *J Bacteriol* 67, 651–657.
- Takechi, M., Kanda, M., Hori, K., Kurotsu, T. & Saito, Y. (1994). Purification and properties of L-ornithine aminotransferase from gramicidin S-producing *Bacillus brevis*. *J Biochem* 116, 955–959.
- Tanaka, H. & Stadtman, T. C. (1979). Selenium-dependent clostridial glycine reductase. Purification and characterization of the two membrane-associated protein components. *J Biol Chem* 254, 5215–5217.
- Tsuda, Y. & Friedmann, H. C. (1970). Ornithine metabolism by *Clostridium sticklandii*. Oxidation of ornithine to 2-amino-4-ketopentonic acid via 2,4-diaminopentanoic acid; participation of B_{12} coenzyme, pyridoxal phosphate, and pyridine nucleotide. *J Biol Chem* 245, 5914–5926.
- Wagner, M. & Andreessen, J. R. (1995). Purification and characterization of threonine dehydrogenase from *Clostridium sticklandii*. *Arch Microbiol* 163, 286–290.
- Walker, J. E., Saraste, M., Runswick, M. J. & Gay, N. J. (1982). Distantly related sequences in the α - and β -subunits of ATP synthase, myosin kinase, and other ATP-requiring enzymes and a common nucleotide binding fold. *EMBO J* 1, 945–951.
- Williams, I. & Frank, L. (1975). Improved chemical synthesis and enzymatic assays of Δ^1 -pyrroline-5-carboxylic acid. *Anal Biochem* 64, 85–97.
- Yagasaki, M. & Ozaki, A. (1998). Industrial biotransformations for the production of D-amino acids. *J Mol Catal B: Enzymatic* 4, 1–11.
- Zinecker, H., Andreessen, J. R. & Pich, A. (1998). Partial purification of an iron-dependent L-serine dehydratase of *Clostridium sticklandii*. *J Basic Microbiol* 38, 147–155.

Received 19 October 1998; revised 21 December 1998; accepted 8 January 1999.