Identification of D-Proline Reductase from Clostridium sticklandii as a Selenoenzyme and Indications for a Catalytically Active Pyruvoyl Group Derived from a Cysteine Residue by Cleavage of a Proprotein*

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Highly active D-proline reductase was obtained from Clostridium sticklandii by a modified purification scheme. The cytoplasmic enzyme had a molecular mass of about 870 kDa and was composed of three subunits with molecular masses of 23, 26, and 45 kDa. The 23-kDa subunit contained a carbonyl group at its N terminus, which could either be labeled with fluorescein thiosemicarbazide or removed by o-phenylenediamine; thus, N-terminal sequencing became feasible for this subunit. L-[14C]proline was covalently bound to the 23-kDa subunit if proline racemase and NaBH₄ were added. Selenocysteine was detected in the 26-kDa subunit, which correlated with an observed selenium content of 10.6 g-atoms in D-proline reductase. No other non-proteinaceous cofactor was identified in the enzyme. A 4.8-kilobase pair (kb) EcoRI fragment was isolated and sequenced containing the two genes prdA and prdB. prdA coding for a 68-kDa protein was most likely translated as a proprotein that was posttranslationally cleaved at a threonine-cysteine site to give the 45-kDa subunit and most probably a pyruvoyl-containing 23-kDa subunit. The gene prdB encoded the 26-kDa subunit and contained an in frame UGA codon for selenocysteine insertion. prdA and prdB were transcribed together on a transcript of 4.5 kb; prdB was additionally transcribed as indicated by a 0.8-kb mRNA species.

D-Proline reductase (EC 1.4.1.6) is involved in amino acid metabolism of several clostridia and catalyzes the reductive ring cleavage of D-proline to 5-aminovalerate (1–3). In a typical Stickland reaction, this reduction is coupled to the oxidation of other amino acids, but an utilization of electron donors like formate is also possible (3, 5, 6). D-Proline reductase catalyzes the cleavage of a carbon-nitrogen bond. A similar reaction is catalyzed by glycine reductase, which cleaves glycine to acetyl phosphate and ammonia (7, 8). Different reaction mechanisms were postulated for the catalytic cascades of these enzymes (2, 3, 7, 9). D-Proline is first activated by an enzyme-bound pyruvoyl group. Then, a nucleophilic attack at the α-carbon of proline was presumed to be carried out by an electron-donating dithiol (like DTT) and the ring is cleaved to give 5-aminovalerate after hydrolytic release from the pyruvoyl adduct. Glycine reductase is composed of the proteins A, B, and C; protein B binds glycine as Schiff base via an enzyme-bound carbonyl group (11). A selenol anion attacks the α-carbon, and subsequently the carbon-nitrogen bond is cleaved to give ammonium and probably a protein B-bound carboxymethylselenoether that is transferred to protein A. By action of protein C, the carbon unit is transformed to a protein C-bound acetyl-enzyme intermediate leaving an oxidized protein A (12). Subsequently, the acetyl group is released as acetyl phosphate from protein C and its energy can be conserved by acetate kinase (13). To start a new reaction cycle, protein A is reduced by the thioredoxin system that obtains its electrons from NADPH (14). The genes encoding protein A, protein B, protein C, thioredoxin, and thioredoxin reductase are organized in an operon-like structure (11, 15). For D-proline reductase, the existence of an analogous high energy acyl-enzyme intermediate has been excluded, indicating that proline reduction is not coupled to energy conservation by substrate level phosphorylation (10). Despite this energetic disadvantage, proline is preferentially utilized from a synthetic growth medium containing both proline and glycine (16).

Clostridium sticklandii is the best characterized Stickland type organism containing both D-proline reductase and glycine reductase (3, 5, 17). In order to learn more about the regulation of proline and glycine reduction on a molecular level, we started to purify D-proline reductase from C. sticklandii. So far, D-proline reductase has been described as a membrane-bound homodecamer of 300 kDa containing a pyruvoyl group in each subunit of 30 kDa (1–3). Evidence was presented that the pyruvoyl moiety is located in a 4.6-kDa peptide that might be connected to the enzyme by an ester bond between a serine and glutamate residue (18, 19). Dithiols acted as electron donor for the purified D-proline reductase, whereas NADH is more effective than dithiols in crude preparations (1). It was suggested that the electrons might be transferred by two proteins from NADH to D-proline reductase, presumably involving an FAD-

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AJ130879.

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The abbreviations used are: DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; MALDI, matrix-assisted laser desorption ionization; kb, kilobase pair(s); bp, base pair(s); ICPMS, inductively coupled plasma mass spectrometry; PTH, phenylthiohydantoin; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; RP, reversed phase.
containing NADH dehydrogenase and a 250-kDa iron-containing protein (3, 20). The specific activity of α-proline reductase was 3 times higher in crude extracts if C. sticklandii was cultivated in the presence of selenite; however, no selenium was detected in the purified enzyme preparation (1).

During our studies, we developed a modified purification procedure for α-proline reductase and characterized the enzyme as a cytoplasmic protein with three subunits and a molecular mass of about 870,000 Da containing selenium in form of selenocysteine and a carbonyl moiety, most probably a pyruvoyl group. These data were supported by sequence analysis of the subunits of α-proline reductase and their corresponding genes and gave a more complex and detailed view of α-proline reductase than described so far (1, 3, 10, 20). The subunits of α-proline reductase were identified to exhibit sequence similarities to the protein B of glycine reductase (11) emphasizing a similar function of these proteins to split a carbon-nitrogen bond.

**EXPERIMENTAL PROCEDURES**

Materials—All chemicals were obtained from commercial sources unless otherwise specified. (750 mg, pH 8.5), and ammonium sulfate between 25% and 60% saturation. Pre-}

### Growth of C. sticklandii—C. sticklandii strain HF, DSM 517 obtained from DSMZ (Braunschweig, Germany), was grown in complex medium containing 50 mM α-proline (21) or in minimal medium at 30 °C as described previously (22); 1 μM selenite was always added. α-Proline reductase was purified from cells harvested in the late logarithmic growth phase. Escherichia coli XL2 blue was obtained from Stratagene (Heidelberg, Germany) and used for cloning purposes. It was grown in LB medium (23) or on agar plates containing 1.5% (w/v) agar. Ampicillin was added (125 μg/ml) if needed; isopropyl-1-thio-β-D-galactopyranoside (40 μg/ml) and 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (48 μg/ml) were added if necessary.

### Enzyme Assay—α-Proline Reductase activity was measured by quantifying the product 5-aminovaleraldehyde with α-phenylaldehyde as described (24). The reaction mixture of 0.5 ml contained 100 mM potassium phosphate buffer, pH 8.0, 10 mM MgCl₂, 20 mM DTT or NADH, 10 mM α-proline, and enzyme. The reaction was incubated at 30 °C and terminated after 30 min by addition of 0.3 ml of 5% HClO₄. After centrifugation to remove precipitated material, 50 μl of the supernatant was added to 1.95 ml of the fluorescence solution and the fluorescence was determined at 455 nm using an excitation at 340 nm. The fluorescence solution was prepared by adding 1 ml of an α-phenylaldoximyaldoxime solution (80 mg/ml ethanol) and 0.2 ml of 2-mercaptoethanol to 100 ml of 0.4 M HNO₃ buffer (pH 9.7, adjusted with KOH). One unit of α-proline reductase activity converted 1 μmol of α-proline to 5-aminovalerate in 1 min under standard reaction conditions.

### Purification of α-Proline Reductase—About 30 g of cells were suspended in 250 ml of 0.1 M Tris–HCl buffer, pH 8.6, containing 1 mM EDTA and 1 mM DTT (TED buffer) and incubated with lysozyme (1 mg/ml), DNaSe (0.2 mg/ml), and PMSF (200 μM) for 30–45 min at 37 °C. This suspension was passed twice at 140 megapascals through a French press cell. Unbroken cells and cell debris were removed by centrifugation at 18,000 × g for 15 min. Subsequently, the membrane fraction was removed by ultracentrifugation at 120,000 × g for 1 h. The resulting supernatant was termed crude extract. It was fractionated with solid ammonium sulfate between 25% and 60% saturation. Precipitates were collected by centrifugation at 20,000 × g for 20 min. The pellet obtained at 60% saturation was dissolved in 50 ml of TED buffer containing 0.7 mM ammonium sulfate. After washing the column with the same buffer, the enzyme was eluted by a linear gradient of ammonium sulfate and then ethylene glycol (0.7–0.0 and 0–5% (v/v), respectively) in 1,150 ml of buffer. The active fractions were combined, concentrated to 30 ml, and dialyzed against TED buffer (3 × 2 liters). The solution was applied to a Superose-6 HR10/30 column (Pharmacia) equilibrated with TED buffer containing 0.4 mM KCl and eluted with the same buffer. The active fractions were collected, concentrated, and applied to a Superose-6 HR10/30 column (Pharmacia) equilibrated with TED buffer containing 0.1 mM KCl. Activity was eluted with a linear KCl gradient (0.1–0.7 mM) in 5 ml. Active fractions were collected, concentrated, and applied to a Superox-6 HR10/30 column (Pharmacia) equilibrated with TED buffer containing 0.4 mM KCl and eluted with the same buffer. The active fractions contained the homogeneous enzyme and were used for further characterization.

### Molecular Mass Determination—The native molecular mass of α-proline reductase was estimated by gel filtration using a fast protein liquid chromatography Superose-6 column equilibrated with TED buffer containing 400 mM KCl and by polyacrylamide gel electrophoresis. Calibration standards were blue dextran (2,900,000), thyroglobulin (686,000), ferritin (440,000), catalase (232,000), aldolase (158,000), myoglobin (17,400), and bovine serum albumin (66,000). The molecular masses of the subunits of α-proline reductase were determined by SDS-PAGE (25) and MALDI mass spectrometry (Bruker-Franzen Analytik, Bremen, Germany).

### Selenite and Metal Determination—Selenium and metal content of α-proline reductase was analyzed by ICPMS using an HP 4500 model (Hewlett Packard, Germany).

Identification of Carbonyl Groups in α-Proline Reductase—Fluorescein thiosemicarbazide was used to detect carbonyl groups (26). About 10 μg of purified protein in 10 ml were incubated with 10 μl of potassium acetate buffer (300 mM, pH 4.6) and 1 μl of fluorescein thiosemicarbazide (0.1% in Me₂SO) for 2 h at room temperature in the dark. Protein was precipitated by chloroform/methanol (27) and separated by SDS-PAGE. Carbonyl groups were identified by UV light on a transilluminator or on a phosphorimager (Molecular Dynamics, Krefeld, Germany).

### Removal of N-terminal α-Keto Acids—α-Keto acids can be selectively removed from the N terminus of proteins by α-phenylenediamine (28). One volume of α-proline reductase (10 μg) was incubated with two volumes of 100 mM α-phenylenediamine (48 μg/ml) and 2.4 cm) equilibrated with TED buffer containing 0.1 M sodium phosphate buffer, pH 8.5; (3) purified α-proline reductase (100 μg) and proline racemase present in crude extracts (0.5 mg) were incubated under standard assay conditions (600 μl) with [14C]proline. After 2 min of incubation at 30 °C, 150 μl of 0.2 M 2-KBH₄ was added to reduce the adduct formed between enzyme and proline resulting in its covalent attachment.

### Electrophoresis—SDS-polyacrylamide gel electrophoresis was performed as described (25). For molecular mass determination, calibration kits from Sigma Chemie (Deisenhofen, Germany) were used as standards. Proteins were stained with Serva Blue (Serva, Heidelberg, Germany).

### Protein Analytical Methods—Protein was determined by the method of Bradford (29) with bovine serum albumin as a standard. N-terminal amino acid sequence determinations were done by automated Edman degradation (16). A synthetic peptide sequence (model 475HA, Applied Biosystems, Weiterstadt, Germany). To get internal amino acid sequence information of proline reductase subunits, two approaches were used to isolate internal peptides: (a) the subunits were separated by SDS-PAGE and digested in the gel overnight at 37 °C with trypsin in 120 mM NH₄HCO₃ buffer, pH 8.5, (b) purified α-proline reductase (1 nmol) was dissolved in 50 μl of 6 M guanidine hydrochloride containing 250 mM Tris-HCl (pH 8.0), reduced with 2-mercaptoethanol to the 5% of the original concentration, dialyzed against 4 M guanidine hydrochloride at 80 °C overnight. After digestion the masses of the peptides were determined by MALDI mass spectrometry on a reflectron-type time-of-flight mass spectrometer (Bruker Franzen). A saturated solution of α-cyano-4-hydroxycinnamonic acid was used as matrix. Peptide separation was done by RP chromatography using an ET 125/2 nucleo-sil 500–5C3 PPN column (Macherey & Nagel, Düren, Germany) and a high pressure liquid chromatography system (Shimadzu, Duisburg, Germany). Protein was precipitated by chloroform/methanol (27); to one volume of protein solution, four volumes of methanol, three volumes of H₂O, and one volume of chloroform were added. After centrifugation (10,000 × g, 1 min), the supernatant was decanted and methanol (three volumes) was added. After another centrifugation, the precipitated protein was dried.

### Identification of Selenocysteine by Pyridylethylation—To derivatize the selenocysteine moiety of α-proline reductase, protein was incubated in a volume of 50 μl containing 6 μM guanidinium chloride, 0.25 mM sodium phosphate buffer, pH 7.0, 1 mM EDTA, 0.3 mg of tricarboxyethylphosphine, and 2 μl of 4-vinylpyridine for 2 h at 37 °C in the dark under an N₂ atmosphere, resulting in a labeling of the selenocysteine residue and all cysteine residues. The selenocysteine-containing peptide was desalted and subjected to Edman degradation. A synthetic peptide con-
taining selenocysteine (obtained from S. Pegoraro, MPI für Biochemie, Munich, Germany) was used to optimize the conditions for labeling and identification.

**Standard Molecular Biology Techniques—**All standard procedures were performed as described (23). Enzymes were used according to the recommendations of the manufacturer. DNA sequence determination was performed by the dideoxy chain-termination method (30) using the Pharmacia ALF system (Pharmacia). Hybridizations were carried out as described by the manufacturer using the DIG system from Boehringer (Mannheim, Germany).

Cloning of the Genes Encoding D-Proline Reductase—The amino acid sequences obtained for D-proline reductase were used to design degenerated primer on the basis of the codon usage for *C. sticklandii* (31). Primer 1 was 5′-AAAGARCATGCNAATGA-3′, primer 2 was 5′-ATCRAAYTCYTCATCATTCATAACYTCAAT-3′. PCR amplification was carried out using Taq DNA polymerase, an annealing temperature of 50 °C, and 30 cycles. A specific PCR fragment was obtained, cloned into the pGEM-T vector (Promega, Mannheim, Germany), and sequenced. To produce a genomic library of *C. sticklandii*, total DNA was isolated (32), partially digested with EcoRI or *Sac*IIIA, and separated in a sucrose density gradient from 5% to 40% (v/v) sucrose for 24 h at 200,000 × g. 5-kb fragments of EcoRI-digested DNA and 3-kb fragments of *Sac*IIIA-digested DNA were isolated, dialyzed against H₂O, and cloned into the vector pBluescript SKII (Stratagene). After transformation of *E. coli* XL2 blue, nearly 2400 EcoRI and 4000 *Sac*IIIA clones were obtained, which were stored as plasmid mixtures at −20 °C.

Isolation of RNA from *C. sticklandii* and Northern Blot Analysis—*C. sticklandii* was grown in 30 ml of minimal medium to mid-logarithmic phase, harvested by centrifugation at 20,000 × g for 5 min, and RNA was isolated using the RNeasy spin columns from Qiagen (Hilden, Germany), and sequenced. To produce a genomic library of *C. sticklandii*, total DNA was isolated (32), partially digested with EcoRI or *Sac*IIIA, and separated in a sucrose density gradient from 5% to 40% (v/v) sucrose for 24 h at 200,000 × g. 5-kb fragments of EcoRI-digested DNA and 3-kb fragments of *Sac*IIIA-digested DNA were isolated, dialyzed against H₂O, and cloned into the vector pBluescript SKII (Stratagene). After transformation of *E. coli* XL2 blue, nearly 2400 EcoRI and 4000 *Sac*IIIA clones were obtained, which were stored as plasmid mixtures at −20 °C.

### RESULTS

**Localization of D-Proline Reductase—**After disruption of cells from *C. sticklandii* by a French press cell and ultracentrifugation, nearly all (99%) of the D-proline reductase activity was recovered in the supernatant, not in the membrane pellet. In the presence of high salt (1 M KCl) or detergents (1.5% Triton X-100 or 1% sodium deoxycholate), 0.2–1% of the total activity was present in the pellet. Similar results were obtained using different methods for cell disintegration (lysozyme, sonication) or preparation of protoplasts before separation of cytoplasmic and membrane fractions (data not shown). Substrate combinations or growth phase using complex or synthetic media had no influence on the localization of D-proline reductase activity being in all cases present in the cytoplasmic fraction (data not shown).

**Purification of D-Proline Reductase—**Compared with previous results (1), an improved purification scheme was established for D-proline reductase (Table 1). Ultracentrifugation, chromatography on phenyl-Sepharose, and affinity chromatography with aminohexane-Sepharose were highly effective for purification. Subsequently, gel filtration on Superose-6 resulted in a homogeneous enzyme preparation as judged by polyacrylamide gel electrophoresis (Fig. 1). However, nearly 90% of the activity was lost during the final gel filtration step. Other methods tried did not remove impurities or give higher yields.

**Pyruvoyl- and Selenium-containing D-Proline Reductase**

| Purification step       | Protein Activity | Specific activity | Purification Yield |
|-------------------------|------------------|-------------------|--------------------|
| Crude extract           | mg/ml            | Units             | units/mg | fold | %    |
| Ammonium sulfate        | 1830             | 6590              | 3.3     | 1.7  | 91.5 |
| Phenyl-Sepharose        | 184              | 2680              | 14.6    | 7.7  | 40.6 |
| Aminohexane-Sepharose   | 17               | 527               | 31.7    | 16.7 | 8.0  |
| Superose-6              | 4.4              | 71                | 16.1    | 1.1  |      |

**FIG. 1. Purification of D-proline reductase from *C. sticklandii*.** An SDS-polyacrylamide gel (12%) electrophoresis was performed with the different preparations obtained during purification of D-proline reductase. Lane 1, marker proteins, the molecular masses are shown in kDa; lane 2, crude extracts (30 μg of protein); lane 3, ammonium sulfate precipitation (30 μg of protein); lane 4, phenyl-Sepharose (20 μg of protein); lane 5 and 6, aminohexane-Sepharose (5 μg of protein); lane 7, Superose-6 (5 μg of protein). The proteins were stained with Serva Blue.
FIG. 2
Nucleotide and amino acid sequence of D-proline reductase operon. Start and stop codons and names of the open reading frames are in bold. Promotors, putative ribosome binding sites, the proposed termination loop, and protein sequences determined by Edmann degradation.
were analyzed by mass spectrometry and identified after the DNA sequence was available (data not shown).

**Cofactor Content of D-Proline Reductase—**Absorption spectra of purified D-proline reductase showed only a peak at 280 nm but no characteristics that might indicate bound cofactors like flavins, pyridoxal phosphate or Fe/S centers. The addition from 1 mM up to 1 mM of Fe$^{2+}$, FAD, FMN, NAD, and pyridoxal phosphate did not stimulate enzyme activity. Mg$^{2+}$ (10 mM) was essential for the reaction, but cations like K$^{+}$ had no effect on enzyme activity. ICPMS was employed to analyze the metal content of D-proline reductase. However, no metal but 10.6 g-atom selenium per 870 kDa was identified by ICPMS.

**Detection of Selenocysteine and Cysteine—**To identify selenocysteine as possible selenium compound, D-proline reductase was pyridylethylated with 4-vinylpyridine and the selenocysteine-containing peptide from Glu140 to Lys 212 of PrdB was isolated. Using normal reducing conditions with 2-mercaptoethanol, no PTH amino acids were detected at position Sec152 and Cys155, although cysteine residues from other peptides were labeled under these conditions and identified during amino acid sequencing. Therefore, tricarboxyethylphosphine was used as reducing agent because it does not react with 4-vinylpyridine and the selenol group can be directly pyridylethylated after its formation. The peptide was desalted and again alkylated, and Edman degradation was repeated (Fig. 2). Now, the pyridylethylated selenocysteine eluted exactly at the position that was revealed for selenocysteine using a synthetic peptide as standard. The retention time was close to proline as standard during amino acid sequencing (35). The cysteine adjacent to selenocysteine became also labeled after the drastic reduction with tricarboxyethylphosphine. This indicates that a mixed selenide-sulfide might be formed between Sec152 and Cys155 preventing pyridylethylation under mild reducing conditions (2-mercaptoethanol) even when the enzyme was denatured.

**Presence of Carbonyl Groups in D-Proline Reductase—**Carbonyl groups were detected in the 23- and 26-kDa subunit using labeling with fluorescein thiosemicarbazide (Fig. 3A). The 23-kDa subunit became susceptible for N-terminal sequencing after treatment with o-phenylenediamine that removes specifically a-keto acids from the N terminus of proteins (25). Subsequently, the amino acid sequence IGPASKRN... (Fig. 2) was determined that corresponded exactly to an internal region of the *prdB* gene product. Cys426 was the amino acid residue upstream of this sequence as identified by DNA sequencing (Fig. 2). Thus, the terminal carbonyl group in the 23-kDa subunit most likely derived from this cysteine residue, which could be transformed to an N-terminal pyruvoyl group. The 23-kDa subunit might therefore correspond to the pyruvoyl-containing 30-kDa subunit previously described for D-proline reductase (1). However, in former studies only one subunit of D-proline reductase was reported (1) and the pyruvoyl moiety was assigned to a small peptide of 4.6 kDa that could be separated from the subunit after treatment with mild alkali (18, 19). We did not obtain such a peptide using similar conditions. The carbonyl function associated with the 26-kDa subunit (Fig. 3A) could so far not be assigned to a specific amino acid.

**Labeling of D-Proline Reductase with [14C]Proline—**In all reaction mechanisms proposed so far for D-proline reductase, the pyruvoyl group is supposed to bind the substrate proline (1–3). By addition of NaBH$_4$, an adduct should be formed that is covalently bound to the enzyme. Because only L-[14C]proline is commercially available, crude extract of *C. sticklandii* containing proline racemase (33) had to be added to allow labeling experiments. The 23-kDa subunit became labeled to a high extent under such conditions, indicating that it reacted with...
Proline (Fig. 3B). Controls using a high molar excess of unlabeled proline (500/1) or no NaBH₄ showed no labeling of the 23-kDa subunit (Fig. 3B). If no NaBH₄ was added, the 26-kDa subunit became slightly labeled, indicating that proline or a reaction derivative might also be bound to this subunit during the reaction cycle (Fig. 3B).

**Cloning of the Genes Encoding 2-Proline Reductase**—The protein sequences obtained from the three subunits were used to isolate the genes encoding 2-proline reductase. A homologous DNA fragment of 1.2 kb was generated by PCR using DNA of *C. sticklandii* as a template and was identified by sequencing to encode part of the prd operon. This fragment was used as a probe to isolate a 4.8-kb EcoRI clone from the genomic library of *C. sticklandii* (Fig. 4). Southern blot hybridization revealed that the genes encoding 2-proline reductase were present as single copy in the genome of *C. sticklandii* (data not shown).

**Identification and Characterization of the prd Operon**—Five open reading frames were identified on the 4.8-kb DNA fragment (Figs. 2 and 4). The open reading frame prdA encoded a protein of 630 amino acids. The amino acid sequences determined for fragments of the 45-kDa subunit of 2-proline reductase corresponded exactly to the deduced sequence from Val¹ to Thr⁴²⁵ (Fig. 2); the amino acid sequences obtained from the deblocked 23-kDa subunit corresponded to the deduced amino acid sequence from Ile⁴²⁷ to Lys⁶³⁰ of prdA (Fig. 2). The C-terminal peptide of the 45-kDa subunit stopped with Thr⁴²⁵. After removal of the N-terminal carbonyl group, the N terminus of the 23-kDa subunit started with Ile⁴²⁷ (Fig. 2). Cys²⁴⁶ was encoded by prdA but not detected in one of the peptides; therefore, this amino acid should be the precursor of the α-keto acid blocking the 23-kDa subunit. The DNA sequence upstream of Cys²⁴⁶ did not contain a typical ribosome binding site or a start codon in a suitable distance. Thus, prdA should encode a proprotein (π subunit) that posttranslationally cleaved to form the 45-kDa subunit and the N-terminally blocked carbon-containing 23-kDa subunit of 2-proline reductase.

The open reading frame prdB encoded a protein of 242 amino acids (25.6 kDa) corresponding to N-terminal and internal protein sequences determined for the 26-kDa subunit of 2-proline reductase (Fig. 2). An in-frame TGA codon was present that should code for selenocysteine as was identified by amino acid determination.

The open reading frame orfX upstream of prdA encoded 237 amino acids of a truncated C-terminal part of a protein that did not correspond to amino acid sequences obtained for subunits of 2-proline reductase. The sequence of orfX stopped at the EcoRI site at the 5’ end of the clone. No function of this protein can be derived from comparisons with sequences deposited in data banks. Two putative open reading frames orfY and orfZ were identified downstream of prdB (Figs. 2 and 4), which exhibited high similarities to N-terminal and C-terminal parts of the prdA gene, respectively (Fig. 5). They were interrupted by a short stretch of nucleotides, which was localized at the Cys²⁴⁶ site, the potential cleavage site of PrdA as became evident from sequence alignments (Fig. 5).

orfX was separated from prdA by 117 bp; the region between prdA and prdB was 287 bp. The start codon of prdB and prdA was preceded by a sequence resembling the Shine-Dalgarno motif for ribosome binding (Fig. 2) (36). The region directly upstream of prdB contained a putative promotor sequence TTAATG-(14 bp)-TAAAT, which was located 32 bp upstream of the start codon. A few bp upstream of this promotor, a sequence motif resembling the structure of a typical *E. coli* ρ₅₄ promotor was identified (Fig. 2). No promotor-like structure was identified upstream of prdA. The sequence located downstream from prdB contained two inverted repeats with the potential to form a hairpin structure. This structure is similar to the Rho-independent transcription termination signal in *E. coli* (37). orfY started downstream of this secondary structure (62 bp downstream of prdB) and was separated from orfZ by a short nucleotide stretch. Both sequences were preceded by a putative ribosome binding site, but no promotor-like structure was identified. An mRNA structure similar to the secondary mRNA structure necessary for selenocysteine incorporation in *E. coli* (38, 39) was not identified in the prdB gene, similar to the situation in prdA and prdB (11) of glycine reductase.

To analyze the transcription of these genes, probes were deduced from prdA and prdB and used in Northern blot experiments for hybridization against total RNA from *C. sticklandii* (Fig. 6). Both probes hybridized with a 4.5-kb mRNA, indicating that prdA and prdB formed a transcription...
In this study, D-proline reductase was found to be located exclusively in the cytoplasm of C. sticklandii. In contrast to former studies (1), no indications were now obtained that the DTT-dependent D-proline reductase might be a membrane-bound enzyme. At least it was readily soluble if associated with the cytoplasmic membrane. This might apply also for the proteins involved in electron transfer from NADH to D-proline reductase. In our studies, these proteins were present in the soluble cytoplasmic fraction after separation by ultracentrifugation, as was also shown by another group (20). An association with the cytoplasmic membrane might be indicated by a report that proline reduction was coupled to the extrusion of H ions in Clostridium sporogenes (40).

The D-proline reductase from C. sticklandii was purified by a modified purification scheme to homogeneity. The enzyme exhibited a molecular mass of about 870 kDa and consisted of three different subunits with molecular masses of 23, 26, and 45 kDa. This is in sharp contrast to earlier studies (1), where D-proline reductase was characterized as homodecamer with a molecular mass of 300 kDa consisting of one pyruvoyl-containing subunit of 30 kDa (1, 2). The now obtained 23-kDa subunit also seems to contain a pyruvoyl group, thus, it might be identical to the reported 30-kDa subunit. However, the amino acid composition reported for the 30-kDa subunit (1) is strikingly different from the amino acid composition of the 23-kDa (or the 26-kDa) subunit characterized in this study.

The three subunits present in our preparation were essential components of active D-proline reductase, as indicated by the fact that the specific activity for purified D-proline reductase was much higher in our preparation (16 m mol min⁻¹ mg⁻¹ protein) than reported before varying between 0.08 to 0.26 m mol min⁻¹ mg⁻¹ using the same assay with DTT as electron donor (1, 20). A more direct evidence was obtained by the organization of the genes prdA and prdB, forming an operon structure. The close relation of the 45- and 23-kDa subunits is also given by the identity of the amino acid sequences of the analyzed peptides to the deduced sequence of prdA indicating their translation as proprotein PrdA.

**DISCUSSION**

In this study, D-proline reductase was found to be located exclusively in the cytoplasm of C. sticklandii. In contrast to former studies (1), no indications were now obtained that the DTT-dependent D-proline reductase might be a membrane-bound enzyme. At least it was readily soluble if associated with the cytoplasmic membrane. This might apply also for the proteins involved in electron transfer from NADH to D-proline reductase. In our studies, these proteins were present in the soluble cytoplasmic fraction after separation by ultracentrifugation, as was also shown by another group (20). An association with the cytoplasmic membrane might be indicated by a report that proline reduction was coupled to the extrusion of H⁺ ions in Clostridium sporogenes (40).
threefold stimulation of the specific activity of δ-proline reductase was observed in extracts if *C. sticklandii* was grown in the presence of selenite (1). No labeling of δ-proline reductase by 75Se selenite was detected in the former final preparation (1). We demonstrated that the 26-kDa subunit (PrdB) actually contained selenocysteine by identifying this particular amino acid as pyridylethylated derivative. An in-frame TGA codon in *prdB* (41) and the selenium content of the holoenzyme as determined by ICPMS were two additional proofs for δ-proline reductase being a selenoprotein.

So far, we have no explanation for these inconsistent data, especially for the fact that the enzyme prepared during earlier studies was still active containing just one subunit. The strikingly lower specific activity of the former preparations (1, 20) might indicate that the other two subunits should have been present in the previous preparation too, perhaps in a much lower non-detected amount; thus, both were not identified as subunits of δ-proline reductase. The dimeric structure previously proposed corresponds to our calculations. However, the reported native molecular mass deviates dramatically for both preparations, being about 300 kDa in former studies (1, 20), but reported native molecular mass deviates dramatically for both preparations, being about 300 kDa in former studies (1, 20), but lower non-detected amount; thus, both were not identified as subunits of δ-proline reductase.

Genes encoding δ-proline reductase have not been cloned before, nor have they been identified during genome sequencing. Thus, for the first time the sequence of a δ-proline reductase is reported. The amino acid sequence data obtained by Edman degradation and mass spectrometry were compared before, nor have they been identified during genome sequencing. Thus, for the first time the sequence of a δ-proline reductase is reported. The amino acid sequence data obtained by Edman degradation and mass spectrometry were compared with the DNA sequence, revealing a highly refined sequence of the *prdB* gene (Fig. 5). So far, no function can be assigned to these putative proteins.

The PrdA protein was translated as a proprotein that is cleaved to the 23- and 45-kDa subunits of δ-proline reductase. The ω-keto acid blocking the new N terminus of the 23-kDa subunit should be a pyruvoyl group because a cysteine residue was detected as the precursor of this group. In previous studies (18), a labeled pyruvoyl group was identified in δ-proline reductase if *C. sticklandii* was grown in the presence of [14C]serine, indicating that either serine or cysteine is the precursor of this group. It is well established (42–45) that all known pyruvoyl-containing enzymes are translated as a preproteins (π subunit) that undergoes a cleavage reaction to generate two subunits with the pyruvoyl group at the N terminus of the former C-terminal part of the proprotein. In all these enzymes, a serine residue has been identified as the precursor of the pyruvoyl group. In mutants of phosphatidylserine decarboxylase of *E. coli* human and human P-adenosymelitonone decarboxylase proenzymes, this serine residue was exchanged by site-directed mutagenesis to threonine or cysteine. In these mutants, processing of the proprotein occurred in vivo and in vitro with 10–200 times decreased processing rates (45, 46). In case of δ-proline reductase, the π subunit is encoded by *prdA* and the precursor of the pyruvoyl group is a cysteine residue according to the derived sequences as observed for GrdB of glycine reductase (11). The cleavage has to occur between Thr46 and Cys48 via a thioester (42, 43) to generate the observed ω-keto acid group. Most likely, the substrate proline reacted with this group, as indicated by the labeling of the 23-kDa subunit with [14C]proline after reduction with borhydride. δ-Proline reductase and protein B of glycine reductase (11) are so far the only known examples of pyruvoyl-containing enzymes where the π subunit is split at the N-terminal site of a cysteine residue and, thus, cysteine is the precursor of the pyruvoyl group. Therefore, both enzymes should be assigned to a new subclass of pyruvoyl-containing enzymes.

Based on structural and sequence data presented in this paper, we postulate a modified reaction mechanism for the reduction of proline involving both selenocysteine and cysteine moieties present in PrdB (Fig. 8, A–E). First, an adduct between the nitrogen of proline and the pyruvoyl group of the 23-kDa subunit is formed (B) (2, 3, 10). The formation of a Schiff base might also be possible (2, 3), but according to Ref. 10 it is not shown. The selenol anion of selenocysteine in PrdB attacks nucleophilically the ω-carbon, resulting in cleavage of the N-C bond of the proline ring (C) similar to protein B of glycine reductase (11). This intermediate would be transformed to the oxidized 26-kDa protein containing a mixed selenide/sulfide group and to the 5-aminovalerate adduct at the 23-kDa protein (D). The final product, 5-aminovalerate, is formed by hydrolysis (E). Subsequently, the selenide-sulfide group of PrdB is reduced by, e.g., artificial electron donors like DTT. The natural electron donating system for δ-proline reductase is quite unknown (3, 20), except that NADH, not NADPH, is

### Pyruvoyl- and Selenium-containing δ-Proline Reductase

| **PrdB** | KGEYTVYKMLPPFVKPKMKADEKMKKEMQVA | 70 |
| **GrdB** | LGEYTVYKMLPPFVKPKMKADEKMKKEMQVA | 264 |
| **PrdB** | DMV---SKEGGDSVDKLVQHSLK | 135 |
| **GrdB** | DLSGSMQGRELKELSDKLQH | 333 |
| **PrdB** | NQKKEVEQDDAVTVLQYQCR | 205 |
| **GrdB** | KLEADGQDDAVTVLQYQCR | 403 |
| **PrdB** | NNEDRARQAIHCTQAKQ | 242 |
| **GrdB** | LGHEKELRKEKVEALALQVTQEEQTVF | 438 |

**Fig. 7.** Alignment of the selenocysteine-containing subunit of δ-proline reductase (PrdB) and the 47-kDa subunit of protein B of glycine reductase. The designations correspond to: PrdB, 26-kDa subunit of δ-proline reductase from *C. sticklandii*; GrdB, selenocysteine-containing subunit of protein B of glycine reductase from *E. acidaminophilum* (11). The selenoprotein A-like motif -UXXC- is underlined, and identical residues are printed in **bold**. The N-terminal extension in GrdB of about 200 amino acids is not shown.
The scheme is based on earlier proposals (2, 3, 8, 36) and on the data presented in this study. Further explanations are given in “Discussion.”

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REFERENCES

1. Seto, B., and Stadtman, T. C. (1976) J. Biol. Chem. 251, 2435–2439
2. Hodgins, S. D., and Abeles, R. H. (1969) Arch. Biochem. Biophys. 130, 274–285
3. Seto, B. (1980) in Diversity in Bacterial Respiratory Systems (Kluyver, J. J., ed) pp. 49–64, CRC Press, Boca Raton, FL
4. Harms, C., Meyer, M. A., and Andreesen, J. R. (1998) Microbiology 144, 793–800
5. Andreesen, J. R., Bahl, H., and Gottschalk, G. (1989) in Clostridia (Minton, N. P., and Clarke, D. P., eds) pp. 27–62, Plenum Press, New York
6. Andreesen, J. R., Wagner, M., Sonntag, D., Kohlstock, M., Harms, C., Gursinsky, T., Jager, J., Kabisch, U., Grantzdorffer, A., Pich, A., and Sohling, B. (1999) BioFactors, in press
7. Andreesen, J. R. (1994) Antonie van Leeuwenhoek 66, 223–237
8. Arkowitz, R. A., and Abeles, R. H. (1989) Biochemistry 28, 4639–4644
9. Arkowitz, R. A., and Abeles, R. H. (1990) J. Am. Chem. Soc. 112, 870–872
10. Arkowitz, R. A., Dhe-Paganon, S., and Abeles, R. H. (1994) Arch. Biochem. Biophys. 311, 457–459
11. Wagner, M., Sonntag, D., Grimm, R., Pich, A., Eckerskorn, C., Sohling, B., and Andreesen, J. R. (1999) Eur. J. Biochem 260, 38–49
12. Arkowitz, R. A., and Abeles, R. H. (1991) Biochemistry 30, 4090–4097
13. Schrider, T., and Andreesen, J. R. (1992) Eur. J. Biochem. 206, 79–85
14. Dietrichs, D., Meyer, M., Rietz, M., and Andreesen, J. R. (1991) J. Bacteriol. 173, 5983–5991
15. Lubbers, M., and Andreesen, J. R. (1993) Eur. J. Biochem. 217, 791–798
16. Uhde, A. (1990) Wachstumsphysiologische Untersuchungen zum Abbau von Aminosäuren und Mögliche Funktion eines Elektronen-transferierenden Flavoproteins bei Clostridium stick landii. Diploma thesis, Universität Göttingen, Germany
17. Stadtman, T. C., and McClung, L. S. (1957) J. Bacteriol. 73, 218–219
18. Seto, B. (1978) J. Biol. Chem. 253, 4525–4529
19. Seto, B. (1980) J. Biol. Chem. 255, 5004–5006
20. Schwartz, A. C., and Müller, W. (1979) Arch. Microbiol. 123, 203–208
21. Schwartz, A. C., and Müller, W. (1979) Arch. Microbiol. 123, 203–208
22. Wagner, M., and Andreesen, J. R. (1995) Arch. Microbiol. 163, 286–290
23. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1998) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
24. Seto, B. (1979) Anal. Biochem. 95, 44–47
25. Baumann, J. G. J., Wiegant, J., and van Duijn, P. (1981) J. Histochem. Cytochem. 29, 257–257
26. Wessel, D., and Flugge, V. J. (1984) Anal. Biochem. 138, 141–143
27. Dixon, H. B. F., and Fields, R. (1972) Methods Enzymol. 25, 409–419
28. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
29. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 5463–5467
30. Garcia, G. E., and Stadman, T. C. (1992) J. Bacteriol. 174, 7080–7089
31. Bertram, J., and Durre, P. (1999) Arch. Microbiol. 170, 551–557
32. Rudnick, G., and Abeles, H. A. (1975) Biochemistry 14, 4515–4522
33. Pu, J., and Reinsberg, J. (1986) FEMS Microbiol. Lett. 7, 153–156
34. Zhong, L., Arner, E. S. J., Ljung, J., Aslund, F., and Holmgren, A. (1998) J. Biol. Chem. 273, 8581–8591
35. Shine, J., and Dalgarno, L. (1974) Proc. Natl. Acad. Sci. U. S. A. 71, 1342–1346
36. D'Aubenton Carafa, Y., Brody, E., and Thermes, C. (1996) J. Mol. Biol. 212, 8453 effective in crude extracts. Thus, in contrast to glycine reductase (7, 11), electrons will not be transferred via the strictly NADPH-dependent thioredoxin system of C. sticklandii (4). Further studies must prove if the protein A-like motif (-U-X-C-) is actually a redox-active site in case of D-proline reductase. However, the resistance of Sec152 and Cys155 against alkylation after reduction with 2-mercaptoethanol but not after reduction using tricarboxyethylphosphine indicates a redox-active function of this group. The presence of an NAD-dependent dihydro-lipoamide dehydrogenase (16) besides an NADPH-dependent thioredoxin system of C. sticklandii (7, 11), electrons will not be transferred via the strictly NADPH-dependent thioredoxin system of C. sticklandii (4) in C. sticklandii would be in line with the observed indication that an FAD-containing protein might be involved in proline reduction (3). In D-proline reductase, no energy-rich intermediate or phosphorylated product has been identified, in contrast to glycine reductase (10, 12, 13). Hence, one main difference between proline and glycine reduction is the capability to conserve energy by substrate level phosphorylation in case of glycine reduction. However, for C. sporo- genes, it has been shown that proline reduction is coupled to the formation of a pH gradient across the cytoplasmic membrane and, thus, proline reduction might be used for energy conservation by a chemiosmotic mechanism (40).

FIG. 8. Proposed reaction mechanism of D-proline reductase.

The scheme is based on earlier proposals (2, 3, 8, 36) and on the data presented in this study. Further explanations are given in “Discussion.”
38. Zinoni, F., Heider, J., and Böck, A. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87**, 4660–4664
39. Hüttenhofer, A., Westhof, E., and Böck, A. (1996) *RNA* **2**, 354–366
40. Lovitt, R. W., Kell, D. D., and Morris, J. G. (1986) *FEMS Microbiol. Lett.* **36**, 269–273
41. Hüttenhofer, A., and Böck, A. (1998) in *RNA Structure and Function* (Simons, R. W., and Grunberg-Manago, M., eds) pp. 603–639, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
42. Hackert, M. L., and Pegg, A. E. (1997) in *Comprehensive Biological Catalysis* (Sinnott, M. L., ed) pp. 201–216, Academic Press, London
43. Perler, F. B. (1998) *Nat. Struct. Biol.* **5**, 249–252
44. Albert, A., Venugopal, D., Genschel, U., Khan, G., Ramjee, M. K., Pulido, R., Sibanda, B. L., Delft, F., Witty, M., Blundell, T., Smith, A. G., and Abell, C. (1998) *Nat. Struct. Biol.* **5**, 289–293
45. Li, Q.-X., and Dowham, W. (1991) *J. Biol. Chem.* **266**, 4111–4115
46. Xiong, H., Stanley, B. A., Tekwani, L., and Pegg, A. E. (1997) *J. Biol. Chem.* **272**, 28342–28348