Purification and Properties of Proline Reductase from Clostridium sticklandii

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Proline reductase of Clostridium sticklandii is a membrane-bound protein and is released by treatment with detergents. The enzyme has been purified to homogeneity and is estimated by gel filtration and sedimentation equilibrium centrifugation to have a molecular weight of 298,000 to 327,000. A minimum molecular weight of 30,000 to 31,000 was calculated on the basis of sodium dodecyl sulfate-acrylamide gel electrophoresis and amino acid composition. Amino acid analysis showed a preponderance of acidic amino acids. No tryptophan was detected in the protein either spectrophotometrically or by amino acid analysis. A total of 20 sulfhydryl groups measured by titration of the reduced protein with 5,5'-dithiobis(2-nitrobenzoic acid) is in agreement with 20 cysteic acid residues determined in hydrolysates of performic acid-oxidized protein. No molybdenum, iron, or selenium was found in the pure protein. Although NADH is the physiological electron donor for the proline reductase complex, the purified 300,000 molecular weight reductase component is inactive in the presence of NADH in vitro. Dithiothreitol, in contrast, can serve as electron donor both for unpurified (putative proline reductase complex) and purified proline reductase in vitro.

EXPERIMENTAL PROCEDURE

Clostridium sticklandii was grown in media described previously (3). For studies on the effects of selenium, the bacteria were cultured in media supplemented with 1 μM levels of Na2SeO3, Na2SeO4, or Na2SeO4.

Purification of Proline Reductase

Step 1: Preparation of Spheroplasts—C. sticklandii cells were subjected to osmotic shock according to the procedure of Nossal and Heppel (5). Spheroplasts were removed by centrifuging the shocked suspension for 1 hour at 27,000 x g at 4°. The pellets were washed with water and then suspended in 50 mM Tricine-KOH, pH 8.6, containing 1 mM each of MgK2EDTA and dithiothreitol.

1Proline reductase has been referred to as NADH-linked proline reductase (EC 1.4.1.6). This enzyme is, in fact, the same as that reported as lipoate-linked proline reductase (EC 1.4.4.1) (1, 2).

2This buffer mixture will be referred to as Tricine buffer.
Proline Reductase: Purification and Properties

Step 1: Solubilization of Proline Reductase—Enzyme activity was solubilized from the pellet suspension (obtained from Step 1) by treatment with 1% sodium deoxycholate and 1.5% Triton X-100. The mixture was sonified for 2 min at 10,000 intervals. The sonicated suspension was gently stirred for 1 hr at 4°C, and then centrifuged at 27,000 x g for 30 min. Although some proline reductase activity remained associated with the pellets, no attempts were made to extract this small amount of residual enzyme. Detergent removal, following solubilization of the enzyme, is necessary before fractionation with ammonium sulfate. Excess detergents were removed from the solubilized enzyme fraction by dialysis, first against water, then against Tricine buffer, and finally by adsorption on Bio-Beads SM-2 as described by Holloway (6).

Step 2: Ammonium Sulfate—Proline reductase was precipitated from the dialyzed extract by the addition of solid ammonium sulfate between 30% and 50% saturation. The 50% saturated ammonium sulfate mixture was incubated in an ice bath for 1 hr to allow complete precipitation of the enzyme. The pellet obtained after centrifugation at 27,000 x g for 20 min was suspended in Tricine buffer, and desalted either by dialysis or Sephadex G-25 filtration.

Step 3: AH-Sepharose 4B Affinity Chromatography—A Sepharose containing bound aminohexane chain was equilibrated and packed in Tricine buffer. Elution of applied proteins was effected by Tricine buffer containing a step gradient of KCl, 0.1 M, 0.3 M, 0.5 M, and 1 M. Detergent-bound proteins which were turbid in appearance were eluted from the column with the equilibrating buffer. Proline reductase activity was recovered in a yellow band which was eluted with 0.5 M KCl.

Step 4: Gel Filtration on Bio-Gel A-1.5m—The yellow colored proline reductase solution was chromatographed on a Bio-Gel A-1.5m column equilibrated with Tricine buffer. The elution buffer was 50 mM Tricine-KOH, pH 8.6, containing 1 mM diithiothreitol. Proline reductase was eluted first, and this was followed by a yellow protein fraction which showed no proline reductase activity. The peak activity fractions were pooled, whereas later fractions which overlapped the yellow protein were applied to a hydroxyapatite column and separated by elution with potassium phosphate buffer, pH 7.2, containing 1 mM diithiothreitol. In the last step, the yellow protein contaminant was completely eluted with 0.0 M potassium phosphate buffer and then the additional proline reductase was recovered by elution with 0.1 M potassium phosphate buffer.

Assay of Proline Reductase Activity
Proline reductase activity was routinely measured by determining the extent of decomposition of n-proline at 30°C with diithiothreitol as the electron donor. The reaction mixture (0.5 ml) consisted of the following: 20 μmol of Tris HCl, pH 8.6; 3 μmol of MgCl₂; 6 μmol of n-proline; 10 μmol of 1,4-dithiothreitol and enzyme. The reactions were carried out in stopped tubes under argon, and usually were terminated after 60 min by the addition of 1 ml of 5% perchloric acid, however, the reaction time was varied depending on enzyme activity. The amount of unreacted n-proline was determined by the acid procedure of Lovenberg et al. (14). Protein samples of 5 mg each were adjusted to pH 1 with 1 N HCl, and then heated at 80°C for 10 min. Molybdenum was determined with the dithion reagent as reported by Clark and Axley (15). Atomic absorption spectrometry was also employed to determine iron, molybdenum, and manganese.

Amino Acid Composition
For amino acid analyses, enzyme samples (25 μg) were desalted by Sephadex G-25 gel filtration and lyophilized to dryness. Hydrolysis was performed in a vacuum in 4 N methanesulfonic acid containing 0.2% 0-(2-aminoethyl)indole for 24, 48, and 72 hours at 110°C as described by Liu and Chang (12). Half-cystine and methionine residues were determined as cysteic acid and methionine sulfone, respectively, after performic acid oxidation as described by Moore (13). Duplicate hydrolyses were performed. Hydrolysis with 6 N HCl was also performed for comparison. Amino acid analyses were performed with a Beckman 120 amino acid analyzer. The areas of the peaks were analyzed by manual integration.

Metal Determination
Total iron was determined by the o-phenanthroline method of Lovengberg et al. (14). Protein samples of 5 mg each were adjusted to pH 1 with 1 N HCl, and then heated at 80°C for 10 min. Molybdenum was determined with the dithion reagent as reported by Clark and Axley (15). Atomic absorption spectrometry was also employed to determine iron, molybdenum, and manganese.

Materials
AH-Sepharose 4B was purchased from Pharmacia Fine Chemicals, Bio-Beads SM-2 from Bio-Rad Laboratories, and [³⁵S]Na₂SeO₃ from Amerham/Deare.

Purification of Proline Reductase
The purification procedures are described under “Experimental Procedure,” and the flow sheet of a typical preparation is presented in Table I. The preparation reported in the table was made from 160 g of freshly harvested C. sticklandii cells, wet weight. The cells were washed twice with water. If cells that had been previously frozen in liquid nitrogen were employed, a substantial amount of protein was released during the water washes. Approximately 9% of the cellular proteins are released from intact cells into the shock suspension during spheroplast preparation. However, only a relatively small percentage of the proline reductase activity is found in this fraction, and consequently, this fraction was not used for subsequent purification.

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Volume</th>
<th>Total Protein</th>
<th>Specific Activity</th>
<th>Total Units</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell suspension</td>
<td>430</td>
<td>66,000</td>
<td>0.23</td>
<td>15,180</td>
<td>100.0</td>
</tr>
<tr>
<td>Shock suspension</td>
<td>488</td>
<td>6,730</td>
<td>0.20</td>
<td>1,344</td>
<td>100.0</td>
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<tr>
<td>Detergent solubility</td>
<td>318</td>
<td>9,685</td>
<td>1.43</td>
<td>10,864</td>
<td>14.7</td>
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<tr>
<td>Ammonium sulfate</td>
<td>239</td>
<td>4,815</td>
<td>2.44</td>
<td>11,749</td>
<td>7.5</td>
</tr>
<tr>
<td>AH-Sepharose 4B</td>
<td>43</td>
<td>1,595</td>
<td>4.73</td>
<td>7,544</td>
<td>24.9</td>
</tr>
<tr>
<td>Bio-Gel A-1.5m</td>
<td>17</td>
<td>249</td>
<td>15.7</td>
<td>3,909</td>
<td>0.38</td>
</tr>
</tbody>
</table>

* The per cent yield was not determined for this fraction because it was not used for the subsequent steps of purification.
As shown in Table I, 91% of the total proline reductase activity of the cells was extracted from the particulate fraction. Apparently, the enzymic activity is stable in the presence of Triton X-100 and sodium deoxycholate. No proline reductase activity was solubilized by treatment with chaotropic agents such as sodium chloride and sodium thiocyanate. The overall recovery of proline reductase activity was about 26%. However, it should be noted that specific activity determinations on the cell suspension varied considerably, and therefore, the calculation of extent of purification of the enzyme from the starting material is only approximate.

### Physical and Chemical Properties of Proline Reductase

#### Molecular Weight Determination and Subunit Structure

The molecular weight of proline reductase as determined by gel filtration on Bio-Gel A-1.5m (Fig. 1) is 327,000. Low speed sedimentation equilibrium centrifugation was also employed to determine the molecular weight of native proline reductase. The data are presented in a plot of ln c versus r², Fig. 2. The plot curves upward, suggesting polydispersity. The least square value of the weight average molecular weight based on the data presented in Fig. 2 is 298,000. That the polydispersity observed was not a result of impurities in the enzyme preparation was shown by the sharp major band obtained on disc gel electrophoresis (Fig. 3). Since the amount of protein used for gel electrophoresis (15 μg) was considerably smaller than that employed for ultracentrifugation (300 μg), it is likely that, at higher concentrations of protein, aggregation is favored.

The subunit molecular weight of proline reductase was determined by sodium dodecyl sulfate gel electrophoresis. A major band of 30,000 was observed. However, two minor bands of 60,000 and 90,000 were consistently found. Amino acid analysis of all three of these bands showed that they have similar compositions. These results further suggest that the polypeptide subunits undergo an equilibrium of association and dissociation.

#### Amino Acid Composition

The amino acid composition of proline reductase is shown in Table II. The residue number of each amino acid per minimum molecular weight was determined by the method of Thornber and Olson (16). The minimum molecular weight thus determined was found to be 31,000. This value was adjusted for the weight of water molecules lost as a result of peptide bond cleavage but does not include the weight contributed by amide nitrogen that might be present. Thus, the minimum molecular weight may be higher than 31,000.

No tryptophan could be detected either by amino acid analysis of methanesulfonic acid hydrolysates (12) or by ultraviolet absorption spectroscopy as described by Donovan (17). However, there are relatively high contents of tyrosine and phenylalanine. These results are confirmed spectrophotometrically by the ultraviolet spectrum of the enzyme shown in Fig. 4. Proline reductase contains a preponderance of acidic amino acids.
Proline Reductase: Purification and Properties

TABLE II
Amino Acid Compositions of Proline Reductase

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>24 hr</th>
<th>48 hr</th>
<th>72 hr</th>
<th>Best value</th>
<th>Residues/minimuma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asx</td>
<td>20.0</td>
<td>17.7</td>
<td>17.8</td>
<td>18.5</td>
<td>31</td>
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<tr>
<td>Thr</td>
<td>11.2</td>
<td>11.1</td>
<td>10.7</td>
<td>11.4b</td>
<td>10</td>
</tr>
<tr>
<td>Ser</td>
<td>9.76</td>
<td>9.76</td>
<td>8.87</td>
<td>10.0b</td>
<td>17</td>
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<tr>
<td>Glx</td>
<td>17.5</td>
<td>20.0</td>
<td>15.1</td>
<td>17.5</td>
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<tr>
<td>Pro</td>
<td>8.86</td>
<td>8.61</td>
<td>7.73</td>
<td>8.40</td>
<td>14</td>
</tr>
<tr>
<td>Gly</td>
<td>15.8</td>
<td>14.2</td>
<td>15.4</td>
<td>15.1</td>
<td>25</td>
</tr>
<tr>
<td>Ala</td>
<td>17.1</td>
<td>15.1</td>
<td>16.0</td>
<td>16.1</td>
<td>27</td>
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<tr>
<td>Cys</td>
<td>1.62</td>
<td>1.23</td>
<td></td>
<td>1.43c</td>
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</tr>
<tr>
<td>Val</td>
<td>12.7</td>
<td>11.1</td>
<td>15.2</td>
<td>15.2b</td>
<td>25</td>
</tr>
<tr>
<td>Met</td>
<td>2.87</td>
<td>2.56</td>
<td>1.32</td>
<td>2.45</td>
<td>4</td>
</tr>
<tr>
<td>Ile</td>
<td>9.56</td>
<td>10.5</td>
<td>14.0</td>
<td>14.0b</td>
<td>23</td>
</tr>
<tr>
<td>Leu</td>
<td>12.5</td>
<td>11.9</td>
<td>14.1</td>
<td>12.8</td>
<td>21</td>
</tr>
<tr>
<td>Tyr</td>
<td>4.26</td>
<td>4.46</td>
<td>4.59</td>
<td>4.44</td>
<td>7</td>
</tr>
<tr>
<td>Phe</td>
<td>3.58</td>
<td>5.34</td>
<td>5.24</td>
<td>4.72</td>
<td>8</td>
</tr>
<tr>
<td>Lys</td>
<td>15.2</td>
<td>15.4</td>
<td>15.2</td>
<td>15.3</td>
<td>25</td>
</tr>
<tr>
<td>His</td>
<td>3.52</td>
<td>3.58</td>
<td>3.53</td>
<td>3.54</td>
<td>6</td>
</tr>
<tr>
<td>Arg</td>
<td>6.27</td>
<td>5.69</td>
<td>5.96</td>
<td>5.97</td>
<td>10</td>
</tr>
<tr>
<td>Trp*</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

a The residue number was determined according to the method of Thornber and Olson (16). Residue number = best value/0.6. Minimum molecular weight is the total weight of all residues minus the weight of water molecules resulting from peptide bond hydrolysis.

b Values obtained from performic acid-oxidized samples. The yield of cysteic acid is 94% as reported by Moore (13). Thus, the values are corrected to 100%.

c Values extrapolated to zero hydrolysis time.

d No trp was detected after methanesulfonic acid hydrolysis according to Liu and Chang (13).

Metal and Sulfhydryl Group Content—No molybdenum, manganese, or iron was found in proline reductase. A sensitive colorimetric procedure (15) that could have detected 0.1 μg of molybdenum/mg of protein was employed. The absence of these metals was confirmed by atomic absorption spectroscopy. Preliminary results obtained from experiments with C. sticklandii grown in media supplemented with selenite suggested that selenium might be present in proline reductase. However, homogeneous proline reductase isolated from radioactive cells grown on [75Se]selenite contained nondetectable quantities of radioactivity. In fact, radioactivity associated with the crude enzyme preparation decreased with each successive purification step, indicating that proline reductase per se contains no selenium.

Sulfhydryl group determination was performed according to Cavallini et al. (18). Proline reductase was exhaustively dialyzed against 50 mM Tricine-KOH buffer, pH 8.6, and 1 mM MgK$_2$EDTA to remove dithiothreitol which is routinely added to all enzyme preparations. The protein was then denatured in urea and reduced with borohydride prior to titration with 5,5'-dithiobis(2-nitrobenzoic acid). A molar extinction coefficient of 1.2 x 10$^4$ M$^-1$ cm$^-1$ (at 412 nm) for the liberated thionitrobenzoic acid was used as reported by Flavin (19). By this method, an average of 20.8 sulfhydryl equivalents per mol of proline reductase was found. The latter result is similar to the total number of cysteic acid residues found by amino acid analysis and corresponds to 2 half-cystine residues per subunit of 31,000 daltons. Experiments with proline reductase that has been alkylated with [14C]iodoacetic acid are in progress in order to corroborate these findings.

Biological Properties

Reduction of Proline—The physiological reduction of proline requires a reductant, electron transport component(s) and proline reductase. NADH is the normal physiological electron donor, and, as shown in Table III, the enzyme system from C. sticklandii is highly specific for NADH. NADPH is inactive as an electron donor. In the in vitro system, dithiothreitol or certain other dithiols (1) can be used as an artificial electron donor to reduce the proline reductase component directly. However, in the unfractionated crude extracts, dithiothreitol is not as efficient a reductant as NADH.

When NADH is used as reductant, electrons must be transferred through a transport system to proline reductase.

**FIG. 4.** The ultraviolet absorption spectrum of proline reductase. Protein concentration was 0.7 mg/ml in 0.05 M Tricine-KOH buffer, pH 8.6. The spectrum was determined with a Cary 15 recording spectrophotometer.

**TABLE III**
Electron donors for proline reductase of different purities

<table>
<thead>
<tr>
<th>Electron donor</th>
<th>Crude extract</th>
<th>Fraction from detergent solubilization</th>
<th>Fraction from AH-Sepharose 4B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dithiothreitol, 20 mM</td>
<td>0.55</td>
<td>1.50</td>
<td>3.20</td>
</tr>
<tr>
<td>40 mM</td>
<td>0.76</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NADH, 20 mM</td>
<td>1.12</td>
<td>0.041</td>
<td>0.039</td>
</tr>
<tr>
<td>40 mM</td>
<td>0.98</td>
<td></td>
<td>0.02</td>
</tr>
<tr>
<td>NADPH, 20 mM</td>
<td>0.009</td>
<td></td>
<td>0.00</td>
</tr>
<tr>
<td>40 mM</td>
<td>0.00</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The crude extract was prepared from 3 g of C. sticklandii wet cell paste suspended in 20 ml of 20 mM potassium phosphate buffer, pH 7.2. The suspension was sonicated with a Branson Sonifier for 2 min at 15-s intervals. Cell debris was removed by centrifugation at 27,000 x g for 20 min.
Preliminary results indicate that the system consists of one or more membrane-bound components and it appears to be labile. Evidence presented in Table III indicates that either the electron transport system is separated from proline reductase by the detergent treatment employed in the purification, or a labile component is inactivated at this step of the procedure. A reconstituted system of purified components has not yet been achieved. Thus, proline reductase activity is determined routinely with dithiothreitol as the reductant.

Proline reductase catalyzes specifically n-proline cleavage, and has no activity on L-proline. However, in crude enzyme preparations, an extremely active proline racemase (1) interconverts L- and n-proline, and thus, proline reductase activity can be measured in most impure enzyme preparations with either isomer as substrate.

**DISCUSSION**

Proline reductase has been successfully solubilized from its associated membrane components and purified to homogeneity. The membrane particles were subjected to detergent treatments to solubilize proline reductase. Undoubtedly, the failure to recognize proline reductase as a membrane-bound protein by previous workers prevented them from achieving a homogeneous protein. The procedures reported in this paper involve the use of a hydrophobic column step in order to take advantage of the physiological affinity of this protein. Ionic interaction between the terminal amino group of the hydrophobic spacer and the acidic residues of the protein further increases specificity to the Sepharose 4B affinity chromatography.

Proline reductase, as isolated by these procedures, has a molecular weight of approximately 300,000. It appears to consist of 10 subunits based on the minimum molecular weight of 31,000 determined from amino acid composition and sodium dodecyl sulfate-polyacrylamide gel electrophoresis. However, direct determination of the subunit structure of proline reductase will be performed by end group analysis.

Experiments designed to determine the metal content of purified proline reductase indicated a lack of iron, molybdenum, or manganese in this enzyme. The absence of iron in the enzyme by added FMN or FAD.

Whereas earlier data showed that NADH can serve as the electron donor in the reduction of n-proline to α-aminovalerate by crude extracts of *C. sticklandii*, dithiothreitol alone can serve as a reductant for the purified proline reductase. Therefore, when NADH is used as the reductant, electrons must be transported through one or more electron carriers in order to catalyze the reductive cleavage of n-proline. In some instances, a stimulatory effect of ferredoxin has been observed with crude preparations of proline reductase, using NADH as an electron donor. Information concerning the nature of the effects on ferredoxin awaits reconstitution of the system from purified proline reductase and isolated electron carrier(s). As discussed in the introduction to the text, 1 μm selenite supplemented to the growth medium of *C. sticklandii* resulted in approximately a 3-fold increase in proline reductase activity. However, cultures grown on Na₂SeO₃ failed to incorporate radioactivity into proline reductase. Thus, the observed stimulatory effect of selenite is not on the reductase component per se, but may be explained by the involvement of selenium in the electron transport system. Separation and characterization of the individual components of the system that link the reductase to the normal electron donor, NADH, will be necessary to resolve this point.

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**REFERENCES**

Purification and properties of proline reductase from Clostridium sticklandii.
B Seto and T C Stadtman


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