Aldose Reductase from Rhodotorula

I. PURIFICATION AND PROPERTIES*

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SUMMARY

A TPN-specific aldose reductase has been purified 1310-fold from yeast (Rhodotorula sp.). Cell growth and preparation of a crude extract are described. Acidification of the extract (pH 5.0) resulted in precipitation of approximately 60% of the inert protein. Further treatment with protamine sulfate, ammonium sulfate fractionation, and negative absorption to calcium phosphate gel produced a 24-fold purification over the crude extract. Electrofocusing (pH 4 to 6 range) gave greater than a 1000-fold purification and subsequent column chromatography on Sephadex G-100 removed the Ampholine and remaining proteins from the reductase enzyme. Recovery of aldose reductase units averaged about 57% of those present in the initial extract.

Homogeneity of the purified enzyme was indicated by both disc gel electrophoretic and immunological criteria. In the presence of 10⁻³ M mercaptoethanol, the enzyme was dissociated into two protein bands by disc gel electrophoresis.

The enzyme has an average molecular weight of 61,000 as estimated by gel filtration, and an isoelectric point of 5.05 (electrofocusing). When TPNH was the varied substrate, competitive inhibition was given by both TPN⁺ (Kᵢ = 2.0 × 10⁻⁴ M) and 2'-AMP (Kᵢ = 5.0 × 10⁻⁴ M).

In a previous report we have described a TPNH-dependent aldose reductase, from a strain of Rhodotorula (1). The yeast had been isolated from soil by enrichment culture and grew rapidly on a simple medium containing D-glyceraldehyde as the sole carbon source. Cell-free extracts of a partially purified enzyme catalyzed the reduction of D-, L-, or Dl-glyceraldehyde to glycerol and some sugars to their corresponding sugar alcohols.

DPNH would not replace TPNH as a coenzyme in this system neither would it inhibit the reaction when added to the complete reaction mixture.

Several partially purified enzymes have been described which catalyze a similar TPNH-dependent reduction of sugars. Differences have been reported in some of their properties, particularly pH optimum, reaction reversibility, and activation of the enzyme by sulfate ions (2-6). Rhodotorula provides not only a stable, convenient, and reproducible source of the cell-free extracts for this purification, but also yields specific activities generally many times greater than extracts from other tissues. This paper describes the purification of aldose reductase to homogeneity and some of its properties.

EXPERIMENTAL PROCEDURE

Materials

Biochemicals of the highest purity available were purchased from the following sources: Dl-glyceraldehyde and calcium phosphate gel from Sigma; N-2-hydroxyethylpiperazine-N'2-ethanesulfonic acid from Calbiochem and protamine sulfate from General Biochemicals. Ampholine (pH 4 to 6 range) was from LKB.

Glyceraldehyde

D- and L-Glyceraldehyde were prepared by lead tetraacetate oxidation of D-fructose and L-sorbose as described by Perlin (7).

Aldose Reductase Assay

The standard assay cuvette contained 0.12 mM TPNH, 10 mM Dl-glyceraldehyde, and HEPES-NaCl buffer (0.05 M HEPES, pH 7.5, and 0.10 M NaCl) to a final volume of 0.975 to 0.995 ml. Aliquots (100 ml) from the stock solutions of TPNH and glyceraldehyde, made fresh daily and kept at 0°C, were added first to cuvettes warmed to 37°C in a water bath. HEPES-NaCl buffer, maintained at 37°C, was added in amounts to give the final volume indicated. The cuvettes were placed in a Gilford model 240 recording spectrophotometer jacketed at 37°C. After a base line was established, the reaction was initiated with 5 to 25 ml of enzyme solution, and the decrease in absorbance (A) at 340 nm was recorded. Initial velocities were generally constant for at least 3 to 4 min and were highly reproducible. One unit of enzyme was defined as that quantity which catalyzed the oxidation of 1

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1 The abbreviation used is: HEPES, N-2-hydroxyethylpiperazine-N'2-ethanesulfonic acid.
μmole of TPNH per min at 37°, and specific activity was expressed as units per mg of protein.

At pH 7.5 there was no detectable nonenzymatic TPNH degradation, nor was there any endogenous TPNH oxidation in the absence of glyceraldehyde at any stage of the purification.

**Other Assays**

Protein was determined by the method of Lowry et al. (8).

**Purification of Enzyme**

**Step 1: Growth of Rhodotorula and Preparation of Cell-free Extract—**Rhodotorula sp. was grown with aeration in a medium containing 0.7% yeast nitrogen base (Difco) and 1.0% glucose as the sole carbon source. The pH was maintained at approximately 7.0 by the addition of 10 N NaOH. After 2 to 3 days, the cells were harvested and washed three times with distilled water. (All subsequent steps were done at 0-4°.) Disruption of the cells was carried out in a 250-ml chamber by suspending the wet cells (about 80 g) with an equal weight of glass beads (0.1-mm diameter) in 1.5 volumes of 0.05 M phthalate buffer, pH 5.8, and homogenizing in an ice bath (Sorvall Omnimixer, run at 0.8 full speed, 30 s on, 15 s off) for 2.5 hours. Cellular debris was removed by centrifugation at 48,000 × g for 10 min.

**Step 2: Acid Precipitation—**The pH of the cell-free extract was lowered to 5.0 with 1 N acetic acid. Stirring was continued for 2 min followed by centrifugation at 48,000 × g for 10 min. The resulting supernatant was adjusted to pH 6.0 and dialyzed for 15 hours against 10 volumes of 5 mM phosphate buffer, pH 7.4.

**Step 3: Protamine Sulfate Treatment—**Protamine sulfate, 1.5 mg per unit of enzyme, was added to the dialyzed supernatant of Step 2 while maintaining the pH at 7.0. After stirring for 15 min, the precipitate was removed by centrifugation at 48,000 × g for 10 min and discarded.

**Step 4: Ammonium Sulfate Fractionation—**Solid ammonium sulfate was added to the supernatant over a period of 90 min until 70% saturation was reached. The precipitate was sedimented (48,000 × g for 10 min) and discarded, and the supernatant was increased to 90% saturation. Throughout this fractionation the pH was continuously monitored and maintained at 7.0 by addition of 1 N NaOH. The 70 to 90% precipitate was collected by centrifugation at 6,000 × g for 30 min and resuspended in about 10 ml of 5 mM phosphate buffer, pH 7.4.

**Step 5: Calcium Phosphate Gel Negative Adsorption—**Calcium phosphate gel was added to this enzyme preparation until the ratio of gel to protein was 3:1 (w/w). Stirring was continued for exactly 15 min while keeping the pH at 5.5. Following a 2-min centrifugation at 50,000 × g, the supernatant was decanted, neutralized to pH 7.0, and dialyzed for 5 hours against distilled water (9).

**Step 6: Electrofocusing—**The electrofocusing column (LKB 8101) was prepared according to the manufacturer’s directions with the pH 4 to 6 Ampholine at a final concentration of 1%. The dialyzed preparation from Step 5 was concentrated to about 4 ml (ultrafiltration), and, after a 10- to 15-hour preliminary focusing of the column, was mixed with an appropriate amount of sucrose and carefully layered at an isodense position near the center of the column by using narrow gauge plastic tubing. Electrofocusing was complete within 24 hours. Two-milliliter fractions were collected and those containing aldose reductase were pooled (10 to 14 ml).

**TABLE I**

*Summary of purification of yeast aldose reductase*

<table>
<thead>
<tr>
<th>Step</th>
<th>Protein</th>
<th>Total protein</th>
<th>Specific activity</th>
<th>Purification</th>
<th>Total units</th>
<th>Yield %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Cell-free extract</td>
<td>4.8</td>
<td>144</td>
<td>0.14</td>
<td>1</td>
<td>42</td>
</tr>
<tr>
<td>2.</td>
<td>pH 5 supernatant</td>
<td>1.9</td>
<td>57</td>
<td>0.35</td>
<td>2.5</td>
<td>40</td>
</tr>
<tr>
<td>3.</td>
<td>Protamine sulfate supernatant</td>
<td>0.77</td>
<td>45</td>
<td>1.16</td>
<td>3.3</td>
<td>3</td>
</tr>
<tr>
<td>4.</td>
<td>Ammonium sulfate</td>
<td>3.3</td>
<td>16.5</td>
<td>2.6</td>
<td>7</td>
<td>19</td>
</tr>
<tr>
<td>5.</td>
<td>Calcium phosphate gel</td>
<td>0.18</td>
<td>12</td>
<td>3.4</td>
<td>24</td>
<td>35</td>
</tr>
<tr>
<td>6.</td>
<td>Electrofocusing</td>
<td>0.018</td>
<td>0.060</td>
<td>153</td>
<td>1090</td>
<td>28</td>
</tr>
<tr>
<td>7.</td>
<td>Sephadex G-100 chromatography</td>
<td>0.027</td>
<td>0.070</td>
<td>184</td>
<td>1310</td>
<td>24</td>
</tr>
</tbody>
</table>

*Protein concentration was estimated from ultraviolet absorption at 280 nm.*

**FIG. 1.** Polyacrylamide disc gel electrophoresis of aldose reductase from Step 7 of the purification procedure. Samples of the enzyme were mixed with sucrose and layered on top of the gels. The amount of protein placed on each gel was; left, 5 μg; center, 50 μg; right, 500 μg. Migration was downward (from cathode to anode).

**Step 7: Column Chromatography—**An upward flow Sephadex G-100 column (5 × 100 cm) with a bed volume of about 1800 ml was equilibrated and eluted with a buffer composed of 0.05 M Tris-HCl, pH 7.5, and 0.10 M NaCl. The pooled fractions from
TABLE II

<table>
<thead>
<tr>
<th></th>
<th>$K_m$</th>
<th>$V_{\text{max}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-Glyceraldehyde</td>
<td>0.9</td>
<td>1.32</td>
</tr>
<tr>
<td>L-Glyceraldehyde</td>
<td>4.0</td>
<td>1.32</td>
</tr>
<tr>
<td>DL-Glyceraldehyde</td>
<td>1.9</td>
<td>1.32</td>
</tr>
</tbody>
</table>

The standard assay procedure was followed except that TPNH concentration was 0.13 mM. All reactions were initiated with 25 μM of the highly purified enzyme. The concentration of the DL mixture is expressed as the sum of the concentrations of the D and L enantiomers.

Fig. 2. Competitive inhibition by TPN⁺. Double reciprocal plots of initial velocities at pH 7.5. Assay cuvettes contained 10 μmoles of glyceraldehyde with TPNH concentrations ranging from 2 to 70 μM and TPN⁺ concentrations as indicated (0 to 150 μM). Competitive $K_i$ for TPN⁺ determined by this method was $2.0 \times 10^{-5}$ M.

Fig. 4. Double reciprocal plots showing the effect of 2'-AMP upon the kinetics of yeast aldose reductase at pH 7.5. DL-Glyceraldehyde concentration in all reactions was 10 mM. The $K_i$ determined by this method is indicated.

Immunization

A white male rabbit was immunized according to the following procedure. Purified TPN-aldose reductase, 50 μg, was emulsified with 1 ml of 0.9% NaCl solution in 1 ml of Freund's complete adjuvant (Difco) and injected subcutaneously (footpads and neck) and intramuscularly (thighs). Freund's incomplete adjuvant was used in identical injections at 2 and 4 weeks. Blood was drawn prior to the initial immunization, 6 weeks later, and twice a week thereafter for 4 weeks. Serum was prepared and stored at −20°C.

A similar procedure was used to immunize another rabbit to the cell-free extract (2 to 3 mg).

Ouchterlony Technique

Ouchterlony immunodiffusion studies were carried out on microscope slides covered with a 2-mm layer of 1.5% (w/v) Noble agar in 0.15 M NaCl and 0.2 M borate, pH 8.0. Slides were incubated at room temperature for 24 hours in a humidity chamber, rinsed in 0.15 M NaCl for 48 hours, and distilled water for 48 hours, before drying at 37°C and staining with Buffalo black.

RESULTS AND DISCUSSION

Purification of TPN-Aldose Reductase—The results of a typical purification are presented in Table I. The cell breakage procedure used was found to solubilize the enzyme leaving few un-
broken cells and negligible activity in the precipitate, which was removed by centrifugation. Lowering the pH of the cell-free extract to 5.0 for a 2-min interval precipitated about 60% of the inert protein without appreciable loss of aldose reductase units from the supernatant.

In Step 3, many of the nucleic acids present were precipitated with protamine sulfate ($A_{400}$:$A_{260}$ increased from 0.7 to 1.2). This step was mandatory for clean-cut fractionation with ammonium sulfate (11).

As shown in Table I, Step 6, preparative electrofocusing produced the greatest increase in specific activity with only a moderate loss of enzyme units. Efforts to determine protein concentration at this stage were greatly hampered by the presence of Ampholine which gave a strong positive reaction to the standard protein assays and had appreciable absorbance at 280 nm. Disc gel electrophoresis following this step showed one major protein band (identified as aldose reductase after extraction from a duplicate gel) and two or three faint bands (not exhibiting aldose reductase activity). Subsequent column chromatography on Sephadex G-100 equilibrated only with 0.05 M Tris-HCl, pH 7.4, did not result in complete removal of Ampholine or further purification (as determined by disc gel electrophoresis). However, when NaCl was added to the eluting buffer to a concentration of 0.1 M (Step 7), the Ampholine was removed and the aldose reductase was obtained in a more purified form.

At any stage of the purification, the enzyme preparation may be stored in a frozen or lyophilized state for months without appreciable loss of activity.

Criteria for Purity—Analytical disc gel electrophoresis of up to 0.5 mg of the purified aldose reductase produced a single protein band which corresponded to the peak of enzymatic activity (Fig. 1).

Antibodies to the cell-free extract and purified enzyme from Step 7 were produced as described under “Experimental Procedure.” The results of Ouchterlony immunodiffusion experiments showed a single precipitation line found between the purified aldose reductase and each antibody preparation as well as between the cell-free extract and the antiserum to the purified enzyme, whereas multiple lines were present between the cell-free extract and the antiserum directed against it. The preliminary immune serum exhibited no precipitation reaction with either enzyme preparation.

Isoelectric Point—Electrofocusing (“Experimental Procedure”) with 1% Ampholine (pH 4 to 6 range) showed the isoelectric point of this reductase to be 5.05.

Molecular Weight—Gel filtration with Sephadex G-200 was used according to the procedure of Andrews (12) to determine the molecular weight of yeast aldose reductase. The value obtained was 60,000. A similar experiment with Sephadex G-100 gave a molecular weight of 61,600.

Stereospecificity for Glyceraldehyde—Initial velocities were studied at various glyceraldehyde concentrations in the presence of 0.13 mM TPNH at pH 7.5. Double reciprocal plots were linear and were used to determine $K_m$ and $V_{max}$ for the D and L isomers and the DL racemic mixture of glyceraldehyde. The results presented in Table II indicate that both the D and L enantiomers are capable of serving as substrate, with a possible slight preference for the D isomer.

Sulfhydryl Reagents—After gel electrophoresis of the highly purified enzyme of polyacrylamide gel only one band of protein was observed. However, when 1.0 mM mercaptoethanol was present in the gel the enzyme dissociated into two bands of approximately equal intensity.

Effect of TPN$^+$—TPN$^+$ was shown to be a noncompetitive inhibitor when glyceraldehyde was the variable substrate ($K_i = 11.2 \times 10^{-4}$ M) and a competitive inhibitor of TPNH ($K_i = 2.0 \times 10^{-8}$ M). Initial velocities were studied at pH 7.5 with TPNH as the variable substrate and initial TPN$^+$ concentrations ranging from 0 to 150 $\mu$M. Fig. 2 is a double reciprocal plot of the data obtained showing TPN$^+$ to exhibit inhibitory properties. A Dixon plot of the same data (Fig. 3A) shows that, although TPN$^+$ exhibits nonlinear inhibition at the lower TPNH concentrations, the nonlinear character of the curve is lost above 45 $\mu$M TPNH and the plots become linear. The slopes of the Dixon plots near the ordinate were determined and plotted against the reciprocal of the TPNH concentration. The points fell on a straight line passing through the origin, showing TPN$^+$ to be a competitive inhibitor (Fig. 3B) (13).

Effect of AMP—The results of three experiments plotted in Fig. 4 show 2-AMP to be a classical competitive inhibitor with $K_i = 5.5 \times 10^{-4}$ M. One experiment was done in the absence of 2-AMP; in the others, the reaction cuvettes contained, in addition to the standard assay contents, 2'-AMP at concentrations of 0.10 and 0.50 mM, respectively. Similar experiments showed 5'-AMP to have a $K_i$ of $46 \times 10^{-4}$ M, while the $K_i$ for 3'-AMP had an intermediate value, 25 $\times 10^{-4}$ M.

Miscellaneous Properties—The enzyme was stored at $-80^\circ$ and could be repeatedly frozen and thawed without loss of activity. Reagents which react with free sulphydryl groups were found to be noncompetitive inhibitors. Inhibition constants for p-hydroxymercuribenzoate and mercuric chloride were 61 and 46 M, respectively. Sulfate was found to be a competitive inhibitor of the reaction at pH 7.5 ($K_i = 44$ M). No cation requirement was observed, nor was the enzyme sensitive to EDTA. Antibodies to the purified enzyme were found to inhibit the in vitro assay up to 75%.

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