Purification and Properties of Histidinol Dehydrogenase from Escherichia coli B

By NAHUM ANDORN* AND JACOB ARONOVITCH

Department of Molecular Biology, Hebrew University, Hadassah Medical School, Jerusalem, Israel

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Histidinol dehydrogenase has been purified from a derepressed mutant of Escherichia coli B. A molecular weight of about 91000 was estimated by gel filtration. The native enzyme seems to be composed of two similar subunits which have a molecular weight of 52000 as determined by sodium dodecyl sulphate–polyacrylamide gel electrophoresis. The pI of the enzyme as determined by isoelectric focusing is 4.75. The enzyme is maximally active at pH 9.5. It is highly specific for NAD+ and histidinol, with a $K_m$ (NAD+) of 0.57 mM and a $K_m$ (histidinol) of 14 μM. Mn$^{2+}$ is required for maximal activity. The enzyme is completely inactivated by 8 M-urea but regains its activity very quickly upon removal of the urea. Mn$^{2+}$ and histidinol protect the enzyme from heat inactivation.

INTRODUCTION

Histidinol dehydrogenase (L-histidinol: NAD$^+$ oxidoreductase, EC 1.1.1.23), which is coded by the structural gene hisD, catalyses the last step in the biosynthesis of histidine, namely the oxidation of histidinol to histidine with the concomitant reduction of 2 mol of NAD$^+$ per mol of histidine formed (Loper, 1968). Histidinol dehydrogenase (HDH) has been partially purified from Arthrobacter histidinolovorans (Dhawale et al., 1972), Neurospora crassa (Minson & Creaser, 1969), yeast (Keese et al., 1979), Bacillus spp. (Lindsay & Creaser, 1977), Salmonella typhimurium (Yorno & Ino, 1968; Loper, 1968) and Escherichia coli K12 (Bitar et al., 1977). The salmonella enzyme has been extensively investigated both genetically and biochemically (Roth et al., 1966; Loper, 1968; Yorno, 1968; Greeb et al., 1971; Bitar et al., 1977). In contrast, most of the work done with the E. coli hisD product has been of a genetic nature. Genetic analysis has demonstrated that the his genes of E. coli constitute an operon very similar to that of S. typhimurium and that the hisD gene of E. coli K12, like the gene of S. typhimurium, is characterized as a single cistron displaying intragenic complementation. However, E. coli F'his fails to recombine with the chromosome when present in S. typhimurium and there is additional evidence of dissimilarity between the two chromosomal regions (Goldschmidt & Cater, 1970; Garrick-Silversmith & Hartman, 1970). It was therefore of interest to compare the hisD gene product of the two related micro-organisms. Bitar et al. (1977) have studied the E. coli K12 HDH produced by an E. coli K12 F-merogenote contained in S. typhimurium. We have studied the enzyme of E. coli B using a mutant with derepressed levels of histidine biosynthetic enzymes. In this paper we describe some of the physical and biochemical properties of the E. coli B HDH and compare them with the properties of the enzyme from other micro-organisms.

METHODS

Source of enzyme. A mutant (R-5) with derepressed levels of histidine biosynthetic enzymes was isolated from E. coli B wild-type after enrichment and selection on minimal medium (Davis & Mingioli, 1950) containing the
histidine analogues 1,2,4-triazole-3-alanine and 3-amino-1,2,4-triazole according to the procedure described by Roth et al. (1966). Preliminary studies of the kinetic properties showed that the R-5 HDH has the same properties as the wild-type enzyme.

**Growth of cells.** Cells were grown in a 500 l fermentor in minimal medium with 1% (w/v) glucose as carbon source. Cells were grown for 8 h at 37 °C with forced aeration. The pH was kept constant with NaOH. Late-exponential phase cells were harvested with a Sharples centrifuge, washed once with 0-01 M-Tris/HCl buffer, pH 7-5, and stored at −20 °C until required.

**Purification of histidinol dehydrogenase (HDH).** All purification steps were carried out at 0 to 4 °C unless otherwise stated. Cells (200 g) were thawed in 0-01 M-Tris/succinate buffer, pH 7-5, containing 1-0 mM-MnCl₂ (TS buffer) supplemented with 0-01 M-MgCl₂, and 2 μg DNAase ml⁻¹. The cells were disrupted in a French press at 10 MPa (1500 lbf in⁻²). After centrifugation at 40000 g for 30 min the supernatant was recovered as the crude extract (step 1, Table 1). The crude extract was heated for 10 min at 60 °C and cooled quickly in an ice bath. After centrifugation as before, the supernatant was recovered (step 2) and brought to pH 5-0 with 0-2 M-HCl. The precipitated proteins were discarded by centrifugation (10 min; 20000 g). The pH of the supernatant was adjusted to 7-5 with 0-2 M-NaOH (step 3) and solid (NH₄)₂SO₄ was added to 0-45 saturation. The precipitated proteins were discarded by centrifugation and the supernatant was brought to 0-65 saturation with (NH₄)₂SO₄. After centrifugation the precipitate was dissolved in a minimal volume of TS buffer (step 4) and applied to a Sephadex G-150 column (2-6 × 50 cm). The enzyme was eluted from the column with TS buffer and the active fractions were combined (step 5). The pooled fractions were then applied to a DEAE-cellulose column (4 × 50 cm) previously equilibrated with TS buffer and the column was washed with the same buffer (0-51). The enzyme was eluted with Tris/succinate buffer containing 1-0 mM-MnCl₂, using a linear gradient from 0-01 M, pH 7-5, to 0-2 M, pH 6-0 (total volume 2 l). Active fractions were combined (step 6) and precipitated with (NH₄)₂SO₄ (0-7 saturation). The precipitate was dissolved in a minimal volume of TS buffer and applied to a Sephadex G-150 column (1 × 50 cm). The enzyme was eluted with the same buffer. The fractions around the peak of activity were electrophoretically homogeneous (step 7).

**Determinations of molecular weight.** The molecular weight of the native enzyme was determined by gel filtration through Sephadex G-150 as described by Andrews (1965) using γ-globulin (mol. wt 150000), lactoperoxidase (90000), bovine serum albumin (67000) and ovalbumin (45000) as molecular weight standards. The molecular weight of the reduced enzyme was determined on 10% (w/v) polyacrylamide gel slabs in the presence of sodium dodecyl sulphate (SDS) by the method of Weber & Osborn (1969) with lactoperoxidase, bovine serum albumin, ovalbumin and chymotrypsinogen A (mol. wt 25000) as standards.

**Isoelectric focusing.** This was performed on thin layers of polyacrylamide gel containing LKB Ampholines in the pH range 3-5-10 or 2-5-6 by the method of Vesterberg (1972) as described in the LKB Application Note 75 (1973). The pH was measured directly on the unstained gel with an LKB surface electrode.

**Polyacrylamide gel electrophoresis.** This was performed on (2 mm) 10% (w/v) polyacrylamide gel slabs by the method of Weber & Osborn (1969) omitting the SDS. Proteins were stained with Coomassie Brilliant Blue R-250, and HDH activity was detected according to Lindsay & Creaser (1977).

**Enzyme assay.** L-Histidinol dehydrogenase activity was measured by following the increase in A₃₄₀ at 40 °C (Lindsay & Creaser, 1977) using a Varian model 635 spectrophotometer equipped with a jacketed cuvette (10 mm light path). Reaction mixtures (1 ml) contained (unless otherwise stated) 200 μmol glycine/NaOH buffer, pH 9-5, 10 μmol NAD⁺, 0-5 μmol MnCl₂ and enzyme. After temperature equilibration, the reaction was initiated by the addition of 2 μmol L-histidinol dihydrochloride. Reaction rates were proportional to the amount of enzyme added and were practically identical whether the reaction was started with enzyme, histidinol or NAD⁺. One unit of enzyme activity (U) is defined as that amount which catalyses the oxidation of 1 μmol histidinol min⁻¹ at 40 °C, assuming 2 μmol NADH is formed per μmol oxidized.

**Protein determination.** Protein concentration was measured by the Lowry method with bovine serum albumin as the standard.

**Materials.** Sephadex G-25, G-100, G-150 and blue dextran 2000 were purchased from Pharmacia, Ampholines from LKB, L-histidinol dihydrochloride, NAD⁺, NADH, NADP⁺, NADPH, 3-acytylpyridine adenine dinucleotide, ovalbumin, bovine serum albumin, chymotrypsinogen A and Coomassie Brilliant Blue R-250 from Sigma. Lactoperoxidase from Boehringer, and DEAE-cellulose (DE-52) from Whatman. Reagents for polyacrylamide gel preparation were from Bio-Rad (Richmond, Calif., U.S.A.). All other chemicals were of analytical grade.

**RESULTS**

**Purification of histidinol dehydrogenase**

A 45-fold purification of the enzyme was achieved with a final yield of 11% of the activity present in the crude extract (Table 1). The loss of activity in the last step of purification was
Histidinol dehydrogenase from E. coli B

Table 1. Purification of histidinol dehydrogenase from E. coli B

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Protein (mg)</th>
<th>Activity (U)</th>
<th>Specific activity [U (mg protein)^{-1}]</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Crude extract</td>
<td>12000</td>
<td>4080</td>
<td>0.34</td>
<td>100</td>
</tr>
<tr>
<td>2. Supernatant after heating</td>
<td>4000</td>
<td>3412</td>
<td>0.85</td>
<td>84</td>
</tr>
<tr>
<td>3. Supernatant after pH 5 treatment</td>
<td>3000</td>
<td>3210</td>
<td>1.07</td>
<td>79</td>
</tr>
<tr>
<td>4. 0.45-0.65 (NH_4)_2SO_4 fraction</td>
<td>900</td>
<td>2709</td>
<td>3.01</td>
<td>66</td>
</tr>
<tr>
<td>5. Gel filtration I</td>
<td>500</td>
<td>2320</td>
<td>4.64</td>
<td>57</td>
</tr>
<tr>
<td>6. DEAE-cellulose eluate</td>
<td>100</td>
<td>1276</td>
<td>12.76</td>
<td>31</td>
</tr>
<tr>
<td>7. Gel filtration II</td>
<td>29</td>
<td>444</td>
<td>15.32</td>
<td>11</td>
</tr>
</tbody>
</table>

due to the fact that the fractions of the ‘DEAE’ step with low activity were discarded, and to loss during precipitation with (NH_4)_2SO_4 prior to the final gel filtration. In comparison with the S. typhimurium LT-7 HDH, which was purified by a similar procedure (Loper, 1968; Yourno & Ino, 1968), we found a greater loss of activity throughout the purification steps. This is probably due to the lower stability of the E. coli enzyme. Only one band with HDH activity was found after electrophoresis of the crude extract and the fractions from the succeeding steps of purification.

**Physicochemical and enzymic properties**

*Molecular weight, subunit composition and pI.* The molecular weight of HDH was determined by gel filtration. A value of 91000 was estimated from a standard curve obtained with proteins of known molecular weight. A similar value was obtained for the enzyme in the crude cell extract. Electrophoresis of the reduced enzyme in the presence of SDS gave a single band, the molecular weight of which was calculated to be 52000. These results indicate that the E. coli HDH contains two similar subunits. From electrophoresis on polyacrylamide gel a pI value of 4-75 was estimated (Fig. 1).

*Substrate specificity and kinetic properties.* The E. coli HDH was found to be specific for NAD^+. No activity could be detected with NADP (20 mM). 3-Acetylpyridine adenine dinucleotide could replace NAD^+, but with reduced velocity (30-40% of that with NAD^+). The enzyme was tested with various substrates other than histidinol. Methanol, ethanol,
Table 2. Effect of various substances on histidinol dehydrogenase activity

An enzyme preparation dialysed overnight against 1000 vol. Mn²⁺-free buffer was used in these experiments. The enzyme was incubated at 40 °C for 3 min in the reaction mixtures containing the various metal ions, and the reaction was started by the addition of histidinol. In inactivation–protection experiments, the inhibitor was added to the reaction mixture containing the enzyme and the protective agent, and after 3 min incubation the reaction was started by the addition of histidinol.

<table>
<thead>
<tr>
<th>Substance added (Concentration, mM)</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100</td>
</tr>
<tr>
<td>MnCl₂ (0.5)</td>
<td>265</td>
</tr>
<tr>
<td>ZnCl₂ (0.5)</td>
<td>120</td>
</tr>
<tr>
<td>CaCl₂ (0.5)</td>
<td>100</td>
</tr>
<tr>
<td>HgCl₂ (0.5)</td>
<td>0</td>
</tr>
<tr>
<td>HgCl₂ (0.05)</td>
<td>21</td>
</tr>
<tr>
<td>Dithiothreitol (1.0)</td>
<td>97</td>
</tr>
<tr>
<td>HgCl₂ (0.05) + dithiothreitol (1.0)</td>
<td>97</td>
</tr>
<tr>
<td>HgCl₂ (0.05) + MnCl₂ (0.5)</td>
<td>160</td>
</tr>
</tbody>
</table>

Ethanolamine and acetaldehyde (at 10 mM, with 100 μM-histidinol as the other substrate) were inactive either as substrates or inhibitors. Histidine and its analogues 1-methylhistidine and 2-thiolhistidine showed no inhibitory effect on the forward reaction and were inactive as substrates in the reverse reaction with either NADPH or NADH (0.5 mM).

The kinetic profile in terms of NAD⁺ and histidinol concentrations exhibited a Michaelian behaviour with a $K_m$ (NAD⁺) of 0.57 mM and a $K_m$ (histidinol) of 14 μM. NADH, but not NADPH, showed inhibition of the forward reaction with a $K_i$ of 50 μM. The optimum pH for HDH activity was about 9.5.

Effect of divalent cations on histidinol dehydrogenase activity. Upon removal of Mn²⁺ (either by dialysis or by gel filtration on Sephadex G-25), the enzyme lost about 60% of its activity. The activity could be restored by the addition of Mn²⁺ to the reaction mixture. Other divalent cations tested did not significantly stimulate the activity (Table 2). HgCl₂ at 0.5 mM completely inactivated the enzyme. Lower concentrations of Hg²⁺ caused partial inactivation which could be lowered by dithiothreitol or by Mn²⁺.

Heat inactivation. For these experiments we omitted the heating and acidifying steps and used the 0.45–0.65 saturation (NH₄)₂SO₄ precipitate as the enzyme source. It was found that the enzyme was more stable at pH 7.5 than at pH 9.4. At pH 7.5, there was almost no loss of activity after 5 min at 65 °C.

Using conditions that cause about 70% heat inactivation, various substances were tested for their protective effect. NAD⁺ only slightly protected the enzyme activity, while Mn²⁺ or histidinol, but not histidine, considerably protected the enzyme activity both at pH 7.5 and at pH 9.4. There was only 20–30% inactivation in the presence of 2 mM-histidinol or 1 mM-MnCl₂.

Effect of urea. Urea added to the reaction mixture caused 50% inhibition at 2.2 M (Fig. 2). In order to test the reversibility of urea inhibition, the enzyme was incubated for 30 min at 25 °C in the presence of various concentrations of urea in TS buffer, pH 7.5. Samples (10 μl) were then assayed for activity, thus diluting the tested concentrations of urea 100-fold to concentrations which caused less than 3% inhibition. It was found that full activity was restored within seconds after diluting the urea (Fig. 2). The inhibitory effect is therefore completely reversible.

**DISCUSSION**

The *his* 4 gene product in yeast and the *his* 3 gene product in *N. crassa* are multifunctional proteins which catalyse the oxidation of histidinol and two other steps in the histidine
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pathway (Keesey et al., 1979; Minson & Creaser, 1969). The molecular weight of the neurospora enzyme is 135000 and it can be separated into subunits by ultracentrifugation, the smallest component with HDH activity corresponding in size to a molecular weight of 40000.

In Enterobacteriaceae and Bacillus spp., the HDH seems to be a unifunctional protein of varied molecular weight. The HDH of a number of bacilli was investigated by Lindsay & Creaser (1977) who found that all the enzymes had a molecular weight of about 29000 as determined by ultracentrifugation. It is not clear, however, if this value represents the molecular weight of the active enzymes or of the subunits. Genetic analysis of HDH mutants of S. typhimurium supports the conclusion that hisD gene is a single cistron producing a single polypeptide (Greeb et al., 1971). By ion-exchange chromatography of the dissociated enzyme and sedimentation equilibrium analysis of the native and dissociated enzyme, Loper (1968) has reached the conclusion that S. typhimurium HDH is a dimer of identical subunits of approximate molecular weight 40000. However, Bitar et al (1977) using ultracentrifugation and SDS–polyacrylamide gel electrophoresis of native and carboxymethylated enzymes have concluded that HDH of S. typhimurium, and probably also the enzyme of E. coli K12 produced in S. typhimurium, is a monomer of molecular weight 47000.

We have found by gel filtration that the native E. coli B enzyme has a molecular weight of 91000. Determination of the molecular weight of the reduced enzyme by SDS–polyacrylamide gel electrophoresis gave a value of about 52000. Only a single band of protein was observed. These findings support the assumption that the native E. coli B enzyme is a dimer of identical or nearly identical subunits. The structure of the E. coli enzyme and the molecular weight of its subunits appear to be very similar to those of the S. typhimurium enzyme as reported by Loper (1968) and Yourno (1968). Although there are differences in molecular weight and organization of HDH in N. crassa, yeast, Bacillus spp., S. typhimurium and E. coli, the catalytic properties of these enzymes are very similar. The pH optimum for the overall reaction from histidinol to histidine is around pH 9.5 and the enzymes are highly specific for histidinol and NAD+. The E. coli B enzyme shows Michaelian kinetics with affinity values for histidinol and NAD+ (Km 14 μM and 0.57 mM, respectively) which are similar to the values reported for the other four enzymes. The E. coli B enzyme, like the salmonella and neurospora enzymes, is activated by Mn2+. The effect of Mn2+ is specific and it also protects the enzyme from inactivation by heat and Hg2+. Dialysis against EDTA buffer does not completely inactivate the enzyme, yielding preparations 25–40% as active as controls dialysed against Mn2+-containing buffer. Taken together, it is therefore assumed that Mn2+ stabilizes the enzyme protein in its catalytically active form rather than acting as a bridging group. Histidinol, but not NAD+ or histidine, also protects the enzyme from heat inactivation.

Many enzymes that have been denatured by urea require slow removal of the urea (e.g. dialysis) for reactivation. In some cases the denatured enzymes regain activity only after prolonged incubation with substrates and certain ions. These conditions are required for the unfolded peptide chains to reassemble and regain the native active conformation (Marangos & Constantinides, 1974; Jaenicke et al., 1979; Yamato & Murachi, 1979). In contrast, although E. coli B HDH is inhibited by high concentrations of urea, it very quickly regains full activity after diluting out the urea (Fig. 2), indicating that the native form of the enzyme is very stable.

Our findings demonstrate a close similarity in the properties of the salmonella and E. coli B HDH enzymes. The chromosomal arrangements of the two histidine operons in these closely related enteric organisms show great similarity. The rarity of interspecies crossing over in this chromosomal region must therefore be ascribed to structural differences.

This work is part of the Ph.D. thesis of N. Andorn to be submitted to the Hebrew University, Jerusalem, Israel.
REFERENCES


