The Purification and Properties of Histidinol Dehydrogenase from *Neurospora crassa*

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1. A procedure is described for the purification of L-histidinol dehydrogenase (L-histidinol-NAD oxidoreductase, EC 1.1.1.23) from *Neurospora crassa*. 2. The enzyme, as purified, has a sedimentation coefficient, S_{20} , of 7.1s and a molecular weight of 81 000. Considerable variation is possible in the state of polymerization of the enzyme, giving rise to observed molecular weights from 40 000 to 240 000. 3. Several kinetic parameters of the enzyme have been determined. The enzyme is maximally active at pH 9.8; the K_m (NAD) is $13.0 \times 10^{-5} M$ and K_m (histidinol) is $8.2 \times 10^{-6} M$. The enzyme is highly specific, does not oxidize a range of amino alcohols and other aliphatic alcohols nor reduce NADP and has no demonstrable affinity for histidine. The turnover number is 49 moles of NAD reduced/min./mole of enzyme (mol.wt. 40 000).

The objective of the work of which this present paper forms part is to correlate the genetic and biochemical observations on the genetic region in *Neurospora crassa* defined as *his-3*. This region has been shown by Catcheside (1960), Webber (1960) and Ahmed, Case & Giles (1964) to control the formation of enzymes that mediate several steps in the biosynthesis of histidine in this organism. This evidence has been interpreted in two ways. Ahmed *et al.* (1964) consider the region to correspond to a bacterial operon with an operator gene and a family of structural genes controlling the formation of several enzymes. Genetical work by Catcheside (1960, 1965) suggested that an alternative was to consider *his-3* as a locus that controlled the formation of a multifunctional protein. Previous work (Creaser, Bennett & Drysdale, 1965) showed that the concept of separate enzymes was unlikely and that postulation of a multifunctional enzyme is consistent with a greater number of facts. To help to resolve this difficulty we have purified the enzyme histidinol dehydrogenase as described below and we hope to assay for the possible other functions when substrates become available.

L-Histidinol dehydrogenase was first observed in yeast by Adams (1955) and also in an organism that he obtained by enrichment culture from soil and named *Arthrobacter histidinolovorans*. Adams showed that there was reduction of 2 moles of NAD

for each molecule of histidinol oxidized to histidine, but no evidence was obtained that histidinal was a free intermediate. However, histidinal could be oxidized to histidine when added to cell-free extracts. A histidinol dehydrogenase has been completely purified from *Salmonella typhimurium* by Loper & Adams (1965), and extracts of *Staphylococcus aureus* and *Saccharomyces cerevisiae* have been shown to have L-histidinol-dehydrogenase activity by Kloos & Pattee (1965) and Fink (1966) respectively.

MATERIALS AND METHODS

Growth and harvesting of organism. *N. crassa* strain Emerson *a* was used throughout this work. The fungus was maintained on slopes of 50 ml. of Vogel's minimal medium (Vogel, 1955) containing sucrose (2%) and solidified with Ionagar (1%). After 4 days' growth at 25% the conidia from one such slope were used to inoculate 8 l. of Vogel's minimal medium containing sucrose (2%). This culture was grown for 3 days at 25–30° with vigorous aeration and the mycelia were harvested, by using an MSE basket centrifuge (31. model) operated at 3000 rev./min. The basket was lined with Whatman no. 3MM filter paper. The resulting cake was broken into small pieces, freeze-dried, ground to a fine powder with a commercial coffee grinder and stored at -10° until required.

Chemicals. All chemicals used in the purification were of reagent or general purpose grade, as there was no advantage in using A.R. or enzyme quality reagents.

Sephadex G-100 was obtained from Pharmacia, Uppsala, Sweden. DEAE-cellulose was prepared from Solka-Flok by the method of Peterson & Sober (1956). For the batch

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procedure described below Cellex D, of either high or low capacity, was suitable (Bio-Rad Laboratories, Richmond, Calif., U.S.A.). TEAE-cellulose* was prepared from our DEAE-cellulose by the procedure of Porath (1957) with ethyl bromide (10%). After use the adsorbents were regenerated by sequential equilibration in *N*-NaOH, *m*-KH₂PO₄ and 0.01 *M*-phosphate buffer, pH 6.8, and finally dried by methylated spirits and acetone.

L-Histidinol dihydrochloride was obtained from Haco A.-G., Berne, Switzerland.

Enzyme assay. L-Histidinol dehydrogenase was assayed by conventional means by using the rate of increase in extinction at 340m μ as a measure of enzyme activity. Increase in extinction was converted into international enzyme units. A spectrophotometer cuvette (20mm. light-path) contained 4.0ml. of glycine-NaOH buffer, pH 9.8 (3.75g. of glycine adjusted to pH 9.8 with NaOH and made to 1l.), containing 0.1% (v/v) of 2-mercaptoethanol, together with 0.2ml. (0.4 μ mole) of a solution of L-histidinol dihydrochloride (0.424 mg./ml.), 0.2ml. (2 μ moles) of a solution of NAD (6.63 mg./ml.) and 0.1ml. of the solution under test. Under these conditions the enzyme was saturated with NAD and L-histidinol and linearity of response over the range 0–0.50 unit of enzyme was observed.

Gel electrophoresis. Gel electrophoresis was performed by the method of Smithies (1955) with a horizontal type apparatus, tris-HCl buffer, pH 9.1 (0.01M), and a starch (Connaught Laboratories, Toronto) concentration of 14%.

* Abbreviation: TEAE-cellulose, triethylaminoethyl-cellulose.

The enzyme was located in the gel after electrophoresis by the procedure of Roberts & Pateman (1964), with histidinol as the substrate for NAD reduction.

Centrifugation. Ultracentrifugation was performed with a Spinco model E analytical ultracentrifuge. Calculations of the sedimentation coefficient and molecular weight by the Archibald procedure were carried out as described by Schachman (1957). A partial specific volume of 0.73 g./ml. was assumed for the enzyme.

Chromatographic apparatus. Chromatographic separations were performed with an LKB drop-counter fraction collector in conjunction with a Uvicord detector and a recorder. Traces from this recorder formed the basis of Figs. 1 and 2, the proportionality of trace to protein concentration being logarithmic. Recycling chromatography was carried out at 8° with an LKB ReCychrom with a jacketed column (95 cm. $\times$ 3.2 cm.) containing Sephadex G-100 equilibrated with 0.10 *M*-phosphate buffer, pH 6.8. The flow rate used was 27 ml./hr. and was the maximum that could be achieved.

RESULTS

Purification of L-histidinol dehydrogenase

During the development of the purification method difficulties were encountered in devising satisfactory procedures because the apparent molecular weight of the enzyme varied between wide limits under different conditions owing to reversible polymerization. To achieve satisfactory results it was necessary to find conditions where only one

Fig. 1. Fractionation of histidinol dehydrogenase on a column of TEAE-cellulose. The 30 cm. $\times$ 1.8 cm. column was eluted by 450 ml. of sodium-potassium phosphate buffer, pH 6.8, of increasing molarity (●····●). —, Trace from the Uvicord recorder, representing protein concentration of the eluate. ○ — ○, Histidinol-dehydrogenase activity located in the eluate.

Fig. 2. Purification of histidinol dehydrogenase by recycling chromatography on Sephadex G-100 gel. A 10 ml. sample was applied to a 95 cm. $\times$ 3.2 cm. column and was eluted at 27 ml./hr. with 0.10 *M*-sodium-potassium phosphate buffer, pH 6.8. Material emerging from the column was monitored and either collected by means of a fraction collector (—) or recycled for further fractionation (·····). After three cycles the sample was completely eluted and the position of the histidinol-dehydrogenase component determined (----).

Table 1. *Purification of Neurospora histidinol dehydrogenase*

Freeze-dried *N. crassa* (200g.), equivalent to 120 g. of protein, was used as starting material. Enzyme activity was measured by the increase in extinction at 340 m μ due to NAD reduction in a system containing NAD, histidinol and glycine-NaOH buffer, pH 9.8. Protein was assayed by the method of Lowry, Rosebrough, Farr & Randall (1951).

Step	Procedure	Total protein after procedure (mg.)	Total enzyme activity after procedure (units)
1	Extraction from freeze-dried mycelium	26100	1200-1800
2	Precipitation of nucleic acids	15800	2180
3	(NH ₄) ₂ SO ₄ fractionation	3360	1800
4	Gel filtration	3050	780
5	Absorption-desorption on DEAE-cellulose	960	760
6	Chromatography on TEAE-cellulose (1)	500	430
7	Recycling gel filtration	80	220
8	Chromatography on TEAE-cellulose (2)	50	220

significant enzyme species was present. This was achieved by keeping the ionic strength of the medium, wherever possible, greater than that of 0.07M-phosphate buffer, pH 6.8, after stage 5. The purification procedure finally devised employs eight steps, the results being summarized in Table 1. All procedures are performed in a cold room (5°).

Step 1: extraction of enzyme. Freeze-dried mycelial powder (200g.) was extracted in 2l. of 0.05M-tris buffer, pH 9.1, containing 0.1% (v/v) of 2-mercaptoethanol at 5° with occasional stirring for 30min. The slurry was then centrifuged for 1hr. at 2000g. This procedure was the most convenient for extraction of large amounts of enzyme from *Neurospora*. Comparable amounts of activity could be obtained by more tedious means such as grinding with glass powder or extraction from powdered acetone-precipitated mycelium. The buffer used was chosen because it seemed to give some selective extraction of histidinol dehydrogenase and the enzyme was stable in it.

Step 2: precipitation of nucleic acids. The supernatant was treated with one-twentieth of its volume of M-manganese chloride solution (Korkes, del Campillo, Gunsalus & Ochoa, 1951) and the precipitate was removed by centrifuging at 2000g for 30min. Precipitation with protamine sulphate could also be used to eliminate nucleic acids but the use of manganese chloride was preferred because it precipitated some unwanted protein and tended to

activate the enzyme. This activation by Mn²⁺ may be similar to that found by Loper & Adams (1965) in the histidinol dehydrogenase from *Salmonella typhimurium*.

Re-extraction of precipitates. The precipitates resulting from the previous two stages contained a large amount of enzyme mechanically trapped in the large sediments of cell debris and nucleic acids. To obtain the maximum yield possible these two precipitates were amalgamated and re-extracted. They were suspended in 2l. of 0.01M-tris buffer, pH 9.1, containing 0.1% (v/v) of 2-mercaptoethanol and subsequently put through steps 1 and 2 again.

Step 3: ammonium sulphate precipitations. To the supernatant resulting from the manganese chloride treatment solid ammonium sulphate was added to bring the concentration to 45% saturation. The precipitate was removed by centrifugation for 1hr. at 2000g and discarded. The supernatant was brought to 65% saturation by means of more solid ammonium sulphate. All the histidinol dehydrogenase in the extract was precipitated at this concentration and was removed by centrifugation for 1hr. at 2000g.

Step 4: batch gel filtration. The precipitate from 65% saturation with ammonium sulphate was dissolved in 200ml. of tris-hydrochloric acid buffer, pH 9.1 (0.05M), and applied to a Sephadex G-100 column (23cm. x 9cm.) previously equilibrated with 0.01M-sodium-potassium phosphate buffer, pH 6.8, saturated with chloroform at room temperature. After the extract had soaked in, the column was eluted with 0.01M-sodium-potassium phosphate buffer, pH 6.8. Histidinol-dehydrogenase activity appeared at the void volume and emerged as a broad peak of 400-600ml. This batch fractionation on gel served to remove only a small amount of protein but this particular protein was shown to be difficult to separate later in the purification. In addition the gel filtration provided a rapid desalting procedure.

Step 5: batch ion-exchange adsorption. The enzymically active fractions from the previous step were diluted to 2l. with distilled water and added to a slurry of 20g. of our own DEAE-cellulose or 20g. of high-capacity or 30g. of low-capacity Cellex D, in 0.01M-phosphate buffer, pH 6.8. After adsorption of the enzyme, which was quantitative, the exchanger was collected by filtration, resuspended in 0.01M-phosphate buffer, refiltered and finally made into a slurry with 0.01M-phosphate and poured into a 3cm.-diam. glass column. Frontal elution with 0.10M-phosphate buffer, pH 6.8, under 10lb./in.² air pressure gave rise to a dark-brown band passing down the column. This band, together with 100ml. of the subsequent eluate, was collected and dialysed for 18hr. against tap water.

Step 6: chromatography on TEAE-cellulose. The

dialysed material was adsorbed on a 30 cm. $\times$ 1.8 cm. column of TEAE-cellulose. After adsorption the column was developed by means of a gradient of increasing concentration of sodium-potassium phosphate buffer, pH 6.8. The gradient was made by a Varigrad device (Peterson & Sober, 1959). The gradient limits were 0.01 and 0.17 M, the nine chambers being filled with buffers of the following molarities: 0.01, 0.01, 0.08, 0.01, 0.12, 0.01, 0.12, 0.01, 0.17; the resulting gradient is shown in Fig. 1. This gradient was the best obtainable in practice; other gradients failed to separate the histidinol dehydrogenase more effectively because although better separation could be achieved over part of the peak, the enzyme was much more spread out and overlapped unwanted proteins to a greater extent. Fig. 1 shows also the protein concentration of the eluate and indicates the position of histidinol dehydrogenase. Earlier experiments with DEAE-cellulose instead of TEAE-cellulose were unsatisfactory in that the activity peak was very diffuse and the degree of separation achieved was much reduced. Whereas histidinol dehydrogenase was eluted at 0.047 M-phosphate when adsorbed on DEAE-cellulose the stronger binding forces of the TEAE-cellulose resulted in elution of the enzyme at 0.069 M-phosphate in a much more compact peak.

Step 7: chromatography on Sephadex G-100. The eluate from the above TEAE-cellulose column (TE-1) was reduced in volume to approx. 10 ml. by means of an LKB Ultrafilter and it was then subjected to recycling chromatography on Sephadex G-100. In this procedure material can be recycled through a gel column to increase the degree of resolution between protein species. Fig. 2 represents the various cycles in this procedure. Even at low temperature diffusion was an important factor and so the highest possible pumping speed was used to minimize separation time. Fig. 2 shows material that recycles through the column and the material bled off at indicated periods. A good degree of purification was achieved by this recycling procedure. Gel electrophoresis showed that the number of components had been reduced to two as compared with six components in the input (Fig. 4).

Step 8: second fractionation on TEAE-cellulose. After the recycling chromatography the last step in the fractionation was simply to dialyse against tap water and repeat the chromatography on TEAE-cellulose as in step 6 with the same gradient. The enzyme was eluted quantitatively as a single peak corresponding to a discrete protein peak in the trace. This material gave a single peak in the ultracentrifuge (Fig. 3) and one band on gel electrophoresis that had enzyme activity (Fig. 4).

Overall yield and purification. The overall yield of histidinol dehydrogenase was normally about 10–15% of the initial activity. If the recoveries at

each step were calculated allowing for losses in the total recovered activity during the various procedures the overall yield would rise to about 30–40%. The protein yield was about 50 mg./200 g. of *Neurospora*. Re-extraction of the precipitates (see step 2 in the purification section) gave an increase of up to 40% in yield, corresponding to about 0.04–0.06% of the cellular protein.

Molecular weight and degree of polymerization of histidinol dehydrogenase. As purified by the above procedure histidinol dehydrogenase gave a single

Fig. 3. Ultracentrifugation of histidinol dehydrogenase preparations. (a) Enzyme prepared as described in the text with TEAE-cellulose. Protein concentration was 1.8 mg./ml. and the material was spun in a 30 mm. cell at 2°. The photograph was taken after 80 min. at 50740 rev./min. (b) Enzyme prepared with DEAE-cellulose. Protein concentration was 1.5 mg./ml. and the material was spun in a 30 mm. cell at 2°. The photograph was taken after 112 min. at 50740 rev./min.

Fig. 4. Starch-gel electrophoretic patterns of different stages in the purification of histidinol dehydrogenase. Samples were taken after the purification stage indicated and were run for 18 hr. at 20 v/cm. in 14% starch containing tris buffer, pH 9.1 (0.01 M). After electrophoresis the bands were made visible by staining with Amido Black. Bands that showed activity in the oxidation of histidinol were located as described in the text; such bands are arrowed.

ultracentrifuge peak with a sedimentation coefficient of 7.1 s (Fig. 4). Determination of the molecular weight by the Archibald procedure gave a molecular weight of $81\,000 \pm 200$. However, the sucrose-density-gradient method of Martin & Ames (1961) gave values of $39\,000 \pm 1000$ and gel-filtration methods similar to that of Andrews (1964) gave values of $250\,000 \pm 50\,000$, both types of measurement being performed on material from early stages of purification. A detailed study of the apparent variation in degree of polymerization is being undertaken and only such points relative to the purification are mentioned here. In early experiments DEAE-cellulose was used in step 6 and it was found this gave a very broad peak. If the leading edge of this peak was taken (about 10–20% of the enzymic activity) the enzyme could be purified by the procedure described in step 7. However, in this case the overall yield of enzyme was extremely low. Ultracentrifugation of histidinol dehydrogenase prepared in this way gave two peaks with sedimentation coefficients of 7 s and 4 s (Fig. 4). Two species could also be separated by starch-gel electrophoresis and both bands had enzyme activity.

Kinetic properties of histidinol dehydrogenase

Optimum pH. This was found to be high; pH 9.8 was the value under the conditions used and de-

Fig. 5. Effect of pH on the activity of histidinol dehydrogenase. Activities were measured in 4 ml. of either 0.05M-glycine-NaOH buffer (○), or a composite buffer, 0.05N-acetic acid-succinic acid-glycylglycine-glycine (●), adjusted to a suitable pH with N-KOH, both containing 0.1% of 2-mercaptoethanol. After addition of 2 μ moles of NAD in 0.2 ml. and 0.1 ml. of enzyme solution, the mixture was preincubated for 2 min. at 35° in the cell before initiation of the reaction by the addition of 0.4 μ mole of L-histidinol in 0.2 ml.

scribed in the Materials and Methods section. The pH-activity curve is shown in Fig. 5 and was asymmetrical, probably owing to the destruction of NAD at elevated pH values. Tris-hydrochloric acid buffer, pH 9.1, did not give as high a reaction velocity as was produced in glycine-sodium hydroxide buffer, pH 9.8, but stability of the enzyme in tris buffer was much greater than in the glycine-sodium hydroxide buffer, pH 9.8. No reaction was found at pH values below neutrality either in the forward or reverse reaction.

Michaelis constants. These can be deduced from the standard plots of Lineweaver & Burk (1934) as shown in Fig. 6, and are K_m (NAD) 13.0×10^{-5} M and K_m (histidinol) 8.2×10^{-6} M. No other substrates for the enzyme have yet been found. The following amino acid alcohols are not oxidized: β -alaninol, DL-alaninol, glycinol, methioninol, phenylalaninol, serinol and tryptophol. Histidinol dehydrogenase has no effect on simple aliphatic alcohols and it does not reduce NADP in the presence of histidinol.

Histidine will not act as a competitive inhibitor of histidinol dehydrogenase and NADH_2 + histidine shows no decrease in the extinction at 340 m μ in the presence of this enzyme. This reverse reaction, histidine + NADH_2 to NAD + histidinol, is undetectable even at low pH whereas the reverse reaction with acetaldehyde can be demonstrated

Fig. 6. Relation between the velocity of histidinol oxidation by histidinol dehydrogenase and the concentration of histidinol and NAD represented in double-reciprocal plots. Activity was measured in 4 ml. of 0.05M-glycine-NaOH buffer, pH 9.8, containing 0.1% of 2-mercaptoethanol. For the determination of K_m (histidinol), 2 μ moles of NAD in 0.2 ml. and 0.1 ml. of enzyme solution were added and the mixture was incubated in the cell for 2 min. before initiation of the reaction by the addition of 0.01–0.1 μ mole of histidinol in 0.1 ml. For the determination of K_m (NAD), 0.2–1 μ mole of NAD in 0.1 ml. and 0.1 ml. of enzyme solution were added and the mixture was incubated at 35° in the cell for 2 min. before initiation of the reaction by the addition of 0.4 μ mole of histidinol in 0.2 ml. Initial velocity, v , is expressed as milliunits. Concentrations are expressed as μ moles/incubation mixture. Values of K_m were estimated by regression analysis (Wilkinson, 1961).

with alcohol dehydrogenase (Wartburg, Bethune & Vallee, 1965). The reaction towards NADH and histidinol went to 95% completion under the standard conditions used, 1.90 moles of NAD being reduced/mole of histidinol utilized. The purified enzyme can catalyse the oxidation of 49 moles of NAD/min./mole. of enzyme, assuming a molecular weight of 40 000.

Activators and inhibitors. Full activity can be restored to aged preparations by small amounts of reducing agents. 2-Mercaptoethanol at 0.1% (v/v) is satisfactory and is normally included in the assay system. Sodium thioglycollate and reduced glutathione have the same effect. High concentrations of 2-mercaptoethanol and sodium thioglycollate at 0.01M or higher inhibit the reaction. *p*-Chloromercuribenzoate (0.1mM) and *o*-iodosobenzoate (1mM) inhibit the reaction, but not instantly, the full effect taking some minutes. The enzyme is activated by Mn^{2+} , as in step 2 of the purification procedure. It has not been possible to reduce the activity of the enzyme by removal of Mn^{2+} from a fully active preparation nor to increase the activity of such a preparation by the addition of more Mn^{2+} . Dialysis of enzyme solutions against EDTA (0.01M) for up to 72 hr. decreases the activity of the enzyme by up to 75% but this could not be restored by addition of any one of a range of bivalent cations tested.

DISCUSSION

Histidinol dehydrogenase of *Neurospora* is similar to the corresponding enzyme from *Salmonella* in that the affinity for histidinol is high and apparently very specific.

It seems that the degree of polymerization of histidinol dehydrogenase preparations desorbed from ion-exchange resins depends on ionic concentration of the eluting solution, and it was also found that little success could be obtained from recycling gel chromatography until the concentration of phosphate used was that which ensured that the enzyme existed as a single species. Fig. 2 shows that the enzyme is retarded when a molarity of 0.1 is used whereas it was excluded from the same gel at lower ionic concentrations (as in step 4 of the purification), indicating a higher state of polymerization. In fact, molecular weights of up to $250\,000 \pm 50\,000$ could be observed under conditions corresponding to step 4 in the purification.

Major problems to be solved are the questions of in what manner the smaller enzyme species are polymerized to give larger molecules and the sub-unit composition of the smallest enzymically active unit. The fact that reducing agents in high concentration have an inhibitory effect indicates that the sub-units may be held together by S-S bridges.

The observed effects of SH inhibitors are not inconsistent with this idea.

Whereas the pure enzyme obtained after TEAE-cellulose chromatography has a sedimentation coefficient of 7.1s, the enzyme obtained after DEAE-cellulose chromatography and purification is made up of two components. These peaks have values of 7s and 4s for their sedimentation coefficient and the working hypothesis is that the 7s unit is made of two of the 4s units. The 4s unit would correspond in size to the enzyme of molecular weight 40 000 found in dilute solution by sucrose density gradient and represents the smallest active component yet found. Preliminary work indicates that 4s unit can be split by reduction by high concentrations of mercaptoethanol into 2.5s and 1.5s units that are probably sub-units.

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