Short Communications

C-Terminal Analysis of Histidinol Dehydrogenase from *Neurospora crassa*

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This communication reports work in pursuance of the object of determining the sub-unit structure of L-histidinol dehydrogenase (L-histidinol-NAD oxidoreductase, EC 1.1.1.23) of *Neurospora crassa* to elucidate the genetic mechanism of its elicitation and preparatory to the determination of its primary sequence. Earlier studies (Creaser, Minson & Bennett, 1967; Bennett & Creaser, 1967) had shown that amino acid analysis and the number of tryptic and chymotryptic peptides produced indicated a minimum chemical unit of approx. 20000 molecular weight. However, oxidative or reductive procedures normally incapable of peptide-bond cleavage produced material with a sedimentation coefficient equivalent to half of this value or less. Attempts to fractionate this material into more than one component have, as yet, proved unsuccessful. Therefore end-group analysis was resorted to in order to attempt a decision as to whether the minimum chemical unit consisted of one or more dissimilar polypeptide chains. *N*-Terminal analysis by the 'dansyl'-labelling technique (Gray & Hartley, 1963) and the cyanate determination procedure (Stark & Smyth, 1963) yielded negative results with L-histidinol dehydrogenase, though giving the expected results with standard proteins having free *N*-termini, presumably indicating the presence of blocking group(s) in common with many other proteins, some of which are listed by Braun & Schroeder (1967). An investigation of *C*-terminal groups by both the use of carboxypeptidase A and the hydrazinolysis method introduced by Akabori, Ohno & Narita (1952) was undertaken, as each method provides corroboration and largely overcomes the limitations of the other.

Materials. Histidinol dehydrogenase was prepared by the method of Creaser, Bennett & Drysdale (1967). Di-isopropyl phosphorofluoridate-treated carboxypeptidase A (three-times-crystallized) was obtained from Worthington Biochemical Corp. (Freehold, N.J., U.S.A.) and prepared for use by the procedure of M. A. Mitz (quoted by Hirs, Moore & Stein, 1960). Ribonuclease and lysozyme were obtained from Worthington Biochemical Corp. and Sigma Chemical Co. (St Louis, Mo., U.S.A.)

respectively. Anhydrous hydrazine was prepared by the method of Kusama (1957) and stored over P_2O_5 .

Methods. (a) Digestion with carboxypeptidase A. Approx. 0.2 mg. desalted freeze-dried samples of protein preparations were dissolved in 1 ml. of water in the stirred vessel, thermostatically controlled at 37°, of a Radiometer pH-stat. The accurate protein concentration was determined spectrophotometrically at 280 m μ ($E_{1\%}^{1\text{cm}}$ 1.211 for histidinol dehydrogenase in distilled water). This determination was checked in certain cases by summation of the contributing residues in samples that were subjected to acid hydrolysis and quantitative amino acid analysis. Such values were within 5% of those determined by spectrophotometric measurement.

The solution was adjusted to and maintained at pH 8.0 by the monitored addition of 0.01 M-NaOH. Then 0.1 ml. of carboxypeptidase solution (20 μ g./0.1 ml.) was added to initiate the hydrolysis and 0.05 ml. of 6 M-HCl to terminate it. The solution was made to a final volume of 2.2 ml. with sodium citrate buffer, pH 2.2 (Beckman amino acid analyser 'sample dilutor'), for quantitative amino acid analysis. No further preparation of the digest was found to be necessary as the protein present caused only slight increase in column back-pressures after 20 analyses.

(b) Hydrazinolysis. Samples (1 ml.) of the protein preparations of accurately known concentration, as used for the carboxypeptidase experiments, were placed in 5 ml. glass stoppered tubes and freeze-dried, and 0.5 ml. of freshly prepared anhydrous hydrazine was added to each. The solution was frozen in liquid N_2 and air displaced with O_2 -free N_2 . The greased stopper was held with a spring clip while hydrazinolysis proceeded at 110° for 48 hr. Hydrazine was removed *in vacuo* over conc. H_2SO_4 for 48 hr. and the residue washed with 0.5 ml. of water three times into a separating funnel. Then 0.5 ml. of benzaldehyde was added to the combined washings and the mixture shaken for 30 min. The upper benzaldehyde layer, which contained the hydrazide dibenzal

derivatives, was separated off and the procedure repeated with the aqueous fraction. The aqueous solution was twice washed with 2ml. of ethyl acetate to remove benzaldehyde. The aqueous fraction was evaporated to dryness in a boiling-water bath under O_2 -free N_2 and made to 2.2ml. with sodium citrate buffer, pH 2.2, for quantitative amino acid analysis.

(c) Amino acid analysis. Analyses were performed by the method of Spackman, Stein & Moore (1958) with a Beckman model 120B amino acid analyser. The analyser was fitted with two 20mm.-path-length-cell high-sensitivity units (Evans Electro-Selenium Ltd., Halstead, Essex) at 440m μ and 570m μ . The 0–5mv range of the Honeywell Elektronik 15 recorder was expanded to a 3.610–4.6385mv range by the replacement, with a Honeywell-manufactured component, of the resistor-panel assembly in the measuring-circuit unit. This system of analysis gives linear scale readings in the 1–10m μ mole range with a standard error of the mean of 4.75%.

Results. (a) Carboxypeptidase method. The prepared carboxypeptidase solution was tested with ribonuclease and lysozyme to determine the overall yield of the analytical procedure described under 'Methods'. *C*-Terminal valine was obtained in 93% yield from ribonuclease and leucine in 95% yield from lysozyme.

Fig. 1 illustrates the liberation of amino acids from histidinol dehydrogenase by carboxypeptidase A. Leucine was the most rapidly released amino acid in an amount corresponding to 189% for a particle weight of 20000. Alanine, serine, valine, glutamic acid and isoleucine were also rapidly released in amounts in excess of 100% for the same particle weight. Significantly, in the earlier stages

of digestion, the relative rates of release of certain residues, e.g. serine and valine, intersected. These observations were repeatably found in three sets of experiments on two separate preparations of histidinol dehydrogenase. In one of these experiments a further addition of carboxypeptidase was made at 1hr. with no detectable result.

(b) Hydrazine method. The hydrazinolytic procedure was tested with ribonuclease and gave a 67% yield of valine, together with small amounts (2–4%) of lysine, aspartic acid, threonine, glycine, alanine and leucine, presumably resulting from peptide-bond cleavage due to residual water in the hydrazine or slight breakdown of hydrazides. Hydrazinolysis of histidinol dehydrogenase gave a 126% yield of leucine for a particle weight of 20000, together with similar concentrations of those contaminating amino acids as were noted with ribonuclease. This value is the average of two determinations on each of two separate protein preparations.

Discussion. Leucine is liberated from histidinol dehydrogenase by hydrazinolysis in an amount in considerable excess of that required for a single *C*-terminus for a minimum chemical molecular weight of 20000. Since this method liberates only *C*-terminal residues as free amino acids, there must be more than one *C*-terminal leucyl residue for this weight. On the other hand, hydrolysis with carboxypeptidase allows the recovery, in amounts approaching quantitative, of those residues having a free α -carboxyl group. Leucine is rapidly released from histidinol dehydrogenase by carboxypeptidase A, and prolonged digestion yields an amount closely approaching that required for 2mol.prop. for a molecular weight of 20000. A -Leu-Leu terminal sequence is ruled out by the yield of leucine by hydrazinolysis. It is therefore concluded that this unit consists of two polypeptide chains with leucine at their *C*-termini. Many of the other amino acids that are susceptible to carboxypeptidase A cleavage are released from histidinol dehydrogenase subsequent to leucine. Alanine, serine and valine are released in amounts in excess of those required for 1mol.prop. for a molecular weight of 20000 and so presumably contribute to both chains, whereas glutamic acid, isoleucine, phenylalanine, aspartic acid and tyrosine are found in smaller amounts than this and presumably contribute to a single chain. It is noted that integral values of amino acids released from the protein by carboxypeptidase are not obtained with the exception of leucine. A possible explanation of this would be that, as the native protein is being used, on removal of the terminal and subsequent amino acids there is a change in configuration such that access by carboxypeptidase to the remaining amino acids becomes hindered, eventually rendering the protein

Fig. 1. Release of amino acids from histidinol dehydrogenase by carboxypeptidase A. Curve 1, leucine; curve 2, alanine; curve 3, serine; curve 4, valine. Amino acids that are released more slowly have been omitted from the Figure.

unsusceptible to further digestion. Such a speculation would accord with the observation that addition of more carboxypeptidase does not result in the further release of amino acids. Obviously, until separation of the two chains is achieved, there is no possibility of determining the sequences of the C-termini.

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