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Amino Acid Substitutions in Mutant Forms of Histidinol Dehydrogenase from *Neurospora crassa*

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Amino acid changes in the enzyme L-histidinol dehydrogenase (L-histidinol–NAD oxidoreductase, EC 1.1.1.23) have been determined between the wild-type *Neurospora crassa* and two temperature-sensitive mutants. Comparison was made between amino acid analyses of peptides of differing electrophoretic and chromatographic mobilities resulting from tryptic and chymotryptic digestion of protein from wild-type and mutant K26, and wild-type and mutant K445 strains, respectively. The analyses demonstrate the substitution of aspartic acid for alanine in mutant K26, and leucine for histidine in mutant K445. The effects of the resulting changes in polarity and charge are discussed in relation to the catalytic functioning of the proteins.

This paper reports further work in pursuance of the object of correlating the mutationally induced amino acid changes of an enzyme protein both within a single organism, and from a range of organisms, with the resulting modifications in its catalytic functioning. Preliminary kinetic studies (Creaser, Bennett & Drysdale, 1965) had revealed a range of mutants in the *his*-3 genetic region of *Neurospora crassa* with altered kinetic parameters in the L-histidinol dehydrogenase function. It therefore became of interest to attempt to determine the amino acid replacements in enzymes from representative mutants. The strains K26 and K445 were chosen from those included in the earlier work since they showed modified affinities for NAD and modified activation energies that were consistent with the differing temperature–growth characteristics from the wild-type strain.

MATERIALS AND METHODS

Organisms. The strains of *N. crassa* used in this work were wild-type Emerson *a* (5297) and two mutants, K26 and K445, kindly supplied by Professor D. G. Catcheside. They had been produced by u.v. irradiation of wild-type strain and isolated by filtration enrichment (Catcheside, 1960). The wild-type organisms were maintained and grown in bulk in Vogel's minimal medium with the addition of 2% sucrose as described by Creaser, Bennett & Drysdale (1967) and the mutants were grown in the same medium with the addition of 300mg. of L-histidine/l.

Proteolytic enzymes and chemicals. Trypsin (freeze-dried, twice crystallized) was obtained from Worthington Biochemical Corp. (Freehold, N.J., U.S.A.), treated by the method of Redfield & Anfinsen (1956) to reduce chymotryptic activity preferentially and screened with ribonuclease (Worthington Biochemical Corp.) to confirm the finding of the expected number of peptides. α-Chymotrypsin (three-times crystallized) was obtained from Worthington Biochemical Corp. and similarly screened.

All chemicals used in the chemical aspects of the work were of A.R. grade; chemicals used for the enzyme purification were reagent quality.

Proteolytic digests. Histidinol dehydrogenase resulting from the final stage of purification (Creaser *et al.* 1967) was freeze-dried, desalted by passage through a Sephadex G-25 column, again freeze-dried and stored over desiccant at $-15°$ until required. It was then oxidized by the method of Moore (1963) to break S·S bridges, to block cysteinyl residues that might otherwise undergo exchange reactions, and to render the protein susceptible to proteolytic digestion. Methionine is converted completely into the stable sulphone, thus preventing ambiguities due to the production of peptides containing methionine residues in different states of oxidation. Tryptophan is destroyed by the oxidation.

Tryptic and chymotryptic digests of 2–10mg. of oxidized protein were performed in a Radiometer pH-stat in 2·5ml. of 0·1M-ammonium acetate buffer, pH 7·9, in a stirred vessel thermostatically controlled at 37° and maintained at pH 7·9 by the monitored addition of 0·1N-NH_3. For all digestions the enzyme/protein weight ratio was 1:50 and the enzyme was added in 0·1ml. of freshly prepared solution. Proteolysis was deemed to be completed when the curve of added OH^- paralleled a previously determined blank curve, due to NH_3 loss and CO_2 uptake, which included all components other than the enzyme. After 4hr. for a tryptic and 12hr. for a chymotryptic digest the digestion was terminated by adjusting the pH to 4·0 with acetic acid.

Peptide separation. The digest was evaporated to dryness in a 100° water bath under a stream of oxygen-free nitrogen and taken up in 1·5N-formic acid–2N-acetic acid buffer,

pH 1·9. Samples corresponding to 2–2·5 mg. of the original proteins were subjected to electrophoresis in this buffer on Whatman no. 3MM paper in a Pherograph cooled-plate high-voltage electrophoresis apparatus. A 0·1 ml. sample of the peptide solution was placed in a narrow 1 cm.-wide band 7·5 cm. from the anode end of a 25 cm. × 59 cm. sheet of paper. Usually two or more samples of either the same or different digests were applied to obtain separation under identical conditions, and were placed at least 5 cm. from either edge of the paper and each other. After electrophoresis for 2·75 hr. at 34 v/cm., 60 mA and −6° to −8°, along the long axis of the paper, the paper was dried in an oven at 100°. Then 5 cm.-wide strips were cut from the paper so that the peptide bands occupied the middle 1–1·5 cm. In initial experiments a 2·5 cm.-wide strip was removed, stained with 0·25% (w/v) ninhydrin in acetone with a few drops of pyridine and developed at 100° to make the peptide bands visible. The technique proved to be entirely reproducible so that removal of the strips for location of bands was not necessary in later work. A 40 cm.-long peptide-containing band was cut from the 5 cm.-wide strip. Further separation was achieved chromatographically by a modification of the method of Richmond & Hartley (1959). The 40 cm. × 5 cm. strip was sewn with a domestic sewing machine (with 2 mm. zig-zag stitches) into a 40 cm. × 50 cm. sheet of Whatman no. 3MM paper (2½ in. from the top, taking care to maintain the overlaps at 2 mm.). The sewing cotton used was previously washed with the chromatography solvent to remove soluble ninhydrin-positive material. The chromatogram was then developed in the second dimension by descending irrigation with freshly prepared butan-1-ol–acetic acid–water (4:1:5, by vol.) (Levy & Chung, 1953). Usually two, three or four chromatograms bearing simultaneously prepared electrophoretic strips were developed in the same tank for the same period of time, to aid subsequent comparability. After chromatography the paper was dried in an oven at 100°. Peptides were located by spraying with 0·25% (w/v) ninhydrin in acetone with a few drops of pyridine, followed by heating at 100° for 7 min.

In two cases, peptides were eluted from excised areas of unstained chromatograms with a solution of pyridine (10%, v/v) in 10% (v/v) propan-2-ol. The eluent was removed at 100° under a stream of oxygen-free nitrogen and the peptides were dissolved in 0·05 ml. of pyridine (10%, v/v)–acetic acid (1%, v/v) buffer, pH 6·5. The peptide solutions were applied together with amino acid markers to a 59 cm. × 20 cm. sheet of Whatman no. 3MM paper and were subjected to electrophoresis in the same buffer for 2 hr. at 20 v/cm., 45 mA and −5°. Spots were located by spraying with 0·25% (w/v) ninhydrin in acetone, followed by heating at 100° for 7 min.

Peptide analysis. Peptides were eluted from excised areas of very lightly ninhydrin-stained chromatograms (0·1%, lightly applied and developed at room temperature overnight) or from the duplicate areas of unstained chromatograms prepared simultaneously under identical conditions, both electrophoretically and chromatographically, to the stained chromatograms. Elution was achieved by successive washing with glass-distilled constant-boiling HCl to a final volume of 0·5 ml. into thick-walled hydrolysis tubes. Peptides (0·01–0·02 μmole) were hydrolysed at 110° for 24 hr. either in a vacuum or under oxygen-free nitrogen and analysed by the method of Spackman, Stein & Moore (1958) and Moore, Spackman & Stein (1958) with a Beckman model 120B amino acid analyser. The analyser was fitted with a 20 mm.-path-length-cell high-sensitivity unit (Evans Electroselenium Ltd., Halstead, Essex), which was initially powered by heavy-duty 12 v batteries. This system of analysis gave a standard error of the mean of 6·4% and a maximum at 13·4% from nine standard runs with a standard mixture containing a 0·025 μmole sample of each amino acid giving log-scale readings of 0·1–0·2 E unit. Base-line values were determined by three blank runs. For later analyses the high-sensitivity unit was powered from the voltage-stabilized power supply of the analyser (by disconnecting the lamp of the alternate 570 mμ colorimeter) and, in addition, expansion of the 0–5 mv range of the Honeywell Electronik 15 recorder to a 3·610–4·6385 mv range by the replacement, with a Honeywell-manufactured component, of the resistor-panel assembly in the measuring-circuit unit. This later system of analysis gave a standard error of the mean of 4·75% and a maximum at 9·95% from seven standard runs with a standard mixture containing a 0·01 μmole sample of each amino acid corresponding to linear scale readings of 20–35% of full-scale deflexion. Base-line values were determined by one blank run.

RESULTS

Histidinol dehydrogenase was prepared from wild-type *N. crassa* by the method of Creaser *et al.* (1967). This procedure was found to be also applicable to the purification of mutant enzymes from strains K26 and K445, with slight modifications in those steps that depended on charge effects. During ammonium sulphate fractionation the enzymically active fraction of strain K445 extracts precipitated over the range 45–57·5% saturation and that of strain K26 over 35–50% saturation, compared with 40–65% saturation for that of the wild-type. After ion-exchange fractionation on triethylaminoethyl-(TEAE-)cellulose the two mutant enzymes emerged over different regions of the elution pattern from the wild-type protein. These differences are illustrated in Fig. 1.

Pure enzyme protein of K26 and K445 mutant strains and the wild-type was subjected to tryptic and chymotryptic digestion and the resulting peptides were analysed as described in the Materials and Methods section. There was no evidence of loss of material due to incomplete digestion or the formation of an acid-insoluble 'core'. Figs. 2 and 3 illustrate traces of the resulting lightly ninhydrin-stained peptide 'maps'. The peptides were designated 'strain-P-T1–14' and 'strain-P-CT1–21' where 'P' indicates that the protein had suffered previous performic acid oxidation, 'T' digestion by trypsin and 'CT' digestion by chymotrypsin.

On comparison of the tryptic peptide maps (Figs. 2*a*, 2*b* and 2*c*) two points were apparent: (i) All but one of the peptides in the three proteins are common. (ii) The wild-type and mutant-K445 proteins differ from the mutant-K26 protein in the position of the peptide T12 (cross-hatched in the Figures).

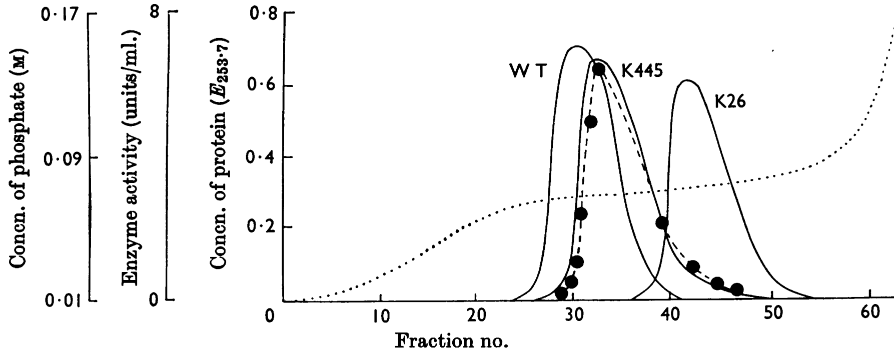

Fig. 1. Final fractionations of histidinol dehydrogenase from wild-type (WT) and mutant K26 and K445 strains on columns of TEAE-cellulose. The 30 cm. × 1·8 cm. column was eluted by 450 ml. of sodium–potassium phosphate buffer, pH 6·8, of increasing molarity. Further experimental details are described in Creaser *et al.* (1967). ——, Protein concentration of the eluates; ······, phosphate concentration gradient; ----, histidinol dehydrogenase activity of the mutant-K445 enzyme located in the eluate. The activities of the eluates from fractionations of the wild-type and mutant-K26 enzymes have been omitted for clarity but were similarly coincident with the protein trace.

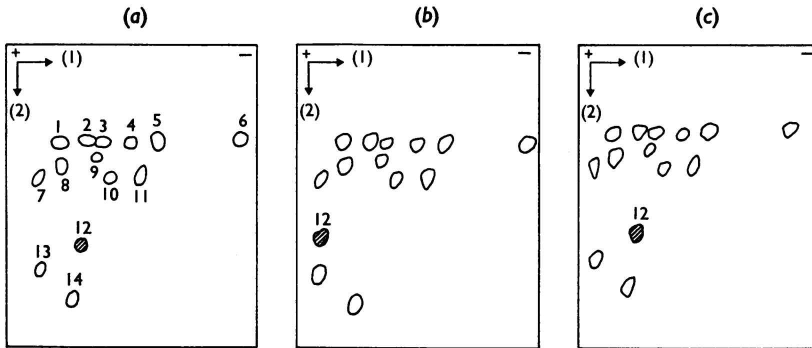

Fig. 2. Traces of wild-type (*a*), mutant K26 (*b*) and mutant K445 (*c*) tryptic peptide 'maps'. Electrophoresis at pH 1·9 (1) proceeded horizontally with the origin and anode in the left-hand corner. After the electrophoretic strip had been sewn into a new sheet of paper, descending chromatography in butan-1-ol–acetic acid (2) proceeded at right angles. The cross-hatching indicates peptides of differing mobility.

Similarly, on comparison of the chymotryptic peptide maps (Figs. 3*a*, 3*b* and 3*c*) equivalent points may be made: (i) All but one of the peptides in the three proteins are common. (ii) The wild-type and mutant-K26 proteins differ from the mutant-K445 protein in the position of the peptide CT3 (cross-hatched in the Figures).

Amino acid analyses of the eluted and hydrolysed peptides WT-P-T12, K26-P-T12, WT-P-CT3 and K445-P-CT3 were determined as described in the Materials and Methods section, and the results are summarized in Table 1. All four peptides were thrice isolated and analysed from digests of the respective performic acid-oxidized protein. Analyses (*b*) and (*c*) of Table 1 were performed on a series of separately purified, oxidized and digested proteins from those under analysis (*a*). Analyses (*b*) and (*c*) in addition utilized the recorder-scale expander of the amino acid analyser (see the Materials and Methods section). Analyses (*a*) and (*b*) utilized peptides eluted from areas of very lightly ninhydrin-stained chromatograms whereas analysis (*c*) utilized peptides obtained from duplicate areas of unstained chromatograms, prepared by electrophoresis and chromatography carried out simultaneously and under identical conditions with those that were stained to give rise to the peptides used in analysis (*b*).

Further fractionation of the peptides was considered to be unnecessary since freshly stained

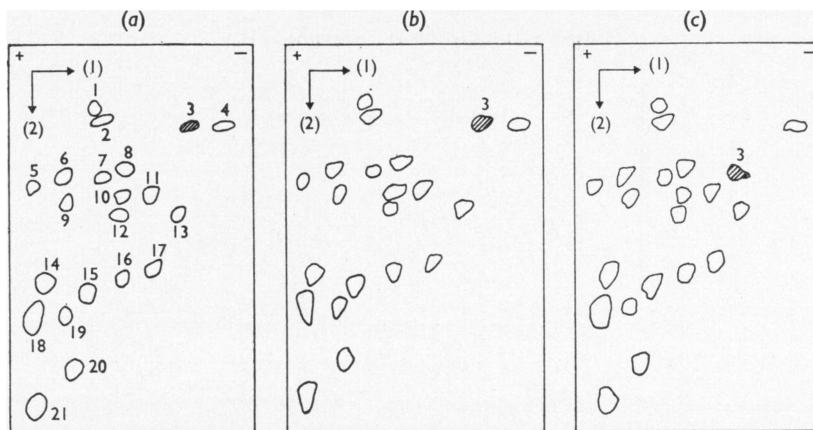

Fig. 3. Traces of wild-type (*a*), mutant K26 (*b*) and mutant K445 (*c*) chymotryptic peptide 'maps'. The conventions used in Fig. 2 apply.

chromatograms showed no ninhydrin-positive material in areas of chromatograms equivalent to positions of peptides of changed mobility. The areas equivalent to peptide WT-P-T12 on map K26-P-T, K26-P-T12 on WT-P-T, WT-P-CT3 on K445-P-CT and K445-P-CT3 on WT-P-CT, when excised, eluted, hydrolysed and analysed for amino acids contained no significant amounts at the mμmole level.

The data are presented as the molar ratios relative to glutamic acid to provide comparison between different concentrations, and are thus subject to analytical error in both the glutamic acid and the individual amino acids. Glutamic acid occurred in all four peptides and is stable and quickly released during acid hydrolysis. All values with a ratio less than 0·1 are omitted, as is tryptophan, which could not be determined. Correction factors have not been applied for amino acids subject to partial destruction and slow release during acid hydrolysis.

With the mutant-K26 and wild-type proteins the T12 peptides were eluted and repurified by electrophoresis at pH 6·5 as described in the Materials and Methods section. Both peptides gave one spot on electrophoresis at this pH and the mobility of the peptide from mutant-K26 enzyme was greater than that of the peptide from the wild-type enzyme (Fig. 4), indicating that the amino acid substituted in mutant-K26 enzyme was aspartic acid rather than asparagine.

DISCUSSION

The peptides have the following compositions: WT-P-T12, (Lys,Asp$_2$,Ser,Glu,Ala); K26-P-T12, (Lys,Asp$_3$,Ser,Glu); WT-P-CT3, (Lys,His,Arg,Asp, Ser,Glu); K445-P-CT3, (Lys,Arg,Asp,Ser,Glu,Leu). They are hexapeptides and have reasonably good stoicheiometric relationships between their constituent amino acids, in the light of the error involved in their analysis and the method of presentation of their contributions as relative molar ratios rather than absolute molar concentrations.

The decrease in mobility of peptide K26-P-T12 during electrophoresis at pH 1·9 compared with that of peptide WT-P-T12 is consistent with the replacement of a neutral alanine residue by an additional aspartic acid residue. Similarly, the replacement of a histidine residue, which is both basic and polar, in peptide WT-P-CT3 by a leucine residue, which is neutral and non-polar, in peptide K445-P-CT3 would tend to decrease its mobility during electrophoresis at pH 1·9 and increase it in the chromatographic solvent system. However, the amino acid changes did not result in detectable changes in any of the wild-type and mutant-K445 tryptic peptides or the wild-type and mutant-K26 chymotryptic peptides in the electrophoretic and chromatographic systems used. Presumably the changes in overall charge and mobility resulting from the amino acid substitution made only a small contribution to the peptides containing them, and therefore peptide differences were not observed. Both of the analysed peptides resulting from chymotryptic cleavage (WT-P-CT3 and K445-P-CT3) lack aromatic residues so that it is probable that either their aspartic acid or their glutamic acid exists as the amine, one of which has acted as a site for cleavage (Desnuelle, 1960).

Although peptide K445-P-CT3 contains a peptide bond with a leucyl residue, which is often slowly split by chymotrypsin, such bonds are protected by the presence of nearby acidic residues (Desnuelle, 1960).

Table 1. *Amino acid compositions of differing normal and mutant peptides*

The amino acid recoveries are reported as molar ratios relative to glutamic acid as 1. See the text for full details.

	Peptide WT-P-T12					Peptide K26-P-T12					Peptide WT-P-CT3					Peptide K445-P-CT3				
	Analysis					Analysis					Analysis					Analysis				
	(a)	(b)	(c)	Mean	Ratio	(a)	(b)	(c)	Mean	Ratio	(a)	(b)	(c)	Mean	Ratio	(a)	(b)	(c)	Mean	Ratio
Lys	1·05	1·03	0·62	0·90	1	0·90	1·01	0·90	0·94	1	0·60	0·70	0·76	0·69	1	0·91	0·72	0·76	0·80	1
His											0·70	0·74	0·70	0·71	1					
Arg											0·76	0·72	0·62	0·70	1	0·97	0·77	0·75	0·83	1
Asp	1·74	2·01	1·73	1·83	2	2·84	2·50	3·35	2·90	3	0·82	0·94	1·13	0·96	1	1·12	1·00	1·29	1·14	1
Ser	0·97	0·92	0·90	0·93	1	0·76	0·81	1·39	0·99	1	0·80	0·81	0·87	0·83	1	0·79	0·84	1·30	0·98	1
Glu	1·00	1·00	1·00	1·00	1	1·00	1·00	1·00	1·00	1	1·00	1·00	1·00	1·00	1	1·00	1·00	1·00	1·00	1
Ala	0·64	1·00	0·99	0·88	1															
Leu																1·07	0·66	0·85	0·86	1

Fig. 4. Trace of the repurification and comparison of the relative mobilities of peptides WT-P-T12 and K26-P-T12 by electrophoresis at pH 6·5.

If the assumption is made that the single amino acid replacement in the polypeptide is due to a single base substitution it is possible from the known genetic code (Söll *et al.* 1965) to examine the nature of the observed replacements. Mutant K26 is characterized by the amino acid replacement of alanine by aspartic acid which would result from a codon change of $GC\begin{pmatrix}U\\C\\G\end{pmatrix}$ to $GA\begin{pmatrix}U\\C\\G\end{pmatrix}$. However, the replacement of alanine by asparagine is possible in theory since a codon for asparagine, AAC, might be achieved from AGC, which trinucleotide is reported by the above-mentioned authors to stimulate the binding of alanyl-transfer-RNA to ribosomes. This possibility, however, is ruled out by the demonstration, by electrophoresis at pH 6·5, of the greater mobility towards the positive pole of the peptide containing the replacement when compared with the peptide containing alanine. This is consistent with an increase in acidity consequent on the presence of aspartic acid rather than asparagine, which would have led to a decrease in mobility towards the positive pole. Similarly a change of $CA\begin{pmatrix}C\\U\end{pmatrix}$ to $CU\begin{pmatrix}C\\U\end{pmatrix}$ would bring about the amino acid replacement of histidine by leucine, which characterizes mutant K445.

It is noteworthy that these base changes involve the replacement of a purine by a pyrimidine and vice versa, i.e. they are 'transversions' according to the terminology of Freese & Yoshida (1965), who view these from collected data as rarer and more drastic events than 'transitions', in which the class of base is conserved.

With regard to the possible functional implications of these amino acid substitutions on the enzymic proteins, the effects of these changes in charge and polarity must be considerable. The

importance of a change from a polar residue to a non-polar and vice versa on the conformation of a protein has been considered by Margoliash (1963) and Epstein (1964). In proteins that have been well characterized, non-polar residues are orientated towards the inside of the molecule in a region of low dielectric constant whereas polar residues are on the outside in a region of high dielectric constant. The classification of alanine is uncertain since it tends to appear at the surface, but a change to the strongly hydrophilic residue of aspartic acid and, even more so, a change from a hydrophilic histidine residue to a hydrophobic leucine one must bring about a large rearrangement of the tertiary structure of the molecule. Additionally, each of these changes results in an alteration of charge. The magnitude of these effects is so large that the replacements must take place in a region distant from the active centre for, although the mutations affect the kinetic parameters of the enzymes (Creaser *et al.* 1965) and the growth characteristics of the auxotrophs, their histidinol dehydrogenase function is retained.

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