Biochem. J. (1974) **137**, 559–566
Printed in Great Britain

Amino Acid Sequence of a Peptide Containing the Active Cysteine Residue of Histidine Ammonia-Lyase

By HAROLD HASSALL and ANNE K. SOUTAR*
Department of Biochemistry, University of Leeds, 9 Hyde Terrace, Leeds LS2 9LS, U.K.

(*Received* 17 *September* 1973)

1. Oxidized (polymerized) histidine ammonia-lyase from *Pseudomonas testosteroni* was activated with dithiothreitol and the reduced disulphide-linked cysteine residues of the native enzyme were carboxymethylated with iodo[^{14}C]acetate. 2. The activity of the carboxymethylated enzyme was similar to that of the polymerized form and approx. 15% of that of the fully reduced form. 3. A tryptic digest of the [^{14}C]carboxymethylated enzyme contained only one radioactive peptide. 4. The amino acid sequence of this peptide was shown to be Gly-Leu-Leu-Asp-Gly-Ser-Ala-Ile-Asn-Pro-Ser-His-Pro-Asn-Cys-(CH_2CO_2H)-Gly-Arg. 5. These findings show that, during polymerization, the disulphide bonds are formed between identical regions of the enzyme, and that the cysteine residue involved is also the one required in the reduced state for full activity of the enzyme.

Histidine ammonia-lyase (histidase, EC 4.3.1.3) readily becomes oxidized during purification, or on aging, to a form that is activated by thiol reagents such as GSH, mercaptoethanol or dithiothreitol (Tabor & Mehler, 1955; Peterkofsky, 1962; Smith *et al.*, 1967). It has been shown that, with the enzyme from *Pseudomonas testosteroni* (Soutar & Hassall, 1969) and from *Pseudomonas* A.T.C.C. 11299b (Klee, 1970), this oxidation is associated with the formation of intermolecular disulphide bonds and the consequent production of polymers of the native tetrameric enzyme. The addition of the activating thiol reagent brings about depolymerization. However, the formation of polymers is not an obligatory consequence of oxidation, since some oxidized non-polymerized enzyme exists that is also activated by thiol reagents (A. K. Soutar & H. Hassall, unpublished work).

All species of oxidized enzyme, polymerized and non-polymerized, retain a low activity. Therefore the presence of the oxidizable thiol group in the reduced form, although leading to a seven- to eight-fold increase in activity under the assay conditions, is not an essential requirement for the enzymic conversion of histidine into urocanate. The part played by this SH group in the reaction mechanism is uncertain, but it has been suggested that it binds the imidazolyl group of the substrate via a metal such as manganese or cadmium (Givot *et al.*, 1970; Klee, 1972). However, the possibility remains that binding of substrate to this group may bring about a conformational change in the enzyme, thereby increasing the affinity of substrate binding at the active centre.

* Present address: Department of Biochemistry, Baylor College of Medicine, Houston, Tex. 77025, U.S.A.

From experiments involving the reduction of histidase with NaB^3H_4 (Wickner, 1969; Givot *et al.*, 1969) it has been suggested that the enzyme contains a dehydroalanine residue that takes part in the reaction mechanism, although it seems possible that this dehydroalanine residue could be formed from the desulphurization of cystine residues (Cecil & McPhee, 1959; Asquith & Carthew, 1972) present in the enzyme.

By using histidase from *P. testosteroni*, we have determined the amino acid sequence of a radioactive peptide isolated from a tryptic digest of thiol reagent-activated enzyme which had been carboxymethylated with ^{14}C-labelled iodoacetate. Since histidase in the polymerized form is not carboxymethylated, and only one radioactive peptide is obtained after carboxymethylation of the depolymerized enzyme, it follows that this peptide contains the thiol group involved in disulphide bond formation.

Klee & Gladner (1972) have isolated a similar peptide from *Pseudomonas* A.T.C.C. 11299b and have determined its amino acid composition.

Materials and Methods

Organism

The organism used was *Pseudomonas testosteroni* (N.C.I.B. 10808). Its characteristics have been described by Coote & Hassall (1973).

Chemicals

DEAE-cellulose (microgranular DE-32 grade) was supplied by Whatman Biochemicals Ltd., Maidstone, Kent, U.K. Trypsin (three times recrystallized) was

obtained from Worthington Biochemical Corp., Freehold, N.J., U.S.A., and thermolysin from Calbiochem, Los Angeles, Calif., U.S.A. Aminopeptidase M, a neutral aminopeptidase, was kindly supplied by Dr. S. G. George and Dr. A. J. Kenny of this Department and was prepared by them from rabbit kidney microsomal fractions (George & Kenny, 1973); 1 mg of enzyme hydrolysed 38 μmol of leucine 2-naphthylamide/min at 37°C. Iodo[2-^{14}C]-acetic acid was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. The remaining reagents were supplied by BDH Chemicals Ltd., Poole, Dorset, U.K. and were AnalaR grade where available.

Methods

Growth of the organism. The organism was grown at 30°C in medium containing (g/litre): L-histidine hydrochloride monohydrate, 3; sodium succinate, 3; KH_2PO_4, 5; $MgSO_4,7H_2O$, 0.2; the pH was adjusted to 7.2 with 5M-NaOH. Cells from a recently prepared nutrient-agar slope were inoculated as a routine into two 100ml samples of medium and grown on a rotary shaker for approx. 14h. These two cultures were used to inoculate two 8-litre volumes of medium, which were in turn incubated overnight (10h) with forced aeration. The 16 litres of cells in late-exponential or early-stationary phase were then added to 84 litres of medium in a 100-litre stainless-steel fermenter (Taylor Rustless Fittings Co. Ltd., Leeds, U.K.) fitted with a stirrer paddle, 30°C thermostat and air supply. The organism grew here without significant lag and with a mean doubling time of 90–100min. When the E_{580} of the culture had reached 1.4 the cells were harvested at a rate of 800ml/min by using two laboratory-type De Laval continuous-flow centrifuges. A further 100 litres of fresh medium were added to the fermenter at the same rate (800ml/min) and under these conditions the E_{580} of the culture was maintained between 1.2 and 1.4. This procedure yielded 550–600g wet wt. of cells, which were stored frozen (−18°C) until required.

Assay of histidase. Histidase was assayed by a modification of the method of Tabor & Mehler (1955). The reaction mixture contained 10 μmol of L-histidine, 10 μmol of GSH, 2.7ml of aminomethylpropanediol buffer (0.1M-aminomethylpropanediol adjusted to pH9.2 with 2M-HCl) and enzyme preparation in a total volume of 3ml. The reaction was started by the addition of L-histidine after preincubation of the enzyme in the presence of GSH for 15min. The formation of urocanate was followed at 277nm at 30°C in a Gilford 2000 multiple-sample absorbance recorder fitted with a Unicam SP. 500 monochromator.

Disruption of cells and purification of histidase. The procedure used for the purification of histidase was based on that of Tabor & Mehler (1955) and was similar to that used by other workers for the purification of the enzyme from other sources (Givot *et al.*, 1969; Klee, 1970; Hassall *et al.*, 1970).

The cells were thawed and resuspended in 5vol. of phosphate buffer (0.02M-KH_2PO_4 adjusted to pH7.2 with 5M-NaOH) at 4°C. The suspension was then passed three times through a Manton–Gaulin homogenizer (Manton–Gaulin Laboratory Homogenizer and Sub-micron Disperser, type 15M-8BA, obtained from A.P.V. Co. Ltd., Manor Royal, Crawley, Sussex, U.K.) used with a pressure difference of approx. 55MPa (8000lbf/in^2) at the outlet. Ribonuclease and deoxyribonuclease were then added (1–2mg/litre), and after 30min the suspension was centrifuged at 38000*g* and 4°C for 45min to remove cell debris and unbroken cells.

The crude extract obtained in this way (usually 2–3 litres) was heat-treated by pumping it at a rate of 20–25ml/min through a coiled vinyl tube (7mm external diam., 5mm internal diam., obtained from Portex Ltd., Hythe, Kent, U.K.) of capacity 320ml submerged in a water bath at 80°C. The suspension was cooled in ice as it emerged from the coil and then centrifuged at 38000*g* for 20min to remove the unwanted precipitate.

The supernatant solution obtained from the previous stage was fractionated at 4°C with solid $(NH_4)_2SO_4$ at 42.5% and 55% saturation respectively. The first precipitate (42.5%) was discarded, and the second (42.5–55%) was taken up in 0.01M-Tris–acetate buffer, pH7.5, and desalted by passage through a 1-litre column of Sephadex G-50 equilibrated and eluted with the same buffer. Fractions containing histidase were pooled and applied to a column (25cm×5cm) of DEAE-cellulose equilibrated with 0.01M-Tris–acetate buffer, pH7.5. The sample was washed into the column with 200ml of this buffer followed by 150ml of 0.1M-Tris–acetate buffer, pH7.5. Protein was then eluted with a linear gradient of 0–0.3M-NaCl in a total of 500ml of 0.1M-Tris–acetate buffer, pH7.5. Histidase was recovered from the column when the NaCl concentration was approx. 0.18M. Those fractions containing activity were pooled and diluted with an equal volume of ice-cold water. This solution was then applied to a second column (20cm×2cm) of DEAE-cellulose equilibrated with 0.05M-KH_2PO_4 buffer (pH7.0), washed into the column with 200ml of the same buffer, and then eluted with a gradient of 0.1–0.3M-NaCl in this buffer.

The enzyme was concentrated by diluting pooled fractions with an equal volume of ice-cold water and running the solution into a column (10cm×1cm) of DEAE-cellulose equilibrated with 0.01M-Tris–acetate buffer, pH8.0. The protein was then removed by frontal elution with 1M-Tris–acetate buffer, pH8.5.

This procedure normally yielded some 175–280mg of histidase (corresponding to 25–45% recovery of enzyme) from 2.5 litres of crude extract. The enzyme was in the oxidized polymerized form (Soutar & Hassall, 1969) as shown by activation with GSH and disc electrophoresis on polyacrylamide gel (Davis, 1964). Electrophoresis of a sample incubated with 10mM-GSH showed only one protein band on staining with Naphthalene Black.

Depolymerization and [^{14}C]carboxymethylation of native histidase. Polymerized enzyme (150mg) in 15.5ml of 1M-Tris–acetate buffer (pH8.5) was depolymerized by the addition of 0.31ml of 0.1M-dithiothreitol and incubation for 30min at 30°C. The exposed free SH groups were then carboxymethylated by adding 0.5ml of 0.2M-iodo[2-^{14}C]acetate (0.5μCi/μmol) and continuing the incubation of the reaction mixture at 30°C. After 60min the carboxymethylation reaction was stopped with 50μl of mercaptoethanol. The solution was then dialysed for a total of 12h against two changes of 2 litres of 0.01M-Tris–acetate buffer (pH8.5) and finally for 5h against 200ml of 1M-Tris–acetate (pH8.5). Both these buffer solutions contained 0.2% (v/v) thiodiglycol.

Denaturation and complete carboxymethylation of histidase. The dialysed solution of [^{14}C]carboxymethylhistidase (15.5ml) was made approx. 6M with respect to guanidine hydrochloride by the addition of 15.5g of the salt, bringing the volume to 27ml. After incubation at 30°C for 4h, 0.54ml of 0.1M-dithiothreitol was added and the solution incubated for a further 30min. Complete carboxymethylation of all thiol groups was then obtained by adding 1.35ml of 0.2M-iodoacetate (non-radioactive). The reaction was stopped after 60min with 0.1ml of mercaptoethanol and the enzyme solution dialysed for 3 days against numerous changes of 0.05M-NH_4HCO_3 (pH8.5) containing 0.2% thiodiglycol. During this time, the solution darkened and the protein precipitated. This suspension of denatured enzyme was finally dialysed overnight against 0.5M-NH_4HCO_3 (pH8.5) to which thiodiglycol had been added (0.2%).

Tryptic digestion of carboxymethylated histidase. The carboxymethylated enzyme was digested with trypsin that had been treated with 1-chloro-4-phenyl-3-L-tosylamidobutan-2-one (TPCK) as described by Carpenter (1967). Trypsin (4mg) was dissolved in 0.5ml of 1mM-HCl and the solution added to the suspension of denatured histidase (40ml) in two portions of 0.25ml each at 3h intervals. After a total incubation time of 6h the protein was completely solubilized. The solution was adjusted to pH5.5 with acetic acid and freeze-dried for 36h to remove acetic acid and ammonium acetate.

Purification of [^{14}C]carboxymethylated peptide. The total tryptic digest of carboxymethylated histidase was applied in a continuous line 10cm away from the short edge of a sheet (55cm×45cm) of Whatman 3MM chromatography paper. The paper was developed in a descending direction in butan-1-ol–acetic acid–water–pyridine (BAWP) (15:3:12:10, by vol.) (Waley & Watson, 1953) until a marker (Phenol Red solution; R_F approx. 0.85) had reached the edge of the paper (18–20h). The position of the radioactive peptide was determined by radioautography for 36h with Kodak Kodirex X-ray film. The radioactive area was then cut from the chromatogram and eluted by descending capillarity with water; the first 3ml of eluate was collected. The peptide was further purified by high-voltage paper electrophoresis in pyridine–acetic acid–water (11:1:89, by vol., pH3.5) (Katz *et al.*, 1959) for 100min at 45V/cm and finally by chromatography on Whatman no. 1 paper in butan-1-ol–acetic acid–water (4:1:5, by vol.). At each stage the radioactive area was located by radioautography and the peptide eluted from the paper with water. This method gave a 15–20% yield of peptide as calculated from a subunit molecular weight of 50000–55000 (Klee, 1970; A. K. Soutar & H. Hassall, unpublished work). In subsequent purifications of the peptide, the strip containing radioactive material from one stage was sewn on to the paper used in the next. In this way, quantitative transfer of the peptide from system to system was achieved and higher recoveries were obtained.

Detection of peptides and free amino acids on paper. As a check on purity and composition of the ^{14}C-labelled peptide, samples were chromatographed in the butan-1-ol–acetic acid–water solvent. Peptides and free amino acids were located by spraying with 0.2% ninhydrin in acetone and heating at 60°C. Histidine and tyrosine were detected by spraying with diazotized 4-chloroaniline (Hall, 1952), a procedure some 5–10 times more sensitive for free histidine than for free tyrosine. Tyrosine was specifically tested for by using the α-nitroso-β-naphthol method of Acher & Crocker (1952), and tryptophan by spraying with Ehrlich's reagent (Smith, 1953). Peptides containing arginine were detected by spraying the papers with a 1:1 (v/v) mixture of 0.2% (w/v) *o*-phenanthroline in ethanol and 10% (w/v) NaOH in 60% (v/v) ethanol.

Amino acid composition of the peptide. Samples of the ^{14}C-labelled peptide were hydrolysed at 110°C for 24h under N_2 in constant-boiling HCl. Amino acids were determined quantitatively by using an automatic analyser (Beckman Unichrom amino acid analyser) fitted with a high-sensitivity flow-cell. The [^{14}C]carboxymethylcysteine content was calculated from the radioactivity of the sample used, since recoveries of carboxymethylcysteine after hydrolysis were low.

Determination of amino acid sequences. The amino acid sequences of peptides were determined by the dansyl–Edman procedure of Gray & Hartley (1963). The peptide was degraded in a stepwise manner from

the *N*-terminal end by reaction with phenylisothiocyanate (Edman, 1950) and the new *N*-terminal residue was identified after its reaction with Dns-chloride (dansyl chloride). The method was essentially as described by Hartley (1970) except that the stated volumes of all reagents were halved and chromatography of Dns-amino acids was carried out on 3.3cm×3.3cm polyamide plates (Woods & Wang, 1967). At each stage of the degradation, two samples of Dns-peptide were hydrolysed in constant-boiling HCl for 3h and 9h respectively. The short hydrolysis time was necessary for the unequivocal detection of proline. Good recoveries of Dns-Cys-CH_2CO_2H were only obtained when the hydrolysis was carried out under N_2 and when 0.2% thiodiglycol was added to the HCl used.

Digestion of peptides with thermolysin. The peptide, usually 20–100nmol in 0.1ml of 2.5mM-$CaCl_2$, 1mM-NH_4HCO_3, pH7.6, was incubated with 1.5–3μl of thermolysin solution (1mg/ml in the $CaCl_2$–NH_4HCO_3 solution). After 2h a second addition of thermolysin (1.5–3μl) was made, and the reaction was finally terminated with 25μl of acetic acid after 4h. On some occasions incubation was prolonged overnight. Thermolysin peptides were purified by paper electrophoresis at pH3.5 in the pyridine–acetic acid–water system. The radioactive peptides were located by radioautography. Other peptides were detected by spraying narrow strips of the paper with the appropriate reagents. All the peptides were eluted with small volumes of water and subjected to sequence analysis.

Differentiation between aspartic acid and asparagine in peptides. Peptides which were shown to contain an aspartic acid or asparagine residue were digested to completion with peptidase M. The peptide (approx. 5nmol) in 30μl of water was incubated with 10μl of 0.1M-KH_2PO_4 (pH7.0) and 5μl of enzyme solution (5.2mg/ml) at 30°C. Samples (10μl) of the reaction mixture were taken at 0, 3 and 10h and freeze-dried. $NaHCO_3$ (5μl, 0.2M) was added and the mixture again freeze-dried to remove traces of NH_3. Each sample was then dansylated as described above and analysed by chromatography on polyamide sheets. Dns-Asp and Dns-Asn are readily identified by this method (but see below).

Measurement of radioactivity. Samples were assayed for ^{14}C in a multichannel Beckman liquid-scintillation system LS-200B with a scintillation fluid consisting of 5g of 2,5-diphenyloxazole and 100g of naphthalene made up to 1 litre with 1,4-dioxan. Scintillation fluid (5ml) was normally added to 0.1ml of aqueous sample. Observed radioactivities (c.p.m.) were corrected to absolute radioactivities (d.p.m.) by using a calibration curve constructed from a plot of internal channels ratio against efficiency. Counting efficiency for ^{14}C was between 90 and 95%.

During Edman degradation of the peptides, at each stage the butyl acetate extract of the phenylthiohydantoin was dried in a scintillation vial and assayed for radioactivity. The radioactivity of the shortened peptide was determined by adding 1μl of the peptide solution to the scintillation system described above. In every case the decrease in radioactivity of the peptide and appearance of radioactivity in the butyl extract corresponded to the removal of the identified Cys-CH_2CO_2H residue.

Results

Effect of reduction and carboxymethylation on the activity of histidase

The enzyme was assayed in the presence and absence of GSH at all stages of the carboxymethylation procedure (Table 1). The relatively low (4.75-fold) activation obtained on addition of GSH to the assay mixture containing native enzyme, compared with that obtained previously (Soutar & Hassall, 1969), showed that the enzyme was not fully oxidized. Treatment with 20mM-dithiothreitol reduced most of the disulphide bonds formed between the reactive SH groups, since there was little increase in activity when this preparation was assayed in the presence of GSH. The carboxymethylated enzyme had 11.1% of the specific activity of the fully reduced enzyme but also showed some activation on addition of GSH. This suggests that the carboxymethylation procedure did not go to completion even though it resulted in an enzyme preparation that had a lower specific activity than the non-activated (non-reduced) native enzyme.

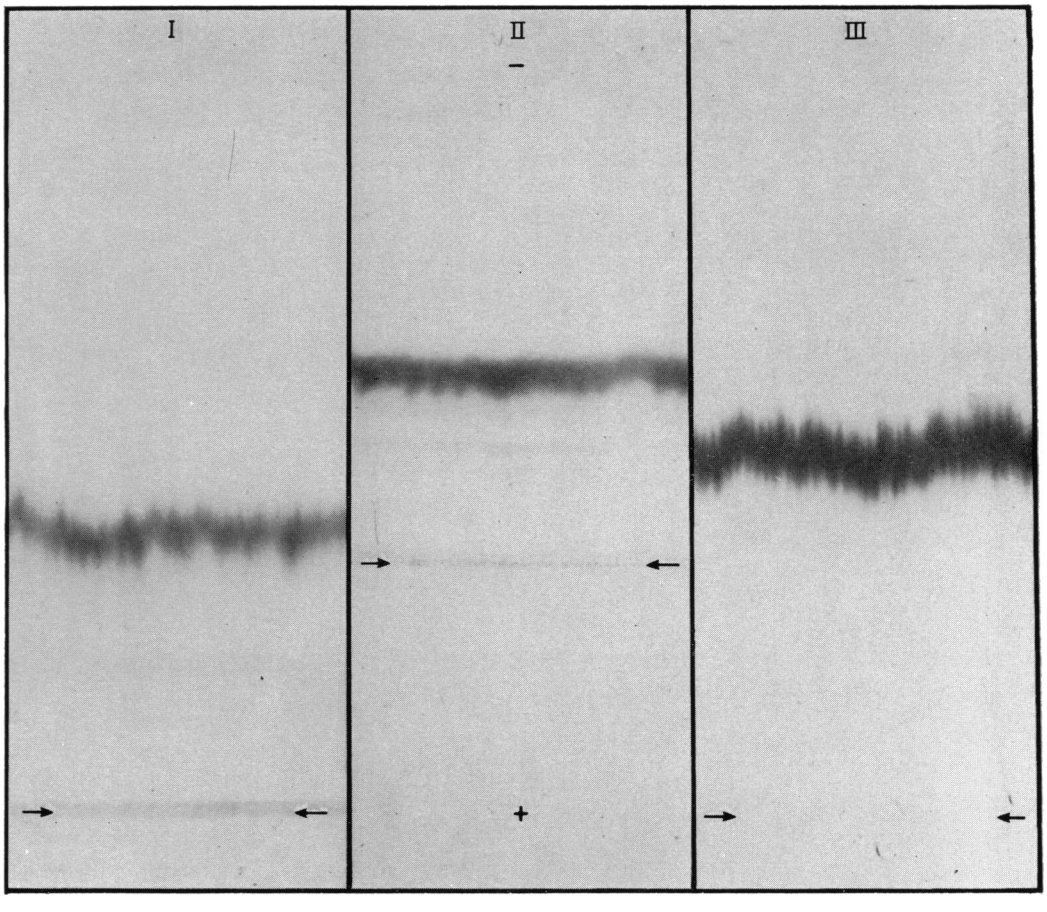
Isolation of the ^{14}C-labelled peptide from a tryptic digest of [^{14}C]carboxymethylated histidase

A ^{14}C-labelled peptide, designated peptide P_1, was isolated from a tryptic digest of 150mg (700–750nmol)

Table 1. *Specific activity of native, reduced and carboxymethylated histidase from P. testosteroni*

Purified histidase (10mg/ml in 1M-Tris–acetate buffer, pH8.5) was reduced with 2mM-dithiothreitol and carboxymethylated with 6mM-iodo[2-^{14}C]acetate. Samples of enzyme were taken at each stage of the procedure and assayed in the absence and presence of 3.3mM-GSH. Protein was measured spectrophotometrically at 280nm.

Sample	Specific activity (μmol·min^{-1}·mg of protein^{-1}) Without GSH	With GSH
Native enzyme	0.98	4.67
Enzyme reduced with dithiothreitol	4.04	4.36
Enzyme carboxymethylated with iodo[^{14}C]acetate	0.52	0.73



EXPLANATION OF PLATE 1

Purification of radioactive tryptic peptide (peptide P_1) from [^{14}C]carboxymethylated histidase

The peptide was purified by: I, paper chromatography of the tryptic digest in butan-1-ol–acetic acid–water–pyridine (15:3:12:10, by vol.); II, paper electrophoresis at pH 3.5 of the radioactive material eluted from chromatogram I; III, paper chromatography in butan-1-ol–acetic acid–water (4:1:5, by vol.) of the radioactive material eluted from electrophoretogram II. Representative areas (52cm × 20cm) of the radioautograms obtained at the three stages are shown. In each case the origin is marked with arrows.

of histidase by using paper chromatography and high-voltage paper electrophoresis as described in the Materials and Methods section. A composite photograph of the radioautograms obtained at different stages of the purification procedure is shown in Plate 1. Only one radioactive peptide was obtained, and the lack of a significant amount of radioactivity remaining at the origin in the first solvent system showed that there was essentially no incorporation of radioactivity into an insoluble tryptic core. The yield of ^{14}C-labelled peptide, as calculated from the initial specific radioactivity of the iodo[2-^{14}C]acetate (0.5μCi/μmol) was 440nmol. However, if, as suggested by the results in Table 1, the initial carboxymethylation step is incomplete, then subsequent total carboxymethylation of the denatured enzyme with non-radioactive iodoacetate would result in a carboxymethylated peptide having a specific radioactivity less than that of the iodo[^{14}C]acetate used. Thus the true yield would be slightly in excess of the apparent yield as calculated from the ^{14}C content.

Properties of the ^{14}C-labelled peptide (peptide P_1)

When strips from a chromatogram of the purified peptide were subjected to the various chemical methods for detection of specific amino acids, strong positive reactions were obtained for histidine and arginine. Negative results were obtained when the strips were sprayed for tryptophan and tyrosine. Amino acid analyses were carried out on three separate batches of the peptide. The results (Table 2) showed the presence of a minimum of 17 amino acid residues. The apparently high recoveries of all amino acids, relative to the calculated amount of [^{14}C]-carboxymethylcysteine, can again be explained in terms of the incomplete initial carboxymethylation of the enzyme with iodo[^{14}C]acetate. The amino acid sequence of the first nine residues from the *N*-terminal end was Gly-Leu-Leu-Asx-Gly-Ser-Ala-Ile-Asx. Treatment of the peptide with carboxypeptidase B gave arginine as the *C*-terminal amino acid residue.

Digestion of peptide P_1 with thermolysin

Thermolysin digestion of peptide P_1 for 4h, followed by high-voltage paper electrophoresis at pH3.5, gave the peptide pattern shown in Fig. 1(*a*). A prolonged digestion (overnight) resulted in the peptides shown in Fig. 1(*b*) and free arginine, the latter having a mobility twice that of the peptide designated T_2.

Properties of the thermolysin fragments obtained from peptide P_1

Peptide T_1. This peptide was non-radioactive and gave the sequence Leu-Asx-Gly-Ser-Ala. Chromatography of a dansylated aminopeptidase M hydrolysate showed the presence of the dansyl derivatives of Leu, Asp, Gly, Ser and Ala; no Dns-Asn was present. Although it is realised that -Asn-Gly- is readily converted into -Asp-Gly-, the absence of even

Table 2. *Amino acid composition of peptide P_1*

Samples from three separate preparations of the peptide were used. The carboxymethylcysteine content was calculated from the radioactivity of the material taken for analysis.

	Amount present relative to Arg				
	Sample				
Amino acid	1	2	3	Mean	Residues
Cys-CH_2CO_2H	0.73	0.85	0.76	0.78	1
Asp	3.20	2.72	2.68	2.87	3
Thr	—	—	—	—	0
Ser	2.15	2.32	1.90	2.12	2
Glu	0.32	0.39	0.29	0.33	0
Pro	1.69	1.72	1.88	1.76	2
Gly	3.02	3.13	2.93	3.03	3
Ala	1.18	1.23	1.27	1.23	1
Val	0.21	0.29	0.16	0.22	0
Met	—	—	—	—	0
Ile	0.97	1.05	0.97	1.00	1
Leu	1.91	2.11	1.73	1.92	2
Tyr	—	—	—	—	0
Phe	—	—	—	—	0
Lys	—	—	—	—	0
His	1.25	1.17	1.20	1.21	1
Arg	1.00	1.00	1.00	1.00	1

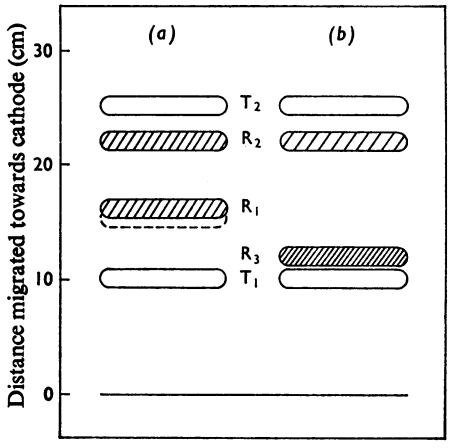


Fig. 1. *Thermolysin fragments of peptide P_1*

Two samples of peptide P_1 were digested (*a*) for 4h and (*b*) overnight respectively. The resulting peptides were separated by paper electrophoresis (45V/cm) at pH3.5 for 3.5h. Radioactive areas, located by radioautography, are shown hatched in proportion to their radioactivity, and the relative position of the parent peptide before digestion is shown by the broken line. All the peptides were ninhydrin positive.

trace amounts of asparagine after enzymic hydrolysis suggests that Asp is the true identity of the Asx residue in this peptide.

Peptide T_2. This was also non-radioactive, gave the sequence Gly-Leu, and showed the presence of no other amino acids when an acid hydrolysate was dansylated and analysed by chromatography on polyamide sheets.

Peptide R_1. This peptide was radioactive and gave Arg as the *C*-terminal amino acid when treated with carboxypeptidase B. The sequence of the first seven residues from the *N*-terminus was Leu-Asx-Gly-Ser-Ala-Ile-Asx.

Peptide R_2. This was radioactive and had the sequence Ile-Asx-Pro-Ser-His-Pro-Asx-Cys-CH_2CO_2H for the first eight residues. The *C*-terminal residue was shown to be Arg. Chromatography of a dansylated aminopeptidase M digest showed the presence of Dns-Asn but no Dns-Asp. Dns-Cys-CH_2CO_2H was obtained, making it reasonably certain that hydrolysis had been sufficiently extensive to liberate both Asx residues. The first five residues from the *N*-terminus were removed by the Edman procedure, but with butyl acetate extraction of the phenylthiohydantoins carried out after the removal of the third and fifth residues only. The new peptide, peptide R_2-5, was subjected to high-voltage paper

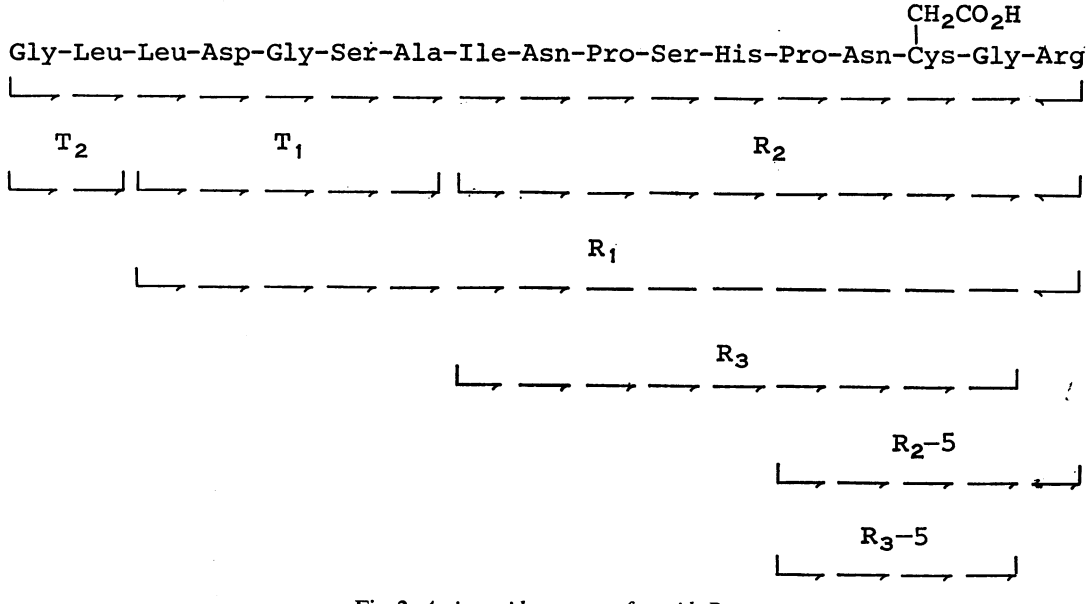


Fig. 2. *Amino acid sequence of peptide P_1*

The symbol → represents the identification of the amino acid as its dansyl derivative during Edman degradation of the peptide; ← represents an amino acid identified as its dansyl derivative after hydrolysis with carboxypeptidase B; —— represents an amino acid for which the position is inferred from the amino acid sequence of other peptides shown. Asp and Asn residues were identified in aminopeptidase M digests as described in the text.

electrophoresis for 2¼h at pH 3.5 and 45 V/cm. Radioautography showed it to have a mobility of 12.5cm towards the cathode. After elution from the paper it was found (by the dansyl–Edman method) to have the sequence:

$$\begin{array}{c} CH_2CO_2H \\ | \\ \text{Pro-Asx-Cys-Gly-Arg} \end{array}$$

Chromatography of a dansylated aminopeptidase M digest showed Dns-Asn and no Dns-Asp.

Peptide R_3. This peptide was obtained on prolonged thermolysin digestion of the parent peptide P_1. It was radioactive but did not stain for arginine. The sequence of the first eight residues was shown to be the same as the sequence of the same region of peptide R_2. Removal of the first five residues and purification of the residual peptide (R_3-5) by high-voltage paper electrophoresis alongside peptide R_2-5 gave a radioactive band with a mobility of 1.5cm towards the anode. On elution from the paper, peptide R_3-5 was found to have the sequence:

$$\begin{array}{c} CH_2CO_2H \\ | \\ \text{Pro-Asx-Cys-Gly} \end{array}$$

Complete sequence of peptide P_1

The complete amino acid sequence of the ^{14}C-labelled carboxymethyl tryptic peptide is given in Fig. 2, together with the sequences of the thermolysin fragments derived from it. The principal sites of thermolysin cleavage are seen to be on the *N*-terminal side of the Leu and Ile residues except that the cleavage did not take place adjacent to the *N*-terminus. The hydrolysis of the Leu-Leu bond by thermolysin is obviously far more rapid than that of the Ala-Ile bond. Prolonged digestion with thermolysin resulted in hydrolysis of the Gly-Arg bond.

Discussion

A radioactive peptide obtained from a tryptic digest of [^{14}C]carboxymethylated native histidase has been shown to have the sequence:

$$\begin{array}{c} CH_2CO_2H \\ | \\ \text{Gly-Leu-Leu-Asp-Gly-Ser-Ala-Ile-Asn-Pro-Ser-His-Pro-Asn-Cys-Gly-Arg} \end{array}$$

This peptide, obtained from histidase purified from *P. testosteroni*, is similar to one isolated from the enzyme from *Pseudomonas* A.T.C.C. 11299b (Klee & Gladner, 1972) in that both have 17 residues and differ in composition by only three amino acids. The one from *Pseudomonas* A.T.C.C. 11299b has one residue each of threonine, histidine and glutamic acid or glutamine, apparently in place of serine, proline and aspartic or asparagine. It is not known, however, at which points in the sequence the amino acid differences occur and they need not, of course, be limited to three.

Now that the amino acid sequence around the 'active' thiol group of histidase has been determined, it will be of interest to investigate further the role of this group in the mechanism of action of the enzyme. The fact that polymerized, oxidized and carboxymethylated forms of the enzyme still retain some activity shows that the presence of the thiol group in the reduced state is not essential for the fundamental reaction mechanism. However, the reduction of this group from disulphide to SH results in a seven-to-eight-fold potentiation of histidase activity. Klee (1972) has shown that the SH group has a high affinity for Mn^{2+} or Cd^{2+}, which are probably involved in the binding of the imidazolyl ring of the substrate.

The function of the postulated dehydroalanine residues in the histidase mechanism is somewhat obscure. Indeed, the ability of cystine residues to give dehydroalanine residues strongly suggests that the [^3H]alanine found in acid hydrolysates of histidase reduced with NaB^3H_4 may arise from the disulphide-linked cysteine residues of oxidized polymerized enzyme. The validity of this hypothesis could be tested by determining whether the site of incorporation of ^3H into alanine in histidase corresponds to the position of carboxymethylcysteine in the peptide.

Since only one radioactive peptide is obtained from a tryptic digest of [^{14}C]carboxymethylated depolymerized histidase, it follows that polymerization of the enzyme occurs by disulphide linkage of identical regions of the polypeptide chains in different tetramers.

We are grateful to the M.R.C. for a studentship for A. K. S. and to the S.R.C. for a research grant. We thank Miss J. L. Ryall-Wilson for assistance with the purification of the enzyme and Dr. K. Brew for carrying out the amino acid analyses.

References

Acher, R. & Crocker, C. (1952) *Biochim. Biophys. Acta* **9**, 704–705

Asquith, R. S. & Carthew, P. (1972) *Biochim. Biophys. Acta* **285**, 346–351
Carpenter, F. H. (1967) *Methods Enzymol.* **11**, 237
Cecil, R. & McPhee, J. R. (1959) *Advan. Protein Chem.* **14**, 255–389
Coote, J. G. & Hassall, H. (1973) *Biochem. J.* **132**, 423–433
Davis, B. J. (1964) *Ann. N. Y. Acad. Sci.* **121**, 404–427
Edman, P. (1950) *Acta Chem. Scand.* **4**, 277–282
George, S. G. & Kenny, A. J. (1973) *Biochem. J.* **134**, 43–57
Givot, I. L., Smith, T. A. & Abeles, R. H. (1969) *J. Biol. Chem.* **244**, 6341–6353
Givot, I. L., Mildvan, A. S. & Abeles, R. H. (1970) *Fed. Proc. Fed. Amer. Soc. Exp. Biol.* **29**, 1590
Gray, W. R. & Hartley, B. S. (1963) *Biochem. J.* **89**, 379–380
Hall, D. A. (1952) *Biochem. J.* **51**, 499–504
Hartley, B. S. (1970) *Biochem. J.* **119**, 805–822
Hassall, H., Lunn, P. & Ryall-Wilson, J. L. (1970) *Anal. Biochem.* **35**, 326–334
Katz, A. M., Dreyer, W. J. & Anfinsen, C. B. (1959) *J. Biol. Chem.* **234**, 2897–2900
Klee, C. B. (1970) *J. Biol. Chem.* **245**, 3143–3152
Klee, C. B. (1972) *J. Biol. Chem.* **247**, 1398–1406
Klee, C. B. & Gladner, J. A. (1972) *J. Biol. Chem.* **247**, 8051–8057
Peterkofsky, A. (1962) *J. Biol. Chem.* **237**, 787–795
Smith, I. (1953) *Nature (London)* **171**, 43–44
Smith, T. A., Cordelle, F. H. & Abeles, R. H. (1967) *Arch. Biochem. Biophys.* **120**, 724–725
Soutar, A. K. & Hassall, H. (1969) *Biochem. J.* **114**, 79P–80P
Tabor, H. & Mehler, A. H. (1955) *Methods Enzymol.* **2**, 228–233
Waley, S. G. & Watson, J. (1953) *Biochem. J.* **55**, 328–337
Wickner, R. B. (1969) *J. Biol. Chem.* **244**, 6550–6552
Woods, K. R. & Wang, K.-T. (1967) *Biochim. Biophys. Acta* **133**, 369–370