# Presence and Quantity of Dehydroalanine in Histidine Ammonia-Lyase from *Pseudomonas putida*†

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ABSTRACT: Dehydroalanine is present in the histidine ammonia-lyase (histidase) from *Pseudomonas putida* ATCC 12633 as shown by reaction of purified enzyme with $K^{14}CN$ or $NaB^3H_4$ and subsequent identification of [$^{14}$C]aspartate or [$^3$H]alanine, respectively, following acid hydrolysis of the labeled protein. When labeling with cyanide was conducted under denaturing conditions, 4 mol of [$^{14}$C]cyanide was incorporated per mol of enzyme ($M_r$ 220 000), equivalent to one dehydroalanine residue being modified per subunit in this protein composed of four essentially identical subunits. In native enzyme, inactivation of catalytic activity by cyanide was complete when 1 mol of [$^{14}$C]cyanide had reacted per mol of histidase, suggesting that modification of any one of the four dehydroalanine residues in the tetrameric enzyme was sufficient to prevent catalysis at all sites. Loss of activity on treatment with cyanide could be blocked by the addition of the competitive inhibitor cysteine or substrate if $Mn^{2+}$ was also present. Cross-linking of native enzyme with dimethyl suberimidate produced no species larger than tetramer, thereby eliminating the possibility that an aggregation phenomenon might explain why only one-fourth of the dehydroalanyl residues was modified by cyanide during inactivation. A labeled tryptic peptide was isolated from enzyme inactivated with [$^{14}$C]cyanide. Its composition was different from that of a tryptic peptide previously isolated from other histidases and shown to contain a highly reactive and catalytically important cysteine residue. Such a finding indicates the dehydroalanine group is distinct from the active site cysteine. Treatment of crude extracts with [$^{14}$C]cyanide and purification of the inactive enzyme yielded labeled protein that released [$^{14}$C]aspartate on acid hydrolysis. This established that dehydroalanyl residues exist normally in the native enzyme and probably arise from a posttranslational modification process.

Dehydroalanine has been implicated as being essential for the function of histidase (histidine ammonia-lyase, EC 4.3.1.3) and phenylalanine ammonia-lyase, EC 4.3.1.5, on the basis of evidence that modification of these enzymes by a variety of nucleophilic reagents led to inactivation (Hanson & Havir, 1973). In both cases, an appropriately modified dehydroalanine residue could be identified. For example, when *Pseudomonas* histidase (Wickner, 1969) or phenylalanine ammonia-lyase from potato tubers (Hanson & Havir, 1970) was inactivated with $NaB^3H_4$, [$^3$H]alanine was recovered after acid hydrolysis. Yeast phenylalanine ammonia-lyase treated with $Na^{14}CN$ gave [4-$^{14}$C]aspartate following acid hydrolysis, presumably as a result of the intermediate formation of $\beta$-cyanoalanine from cyanide addition to dehydroalanine (Hodgins, 1971). Also, nitro[$^{14}$C]methane inactivated histidase and produced a labeled enzyme that, when subjected to catalytic hydrogenation and acid hydrolysis, released 2,4-diamino[$^{14}$C]butyrate as would be expected if 2-amino-4-nitrobutyrate were the product of $CH_3NO_2$ addition to dehydroalanine (Givot et al., 1969). These data provide a reasonable argument for the existence and catalytic importance of dehydroalanine in this pair of ammonia-lyases, but little detailed information on the mode of attachment of dehydroalanine and its precise function in catalysis has been obtained thus far.

Several studies have pointed out the importance of sulfhydryl groups to activity, both in *Pseudomonas* sp. ATCC 11299 (Klee, 1970) and *Pseudomonas testosteroni* (Soutar & Hassall, 1969). In these reports, evidence was presented that a critical thiol group becomes involved in disulfide bond formation in polymerized (less active) histidase. Moreover, native enzyme labeled with iodo[$^{14}$C]acetate and digested with trypsin gave rise to a single peptide containing labeled (carboxymethyl)cysteine, which was believed to correspond to the active cysteine residue. Recognition of the importance of this cysteinyl residue, coupled with the possibility that a disulfide-bonded cysteine in oxidized, polymerized histidase could undergo $\beta$-elimination to produce dehydroalanine, led Hassal & Soutar (1974) to suggest this route for the origin of dehydroalanine in histidase and, by implication, raised doubts as to the catalytic significance of dehydroalanine.

There have been conflicting reports on the quantity of dehydroalanine present in *Pseudomonas* sp. ATCC 11299 histidase. Givot et al. (1969) found that 2 mol of labeled nitromethane was incorporated per 210 000 g of protein, whereas Wickner (1969) obtained incorporation of 1 mol of H/mol of histidase ($M_r$ 210 000) upon $NaBH_4$ reduction. While these findings would suggest either 1 or 2 mol of dehydroalanine/mol of enzyme, Klee (1970) has shown that histidase of the same organism contains four subunits, apparently identical with respect to size and amino acid content.

The organism used in many of these earlier studies on dehydroalanine in histidase, namely, *Pseudomonas* sp. ATCC 11299, has been variously referred to as *Pseudomonas fluorescens* (Peterkofsky, 1962) or *Pseudomonas putida* (Givot et al., 1969) but now is classified as *Pseudomonas acidovorans* (*American Type Culture Collection Catalog of Strains I*, 1982). In order to take advantage of knowledge regarding the genetic systems and chromosomal gene locations in better

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characterized *Pseudomonas* species (Dean & Morgan, 1983), we have used *P. putida* ATCC 12633 in an investigation aimed ultimately at understanding how dehydroalanine is formed in histidase and how it functions in catalysis.

A recent report by Rokosu (1983) has indicated that antiserum directed against histidase from *P. testosteroni* would cross-react well with histidase from other members of the acidovorans group but did not inhibit action of histidase from fluorescent pseudomonads, including *P. putida*. Thus, the revised classification status of *Pseudomonas* sp. ATCC 11299 as *P. acidovorans* raises some uncertainty with regard to the comparative characteristics of histidase from this organism and *P. putida*. This study addresses the question of the existence and quantity of dehydroalanine contained in the histidase of *P. putida*, relates loss of dehydroalanine residues with reduction in catalytic activity, and provides structural information needed for a more detailed analysis of the active site.

## Experimental Procedures

*Purification and Assay of Histidase.* Enzymatic activity was measured according to the procedure of Rechler & Tabor (1971) in which the conversion of histidine to urocanate is followed at 277 nm and 25 °C in a recording spectrophotometer. Histidine concentration (4 mM) in this assay is not saturating but the procedure was followed to facilitate comparison with older results. Protein determinations were performed according to the method of Bradford (1969) with ovalbumin as the standard.

Histidase was obtained from a strain of *P. putida* ATCC 12633 that was constructed in this laboratory to be constitutive for the production of histidase. Cells were grown with aeration at 30 °C in 100 L of medium that consisted of 1% NZ-Amine (type A) 0.5% yeast extract, 0.5% $K_2HPO_4$, and 0.02% $H_3PO_4$. During growth, the pH was maintained between pH 7.0 and 7.3 by addition of $H_3PO_4$. Cells were harvested after 6 h by centrifugation and stored at −70 °C.

Histidase was purified essentially by the method of Klee (1970) but with inclusion of the heat step recommended by Rechler & Tabor (1971). Purified enzyme was stored in liquid $N_2$.

*Inactivation and Labeling of Purified Histidase.* The time course of inactivation of histidase by $K^{14}CN$ was determined under the following conditions. A 2.7-mL reaction contained 19 mg of enzyme, 26 mM $K^{14}CN$ of sp act. 1.5 mCi/mmol, and 0.1 M diethanolamine–HCl, pH 9.5. At various times between 0 and 90 min, a 0.3-mL sample was withdrawn and the inactivation terminated by the addition of 0.1 mL of 1 M potassium phosphate, pH 7.0. The labeled product was separated from $K^{14}CN$ by passing the sample through a 0.9 × 40 cm column of Sephadex G-25 equilibrated with 50 mM potassium phosphate, pH 7.3. The eluted protein fractions were pooled and assayed for residual enzymatic activity, protein, and the amount of radioactivity incorporated.

In one experiment, histidase was inactivated by nitromethane prior to reaction with $K^{14}CN$. This treatment was performed under the conditions of 7.5 mg of protein, 40 mM nitromethane, and 0.1 M diethanolamine–HCl, pH 9.5, in a final volume of 0.25 mL. Incubation with nitromethane was conducted until the enzymatic activity was less than 1% of the original activity. The reaction mixture was dialyzed against three 250-mL changes of 50 mM potassium phosphate, pH 7.3. A portion of the nitromethane-treated protein was then incubated with $K^{14}CN$ in the usual fashion for a period of 180 min and separated from $K^{14}CN$ as described above. The amount of radioactivity incorporated into histidase was determined from the pooled protein fractions.

For labeling of fully denatured enzyme with [$^{14}$C]cyanide, histidase at a concentration of 30 mg/mL was denatured in the presence of 1% mercaptoethanol and 1% sodium dodecyl sulfate at 100 °C for 2 min prior to $K^{14}CN$ exposure. A 90-min incubation with $K^{14}CN$ and isolation of the modified protein were conducted as described above, except that 0.1% sodium dodecyl sulfate was incorporated in the gel filtration buffer.

Reduction of histidase with $NaB^3H_4$ was conducted in a closed vial at room temperature. The reaction consisted of 30 mg of protein in 1.0 mL of 50 mM potassium phosphate, pH 7.3. $NaB^3H_4$, 3.0 mCi dissolved in 0.25 mL of 1 mM NaOH, was added to the protein over a 15-min period. After an additional 30 min, the reaction was terminated by the addition of 20 μL of acetone. the reduced protein was separated from the other products by fractionation over a 1.6 × 60 cm Sephadex G-25 column with 1% $NH_4HCO_3$ as the eluting solution. The protein fractions were combined and lyophilized. The specific activity of the $NaB^3H_4$ was 62 mCi/milliatom of H as determined by the method of George & Phillips (1970).

*Purification of Labeled Protein from Crude Extracts. P. putida* cells, 4 g, were suspended in 8 mL of 0.1 M diethanolamine–HCl, pH 9.5. The cells were sonically disrupted at 4 °C, and the extract was centrifuged at 43000*g* for 20 min. $K^{14}CN$, 5.2 mg, 0.27 mCi, was added to the supernatant, and the resulting solution was incubated at room temperature until less than 1% of the original enzymatic activity remained. The protein was separated from $K^{14}CN$ by gel filtration on a 2.5 × 46 cm Sephadex G-25 column equilibrated with 50 mM potassium phosphate, pH 7.3, the appropriate fractions were pooled, and solid ammonium sulfate was added at 0 °C to a final concentration of 60%. The solution was centrifuged as before and the pellet dissolved in 4 mL of 25 mM potassium phosphate, pH 7.3. The solution was heated with stirring at 80 °C for 10 min and centrifuged, and the supernatant was dialyzed against two 4-L changes of 25 mM potassium phosphate, pH 7.3. The dialyzed extract was then passed over a 0.7 × 5.5 cm column of anti-histidase–Sepharose. The column was washed with 8 mL of 25 mM potassium phosphate, pH 7.3, and the labeled histidase eluted with 6 mL of this buffer containing 6 M urea. The protein fractions were pooled and dialyzed against two 4-L changes of 1% $NH_4HCO_3$. A total of 2.7 mg of protein was isolated, and the preparation was judged to be greater than 90% pure by sodium dodecyl sulfate gel electrophoresis. The protein was hydrolyzed with HCl (as described in a later section) in order to establish the identity of the labeled component.

For the preparation of anti-histidase–Sepharose, rabbit serum from animals injected with purified histidase in Freund's adjuvant was enriched for IgG by $(NH_4)_2SO_4$ fractionation and DEAE-cellulose chromatography according to the method of Garvey et al. (1977). This partially purified fraction was then coupled to cyanogen bromide activated Sepharose 4B as recommended by Pharmacia.

*Identification of Labeled Products.* The labeled compound from either the $^3$H- or $^{14}$C-modified protein was identified after hydrolysis of the protein and separation of the released amino acids by two-dimensional thin-layer chromatography. Prior to hydrolysis, the labeled protein was dialyzed against 1% $NH_4HCO_3$ and lyophilized. A 0.5-mg sample of protein was hydrolyzed under vaccum for 24 h at 110 °C in 400 μL of 6 N HCl containing 0.1 mg of phenol. After HCl was removed, the residue was dissolved in 500 μL of double-distilled water,

and the solution was lyophilized. The residue was then dissolved in 10–20 μL of a standard amino acid solution (15 mM each amino acid), and 5 μL of the resulting solution was spotted on a cellulose thin-layer plate. The chromatogram was developed by the method of Jones & Heathcote (1966) with two slight modifications. First, the proportions of 2-propanol–formic acid–water in solvent system I were changed to 44:4:10. Second, in order to resolve cleanly aspartic and glutamic acids, the chromatogram was developed twice in the first dimension. The separated amino acids were visualized with ninhydrin as described by Jones & Heathcote (1966). The ninhydrin-positive spots were cut out of the chromatogram and soaked in 1.0 mL of water overnight, after which each sample was counted for radioactivity. The overall recovery was approximately 80% as judged by chromatographing and counting a known amount of [U-$^{14}$C]threonine.

*Incorporation of [$^{14}$C]Serine into Histidase.* A serine auxotroph of *P. putida* was obtained from B. J. Andersen of this laboratory and grown on a minimal medium containing histidine as sole carbon source (Phillips & Mulfinger, 1981), supplemented with 100 μg of L-serine/mL (250 μCi in a total volume of 1 L). Histidase was purified from 4 g of cells to approximately 90% purity. The enzyme was then treated with unlabeled KCN and subsequently acid hydrolyzed to release free amino acids. A portion of the hydrolyzate was analyzed for its content of labeled aspartic acid by thin-layer chromatography as described above.

*Isoelectric Focusing of Histidase.* Isoelectric focusing was performed by the method of O'Farrell (1975) with ultrapure urea deionized as described by Marglin & Merrifield (1967). To determine the isoelectric point of a band, nonfixed gels were sliced into 2-mm portions and soaked in 1 mL of water prior to measurement of the pH.

*Cross-Linking of Histidase.* Histidase was cross-linked with dimethyl suberimidate for 180 min at room temperature in 0.1 M triethanolamine–HCl, pH 8.5. The total reaction volume was 100 μL at a final protein concentration of either 2 or 6 mg/mL and a final dimethyl suberimidate concentration of either 0.75 or 1.5 mg/mL. The reaction was stopped by the addition of 20 μL of 0.5 M tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 6.8. The protein was analyzed on 1.5 mm sodium dodecyl sulfate–polyacrylamide gels that contained 5% acrylamide with 2.7% cross-linking (Laemmli, 1970). Gels were stained with 0.2% Coomassie blue R-250.

*Amino Acid Analysis of Histidase.* Histidase was dialyzed against 0.5% $NH_4HCO_3$ in double-distilled water and lyophilized prior to hydrolysis. Protein samples of 1 mg each were hydrolyzed under vacuum in 0.2 mL of 6 N HCl plus 10 μL of 1% phenol for 22, 48, or 72 h at 110 °C. After hydrolysis, the HCl was evaporated and the residue dissolved in 1 mL of 0.2 M sodium citrate, pH 2.2. Total nitrogen content was measured on each sample (Jaenicke, 1974). Duplicate samples, each containing 10–15 μg of protein, were analyzed on a Glenco amino acid analyzer operated with a three-buffer program (PICO II system, Pierce Chemical Co.). The amino acids were detected with *o*-phthalaldehyde. For proline determinations, detection was changed to ninhydrin. Only analyses with at least 95% recovery of total nitrogen were used in calculating the amino acid composition for histidase. To establish the number of half-cystine residues, histidase was reduced and carboxymethylated with iodoacetic acid (Hirs, 1967), or oxidized by performic acid (Glazer et al., 1976), and then analyzed for either (carboxymethyl)cysteine or cysteic acid. The modified protein was hydrolyzed in HCl as described above. In addition to these chemical modifications, the total cysteine content of borohydride-reduced histidase was also quantitated by titration with dithionitrobenzoic acid as described by Cavallini et al. (1966). Tryptophan was determined by amino acid analysis following hydrolysis of the protein in 3 N *p*-toluenesulfonic acid, as described by Liu & Chang (1971).

*Tryptic Peptide Isolation.* A sample of histidase was inactivated by treatment with [$^{14}$C]cyanide and the extent of incorporation determined as described above. A 70-mg sample of this material containing 1.6 mol of cyanide/mol of tetramer was carboxymethylated (Hirs, 1967) and dialyzed overnight against two 4-L volumes of 1% $NH_4HCO_3$. The carboxymethylated, labeled protein was then digested with trypsin (3 mg, Sigma No. T8642) for 18 h at 37 °C. The cleaved product was subjected to gel filtration on Sephadex G-50 (1 × 200 cm column) equilibrated with 50 mM $NH_4HCO_3$ and the major labeled product isolated. This material, containing $6.3 \times 10^5$ cpm, was then purified on a Waters HPLC unit with a Supelco C-18 column and a gradient consisting of 0.04% heptafluorobutyric acid and acetonitrile, followed by rechromatography with a 20 mM potassium phosphate, pH 7.0, and acetonitrile gradient.

*Molecular Weight Determinations.* Native enzyme in 50 mM potassium phosphate, pH 7.3, with or without 0.2 mM mercaptoethanol, was subjected to analytical ultracentrifugation at 10 °C and 7700 rpm. Sedimentation equilibrium was attained after 48 h, following which data were collected with a photoelectric scanner operated at 280 nm. Determination of partial specific volume by the $H_2O$–$D_2O$ ultracentrifugation method of Edelstein & Schachman (1967) was accomplished in a similar fashion except enzyme was dialyzed against 50 mM potassium phosphate, pD 7.5, prepared in 99% $D_2O$, in addition to samples prepared as described above. All densities were determined in a 1-mL pycnometer.

Molecular weights of protein subunits were determined by sodium dodecyl sulfate gel electrophoresis according to the procedure of Weber & Osborn (1969). Standard proteins examined along with histidase included bovine serum albumin ($M_r$ 68 000), catalase (58 000), glutamate dehydrogenase (53 000), ovalbumin (43 000), fructosebisphosphate aldolase (40 000), glyceraldehyde-3-phosphate dehydrogenase (36 000), α-chymotrypsinogen (26 000), and myoglobin (17 000).

*Chemicals.* Radioisotopes were purchased from ICN, while deuterated water and electrophoresis reagents were obtained from Bio-Rad. Most biochemical products were supplied by Sigma Chemical Co. Precoated cellulose thin-layer plastic sheets were from Merck. NZ-Amine, type A, was a product of Humko-Sheffield Co.

## RESULTS

*Properties of Histidase Purified from P. putida.* Histidase was purified 100-fold from 200 g of *P. putida* ATCC 12633 cells in a 50% yield. The product was at least 95% pure on the basis of the results of polyacrylamide gel electrophoresis at pH 8.3 (Davis, 1965) and by sodium dodecyl sulfate gel electrophoresis (Laemmli, 1970) when 15 μg of protein was applied and the visual limit of detecting a band with Coomassie blue R-250 was 0.2 μg. The enzyme had a specific activity of 11 ± 0.5 μmol of urocanate formed $min^{-1}$ $mg^{-1}$, a value identical with that reported for this enzyme from other pseudomonad sources (Klee, 1970; Rechler, 1969; Hassall et al., 1970) when assayed under the similar conditions of 4 mM histidine and 25 °C. The $K_m$ for histidine was 5.3 mM at pH 9.0. At saturating histidine concentration, the pure enzyme had a specific activity of 25 μmol $min^{-1}$ $mg^{-1}$.

Table I: Characteristics of the Inhibition of Histidase by KCN

| addition[a] | % of control[b] |
|---|---|
| none | (100) |
| KCN | 6 |
| KCN + $MnCl_2$ | 10 |
| KCN + histidine | 6 |
| KCN + $MnCl_2$ + histidine | 101 |
| KCN + cysteine | 4 |
| KCN + $MnCl_2$ + cysteine | 20 |
| KCN + $MnCl_2$ + glycine | 5 |

[a] Reactions were conducted in 0.1 M diethanolamine–HCl, pH 9.5, with the concentration of additions being 100 mM for L-histidine, cysteine, or glycine, 40 mM for KCN, and 2.5 mM for $MnCl_2$. Total reaction volume was 150 μL. After 20 min, reaction mixtures were diluted 100-fold into diethanolamine–HCl, pH 9.0, and then assayed by the usual procedure for histidase activity. [b] Control activity was 1.05 units/150 μL, equivalent to a histidase concentration of 0.67 mg/mL.

Cysteine and glycine acted as competitive inhibitors with $K_i$ values of 1.6 and 4.8 mM, respectively. Imidazole was a noncompetitive inhibitor with a $K_i$ of 120 mM, in contrast to linear uncompetitive behavior and a $K_i$ of 26 mM reported by Givot et al. (1969).

*Estimation of Native Enzyme and Subunit Molecular Weights.* Klee (1970) observed that histidase purified from *Pseudomonas* sp. ATCC 11299 is a mixture of active polymeric species joined by disulfide bridges. The monomeric form has a $M_r$ of 210 000 and can be dissociated into subunits of $M_r$ 53 000 (Klee, 1970). Sedimentation studies of the *P. putida* histidase gave a $M_r$ of 219 000 for native enzyme with a standard deviation of 9000 for six samples. We observed no indication of polymeric forms either in four samples analyzed without reductant or in two samples containing 0.2 mM mercaptoethanol. Partial specific volume was determined to be 0.742 mL $g^{-1}$ from centrifugation data in $H_2O$ and $D_2O$ solutions, while calculation from amino acid analysis results (presented below) by the method of Cohn & Edsall (1943) gave a value of 0.740 mL $g^{-1}$.

Molecular weight of histidase subunits was 56 000 by sodium dodecyl sulfate gel electrophoresis in the presence of reducing agents. This indicated the native enzyme is a tetramer composed of identical size subunits, and results that follow on the stoichiometry of active sites support this conclusion.

*Irreversible Inactivation of Histidase.* When *P. putida* histidase was incubated with either 4 mM nitromethane, 40 mM sodium bisulfite, or 20 mM sodium borohydride for a reaction period of 20 min, the enzymatic activity decreased to 9%, 31%, or 16%, respectively, of the original activity. Histidase was almost completely inactivated after a 20-min incubation with 40 mM KCN (Table I). This inactivation by KCN could be prevented by the addition of histidine and partially by the competitive inhibitor cysteine (but not glycine) if $Mn^{2+}$ was also present. These experiments indicate that the action of cyanide on histidase from *P. putida* is very likely directed toward the enzyme's active site because inactivation was markedly reduced in the presence of substrate or a compound (cysteine) competitive with substrate. The necessity for a divalent cation in order to have effective protection is in accord with the conclusion of Hanson & Havir (1973) that a metal ion ($Mn^{2+}$, $Fe^{2+}$, or $Cd^{2+}$) is involved in coordination with the substrate and the enzyme.

Loss of activity upon reaction with potassium cyanide was found to be directly related to the concentration of reagent and time of reaction. Figure 1 illustrates the pseudo-first-order reaction kinetics for activity loss at various levels of cyanide. Figure 2 shows that inactivation by cyanide could be halted immediately upon dropping the pH from 9.5 to 7.0. These

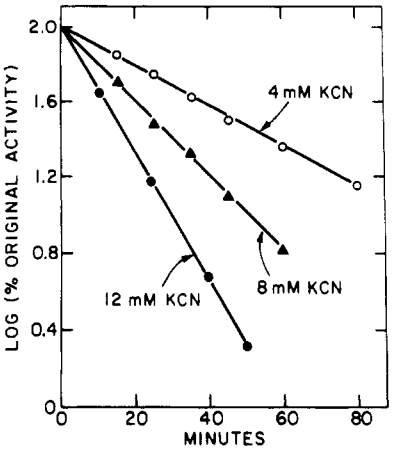


FIGURE 1: Kinetic characteristics of cyanide inhibition. The reactions were conducted in 0.1 M diethanolamine–HCl, pH 9.5, in the presence of the indicated concentration of KCN; temperature of incubation was 25 °C in a volume of 150 μL. Samples were taken at the indicated times, diluted at least 100-fold, and assayed by the standard assay method.

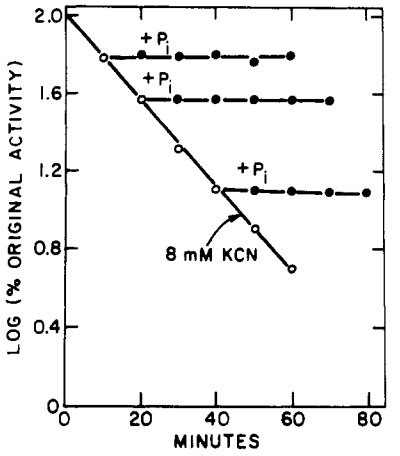


FIGURE 2: Termination of cyanide inactivation by pH shift. Incubations were identical with those conducted in Figure 1, except that a single KCN concentration was employed (8 mM) for all reactions. At the indicated times, 50 μL of 1 M potassium phosphate, pH 7.0, was added to drop the pH from 9.5 to between 7.0 and 7.2. Portions of the reaction mixture were taken for dilution and determination of remaining histidase activity throughout the incubation period.

data are fully consistent with the idea that an active site group becomes modified through nucleophilic attack by cyanide ion and this leads to loss of catalytic activity.

*Quantification of Cyanide-Sensitive Sites.* Histidase was treated with $K^{14}CN$ in order to determine the extent of incorporation under various conditions. When native histidase was incubated with KCN for periods extending to 90 min, a total of 1 mol of cyanide was incorporated per mol of tetrameric enzyme (Figure 3). Modification by cyanide resulted in the loss of enzymatic activity strictly proportional to the extent of labeling, with total inactivation being achieved at an average of 1 mole of cyanide bound per tetramer.

Histidase was denatured in the presence of sodium dodecyl sulfate plus mercaptoethanol and then treated with $K^{14}CN$. In this case, a stoichiometry of four sites labeled per tetramer was observed, and this value did not increase with reaction time beyond 20 min. We interpreted these combined results as meaning that there are four cyanide-reactive sites in histidase but only one of these can be readily modified under nondenaturing conditions. When this more reactive site was attacked by cyanide, the enzyme was completely inactivated.

Additional support for this conclusion came from observa-

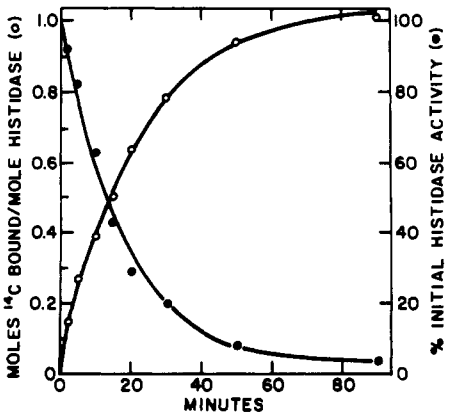


FIGURE 3: Correlation of activity loss with the amount of [$^{14}$C]cyanide incorporated into native histidase: (O) moles of [$^{14}$]cyanide bound per mole of tetramer; (●) % original histidase activity. The reaction mixture (2.7 mL) contained 19 mg of pure histidase (0.09 μmol of tetramer) and 70 μmol of K$^{14}$CN (1.5 mCi/mmol) in 0.1 M diethanolamine–HCl, pH 9.5, to give a cyanide to histidase tetramer ratio of 780:1. Samples were taken at the indicated times for activity measurement and $^{14}$C incorporation as described in the text.

tions that under the incubation conditions (pH 9.5, 25 °C) used in the cyanide experiments, the native enzyme was stable for approximately 90 min in the absence of cyanide, after which a slow, pH-dependent loss of enzymatic activity occurred with a half-life of roughly 70 min. If the inactivation was due to an unfolding of the protein, this raised the possibility that some additional cyanide-sensitive sites in the enzyme might be exposed as a function of time. Incubation of histidase with cyanide for a total period of 180 min revealed that 1.8–2.0 mol of [$^{14}$C]cyanide was incorporated per mol of protein. Thus extending the incubation period did unmask some additional cyanide-reactive sites. In a control experiment, histidase was completely inactivated with nitromethane and then incubated for 180 min with cyanide. Under these conditions, only 0.2 mol of cyanide was bound, compared to the usual 1.8 mol/mol of tetrameric protein found for extended incubations. This result illustrates that cyanide bound to the same functional sites as were modified by nitromethane and further suggests a single type of cyanide-reactive site whose modification depended upon its degree of exposure.

*Identification of Labeled Product.* The product of the extended (180-min) K$^{14}$CN treatment of histidase was identified in order to confirm that dehydroalanine was the material being modified. The labeled protein was hydrolyzed in 6 N HCl and dried under $N_2$. Over 80% of the radioactivity was recovered. The hydrolyzed products were separated by two-dimensional thin-layer chromatography and the predominant $^{14}$C material was found to comigrate with aspartic acid (Table II). This indicates that cyanide reacted with a dehydroalanine unit to form β-cyanoalanine, which upon hydrolysis yielded [$^{14}$C]aspartic acid. To confirm this result, the enzyme was reacted with NaB$^3$H$_4$. If a dehydroalanine really existed, then the reduced protein should yield [$^3$H]alanine upon hydrolysis. As shown in Table II, the radioactivity from borohydride-reduced enzyme did largely comigrate with alanine.

*Amino Acid Composition of Histidase and an Active Site Tryptic Peptide.* The amino acid composition for histidase is presented in Table III. The results are based on averages obtained from duplicate hydrolyses of unmodified histidase, all conducted in HCl for 22, 48, and 72 h plus duplicate hydrolyses of histidase in *p*-toluenesulfonic acid for 22, 48, and 72 h. The error represents one standard deviation. The values for threonine and serine were obtained by extrapolation to zero time while the values for valine, leucine, and isoleucine were obtained from the averages of the 72-h time points. Shown also in this table are comparison values for histidase isolated from another strain of *P. putida* (Rokosu, 1979). Since the values of Rokosu were based on a different molecular weight, we have presented the latter results recalculated to a $M_r$ of 220 000. While the two data sets are overall fairly similar, there are a few major differences, the largest of which involve half-cystine, serine, and methionine. Because these three are among the most difficult amino acids to quantify accurately, we believe analytical differences are as likely an explanation as might be strain variations.

[$^{14}$C]Cyanide-treated histidase was carboxymethylated prior to digestion with trypsin and the major radioactive peptide

Table II: Identification of Labeled Products after Hydrolysis of Modified Histidase

| amino acid | radioactivity (cpm) in sample[a]: purified histidase[b] + K$^{14}$CN | purified histidase[c] + NaB$^3$H$_4$ | crude histidase[d] + K$^{14}$CN |
|---|---|---|---|
| origin | 130 | 390 | 20 |
| aspartate | 1290 | 150 | 920 |
| glutamate | 170 | 0 | 50 |
| arginine | 0 | 200 | 0 |
| lysine | 0 | 30 | 0 |
| glycine | 30 | 70 | 10 |
| histidine | 120 | 0 | 10 |
| alanine | 110 | 2250 | 0 |
| proline | 0 | 100 | 0 |
| tyrosine | 0 | 170 | 10 |
| threonine | 20 | 0 | 20 |
| phenylalanine | 80 | 0 | 10 |
| serine | 90 | 50 | 0 |
| valine, methionine, leucine, isoleucine | 0 | 0 | 0 |

[a] Results were corrected for background radioactivity (50 cpm). [b] Extent of modification was 1.8 mol of cyanide bound/mol of protein, with 2300 cpm applied to the chromatography plate. [c] Total cpm applied to plate was 3900, and incorporation of $^3$H into histidase was 0.9 mol of H/mol of enzyme (tetramer). [d] Total cpm applied to plate was 1000.

Table III: Amino Acid Composition of Histidase

| amino acid | mol/mol of protein: this study[a] | data of Rokosu[b] |
|---|---|---|
| aspartic acid (+Asn) | 176 ± 9 | 202 (182) |
| half-cystine[c] | 16 ± 1 | 31 (28) |
| threonine | 82 ± 3 | 70 (63) |
| serine | 131 ± 4 | 84 (76) |
| glutamic acid (+Gln) | 214 ± 10 | 219 (198) |
| glycine | 169 ± 7 | 182 (164) |
| alanine | 342 ± 12 | 303 (273) |
| valine | 143 ± 4 | 161 (145) |
| methionine[d] | 35 ± 1 | 24 (22) |
| isoleucine | 99 ± 5 | 103 (93) |
| leucine | 304 ± 8 | 278 (251) |
| tyrosine | 31 ± 2 | 30 (27) |
| phenylalanine | 49 ± 2 | 59 (53) |
| lysine | 74 ± 3 | 84 (76) |
| histidine | 55 ± 2 | 54 (49) |
| arginine | 124 ± 5 | 140 (126) |
| tryptophan[e] | 7 ± 1 | ND[f] |
| proline | 76 ± 3 | 78 (71) |

[a] Average ± SD and based on a $M_r$ of 220 000. [b] Data from Rokosu (1979). Values shown in parentheses are original results based on a $M_r$ of 200 000 while each accompanying value has been recalculated to a $M_r$ of 220 000 for comparison to the results of the present study. [c] Average of results obtained from analysis as (carboxymethyl)cysteine, as cysteic acid, or by titration with dithionitrobenzoic acid. [d] Average of results obtained from analysis as methionine or as methionine sulfone. [e] Determined from hydrolyses with *p*-toluenesulfonic acid. [f] ND, not determined.

isolated by a combination of gel filtration and HPLC steps. Amino acid analysis of this peptide gave the following result: $Ala_6Asp_1Glx_4Gly_2Leu_5Lys_1Pro_1Ser_1Thr_2Trp_1Val_1$.

It is assumed that the single aspartic acid residue arose from hydrolysis of $\beta$-cyanoalanine, which itself was formed by the reaction between cyanide and dehydroalanine. From the composition obtained, it can be seen that the peptide contained one of the two tryptophan residues present in the histidase monomer and that there was no (carboxymethyl)cysteine present. This is important because Hassal & Soutar (1974) reported the sequence of a 17-residue tryptic peptide containing the active cysteine residue of histidase from *P. testosteroni* and earlier Klee & Gladner (1972) described an almost identical peptide from the *Pseudomonas sp.* ATCC 11299 histidase. These peptides had the following composition: for *P. testosteroni*, $Ala_1Asx_3Arg_1CM\text{-}Cys_1Gly_3His_1Ile_1Leu_2Pro_2Ser_2$; for *Pseudomonas sp.* ATCC 11299, $Ala_1Asp_2Arg_1CM\text{-}Cys_1Gly_2His_2Ile_1Leu_2Pro_1Ser_2Glu_1Thr_1$. We conclude the cyanide-labeled peptide is completely different from the cysteine-containing peptides studied by these other groups.

*Labeling of Histidase in Crude Extracts.* Crude extracts containing histidase were treated with $K^{14}CN$ to demonstrate that the cyanide-sensitive sites on histidase were present before purification rather than being due to an artifact that might be introduced during purification. This inactive labeled protein was then purified by a combination of conventional steps plus use of an antibody affinity column and subjected to hydrolysis in 6 N HCl. The products of hydrolysis were chromatographed as above. The results, shown in Table II, indicate that the radioactivity migrated with aspartic acid. These data therefore support the position that the product of the modified protein originated from a dehydroalanine and that this dehydroalanine existed in the protein prior to purification.

*Test for Serine as a Precursor of Dehydroalanine.* Enzyme that had been labeled with [$^{14}$C]serine during biosynthesis was examined for whether or not dehydroalanine also became labeled due to serine serving as its precursor. The purified protein was treated with KCN to inactivate the enzyme and then hydrolyzed to convert the modified dehydroalanine residues to aspartate. Upon examination of the hydrolysate for the presence of labeled aspartate, no appreciable radioactivity above that found in other unrelated amino acids was noted in the aspartate fraction, and only serine plus glycine were labeled to a large degree, although significant label was present in the regions corresponding to tyrosine and phenylalanine and in the origin area where cystine migrated. On the basis of this negative finding, we conclude for the present that it is unlikely dehydroalanine arises by a direct dehydration of serine.

*Cross-Linking of Histidase.* Cross-linking of histidase with dimethyl suberimidate was performed in order to explore the possibility that the enzyme existed as a large, multimolecular aggregate and this polymeric structure rendered some of the potentially reactive sites inaccessible to cyanide. It has previously been shown that histidases from *P. testosteroni* (Soutar & Hassall, 1969) and from *Pseudomonas sp.* ATCC 11299 (Klee, 1970) undergo reversible polymerization to produce a range of species much larger than the native tetrameric enzyme, although our sedimentation equilibrium analyses did not indicate such multimolecular aggregates for the *P. putida* enzyme. In order to distinguish between these possibilities of aggregates vs. normal tetramer under conditions where labeling had been conducted, histidase was cross-linked with dimethyl suberimidate in the presence or absence of KCN. Upon denaturation of the protein and separation of the cross-linked species, only tetramer or smaller species ($M_r$ of 220 000, 165 000, 110 000, and 55 000) were detected on sodium dodecyl sulfate–polyacrylamide gel electrophoresis. No higher molecular forms were produced upon increasing the dimethyl suberimidate or protein concentration or time of incubation. It should be noted that the protein concentrations used in these experiments were within the range in which Klee (1970) observed polymerization of histidase. This suggests that aggregation of histidase from *P. putida* does not readily occur and that the enzyme exists principally in a form no larger than a tetramer.

Table IV: Isoelectric Focusing of [$^{14}$C]Cyanide-Modified Histidase[a]

| mol of $^{14}$C bound/mol of protein | cpm contained in species with | |
|---|---|---|
| | p$I$ = 5.75 | p$I$ = 5.70 |
| 1.0 | 1160 | 960 |
| 1.8 | 1790 | 1720 |
| 3.9 | 3900 | 3860 |

[a] Histidase was labeled to varying extents with $K^{14}CN$ as described under Experimental Procedures. The sample labeled to 3.9 mol of [$^{14}$C]cyanide/mol of tetrameric protein was denatured in 8 M urea prior to labeling rather than in sodium dodecyl sulfate. In all cases, a 10-$\mu$g portion of sample was subjected to denaturing isoelectric focusing gels. After the gels were stained, bands were sliced out, soaked in 1.0 mL of water overnight, and counted for radioactivity.

*Evidence for Identical Subunits in Histidase.* A series of isoelectric focusing experiments were conducted to determine if histidase consisted of identical subunits. On both denaturing gel electrophoresis and nondenaturing isoelectrofucusing gels, only one protein component could be detected. This suggested that the native protein contained identical molecular weight subunits and was overall homogeneous in charge. However, when histidase was subjected to isoelectric focusing under the denaturing conditions described by O'Farrell (1975), two major bands of approximately equal intensity were detected. The p$I$ values of these bands were 5.70 and 5.75. When histidase was labeled with KCN to various extents and then analyzed on denaturing isoelectric focusing gels, the two bands were found to be labeled to approximately the same level (Table IV). Thus, the minor difference in charge between these two bands is probably due to microheterogeneity rather than being the result of two different subunits; a differential of only four charges needs to occur to have a p$I$ difference of 0.05. This value was calculated as described by Sherbet (1978), assuming the deamidation of asparagine to aspartate. We, therefore, believe the histidase subunits are essentially identical and that the labeling of only one to two of the four possible cyanide-sensitive sites cannot be explained in terms of different subunits.

## Discussion

A comparison of size and subunit structure among the various pseudomonad histidases studied thus far reveals the *P. putida* enzyme to be quite similar to others in those respects. Like the *Pseudomonas* sp. ATCC 11299 histidase, the *P. putida* enzyme contains an electrophilic center that appears to be a dehydroalanine residue on the basis of experiments involving cyanide and sodium borohydride treatment. This dehydroalanine can now be stated as existing in unpurified enzyme, and its presence appears to be essential for catalysis. The proposal of Hassal & Soutar (1974) that dehydroalanine forms in histidase as a consequence of desulfurization of cystine following oxidation of an essential active site cysteine seems incompatible with our observations that dehydroalanine occurs in unpurified enzyme and is itself necessary for enzymatic activity. Also the compositional differences observed between

the cysteine-containing active site tryptic peptide, reported by Hassall & Soutar (1974) and Klee & Gladner (1972), and the tryptic peptide we isolated containing a modified dehydroalanine argue against homology between these peptides and instead suggest that the reactive cysteine is distinct from dehydroalanine in histidase. This does not, however, eliminate the possibility that dehydroalanine arises normally in histidase by a mechanism involving sulfur elimination. At this time, our inability to incorporate radioactivity from serine into dehydroalanine would seem only to rule out serine as a precursor for dehydroalanine.

Since inactivation by [$^{14}$C]cyanide correlated very well with isotope incorporation and could be prevented by the addition of $Mn^{2+}$ and substrate, it is likely that the dehydroalanine is located at the active site of histidase. Our data indicated that $Mn^{2+}$ was required for substrate or inhibitor binding in order to block cyanide action, just as $Mn^{2+}$ is necessary for activity (Klee, 1972). It was interesting to note from Table I that glycine did not protect against cyanide inactivation while cysteine could. If the dehydroalanine is located near the imidazole binding site, then it can be concluded that the $-CH_2SH$ group of cysteine spans some of the imidazole binding site while glycine occupies only the carboxyl and amino binding sites. Moreover, the sulfur atom may coordinate to the metal as has been proposed for the imidazole group (Hanson & Havir, 1973), and this may further enhance inhibition by cysteine.

Molecular weight determinations have shown the $M_r$ of the native protein to be 220 000, with a subunit $M_r$ of 55 000. Although we obtained isoelectric focusing data indicating two types of subunits based on charge differences, it seems most reasonable to conclude that the subunits are identical and that the minor charge difference noted is due to a slight microheterogeneity. This conclusion is supported by data from a restriction enzyme map of the histidase gene,[1] which has indicated that the size of DNA coding for histidase is 1.7-kilobase pairs. This would be the expected size for a single polypeptide of $M_r$ near 60 000. Moreover, even under conditions where a single dehydroalanine was modified per tetramer by [$^{14}$C]cyanide, the $^{14}$C incorporation into the two charge forms was equivalent (Table IV).

Our findings that indicate the presence of dehydroalanine in histidase from *P. putida* may be considered to confirm and extend the results presented by other groups who have obtained evidence for dehydroalanine in various histidases [reviewed by Hanson & Havir (1973)]. We believe the conflicting reports on the number of dehydroalanine residues present in histidase (Wickner, 1969; Givot et al., 1969) can now be explained by variations in the experimental conditions used for the modification studies since our own results indicate that the enzyme may become unstable during the course of a lengthy exposure to a modifying agent, with a tendency toward unfolding and exposure of additional dehydroalanine residues. Givot et al. (1969) have shown that nitro[$^{14}$C]methane inactivated histidase and up to 2.4 mol of nitromethane was incorporated per mol of enzyme during the process. Although that work did not present extensive data correlating degree of inactivation with extent of labeling, it is clear that the observed stoichiometry could be in excess of the amount of nitromethane necessary simply to inactivate the enzyme; had it been possible to quench the incorporation by a method other than dialysis, perhaps a lower stoichiometry would have been observed.

[1] M. W. Consevage and A. T. Phillips, unpublished results.

The fact that we could modify a total of four dehydroalanine residues in denatured histidase but needed to modify only one of these to inactivate the enzyme leads to several interesting possibilities for substrate catalysis. Since no polymeric species of histidase were detected, it is possible that only one functionally active site exists per tetramer, with the remaining three dehydroalanine groups being shielded inside the tetrameric structure. On the other hand, there may be four equivalent active sites per tetramer, but modification of or binding to any one of these prevents catalysis at the other sites. A more thorough examination of reaction kinetics may provide some evidence for negative cooperativity. In any case, modification of more than one dehydroalanine group by cyanide can only be observed under conditions where conformational changes are likely to produce an altered environment around the remaining dehydroalanine residues.

Experiments on sequencing active site tryptic and chymotryptic peptides containing the dehydroalanine residue are in progress. These data not only should give an insight into the possible origins of dehydroalanine but also could give an indication as to the role of dehydroalanine in catalysis. The mechanistic proposal by Givot et al. (1969) for how dehydroalanine reacts with $CH_3NO_2$ considers that the amino group of dehydroalanine forms a Schiff base with an unidentified carbonyl compound. On the basis of this, we might expect to find the dehydroalanine located at the $NH_2$ terminus of the protein or attached to the side chain of some amino acid in the main protein sequence. Regardless of whether these prove to be the case or whether instead dehydroalanine is found in peptide linkage at some interior position, the result will have implications for how dehydroalanine functions in catalysis.

**Registry No.** Histidine ammonia-lyase, 9013-75-6; dehydroalanine, 1948-56-7; cyanide, 57-12-5.

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# Structures of Manganese(II) Complexes with ATP, ADP, and Phosphocreatine in the Reactive Central Complexes with Creatine Kinase: Electron Paramagnetic Resonance Studies with Oxygen-17-Labeled Ligands†

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ABSTRACT: Coordination of Mn(II) to the phosphate groups of the substrates and products in the central complexes of the creatine kinase reaction mixture has been investigated by electron paramagnetic resonance (EPR) spectroscopy with regiospecifically $^{17}O$-labeled substrates. The EPR pattern for the equilibrium mixture is a superposition of spectra for the two central complexes, and this pattern differs from those observed for the ternary enzyme–Mn(II)–nucleotide complexes and from that for the dead-end complex enzyme–Mn(II)ADP–creatine. In order to identify those signals that are associated with each of the central complexes of the equilibrium mixture, spectra were obtained for a complex of enzyme, Mn(II)ATP, and a nonreactive analogue of creatine, 1-(carboxymethyl)-2-iminoimidazolidin-4-one, which is a newly synthesized competitive inhibitor. This inhibitor permits an unobstructed view of the EPR spectrum for Mn(II)ATP in the closed conformation of the active site. The EPR spectrum for this nonreactive complex with Mn(II)ATP matches one subset of signals in the spectrum for the equilibrium mixture, i.e., those due to the enzyme–Mn(II)-ATP–creatine complex. Chemical quenching of the samples followed by chromatographic assays for both ATP and ADP indicates that the enzyme–Mn(II)ADP–phosphocreatine and the enzyme–Mn(II)ATP–creatine complexes are present in a ratio of approximately 0.7 to 1. A similar value for the equilibrium constant for enzyme-bound substrates is obtained directly from the EPR spectrum for the equilibrium mixture. Spectra for samples of the equilibrium mixture set up initially with creatine and either [$\alpha$-$^{17}O$]ATP, [$\beta$-$^{17}O$]ATP, or [$\gamma$-$^{17}O$]ATP show equivalent inhomogeneous broadening of signals from both central complexes irrespective of the position of the $^{17}O$ label. Similar measurements with the nonreactive complex of 1-(carboxymethyl)-2-iminoimidazolidin-4-one and the $^{17}O$-labeled forms of ATP show that Mn(II) is coordinated to all three phosphate groups of ATP in this complex. These results show that an $\alpha,\beta,\gamma$-tridentate complex of Mn(II)ATP is the substrate in the forward direction and Mn(II) remains coordinated to the $\alpha$- and $\beta$-phosphates of ADP and to phosphocreatine in the product complex. A definitive model for the involvement of the divalent metal ion in the entire reaction sequence emerges from these results.

The mode of coordination of metal ions to nucleotides in free solution and in complexes with enzymes has been a topic of considerable interest because metal ion–nucleotide complexes are the reactive species in virtually all enzyme-catalyzed reactions that involve nucleotides. Nucleotides possess several functional groups that are potential ligands for metal ions, and knowledge of the isomer of the metal–nucleotide complex that is favored by a given enzyme is necessary to understand the role of the metal ion in the catalytic cycle.

Creatine kinase (EC 2.7.3.2) catalyzes the reversible reaction

$$MgATP^{2-} + \text{creatine} \rightleftharpoons MgADP^{-} + \text{phosphocreatine}^{2-} + H^{+}$$

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