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On the Nature of the Irreversible Inhibition of Histidine Ammonia Lyase by Cysteine and Dioxygen

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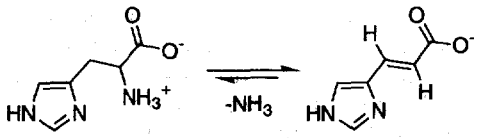
Abstract—The irreversible inhibition of histidine ammonia lyase by L-cysteine and dioxygen has been reexamined. After denaturation and consecutive digestion of the inhibited enzyme by trypsin and endoproteinase Glu-C, the generated chromophoric system ($\lambda_{max}=340$ nm) remained intact and was isolated in an octapeptide containing amino acids 138–145, as previously described (Hernandez, D.; Stroh, J. G.; Phillips, A. T. *Arch. Biochem. Biophys.* **1993**, *307*, 126). Conducting the inhibition in the presence of $^{18}O_2$ did not result in the incorporation of the heavy isotope into the isolated octapeptide. Total hydrolysis followed by amino acid analysis of the octapeptide revealed the presence of one arginine in addition to those expected from the deduced sequence (G_3SVAD). ^1H NMR spectroscopy of the octapeptide confirmed the presence of the amino acids GSVAD and showed no signals for olefinic or aromatic protons. To account for the excess mass of the octapeptide, we propose an oxidative degradation of the dehydroalanine prosthetic group, followed by reaction of the resulting dicarbonyl system with a nearby arginine residue. Copyright © 1996 Elsevier Science Ltd

Introduction

Histidine ammonia lyase (HAL; EC 4.3.1.3) catalyses the first step in histidine degradation in most cells by converting it into urocanic acid (Scheme 1).



Scheme 1. HAL reaction.

Its failure in humans causes histidinaemia.[1] The HALs from different sources are homotetramers and show a high degree of sequence homology.[2] For the enzyme from *Pseudomonas putida*, it has been shown by site-directed mutagenesis that serine 143 is posttranslationally converted into the catalytically-essential dehydroalanine.[3] This rare prosthetic group occurs in all HALs and is shared only by the important plant enzyme phenylalanine ammonia lyase (PAL).[4] Recently it has been proposed that catalysis begins with an electrophilic attack of the dehydroalanine prosthetic group at the imidazole (phenyl) ring and the transiently electron deficient nucleus facilitates deprotonation at the β-carbon.[5,6] A similar effect can be reached by a nitro substituent at an appropriate position of the substrate, thus eliminating the necessity of the dehydroalanine prosthetic group.[5,6]

Cysteine is a competitive inhibitor of HAL and at pH >10 in combination with oxygen irreversibly inactivates HAL with concomitant formation of a chromophore ($\lambda_{max}=340$ nm).[7] This chromophore does not contain cysteine [8] and remains intact after digestion of the modified protein with trypsin and endoproteinase Glu-C.[9] Here we describe experiments that led us to a proposal for the structure of the 340 nm chromophore and for the mechanism of the irreversible inactivation.

Results

HAL was isolated from an overproducing strain of *E. coli* harboring the HAL gene from *P. putida* cloned into the expression vector pT7-7.[10] One liter of an overnight culture of this overproducing strain yielded, after purification, about 80 mg of pure HAL exhibiting a specific activity of 35–40 units/mg.[10] This preparation was treated with a solution of L-cysteine in a sodium hydrogen carbonate buffer (pH 10.5), either in the presence of air or in an atmosphere of $^{18}O_2$. The reaction was monitored by appearance of the absorption at 340 nm. Partial proteolysis was carried out as described by Hernandez et al.[9] Tryptic digestion was followed by two consecutive HPLC separations. N-terminal sequencing of the resulting modified oligopeptide showed GSVG in agreement with the literature.[9] Additional proteolysis with endoproteinase Glu-C was followed by HPLC which separated two peptides, of which only the faster eluted one absorbed at 340 nm and was further examined. The N-terminal sequence of this modified peptide was again GSVG. Electrospray mass spectrometry showed a species with a *m/z* 790 ($[M+H]^+$) in agreement with published results (Fig. 1).[9] On the other hand, we could not detect a peak at *m/z* 833.4 reported in the same paper.[9] Similar results were obtained with the octapeptide when HAL was inhibited with L-cysteine in the

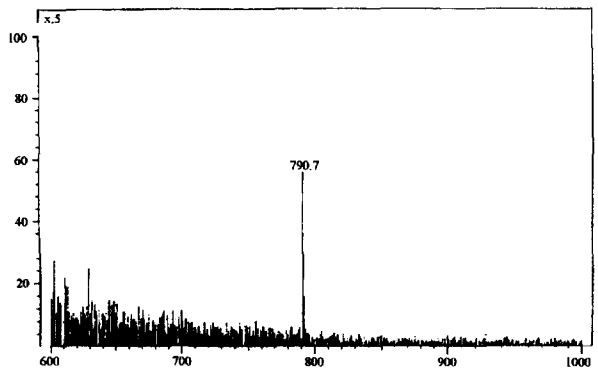


Figure 1. ES mass spectrum of the modified octapeptide.

presence of $^{18}O_2$, excluding incorporation of oxygen atoms from dioxygen into the final product absorbing at 340 nm. The ^1H NMR spectrum of the modified octapeptide confirmed the presence of the amino acid residues G, S, V, A, D (Table 1). Additional signals appeared and could be assigned to lactate, acetate and formate as contaminations. The signals for arginine are more difficult to assign because of the more complex ^1H–^1H couplings.

Total hydrolysis of the final octapeptide followed by amino acid analysis revealed, besides the expected amino acids (deduced from the N-terminal sequencing and from the known DNA sequence),[11] one arginine. The measured ratios for the amino acids were: 1.30 A, 1.30 D, 3.00 G, 0.93 V, 0.58 R, 0.3 citrulline, and 0.1 ornithine. Since under the hydrolysis conditions (6 N HCl, 110 °C, 30 h) arginine is partially degraded to citrulline and ornithine,[12] at least 0.98 arginines were present in the modified oligopeptide. The analysis revealed 0.49 L (flanking the octapeptide on both sides). The values for T and I were about 0.2 and for F and Q about 0.1. In another analysis, under slightly harsher hydrolysis conditions, less arginine (0.43) and more citrulline (0.54) were found, but their sum was similar.

As a model reaction, L-arginine was reacted with phenylglyoxal under conditions previously described.[13] Analysis of the products by HPLC revealed several products but only one minor product showing absorption maximum at 340 nm.

Discussion

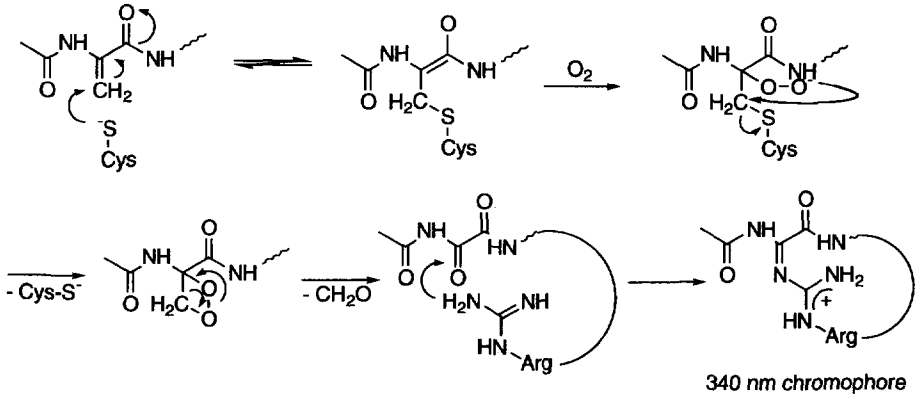
The irreversible inhibition of HAL by L-cysteine under aerobic conditions and at high pH (>10) was first reported by Klee.[7] She also observed the concomitant formation of a chromophore absorbing at 340 nm which remained stable after proteolytic digestion. Moreover, using [^{14}C]- and [^{35}S]cysteine the originally incorporated radioactivity was released after denaturation.[8] Hernandez et al. confirmed these results and in addition showed that the modification occurs at serine 143.[9] Furthermore, they obtained, after digestion with trypsin and endoproteinase Glu-C, an octapeptide to which they assigned the sequence GSVGAS*GD comprising the amino acids 138–145 of HAL. Serine 143 (labelled above with an asterisk) carried the posttranslational modification and the 340 nm chromophore. Mass spectrometry showed signals at *m/z* 833.7 and 790, so that the molecular mass of the modified octapeptide was 184 and 141 Da higher, respectively, than calculated for the above sequence. The authors speculated that serine 143 carried an electrophilic prosthetic group of unknown nature possibly attached by an ester bond.[9] The importance of serine 143 for catalysis was also manifested by site-directed mutagenesis.[10,14] More recently it was shown that the prosthetic group, of which serine 143 is the precursor, can be substituted by attaching a nitro group to position 5 of the substrate, i.e. 5-nitrohistidine reacts with mutant HALs lacking the prosthetic group.[5] As discussed elsewhere,[3] the hypothesis concerning the attachment of an electrophilic group by an ester bond to serine 143[9] cannot be correct. On the contrary, there is sufficient new evidence in support of the original assumption, i.e., that the essential electrophilic group of HAL[3,15] and PAL[4,6,16] is a dehydroalanine residue formed posttranslationally from serine 143 and 202, respectively. To reconcile the findings of Hernandez et al.[9] with the existence of dehydroalanine at position 143, the Karlsruhe group recently proposed possible initial steps for the inhibition by L-cysteine and O_2 leading to the 340 nm chromophore.[3] In the present work we essentially confirmed and extended the experimental results of Hernandez et al.,[9] which allow a more detailed proposal for the structure and mechanism of formation of the 340 nm chromophore (Scheme 2).

Table 1. ^1H NMR data of the amino acid residues

δ_H (ppm)	G	S	V	A	D
α-C	3.97	4.5	4.18	4.35	4.76
β-C		3.88	2.13	1.39	2.84
γ-C			0.97		
			0.94		

L-Cysteine is a competitive inhibitor of HAL, even at neutral pH values and under anaerobic conditions.[8,17] Since other L-amino acids are much less inhibitory, we propose that the thiol group of the enzyme-bound cysteine is in the correct position for nucleophilic attack at the dehydroalanine prosthetic group. While this reaction is reversible under anaerobic conditions,[8] the highly nucleophilic intermediate may be irreversibly trapped by dioxygen to form a hydroperoxide anion. Such a reaction has its precedence in photorespiration catalysed by rubisco,[18] and in the oxidation of the dihydroquinone form of vitamin K catalysed by an oxygen-dependent carboxylase.[19,20]

In the next step nucleophilic substitution by the hydroperoxide anion furnishes a dioxetane under elimination of L-cysteine. Dioxetanes can undergo fragmentation;[21] in our case, to formaldehyde and an imide carbonyl group. The resulting glyoxylic acid diamide is normally



Scheme 2. Proposed mechanism of formation and structure of the 340 nm chromophore

a stable unit but to account for the extra weight of the isolated octapeptide as well as for the 340 nm chromophore we must assume further reactions to occur. It is known that α-dicarbonyl compounds specifically react with arginine.[13,22,23] Indeed, an arginine residue may be in close vicinity of the active site due to specific folding of the peptide chain. Since the imide carbonyl is more electrophilic than its amide carbonyl neighbor it should be preferentially attacked by the deprotonated (pH >10) guanidino group of arginine. The so formed imine, conjugated both with the amide carbonyl and guanidino imine group, could account for the 340 nm chromophoric system. The proposed binding mode of the arginine guanidino group to the dicarbonyl system present in the modified octapeptide can be explained by the lower reactivity of the amide carbonyl group relative to a ketone or aldehyde group. The electrophilicity of the imide carbonyl group is intermediate and this group may react with the guanidino group, especially in an intramolecular fashion. In contrast to dicarbonyl compounds as phenylglyoxal or butane-2,3-dione which form a ring by a further condensation (Scheme 3) we propose the reaction with the glyoxylic amide unit to stop at this stage. Tryptic digestion of the modified protein could carve out the arginine residue, provided that it is flanked by a further arginine or lysine at its N-terminal side.[†] Examination of the HAL sequence identifies three dipeptides meeting this criterion (see Table 2). Table 2 also shows four further arginines flanked by aspartic or glutamic acids at their N-terminal side. These arginines would be carved out only after the second digestion, i.e., by endoproteinase Glu-C. Were this the case, the tryptic oligopeptide would exhibit two N-termini. Since sequencing of the purified tryptic oligopeptide revealed the same sequence as that of the doubly digested final octapeptide, namely GSVG (these amino acids appeared in >95% purity), only arginines 116, 355, and 426 are candidates.

Further experimental support for the proposed structure of the 340 nm chromophore is provided by the molecular mass of the modified octapeptide. The measured value, 789 Da, is exactly the one that would be expected by substitution of the hydroxymethyl group of serine 143 by arginine.[‡] Furthermore, the presence of arginine in this oligopeptide could be shown by amino acid analysis after total hydrolysis with 6 N HCl. The proposed structure is also consistent with the lack of signals for aromatic and olefinic protons in the ^1H NMR spectrum, and with the absence of ^{18}O in the final product, when the inhibition reaction is conducted under an $^{18}O_2$ atmosphere.[§]

Conclusion

Reexamination of the inhibition of HAL by L-cysteine and dioxygen led to a proposal for the structure and mechanism of formation of the chromophore absorbing at 340 nm. In contrast to a previous interpretation of the structural data,[9] we propose a novel chromophoric structure which is in agreement with all former results and some novel ones presented in this paper. The postulated structure is also supported by a chemically plausible sequence of reactions leading to its formation.

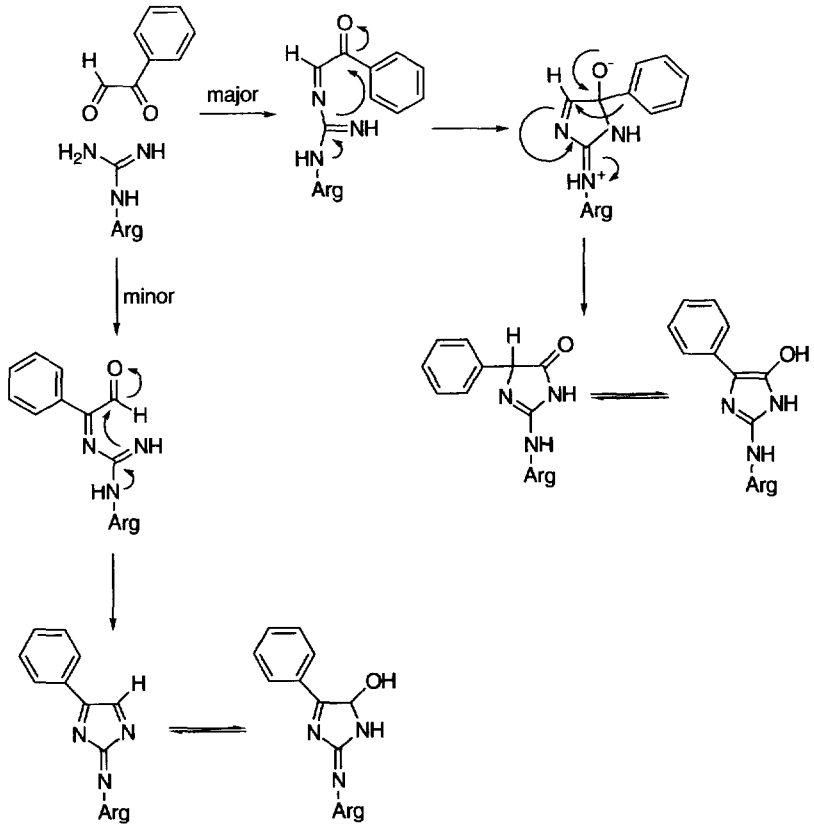
Table 2. Suitable dipeptides for release of arginine

Trypsin	Endoproteinase Glu-C
Arg 115–Arg 116	Asp 49–Arg 50
Arg 354–Arg 355	Glu 353–Arg 354
Lys 425–Arg 426	Glu 476–Arg 477
	Glu 478–Arg 479

[†]Although arginines bound to dicarbonyl are not attacked by trypsin, this may not be the case for this half-bound arginine which has still a positive charge.

[‡]The calculated molecular mass for ADG_3S_2V is 648, for arginine 174. By the proposed oxidation and condensation reactions serine 143 and the arginine residue lose the elements of methanol +1 H amounting to a mass of 33.

[§]Between submission and revision of this paper it could be shown (Merkel, D.; Friedl, K.; Rétey, J., unpublished results) that treatment of HAL with butane-2,3-dione or phenylglyoxal hardly inhibits the enzyme, but further treatment with cysteine and O_2 at pH 10.5 leads to irreversible inhibition without generation of absorption at 340 nm.



Scheme 3. Mechanism of the condensation of phenylglyoxal with arginine.[22]

Experimental

Modification of histidase

Histidase was purified from a 1 L culture of *E. coli* BL21 (DE) (pT7-7 hut H) containing the histidase gene from *P. putida* as described earlier.[10] The usually obtained 70–80 mg of purified enzyme with a typical specific activity of 35 U/mg was dissolved in 50 mM Na_2CO_3/$NaHCO_3$ buffer (pH 10.5) and concd to 0.5 mL. This enzyme soln was slowly added to 10 mL of the same buffer containing 10 mM L-cysteine. After stirring for 5 h under aerobic conditions, the enzyme showed complete loss of catalytic activity and strong absorbance at 340 nm.

For modification of histidase under $^{18}O_2$, the 0.5 mL of enzyme solution and the buffer containing L-cysteine were evacuated $\times 5$ and flushed with nitrogen. While a slow stream of $^{18}O_2$ was injected into the reaction vial containing the L-cysteine solution, the enzyme was added through a septum to avoid air contact. After 30 min of a bubbling $^{18}O_2$ stream, the vial was closed tightly and the enzyme solution was stirred under the $^{18}O_2$ atmosphere for 5 h. Again, the loss of biological activity was accompanied by a strong absorbance at 340 nm.

Isolation of the modified oligopeptide

From the resulting enzyme solution, the L-cysteine was removed by using a stirred cell, where the inactivated enzyme was dissolved and concd several times in 20 mM potassium phosphate buffer (pH 7.3) to a final volume of 2 mL. After the addition of urea (8 M), the enzyme was denatured by heating for 2 min at 100 °C. For reductive carboxymethylation, a 50-fold molar excess of iodoacetate was added, followed by four portions of a 50-fold molar excess of $NaBH_4$ during 1 h. Excess reagent was destroyed by adding 50 µL of acetone and 10 µL of mercaptoethanol. Using a stirred cell, the solvent was changed to 25 mM NH_4HCO_3 (pH 7.8) and the enzyme solution was freed of the reagents used in the reductive carboxymethylation step. For the first enzymatic digestion a solution of 3.5 mg lyophilized trypsin in 400 µL buffer was added to the resulting volume of 5 mL enzyme solution. The trypsin solution was added in four portions over a period of 24 h. During this time the temperature was held constant at 37 °C.

To isolate the oligopeptide containing the modified active site of HAL, the resulting peptide solution was separated by HPLC using a Hewlett-Packard Ti-Series 1050 liquid chromatograph with a RP-HPLC column Nucleosil $7C_{18}$ from Macherey–Nagel. As solvent system 20 mM potassium phosphate, pH 7.3 (A) and 60% acetonitrile in 20 mM potassium phosphate, pH 7.3 (B) was used. Chromatography was carried out with a linear gradient from 0 to 100% B over 2 h with a flow rate of 5 mL/min. Detection was monitored at 340 nm. The main fraction with the highest absorbance at 340 nm was eluted after 65 min. To further purify the

isolated peptide, this fraction was rechromatographed using H_2O (0.1% TFA) (A) and 60% acetonitrile in water (0.1% TFA) (B) on the same column. The fraction with the highest absorbance at 340 nm was eluted after 125 min and lyophilized. N-Terminal amino acid sequencing was performed at the Zentrum für Molekularbiologie der Universität Heidelberg. The first four amino acids were identified as glycine, serine, valine, and glycine, followed by a much smaller signal for alanine. Further sequence data for the modified oligopeptide could not be obtained.

Isolation of the modified octapeptide

The second enzymatic digest was carried out using endoproteinase Glu-C as the proteolytic enzyme. After dissolving the lyophilized oligopeptide in 500 µL of 50 mM potassium phosphate (pH 7.3), a solution of 0.1 mg endoproteinase in 100 µL water was added and incubated for 24 h at 37 °C. Endoproteinase Glu-C cleaves the amide bond of the carboxyl group of aspartate to release the modified octapeptide. The separation of the two newly formed peptides was carried out on the same HPLC system as before. A solvent system composed of H_2O (0.1% TFA) (A) and 40% acetonitrile in H_2O (0.1% TFA) (B) was used. The gradient was raised from 0 to 100% B over 200 min with a flow rate of 5 mL/min. The absorbance of the fractions was monitored at 340 nm. The modified octapeptide was eluted with a retention time of 77 min (typically 0.5 mg after lyophilization).

N-terminal amino acid sequencing led to the same result as for the first oligopeptide.

ES mass spectrometry of the modified octapeptide was carried out at the Deutsches Krebsforschungszentrum Heidelberg. It revealed the molecule ion peak for the $[M+H]^+$ species at *m/z* 790, indicating the molecular weight of the modified octapeptide to be 789 Da. Identical results were obtained under aerobic and under $^{18}O_2$-conditions.

^1H NMR spectroscopy in D_2O showed signals for the expected amino acid residues (Table 1), but no signals in the region of olefinic or aromatic protons.

Chemical model for the enzymatic modification

To a solution of 100 mg of arginine hydrochloride (0.5 mmol) in 5 mL of 0.2 M *N*-ethylmorpholin-acetate-buffer (pH 8) were added dropwise a soln of 160 mg phenylglyoxal monohydrate (1 mmol) in 2.5 mL of 0.2 M *N*-ethylmorpholin-acetate-buffer and 2.5 mL of methanol. After stirring for 1 h at 25 °C, the reaction was terminated by adding 2 mL acetic acid. Polymeric reaction products were separated by centrifugation and the resulting clear solution was chromatographed with the same HPLC system used for the peptide isolations. For elution, H_2O (A) and acetonitrile (B) was used with a linear gradient from 0 to 80% B within 160 min and a flow rate of 4 mL/min. Only one product absorbed at 340 nm and was eluted with a retention time of 138 min.

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