Further Insight into the Mechanism of the Irreversible Inhibition of Histidine Ammonia-Lyase by l-Cysteine and Dioxygen

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Histidine ammonia-lyase (HAL) was irreversibly inhibited by l-cysteine at pH 10.5 under aerobic conditions. The inhibited enzyme, still in its intact conformation, showed an absorption maximum at 338 nm. Upon denaturation, followed by pronase digestion, two main chromophoric products 1 and 2 (Figs 4 and 5, resp.) could be isolated with absorption maxima at 335 and 332 nm, respectively. As determined by MALDI-TOF mass spectrometry and 1H-NMR spectroscopy, in product 1 one of the methylidene H-atoms of the 3,5-dihydro-5-methylidene-4H-imidazol-4-one (formerly called 4-methylideneimidazol-5-one; MIO) prosthetic group was substituted by one of the amino groups of l-cystine, while in product 2 the ε-amino group of l-lysine was the analogous substituent. Acid-catalyzed hydrolysis of product 1 gave compound 3 whose chromophore (λmax 310 nm) was that of 3,5-dihydro-5-(4-hydroxymethylidene)-4H-imidazol-4-one, i.e., of a vinylogous acid. These results support our previous proposal that, in the first step, the l-cysteine S-atom attacks the prosthetic electrophile (Scheme 2). The resulting nucleophilic enolate captures O2 to form a peroxide. On the basis of the present results, we postulate that the observed products 1–3 arise from a vinylogous thioester 4, which is formed in the conformationally intact inhibited enzyme by an electrocyclic reaction eliminating H2O2.

Introduction. – Histidine ammonia-lyase (HAL; EC 4.3.1.3) catalyzes the first step in the degradation of histidine by converting it into urocanic acid (Scheme 1).

Scheme 1. The HAL Reaction

HAL contains an electrophilic moiety which has been shown to be 3,5-dihydro-5-methylidene-4H-imidazol-4-one (formerly called 4-methylidene-imidazol-5-one; MIO) by X-ray crystal-structure analysis [2]. MIO is formed autocatalytically from the tripeptide portion Ala142Ser143Gly144 by cyclization, concomitant with the consecutive elimination of two molecules of H2O. Mutation of Ser143 (except for Cys) or chemical modification of the MIO group by nucleophiles lead to dramatic loss of the catalytic activity [3]. A further inhibitor of HAL is l-cysteine, and, some years ago, Weber and Rétye proposed that its SH group adds to the electrophilic catalytic centre [4]. In addition to other evidence, the specific inhibition by l-cysteine gave the hint to a novel mechanism of the HAL reaction, which is initiated by the attack of the

1) Part of the Ph.D. Thesis.
electrophilic group at the imidazole ring of histidine [5]. Whereas, at neutral pH, L-
cysteine is a competitive inhibitor, above pH 10 and in the presence of O₂ it causes
irreversible inhibition with simultaneous formation of a chromophore at 340 nm [6].
This chromophore survives proteolytic digestion of the inhibited enzyme [4][7][8].
When L-[¹⁴C]cysteine or L-[³⁵S]cysteine were used as inhibitors, the chromophore
containing denatured protein or proteolytic product, was reported to have lost most of
its radioactivity [7][8]. Proposals for the nature of this chromophore had appeared
before the MIO structure was known [4]. A recent communication on the structure of
the chromophore of inhibited HAL prompted us to describe our results, which partially
confirm and extend those of Galpin et al. [9].

Results. – Isolation and UV/VIS Spectroscopy of the Chromophores. Highly purified
histidine ammonia-lyase was inhibited by addition of either unlabelled L-cysteine or L-
[³⁵S]cysteine at pH 10.5 under aerobic conditions (cf. Exper. Part). The irreversible
inhibition was monitored by enzyme assays and measuring the UV spectrum at 338 nm.
After denaturation under drastic conditions and removal of the denaturing agents, the
inhibited enzyme was digested by pronase for 24 h. The resulting reaction mixture was
separated by HPLC. The first chromatography on a reversed-phase (RP) column
showed two major peaks absorbing at 340 nm (Fig. 1). Chromophore 1 was eluted at
38.3 min, chromophore 2 at 41.6 min. They were further purified on reversed-phase and
Biosep SEC 2000 columns. UV Spectroscopy of the pure chromophoric peptides

![Fig. 1. First chromatography of the pronase digest of irreversibly inactivated HAL registered at wavelength of
215 (above) and 340 (below) nm. The peaks appearing at 38.3 and 41.6 min represent chromophores 1 and 2,
respectively.](image-url)
showed absorption maxima at 335 and 332 nm, respectively. This meant a shift compared to the absorption maximum of 338 nm of the inhibited enzyme before denaturation and pronase treatment.

During isolation of the chromophoric peptide 1, it was noted that it is unstable under the acidic conditions of the reversed-phase chromatography (Fig. 2). The decomposition product 3 was also isolated, it was less hydrophobic than the chromophore 1, and had an absorption maximum at 310 nm.

![Fig. 2. Reversed-phase rechromatography of the pure chromophore 1 showing partial decomposition to chromophore 3 (shown by arrow)](image)

When L-[35S]cysteine was used for the irreversible inhibition, the isolated chromophore 1 carried the radioactive label, whereas chromophores 2 and 3 were void of radioactivity.

**MALDI-TOF Mass Spectrometry.** The mass of the main chromophore containing products of the pronase digest was measured by using dihydroxybenzoic acid as the matrix for MALDI-TOF mass spectrometry. For chromophore 1, MH+ was detected at 608.3 Da, and for its decomposition product 3 MH+ appeared at 386.6 Da. Chromophore 2 showed MH+ at 514.8 Da (Fig. 3).

**1H-NMR Spectroscopy.** The 1H-NMR spectra (600 MHz, 2H2O, pH 6.8) of the two main products 1 and 2 showed several similar signals and also some differences (see Exper. Part). In both spectra, 1H signals for Gly141 and Asp145, as well as the signals of two further amino acids, appeared from which the imidazolone moiety had been formed, i.e., Ala142 and Gly144. In addition, both spectra showed two characteristic singlets at 7.8 and 8.0 ppm, of which the total integral corresponded to one proton, indicating the presence of two diastereoisomers for each chromophore carrying peptide. Beside these similar 1H-NMR signals, different signals could be also detected.
Compound 2 showed the signals of a lysine residue, while compound 1 those of two cysteine residues.

Discussion. – On the basis of the recently elucidated structure of the catalytically essential electrophilic moiety of HAL as 3,5-dihydro-5-methylidene-4H-imidazol-4-one [2], the mass and 1H-NMR spectra, as well as the incorporation of L-[35S]cysteine into chromophore 1 and its absence in chromophore 2, we suggest the diastereoisomeric structures depicted in Figs. 4 and 5. Although we initially used more specific proteinases for digestion of the inhibited HAL [1][4], prolonged digestion with pronase resulted in smaller chromophoric peptide fractions, of which the 1H-NMR

![Fig. 3. MALDI-TOF Mass spectrum of chromophore 2](image)

![Fig. 4. Chromophore 1 after pronase digest](image)
spectra were easier to interpret. Moreover, the digestion product mixture contained a large number of free amino acids, among them lysines, whose reaction with the initially formed chromophore afforded the chromophoric peptide 2.

What may be the structure of the initially formed chromophore that exists before denaturation and digestion of the inhibited HAL? Since only L-cysteine and L-homocysteine, and no other amino acids, inhibit the enzyme, we suggested that the SH group of these amino acids attacks the catalytic electrophile that had been assumed to be dehydroalanine [10][11]. Such an attack is consistent with the proposal that, in the catalysis by HAL, the imidazole group of histidine reacts with the electrophilic group of the enzyme, since the SH group of cysteine is at about the same distance from the α-amino-acid partial structure as the imidazole group of the natural substrate [5].

Superficially, our finding and the recent report of Galpin et al. [9] would support the previously favored mechanism, in which the α-amino group of histidine is the nucleophile reacting with the electrophilic group at the active site of HAL. However, we believe [1], as do Galpin et al., that the isolated chromophores of type 1 are the result of a rearrangement by intramolecular aminolysis of the originally formed vinylogous thioester (Scheme 2). The formation of the latter can be explained by attack of the thiolate at MIO, affording a highly nucleophilic enolate which, like in the photorespiration mechanism of rubisco [12], may react with O₂ to give a peroxide anion which, by an electrocyclic reaction coupled with a 1,3 C → O H-shift, could form the vinylogous thioester 4 as the primary product. The existence of 4 is supported by the λ_max of 338 nm of the inhibited enzyme still in its natural folding. After denaturation and proteolytic digestion, either intramolecular or intermolecular aminolysis of the vinylogous thioester 4 may occur affording 1 or 2, respectively (Schemes 2 and 3). The aminolysis is concomitant with a shift of λ_max of the chromophore to 335 nm in the case of 1 and even to 332 nm in the case of 2. The formation of 2, which is the main chromophoric product, is possible, because, after pronase digestion, many free lysine molecules are present in the reaction mixture. Moreover, while the formation of 2...
seems to be irreversible under the reaction conditions, the formation of the vinylogous amide 1a from 4 is likely to be reversible (Scheme 2). In the tetrahedral intermediate of the aminolysis, rotation around the C–C bond may occur, leading to the (Z)/(E) isomers of 1 and 2, as observed in their ¹H-NMR spectra. Such a rotation is consistent with the assumption that aminolysis occurs after denaturation of the inhibited enzyme.

Scheme 2. Proposed Mechanism of the Irreversible Inhibition of HAL by l-Cysteine and Oxygen

Finally, the instability of chromophore 1 under acidic conditions deserves comment. This may be explained by a further rearrangement with participation of the COOH group of the first cysteine molecule followed by hydrolysis to chromophore 3 (Scheme 4). As judged by its mass spectrum (MH⁺ 386.6 Da), 3 is the corresponding vinylogous acid or a reductone, whose UV absorption (λₘₐₓ 310 nm) is also consistent
Scheme 3. *Formation of the Lysine-Containing Chromophore 2 by Intermolecular Aminolysis of the Vinylogous Thioester 4*

with the proposed structure (intact MIO has an absorption maximum in a similar region, namely at 305 nm [13]).

Our finding that L-[\textsuperscript{35}S]cysteine is incorporated into chromophore 1 seems to be in conflict with previous results of two research groups [7][8]. The most plausible explanation for this deviation could be that, in the former works, the primarily formed unstable chromophore 1 was converted into some other chromophore, leading to products void of cysteine. This could occur by reactions similar to those shown in Schemes 3 and 4.
Scheme 4. Formation of the Decomposition Product ([2-(1-(Glycylamino)ethyl)-4,5-dihydro-4-(hydroxymethylidene)-5-oxo-1H-imidazol-1-yl]acetyl)-L-aspartic Acid (3) of the Acid-Labile Chromophore 1

In conclusion, we isolated and characterized chromophores 1–3. Chromophore 1 has a structure analogous to that of the compound recently found by Galpin et al. [9]. The isolation of compounds 2 and 3, as well as the observed shifts in the chromophore’s UV maxima, support the initial formation of the vinylogous thioester 4, which is stable only in the correctly folded protein. Upon denaturation and hydrolysis thereof, aminolysis and eventual hydrolysis lead to the observed products.

We thank Dr. M. Spraul, Bruker Analytic, Rheinstetten-Karlsruhe, for the 600-MHz 1H-NMR spectra. D. Merkel thanks the Land Baden-Württemberg for a graduate scholarship. Financial support by the Deutsche Forschungsgemeinschaft and the Fonds der Chemischen Industrie is gratefully acknowledged.
Experimental Part

Preparation of l-Cysteine-Inactivated Enzyme. HAL was purified from a 2-l culture of E. coli BL21 DE3 (pT7-7 hut H) containing the histidase gene from P. putida as described in [3]. Under these conditions, 150–200 mg of enzyme of a specific activity of 35 U/mg could be obtained. HAL was dissolved in 20 ml of 50 mM Na2CO3/NaHCO3 buffer (pH 10.5), and l-cysteine was added to a final concentration of 10 mM. After stirring for 3 h under aerobic conditions, total inactivation was achieved, and the UV/VIS spectra of the enzyme showed an absorption at \( \lambda_{\text{max}} \) 338 nm.

\[ ^{13}\text{S} \] Labelling of the inhibited enzyme was achieved by using l-\(^{13}\text{S}\)Cysteine (10 mCi/ml, ICN), diluting with unlabelled l-cysteine to a final activity of 0.5 MBq.

Denaturation of the Modified HAL and Digestion by Pronase. The inhibited HAL in 0.1m Tris buffer (pH 7.5), containing 10 mM CaCl\(_2\), was concentrated to 2 ml. It was denatured by addition of 6M urea and 0.5% SDS, and heating to 100° for 5 min. The soln. was dialyzed against 0.1m Tris buffer (pH 7.5) and supplemented with 10 mM CaCl\(_2\) to remove the denaturing agents. Digestion was carried out with a final concentration of 1 mg/ml pronase at 37° for 36 h.

Isolation of the Chromophore. To isolate the peptides, which contain the 340-nm chromophore, the pronase digest was separated by HPLC (Hewlett-Packard-Ti series 1050 liquid chromatograph). First, the mixture was applied to a reversed-phase HPLC column (Nucleosil 100 7C18 Macherey-Nagel) and eluted with H\(_2\)O (0.1% CF\(_3\)COOH) (A) and MeCN (0.1% CF\(_3\)COOH) (B). After 10 min washing with 100% A, a linear gradient 0 → 30% B was applied for 90 min at a flow rate of 5 ml/min. The eluate was monitored at 340 nm. Under these conditions, the two main chromophoric products 1 and 2 were eluted after 38 and 42 min, resp. For a further separation, the same column was used, and the two chromophores were isolated with a gradient 0 → 10% B in 60 min and 10 → 40% B up to 90 min. Chromophores 1 and 2 were obtained in sufficient purity for NMR spectroscopy by size-exclusion chromatography (Biosep SEC 2000 column, Phenomenex) with 50 mM phosphate buffer pH 6.8 under isocratic conditions. Finally, the products were desalted using a Grom-Sil 100 ODS-0 AB column (Grom) with a linear gradient to 30% MeCN (0.1% CF\(_3\)COOH) in 30 min.

Spectroscopy. UV Spectra: Cary 3 E UV/VIS spectrometer (Varian) and Lambda 2 UV/VIS spectrometer (Perkin Elmer) for the enzyme assay, and the measurements of the spectra of the irreversibly inhibited HAL.

\(^{1}H\)-NMR spectra: Bruker model DRX-600 in \(^{1}H\)O (pH 6.8).

\((\text{4-L-Cystin-N\textsuperscript{-2}}\text{-yl}methylideneyl)-2-[(\text{glycylamino}ethyl]-4,5-dihydro-5-oxo-1H-imidazol-1-yl]acetyl]-l-aspartic Acid (1). \(^{1}H\)-NMR (600 MHz, \(^{1}H\)O, pH 6.8): 1.62 (d, 3 H, Ala\(_{3}\)); 3.04 (m, 2 H, Asp\(_{3}\)); 3.25 (m, 1 H, Cys); 3.34 (m, 1 H, Cys); 3.43 (m, 1 H, Cys); 3.52 (m, 1 H, Cys); 3.89 (dd, 2 H, Gly\(_{4}\)); 4.27 (m, 1 H, Cys); 4.60 (m, 1 H, Cys); 4.75 (d, 2 H, Gly\(_{4}\)); 4.87 (m, 1 H, Asp\(_{3}\)); 5.12 (q, 1 H, Ala\(_{3}\)); 7.81 + 7.98 (s, 1 H).

\((\text{4-L-Lysin-N\textsuperscript{-2}}\text{-yl}methylideneyl)-2-[(\text{glycylamino}ethyl]-4,5-dihydro-5-oxo-1H-imidazol-1-yl]acetyl]-l-aspartic Acid (2). \(^{1}H\)-NMR (600 MHz, \(^{1}H\)O, pH 6.8): 1.56 (m, 2 H, Lys); 1.62 (dd, 3 H, Ala\(_{3}\)); 1.78 (m, 2 H, Lys); 1.98 (m, 2 H, Lys); 3.02 (m, 2 H, Asp\(_{3}\)); 3.62 (t, 2 H, Lys); 3.88 (dd, 2 H, Gly\(_{4}\)); 3.91 (m, 1 H, Lys); 4.72 (d, 2 H, Gly\(_{4}\)); 4.87 (m, 1 H, Asp\(_{3}\)); 5.11 (q, 1 H, Ala\(_{3}\)); 7.81 + 7.98 (s, 1 H).

MALDI-TOF-MS: Voyager Biospectrometry workstation (PerSeptive Biosystems); dihydroxybenzoic acid as matrix.

Radioactivity Measurements. For the radioactivity measurements, we used a Tri-Carb 2100 TR scintillation counter (Packard).

REFERENCES


*Received February 12, 2000*