The Inactivation of Histidine Ammonia-Lyase by L-Cysteine and Oxygen: Modification of the Electrophilic Center

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Histidine ammonia-lyase (HAL) catalyzes the β-elimination of ammonia from L-histidine and produces urocanic acid in the first step of histidine metabolism (Figure 1). The mechanism employed by this enzyme, along with that of the related phenylalanine ammonia lyase (PAL), is of considerable interest since the proton removed during the elimination event is very nonacidic.[1,2] Crucial to the understanding of this enzyme is the observation that it utilizes an electrophilic prosthetic group in catalyzing the elimination reaction.[3] This prosthetic group has often been thought of as a simple dehydroalanine residue[4−7] despite early evidence that the true species contains additional unsaturation.[8,9] Very recently, the X-ray crystal structure of HAL from *Pseudomonas putida* provided very strong support in favor of a 4-methylidene-imidazol-5-one moiety, MIO, as the electrophilic species (Figure 1).[10] This species is formed by an intrachain cyclization event between Ala142 and Gly144, accompanied by the dehydration of Ser143.

Despite the better understanding of the electrophilic species, the full details of the catalytic mechanism remain unclear. One mechanism proposed by Hanson and Havir suggests that the electrophilic center serves to increase the leaving group ability of the amine (Figure 1, path A).[8] The α-amine of histidine attacks the methylidene carbon of MIO and then the ring unsaturation is isomerized to an exocyclic position ultimately giving species **1**. A subsequent elimination step produces urocanate and the covalently bound ammonia-enzyme adduct **2**. The active form of the catalyst is finally regenerated by the elimination of ammonia from the prosthetic group. This mechanism helps to explain the ordered release of products (ammonia is released last); however, it fails to explain how the β-hydrogen is activated for removal. Recently, Rétey and co-workers have proposed an alternative mechanism in which the electrophilic center serves to acidify the β-proton of histidine (Figure 1, path B).[4,10] The imidazole ring of histidine first attacks the electrophilic center of HAL generating the immonium intermediate **3**. The β-proton is now acidified and an "E1-cB-like" elimination of ammonia occurs to give **4**. Finally, a fragmentation of **4** produces urocanate and reforms the electrophilic catalyst. This mechanism demands that aromaticity

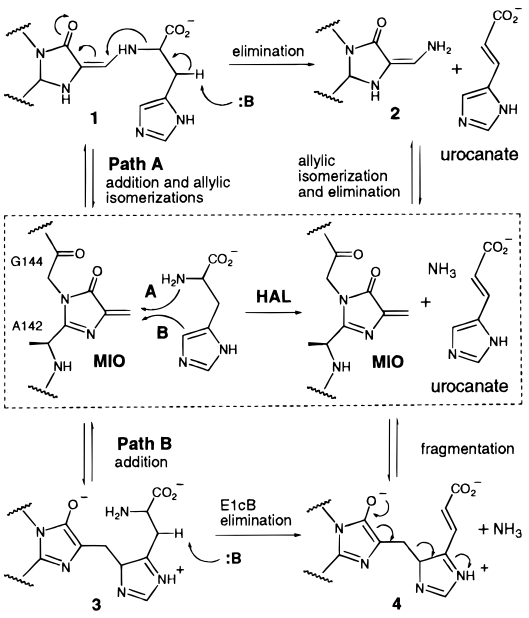

Figure 1. Proposed mechanisms for the reaction catalyzed by histidine ammonia-lyase.

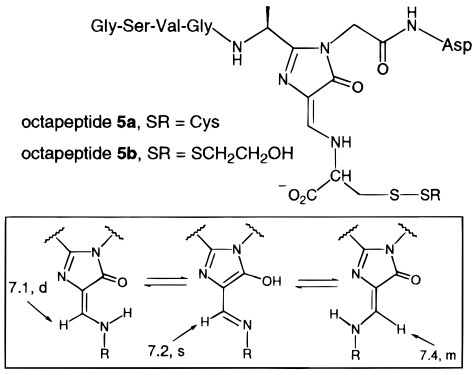

Figure 2. Structure of isolated octapeptides. Inset shows possible conformers/tautomers.

is lost in the substrate side chain (which is particularly troubling in the case of PAL), but this barrier may be somewhat compensated for by the aromatization of the MIO ring in adducts **3** and **4**. Further insights into the nature of this reaction may be gained by investigating the reactivity of the enzyme with substrate analogues. In this paper we report that the inactivation of HAL by treatment with cysteine in the presence of O_2 generates a covalently modified enzyme containing the peptide **5** (Figure 2).

Early reports indicated that the incubation of HAL with the combination of L-cysteine, oxygen, and basic pH leads to an irreversible inactivation of the enzyme.[11,12] This inactivation was accompanied by the appearance of an enzyme-bound chromophore absorbing at 335 nm. Further studies showed that the chromophore survived proteolytic digestion and that it could be isolated as part of an octapeptide containing the modified Ser143.[13,14] We felt that the large-scale preparation of this adduct and its structural determination would reveal the nature of the modification to the electrophilic center in HAL.

Toward this end we have purified 3.6 g of recombinant *P. putida* HAL from 100 L of *E. coli*.[15] The enzyme was incubated

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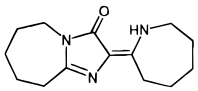
with 50 mM L-cysteine at pH 10.5 for 2 h and the loss of activity was accompanied by the formation of a chromophore centered at 335 nm. The reductive carboxymethylation steps that had been employed in the previous work[13,14] were omitted and the digestions were performed directly on the inactivated enzyme. Digestion with trypsin produced two chromophoric 24-residue peptides that were isolated using HPLC. Each of these peptides was digested with V-8 protease and two new chromophoric octapeptides were isolated. We have assigned the structures **5a** to the minor species and **5b** to the major species (Figure 2).[16] Presumably **5a** is initially formed during the inactivation reaction and disulfide exchange with β-mercaptoethanol takes place during the digestions steps to generate **5b**.[17]

In both cases N-terminal sequencing data showed GSVG as the first four amino acids and the fifth position appeared to be blocked. Total amino acid analysis of the peptides showed peaks for G, A, S, V, D, and C in addition to three unidentified peaks. High-resolution LSI-mass spectra were obtained for both **5a** + H^+ (calcd for $C_{30}H_{47}N_{10}O_{15}S_2$ 851.2667, found 851.2675) and **5b** + H^+ (calcd for $C_{29}H_{46}N_9O_{14}S_2$ 808.2609, found 808.2620) in agreement with the proposed structures. Compound **5b** has been fully characterized by NMR spectroscopy (COSY, HMQC, HMBC) and appears to exist as three conformers/tautomers that are slowly interconverting on the NMR time scale (potential structures are shown in Figure 2 inset).[18] This is most apparent in the three signals attributed to the vinylic proton (7.1, 7.2, and 7.4 ppm in DMSO-d_6). Both peptides were treated with 4 mM dithiothreitol (20 mM phosphate buffer, pH 7.0) to reduce the disulfide bond and generate the free thiols. In the case of **5a**, but not **5b**, released cysteine was detected.[19]

Precedence for the location of the absorbance maximum at 335 nm can be found in compound **6** that contains a nearly identical



6 uv max. = 336 nm

chromophore.[20] In addition, precedence can be found for the slowly interconverting conformers in studies on closely related compounds containing the 4-alkylaminomethylidene-imidazol-5-one structure.[21]

We assume that cysteine is bound in the active site of HAL in a fashion similar to histidine since it acts as a competitive inhibitor at pH 8.6 and since other thiols do not inactivate the enzyme.[9,11] Any proposed mechanism for the formation of **5a** must also explain the role of the thiol since serine, *S*-methylcysteine, and cystine all fail to inactivate the enzyme. An attractive possibility is that at higher pH the thiolate acts as a nucleophile (Figure 3) in a manner analogous to the histidine side chain in the Retey

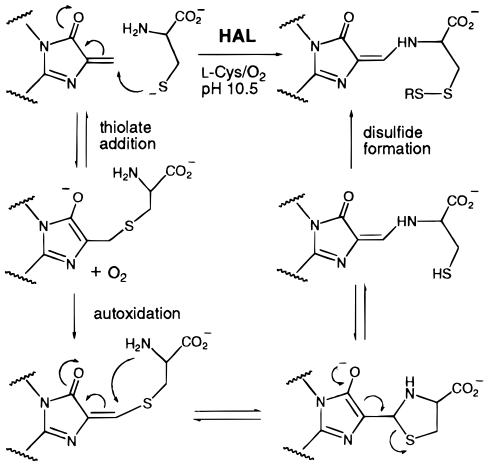


Figure 3. A potential mechanism for inactivation.

mechanism (Figure 1, path B). The addition product is then autoxidized and the exocyclic unsaturation is introduced.[22] Precedence for this transformation is found in the nonenzymatic autoxidation of imidazol-5-ones at the 4-position,[20,23] and in the formation of the green fluorescent protein chromophore.[24] A subsequent S-to-N rearrangement takes place and a final disulfide bond formation generates the observed chromophore. A similar mechanism could be drawn with an initial attack by the α-amine (analogous to the Hanson−Havir mechanism) and without an S-to-N rearrangement;[25] however, it is then more difficult to explain why other amino acids do not inactivate the enzyme. One possibility is that the thiolate serves to position the amino acid via coordination to a metal ion in the active site.[26] Metal ions such as Mn^{2+} and Zn^{2+} have been shown to increase the activity of the enzyme (reduced form) by 5−10-fold[9,15,27] and experiments supporting coordination via the imidazole side chain of histidine have been reported.[28,29] In structural studies on the free enzyme, no metal ion was located in the active site, presumably due to the absence of substrate.[10]

These observations support the assignment of the MIO structure and can be explained in the context of the Rétey mechanism. Further work is underway in an attempt to clearly distinguish between the possible mechanisms for inactivation.

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Supporting Information Available: ^{1}H NMR, COSY, HMQC, and HMBC spectral data and assignments for **5b** (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(16) A structural assignment made previously (see ref 13) involved an adduct with arginine and appears to be incorrect. In that paper reductive carboxymethylation conditions were used prior to protein digestion. Their reported mass of the isolated peptide is consistent with structure **5** bearing a thioacetate in place of the disulfide we isolated.

(17) The digestions were carried out in 20 mM phosphate buffer (pH 7.8 for trypsin, pH 7.3 for V-8 protease) containing 2 mM β-ME and 3 M urea. β-ME was also present in the enzyme storage buffer and traces could have been present during the inactivation.

(18) At this point we cannot rule out the possibility that one of the species is actually a diastereomer obtained upon racemization of the cysteine adduct (the α-proton would be relatively acidic in one of the tautomers (central species in Figure 2, inset)).

(19) The cysteine was detected by PITC derivatization and HPLC retention time analysis. Treatment of both octapeptides in this fashion also gave an as yet unidentified species with a mass of 610 g/mol and a UV max at 326 nm. This byproduct was also observed at various levels during the normal isolation of **5a** and **5b**.

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(25) The inactivation is carried out at pH 10.5 where the cysteine could be bound with its α-amine in a deprotonated form. Another explanation for the requirement of basic conditions could be that it accelerates the audoxidation step through the base-catalyzed elimination of hydrogen peroxide from a hydroperoxide intermediate (in either inactivation mechanism).

(26) Another possible role for the thiolate in such a mechanism is that it could react with oxygen to generate a thiol radical and an O_2^- radical (Wallace, T. J.; Schriesheim, A.; Bartok, W. *J. Org. Chem.* **1963**, *28*, 1311−1314). This could play a role in the oxidation of the cofactor by promoting a hydrogen atom abstraction.

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