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FORMATION OF THE MICHAELIS COMPLEX WITHOUT INVOLVEMENT OF THE PROSTHETIC GROUP DEHYDROALANINE OF HISTIDINE AMMONIA-LYASE

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Abstract

The dehydroalanine-less S143G mutant of histidine ammonia-lyase was constructed and used for kinetic measurements with 5′-nitro-histidine as a substrate. The natural substrate histidine turned out to be a competitive inhibitor of the mutant enzyme and exhibited a K_i value which was similar to its K_m value with the wild-type enzyme. Thus the dehydroalanine prosthetic group does not play a role in formation of the Michaelis complex.

Introduction

Histidine ammonia-lyase [histidase, HAL; EC 4.3.1.3] catalyses the conversion of L-histidine into urocanate by the elimination of ammonia (Fig. 1). 5′-Nitro-histidine is a moderately good substrate of the wild-type enzyme[1,2]. HAL from *Pseudomonas putida* contains dehydroalanine as an electrophilic prosthetic group which is biosynthesised by dehydration of serine 143 of the polypeptide chain[3]. HAL mutants S143A and S143T are virtually inactive with L-histidine as substrate, but convert 5′-nitro-histidine into 5′-nitro-urocanate at the same rate as does the wild-type enzyme[2]. This suggested that the nitro-group fulfils the same role as the prosthetic dehydroalanine and makes it unnecessary. The obvious role of both of them is the activation of the ß-proton of histidine. Abstraction of this proton by an enzymic base must precede the elimination of the amino group. These considerations led us to postulate a novel mechanism for the HAL reaction which includes nucleophilic attack of the imidazole ring at the dehydroalanine residue as a key step. The now electron deficient heterocycle activates the ß-proton for abstraction by an enzymic base (Fig. 1).

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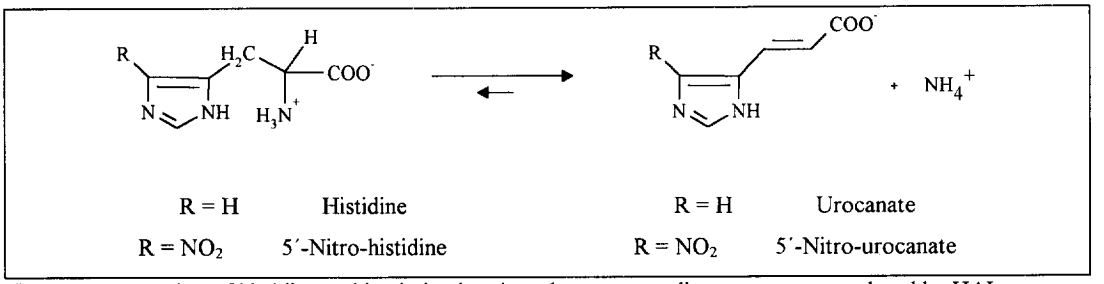

figure 1: Conversion of histidine and its derivatives into the corresponding urocanates catalysed by HAL

In the assumption that L-5′-nitrohistidine occupies the same binding site as does L-histidine we surmised that the S143G mutant would exhibit less steric encumbrance with the nitro group of the alternative substrate than do the wild-type enzyme and the mutants S143A and S143T. This turned out to be the case (vide infra). Here we describe the construction of the mutant HAL S143G and use it to investigate the role of dehydroalanine in the substrate binding. With this mutant we describe inhibition kinetic measurements where the inhibitor is the natural substrate L-histidine and the alternative substrate is 5′-nitro-histidine.

Results and discussion

It has been previously reported that expression of the HAL mutant S143C in *E. coli* BL21(DE3) cells leads to a high level of recombinant active enzyme[4]. For preparation of the mutant S143G site-directed mutagenesis was started using a DNA isolate from the mutant S143C. This mutant was chosen instead of wild-type HAL gene because here only one base had to be changed and the codon usage of *E. coli* was also appropriate. The mutant S143G was constructed and overexpressed using the same expression system as for wild-type HAL and its other mutants[3]. The rate of expression of the mutant S143G was comparable to that of wild-type HAL, i.e., 80-90 mg per liter of *E. coli* culture. SDS/PAGE gel electrophoresis and Western blot analysis of the wild-type HAL[2] and the mutant S143G showed that both proteins are indistinguishable by their molecular masses (figure 2).

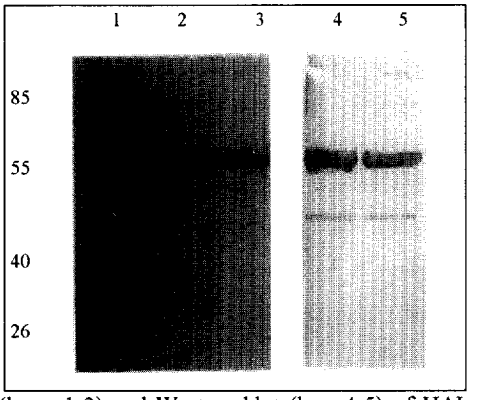

figure 2: SDS/PAGE (lanes 1-3) and Western blot (lane 4-5) of HAL and mutant S143G. Lane 1: molecular mass standard; lane 2: wild-type HAL; lane 3 mutant S143G; lane 4: wild-type HAL; lane 5: mutant S143G

[2] The mutant S143C is processed to wild-type HAL[4].

The kinetic constants K_m and V_{max} with L-histidine as substrate of the mutant S143G were estimated and compared to those of recombinant wild-type HAL and the mutants S143A and S143T (table 1). As previously reported[3,4], the mutant S143A and S143T showed similar K_m-values as wild-type HAL whereas their V_{max}-values were 1170 and 53100 times lower, respectively. These results identified serine 143 to be the precursor of the prosthetic dehydroalanine. As expected, the mutant S143G shows a similar K_m-value (5.3 mM) and a V_{max} value of 0.0047 IU/mg that is 5310 times lower than that of fully active wild-type HAL. This mutant is also unable to form dehydroalanine resulting in a virtually inactive enzyme.

Table 1: Comparison of the kinetic parameters from wild-type (wt) HAL and mutants S143A, S143T and S143G with L-hisidine as substrate

enzyme	K_m [mM]	V_{max} [IU/mg]	V_{wt}/V_{mutant}
wt	5.2	25	
S143A	7.5	0.0214	1170
S143T	3.1	0.00047	53100
S143G	5.3	0.0047	5310

To test the reactivity of the mutant S143G with 5′-nitro-histidine as substrate the corresponding K_m- and V_{max}-values were determined. The kinetic constants were compared to those of wild-type HAL and its other mutants. Table 2 shows the results of the kinetic analyses. Depending on the size of the active site residue S143T, S143A, wild-type HAL and S143G the K_m-value for 5′-nitro-histidine decreases from 12.8 mM to 9.8 mM to 7.7 mM and 4.0 mM. On the other hand, the V_{max} values are about the same. It can be concluded that in agreement with the mechanism postulated by Langer et al.[2] the nitro group of the substrate fulfils the same task as the dehydroalanine prosthetic group.

Table 2: Comparison of the kinetic parameters from wild-type HAL and the mutants S143A, S143T and S143G with 5′-nitro-histidine as substrate

enzyme	K_m [mM]	V_{max} [IU/mg]
wt	7.7	0.85
S143A	9.8	0.87
S143T	12.8	0.87
S143G	4.0	0.52

To investigate the role of dehydroalanine in the substrate binding mutant S143G was assayed with 5′-nitro-histidine as a substrate and histidine as an inhibitor. The K_i value was determined spectrophotometrically at 378 nm. The double reciprocal plot is depicted in figure 3. In this set-up histidine acts as a competitive inhibitor of mutant S143G. The K_i-value was estimated to 5.3 ± 0.4 mM which is, within the limit of error, identical with the K_m-value measured with wild- type HAL for L-histidine as the substrate (table 3)[3,5].

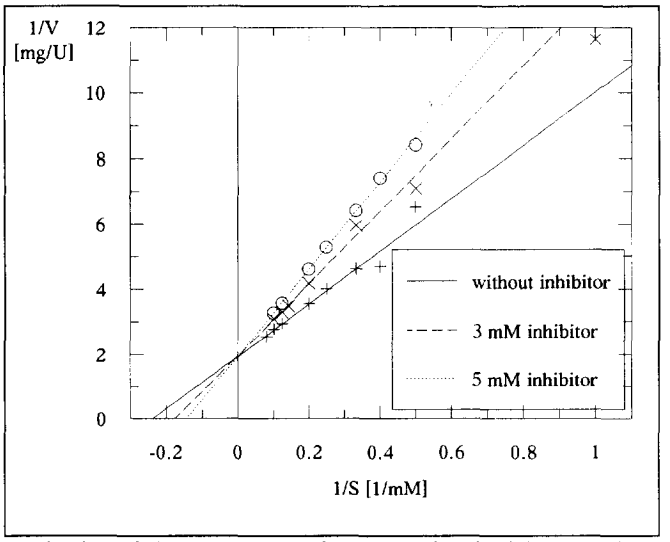

figure 3: Lineweaver-Burk plot of the mutant S143G with 5′-nitro-histidine as substrate and L-histidine as inhibitor. The rate of reaction was determined spectrophotometrically at $\lambda = 378$ nm. The K_i-value was estimated to 5.3 ± 0.4 mM.

Table 3: Comparison of the K_m values measured with wild-type HAL[2], recombinant wild-type HAL[3] and mutant S143G for L-histidine as substrate and the K_i value measured with mutant S143G for 5′-nitro-histidine as substrate and L-histidine as competitive inhibitor

	kinetic constants of enzyme	
histidases	K_m [mM]	K_i [mM]
wild-type *Pseudomonas* ATCC 11299b	4.0	
recombinant wild-type *E. coli* BL21	5.2	
HAL mutant S143G	5.3	5.3 ± 0.4

This result clearly indicates that the prosthetic dehydroalanine is only important for enzyme catalysis where its abrogation leads to a 1000 - 53000 fold reduction of V_{max} measured with L-histidine. However the substrate binding in the Michaelis complex is independent of the presence of the prosthetic dehydroalanine. To our knowledge it is the first time that a natural substrate has been used as inhibitor of a mutant enzyme.

Biological Assays

Bacterial Strains, Plasmids, Culture Conditions and Substrates.

Escherichia coli SURE cells (purchased from Stratagene) were used for the isolation of single-stranded (ss) DNA from M13mp 19 phage (Boehringer Mannheim) to carry out site-specific mutagenesis. Cells were grown and infected as described in the lab manual[6].

E. coli BL21 (DE3) cells served for the expression of either wild-type or mutant histidase. For overexpression, cells were grown as described by Langer et al.[3]

pGEM 11-Zf-(-) (Promega) was used as subcloning vector.

The expression vector pT7.7 was generously provided by Stanley Tabor[7].

5-Nitro-histidine was synthesised as described by Klee[1] and its quality was checked by ^{1}H NMR spectroscopy. L-histidine was purchased from Fluka.

Site-Directed Mutagenesis.

Instead of recombinant wild-type HAL the recombinant mutant S143C was used to subclone a *Xba*I-*Sal*I fragment into M13mp 19. The exchange from cysteine 143 to glycine was performed starting from this construct, so only one base had to be changed. Site-specific mutagenesis was performed by following the protocol of Amersham mutagenesis kit (Sculptor)[8]. HAL S143G was constructed as described by Langer[3]. The oligonucleotide sequence used in this mutagenesis reaction was 5′-GTGGGTGCT*G*GCGGCGACCTG-3′.

Purification, SDS/PAGE, Western Blot Analysis, and Protein Assays.

Purification of recombinant histidase was carried out as described in detail by Langer et al.[3].

SDS/PAGE was performed according to Laemmli[9] using 10% polyacrylamide gels. For staining of the gels Coomassie Brillant Blue R 250 was used. Western blotting was carried out as described by Symingteon et al.[10] using nitrocellulose as blotting filter. Histidase was detected with a polyclonal antibody that was raised in rabbits against wild-type histidase isolated from *Pseudomonas putida*. Protein determinations were performed by measurement of A_{260} and A_{280} according to Warburg and Christian as described by Lane[11].

Enzyme Assay

The assay used in this work was described by Klee[1,5]. The enzyme was reduced in the presence of 0.001 M glutathione. Excess thiol was removed by washing the enzyme solution with 100 volumes of phosphate buffer by the use of a stirred cell with a nominal molecular mass limit of 30 kDa. The reduced enzyme was kept under

nitrogen. Measurements were performed at 25 °C at 378 nm, and each value was calculated from 5-8 individual measurements. The extinction coefficient ($\varepsilon_{378\ nm}$) of nitro-urocanic acid is 8680 l mol^{-1} cm^{-1}. K_m and V_{max} values were determined using the double-reciprocal plot.

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