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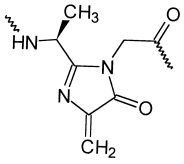
Spectroscopic Evidence for a 4-Methylidene Imidazol-5-one in Histidine and Phenylalanine Ammonia-Lyases**

Dagmar Röther, Dietrich Merkel, and János Rétey*

Histidine ammonia-lyase (HAL, histidase, EC 4.3.1.3) and phenylalanine ammonia-lyase (PAL, EC 4.3.1.5) catalyse the nonoxidative deamination of their amino acid substrates to form *trans*-urocanate and *trans*-cinnamate, respectively.[1] HAL initiates histidine degradation both in bacteria and in animals.[2, 3] Its deficiency in humans causes the disease histidinemia.[4] PAL is an important plant enzyme at the crossroads of primary and secondary metabolism. Its product, cinnamate, is the precursor of lignins, flavonoids, and coumarins.

The two enzymes are highly homologous in their amino acid sequence and a prosthetic dehydroalanine was postulated at their active sites.[5–7] Overexpression in *E. coli* combined with mutagenesis experiments showed that the prosthetic group is formed autocatalytically from serines 143 and 202 of HAL and PAL, respectively.[8–10] Recently Schwede et al. solved the 3D structure of HAL by X-ray crystallography.[11] The structure revealed that the prosthetic group is not dehydroalanine but 4-methylidene imidazol-5-one (MIO, **1**). This novel electrophilic group is formed by autocatalytic cyclisation of an Ala–Ser–Gly tripeptide portion of the protein precursor, a process which is concomitant with the elimination of two molecules of water.



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We investigated the UV spectra of wild-type HAL and of the mutant S143A which lacks the MIO group to provide spectroscopic evidence for such a chromophore. Figure 1a shows the UV difference spectra of HAL mutant S143A and wild-type HAL from 240 to 360 nm, measured at enzyme concentrations of 0.2, 0.4, 0.8, and 1.6 mg mL^{-1}. The UV spectrum of wild-type HAL exhibits a discrete maximum between 305 and 310 nm whose intensity grows with increasing enzyme concentration. We propose that this maximum originates from the MIO group, which contains a cross-conjugated double bond system. The mutant lacks this conjugated system and, therefore, it shows no absorption maximum around 308 nm.

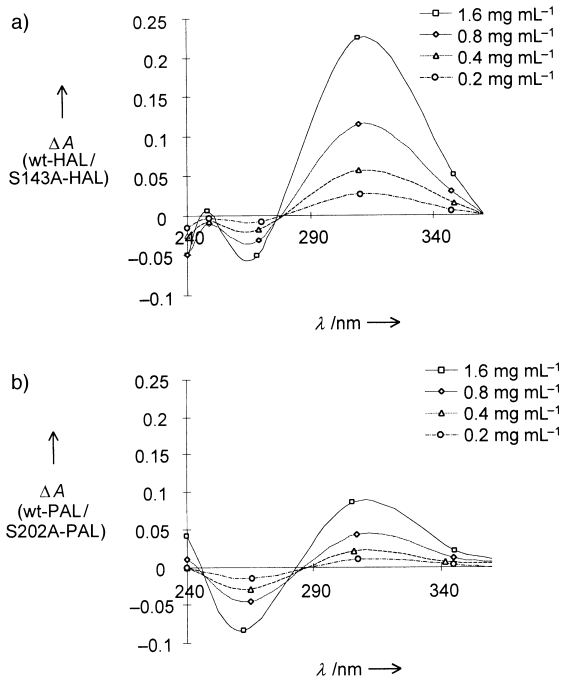

Figure 1. a) UV difference spectra of HAL mutant S143A and wild-type HAL; b) UV difference spectra of PAL mutant S202A and wild-type PAL. wt = wild-type.

[*] Prof. Dr. J. Rétey, D. Röther, Dr. D. Merkel
Institut für Organische Chemie und Biochemie der Universität
Richard-Willstätter-Allee, 76128 Karlsruhe (Germany)
Fax: (+49) 721-608-4823
E-mail: biochem@ochhades.chemie.uni-karlsruhe.de

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The X-ray structure of PAL has not yet been solved. We performed UV spectrophotometric measurements with the wild-type enzyme and the mutant S202A to decide whether PAL also has an MIO group at the active site. Figure 1b shows the UV difference spectra of mutant S202A and wild-type PAL, measured at enzyme concentrations of 0.2, 0.4, 0.8, and 1.6 mg mL^{-1}. As in the HAL experiment one observes a maximum whose intensity grows with increasing enzyme concentration. The maximum is positioned between 305 and 310 nm.

Comparison of Figures 1a and 1b shows that the UV difference spectra of HAL and PAL are very similar, but PAL, at the same protein concentration, shows somewhat lower absorbance; this could be due to the higher molecular weight of PAL (312 relative to 215 kDa for HAL). In addition, PAL is much less stable than HAL. As a consequence, it is partially inactivated during the concentration of the solution by ultrafiltration, a process which is necessary for measuring the UV difference spectra. It has been noted that MIO, as a strong electrophile, is only stable within the intact active site. Before the concentration procedure both the wild-type and the mutant PAL samples were homogeneous in electrophoresis and were treated identically in every respect.

An absorbance maximum of wild-type HAL at approximately 315 nm was previously reported by Klee[12] but no explanation was given for this phenomenon. To exclude the possibility that the difference spectra just show relative differences in absorbance, we performed partial digestion of HAL and PAL to locate the chromophore in smaller peptides. First the enzymes were denatured with 6 M urea followed by dialysis and then incubation with pronase. The digest was applied to a reversed phase Nucleosil 100 C18 column. The peptide fragments were separated with a gradient of H_2O/CH_3CN, supplemented with 0.1% trifluoroacetic acid (TFA). In the digest of wild-type HAL we found a few peaks showing absorbance between 300 and 310 nm (Figure 2). No peptide fragment of the HAL mutant S143A showed this absorbance during the whole separation. Peptide fragments of wild-type PAL also exhibit an absorbance around wave lengths comparable to the absorbance in the UV difference spectra (Figure 2). No such maximum could be observed in any fragment of the PAL mutant S202A. Although repeated experiments led to the same results, in the case of wild-type HAL and wild-type PAL we also found peptide fragments which showed absorption maxima at higher wavelengths ($\lambda_{max} = 335$ nm). These fragments contained a modified MIO group arising from reaction with certain amino acids, such as lysine, which are present in the pronase digest.[13a] Compounds carrying a very similar chromophore to (substituted) MIO are known and exhibit λ_{max} values in the same region.[13b]

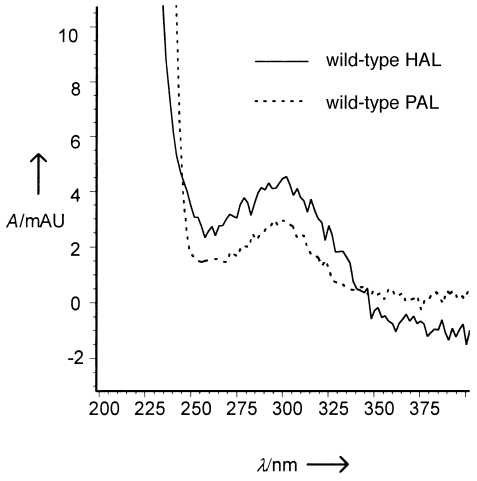

Figure 2. UV spectra of two of the peptide fragments separated after pronase digestion. The solid line shows the UV spectrum of a separated fragment (retention time of 65 min) of digested wild-type HAL. The dashed line shows the UV spectrum of a separated fragment (retention time of 14 min) of digested wild-type PAL.

We conclude that HAL and PAL are closely related enzymes which both contain the MIO group at their active sites. This also indicates that the mechanisms of action of HAL and PAL are analogous, as recently postulated.[14, 15]

Experimental Section

Bacterial strains and plasmids: HAL and PAL were overexpressed in *E. coli* BL21(DE3) cells. The gene for HAL from *Pseudomonas putida* was subcloned in the expression vector pT7-7.[8] The gene for PAL from *Petroselinum crispum* was changed to the codon usage of *E. coli* and cloned in vector pT7-7, followed by a transformation in *E. coli* BL21(DE3) cells containing vector pREP4-GroESL.[16]

Site-directed mutagenesis: HAL mutant S143A was produced by Langer et al.[8] PAL mutant S202A was produced by following the instruction manual of the QuickChange site-directed mutagenesis kit (Stratagene).[17] The oligonucleotides used in the mutagenesis reaction were as follows: S202A(+): 5′-CATCACTGCTGCCGGCGACCTGG-3′; S202A(−): 5′-CCAGGTCGCCGGCAGCAGTGATG-3′. The mutation was checked by sequence analysis using the dideoxynucleotide chain termination method.[18]

Protein expression and purification: Both HAL and PAL were expressed and purified as described in Ref. [8, 10, 16].

Enzyme activity and protein determination: The activities of HAL and PAL were measured spectrophotometrically.[19, 20] Determination of protein concentration was carried out according to Warburg and Christian[21] as modified by Layne.[22]

Spectroscopic measurements: UV difference spectra were measured at enzyme concentrations of 1.6, 0.8, 0.4, and 0.2 mg mL^{-1} in 10 mm Tris-HCl buffer (pH 7.2, Tris = Tris(hydroxymethyl)methylamine) from 240 to 360 nm using 1 cm quartz cuvettes. A blank experiment containing the mutant lacking the 4-methylidene imidazole-5-one was measured first, followed by a scan of the wild-type enzyme.

Enzyme denaturation and pronase digest: Enzyme (30 mg) was dialysed against 100 mm Tris-HCl buffer (pH 7.5) for 15 h and then denatured with 6 M urea and 0.5% sodium dodecylsulfate (SDS) for 10 min at 100 °C. The denatured enzyme was dialysed against 100 mm Tris-HCl buffer (pH 7.5) containing 10 mM $CaCl_2$, followed by concentration of the solution to 1.5 mL by ultrafiltration through a 10 kDa concentration unit (Millipore). After addition of 4 mg pronase (Roche Diagnostics), the mixture was incubated for 48 h at 37 °C. Pronase was eliminated by ultrafiltration.

Separation of the digest by HPLC: The pronase digest was separated by HPLC using a Hewlett–Packard Ti-Series 1050 liquid chromatography system. The solution was applied to a preparative-grade reverse-phase Nucleosil 100 C18 column (Macherey-Nagel) equilibrated with solvent A (twice-distilled H_2O containing 0.1% TFA). After washing with solvent A for 10 min, elution was performed at a flow rate of 5 mL min^{-1} with a linear gradient of increasing concentration of solvent B (CH_3CN containing 0.1% TFA) over a period of 90 min (0–30% B). Absorbance signals were measured at 215, 315, and 340 nm and the UV spectrum of each peak was monitored with a diode array detector.

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Tris(pyrazolyl)methanesulfonates: A Novel Class of Water-Soluble Ligands

Wolfgang Kläui,* Michael Berghahn, Gerd Rheinwald, and Heinrich Lang

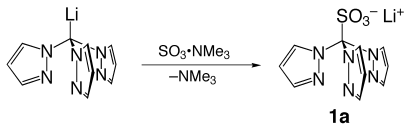
In 1966 Trofimenko introduced a new tripodal nitrogen-donating ligand, hydrotris(pyrazolyl)borate, into coordination chemistry. Soon these anions found their way into coordination chemistry as versatile nitrogen-based chelating ligands. Nowadays the substituted hydrotris(pyrazolyl)borates (in the following abbreviated as Tp) are the most important class of N_3 tripodal ligands.[1] The possibility of increasing the steric demands of the ligand through bulky substituents in the 3-position of the pyrazole ring is an important aspect in the chemistry of Tp ligands.[2] Such sterically demanding Tp ligands play an important role in the synthesis of various enzyme models.[3]

Vahrenkamp and co-workers were able to obtain a Zn–hydroxide complex with $Tp^{Cum,Me}$ ligands[4] which showed, for example, hydrolytic activity towards activated esters, amides, and nonactivated phosphate esters, and thus functioned as a stoichiometric model for esterases, peptidases, and phosphatases.[5] However, due to the insolubility of this particular complex in water it is neither possible to determine a pK_s value for a zinc-bound water molecule in aqueous solution, nor is it possible to add water during the hydrolysis reaction to regenerate the $Tp^{Cum,Me}$-Zn-OH complex. Therefore, the hydrolysis reaction shows only stoichiometric not catalytic behavior.[6] Another problem is the sensitivity of the B–N bond in hydrotris(pyrazolyl)borate ligands to hydrolysis. Even by substituting the proton in the 5-position of the pyrazolyl ring with a methyl group, the hydrolysis of the B–N bond cannot be completely avoided.[7]

Especially in the field of enzyme models it is of great interest to obtain complexes that are soluble and stable under physiological conditions. The introduction of functional groups that would generate water solubility in hydrotris(pyrazolyl)borate is still an unsolved problem to date. With this in mind, our aim was to find a ligand that would prove to be water-soluble and stable towards hydrolysis, and thus could be used as an alternative to hydrotris(pyrazolyl)borates.

A suitable ligand system should have a similar arrangement of the donor centers as in hydrotris(pyrazolyl)borate and offer the possibility to introduce hydrophilic groups in a straightforward manner. We have found that the isosteric and isoelectronic tris(pyrazolyl)methane ligand proved to be a suitable starting material. In this class of ligands, first published by Hückel and later made more easily accessible by a more facile preparation reported by Juliá, the B–N bonds are substituted by C–N bonds.[8, 9] The methine proton of tris(pyrazolyl)methane is sufficiently acidic to be removed by butyllithium, and the resulting reactive intermediate readily reacts with electrophiles.[10]

Since our goal was the introduction of a hydrophilic moiety, we added the lithiated tris(pyrazolyl)methane to a sulfurtrioxide–trimethylamine complex and obtained the lithium salt of tris(pyrazolyl)methanesulfonic acid (**1a**; LiTpms) as shown in Equation (1). A metathesis reaction of **1a** with potassium carbonate gave the corresponding potassium salt (**1b**; KTpms).



1a (1)

In contrast to tris(pyrazolyl)methane, **1b** is almost exclusively soluble in water and only moderately soluble in methanol. Another distinct difference to tris(pyrazolyl)methane[11] and especially to hydrotris(pyrazolyl)borate is the fact that the reported ligand Tpms (**1**) is stable over a wide range of pH values in aqueous solution. At pH 0 only small amounts of pyrazole—the product resulting from a hydrolysis

[*] Prof. Dr. W. Kläui, M. Berghahn
Institut für Anorganische Chemie und Strukturchemie der
Heinrich-Heine-Universität Düsseldorf
Universitätsstrasse 1, 40225 Düsseldorf (Germany)
Fax: (+49) 211-81-12287
E-mail: klaeui@uni-duesseldorf.de
Dr. G. Rheinwald, Prof. Dr. H. Lang
Institut für Chemie
Lehrstuhl für Anorganische Chemie
Strasse der Nationen 62
09107 Chemnitz (Germany)

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