NMR STUDIES ON THE MECHANISM OF ACTION OF UROCANASE

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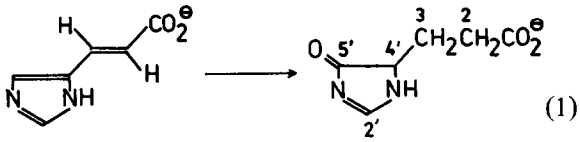
1. Introduction

Urocanase catalyses the conversion of urocanate into imidazolone propionate (eq. (1)):



(1)

We showed in [1] that one solvent proton is incorporated into each of the positions 2, 3 and 4′ of the product. The addition of these protons takes place stereospecifically at C-2 and C-3, whereas it is non-stereospecific at C-4′. Direct monitoring of the urocanase reaction by ^{1}H NMR spectroscopy indicated the intermediacy of an enol form of the final product. It has been shown in [2,3] that urocanase contains a firmly bound NAD^+ molecule which is essential for catalytic activity. The exact role of this NAD^+ in the catalysis is however not yet clear, since no direct hydrogen transfer from C-5′ of urocanate to any position of the product could be detected.

Here we present evidence that urocanase catalyses the exchange of the 5′-H atom of urocanate faster than it promotes the overall reaction. Moreover the competitive inhibitor, imidazole propionate, will also exchange its 5′-H atom upon incubation with urocanase.

2. Materials and methods

Urocanase has been isolated from *Pseudomonas putida* (ATCC 11299 or 12633) as in [3]. Urocanic and imidazole propionic acids were prepared according to [4] and [5], respectively. 2′-Mercapto-urocanic acid was synthesised from ergothioneine (Roth, Karlsruhe) as in [6] and was spectroscopically characterized.

The ^{1}H NMR spectra were recorded with Bruker WH 90 or WH 300 spectrometers.

For preparation of the samples potassium phosphate buffer (0.2 M, pH 7.5) was repeatedly lyophilized in ^2H_2O (99.98%) in order to exchange all protium against deuterium. Urocanic acid was recrystallized from deuterium oxide, urocanase (spec. act. 1.3 U/mg) precipitated with ammonium sulphate, centrifuged, the pellet carefully washed with deuterium oxide and dissolved in the deuterated phosphate buffer (vide supra).

For the NMR experiments the following mixtures were prepared:

(i) 12 mg urocanic acid × 2 ^2H_2O; 4.8 mg K_2CO_3; 0.15 ml enzyme solution (0.72 U) and 1 ml deuterated phosphate buffer;
(ii) 5 mg imidazole propionic acid; 2 mg K_2CO_3; 0.2 ml enzyme solution (0.89 U) and 0.5 ml deuterated phosphate buffer;
(iii) 5 mg 2′-mercapto-urocanic acid; 2 mg K_2CO_3; 0.2,ml enzyme solution (0.89 U) and 5 ml deuterated phosphate buffer.

The solutions in the NMR tubes were charged with argon and the spectra were recorded in about 20 min intervals (fig.1a–e). The exchange experiments with imidazole propionate [^{1}H NMR: δ = 2.53 ppm (*t*,*J* = 7 Hz, 2 H), δ = 2.92 ppm (*t*,*J* = 7 Hz, 2 H), δ = 7.06 ppm (*s*, 1 H) and δ = 8.18 ppm (*s*, 1 H)] and with 2′-mercapto-urocanate [^{1}H NMR: δ = 6.30 ppm (*d*,*J* = 16 Hz, 1 H), δ = 7.11 ppm (*d*,*J* = 16 Hz, 1 H), δ = 7.2 ppm (*s*, 1 H)] were monitored in a similar way but at 90 MHz.

3. Results

In the ^{1}H NMR spectrum of urocanate at 300 MHz

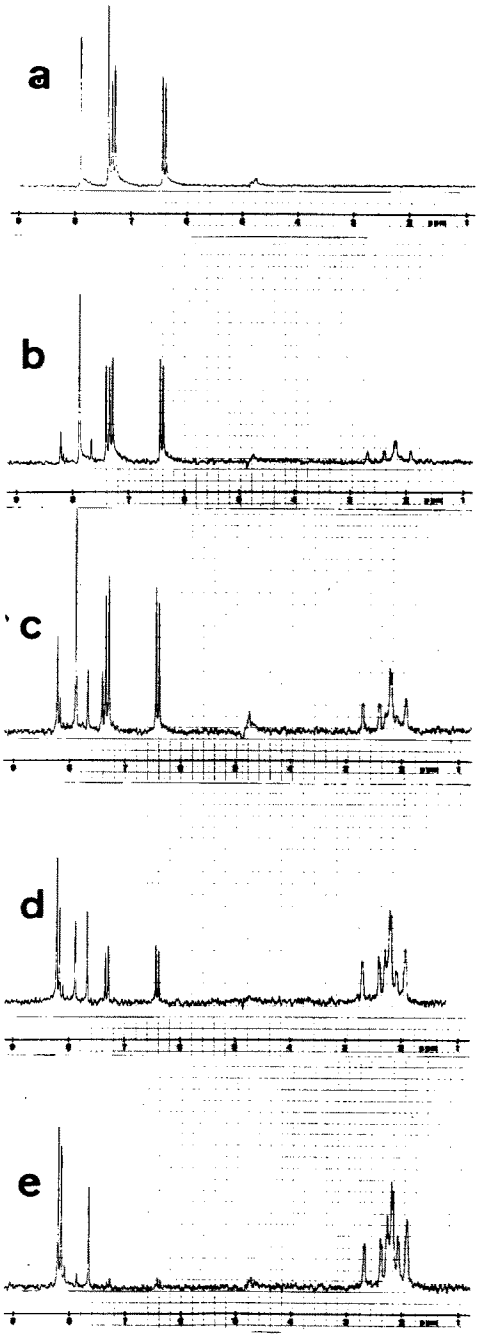


Fig.1. Kinetics of the urocanase reaction in deuterium oxide as monitored by ^{1}H NMR spectroscopy at 300 MHz. The spectra (200 scans) were recorded at 0 (a), 20 (b), 40 (c), 66 (d) and 89 (e) min after starting the reaction. The water peak was eliminated by gated decoupling. The residue of this peak was arbitrarily taken as 4.86 ppm. For further details see section 2.

the AB signal arising from the olefinic protons is cleanly separated from the singulets at 7.41 and 7.90 ppm corresponding to the protons at carbon 5′ and 2′ of the imidazole ring (fig.1a). Upon incubation with a trace of urocanase the characteristic signals of urocanate slowly decrease and new signals appear (fig.1b–d). However, the singulet arising from the 5′-H atom diminishes much faster than the signals of the remaining 3 carbon-bound urocanate protons. After 66 min of incubation this signal has completely vanished although a considerable portion of the starting material is still present.

In a non-enzymic experiment it has been shown that none of the carbon-bound urocanate protons will be exchanged under the conditions of the above NMR experiment. Heating a solution of urocanic acid (90°C, 2 h) in deuterium oxide led to exchange of the 2′-proton but not of the 5′-proton. When a sample of [2′-^{2}H$_1$]-urocanate thus obtained was incubated with urocanase in deuterium oxide no ^{1}H NMR signals could be detected between 7.7–8.2 ppm, indicating that the singulets normally seen in this frequency range (spectra a–e in fig.1) arise from the 2′-H atoms of the substrate, of the product and of some intermediates.

Urocanase-catalysed proton exchange also in position 5′ of imidazole propionate as revealed by ^{1}H NMR spectroscopy. This exchange was however somewhat slower. No such exchange could be observed with 2′-mercapto-urocanic acid which is neither a substrate nor an inhibitor of urocanase.

4. Discussion

Although the mechanistic significance of the urocanase-promoted exchange of the 5′-H-atom of urocanate with solvent protons is not yet apparent, it has already some implications in connection with previous findings. In particular the observed lack of hydrogen transfer from position 5′ of the substrate to any other position of the product [1], that made a redox function of the enzyme-bound NAD$^+$ doubtful [2,3], can now be readily explained by the prior exchange of this proton. The role of the coenzyme is thus again open for discussion. It is noteworthy that the competitive inhibitor of urocanase, imidazole propionate, is subject to a similar exchange, whereas 2′-mercapto-urocanate is inert. It is tempting to speculate that imidazole propionate is able to undergo the first part of the urocanase reaction consisting of reversible

oxidation of the imidazole ring by NAD^+ followed by tautomerization. The second portion of the urocanase reaction would then be the reduction of the side chain double bond by NADH. Experiments to prove or disprove such a mechanism are under way.

Acknowledgements

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