Proposal for the Mechanism of Action of Urocanase. Inference from the Inhibition by 2-Methylurocanate

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Dedicated to Professor Helmut Simon on the occasion of his 60th birthday

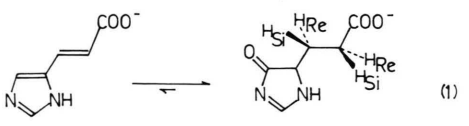
Urocanase Mechanism, Novel Role of NAD

Incubation of urocanase with 2-methylurocanate leads, after an initial normal reaction, to a time dependent inactivation of the enzyme. It is suggested that a tautomeric form (**1**) of the product, 2-methyl-imidazolone propionate, is the actual inhibitor. On the basis of these and of published experimental data a novel mechanism is proposed for the urocanase reaction. The crucial and initial step is the electrophilic addition of enzyme-bound NAD to the 2-position of the imidazole nucleus of urocanate.

Introduction

The second step in histidine degradation is the hydration of urocanic acid by the enzyme urocanase yielding imidazolone propionic acid (Eqn. (1)). Inspite of many suggestions [1–5] the mechanism of this unique transformation remains a chemical challenge. The enzyme faces the problem of attacking the electron-rich imidazole ring with the weak nucleophile water. (The electron deficient side chain of urocanic acid is not sufficient to change the nucleophilic character of the imidazole nucleus.) In fact it is easy to achieve electrophilic substitutions in positions 2, 4 and 5 of imidazoles under conserving the aromatic character of the nucleus.

Since a tightly bound NAD is not only a constituent of urocanase but is also essential for its activity it is at hand to speculate that NAD is the electrophile that first attacks the imidazole ring of the substrate. A mechanism starting with this step has been recently proposed [4]. Here we describe the inhibitory nature of the substrate analogue, 2-methylurocanate and delineate a novel mechanism both consistent with all known experimental facts and chemically plausible.



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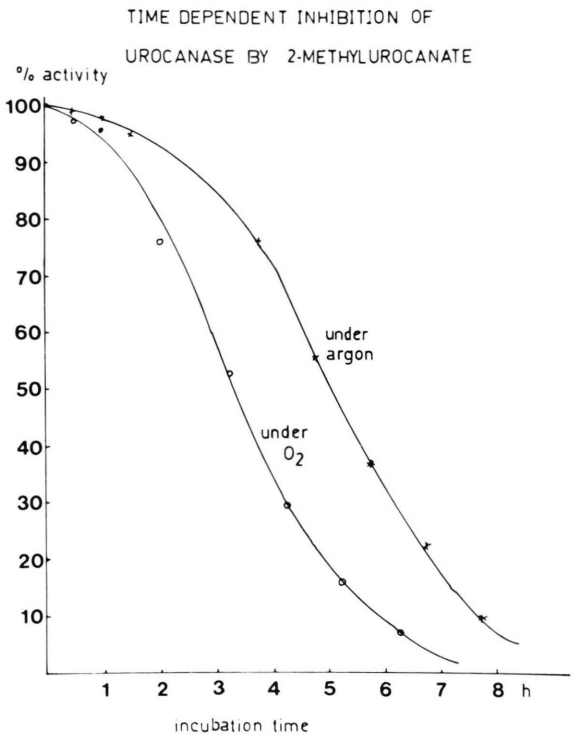
Materials and Methods

Urocanase (spec. activity ~ 2 U/mg) has been isolated from *Pseudomonas putida* (ATCC 11299) as in [3]. Urocanic and 2-methylurocanic acids were prepared according to [6] and [1, 7], respectively.

The inhibition measurements were carried out as described in the figure legend. After 95% inhibition the incubation mixtures were dialysed against potassium phosphate buffer (0.05 M, pH 7.5, 5 l) overnight to remove excess 2-methylurocanate and 2-methylimidazolone propionate. The urocanase assay showed only 1% of the original activity indicating irreversible inhibition both in the anaerobic and aerobic incubation.

Results and Discussion

2-Methylurocanate has recently been shown to be a slowly reacting substrate for urocanase from *Pseudomonas putida* by ^{1}H NMR spectroscopy [7]. A thorough analysis of the ^{1}H NMR spectra suggested that the enzyme was inactivated during the reaction. In this paper we show (Fig. 1) that the inactivation is time-dependent with a pronounced lag period. As expected, the inactivation is dependent on the concentration of 2-methylurocanate (not shown). With 15 mM 2-methylurocanate 95% inactivation was reached after 8 h under anaerobic conditions. In the presence of oxygen this inactivation period was shortened to 6 h but the shape of the curve was similar. The inactivation was irreversible, *i.e.*, prolonged dialysis against dilute buffer did not result in reactivation. Furthermore, using [α-^{14}C]-2-methylurocanate radioactivity was shown to be associated with



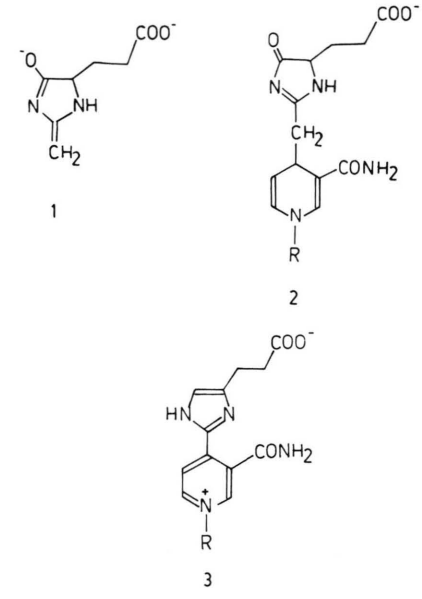
The incubation mixtures contained: 2-methylurocanate (30 μmol), urocanase from *P. putida* (12.5 U), potassium phosphate buffer (0.05 M, pH 7.5) in a total volume of 2 ml. The incubations were carried out either under argon or oxygen at 25 °C. Aliquots (50 μl) were withdrawn initially in 0.5 h intervals, later as indicated on the diagram. The urocanase assays were performed after a 20:1 dilution with potassium phosphate buffer (0.05 M, pH 7.5) and using urocanate as substrate and following the decrease of absorption at 277 nm [16].

the urocanase protein (J. Herman and J. Rétey, unpublished).

All these results indicate that not the substrate but some form of the product was the inhibitory species.

Before the next conclusion can be drawn we have to recall that the methyl protons of 2-methylimidazolone propionate undergo a slow spontaneous exchange with solvent deuterium at pH 7 [7]. In this exchange the enolic intermediate **1** was implicated, which is a potent nucleophile. If **1** occurs at the active site of urocanase it could react with the tightly bound NAD leading to irreversible inhibition.

For the product of the inhibition we tentatively assign structure **2**. If the nucleophilic species **1** adds to the 4-position of the nicotinamide ring at the active site, the further inference is at hand that the tightly bound NAD is situated close to position 2 of the imidazole ring. For the normal reaction we therefore postulate the addition of NAD to C-2 of the imidazole ring of urocanate as a starting chemical step. Before developing the further steps of a novel mechanism we briefly recapitulate all published experimental data relevant to the mechanism.



Survey of experimental data

Labelling experiments showed that both the carbonyl oxygen and the two vicinal side chain H_{Re}-atoms of the product originate from water [8, 9]. ^1H NMR monitoring of the reaction revealed furthermore an enzyme-catalyzed exchange of the 5-H atom of the imidazole with solvent protons [4, 5, 10, 11]. This exchange is substantially faster than the overall reaction [4, 5, 10, 11].

The same exchange was observed also with imidazole propionic acid which is a competitive inhibitor of urocanase, but not with 2-mercapto-urocanate which is neither a substrate nor a competitive inhibitor [10]. 2-Methylurocanate, a reluctant substrate of urocanase, is subject of the same exchange [7].

Urocanase from *Pseudomonas putida* and beef liver contains a tightly bound NAD that is necessary for catalytic activity [3, 12]. Its reduction ($NaBH_4$, sodium dithionite) abolishes urocanase activity and so does the presence of some other nucleophilic reagents (NH_2OH, NH_2OCH_3, $HSO_3^{\ominus}$ etc.). Most of these inhibitions are at least partially reversible upon regenerating the enzyme-bound NAD.

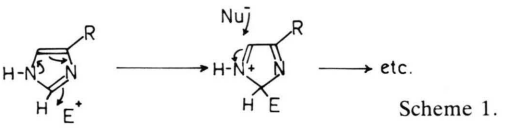
Both the reduction of urocanase and its reaction with nucleophilic reagents result in an increase of the absorption at about 330–340 nm [3, 12]. This can be attributed to enzyme-bound NADH and to the NAD-nucleophile adduct, respectively. Hug *et al.* [13] observed a similar increase of the absorption at 331 nm upon binding the competitive inhibitor, imidazole-propionate to urocanase. More recently Matherly *et al.* [14] isolated the corresponding product from urocanase the ^{1}H NMR spectrum of which indicated that it was an adduct between imidazole-propionate and NAD. To obtain this adduct it was necessary to denature the enzyme with SDS and stabilize the adduct by oxidation with phenazine methosulphate. If this treatment did not cause an artefact then adduct formation occurred at the active site of urocanase and may be analogous to the reaction between substrate and enzyme-bound NAD.

More doubt emerges concerning the interpretation of the ^{1}H NMR spectrum of the isolated adduct. The argument for the alleged attachment between position 4 of the nicotinamide ring and the τ nitrogen of the imidazole is weak. Furthermore the lack of a signal for the 2-H-atom of the imidazole ring is not satisfactorily explained.

On the basis of their conclusions for the structure of the NAD-imidazole propionate adduct Egan, Matherly and Phillips [4] proposed a mechanism whose initial steps involve nucleophilic attack of the τ imidazole nitrogen of urocanate at the 4-position of NAD. It is not obvious why the postulated adduct formation should facilitate the subsequent reaction steps. Neither is the explanation for the enzyme-catalyzed proton exchange as a side reaction plausible.

The mechanism proposed

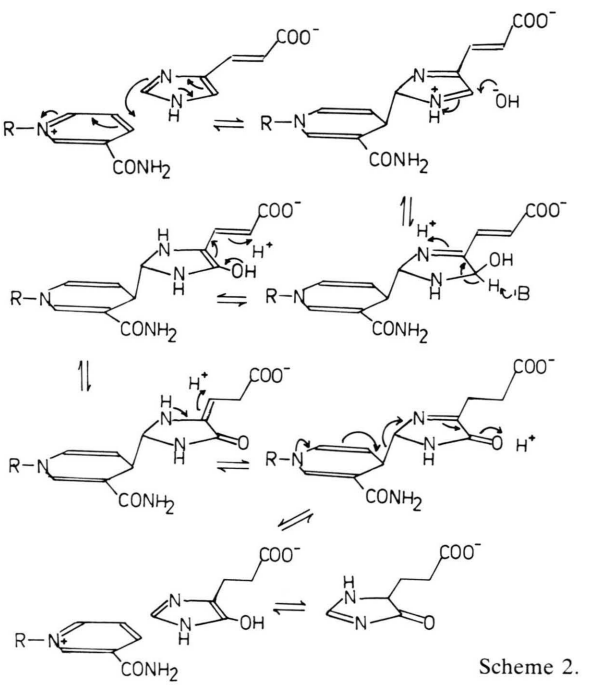
In our opinion an initial electrophilic attack at the 2-position of the imidazole ring makes much more sense for a reasonable mechanism. Not only has such an attack a chemical analogy (*e.g.* diazotation of imidazoles [15]) but it causes immediately an "Umpolung" of the imidazole nucleus (Scheme 1).



Scheme 1.

In 1981 Sawada *et al.* [5] proposed protonation of the imidazole 2-position as an initial step with simultaneous addition of a hydroxyl ion to the 5-position. In the light of the results of Matherly *et al.* [14] we propose that the electrophile in question be the enzyme-bound NAD (Scheme 2).

The NAD-imidazole propionate adduct isolated by Matherly *et al.* [14] would have structure **3**.



Scheme 2.

Acknowledgements

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