TRANSITION-STATE ANALOGS OF AN ALIPHATIC AMIDASE

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1. Introduction

The use of transition-state analogs in the elucidation of enzyme mechanisms has assumed importance in recent years [1, 2]. The idea that synthetic compounds resembling the proposed transition-state should be extremely effective inhibitors compared to ground-state analogs, and should bind more effectively even than the substrate, has been substantiated. Recent studies with papain [3] and elastase [4] showing that aldehydes with the appropriate peptide side chains are extremely effective inhibitors has led to the proposal that the free aldehyde reacts with an amino acid residue (cysteine–papain; serine–elastase) in the active centre to form a hemithioacetal or hemiacetal; this being the 'true' transition-state analog on the reaction pathway to acylenzyme formation. Aldehydes, however, exist in appreciable amounts in aqueous solution as hydrates formed rapidly by a general base catalysed reaction [5]. At the present time there is no way of determining whether the true inhibitor is the free aldehyde or its hydrate.

This paper presents evidence that, at least for the aliphatic amidase from *Pseudomonas aeruginosa*, it is the hydrated aldehyde that is the true inhibitor. This conclusion is substantiated by the observation that acetaldehyde–ammonia is a potent inhibitor and might be considered to be a transition-state analog for amide hydrolysis.

2. Materials and methods

A partially purified preparation (Stage III) of the amidase from *Pseudomonas aeruginosa* (PAC 111) was obtained from Dr. P.H. Clarke of University College London; it was purified by linear gradient elution (0.15–0.35 M KCl in 0.1 M Tris buffer pH 7.2) from a DEAE-Sephadex column [6]. Acetohydroxamate and propionohydroxamate were prepared by the method of Fishbein et al. [7]. Acetaldehyde and propionaldehyde were freshly distilled prior to use. Formaldehyde was obtained as a commercial aqueous solution and used without further treatment. Acetamide, propionamide, formamide and ethylamine were obtained from B.D.H, (Poole, England); acetaldehyde–ammonia was obtained from R.N. Emanuel (London, England).

The kinetic constants for acetamide and propionamide hydrolysis were determined by measuring the ammonia released by the Berthelot procedure [8]. In the case of formamide the ninhydrin method described by Brown et al. [6] was used. The kinetic constants for acylhydroxamate hydrolysis and inhibition constants (acetohydroxamate as the substrate) were measured by the ferric chloride method [9]. Kinetic data were fitted directly to the Michaelis–Menten equation by the method of least squares [10].

3. Results and discussion

The kinetic constants for the amide and hydroxamate substrates are shown in table 1. Although an accurate value for k_{cat}/K_m was obtainable for formamide, due to the apparently high K_m with attendant substrate inhibition it was not possible to obtain values for K_m and k_{cat} and only lower and upper limits respectively are recorded. The results show that although there is a factor of approximately 2000 in the range of K_m and k_{cat}/K_m, k_{cat} itself only varies by a factor of about 17.

Table 1

Kinetic and inhibition constants for *Pseudomonas aeruginosa* amidase. These were determined in 0.05 M potassium phosphate buffer at pH 7.2 and 25°C.

Substrate or inhibitor	K_{eq}	K_i (M)	K_m (M)	k_{cat} (sec^{-1})	k_{cat}/K_m ($M^{-1}sec^{-1}$)
Formamide	–	–	> 2	< 312	6.25×10^2
Formaldehyde*	1000**	3.8×10^{-4}	–	–	–
Methanol*	–	0.7	–	–	–
Acetamide	–	–	8.3×10^{-4}	975	1.17×10^6
Acetohydroxamate	–	–	6.7×10^{-3}	85	1.27×10^4
Acetate	–	5.7×10^{-3}****	–	–	–
Ethylamine	–	2.3×10^{-2}	–	–	–
Ethanol	–	1.1	–	–	–
Acetaldehyde	0.93**	1.1×10^{-4}	–	–	–
Acetaldehyde–NH_3	11.9***	1.6×10^{-5}	–	–	–
Propionamide	–	–	7.8×10^{-3}	2245	2.9×10^5
Propionohydroxamate	–	–	7.5×10^{-2}	133	1.78×10^3
Propionaldehyde	0.69**	1.2×10^{-4}	–	–	–

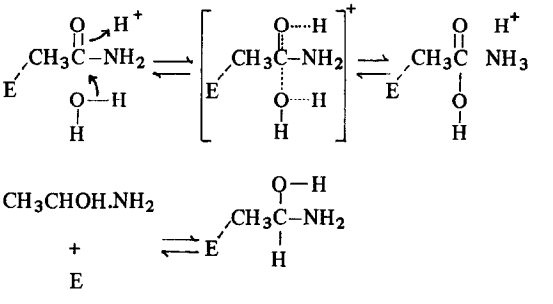
* Methanol (10%) is a normal additive of commercial formaldehyde.
** K_{eq} = [hydrated aldehyde]/[aldehyde]. Data from [11].
*** K_{eq} = [acetaldehyde–NH_3]/[aldehyde]. Value from [12] at pH 9.4.
**** At pH 5.5.

All the inhibitors gave linear competitive inhibition and here the range in the inhibition constants was of the order of 70 000. All the aldehydes were very effective inhibitors whereas related compounds such as ethylamine and ethanol were poor inhibitors. Acetate, a product of the reaction, was a good inhibitor but was only effective in the acid form.

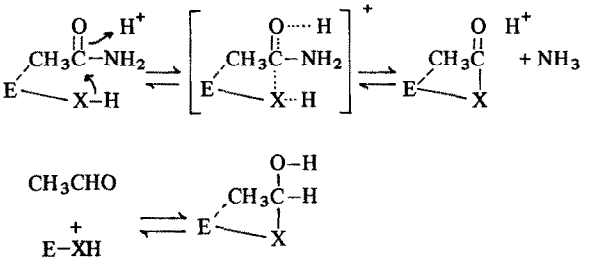
The observation that formaldehyde was a potent inhibitor and the fact that this aldehyde exists almost exclusively in the hydrated form in aqueous solution [11] strongly suggests that it is the hydrated aldehyde that is the true inhibitor. If the free aldehyde was the inhibitor the K_i would be 3.8×10^{-7} M and this seems extremely unlikely in view of the apparently very high K_m for formamide. Assuming that the hydrated aldehyde is the effective inhibitor then the true K_i values for propionaldehyde, acetaldehyde and formaldehyde are 4.9×10^{-5}, 5.3×10^{-5} and 3.8×10^{-4} M respectively. There appears to be little correlation between these inhibition constants and the kinetic constants; the closest being with the k_{cat} values. The lack of correlation is not surprising in view of the fact that even in a relatively simple mechanism, such as the substitution (ping pong) mechanism of many hydrolases, kinetic constants are composites of a number of rate constants and at least four transition-states occur during one cycle of enzyme activity.

The assumption that the hydrated aldehyde is the true inhibitor is substantiated by the observation that acetaldehyde–ammonia is the most potent inhibitor of all (table 1). There is some doubt as to the K_{eq} for acetaldehyde–ammonia dissociation in aqueous solution at pH 7.2 [12], however, the K_i at pH 9.0 was found to be 2.7×10^{-5} M clearly implicating the addition compound rather than the free aldehyde as the inhibitor.

These conclusions have an important effect on the interpretation of the mechanism of action of the amidase. The results suggest that the reaction proceeds through a ternary complex (sequential mechanism) involving the elimination of ammonia by water (Scheme 1) rather than a substitution mechanism involving only



Scheme 1



Scheme 2

binary complexes in which an acyl–enzyme compound is formed as an intermediate (Scheme 2). This conclusion is supported by the work of Evans and Wolfenden [13] who showed that the tetrahedral compound 1,6-dihydro-6-hydroxymethylpurineribonucleoside was a possible transition-state analog for adenosine aminohydrolase and later they provided convincing evidence [14] that this enzyme most probably proceeds via a sequential or elimination mechanism.

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