ON THE USE OF *p*-NITROANILIDES AS SUBSTRATES FOR PROTEOLYTIC ENZYMES

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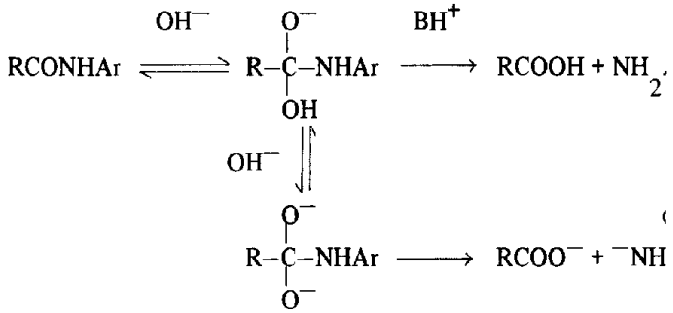
1. Introduction

p-Nitroanilides have become increasingly common recently as substrates for proteolytic enzymes due to the obvious advantage of allowing amide bond cleavage to be followed spectrally. They have been used as substrates for trypsin [1], chymotrypsin [2–5], elastase [6,7], aminopeptidase M [8], papain [1,9] and subtilisn [10]. It is unclear, however, whether *p*-nitroanilides are normal substrates for these enzymes. For example, although a good correlation exists between the acylation rates (k_{cat}) of chymotrypsin by a series of ring substituted *N*-acetyl-tyrosine anilides and σ^-, the rate constant for the corresponding *p*-nitroanilide is fasted than predicted by a factor of over 100 [3]. In addition, acylation of chymotrypsin by nitro-substituted *N*-benzoyltyrosine anilides shows a rate enhancement as electron-withdrawal increases [5], whereas for other substituents the rate is increased by electron-donating groups [2–4].

Furthermore, in the non-enzymatic hydrolysis of *p*-nitro-substituted anilides, it has been postulated that a different mechanism is involved from that operating with other anilides [11,12]. This mechanism involves loss of *p*-nitroanilide ion from the dianion of a tetrahedral intermediate (schema 1b),



rather than the generally accepted pathway for other substituted anilides, loss of free aniline from the monoanion (schema 1a). Since *p*-nitroanilides appear to hydrolyze through a different pathway from other anilides in aqueous solution, it is reasonable to suppose that their enzymatic hydrolyses may also be anomalous.

We and other [13,14] have recently commented on the apparent deviation of the rate of acylation of chymotrypsin by *p*-nitroanilides and have concluded that the initially observed correlation is fortuitous and *N*-acyltyrosine-*p*-nitroanilides are not, in fact, abnormally reactive.

The only remaining drawback then to the acceptance of *p*-nitroanilides as normal substrates for proteases is their unusual mechanism of alkaline hydrolysis. However, use of this process as a model for the acylation of proteases suffers from the defect that expulsion of the leaving group occurs from a dianion, which is obviously not possible in the enzymatic case. A better model would be the cleavage of the monoanion of the tetrahedral intermediate (I), since it corresponds more closely to the postulated enzymatic

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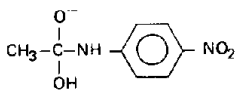
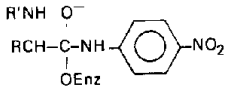


I



II

tetrahedral intermediate (II). It is of interest then to determine the identity of the leaving group from the monoanion intermediate. In a recent publication, we showed that there are two pathways for the alkaline hydrolysis of 2,2,2-trifluoro-*p*-nitroacetanilide [15]. At high pH (>8), the reaction proceeds via the dianion intermediate with expulsion of the anilide ion, similar to the high pH hydrolysis of *p*-nitroacetanilide. At lower pHs, however, the reaction proceeds through the monoanion of the intermediate with general acid catalyzed expulsion of neutral *p*-nitroaniline (scheme 1a). This result shows that breakdown from the monoanion is not anomalous and suggests that the analogous cleavage of the tetrahedral intermediate for the acylation of chymotrypsin proceeds through loss of neutral *p*-nitroaniline, similar to acylation by other anilides, and that acylation of chymotrypsin by *p*-nitroanilides does not involve an unusual mechanism.

In this report, we wish to extend our prior work to *p*-nitroacetanilide itself, since *p*-nitrotrifluoroacetanilide is a poor model for the anilide substrates generally used for proteases, due to its extreme lability to alkaline hydrolysis. *p*-Nitroacetanilide should be a much better model, since its hydrolytic lability is more like that of the enzyme substrates.

2. Experimental

p-Nitroacetanilide was synthesized by acylation of *p*-nitroaniline with acetic anhydride. m.p. 214°C; lit. m.p. 214–215°C [11].

The rate constants were determined by an initial rate technique as described elsewhere [11].

3. Results and discussion

The rate of hydrolysis of *p*-nitroacetanilide was examined at pH values of 10.1–11.0 at 25.0°C in sodium bicarbonate–sodium carbonate buffers. At each pH, runs were done at sodium carbonate concentrations ranging from 0.06 M to 0.3 M with the ionic strength kept at 1.0 by the addition of NaCl. Plots of k^{obs} vs. carbonate concentration were linear. The slopes of these plots gave the catalytic constant, k_B, and the intercepts gave the hydroxide catalyzed rate constants, k_{OH^-}. The reaction was also found to be generally catalyzed by *n*-propylamine buffers at 45.0°C (total amine concentration 0.1 M–0.5 M) and a similar analysis gave k_B for propylamine. The rate constants for these processes are collected in table 1. These values of k_{OH^-}, along with the rate constant at pH 11.58 [11], are plotted in fig. 1. Least squares analysis of the data given a slope of 1.07 ± 0.1 at 25°C and 1.10 ± .09 at 45°C, showing that the reaction is of the first-order in hydroxide ion. The calculated value of the second-order rate constant for this process at 25°C is $1.39 \pm .36 \times 10^{-4} \cdot M^{-1} \cdot sec^{-1}$. The corresponding rate constant for acetanilide is about $7 \times 10^{-6} \cdot M^{-1} \cdot sec^{-1}$ [16, 17].

Table 1

Rate constants for hydrolysis of *p*-nitroacetanilide by hydroxide ion, carbonate ion and *n*-propylamine.

pH*	$k_B(10^7\ sec^{-1})$**	$k_{OH^-}(10^8\ sec^{-1})$**
Carbonate (25.0°C)		
10.11	4.06 ± 0.14	2.14 ± 0.16
10.13	4.01 ± 0.15	1.64 ± 0.17
10.51	3.23 ± 0.19	5.1 ± 0.3
10.52	3.36 ± 0.32	3.4 ± 0.5
10.59	3.16 ± 0.31	3.37 ± 0.05
11.01	2.29 ± 0.84	18.9 ± 1.7
Propylamine (45.0°C)		
10.52	10.5 ± 1.1	17.2 ± 0.15
10.52	9.99 ± 1.70	22.3 ± 0.24
11.08	9.93 ± 1.13	78.9 ± 0.34
11.10	8.13 ± 1.00	82.9 ± 0.30
11.51	7.39 ± 1.05	317 ± 5
11.55	12.9 ± 0.6	211 ± 3

* All pH measurements at 23°C.

** Rate constants were obtained by weighted least squares analysis of plots of k^{obs} vs. [*B*]. Errors are standard deviations.

These results may be analyzed by the generally accepted scheme for hydrolysis of other substituted anilides (scheme 1a). The slope of 1.0 for the plot of log *k* vs. pH indicates that the intermediate is the monoanion, and general catalysis implies a proton transfer in the slow step. In addition, the small rate acceleration for *p*-nitroacetanilide over acetanilide

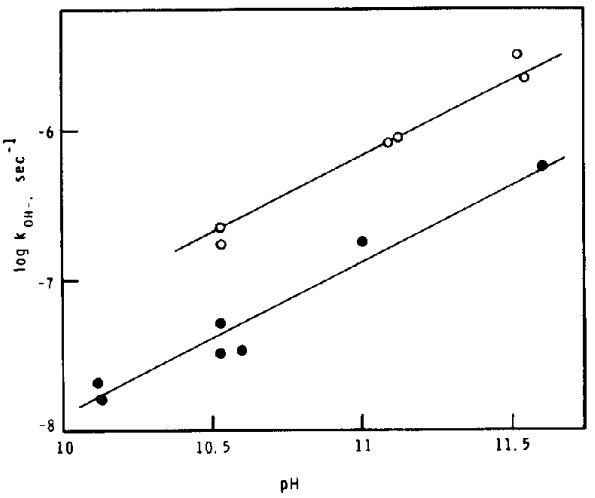


Fig. 1. pH dependence of the pseudo first-order rate constant for the alkaline hydrolysis of *p*-nitroacetanilide.

(~ 20-fold) is consistent with the generation of little or no negative charge on the nitrogen in the transition state. In contrast, the high pH mechanism which involves loss of the negative *p*-nitroanilide from the dianion ion shows a very large effect of ring substituent on the rate ($k_{p\text{-}NO_2}/k_H$ = 7000) [11,17].

The above results suggest that the first-order process for the alkaline hydrolysis of *p*-nitroacetanilide is identical to that for other anilides, even though the second-order process is anomalous. Since it is the first-order process which provides the best model for acylation of proteases, there is no longer any reason from model studies to suspect that *p*-nitroanilides are anomalous substrates for enzymes. These results, coupled with our previous explanation of the apparent anomaly of *N*-acetylt-tyrosine-*p*-nitroanilide acylation of chymotrypsin, indicate that the use of *p*-nitroanilides as enzymatic substrates is entirely appropriate.

Acknowledgement

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