Detection of covalent enzyme-substrate complexes of nitrilase by ion-spray mass spectroscopy+

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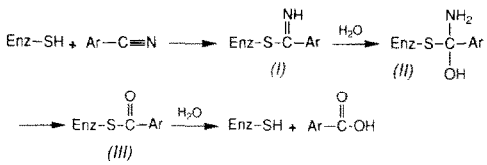
Nitrilase from *Rhodococcus* ATCC 39484 was found to consist of two species of M_r 40258±2 and 40388±2 Da. When the enzyme was incubated with nitrile substrates and the reaction quenched with acid, higher M_r species were observed. The mass differences were consistent with addition of a substrate molecule to each species. These results represent the first reported demonstration that this, or any other nitrilase forms a covalent intermediate with its substrates. The observation that the intermediate, suggested to be either a thioimidate or an acylenzyme, can be trapped by acidification indicates that the rate of breakdown of the intermediate is rate-limiting.

Nitrilase; Mass spectroscopy; Nitrile hydrolysis; Covalent catalysis

1. INTRODUCTION

There has been much interest in recent years in the use of nitrilases to produce carboxylic acids from their readily-available nitrile analogues [1,2]. The enzymic route benefits from mild conditions which allow hydrolysis of nitriles containing other functional groups. There have been few reports in the literature on the mechanism of these enzymes and the only information available is that all nitrilases characterized so far appear to contain a catalytically-essential thiol [3–10]. On the basis of the known reaction between nitriles and thiols to form thiomidates, nitrile hydrolysis by nitrilases has been proposed to proceed through a thioimidate intermediate [4,5,11]. The first step of the reaction would be attack of the thiol on the carbon of the nitrile (Scheme 1), with concomitant protonation of the nitrogen to form the thioimidate (i). Attack by water, accompanied by further protonation on nitrogen (thioimidate hydrolysis is acid-catalysed; base catalysis brings about an elimination to give the free nitrile and thiol [12]) would lead to the tetrahedral intermediate (ii). Collapse of the tetrahedral intermediate would lead to ammonia and an acyl-enzyme (iii) which would react with water to form the acid product. The thiol protease papain has been shown to form a thioimidate in the presence of peptide-nitriles, which are efficient inhibitors of the enzyme [13], lending weight to the idea that nitrilases operate via a thioimidate.



Scheme 1.

In the present work we have used ion-spray [14] (pneumatically assisted electrospray [15,16]) mass spectroscopy (MS) to probe for the accumulation of covalent intermediates in nitrilase-catalyzed nitrile hydrolysis.

2. MATERIALS AND METHODS

2.1. *Chemicals*

Chemicals were obtained from Aldrich (Milwaukee, Wisconsin, USA), Anachemia (Montreal, Quebec, Canada), or Sigma (St Louis, Missouri, USA).

2.2. *Enzyme Production and Purification*

Nitrilase was obtained from *Rhodococcus* ATCC 39484 [17], essentially using the methods of Kobayashi et al. [10] to induce production of the enzyme and purify it to homogeneity. Electrophoretic analysis under denaturing conditions [18], at high sample loading, showed one strong band of M_r 41.5 kDa plus some very faint impurity bands.

2.3. *Mass Spectral Analysis*

Purified nitrilase was dialysed (into 5 mM ammonium acetate pH 7.5, containing 1 mM DTT) and concentrated to 3 mg/ml using an Amicon ultrafiltration cell (Amicon, Danvers, Massachusetts, USA). The resulting solution was stored frozen at −30°C in 90 μl portions until needed. Thawed nitrilase solution was acidified with glacial acetic acid (10 μl) and infused directly into an ion-spray interface [19] fitted to a triple quadrupole mass spectrometer (Sciex, Thornhill, Ontario, Canada). The effect of aromatic compounds on the spectrum was determined by their addition to the enzyme solution, thorough

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shaking and incubation at 25°C for 30 s before addition of the glacial acetic acid. Liquid compounds were added neat (0.5 μl) and solids as a saturated solution in methanol (1 μl).

3. RESULTS AND DISCUSSION

Fig. 1a shows the low-resolution ion-spray spectrum obtained from nitrilase. Fig. 1b shows the partial spectrum for the 24 charge-state ions at M/z 1675–1695. Data collected under high-resolution conditions (not shown) yielded masses for the two species of 40 258 ± 2 (1) and 40 388 ± 2 (2) Da, a difference of 130 ± 2 Da. There are a number of possible explanations for the two species: proteolysis of a terminal residue during purification (Glu, Gln, Lys or Met fit within the error limits), two isoenzymes with different sequences or fractional post-translational modification. The presence of two polypeptides complicates the analysis of results but is not a serious problem. Fig. 1c shows the signal obtained after reaction with benzonitrile (M_w 103). Peak (1) has reduced intensity due to formation of a higher molecular weight adduct which overlaps the signal from peak (2), increasing its apparent intensity. The higher M_r polypeptide has formed the same adduct, leading to a third peak. The separation between the first and third peak is 237 ± 4, in good agreement with the expected value of 233 (130 + 103). The spacings between adjacent peaks (114 ± 4 and 123 ± 4 Da) are intensity weighted averages of the spacing due to the adduct (103) and the difference between the polypeptides (130); the spectrometer was unable to resolve the overlapping two middle peaks. In control samples in which the enzyme had been denatured by heating (70°C, 10 min) before adding benzonitrile, or the acetic acid was added before the substrate, no adduct formation was observed. Other aromatic compounds with polar substituents (benzaldehyde and methyl benzoate) showed no adduct formation under the conditions used. These controls indicate that adduct formation requires an intact active-site and that non-covalent adducts are not formed. It is thus very likely that the procedure has trapped a covalent enzyme-substrate complex which forms faster than it breaks down. Fig. 1d shows the result obtained with 3-chlorobenzonitrile (M_w 137.5). The result is essentially the same but the peak separations are now 135 ± 4 and 137 ± 4 Da. Fig. 1e shows the result obtained with 2-methoxybenzonitrile (M_w 133). Again, an adduct is seen and the peak separations are 131 ± 4 and 139 ± 4 respectively. This result is very interesting because, while benzonitrile and its 3-chloro derivative are good substrates for *Rhodococcus* nitrilase, 2-substituted benzonitriles are very poor substrates [10]. The detection of a covalent adduct between 2-methoxybenzonitrile and nitrilase indicates that it is the rate of breakdown of the covalent intermediate which determines the rate, for both good and poor substrates, rather than Michaelis-complex formation or

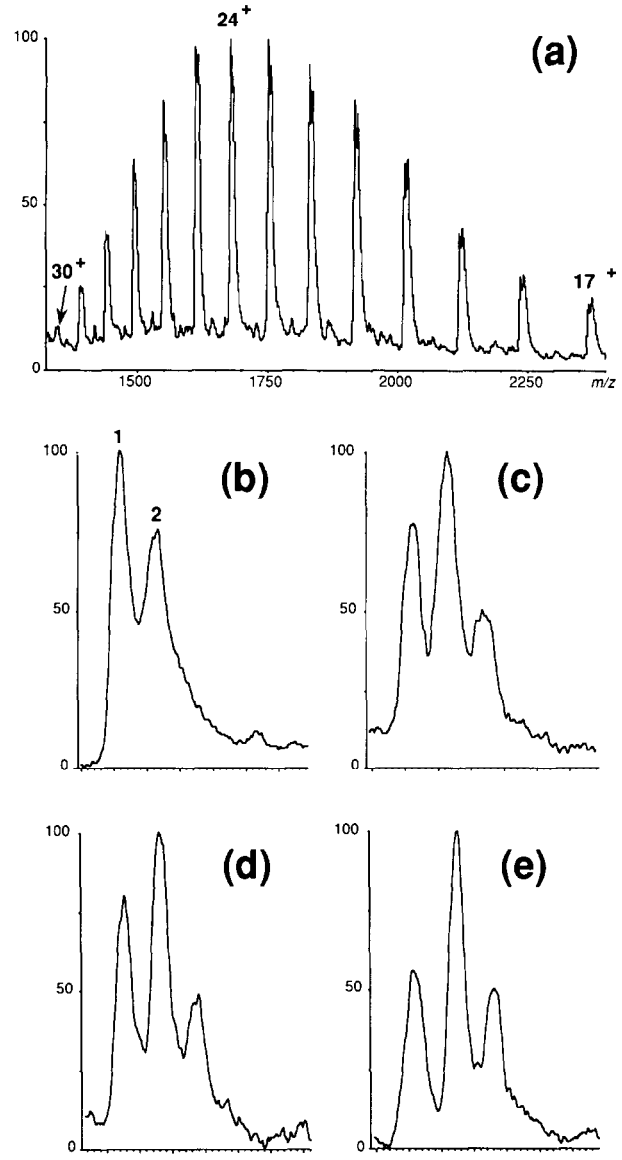

Fig. 1. (a) The ion-spray spectrum of nitrilase; the multiply charged protein ions having 17–30 of the basic amino acid residues (Arg, Lys, His) protonated, have been detected. (b) The partial spectrum of the enzyme from the 24 charge-state region. Peaks (1) and (2) correspond to the two polypeptides. The partial spectra of the enzyme, after incubation with benzonitrile (c), 3-chlorobenzonitrile (d) or 2-methoxybenzonitrile (e), followed by quenching with acid, are shown for comparison.

covalent bond formation. It is possible that the enzyme could bind to and carry out at least the first step of the reaction with many different nitriles but later steps are fast only with 'good' substrates.

It is not possible to distinguish between the two covalent intermediates proposed to exist in the nitrilase reaction, thioimidate (i) and acylenzyme (iii) (M_r difference 1 Da) at the current instrumental precision. We are currently studying the mechanism of the enzyme in more detail, in order to elucidate the structure of the covalent intermediate.

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