Detection and accumulation of tetrahedral intermediates in elastase catalysis

(serine proteases/enzyme mechanisms)

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ABSTRACT Tetrahedral intermediates in the reaction of elastase with specific di- and tripeptide p-nitroanilide substrates have been detected, accumulated, and stabilized at high pH by using subzero temperatures and fluid aqueous/organic cryosolvents. The tetrahedral adducts are characterized by spectra with \( \lambda_{\text{max}} \) of 359 ± 2 nm, compared with that of 380 nm for p-nitroaniline and 315-320 nm for the substrates. The maximal concentration of intermediate that could be accumulated varied with the different substrates from 40 to 100% of the active enzyme present. The pH dependence of the reactions indicated that formation of the tetrahedral intermediates was rate-limiting at low pH (pK\text{a} = 7.0 at -39°C) and that collapse to the acyl-enzymes was rate-determining at high pH. When corrected for the effect of temperature and cosolvent, the rate of intermediate formation was in good agreement with that measured at 25°C in aqueous solution by stopped-flow techniques.

The existence of tetrahedral intermediates in the enzyme-catalyzed reactions of esters and amides has been a long-standing question of major concern in mechanistic enzymology. That such intermediates are on the reaction pathway has been predicted on the basis of their existence in corresponding nonenzymatic systems (1) and the presence of "oxyanion holes" (detected by x-ray crystallography) that can stabilize the incipient tetrahedral oxyanion by H bonding (2-4). There has been some question, however, as to whether they would exist as discrete intermediates or as transition-state complexes (5, 6). Considerable indirect kinetic evidence suggests that they may be discrete intermediates (7-12). Recently Richards and co-workers (13) have reported direct observation of such a species in a stopped-flow spectrophotometric study of the elastase-catalyzed hydrolysis of a p-nitroanilide (PNA) substrate, Ac-Ala-Pro-Ala-PNA. A similar observation has now been made in the case of trypsin and N\text{a}-Ac-L-Lys-PNA (14).

We have been exploring the use of subzero temperatures as a means of allowing the detection, accumulation, and stabilization of normally transient intermediates in enzymic catalysis (15-17). As a part of a detailed study of the mechanisms of action of proteases, we have examined the reactions of PNA substrates, utilizing the chromophoric group attached to the bond being cleaved as a sensitive probe of events at that site (18, 19).

In the present work the reaction of elastase with Ac-Ala-Pro-Ala-PNA (I), Ac-Pro-Ala-PNA (II), and succinyl (Suc)-Ala-Ala-PNA (III) has been investigated at subzero temperatures by using aqueous methanol cryosolvents. It previously had been found (ref. 20; unpublished data) that cosolvent concentrations as high as 90% methanol have no adverse effects on the catalytic or structural properties of the enzyme at 0°C and below. It also has been shown (20), by active-site titrations, that the concentration of elastase in methanol cryosolvents at subzero temperatures is the same as that determined in aqueous solution at 25°C (21).

Because our observations for all three PNA substrates are similar, we will present mostly representative data for reactions of elastase with I. The significance of our findings is 2-fold. First, the accord between the subzero temperature kinetics relating to tetrahedral intermediate formation and those in aqueous solution at 25°C indicates that the catalytic mechanism is similar at the low temperatures. Second, the ability to stabilize the tetrahedral adduct at subzero temperatures for prolonged time periods means that structural characterizations requiring long data collection periods are feasible. The data obtained in this investigation are necessary prerequisites for a crystallographic cryoenzymological study.

EXPERIMENTAL PROCEDURES

Chromatographically purified elastase (Sigma; type III) was used without further purification. Enzyme activity was measured by standard procedures using carbobenzyx (CBZ)-Gly p-nitrophenyl ester (21). N-CBZ-L-Gly-PNA was purchased from Vega-Fox (Tucson, AZ). Ac-L-Ala-L-Pro-L-Ala-PNA was a gift from J. H. Richards (California Institute of Technology). Suc-L-Ala-PNA was obtained from Calbiochem. Ac-L-Pro-L-Ala-PNA was synthesized by standard procedures.

Aqueous methanol cryosolvents were made up on a vol/vol basis as described (17, 18). The apparent protonic activity, pH*, was determined from measurements with a glass electrode at 25°C and corrected for temperature effects (22). The general protocols for the low-temperature experiments have been described (17).

RESULTS

The large difference between \( \lambda_{\text{max}} \) values for the PNA substrates, 315-320 nm, and the product p-nitroaniline, 380 nm, means that at wave-lengths >400 nm the substrate contribution to the absorbance is negligible, but both tetrahedral adduct and p-nitroaniline absorb strongly. Thus, a typical experiment involved mixing enzyme and substrate at the desired low temperature and monitoring the reaction by absorbance changes at 400 nm or by repetitive spectral scans. At higher subzero temperatures, turnover was observed and was unambiguously identified by allowing the reaction to proceed to completion and measuring the amount of p-nitroaniline released. The energy of activation for the turnover reaction of I in 70% aqueous methanol was 15.8 ± 1.5 kcal-mol\(^{-1}\) (1 kcal = 4.18 kJ).

When elastase and substrate were mixed at progressively lower temperatures (at pH* >6) an initial faster reaction was detected prior to turnover (Fig. 1). This reaction was manifested as an increase in absorbance in the 350-420 nm range and is

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Abbreviations: TI, tetrahedral intermediate; PNA, p-nitroanilide; Suc, succinyl; CBZ, carbobenzyx.
ascribed to formation of a tetrahedral adduct. By varying pH*, temperature, and enzyme and substrate concentrations, it was possible to find conditions under which the turnover reaction could be stopped and the tetrahedral intermediate (TI) could be accumulated. At pH* 9.4, 70% methanol, [E0] = 5.0 μM, and [S0] > 0.1 mM, the temperatures at which turnover was negligible relative to TI formation were ≤−50°C for I, ≤−40°C for II, and ≤−75°C for III.

The rates of formation of TI followed good first-order kinetics and exhibited a sigmoidal pH* dependence with pK* = 7.0 ± 0.3 at −39°C (Fig. 2). The pK* for turnover in 70% methanol was 6.9 ± 0.1 at 0°C. The spectrum of the accumulated TI (Fig. 3) has λmax of 359 ± 2 nm. The amount of trapped TI increased with increasing pH*, to a maximum around pH* 9.4, and increased with decreasing temperature. As a first approximation in determining the concentration of TI accumulated, the assumption was made that its extinction coefficient would be similar to that of p-nitroaniline (19). On this basis, the maximal amount of tetrahedral intermediate accumulated under conditions of excess substrate varied from 30 to 40% of the enzyme present for I to 100% for III.

Spectrophotometric evidence for an intermediate preceding the TI has also been observed. For example under nonturnover conditions at wavelengths around 300–320 nm, a reaction is observed as an increase in absorbance with a rate several times faster than that observed for TI formation (monitored at 400 nm). The spectrum of this intermediate is somewhat blue-shifted, and hyperchromic, relative to the substrate. The initial, noncovalent, Michaelis complex is formed more rapidly than this intermediate, being a diffusion-controlled process. The kinetics of interconversion between the TI and the preceding intermediate are such that, with I, appreciable concentrations of both species are present under most conditions.

In control experiments, in which either enzyme or substrate was omitted or in which enzyme inhibited by p-methoxyphenylsulfonyl fluoride was used, no time-dependent absorbance changes were observed. It was noted that at high enzyme concentrations (especially at ionic strength ≥0.1 M) the enzyme tended to aggregate in the cryosolvent, particularly at very low temperatures.

**DISCUSSION**

The key chemical steps in catalysis by the serine proteases involve nucleophilic attack by the hydroxyl of the active-site serine aided by the imidazole-aspartate system of pK 7 to form the putative tetrahedral intermediate. Subsequent collapse to the acyl-enzyme involves acid-catalyzed departure of the leaving group (Fig. 4). Although controversy exists with regard to which group in the active site is responsible for the observed pK (24, 25), there is no question that the formation of the tetrahedral intermediate would be expected to exhibit a pK around 7. Furthermore, the accumulation of the tetrahedral intermediate would be expected to be favored by high pH at which the imidazole-aspartate would be unable to function as a general acid catalyst to assist the departure of the leaving group.

Our interpretation that the reaction preceding turnover corresponds to the formation of a tetrahedral intermediate is based on the following lines of reasoning. The rate of hydrolysis of the PNA substrate is much slower than that of the corresponding p-nitrophenyl ester substrate (13). Because decylation is well established as being rate-limiting for the p-nitrophenyl esters, the overall rate-determining step with the PNA
substrates must be prior to decylation—that is, one of the steps leading to acyl-enzyme formation. Increasing the enzyme concentration increased the rate of the turnover reaction and the magnitude of the absorbance change associated with the formation of the tetrahedral intermediate, whereas increasing the substrate concentration increased the rate of formation of the tetrahedral adduct but not the rate of turnover (15). Similarly, the amplitudes of the absorbance increases associated with TI formation are of the appropriate magnitude based on the extinction coefficient for p-nitroaniline and the limiting concentration of enzyme or substrate.

The experimental data are inconsistent with a rate-limiting conformation change following formation of a tight acyl-enzyme-p-nitroaniline complex. Three arguments in particular argue against such a possibility. The spectrum of p-nitroaniline is not significantly perturbed in the presence of the enzyme ($\lambda_{\text{max}} = 381$ nm) or in solvents of high or low polarity ($\lambda_{\text{max}} = 394$ nm in formamide, 579 nm in acetone). The large difference between the $k_{\text{cat}}$ values for p-nitrophenyl and PNA substrates make it exceedingly unlikely that a simple desorption process is rate-limiting in catalysis because the similar size and structure of the leaving groups would be expected to give similar desorption rates. Furthermore, neither p-nitroaniline nor p-nitrophenol inhibit substrate hydrolysis.

The $\lambda_{\text{max}}$ values of the intermediates are similar to the $\lambda_{\text{max}}$ of p-nitroaniline but blue-shifted by 20 nm. This is entirely in accord with predictions for such a species, as well as being consistent with the increase in absorbance at 410 nm observed by Hunkapiller et al. (13) in the elastase case under normal conditions. The elastase TI spectra are also similar to those of a PNA TI formed with papain ($\lambda_{\text{max}} = 365$ nm) (19) and trypsin and subtilisin (unpublished data). The spectrum of the intermediate preceding the tetrahedral adduct resembles that of the substrate, making it exceedingly unlikely that the active-site environment alone could cause the observed 45-nm red-shift. The similar $\lambda_{\text{max}}$ values for p-nitroaniline in high- and low-polarity solvents indicate that active-site microenvironmental effects involving polarity cannot be responsible for the observed spectral shift. Furthermore, if the 45-nm red-shift of the intermediate is attributed to substrate distortion in the active site, the magnitude of the spectral shift implies substantial distortion from the ground-state trigonal structure. Such a distorted species would be expected to be of high energy and thus more likely to be a transition state rather than an intermediate whose breakdown was rate-limiting.

The concentration of TI accumulated is determined by the ratio $k_2/(k_{-2} + k_3)$ (see Fig. 4). The observation that the concentration of accumulated intermediate (at high pH) decreases with increasing temperature is readily accounted for in terms of the increased magnitude of $k_3$ under conditions such that turnover occurred. The pH dependence of the formation and breakdown of the tetrahedral adducts indicates that the formation of the intermediate is rate-limiting at low pH and that breakdown is the rate-limiting step at high pH. These findings are consistent with previous suggestions (12).

From the energies of activation for the rate of formation of the TI and the known dependence of $K_m$ on cosolvent, it was possible to estimate the expected rate of TI formation in aqueous solution at 25°C. The extrapolated value (11 ± 6 sec$^{-1}$) is in good agreement with that reported by Hunkapiller et al. (13) for such conditions (17 sec$^{-1}$). Such observations are strongly indicative that the catalytic mechanism is not significantly affected by the presence of cosolvent and subzero temperatures. Balny and Bieth (26) investigated the reaction of elastase with III in aqueous ethylene glycol at subzero temperatures and reported spectral evidence for a transient intermediate preceding turnover. However, their attempts to trap the intermediate were apparently unsuccessful.

The results reported here, in conjunction with many previous studies, indicate that discrete tetrahedral adducts may occur on the productive catalytic pathway in protease catalysis but can only be detected or accumulated in certain cases in which the relative ratios of rate constants controlling the rates of formation and breakdown of the intermediates are appropriate. For example, we have been unable to detect a TI in the reaction of chymotrypsin with Ac-Phe-PNA (18). In the case of elastase, however, by using the same leaving group (p-nitroaniline), whose electronic properties would be expected to result in a particularly stable tetrahedral adduct, and by carrying out the reaction at appropriately low temperature, it is possible to accumulate and stabilize TIs for prolonged periods.

Our results support the mechanism depicted in Fig. 4. We believe the intermediate preceding the TI arises from a conformational change in the active-site involving movement of the imidazole toward the serine so as to align the substrate and catalytic triad most effectively for the bond breaking and formation.

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