0273-2289/04/118/0171/$25.00

Insight Into Catalytic Mechanism of Papain-Like Cysteine Proteinases

The Case of D^{158}

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Received May 21, 2003; Revised August 20, 2003; Accepted August 28, 2003

Abstract

We studied the role of D^{158} in papain-like cysteine proteinases by using subtilisin Carlsberg, and its chemically modified analog thiolsubtilisin, by applying the proton inventory (PI) method and also by taking into account the pH profiles of the k_{cat}/K_m parameter. In the case of thiolsubtilisin, we estimated large inverse solvent isotope effects for k_{cat}/K_m, as in papain, whereas for subtilisin we found "dome-shaped" PI, suggesting a completely different mechanism. Finally, the kinetic behavior of thiolsubtilisin presented similarities as well as differences, compared to papain, suggesting a possible role for D^{158} as part of a catalytic triad in papain-like cysteine proteinases.

Index Entries: Cysteine proteinases; proton inventory; catalytic mechanism; aspartate[158]; thiolsubtilisin.

Introduction

Serine proteinases hydrolyze amide or ester substrates by means of a charge-relay system formed by D^{102}, H^{57}, and S^{195} (chymotrypsin numbering) *(1)*, whereas papain-like cysteine proteinases carry out catalysis by an ion pair (C^{25}-S^-/H^{159}-Im^+H; papain numbering). Although in both cases the minimal mechanism could be presented by Scheme 1 *(2)*, the ambiguous role of D^{158} in papain-like cysteine proteinases has been argued *(3–6)*, and, therefore, it should be elucidated:

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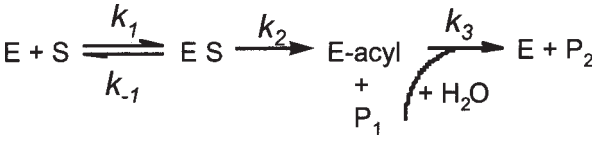

Scheme 1: Minimal mechanism of hydrolysis of serine and cysteine proteinases.

Recently, Theodorou et al. *(7)* refined the catalytic mechanism of papain via proton inventory (PI) experiments, and large inverse solvent isotope effects (SIEs) were found for the k_{cat}/K_m parameter suggesting a nucleophilic attack of the ion pair on substrates. Then, important rate constants (k_1, k_{-1}, and k_2) and relations ($k_{-1} << k_2$ and $k_{cat}/K_m = k_1$) were estimated implying that the enzyme-substrate complex represents a tetrahedral adduct *(7)*.

In the present work, we found large inverse SIEs for k_{cat}/K_m also in the case of thiolsubtilisin, whereas we estimated comparable values for the k_{-1} and k_2 rate constants implying that the relation $k_{cat}/K_m = k_2/K_S$ is more likely to be valid in that case.

Materials and Methods

All reagents were purchased from Sigma or Bachem. Thiolsubtilisin was synthesized from subtilisin Carlsberg *(8)*. Phosphate buffers were prepared allowing different values of deuterium atom fraction *n* in the solvent, and reaction mixtures contained 5% dimethyl sulfoxide and/or 2 m*M* dithiothreitol *(7)*. All kinetic measurements were performed spectrophotometrically at 410 nm, at 30°C, and by initial velocities. In a typical kinetic run, the enzyme solution was diluted into the appropriate quantity of buffer contained in a glass cuvet of 1-cm path length, and it was thermostated for 5 min. Then, the reaction was initiated by addition of the appropriate substrate solution to the cuvet, and the release of the leaving group was recorded *(7)*. Eleven different values of *n* ranging from 0 to 0.99 were used for each substrate, and eight substrate concentrations were used, per *n* value, to measure the kinetic parameters $(k_{cat})_n$ and $(K_m)_n$, with each single kinetic measurement repeated eight times. The synthetic substrates SucAAFpNA and SucAAFONPh were used for subtilisin and thiolsubtilisin. Additional measurements were performed in the pH range of 3.5–10.5, for all enzymes, and buffers of 0.1*M* ionic strength were prepared from citrate (pH 3.5), acetate (pH 4.0 to 5.0), phosphate (pH 5.5–8.0), borate (pH 8.5–10.0), and carbonate (pH 10.5).

Results and Discussion

All parameters $(k_{cat})_n$ and/or $(K_m)_n$ were estimated by nonlinear fitting *(9)* of Michaelis-Menten equation, whereas the PIs were determined by nonlinear fitting *(9–11)* of Eqs. 2–6 to the series of experimental data and

by applying the same statistical tests *(12–16)* as detailed exhaustively by Theodorou et al. *(7)* and other *(10,13,14,17)*. Equations 2–6 are simplified forms of the general Eq. 1 *(7,17–19)*; ϕ_i^T and ϕ_j^G are the isotopic fractionation factors of the *i*th transition state proton and the *j*th ground state proton, respectively, which reveal the effect of solvent in the process from a reactant state to a transition state.

$$k_n = k_0 \frac{\prod_{i=1}^{\mu} (1 - n + n\phi_i^T)}{\prod_{j=1}^{\nu} (1 - n + n\phi_j^G)} \tag{1}$$

$$k_n = k_0 \frac{1 - n + n\phi^T}{1 - n + n\phi^G} \tag{2}$$

$$k_n = k_0 \frac{(1 - n + n\phi_1^T)(1 - n + n\phi_2^T)}{(1 - n + n\phi_1^G)(1 - n + n\phi_2^G)} \tag{3}$$

$$k_n = k_0 (1 - n + n\phi^T) \tag{4}$$

$$k_n = k_0 (1 - n + n\phi_1^T)(1 - n + n\phi_2^T) \tag{5}$$

$$k_n = k_0 \frac{(1 - n + n\phi^T)^2}{(1 - n + n\phi^G)^2} \tag{6}$$

Since it has been accepted *(17,19)* that deacylation of acyl-enzymes is the overall rate-determining step of hydrolysis of ester substrates, we introduced an approximation, $k_{cat} \approx k_3$, assuming also that amide substrates have equal k_3 rate constants *(7)*. Accordingly, we calculated all $(k_2)_n$ and $(K_S)_n$ values for amide substrates using equation 6 in ref. *7*. The pH dependence of the k_{cat}/K_m parameter for both subtilisin and thiolsubtilisin was analyzed according to Ménard et al. *(20)* by means of Eq. 7.

$$(k_{cat}/K_m)_{obs} = \frac{(k_{cat}/K_m)_{lim}}{\frac{[H^+]}{K_1^{app}} + 1 + \frac{K_2}{[H^+]}} \tag{7}$$

The PIs of subtilisin for k_{cat}/K_m were found to be "dome shaped," exhibited small normal SIEs, and were best fitted by Eq. 3, whereas the PIs for k_2 and k_3 (or k_{cat}) were "bowed downward" in shape and were best fitted

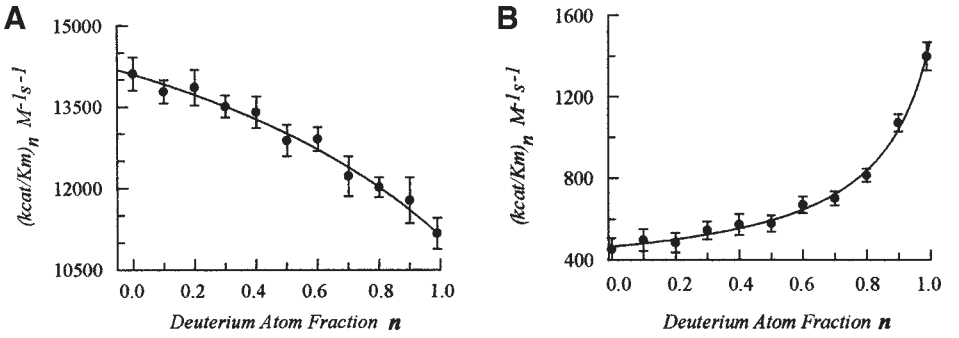

Fig. 1. Fitting of **(A)** Eq. 3, and **(B)** Eq. 2 of the experimental data for k_{cat}/K_m for subtilisin and thiolsubtilisin, respectively.

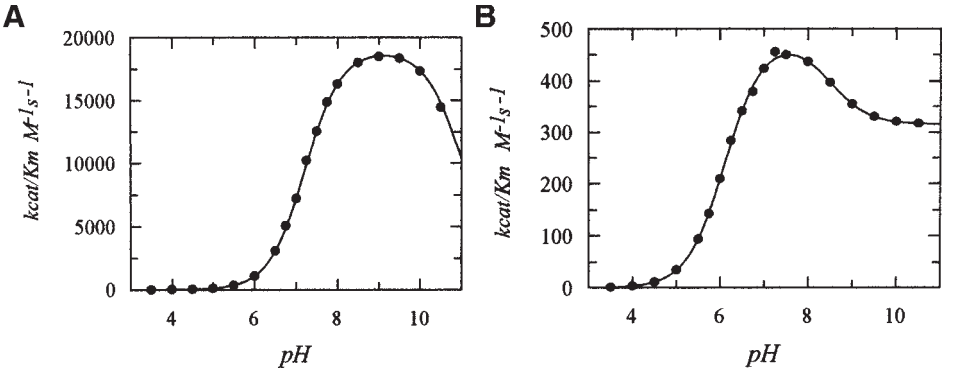

Fig. 2. pH dependencies of k_{cat}/K_m for **(A)** subtilisin and **(B)** thiolsubtilisin catalyzed hydrolysis of SucAAFpNA. The lines were drawn according to Eq. 7, in which $(k_{cat}/K_m)_{lim}$ = 19,110 ± 126 and 486 ± 4 M^{-1} s^{-1}, pK_1^{app} = 7.21 ± 0.01 and 6.12 ± 0.01, and pK_2 = 10.95 ± 0.36 and 8.49 ± 0.05 for subtilisin and thiolsubtilisin, respectively.

by Eq. 5, giving normal SIEs. Furthermore, for subtilisin we estimated $k_2 << k_{-1}$. Therefore, $k_{cat}/K_m = k_1k_2/k_{-1}$, and, hence, $K_S = k_{-1}/k_1$ (i.e., the instability constant of the Michaelis-Menten complex). Conversely, the PIs of thiolsubtilisin for k_{cat}/K_m were found to be "bowed downward" in shape, exhibited large inverse SIEs, and were best fitted by Eq. 2, whereas the PIs for k_2 and k_3 (or k_{cat}) were found to be "linear" in shape and were best fitted by Eq. 4, also giving normal SIEs. However, in the latter case, we estimated comparable values for the k_{-1} and k_2 rate constants, and, therefore, the relation $k_{cat}/K_m = k_1k_2/(k_{-1} + k_2)$ is valid; hence, $K_S = (k_{-1} + k_2)/k_1$ (i.e., a complex thermodynamic equilibrium constant). Examples of these results are shown in Fig. 1.

Conclusion

The PI studies showed that acylation in thiolsubtilisin occurs by means of a nucleophilic attack from an ion pair formed at the catalytic site of this

enzyme. Consequently, this implies a completely different mechanism *(7)* from that of general acid-base catalysis through a "charge-relay system" suggested by the PI found for subtilisin. However, the observed differences in the relation between k_{-1} and k_2 rate constants for thiolsubtilisin and papain *(7)* could be explained. The estimated comparable values for these rate constants vs $k_2 >> k_{-1}$ *(7)* and, therefore $k_{cat}/K_m = k_1k_2/(k_{-1} + k_2)$ vs $k_{cat}/K_m = k_1$ could consider a specific role for D^{32} and D^{158}, found closer than 3 Å and at about 7 Å from the ion pair in thiolsubtilisin and papain, respectively. Moreover, the observed shift toward more acidic values of the pK_1^{app} in the pH dependency of the k_{cat}/K_m parameter for thiolsubtilisin vs that of subtilisin (Fig. 2) could demonstrate a case in which D^{32} in thiolsubtilisin is found in a less hydrophobic environment owing to the ion pair, whose function is also strongly affected *(7,21)*. Therefore, it seems reasonable to accept, based on the experimental evidence, that D^{158} (papain numbering) is part of a "catalytic triad" in papain-like cysteine proteinases *(5,22)*.

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