Characterization of the active site thiol group of rhinovirus 2A proteinase

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Abstract Picornains 2A are cysteine proteases of picornaviruses, a virus family containing several human and animal pathogens. The pH dependencies of the alkylations of picornain 2A of rhinovirus type 2 with iodoacetamide and iodoacetate show two reactive thiol forms, namely the free thiolate ion at high pH and an imidazole assisted thiol group at low pH. Kinetic deuterium isotope effects do not support general base catalysis by the imidazole group, but rather the existence of a catalytically competent thiolate–imidazolium ion-pair. The nature of the ion-pair differs from that of papain, the paradigm of cysteine proteases. The ion-pair is confined to the same, unusually narrow pH range in which the enzyme exhibits catalytic activity.

Key words: Cysteine protease; Rhinovirus protease; Picornain 2A; Active site reactivity; Kinetic deuterium isotope effect

1. Introduction

Picornaviruses constitute a large family of single-stranded, positive-sense RNA viruses. They cause a wide variety of diseases in humans and animals, ranging from the mild and widespread common cold to hepatitis A and poliomyelitis. The mRNA-like genome of picornaviruses is translated in the host cell into a large polyprotein, which is then cleaved by specific viral proteinases into structural and non-structural proteins [1–4]. In entero- and rhinoviruses, the 2A and 3C proteins process the polyproteins. Most cleavages are mediated by the larger 3C proteinase, designated picornain 3C. However, processing on the polyprotein is initiated by picornain 2A, which cleaves between the C-terminus of VP1 and its own N-terminus. This cleavage, the only one in fact performed by picornain 2A, separates the capsid protein precursor from the non-capsid protein precursor. In addition to this cleavage, picornain 2A cleaves the cellular protein eukaryotic initiation factor 4G, which serves to down-regulate the translation of capped host cell mRNAs. Viral protein synthesis is unaffected as it initiates internally at an internal ribosomal entry site. The cleavage sites recognized by the rhinovirus and enterovirus picornains 2A are characterized by a P1′ glycine and preferences for P2 threonine, serine or asparagine and P2′ proline or phenylalanine.

The structures of picornains 2A and 3C show a marked relationship to serine peptidases, despite the fact that they are cysteine peptidases. This feature warranted their grouping in a new family of cysteine peptidases, namely family C3 in clan CB. The three-dimensional structures of the picornains 3C from hepatitis A virus [5], rhinovirus [6] and poliovirus [7] have been determined, while that of the first picornain 2A (human rhinovirus type 2 (HRV2)) has recently been solved [8]. Notwithstanding the obvious similarities to picornains 3C, the 2A proteinases are zinc proteins, and the tightly bound zinc ion presumably plays a role in stabilizing the three-dimensional structure [8,9]. From sequence comparisons and site-directed mutagenesis experiments, Cys106 had been postulated to be the active site residue in HRV2 protease 2A, with His18 and Asp35 acting as the additional members of the catalytic triad [10]. This has been confirmed by the three-dimensional structure determination [8].

There is little experimental evidence for the catalytic mechanism of picornain 2A. Notably, picornains are structurally distinct from the most extensively studied cysteine proteases of the papain family. Papain is known to have a catalytically competent thiolate–imidazolium ion-pair [11] and an asparagine residue, as the third member of the catalytic triad. In contrast, picornain 2A displays a charged aspartic acid, which may significantly influence the interaction between the thiol and imidazole groups. This work is aimed at characterization of the catalytic entity of HRV2 protease 2A.

2. Materials and methods

2.1. Expression and purification of picornain 2A

HRV2 picornain 2A was expressed in *Escherichia coli* BL21(DE3)-pLysE from pET3d/2A (essentially identical with pET3c/ATG-2A described earlier [12]). Cells were grown in six 2000 ml flasks, each containing 500 ml LB, at 37°C to an OD at 600 nm of 0.5–0.6. 0.4 mM isopropyl-β-D-thiogalactopyranoside and 50 μg/ml ampicillin were added and the incubation continued for about 15 h. Cells were harvested, suspended in 60 ml buffer (50 mM Tris–HCl, pH 8.0, 50 mM NaCl, 1 mM ethylenediaminotetraacetic acid (EDTA), 5 mM 2-mercaptoethanol and 5% glycerol) and sonicated.

The cell extract was centrifuged for 30 min at 18 000×*g* and applied to a Whatman DE32 cellulose column (4.7×27 cm) equilibrated with equilibration buffer (30 mM Tris–HCl, pH 8.0, 0.2 M NaCl, 1 mM EDTA and 5 mM 2-mercaptoethanol, EB). After washing with one column volume EB, the enzyme was eluted with a linear salt gradient (1000 ml of EB, and 1000 ml of 1.4 M NaCl in the EB).

Active fractions eluted from the DE32 column were combined (400 ml), precipitated with ammonium sulfate at 50% saturation overnight at 4°C. The precipitate was centrifuged and dissolved in 30 mM Tris–HCl buffer, pH 8.0, containing 1 mM EDTA and 5 mM 2-mercaptoethanol (buffer A) to a final volume of 40 ml. The clear solution was gel-filtered on a Sephadex G25 column equilibrated with buffer A, and

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Abbreviations: HRV2, human rhinovirus type 2; EDTA, ethylenediaminotetraacetic acid; Mes, 2-(morpholino)ethanesulfonic acid; Abz, 2-aminobenzoic acid; Phe(NO_2), 4-nitrophenylalanine; Nbs_2, 5.5′-dithio-bis(2-nitrobenzoic acid)

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concentrated by ultrafiltration on an Amicon PM10 membrane to 6.5 ml.

An aliquot (0.5 ml) of the concentrated solution was applied to a Q-Sepharose High Performance column (HR10/30) equilibrated with buffer A. The enzyme was eluted with a linear gradient developed with buffer B (1.0 M NaCl in buffer A) between 40 and 80% buffer B for 60 min. The flow rate was 1.0 ml/min, and the enzyme eluted at about 60% buffer B.

Three runs from the Q-Sepharose column were combined and further purified on a MonoQ HR10/10 column with a linear gradient of 30–60% buffer B for 60 min. The flow rate was 1.0 ml/min and the enzyme eluted at 41% buffer B. From 3000 ml broth, 20.1 mg picornain 2A was obtained, with a k_{cat}/K_m of 22 150 M^{-1} s^{-1}.

Thiol group concentrations were determined by titration with 5.5′-dithio-bis(2-nitrobenzoic acid) (Nbs_2) at pH 6.8 [13].

2.2. Kinetic measurements

Picornain 2A activity was measured fluorometrically with 2-aminobenzoic acid (Abz)-Arg-Pro-Ile-Ile-Thr-Thr-Ala-Gly-Pro-Ser-4-nitrophenylalanine (Phe(NO_2))-Ala-OH, an internally quenched fluorescence substrate that is cleaved at the Ala-Gly bond. Pseudo first-order rate constants were determined at 25°C using a Jasco FP 777 spectrofluorometer at 338 nm and 420 nm excitation and emission wavelengths, respectively. The second-order rate constant (k_{cat}/K_m) was calculated by dividing the pseudo first-order rate constant by the enzyme concentration(calculated from the molar extinction coefficient of 20 000 M^{-1} cm^{-1} at 280 nm (M_r = 16 209) [14]).

The pH dependence of the rate constants was measured at 25°C in a buffer containing 25 mM acetic acid, 25 mM 2-(morpholino)ethanesulfonic acid (Mes), 25 mM glycine, 75 mM Tris, 1 mM EDTA and 1 mM DTE (standard buffer), adjusted to the required pH by the addition of 1 M NaOH or 1 M HCl. The data were fitted with the GraFit software [15] to the appropriate equation (Eqs. 1–3), where $k(\text{limit})_1$ and $k(\text{limit})_2$ are pH independent rate constants, and pK_1, pK_2 and pK_3 stand for the pK_a values of catalytically competent functional groups.

$$k = k(\text{limit})_1[1/(1+10^{pK_1-pH}+10_2^{pH-pK})] + k(\text{limit})_2[1/(1+10^{pK_2-pH})] \quad (1)$$

$$k = k(\text{limit})_1[1/(1+10^{pK_1-pH}+10_2^{pH-pK})] + k(\text{limit})_2[1/(1+10^{pK_2-pH}+10^{pK_2+pK_3-2pH})] \quad (2)$$

$$k = k(\text{limit})[1/(1+10^{pK_1-pH}+10_2^{pH-pK})] \quad (3)$$

The reaction of HRV2 protease 2A with iodoacetamide or iodoacetate was followed under pseudo first-order conditions by taking aliquots from the reaction mixture at appropriate times and by measuring the decrease in the initial rate. The second-order rate constant was calculated by dividing the first-order rate constant by the concentration of the alkylating agent. The reaction mixture contained 6.0–7.9 μM enzyme in a buffer composed of 25 mM acetic acid, 25 mM Mes, 25 mM glycine, 75 mM Tris, 1 mM EDTA and 0.165–1.90 mM iodoacetamide or 0.220–6.55 mM iodoacetate.

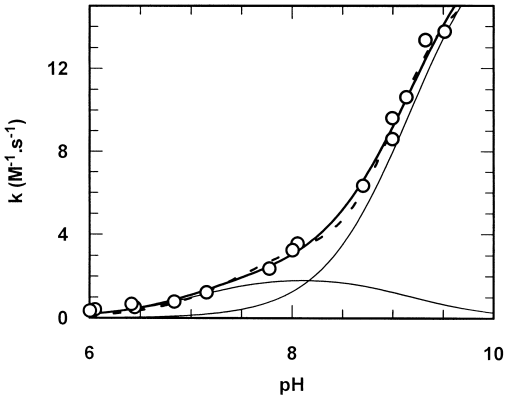

Fig. 1. Alkylation of picornain 2A with iodoacetamide. The data were fitted to Eq. 1 (solid line) and Eq. 2 (broken line). Each point was determined from a separate reaction mixture. The thin lines show the contributions of the different reactive enzyme forms.

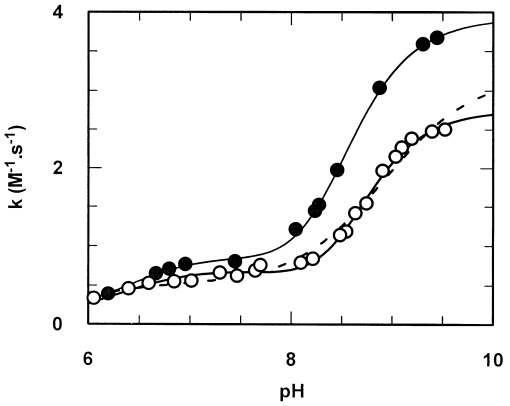

Fig. 2. Rate constants for inactivation of picornain 2A with iodoacetate. The data (○) were fitted to Eq. 2 (solid line) and Eq. 1 (broken line). The points marked with (●) signs were determined in deuterium oxide and fitted to Eq. 2.

General base catalysis was tested in heavy water (99.9%). The deuterium oxide content of the reaction mixture was at least 95%. The p^2H of deuterium oxide solution can be obtained from pH meter readings according to the relationship p^2H = pH(meter reading)+0.4 [16].

3. Results

Cysteine enzymes, of which papain is the most extensively studied, are often remarkably inactivated by oxidation during preparation and storage. It is therefore essential that the active concentration of the enzyme is precisely understood when measuring rate constants. Titration with Ellman's reagent, Nbs_2, is a useful way of determining the thiol content of enzymes [13]. Picornain 2A has seven thiol groups which have indeed been detected by titration in 8 M urea. Titration of the native enzyme at pH 6.8, however, gave 0.97 ± 0.03 mol of free thiol group per mol of enzyme. This indicates that the active site thiol is considerably more reactive under the conditions employed than the remaining six thiol groups, and also shows that the enzyme is fully active, as its catalytically competent thiol group is practically all in the reduced form. Compared to papain preparations, which often contain irreversibly oxidized thiols, this is an important and distinct feature of picornain 2A.

While only the active site thiol group is titrated with Nbs_2 at pH 6.8, additional thiol groups react at alkaline pH although at a much lower rate. Thus, at pH 9.2, the four free thiols, not bound to the zinc ion, can react, but completion of the reaction requires about 50 min, whereas the active site thiol can be titrated in 2 min even at pH 6.8. It may be noted that at high pH, the Ellman's reagent slowly decomposes, which should be taken into account. It appears that the three free thiols, in particular two of them, are not readily accessible for Nbs_2, and the slow reaction suggests that the enzyme structure is fairly stable at pH 9.2. The remaining three thiol groups bound to the zinc ion do not react at pH 9.2 because the Zn–S bond is stable at slightly alkaline pH. Above pH 9.5, the Zn-bound thiol groups tend to react with Nbs_2, apparently because of slow denaturation of the protein.

As expected, all the seven thiol groups can readily be titrated in 6 M guanidinium chloride at pH 7.0. These results indicate that the reactivity of the non-catalytic thiol groups is negligible even at high pH with respect to the active site thiol group.

The reactivity of the essential thiol group of HRV2 protease 2A has been explored by measuring the rate constant for a simple alkylation with iodoacetamide. Since only the dissociated form of the thiol group can react with an appreciable rate, the pH dependence of the alkylation reaction provides useful information about the chemical reactivity of the thiol group, which is crucial in understanding the catalytic activity. It is apparent from Fig. 1 that the pH dependence does not follow a simple dissociation curve. The data conform to an ionization curve composed of bell-shaped and sigmoid terms (Eq. 1, thin lines in Fig. 1). At high pH, the curve approximates a simple dissociation corresponding to that of the thiol group, while at low pH a substantial deviation can be observed. Similar results are obtained with the negatively charged iodoacetate, except that at high pH the points are clearly incompatible with the single ionization (Fig. 2). The data better fit to a curve that considers the contribution of an additional base (Eq. 2). This second ionization is probably involved in the iodoacetamide reaction as well, but its effect on the neutral compound is not significant and the curves calculated with single and double ionizations agree within experimental error (Fig. 1). The deviation at low pH is presumably due to the participation of His18 and originates either from a simple general base catalysis or from the formation of a thiolate–imidazolium ion-pair. It should be pointed out that the slow denaturation at high pH does not interfere with the fast alkylation reactions, which have been completed in less than 6 min.

General base catalysis by His18 was probed by measuring kinetic deuterium isotope effects. It is known that general base-catalyzed steps proceed slower by a factor of about three in deuterium oxide than in water, as indeed found in the case of serine proteases. On the other hand, such an effect is not existent in the alkylation or acylation of papain, where the reactive nucleophile is a thiolate–imidazolium ion-pair, i.e. the proton of the thiol group has been transferred to the imidazole ring in the ground state of the reaction, prior to the nucleophilic attack [11,17]. It can be seen in Fig. 2 that the rate constants are higher in heavy water, which is not consistent with general base catalysis by the His18 residue. Similar inverse kinetic isotope effects are associated with the thiolate ion at high pH, where facilitation by the imidazole group is not needed. This suggests that picornain 2A possesses a thio-

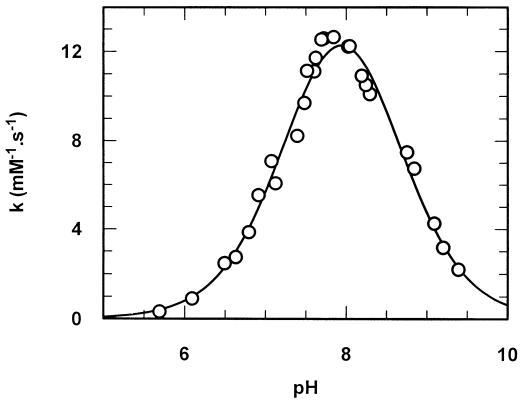

Fig. 3. The pH–k_{cat}/K_m profile for the reaction of picornain 2A with the oligopeptide substrate, Abz-Arg-Pro-Ile-Ile-Thr-Thr-Ala-Gly-Pro-Ser-Phe(NO_2)-Ala-OH. The points were fitted to Eq. 3 using the following parameters: k(limit) = 18.5 ± 1.0 mM^{-1} s^{-1}, pK_1 = 7.34 ± 0.05, pK_2 = 8.54 ± 0.06.

Table 1
Rate constants for alkylation of HRV2 picornain 2A and related thiol reactants

	Iodoacetamide	Iodoacetate (A)	Iodoacetate in ^2H_2O (B)	A/B
HRV2-2A, Eq. 1				
k(limit)$_1$ (M^{-1} s^{-1})	2.13 ± 0.50	0.52 ± 0.06		
k(limit)$_2$ (M^{-1} s^{-1})	19.55 ± 1.42	3.19 ± 0.16		
pK_1	7.06 ± 0.30	5.68 ± 0.40		
pK_2	9.16 ± 0.09	8.93 ± 0.08		
HRV2-2A, Eq. 2				
k(limit)$_1$ (M^{-1} s^{-1})	4.42 ± 1.06	0.78 ± 0.05	0.95 ± 0.05	0.82
k(limit)$_2$ (M^{-1} s^{-1})	16.75 ± 1.78	2.75 ± 0.07	3.94 ± 0.05	0.70
pK_1	7.56 ± 0.25	6.31 ± 0.11	6.34 ± 0.08	
pK_2	8.83 ± 0.31	8.46 ± 0.11	8.37 ± 0.06	
pK_3	8.92 ± 0.42	8.75 ± 0.13	8.49 ± 0.08	
Papain[a]				
k(limit)$_1$ (M^{-1} s^{-1})	14.5	1100		
k(limit)$_2$ (M^{-1} s^{-1})	976	20		
pK_1	4.0	3.7		
pK_2	8.4	8.9		
Thiolsubtilisin[a]				
k(limit)$_1$ (M^{-1} s^{-1})	6.3	0.84		
k(limit)$_2$ (M^{-1} s^{-1})	118	0.2		
Bean endopeptidase (pH 6.0)[b]				
k (M^{-1} s^{-1})	7.9	0.11		
Clostripain (pH 7.5)[c]				
k (M^{-1} s^{-1})	5.2	0.79		
Glutathione[a]				
k(limit) (M^{-1} s^{-1})	27	2.8		

[a]From [21].
[b]From [23].
[c]From [24].

late–imidazolium ion-pair, as found with other cysteine enzymes, although the virus protease has an unrelated structure and a distinct catalytic triad. It should be noted that inverse kinetic isotope effects have already been observed with papain reactions [17–20]; these effects may have their origin in hydrogen bond formation between the enzyme and the carboxylate ion or the carboxamide group of the alkylating reagent.

The pH dependence of the reaction with the oligopeptide substrate, Abz-Arg-Pro-Ile-Ile-Thr-Thr-Ala-Gly-Pro-Ser-Phe(NO_2)-Ala-OH, was also determined and shown in Fig. 3.

4. Discussion

The alkylations of picornain 2A reported here are very different from those obtained with papain [17,18,20,21]. In the iodoacetamide reaction $k(\text{limit})_2$, which corresponds to the reactivity of the free thiolate ion, is more than 50 times greater for papain than for picornain 2A (Table 1). The thiol group of papain is apparently located in an environment which facilitates the reaction towards iodoacetamide, whereas the reactivity of picornain 2A is very close to that of the model compound, glutathione (Table 1). The $k(\text{limit})_1$, which reflects the reactivity of the catalytically competent state of the enzyme, only differs by a factor of 3.5–7, depending on the equation (Eq. 1 or Eq. 2) used.

Another difference from papain is found in the iodoacetate reaction. This charged compound has an inherently lower reactivity compared with that of iodoacetamide, as manifested in the reaction with glutathione (Table 1). The lower reactivity also holds with picornain 2A over the entire pH range studied. On the other hand, iodoacetate exhibits strikingly higher $k(\text{limit})_1$ for papain than for the virus protease (Table 1). This is explained by participation of the imidazolium moiety of the ion-pair, which offers an excellent binding site for the carboxylate ion, and thus produces a bell-shaped curve in the catalytically competent pH range [21]. This phenomenon, however, is not a general trait of thiol enzymes because the conformation and the accessibility of the ion-pair may differ in the different enzymes. For example, in the case of thiolsubtilisin, where the existence of the thiolate–imidazolium ion-pair has unequivocally been demonstrated [22], $k(\text{limit})_1$ is lower with iodoacetate than with iodoacetamide (Table 1). A similar relationship was observed with a bean endopeptidase [23] and clostripain [24], as shown in Table 1. The bean endopeptidase, now called legumain, constitutes the new clan CD of cysteine peptidases which also includes caspases, clostripain and gingipain [25]. In terms of catalysis, picornain 2A resembles this new clan, although it is distinct both structurally and evolutionarily.

An additional difference is found in the pH range of histidine participation. With papain, the ion-pair exists in a wide pH range defined by $pK_1 \sim 4$ and $pK_2 \sim 8.5$ (Table 1). With picornain 2A, on the other hand, the assistance by histidine is restricted to a much narrower pH interval characterized by $pK_1 \sim 7$ and $pK_2 \sim 9$, which suggests a less intimate interaction between the thiol and imidazole groups.

The catalytic activity of picornain 2A is confined to a similarly narrow pH range (Fig. 3), as that found for the imidazole participation in the alkylation reactions. While in the papain catalysis the hydrolytic reaction considerably extends into the acidic pH range, picornain 2A, probably because it is a zinc-containing enzyme, is unstable in acidic medium and operates at neutral and slightly alkaline pH.

The recently published three-dimensional structure of HRV2 picornain 2A is consistent with the ion-pair formation in this proteinase [8]. It also offers an explanation for the participation of two basic groups in the alkylation of Cys106, as interpreted from the results in Fig. 2. One is the free thiolate ion itself, which is formed upon the decomposition of the thiolate–imidazolium (supplied by His18) ion-pair with increasing pH. The second basic group is very likely His63, which forms an ion-pair with Asp105, the adjacent residue to Cys106. The pK_a of His63 is apparently increased in the ion-pair with Asp105, just like that of His18 in the thiolate–imidazolium ion-pair.

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