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Interactions of papaya proteinase IV with inhibitors

David J. Buttle[1], Anka Ritonja[2], Pamela M. Dando[1], Magnus Abrahamson[3], Elliot N. Shaw[4], Peter Wikstrom[4], Vito Turk[2] and Alan J. Barrett[1]

[1]*Biochemistry Department, Strangeways Research Laboratory, Worts Causeway, Cambridge CB1 4RN, England,* [2]*Department of Biochemistry, J. Stefan Institute, Jamova 39, 61000 Ljubljana, Yugoslavia,* [3]*Department of Clinical Chemistry, University Hospital, University of Lund, Lund S221 85, Sweden* and [4]*Friedrich-Miescher Institute, CH4002 Basel, Switzerland*

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Papaya proteinase IV (PPIV) is not inhibited by chicken cystatin, or human cystatins A or C, unlike most other proteinases of the papain superfamily. The enzyme inactivates chicken cystatin and human cystatin C by limited proteolysis of the glycyl bond previously shown to be involved in the inhibitory inactivity of the cystatins, but has no action on cystatin A. Contamination of commercial crystalline papain with PPIV accounts for the limited proteolysis of cystatins by 'papain' reported previously. PPIV is slowly bound by human α_2-macroglobulin. The enzyme is irreversibly inactivated by E-64, and by peptidyl diazomethanes containing glycine in P_1 and a hydrophobic side-chain in P_2. The reaction of PPIV with iodoacetate is extremely slow. PPIV is inhibited by peptide aldehydes despite the presence of bulky sidechains in P_1, suggesting that these reversible inhibitors do not bind as substrate analogues.

Macroglobulin, α_2-; Cystatin; Compound E-64; Iodoacetate; Iodoacetamide; Papain; Peptide aldehyde; Peptidyl diazomethane

1. INTRODUCTION

Papaya proteinase IV (PPIV) was recently discovered to be a major component of the latex of the unripe papaya (*Carica papaya*) fruit [1]. The amino acid sequence shows the enzyme to be closely related to papain and other cysteine proteinases of papaya [2], but substitution of certain residues in the active site cleft confers narrow substrate specificity, largely confined to the cleavage of glycyl bonds [3]. PPIV resembles most other members of the papain superfamily in being irreversibly inactivated by E-64 [1], but it is not inhibited by the protein inhibitor of cysteine proteinases, chicken cystatin [1]. Earlier work on the interaction of commercial papain with cystatins led to the discovery of proteolytic inactivation of these inhibitors by cleavage of a glycyl bond near the N-terminus. At the time, this was attributed to the action of an abnormal form of papain [4]. The purpose of the present paper is to provide an alternative explanation for this phenomenon, and to describe further aspects of the interaction of PPIV with protein and small-molecule inhibitors.

Correspondence address: D.J. Buttle, Biochemistry Department, Strangeways Research Laboratory, Worts Causeway, Cambridge CB1 4RN, England

Abbreviations: α_2M, human α_2-macroglobulin; Boc, *t*-butyloxycarbonyl; Bz, benzoyl; E-64, L-3-carboxy-2,3-*trans*-epoxypropionylleucylamido(4-guanidino)butane; NHMec, 7-(4-methyl)coumarylamide; $NHPhNO_2$, *p*-nitroanilide; PPIII, papaya proteinase III; PPIV, papaya proteinase IV; Z, benzyloxycarbonyl; I, total inhibitor concentration; $K_{i(app)}$, apparent dissociation constant of enzyme and inhibitor in the presence of substrate; K_i, inhibition constant; k_{obs}, apparent pseudo-first-order rate constant for inactivation; $k_{2(app)}$, apparent second-order rate constant for inactivation in the presence of substrate; k_2, second-order rate constant for inactivation

2. EXPERIMENTAL

2.1. *Materials*

Proteins were prepared as previously described: PPIV and papain [1,2], chicken cystatin [4], human cystatin A (also called stefin A: [5]) [6] and recombinant human cystatin C [7]. Human α_2M was a gift from Dr J.S. Mort, Shriners Hospital for Crippled Children, Montreal, Canada. Papain (2 × cryst., cat. no. P3125) and leupeptin were purchased from Sigma. Boc-Val-Gly-CHN_2, Z-Leu-Val-Gly-CHN_2, Z-Leu-Gly-CHN_2, Z-Met-Gly-CHN_2, Z-Phe-Gly-CHN_2, Z-Gln-Gly-CHN_2, Z-Leu-Gly-Gly-CHN_2 and Z-Leu-Phe-Gly-CHN_2 were synthesized by standard methods [8,9]. Z-Phe-Phe-CHN_2 and Z-Phe-Ala-CHN_2 were purchased from Bachem Feinchemikalien AG, Bubendorf, Switzerland. Chymostatin was titrated with papain which had previously been titrated with E-64 [10]. Other compounds were as described previously [3].

2.2. *Automated amino acid sequencing*

This was as described [4].

2.3. *Radial immunodiffusion assay of PPIV*

Specific immunoglobulin G antibody against PPIV was incorporated into agarose gel at 100 μg/ml for use in single radial immunodiffusion analysis otherwise as described in [1]. Commercial papain was carboxymethylated [10] before application to the immunodiffusion plate, and its concentration was determined on the basis of $A_{280,1\%} = 25.0$.

2.4. *Treatment of cystatins with PPIV*

Chicken cystatin (2 mg) was incubated with 4 μg of PPIV in 3.0 ml

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of 0.05 M sodium phosphate buffer, pH 7.8, containing 0.67 mM dithiothreitol for 30 min at 30°C. Recombinant human cystatin C (0.2 mg) was incubated with 0.2 μg of PPIV in 0.1 ml of 0.1 M sodium phosphate buffer, pH 6.5, containing 1 mM dithiothreitol and 1 mM EDTA for 60 min at 37°C. The modified protein was then separated by agarose gel electrophoresis [4], and blotted onto a polyvinylidene difluoride membrane for amino acid sequencing [11]. Human cystatin A (1.2 mg) was incubated with PPIV (96 μg) in 1.0 ml of 50 mM sodium acetate buffer, pH 5.0, containing 2 mM dithiothreitol, for 8 h at 22°C.

2.5. *Interaction with α_2M*

Papain or PPIV (430 pmol) was activated in 100 μl of 50 mM sodium phosphate, 1 mM EDTA, 2 mM cysteine, pH 6.5, for 30 min at 30°C. α_2M (1.2 nmol) was then added and the mixture was incubated for 15, 120 or 240 min at 30°C before application to a TSK-G 2000SW (LKB) gel chromatography column (7.5 × 600 mm) which had been equilibrated in 50 mM Tris/HCl, 0.5 M NaCl, 0.1% Brij 35 in 20% glycerol, pH 7.5. Eluted fractions (0.5 ml) were assayed with Bz-Arg-NHPhNO_2 (for papain) or Boc-Ala-Ala-Gly-NHPhNO_2 (for PPIV). The activated enzymes, and α_2M, were also run individually on the column.

2.6. *Determination of kinetic parameters*

Molar concentrations of PPIV, papain and cystatins were determined by stoichiometric titration [3]. Continuous rate assays with Boc-Ala-Ala-Gly-NHMec (50 μM) as substrate for PPIV or Z-Phe-Arg-NHMec (10 μM) for papain, in 100 mM sodium phosphate, 1 mM EDTA, 0.005% Brij 35, 2 or 4 mM cysteine, pH 6.8, 30°C, were used to analyse the kinetics of reversible inhibition and irreversible inactivation. For the determination of $K_{i(app)}$ the rate of substrate hydrolysis in the absence of inhibitor was recorded, after which inhibitor was added in a negligible volume and the new steady state was monitored. Non-linear regression analysis of the replot of fractional activity vs I (Enzfitter, Elsevier-Biosoft, Cambridge, England) gave $K_{i(app)}$. Rate constants for inactivation were found by non-linear regression analysis of the apparent pseudo-first-order curves of inactivation utilizing the FLUSYS software package [12], giving k_{obs}. $k_{2(app)}$ was calculated as k_{obs}/I. K_i and k_2 were then found by the standard correction for the effect of competition by substrate.

3. RESULTS AND DISCUSSION

3.1. *Lack of inhibition of PPIV by cystatins*

There was no detectable inhibition of PPIV by human cystatin A (152 nM) or recombinant human cystatin C (1 nM). The lack of inhibition of PPIV by chicken cystatin has been reported earlier [1]. PPIV is one of the few plant cysteine proteinases that are scarcely inhibited by chicken cystatin [1], others being stem bromelain [13] and fruit bromelain [14].

3.2. *Proteolytic modification of cystatins*

Automated amino acid sequencing of chicken cystatin modified by PPIV, and re-isolated by FPLC [4], showed the single sequence: Ala-Pro-Val-Pro-Val-Asp-, revealing that cleavage had occurred only at the Gly^9-Ala^{10} position. Recombinant human cystatin C was likewise found to be modified by the cleavage of the homologous Gly^{11}-Gly^{12} bond, the detected sequence being Gly-Pro-Met-Asp-Ala-. In contrast, incubation of human cystatin A with PPIV did not result in the appearance of any new chromatographic components or N-terminal sequence; this is attributable to the presence of Pro in the P_2 position relative to the homologous Gly^4 [15], which is not accepted by PPIV [3].

Cleavage of the Gly^9-bond of chicken cystatin and the Gly^{11}-bond of cystatin C were previously seen with a commercial papain preparation [4]. Failure of the cystatins to inhibit PPIV is apparently not due to the rapid degradation seen with chicken cystatin and cystatin C, because cystatin A, which is also not inhibitory for PPIV was not a substrate for the enzyme. Presumably, the restricted active site cleft of PPIV [3] will not accommodate the inhibitory 'wedge' [16] of the cystatins.

3.3. *Presence of PPIV in commercial papain*

The assay of commercial papain (Sigma) for PPIV by single radial immunodiffusion analysis showed the presence of PPIV at a level of 2.96 ± 0.15% (w/w; mean ± SE, n = 6).

3.4. *Interaction with α_2M*

The percentages of total PPIV associated with α_2M after various incubation times (see section 2) were 0% (0 min), 25% (15 min), 40% (120 min), and 50% (240 min). Corresponding values for papain were 0% (0 min) and 80% (15 min). We conclude that PPIV is bound by α_2M, like the great majority of endopeptidases, but at least one order of magnitude more slowly than papain.

3.5. *Irreversible inactivation by active-site-directed reagents*

The second order rate constants for inactivation of PPIV by iodoacetate, iodoacetamide, E-64 and a series of peptidyl diazomethanes are shown in table 1. The very low rate of inactivation of PPIV by iodoacetate is

Table 1

Rate constants for the inactivation of PPIV

Inactivator	k_2 ($M^{-1} \cdot s^{-1}$)	
	PPIV	Papain
Iodoacetate	2.2	1000
Iodoacetamide	0.5	46
E-64	58000	1160000
Z-Phe-Phe-CHN_2	<10	5500
Z-Phe-Ala-CHN_2	60	43000
Z-Gln-Gly-CHN_2	100	50
Boc-Val-Gly-CHN_2	5000	12000
Z-Leu-Gly-CHN_2	5500	1600
Z-Met-Gly-CHN_2	5500	1700
Z-Phe-Gly-CHN_2	14500	12000
Z-Leu-Gly-Gly-CHN_2	<50	<50
Z-Leu-Phe-Gly-CHN_2	630000	62000
Z-Leu-Val-Gly-CHN_2	3500000	460000

Rate constants were determined as described in section 2 at pH 6.8 and 30°C

of a similar order to that of an enzyme from germinating bean cotyledons [17], and stem bromelain also reacts very slowly [18]. The structural basis for the great differences in reactivity of the catalytic site cysteine in homologous proteinases has yet to be explained.

The rate constant for inactivation of PPIV by E-64 was previously estimated to be 15300 $M^{-1} \cdot s^{-1}$ [1], but the value of 58000 $M^{-1} \cdot s^{-1}$ now obtained by use of the sensitive fluorimetric substrate is likely to be more accurate. The inhibitor probably binds with the leucine sidechain in the S_2 subsite (in the terminology of [19]), leaving the S_1 subsite essentially unoccupied, as is the case with papain [20].

The data for the peptidyl diazomethane inhibitors (table 1) reflect the primary specificity of PPIV for Gly in S_1 [3], but also reveal a strong preference for a hydrophobic side-chain in S_2; Leu, Val, Met and Phe all being preferred over Gln in P_2. This is a preference also seen with papain. The occupation of the S_3 binding pocket of PPIV by Leu (and possibly S_4 by the Z group) in Z-Leu-Phe-Gly-CHN_2 and Z-Leu-Val-Gly-CHN_2 led to further large increases in k_2 of over one and almost three orders of magnitude, respectively, when compared with corresponding dipeptide derivatives. The effects were smaller with papain. The structure of the fastest-reacting inhibitor of PPIV, Z-Leu-Val-Gly-CHN_2, had been modelled on the cleavage site of cystatin C [21]. The increase in k_2 afforded by the extra amino acid residue indicates that PPIV, like papain [19], has an extended binding site. Even so, no significant inhibition of either PPIV or papain was seen with Z-Leu-Gly-Gly-CHN_2, which emphasises the importance of the hydrophobic side-chain in S_2.

3.6. *Reversible inhibition*

The inhibition of PPIV by two peptide aldehydes was studied. The K_i with leupeptin was found to be 20 nM, and with chymostatin 53 nM. These interactions are weaker than those of the aldehydes with papain (1.6 nM and 0.73 nM, respectively) but nevertheless surprisingly strong in view of the presence of arginine and phenylalanine sidechains in what could correspond to the P_1 position. The possibility has to be considered that the aldehydes do not bind in a substrate-like way, however.

In conclusion, our results show that the earlier finding of cleavage of crucial glycyl bonds in two type 2 cystatins by 'papain' can be explained by the presence of PPIV in the commercial enzyme preparation. They also confirm the primary specificity of PPIV for glycyl bonds, and indicate that subsites S_2 and S_3 preferentially accommodate hydrophobic sidechains.

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