Evidence by chemical modification that tryptophan-104 of the cysteine-proteinase inhibitor chicken cystatin is located in or near the proteinase-binding site

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The single tryptophan residue Trp-104 of chicken cystatin was modified with a 2-hydroxy-5-nitrobenzyl group. The change of the absorption spectrum of this group on binding of the modified cystatin to papain indicated a decreased environmental polarity of the probe. The modified inhibitor had about a 10^5 -fold lower affinity for papain than had intact cystatin, this being due to a higher dissociation rate constant. These results show that Trp-104 of cystatin is located in or near the proteinase-binding site of the inhibitor, in agreement with a model proposed from computer docking experiments.

INTRODUCTION

The binding of the cysteine-proteinase inhibitor cystatin from chicken egg-white to papain is being studied as a model for the general mode of action of such inhibitors. Cystatin forms a tight (K_d approx. 60 fM) complex with papain, in which the active site of the proteinase is blocked (Anastasi *et al.*, 1983; Nicklin & Barrett, 1984; Lindahl *et al.*, 1988; Machleidt *et al.*, 1989; Björk *et al.*, 1989). The complex appears to be formed with minimal conformational adaptation of either protein (Lindahl *et al.*, 1988; Björk *et al.*, 1989; Björk & Ylinenjärvi, 1990). The kinetics of the reaction are best described by a simple reversible bimolecular mechanism with an association rate constant of approx. $10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$, compatible with the rate being limited by the frequency of encounter between the binding regions of the two proteins (Björk *et al.*, 1989). From computer docking experiments based on the X-ray structures of cystatin and papain it has been suggested that three regions of the inhibitor participate in the interaction with the enzyme (Bode *et al.*, 1988). One of these regions comprises a loop of the polypeptide chain around the only tryptophan residue of cystatin, Trp-104. In the present paper we show that chemical modification of this tryptophan residue with a 2-hydroxy-5-nitrobenzyl group markedly affects the binding of cystatin to papain, in strong support of the proposed binding mechanism.

MATERIALS AND METHODS

Cystatin was purified as described previously (Anastasi *et al.*, 1983; Lindahl *et al.*, 1988). Cystatin 2, which differs from cystatin 1 only in being phosphorylated at Ser-80 (Laber *et al.*, 1989), was used in all experiments. The two forms have been shown to interact with active and inactivated papains in an identical or highly similar manner (Nicklin & Barrett, 1984; Lindahl *et al.*, 1988; Björk *et al.*, 1989; Björk & Ylinenjärvi, 1989; Machleidt *et al.*, 1989).

The purification of papain and actinidin, the storage of the two enzymes as their *S*-methylthio derivatives and the activation of these derivatives before use have been described previously (Burke *et al.*, 1974; Roberts *et al.*, 1986; Lindahl *et al.*, 1988; Björk & Ylinenjärvi, 1990). The *S*-carboxymethyl derivatives of

papain and actinidin were prepared by activation of the *S*-methylthio derivatives at a concentration of 100 μM for 15 min with 1 mM-dithiothreitol in 0.1 M-NaCl/100 μM -EDTA/0.1 M-Tris/HCl buffer, pH 8.0, followed by addition of iodoacetic acid to a concentration of 3 mM. After another 15 min, excess reagents were removed by chromatography on Sephadex G-25 (Pharmacia, Uppsala, Sweden). *S*-Carboxymethyl-actinidin was coupled to CNBr-activated Sepharose (Pharmacia) with a resulting protein content of 7 mg/ml of gel.

Cystatin (final concentration 100 μM) in 0.1 M-sodium acetate buffer, pH 4.5, was modified with dimethyl-(2-hydroxy-5-nitrobenzyl)sulphonium bromide (Serva, Heidelberg, Germany) (Horton & Tucker, 1970) at a 15-fold molar ratio to the protein by the addition of a freshly made solution of 25 mM reagent. After incubation for 20 min at room temperature, the reaction mixture was cooled to 0 °C, and unbound reagent was removed by gel chromatography at 4 °C on a column (2.6 cm $\times$ 17 cm) of Sephadex G-25, equilibrated with the reaction buffer. The protein pool was extensively dialysed against 0.5 M-NaCl/0.05 M-Tris/HCl buffer, pH 7.4. The modified cystatin was separated from a small amount of unmodified inhibitor by affinity chromatography on a column (13 cm $\times$ 1.6 cm) of *S*-carboxymethyl-actinidin-Sepharose, equilibrated with the dialysis buffer. The modified protein did not bind to the column, whereas the unmodified protein bound strongly. The modified cystatin was detected by its absorbance at both 280 and 410 nm.

Protein concentrations were obtained by absorption measurements at 280 nm. Absorption coefficients of 2.39 and 0.87 litre $\cdot$ g $^{-1} \cdot$ cm $^{-1}$ were used for both active and inactivated papains and unmodified cystatin respectively (Lindahl *et al.*, 1988; Björk & Ylinenjärvi, 1989). An absorption coefficient at 280 nm of 1.08 ± 0.03 litre $\cdot$ g $^{-1} \cdot$ cm $^{-1}$ (mean $\pm$ S.D., $n = 3$) was determined for the tryptophan-modified cystatin by the Coomassie Brilliant Blue G-250 dye-binding method (Bradford, 1976) with the use of a commercial reagent (Bio-Rad Protein Assay kit; Bio-Rad Laboratories, Richmond, CA, U.S.A.), with unmodified cystatin as standard. M_r values of 23400 for active or inactivated papain and 13100 for unmodified or modified cystatin were used in calculations of molar concentrations (Lindahl *et al.*, 1988). Molar ratios of the bound 2-hydroxy-5-nitrobenzyl (HNB) group to cystatin were determined at pH 12 by absorbance measure-

Abbreviation used: HNB, 2-hydroxy-5-nitrobenzyl.

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ments at 410 nm and were calculated from an absorption coefficient for the HNB chromophore of $18\,500\text{ M}^{-1}\cdot\text{cm}^{-1}$ (Loudon & Koshland, 1970).

Absorption difference spectra for the interaction between HNB-cystatin and papain were measured basically as described previously (Lindahl *et al.*, 1988). However, the measurements were done in the 300–500 nm wavelength region with $20\text{ }\mu\text{M}$ -HNB-cystatin, to which papain was added at a molar ratio to the modified inhibitor of 1.3:1. This ratio was sufficient to give at least 99 % saturation of HNB-cystatin with papain, as calculated from the equilibrium constant presented below.

Corrected fluorescence emission spectra of HNB-cystatin, papain and the complex between HNB-cystatin and papain were measured in the 300–400 nm wavelength region as described previously for the interaction between unmodified cystatin and papain (Lindahl *et al.*, 1988). The concentration of papain was $1\text{ }\mu\text{M}$ and the molar ratio of HNB-cystatin to papain was 1.3:1, giving approx. 94 % saturation of the proteinase. Fluorescence difference spectra were calculated from the measured spectra as detailed previously (Lindahl *et al.*, 1988).

Stoichiometries and dissociation equilibrium constants for the interaction between HNB-cystatin and active or inactivated papains were measured by titrations of enzyme with modified inhibitor. These titrations were monitored by the decrease in tryptophan fluorescence accompanying the interactions, as described previously (Lindahl *et al.*, 1988; Björk & Ylinenjärvi, 1989). The stoichiometries and dissociation constants were obtained by non-linear regression of the titration curves (Nordenman & Björk, 1978; Lindahl *et al.*, 1988).

The kinetics of the reaction between HNB-cystatin and papain were studied under pseudo-first-order conditions in a Hi-Tech SF4 stopped-flow spectrophotometer (Hi-Tech, Salisbury, Wilts., U.K.) by measurements of the changes of tryptophan fluorescence induced by the interaction (Björk *et al.*, 1989).

All analyses were carried out at $25\text{ }^{\circ}\text{C}$ in 0.1 M -NaCl/ $100\text{ }\mu\text{M}$ -EDTA/ 0.05 M -Tris/HCl buffer, pH 7.4, unless otherwise indicated.

RESULTS

Cystatin was modified by reaction with the tryptophan-specific reagent dimethyl-(2-hydroxy-5-nitrobenzyl)sulphonium bromide (Koshland *et al.*, 1964; Horton & Koshland, 1965; Horton & Tucker, 1970) at pH 4.5, resulting in covalent attachment of the HNB group to the only tryptophan residue of the protein, Trp-104. The molar ratio of the bound HNB group to cystatin in preparations of the modified inhibitor purified by affinity chromatography was 1.12 ± 0.03 (mean $\pm$ s.d., $n = 4$), consistent with complete modification of the tryptophan residue. The observed molar ratio of somewhat over 1.0:1 most probably is due to the uncertainty involved in the use of the same absorption coefficient for the protein-bound as for the free chromophore and to a possible error in the concentration determinations of the modified protein. Substitution of other residues than tryptophan is unlikely because of the high specificity of the reagent for this residue under acidic conditions (Koshland *et al.*, 1964; Horton & Koshland, 1965; Horton & Tucker, 1970). However, an occasional binding of two HNB groups to one tryptophan residue (Loudon & Koshland, 1970) cannot be excluded. The binding of the purified HNB-cystatin to papain was characterized by spectroscopic methods and by measurements of equilibrium and kinetic constants for the interaction.

The absorption difference spectrum in the visible region measured between the complex of HNB-cystatin with active papain and the free proteins showed that the environment of the HNB group was perturbed when the modified inhibitor bound to

Fig. 1. Absorption difference spectrum measured between the complex of HNB-cystatin with papain and the free proteins

Table 1. Dissociation equilibrium constants at $25\text{ }^{\circ}\text{C}$ at pH 7.4 and 1.015 for the interaction between HNB-cystatin and active or inactivated papains

The analyses were done at concentrations of $0.1\text{ }\mu\text{M}$ for active papain and *S*-methylthio-papain and 1.0 – $1.5\text{ }\mu\text{M}$ for *S*-carboxymethyl-papain. The values are given as means $\pm$ s.d. with the numbers of measurements in parentheses. The dissociation constants for the binding of unmodified cystatin to the three papain forms, shown for comparison, are taken from Björk *et al.* (1989) and Björk & Ylinenjärvi (1989).

Papain form	Dissociation constant (M)	
	HNB-cystatin	Unmodified cystatin
Active	$(2.4 \pm 1.2) \times 10^{-8}$ (4)	6×10^{-14}
<i>S</i> -Methylthio-	$(2.4 \pm 0.8) \times 10^{-8}$ (5)	7.1×10^{-13}
<i>S</i> -Carboxymethyl-	$(1.6 \pm 0.1) \times 10^{-6}$ (4)	5×10^{-10}

the enzyme (Fig. 1). The difference spectrum thus had a minimum at approx. 412 nm ($\Delta\epsilon$ approx. $-1500\text{ M}^{-1}\cdot\text{cm}^{-1}$) and a maximum at approx. 312 nm ($\Delta\epsilon$ approx. $300\text{ M}^{-1}\cdot\text{cm}^{-1}$), changes that are compatible with previous studies of the effect of a decreased solvent polarity on HNB absorption (Peterson & Blackburn, 1987).

The fluorescence-emission spectrum of HNB-cystatin had a maximum at approx. 305 nm, compared with approx. 340 nm for the unmodified protein (Lindahl *et al.*, 1988), and was thus consistent with tryptophan modification. The interaction of HNB-cystatin with active papain was accompanied by a shift of the emission maximum to a shorter wavelength and a decrease in intensity, together resulting in a maximal decrease in fluorescence emission at approx. 350 nm (results not shown). These changes were similar to those observed for the interaction between unmodified cystatin and papain (Lindahl *et al.*, 1988), indicating that the changes arise primarily from tryptophan residues in papain.

Stoichiometries and affinities for the interaction of HNB-cystatin with active papain and two inactivated forms of the enzyme were determined by titrations of the papains with the modified inhibitor, monitored by the decrease in tryptophan fluorescence accompanying the binding. Stoichiometries of 1.0 ± 0.1 (mean $\pm$ s.d., $n = 4$) and 0.95 ± 0.05 (range, $n = 2$) were obtained for the binding of HNB-cystatin to active papain and *S*-methylthio-papain respectively. However, the stoichiometry for

Fig. 2. Observed pseudo-first-order rate constants (k_{obs}) at 25 °C at pH 7.4 and 1/0.15 for the binding of HNB-cystatin to active papain as a function of the concentration of the modified inhibitor

The molar ratio of HNB-cystatin to active papain was maintained at 10:1 in all analyses. The vertical bars represent the standard deviations computed from six individual experiments.

the binding to *S*-carboxymethyl-papain could not be determined with reasonable accuracy, owing to the low affinity of this interaction. Measurements of dissociation constants showed that attachment of the HNB group to Trp-104 of cystatin resulted in about a 4×10^5 -fold lower affinity of the inhibitor for active papain and about 3×10^4 -fold and 3×10^3 -fold lower affinities for the *S*-methylthio and *S*-carboxymethyl derivatives of the enzyme respectively (Table 1).

The kinetics of the binding of HNB-cystatin to active papain were investigated by stopped-flow fluorimetry under pseudo-first-order conditions, i.e. with an excess of modified inhibitor. All reactions showed simple exponential behaviour. The resulting plot of the observed pseudo-first-order rate constants versus HNB-cystatin concentration was linear (Fig. 2) and gave a second-order association rate constant of $4.0 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$ at 25 °C. This value is somewhat lower than that of $9.9 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$ measured for the interaction of unmodified cystatin with papain (Björk *et al.*, 1989). Together, the dissociation equilibrium (Table 1) and association rate constants gave a dissociation rate constant for the HNB-cystatin–papain complex of approx. 0.1 s^{-1} , compared with the value of approx. $5.7 \times 10^{-7} \text{ s}^{-1}$ for the complex of unmodified cystatin with the enzyme (Björk *et al.*, 1989).

DISCUSSION

The HNB group attached to tryptophan residues in proteins serves as a spectral probe that is sensitive to changes in the local environment (Koshland *et al.*, 1964; Horton & Koshland, 1965). In the present work, the only tryptophan residue, Trp-104, of chicken cystatin was labelled with this group. The features of the difference spectrum measured on interaction of the modified inhibitor with papain indicate a decreased environmental polarity of the probe in the complex with the enzyme (Peterson & Blackburn, 1987). The attachment of the HNB group to Trp-104 also results in a large decrease of the affinity of cystatin for papain, due to an increased dissociation rate constant. Together, these observations indicate that Trp-104 of cystatin either is directly involved in the interaction with the proteinase or, alternatively, is located close to the proteinase-binding region of the inhibitor, the modifying group affecting the interaction because of its size. In either case, the HNB group presumably is 'buried' as a result of making contact with papain in the complex, but is sufficiently large that it displaces the inhibitor out of the tight fit with the enzyme, most probably preserving the

general mode of interaction but leading to lower affinity. The effect of attachment of the HNB group to Trp-104 of cystatin thus resembles the effect of introduction of substituents on the reactive cysteine residue of papain, which leads to a similar decrease in the stability of the complex with unmodified cystatin (Björk & Ylinenjärvi, 1989). In contrast with the unmodified inhibitor, HNB-cystatin shows about the same affinity for *S*-methylthio-papain as for active papain, although, like unmodified cystatin, it has a much lower affinity for *S*-carboxymethyl-papain. This finding is consistent with the presence of the HNB group leading to a sufficiently 'loose' complex with papain that there is space for a small, but not a large, inactivating group on the reactive cysteine residue of the enzyme without any further decrease in affinity.

The results strongly support the model for the interaction of chicken cystatin with papain proposed from computer docking experiments (Bode *et al.*, 1988). In this model, three regions of the inhibitor are suggested to interact with the enzyme, namely the segment of the chain around Gly-9, the Gln-Leu-Val-Ser-Gly sequence at residues 53–57 and the loop around Trp-104. The participation of the first two of these regions in the interaction is in agreement with other data (Ohkubo *et al.*, 1984; Barrett *et al.*, 1986; Abrahamson *et al.*, 1987; Machleidt *et al.*, 1989), but only circumstantial evidence has been presented for the involvement of the last-mentioned region (Lindahl *et al.*, 1988). The findings presented here show the importance of this part of the molecule also. In the proposed model, Trp-104 of cystatin interacts primarily with two tryptophan side chains in the active-site cleft of papain, Trp-177 and Trp-181, in such a manner that the indole ring of Trp-104 stacks on the side chain of Trp-177 and lies edge-on with the indole ring of Trp-181 (Bode *et al.*, 1988). It is apparent that attachment of an HNB group to Trp-104 should severely perturb these interactions, leading to lower binding affinity, in agreement with the results obtained. However, the interactions remaining in the other two putative binding regions apparently are sufficiently strong that they confer appreciable stability on the complex between the modified inhibitor and papain, as evident by the substantial affinity measured. Possibly, this affinity, corresponding to about 60 % of the total free energy of binding of intact cystatin to papain, predominantly reflects the interaction energy contributed by these other two regions.

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