Papain labelled with fluorescent thiol-specific reagents as a probe for characterization of interactions between cysteine proteinases and their protein inhibitors by competitive titrations

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Papain was labelled by attachment of the fluorescent groups 2-(4'-acetamidoanilino)naphthalene-6-sulphonic acid (AANS) or *N*-(acetylaminomethyl)-8-naphthylamine-1-sulphonic acid (AEDANS) to the active-site cysteine residue, with the aim of using the labelled papains as probes in competitive titrations of unlabelled cysteine proteinases with their inhibitors. The interaction between the labelled papains and cystatins was accompanied by an increase in fluorescence emission of up to 38-fold for AANS-papain and ~3.5-fold for AEDANS-papain. Fluorescence titrations gave dissociation equilibrium constants of 3.1 and 0.6 μM for the binding of chicken cystatin and recombinant human cystatin C respectively to AANS-papain and of 11.9 μM for the binding of chicken cystatin to AEDANS-papain. The kinetics of interaction of chicken cystatin with AANS-papain showed an unusual biphasic dependence of the observed pseudo-first-order rate constant on inhibitor concentration, consistent with the reaction occurring via both pathways of a general two-step binding mechanism. AANS-papain was selected as the most suitable probe for competitive titrations of unlabelled active or inactivated cysteine proteinases with inhibitors. This technique, which provides stoichiometries and dissociation constants for the interaction between unlabelled enzyme and inhibitor, allows monitoring of the interactions by a large fluorescent signal in a wavelength region where the interacting proteins do not contribute to the observed fluorescence. Such competitive titrations of active papain or actinidin with chicken cystatin or recombinant human cystatin C all gave inhibitor/enzyme stoichiometries of close to 1.0. A dissociation constant of 1.8 μM for the reaction of chicken cystatin with a papain derivative, *S*-[*N*-(3-carboxypropyl)succinimidyl]-papain, was also determined by the same technique. These results show the usefulness of the fluorescent papains for the characterization of interactions between cysteine-proteinase inhibitors and their target enzymes.

INTRODUCTION

Most protein inhibitors of cysteine proteinases found in mammalian tissues and plasma belong to the cystatin superfamily of evolutionary related proteins, which comprises three distinct individual families, namely I, II and III (Barrett *et al.*, 1986). Members of the first two of these families, the cystatins, are small proteins of ~100 amino acid residues (M_r 12000–14000) with no carbohydrate, whereas inhibitors of the third family, the kininogens, are larger glycoproteins (M_r 60000–120000) containing three low- M_r cystatin-like domains. The inhibitors of the cystatin superfamily inhibit both mammalian cysteine proteinases, e.g. cathepsins B, H and L, and several structurally related plant enzymes, such as papain and actinidin (Barrett *et al.*, 1986; Lindahl *et al.*, 1988; Björk & Ylinenjärvi, 1990). The mechanism of these interactions has been investigated by studies of reactions between the different cystatins and various cysteine proteinases and derivatives thereof (Barrett *et al.*, 1986; Abrahamson *et al.*, 1987; Bode *et al.*, 1988; Machleidt *et al.*, 1989; Björk *et al.*, 1989; Nikawa *et al.*, 1989; Björk & Ylinenjärvi, 1990; Stubbs *et al.*, 1990). The reaction mechanism appears to differ from that of serine-proteinase inhibitors in many respects. The kinetics of binding of chicken cystatin to several cysteine proteinases thus are best described by a simple reversible bimolecular mechanism, in contrast with the more complicated kinetics of interaction of

serine-proteinase inhibitors with their target enzymes (Laskowski & Kato, 1980; Björk *et al.*, 1989; Björk & Ylinenjärvi, 1990). Moreover, in contrast with serine-proteinase inhibitors, chicken cystatin binds also to enzymes which have been inactivated by attachment of bulky reagents to the active-site amino acid residue (Anastasi *et al.*, 1983; Björk & Ylinenjärvi, 1989). Computer docking experiments, based on the X-ray structures of a variant of chicken cystatin and of papain (Bode *et al.*, 1988), and the recently determined X-ray structure of a complex between human cystatin B (also called stefin B) and papain (Stubbs *et al.*, 1990) also demonstrate that the inhibitor binds to the enzyme in a manner different from that of a substrate, in contrast with serine-proteinase inhibitors.

In the present study we attached fluorophores to the active-site cysteine residue of papain with the aim of using these labelled papains as probes in competitive titrations for the characterization of interactions between unlabelled cysteine proteinases and their protein inhibitors. The fluorescence of the labelled papains were shown to increase markedly on interaction with inhibitors. Characterization of the kinetics of one such interaction revealed a unique binding mechanism in which the inhibitor induced a change of the conformation of the labelled proteinase to a state more favourable for binding by both pathways than is possible for a two-step binding process. Experiments demonstrated the usefulness of the labelled papains in competitive titrations for the

Abbreviations used: AANS, 2-(4'-acetamidoanilino)naphthalene-6-sulphonic acid; AEDANS, *N*-(acetylaminomethyl)-8-naphthylamine-1-sulphonic acid; ANBD, 4-[*N*-(acetoxymethyl)-*N*-methylamino-7-nitrobenz-2-oxa-1,3-diazole]; IAANS, 2-(4'-iodoacetamidoanilino)naphthalene-6-sulphonic acid; IAEDANS, *N*-(iodoacetylaminomethyl)-8-naphthylamine-1-sulphonic acid; IANBD, 4-[*N*-(iodoacetoxymethyl)-*N*-methylamino-7-nitrobenz-2-oxa-1,3-diazole].

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determination of the stoichiometry of binding of cystatins to enzymes with high affinity for the inhibitors, as well as for measurements of dissociation constants for weaker interactions of intact or modified inhibitors with enzymes or enzyme derivatives. A major advantage of the probes in such studies is that they give strong fluorescent signals by which the interactions can be monitored under conditions where the background tryptophan fluorescence of the proteins is negligible. Moreover, the probes can be used to characterize interactions which do not result in any measurable changes of intrinsic protein fluorescence.

MATERIALS AND METHODS

Chicken cystatin was isolated by affinity chromatography on matrix-linked carboxymethylpapain (Anastasi *et al.*, 1983; Lindahl *et al.*, 1988). Form 1 of the inhibitor was used in all experiments. The non-phosphorylated and phosphorylated forms of chicken cystatin, 1 and 2 (Anastasi *et al.*, 1983; Laber *et al.*, 1989), have been shown previously 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). Chicken cystatin was coupled to Affi-Gel 10 (Bio-Rad, Richmond CA, U.S.A.) with a resulting protein content of 5 mg/ml of gel. Recombinant human cystatin C, expressed in *Escherichia coli* (Abrahamson *et al.*, 1988), was kindly provided by Dr. A. Grubb and Dr. M. Abrahamson, Department of Clinical Chemistry, University of Lund, Lund, Sweden.

Papain (EC 3.4.22.2) and actinidin (EC 3.4.22.14) were purified from papaya (*Carica papaya*) latex (Sigma) and kiwi fruit (*Actinidia chinensis*) respectively by methods described previously (Burke *et al.*, 1974; Brocklehurst *et al.*, 1981; Lindahl *et al.*, 1988; Björk & Ylinenjärvi, 1990). The enzymes were stored as their inactive *S*-methylthio derivatives and were activated with 1 mM-dithiothreitol before use (Roberts *et al.*, 1986; Lindahl *et al.*, 1988). Papain was inactivated with *N*-(3-carboxypropyl)-maleimide as described by Björk & Ylinenjärvi (1989). The inactivated protein was applied to a column of matrix-linked cystatin in 0.05 M-Tris/HCl/0.1 M-NaCl/100 μ M-EDTA, pH 7.4, and was eluted with 0.05 M-Na₂CO₃/0.5 M-NaCl/100 μ M-EDTA, pH 9.5. This treatment removed a small amount of residual active papain, which binds more tightly to cystatin than does the inactivated enzyme (Björk & Ylinenjärvi, 1989) and therefore was retained on the column.

Papain (66–106 μ M in 0.05 M-Tris/HCl/0.1 M-NaCl/100 μ M-EDTA, pH 7.4) was labelled by reaction at 20–22 °C with the fluorescent thiol group reagents 2-(4'-iodoacetamidooanilino)-naphthalene-6-sulphonic acid (IAANS) (Molecular Probes, Eugene, OR, U.S.A.), *N*-(iodoacetylaminooethyl)-8-naphthylamine-1-sulphonic acid (IAEDANS) (Serva, Heidelberg, Germany) and 4-[*N*-(iodoacetoxy)ethyl-*N*-methyl]amino-7-nitrobenz-2-oxa-1,3-diazole (IANBD) (Molecular Probes). The reactions were monitored by measurements of papain activity against *N*- α -benzoyl-L-arginine *p*-nitroanilide, essentially as described by Björk & Ylinenjärvi (1989), and were continued until this activity was $< \sim 1\%$ of that measured for the active enzyme. The reactions with IAANS and IAEDANS were allowed to proceed for 30 and 90–120 min at reagent concentrations of 200 and 375–500 μ M respectively, whereas the reaction with IANBD was allowed to proceed for 45–65 min, 1 mg of reagent being added to 1 ml of papain solution. In all labelling reactions, iodoacetamide was added in a molar ratio to papain of ~ 0.5 after the times indicated to inactivate traces of active papain possibly remaining. After a further incubation of ~ 5 min, excess reagents were removed by chromatography on Sephadex G-25 (Pharmacia, Uppsala, Sweden) in 0.05 M-Tris/HCl/0.1 M-NaCl/

100 μ M-EDTA, pH 7.4. In the case of IAANS, the solution was also dialysed against this buffer and the small amount of precipitate that appeared was removed by filtration through a 0.45 μ m-pore-size filter (Millipore Corp., Bedford, MA, U.S.A.) before further analyses. The molar ratios of reagents bound to papain were calculated from absorbance measurements; in these calculations, ϵ_{325} 24900 (Johnson *et al.*, 1980), $\epsilon_{342.5}$ 6300 (Hudson & Weber, 1973) and ϵ_{490} 24000 (Lancet & Pecht, 1977; Dunn *et al.*, 1983) M⁻¹·cm⁻¹ were used for the 2-(4'-acetamidooanilino)-naphthalene-6-sulphonic acid (AANS), *N*-(acetylaminooethyl)-8-naphthylamine-1-sulphonic acid (AEDANS) and 4-[*N*-(acetoxo)-ethyl-*N*-methyl]amino-7-nitrobenz-2-oxa-1,3-diazole (ANBD) groups respectively.

Fluorescence emission spectra were measured in an SLM 4800S spectrofluorimeter (SLM-Aminco, Urbana, IL, U.S.A.) essentially as described by Lindahl *et al.* (1988). The excitation wavelengths used in these and other fluorescence measurements were 328, 342 and 495 nm for the AANS, AEDANS and ANBD derivatives of papain respectively. The excitation and emission bandwidths were 4 and 4–8 nm respectively in all measurements. The spectra were obtained with 3–15 μ M labelled papain and 40–99 μ M-cystatin, conditions which gave $\geq 88\%$ saturation of the labelled enzymes with the inhibitor, as calculated from the dissociation constants given below. The spectra were corrected for inner-filter effects ($< 5\%$) and for the wavelength dependence of the photomultiplier tube.

Acrylamide quenching (Eftink & Ghiron, 1976) of the fluorescence of AANS-papain and AEDANS-papain was analysed by successive additions of 10 μ l of 6.3 M-acrylamide (Eastman-Kodak, Rochester, NY, U.S.A.) to 2 ml of 3 μ M-AANS-papain or 12 μ M-AEDANS-papain, with or without cystatin at a 10-fold molar ratio to the labelled enzyme. The fluorescence decrease was measured at the wavelength of the maximum of the uncorrected emission spectrum of the sample. The data were corrected for inner-filter effects ($< 2\%$) and dilution ($< 8\%$) and were plotted according to the Stern–Volmer equation (Eftink & Ghiron, 1976).

Dissociation equilibrium constants for the interaction between cystatin and the AANS and AEDANS derivatives of papain were measured by titrations of labelled enzyme with inhibitor. The initial concentrations of AANS-papain and AEDANS-papain were 1.0–6.0 and 5–20 μ M respectively. The titrations were monitored by the increase in fluorescence intensity accompanying the interaction (measured at the wavelength of the maximum of the uncorrected emission spectrum of the complex formed) and were continued until the degree of saturation of enzyme with inhibitor was $\geq 85\%$. The data were corrected for inner-filter effects ($< 5\%$) and dilution, the latter being $< 10\%$ in most experiments, but up to $\sim 30\%$ in some experiments with the weakly binding AEDANS-papain. Dissociation constants were obtained by non-linear least-squares regression of the titration data to the equation derived for formation of an equimolar complex between the two reactants (Nordenman & Björk, 1978; Lindahl *et al.*, 1988). The data are presented as $\Delta F/\Delta F_{\max}$, where ΔF is the increase of the fluorescence intensity of the labelled papain on addition of inhibitor and $\Delta F_{\max}$ is the value of ΔF at saturation of the labelled papain, obtained from the regression analysis. In this manner the maximal fluorescence change in all titrations is normalized to 1.

The stoichiometries for the binding of cystatin to active papain or actinidin and the dissociation equilibrium constant for the interaction of cystatin with the inactive papain derivative *S*-[*N*-(3-carboxypropyl)succinimidyl]-papain were measured by competitive titrations (Bock & Shore, 1983) with AANS-papain as a probe. In such a titration a mixture of AANS-papain and competing active or inactivated non-fluorescent enzyme was

titrated with the inhibitor. A volume (1.8 ml) of 1.5 μM AANS-papain with or without 6 μM -papain or actinidin was titrated with cystatin in the stoichiometry measurements, and the same volume of 1 μM -AANS-papain with or without 10–18.5 μM -S-[N-(3-carboxypentyl)succinimidyl]-papain was used in the measurements of dissociation equilibrium constants. The fluorescence intensities after each addition, measured as in the titrations of the labelled enzyme alone (see above), were corrected for inner-filter effects (< 2%) and dilution (< 13%). The stoichiometries and dissociation constants were obtained by non-linear least-squares fitting of the data to an equation relating the increase of the fluorescence of the labelled papain to the concentration of added cystatin for a competitive titration, in which the competing unlabelled proteinase or proteinase derivative binds to the inhibitor but does not contribute to the measured fluorescence increase. Such a titration is described by the following equilibria:

where P^* is the labelled papain, P is the unlabelled competing proteinase or proteinase derivative, I is the inhibitor, m and n are the stoichiometries of binding of the inhibitor to the labelled papain and competing proteinase respectively, and K_{P^*} and K_{P} are the dissociation constants for these interactions. By algebraic manipulation of the relationship derived by Bock & Shore (1983) for a similar kind of titration, the following equation can be derived:

$$C_1 \cdot (\Delta F / \Delta F_{\text{max}})^3 + C_2 \cdot (\Delta F / \Delta F_{\text{max}})^2 + C_3 \cdot (\Delta F / \Delta F_{\text{max}}) + C_4 = 0 \quad (1)$$

with the coefficients:

$$\begin{aligned} C_1 &= m \cdot [\text{P}^*]_{\text{T}} \cdot [(K_{\text{P}} / K_{\text{P}^*}) - 1] \\ C_2 &= [\text{I}]_{\text{T}} \cdot [1 - (K_{\text{P}} / K_{\text{P}^*})] + m \cdot [\text{P}^*]_{\text{T}} \cdot [1 - 2(K_{\text{P}} / K_{\text{P}^*})] \\ &\quad + K_{\text{P}^*} - K_{\text{P}} - n \cdot [\text{P}]_{\text{T}} \\ C_3 &= [\text{I}]_{\text{T}} \cdot [2(K_{\text{P}} / K_{\text{P}^*}) - 1] + m \cdot [\text{P}^*]_{\text{T}} \cdot (K_{\text{P}} / K_{\text{P}^*}) + K_{\text{P}} + n \cdot [\text{P}]_{\text{T}} \\ C_4 &= -[\text{I}]_{\text{T}} \cdot (K_{\text{P}} / K_{\text{P}^*}) \end{aligned}$$

In this equation, ΔF and ΔF_{max} have the meanings indicated above, and the subscript T denotes total concentration. In the fitting procedure, the cubic equation was solved by the Newton-Raphson method. The fitted parameters were ΔF_{max} , n and K_{P} , and m was taken as 1 (Lindahl *et al.*, 1988; Björk & Ylinenjärvi, 1989); K_{P^*} was that obtained from titrations of the labelled enzyme alone. The procedure thus gives the stoichiometry and dissociation constant for the interaction of unlabelled proteinase with inhibitor. The titration data are presented as $\Delta F / \Delta F_{\text{max}}$.

The kinetics of the reaction between chicken cystatin and AANS-papain were studied by fluorescence measurements in a Hi-Tech SF4 stopped-flow spectrophotometer (Hi-Tech, Salisbury, Wilts., U.K.), essentially as described by Björk *et al.* (1989). Excitation was at 328 nm and emission was observed through a cut-off filter with 50% transmission at ~ 350 nm.

All analyses were made at 25 $^{\circ}\text{C}$ in 0.05 M-Tris/HCl/0.1 M-NaCl/100 μM -EDTA, pH 7.4. Protein concentrations were obtained from absorption measurements at 280 nm with the use of absorption coefficients and relative molecular masses reported previously (McDowall, 1970; Cejka & Fleischman, 1973; Carne & Moore, 1978; Lindahl *et al.*, 1988; Björk & Ylinenjärvi, 1989; Abrahamson *et al.*, 1988). Before concentrations of the labelled papains were calculated, the contribution from the labelling group to the observed absorbance at 280 nm was subtracted. This contribution ($\sim 25\%$ of the observed absorbance for AANS-papain and $< 3\%$ for the other two labelled papains) was obtained from the absorption spectra of the reagents alone.

Fig. 1. Corrected fluorescence emission spectra of AANS-papain or AEDANS-papain, with or without chicken cystatin

(a) Curve 1, AANS-papain alone; curve 2, AANS-papain with cystatin. The AANS-papain and cystatin concentrations were 3 and 40 μM respectively, giving 92% saturation of the labelled papain. (b) Curve 1, AEDANS-papain alone; curve 2, AEDANS-papain with cystatin. The AEDANS-papain and cystatin concentrations were 10 and 99 μM respectively, giving 88% saturation of the labelled papain. The spectra in both (a) and (b) have been normalized to a relative fluorescence of 1.0 for the labelled papains alone at the wavelength of the emission maximum.

RESULTS

Labelling of papain

Fully active papain, containing about 1 mol of thiol groups/mol of protein (Burke *et al.*, 1974; Lindahl *et al.*, 1988), was labelled by reaction with three different fluorescent thiol reagents, IAANS, IAEDANS and IANBD, resulting in covalent attachment of the fluorophores to the active-site cysteine residue of the enzyme. The molar ratios of bound reagent to protein were 0.96 ± 0.11 (s.d., $n = 4$), 0.85 ± 0.09 (s.d., $n = 3$), and 0.81 ± 0.04 (range, $n = 2$) for the AANS, AEDANS and ANBD derivatives of papain respectively.

Characterization of the interaction between the labelled papains and cystatins

The interaction between the labelled papains and cystatins was studied as a prerequisite for the use of the labelled enzymes as probes for interactions between unlabelled cysteine proteinases and inhibitors. Corrected fluorescence emission spectra of AANS-papain and AEDANS-papain, alone and $\geq 88\%$ saturated with chicken cystatin, showed that the emission intensity of the protein-bound fluorophores increased markedly on interaction of the labelled papain with the inhibitor (Fig. 1). The increase of the intensities at the emission maxima, recalculated to reflect saturation of the labelled enzyme, was ~ 18 - and ~ 3.6 -

Fig. 2. Titrations of AANS-papain or AEDANS-papain with chicken cystatin, monitored by fluorescence measurements at 25 °C

(a) AANS-papain with initial concentrations: ○, 6 μM; △, 3 μM; □, 1 μM. (b) AEDANS-papain with initial concentrations: ●, 20 μM; ▲, 10 μM; ■, 5 μM. The continuous lines represent the computer fits of the data to the theoretical binding equation. The dissociation constants (assuming equimolar stoichiometry between enzyme and inhibitor) giving these lines were 3.1 μM for all curves in (a) and 11.5–11.6 μM for the curves in (b). ΔF , Difference in fluorescence intensity between the mixture of labelled papain with cystatin and labelled papain alone; $\Delta F_{\max}$, the value of ΔF at saturation of the labelled papain, obtained from the regression analyses.

fold for AANS-papain and AEDANS-papain respectively. The binding of cystatin also shifted the wavelength of the emission maximum from 459 to 444 nm for AANS-papain and from 513 to 493 nm for AEDANS-papain. The corresponding spectra for ANBD-papain showed only about a 1.5-fold increase in emission intensity, and no wavelength shift, on binding of chicken cystatin to the labelled enzyme (results not shown). Because of this low fluorescence enhancement, ANBD-papain was not used in further studies.

The dissociation equilibrium constants for the binding of chicken cystatin to AANS-papain or AEDANS-papain were determined by fluorescence titrations (Fig. 2). The data were evaluated on the basis of an equimolar binding stoichiometry, as has been demonstrated previously for the interaction of the inhibitor with both active and several inactivated papains (Lindahl *et al.*, 1988; Björk & Ylinenjärvi, 1989). Dissociation constants of 3.1 ± 0.1 (S.D., $n = 7$) and 11.9 ± 1.0 ($n = 5$) μM were obtained for the binding of chicken cystatin to AANS-papain and AEDANS-papain respectively. A similar fluorescence titration of AANS-papain with recombinant human cystatin C (not shown), also evaluated on the basis of an equimolar binding

Fig. 3. Observed pseudo-first-order rate constants (k_{obs}) for the binding of cystatin to AANS-papain at 25 °C as a function of cystatin concentration

The cystatin/AANS-papain ratio was maintained at 10:1 in all measurements. The error bars show the 95% confidence intervals computed from four to six individual experiments. The continuous line represents the computer fit of the data to eqns. (2) and (3), resulting in the values for the parameters given in the Discussion section.

stoichiometry (see below), gave a dissociation equilibrium constant of 0.6 μM. The human inhibitor thus bound somewhat tighter than did chicken cystatin to AANS-papain. The fluorescence increase at saturation of labelled enzyme with inhibitor was ~38-fold, i.e. about twice the value given by chicken cystatin.

The fluorescence of AANS-papain complexed with chicken cystatin was quenched by acrylamide to a somewhat greater extent than that of the free labelled enzyme (55 as against 42% at 0.4 M-acrylamide). However, no difference in acrylamide quenching of the fluorescence of free and complex-bound AEDANS-papain could be observed ($31 \pm 1\%$ at 0.45 M-acrylamide). All Stern–Volmer plots were linear, except that for the cystatin–AANS-papain complex, which showed a slight upwards curvature, compatible with either a heterogeneity of the fluorescent species or a contribution from static quenching (Eftink & Ghiron, 1976).

The kinetics of binding of chicken cystatin were studied only with AANS-papain. The experiments were done by stopped-flow fluorimetry under pseudo-first-order conditions, i.e. with excess inhibitor. First-order reactions were observed throughout the range of cystatin concentrations covered. Amplitudes were approximately proportional to the expected concentrations of complex of inhibitor with labelled enzyme, calculated from the dissociation constant given above. The resulting plot of the observed pseudo-first-order rate constant against cystatin concentration was unusual in that k_{obs} decreased at low inhibitor concentrations to a minimum at ~1 μM-cystatin and increased again to a limiting rate at higher concentrations (Fig. 3).

Use of labelled papains as probes for cysteine proteinase–inhibitor interactions

The AANS-derivative of papain, which gave the largest fluorescence increase on interaction with chicken cystatin, was selected to demonstrate the usefulness of the labelled papains as probes in studies of the binding of unlabelled cysteine proteinases to their protein inhibitors by competitive titrations. In one set of experiments, several interactions of inhibitors with active, unlabelled proteinases were characterized by such titrations of mixtures of AANS-papain and unlabelled proteinase with inhibitor (Fig. 4). The stoichiometry for the binding of the inhibitor to the unlabelled enzyme could be obtained from these analyses by non-linear least-squares fitting of the data to a theoretical

Fig. 4. Competitive titrations of mixtures of AANS-papain and active cysteine proteinases with chicken cystatin or recombinant human cystatin C, monitored by fluorescence measurements at 25 °C

○, AANS-papain and papain titrated with chicken cystatin; △, AANS-papain and actinidin titrated with chicken cystatin; □, AANS-papain and papain titrated with recombinant human cystatin C. Initial concentrations were 1.5 and 6 μM for AANS-papain and active enzyme respectively. The continuous lines represent the computer fits of the data to eqn. (1). The stoichiometries of binding of inhibitor to active enzyme giving these lines were 1.0 for chicken cystatin/papain and chicken cystatin/actinidin and 0.9 for recombinant human cystatin C/papain. For an explanation of the label on the ordinate, see the legend to Fig. 2.

Fig. 5. Competitive titration of a mixture of AANS-papain and *S*-[*N*-(3-carboxypropyl)succinimidyl]-papain with chicken cystatin, monitored by fluorescence measurements at 25 °C

The initial concentrations of AANS-papain and *S*-[*N*-(3-carboxypropyl)succinimidyl]-papain were 1 and 10 μM respectively. The continuous line represents the computer fits of the data to eqn. (1). The stoichiometry and dissociation constant for the binding of cystatin to *S*-[*N*-(3-carboxypropyl)succinimidyl]-papain (assuming equimolar stoichiometry and a dissociation constant of 3.1 μM for the interaction between the inhibitor and AANS-papain) giving this line were 0.5 and 1.8 μM respectively. For an explanation of the label on the ordinate, see the legend to Fig. 2.

equation relating the observed fluorescence increase to the cystatin concentration (see the Materials and methods section). However, because of the high affinity of the interactions the dissociation constants were undefined by the data and could not be evaluated. Besides by curve-fitting, the binding stoichiometries could also be obtained with good accuracy, and more simply, by visual inspection of the titration curves. Because the inhibitor binds considerably more tightly to the unlabelled enzyme than to AANS-papain, essentially no inhibitor will bind to the AANS-papain until the unlabelled enzyme has been saturated. An increase of AANS fluorescence is thus observed only when the molar ratio between inhibitor and unlabelled proteinase cor-

responding to the binding stoichiometry between the two proteins is exceeded (Fig. 4). Titrations with chicken cystatin as inhibitor and papain as unlabelled enzyme gave a stoichiometry of binding of inhibitor to unlabelled enzyme of 1.0. This value is in agreement with earlier results (Lindahl *et al.*, 1988) and thus verifies the validity of the method. Similar titrations with chicken cystatin and actinidin and with recombinant human cystatin C and papain gave corresponding stoichiometries of 1.0 and 0.9 respectively.

In other experiments the interaction of chicken cystatin with an enzyme derivative binding less tightly to the inhibitor, *S*-[*N*-(3-carboxypropyl)succinimidyl]-papain, was characterized by the same competitive titration technique (Fig. 5). A binding stoichiometry of ~ 0.5 and a dissociation constant of $1.8 \pm 0.3 \mu\text{M}$ (S.D., $n = 3$) was obtained by non-linear least-squares fitting of the data. The dissociation constant is in good agreement with the value of 1.7 μM obtained previously for this interaction (Björk & Ylinenjärvi, 1989). Although in this case the stoichiometry was not well defined by the data, the somewhat low value may indicate the presence in the preparation of the papain derivative of some material unable to bind to cystatin.

DISCUSSION

Papains labelled with the AANS or AEDANS groups were found to be suitable fluorescent probes for characterization of the binding of unlabelled cysteine proteinases to their protein inhibitors by competitive titrations. A basis for a valid use of the probes was established by studies with cystatins of family II, mainly chicken cystatin, of the nature of the interactions between the inhibitors and the labelled enzymes alone. The blue shift and large fluorescence enhancement accompanying these reactions indicate that the interaction occurs in a manner that renders the environment around the fluorophore more hydrophobic. However, the similar, or in the case of AANS-papain even somewhat increased, quenching by acrylamide of the fluorescence of bound compared with that of free labelled papain suggests that the fluorophore is only partially buried and remains relatively freely accessible to solvent. This conclusion is consistent with the appreciably higher dissociation constants for the binding of the labelled papains than of active papain to the inhibitor (Björk *et al.*, 1989), reflecting a comparatively 'loose' interaction. These dissociation constants are of the same order of magnitude as those observed with papains inactivated with non-fluorescent groups of similar size (Björk & Ylinenjärvi, 1989). The results are thus in agreement with earlier conclusions, based on experimental binding data (Björk & Ylinenjärvi, 1989) and computer-modelling studies (Bode *et al.*, 1988), that only small inactivating groups can be accommodated in the space suggested by the modelling studies to occur around the reactive cysteine residue in the complex. Larger inactivating groups displace the inhibitor out of the tight fit with the enzyme, preserving the general mode of interaction, but leading to lower affinity.

Most work was done with AANS-papain, because of the larger fluorescence increase and higher affinity of the interaction of this papain derivative with the inhibitors tested. The kinetics of binding of chicken cystatin to AANS-papain were found to be highly unusual and appreciably different from those of the binding of the inhibitor to active papain or papain inactivated with smaller, non-fluorescent, groups. The latter reactions can be best described as simple reversible bimolecular reactions with second-order rate constants approaching that expected for a rate controlled by macromolecular diffusion (Björk & Ylinenjärvi, 1989). In contrast, the biphasic dependence of the observed pseudo-first-order rate constant on cystatin concentration, showing a minimum and leading to a limiting rate at high

concentrations, indicates that the reaction with AANS-papain is more complicated and must involve more than a single step. This peculiar dependence of k_{obs} on inhibitor concentration, together with the observed first-order behaviour of all reactions, can be explained most simply by the following general two-step binding mechanism:

In one pathway (A) of this mechanism, the inhibitor (I) can bind to the labelled papain (P*) by first forming a weak complex with the latter (P*I) and then inducing a conformational change of the labelled papain in this complex (P*†I), thereby giving rise to the observed fluorescence change. In the other pathway (B) the inhibitor can also shift an equilibrium between two conformational states of free, labelled papain (P* and P*†, the latter being the same state as that induced by the inhibitor in pathway A) by binding preferentially to P*† with the same overall fluorescence change as in pathway A. In both pathways the binding steps are assumed to be in rapid equilibrium with dissociation constants K_1 and K_4 . That both pathways are necessary to explain the observed dependence of the rate constant on inhibitor concentration follows from the fact that pathways A or B alone predict that k_{obs} should either increase or decrease respectively in a unidirectional fashion to a limiting value (Fersht, 1985). As derived in the Appendix, the proposed general binding mechanism results in single exponentials with the following dependence of k_{obs} on $[\text{I}]_{\text{T}}$ for reactions under pseudo-first-order conditions ($[\text{I}]_{\text{T}} \gg [\text{P}^*]$):

$$k_{\text{obs}} = [(k_3 K_1 / [\text{I}]_{\text{T}}) + k_2] / (1 + K_1 / [\text{I}]_{\text{T}}) + [k_{-3} + (k_{-2} [\text{I}]_{\text{T}}) / K_4] / (1 + [\text{I}]_{\text{T}} / K_4) \quad (2)$$

where a subscript T denotes total concentration. Of the six parameters of this equation, only five are independent, since the parameters are related by the equation:

$$K_1 / K_4 = (k_2 \cdot k_{-3}) / (k_{-2} \cdot k_3) \quad (3)$$

A fit of the data to eqns. (2) and (3) well described the observed variation of k_{obs} with cystatin concentration (Fig. 3) and gave K_1 $11.0 \pm 1.2 \mu\text{M}$, k_2 $0.64 \pm 0.04 \text{ s}^{-1}$, k_{-2} $0.21 \pm 0.05 \text{ s}^{-1}$, k_3 $0.033 \pm 0.023 \text{ s}^{-1}$, k_{-3} $0.73 \pm 0.18 \text{ s}^{-1}$ and K_4 $0.16 \pm 0.09 \mu\text{M}$. An overall dissociation constant, $K_{\text{eq}} = (k_{-2} K_1 + k_2 K_4) / (k_2 + k_{-2})$ (see the Appendix), of $2.9 \mu\text{M}$ was calculated from these data, in good agreement with the value of $3.1 \mu\text{M}$ determined by fluorescence titrations.

The binding of cystatin to AANS-papain thus is most simply described by the general two-step-binding mechanism delineated above. In particular, a conformational change of the fluorescent papain appears to occur both when the latter is free and in an initial complex with the inhibitor. This change allows the labelled papain to interact much more tightly with cystatin, with an affinity ~ 20 -fold higher than the overall affinity actually measured (cf. K_4 with K_{eq}). A plausible model for the conformational change is a rearrangement of the fluorescent group to a position or conformation that sterically interferes less with inhibitor binding, although an alteration of the protein structure in the vicinity of the fluorescent group, leading to tighter binding, cannot be excluded. Such a model would be consistent with the observation that the fluorophore is accessible to acrylamide quenching in the complex of labelled proteinase with inhibitor.

Moreover, the contribution of both conformational states of the fluorophore to the fluorescence of the complex (as indicated by the conformational equilibrium constant for the complex, $k_2/k_{-2} \sim 3$) may explain the observed deviation from linearity of the Stern-Volmer plot for acrylamide quenching of the complex. The fitted values of k_3 and k_{-3} ($k_{-3} \gg k_3$) indicate the most of the free labelled papain exists in the 'native' conformation, P*. Nevertheless, the pre-equilibrium pathway (B) dominates at low cystatin concentrations, owing to the much stronger binding of the inhibitor to the altered conformation, P*†, than to the 'native' conformation (as evident from $K_4 \ll K_1$), resulting in low amounts of the P*I species under these conditions. However, the pathway involving an induced conformational change (A) is the major contributor at higher cystatin concentrations, where the P†I species becomes predominant. This shift between the two pathways with increasing cystatin concentrations results in the biphasic dependence of k_{obs} on inhibitor concentration. The observed kinetic behaviour provides a unique example of a two-step binding process involving a conformational change in which both pathways that are possible for such a process contribute to the reaction mechanism.

The possibility that the conformational change observed to accompany the interaction of labelled papain with cystatin originates from a movement of the fluorescent label rather than from the proteinase has general implications for the use of probe molecules as reporters of protein-ligand interactions. Conformational changes indicated by such extrinsic probes thus may not reflect the properties of the interaction of the native protein with the ligand, but may instead reflect conformational changes of the probe which affect the interaction being studied. However, this possibility in no way invalidates the use of labelled proteins as probes of interactions involving unlabelled proteins by competitive binding titrations in the manner used here.

Several experiments with AANS-papain showed that papains labelled with fluorescent groups can be used to advantage in two different aspects of the characterization of interactions between unlabelled cysteine proteinases and their protein inhibitors. Stoichiometries of such interactions can thus easily be determined by competitive titrations with the papain derivatives as a probe. Since either an equimolar complex or, for kininogens, a complex of two enzymes per inhibitor can be expected to be formed (Anastasi *et al.*, 1983; Salvesen *et al.*, 1986; Lindahl *et al.*, 1988; Higashiyama *et al.*, 1986; Björk & Ylinenjärvi, 1990), a major use of these determinations is the assessment of the fraction of protein with binding ability in preparations of either proteinase or inhibitor from the apparent stoichiometry obtained in titrations with a well-characterized titrant, e.g. chicken cystatin or papain (Lindahl *et al.*, 1988). This application is illustrated by the studies with actinidin and recombinant human cystatin C, which show that these two proteins are essentially fully active in binding to inhibitor and target enzyme respectively. Competitive titrations with labelled papains as probes can also be used to obtain binding affinities, although only for interactions with affinities not too different from that between the probe and the inhibitor investigated. Those interactions between inhibitors and active enzymes studied so far have dissociation constants of $\sim 10^{-9}$ – 10^{-13} M (Björk *et al.*, 1989; Björk & Ylinenjärvi, 1990) and are thus inaccessible by this method, as well as by other equilibrium binding methods (Lindahl *et al.*, 1988). However, affinities of weaker interactions of inhibitors or inhibitor derivatives with active enzymes and of interactions of inhibitors with most inactivated enzymes (Björk & Ylinenjärvi, 1989) can be characterized, as exemplified by the studies with papain inactivated with the *S*-[*N*-(3-carboxypropyl)succinimidyl] group. One potential use of the labelled papains in this respect is in studies involving mapping of proteinase-binding sites of in-

inhibitors by chemical modification or site-directed mutagenesis, in which lower affinities for proteinases than those shown by the intact inhibitors would be expected.

In those cases discussed above where competitive titrations with labelled papains can give useful information, this method has several advantages over other techniques. The labelled papains can thus be used to characterize proteinase-inhibitor interactions which do not result in any measurable change in the intrinsic spectroscopic properties, e.g. tryptophan fluorescence, of the interacting proteins. Moreover, they give much larger fluorescence changes than those of intrinsic tryptophan fluorescence that have been observed so far (Lindahl *et al.*, 1988; Björk & Ylinenjärvi, 1989, 1990). Finally, the fluorescence of the labelled papains is measured at a wavelength at which the protein fluorescence is negligible, so that problems with inner-filter effects and contributions from the fluorescence of excess titrant are minimized. Together, the latter two advantages result in higher precision of the measurements.

This work was supported by research grants from the Swedish Medical Research Council (Project 4212), the Swedish Council for Forestry and Agricultural Research (Project 546/88), the Knut and Alice Wallenberg's Foundation and Svenska Sällskapet för Medicinsk Forskning.

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Received 10 December 1990; accepted 16 January 1991

APPENDIX

DERIVATION OF THE RATE EQUATION FOR A GENERAL TWO-STEP BINDING MECHANISM

The general two-step mechanism for the binding of an inhibitor, I, to a protein, P, involving a conformational change of the protein to the state P⁺, can be written:

in which it is assumed that the binding steps are rapidly equilibrated. The overall differential equation which describes this system is given by:

$$\frac{-d([P] + [PI])}{dt} = \frac{d([P^+] + [P^+I])}{dt} = k_2[PI] - k_{-2}[P^+I] + k_3[P] - k_{-3}[P^+] \quad (1)$$

With the use of the expressions for the dissociation equilibrium constants, K_1 and K_4 , valid for the pseudo-first-order condition, $[I]_T \gg [P]_T$:

$$K_1 = \frac{[P][I]_T}{[PI]}, \quad K_4 = \frac{[P^+][I]_T}{[P^+I]} \quad (2)$$

the differential equation for species P⁺I can be obtained from differential eqn. (1):

$$\frac{d([P^+] + [P^+I])}{dt} = \left(1 + \frac{K_4}{[I]_T}\right) \frac{d[P^+I]}{dt} = k_3[P] - \frac{k_{-3}[P^+I]K_4}{[I]_T} + \frac{k_2[P][I]_T}{K_1} - k_{-2}[P^+I] \quad (3)$$

From the conservation equation for the different protein species:

$$[P]_T = [P] + [P^+] + [PI] + [P^+I] \quad (4)$$

together with the expressions for the dissociation equilibrium constants, the concentration of species P can be expressed in terms of the concentration of species P†I as follows:

$$[P]_T = [P] + \frac{[P†I]K_4}{[I]_T} + \frac{[P][I]_T}{K_1} + [P†I] \quad (5)$$

$$[P] = \frac{([P]_T - \left(1 + \frac{K_4}{[I]_T}\right)[P†I])}{\left(1 + \frac{[I]_T}{K_1}\right)} \quad (6)$$

Substitution of eqn. (6) into the differential eqn. (3) and rearrangement then yields:

$$\frac{d[P†I]}{dt} + \left[\frac{\left(k_3 + k_2 \frac{[I]_T}{K_1}\right)}{\left(1 + \frac{[I]_T}{K_1}\right)} + \frac{\left(k_{-3} \frac{K_4}{[I]_T} + k_{-2}\right)}{\left(1 + \frac{K_4}{[I]_T}\right)} \right] [P†I] = \frac{\left(k_3 + k_2 \frac{[I]_T}{K_1}\right)[P]_T}{\left(1 + \frac{K_4}{[I]_T}\right)\left(1 + \frac{[I]_T}{K_1}\right)} \quad (7)$$

Eqn. (7) is a first-order differential equation of the form:

$$\frac{dy}{dt} + Ay = B \quad (8)$$

where A and B are constants. For the boundary condition, $y = 0$ at $t = 0$, this has the solution:

$$y = \frac{B}{A}(1 - e^{-At}) \quad (9)$$

where B/A is equal to the limiting equilibrium concentration of species P†I and A is the observed pseudo-first-order rate constant ($k_{obs.}$) for the approach of species P†I to its final equilibrium concentration. $k_{obs.}$ is thus given by:

$$k_{obs.} = \frac{\left(k_3 K_1 + k_2\right)}{\left(1 + \frac{K_1}{[I]_T}\right)} + \frac{\left(k_{-3} + k_{-2} \frac{[I]_T}{K_4}\right)}{\left(1 + \frac{[I]_T}{K_4}\right)} \quad (10)$$

The limiting values of $k_{obs.}$ as $[I]_T$ approaches 0 or ∞ are $k_3 + k_{-3}$ and $k_2 + k_{-2}$ respectively, consistent with the pre-equilibrium pathway predominating at low inhibitor concentrations and the induced conformational change pathway becoming dominant at high inhibitor concentrations. Also, when the pre-equilibrium pathway does not contribute, $k_3 = k_{-3} = 0$ and $K_4 = 0$, and $k_{obs.}$ reduces to the equation characterizing the induced conformational change pathway:

$$k_{obs.} = \frac{k_2}{\left(1 + \frac{K_1}{[I]_T}\right)} + k_{-2} \quad (11)$$

Similarly, when the induced conformational change pathway does not contribute, $k_2 = k_{-2} = 0$ and $K_1 = \infty$, and $k_{obs.}$ reduces to the equation characteristic of the pre-equilibrium pathway:

$$k_{obs.} = k_3 + \frac{k_{-3}}{\left(1 + \frac{[I]_T}{K_4}\right)} \quad (12)$$

[See Fersht (1985) for eqns. 11 and 12].

The six parameters involved in eqn. (10) are related by:

$$K_1 \frac{k_{-2}}{k_2} = K_4 \frac{k_{-3}}{k_3}$$

The measured overall dissociation equilibrium constant for this system is given by:

$$\begin{aligned} K_{eq.} &= \frac{([P] + [P†])[I]_T}{[PI] + [P†I]} = \frac{[P][I]_T}{[PI] + [P†I]} + \frac{[P†][I]_T}{[PI] + [P†I]} = \\ &= \frac{1}{\frac{[PI]}{[P][I]_T} + \frac{[P†I]}{[P][I]_T} \times \frac{[PI]}{[P†I]}} + \frac{1}{\frac{[PI]}{[P†I]} \times \frac{[P†I]}{[P†I]} + \frac{[P†I]}{[P†I]} \times \frac{[P†I]}{[P†I]}} = \\ &= \frac{1}{\frac{1}{K_1} + \frac{1}{K_1} \times \frac{k_2}{k_{-2}}} + \frac{1}{\frac{1}{K_4} \times \frac{k_{-2}}{k_2} + \frac{1}{K_4}} = \frac{k_{-2}K_1 + k_2K_4}{k_2 + k_{-2}} \end{aligned} \quad (13)$$

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