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Saxiphilin, a Saxitoxin-binding Protein with Two Thyroglobulin Type 1 Domains, Is an Inhibitor of Papain-like Cysteine Proteinases*

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The type 1 domain of thyroglobulin is a protein module (Thyr-1) that occurs in a variety of secreted and membrane proteins. Several examples of Thyr-1 modules have been previously identified as inhibitors of the papain family of cysteine proteinases. Saxiphilin is a neurotoxin-binding protein from bullfrog and a homolog of transferrin with a pair of such Thyr-1 modules located in the N-lobe. Saxiphilin is now characterized as a potent inhibitor of three cysteine proteinases as follows: papain, human cathepsin B, and cathepsin L. The stoichiometry of enzyme inhibition reveals that both Thyr-1 domains of saxiphilin inhibit papain (apparent K_i = 1.72 nM), but only one of these domains inhibits cathepsin B (K_i = 1.67 nM) and cathepsin L (K_i = 0.02 nM). Physical association of saxiphilin and papain blocked from turnover at the active-site cysteine residue can be detected by cross-linking with glutaraldehyde. The rate of association of saxiphilin and cathepsin B is strongly pH-dependent with an optimum at pH 5.2, reflecting control by at least two H^+-titratable groups. These results further demonstrate that various Thyr-1 domains are selective inhibitors of cysteine proteinases with utility in the study of protein interactions and degradation.

Saxiphilin is a 91-kDa soluble protein isolated from plasma of the North American bullfrog, *Rana catesbiana.* Saxiphilin binds the neurotoxin, saxitoxin (STX),[1] and various STX derivatives with high affinity (K_D ≃0.2 nM STX) and specificity (1–3). STX is a tricyclic organic cation produced by various dinoflagellates and cyanobacteria (4). Paralysis caused by STX intoxication is due to blockage of voltage-sensitive sodium channels (5). Molecular characterization of saxiphilin revealed that it is homologous to transferrins, a family of high affinity Fe^{3+}-binding proteins (3, 6, 7). Despite this structural similarity, saxiphilin differs from the transferrin family in two major respects as follows: (i) 9 of 10 highly conserved Fe^{3+} site residues in transferrin are substituted in saxiphilin, accounting for a lack of Fe^{3+} binding (6, 8); (ii) the larger size of saxiphilin (91 kDa) *versus* transferrin (~80 kDa) is primarily due to an insertion of 143 residues in the N-lobe. This insertion corresponds to a tandem duplication of a thyroglobulin type 1 domain (Thyr-1) (9–11).

Thyr-1 domains are recognized as a unique sequence motif of ~60–80 residues, usually containing six conserved cysteine residues (12). These domains or modules are present in diverse families of proteins that include mosaic proteins such as thyroglobulin (11), membrane proteins such as p41 invariant chain (13), and unrelated binding proteins such as saxiphilin (6, 9) and insulin-like growth factor-binding protein (14). A few years ago, it was discovered that some proteins containing Thyr-1 modules function as inhibitors of the papain family of cysteine proteinases that include cathepsins B and L, *e.g.* p41 invariant chain fragment (15), chum salmon egg cysteine proteinase inhibitor (16), and equistatin (17). A common feature of these latter proteins containing either one (p41, egg cysteine proteinase inhibitor) or three (equistatin) Thyr-1 modules is an ability to inhibit a variety of papain-related cysteine proteinases with K_i values in the picomolar to nanomolar range. They have thus been classified as thyroglobulin type 1 proteinase inhibitors, also called thyropins (19). In a recent development to this emerging picture of thyropin function, it was found that the sea anemone protein, equistatin, also inhibits an aspartic proteinase (20). Interestingly, the distinct proteinase inhibitor activities of equistatin reside on different Thyr-1 domains. Inhibition of cysteine proteinase activity is associated with the N-terminal Thyr-1 domain of equistatin, and inhibition of an aspartic proteinase, cathepsin D, is associated with one of the two C-terminal Thyr-1 domains (20). In contrast, thyroglobulin, a protein with 11 Thyr-1 modules, and mouse nidogen (or entactin) with one Thyr-1 module, reportedly do not inhibit papain (16). These results suggest that various Thyr-1 modules contain specific structural features that determine anti-proteinase activity against certain papain-related or cathepsin D-related enzymes.

The inhibitory mechanism of a thyroglobulin type 1 domain was revealed by the crystal structure of p41 invariant chain fragment in complex with cathepsin L (21). The wedge shape of the inhibitor binds into the active site cleft. The nonspecific interactions at the bottom of the cleft are mediated by three loops, whereas the specific interactions are mediated via side chains on one side of the p41 fragment and the loops embracing the substrate-binding sites S2 and S1′ of cathepsin L.

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[1] The abbreviations used are: STX, saxitoxin; Thyr-1, thyroglobulin type 1; R-sax, recombinant saxiphilin; Z, benzyloxycarbonyl; AMC, 4-methyl-7-coumarylamide; DTT, dithiothreitol; PEG, polyethylene glycol; MES, 4-morpholineethanesulfonic acid; IAA, iodoacetamide; PAGE, polyacrylamide gel electrophoresis.

Identification of Thyr-1 domains as inhibitors of cysteine or aspartic proteinases suggests that saxiphilin may have other biochemical activities beside its known binding affinity for STX. In this work, we examined the interaction of recombinant saxiphilin with three papain-related cysteine proteinases (papain and cathepsins B and L) to determine whether saxiphilin is an inhibitor of such enzymes and to characterize further the specificity of Thyr-1 domains. Our results indicate that both Thyr-1 domains of saxiphilin are functionally capable of inhibiting papain, but only one of the two domains is an active inhibitor of cathepsins B and L.

EXPERIMENTAL PROCEDURES

Materials—Z-Phe-Arg-4-methyl-7-coumarin (AMC) was obtained from Serva (Germany) and Z-Phe-Arg-*p*-nitroanilide from Bachem (Switzerland). Stock solutions of these latter substrates were prepared in dimethyl sulfoxide (Merck). Papain, 2× crystallized (Sigma), was further purified by affinity chromatography (22). Recombinant human cathepsin B and cathepsin L were prepared as described previously (23, 24).

Purification of Saxiphilin—Recombinant saxiphilin (R-sax) was produced by expression in suspension cultures of High Five insect cells infected with baculovirus as described previously (10). This expression system provided a starting medium containing 44 ± 12 pmol of R-sax per ml (mean ± S.D., n = 10 batches). Purification of milligram quantities of R-sax from insect cell medium was accomplished by the following procedure adapted from a protocol for purification of the native protein from frog plasma (3). Insect cell medium containing R-sax was supplemented with 1 μM proteinase inhibitors (benzamidine, leupeptin, and pepstatin) and stored frozen at −20 °C until used for purification. Five liters of medium containing >20 pmol of R-sax/ml was thawed and centrifuged at 100,000 × g for 45 min to remove cell debris and virus particles. The supernatant was concentrated to 200 ml with a tangential flow device (Pall Filtron, Northborough, MA) followed by ultrafiltration (Amicon, Beverly, MA) using membranes with a 30-kDa cutoff. The concentrated sample was subjected to precipitation at 0 °C by polyethylene glycol with an average M_r of 400 (PEG-400, from Sigma). PEG-400 was added dropwise with stirring to a final concentration of 8%. The sample was stirred for 2 h, centrifuged at 100,000 × g, and the pellet discarded. The supernatant was passed through a 0.22-μm filter and purified on a column (0.8 × 10 cm) of POROS 20HE heparin affinity chromatography media (Perspective Biosystems, Framingham, MA). A sample of the PEG-400 supernatant containing 25 mg of protein was applied to the column in buffer (10 mM MES, 30 mM sodium acetate, 5 mM EDTA, pH 6.0) at a flow rate of 2 ml/min. The column was washed with buffer plus 20 mM NaCl for 10 min and eluted with a linear gradient (10 min) of 50–300 mM NaCl in buffer at a flow rate of 6 ml/min. Fractions containing [^{3}H]STX-binding activity were pooled, concentrated to 50 ml by ultrafiltration, and dialyzed against deionized water. This sample was purified by chromatofocusing on a column (2.5 × 20 cm) of PBE 118 anion exchange resin (Amersham Pharmacia Biotech). The column was pre-equilibrated with 25 mM triethanolamine, pH 10.5. After loading the protein sample, the column was eluted at 0.2 ml/min with the following solution: 17.8 ml Polybuffer 96 (Amersham Pharmacia Biotech) and 3.6 ml Pharmalyte 8–10.5 (Amersham Pharmacia Biotech), adjusted to pH 8.0 with HCl, and brought to a final volume of 500 ml with deionized water. Fractions containing [^{3}H]STX binding activity were pooled and ampholytes removed by gel filtration chromatography on a column of Sephacryl S-300 (Amersham Pharmacia Biotech) eluted with 150 mM NaCl, 1 mM Hepes-NaOH, pH 7.4. Pure R-sax was visualized by silver staining as a single band on SDS-PAGE. Samples of purified R-Sax were stored in a solution containing 35% glycerol at −20 °C. This method typically achieved a yield of ~25% or ~5 mg of saxiphilin per 5 liters of starting medium.

SDS-PAGE and Glutaraldehyde Cross-linking—SDS-PAGE was performed according to the method of Laemmli (25) using precast 10% polyacrylamide gels from Bio-Rad. Physical association of saxiphilin and papain was investigated by size analysis of protein complexes trapped by glutaraldehyde cross-linking (26). Papain used for this experiment was inactivated by a treatment with 2 mM cysteine for 10 min followed by 100 mM iodoacetamide (IAA) for 1 h in 50 mM NaP_i, 1 mM EDTA, pH 8.0, at 22 °C. IAA-inactivated papain was dialyzed overnight at 4 °C against 20 mM NaP_i, pH 6.5. A fixed amount of saxiphilin (3 μg) was titrated with increasing amounts of IAA-inactivated papain in 30 μl of buffer (100 mM sodium acetate, pH 5.0). The samples were incubated on ice for 10 min. Glutaraldehyde (Sigma grade I) was added to a final concentration of 20 mM and allowed to react at 22 °C for 20 min. The samples were subjected to SDS-PAGE, and proteins bands were visualized by staining with Coomassie Blue (27).

Protein Determination—Protein concentration of saxiphilin was measured by the bicinchoninic acid method (28) using bovine serum albumin as a standard. The concentration of [^{3}H]STX-binding sites in samples of saxiphilin was assayed according to the method described (10). The protein concentration of papain and cathepsins B and L was determined by absorption measurements at 280 nm using the molar absorption coefficients of 56,200 (29), 44,000 (30), and 44,300 M^{-1} cm^{-1} (31), respectively.

Active-site Titrations—For purified papain, a thiol content of 0.92 ± 0.05 mol/mol of enzyme was determined by reaction with 5,5′-dithiobis(2-nitrobenzoic acid). For cathepsins B and L the active concentrations were determined by active-site titration with the inhibitor Ep-475 as described previously (32).

The active-site titrated enzymes were then used to titrate saxiphilin as follows. Papain (0.1 μM final concentration) was added to 200 μl of 0.1 M phosphate buffer, pH 6.0, containing 5 mM dithiothreitol and 1 mM EDTA, followed 5 min later by the addition of 200 μl of increasing concentrations of saxiphilin. After 20 min incubation at 25 °C, 600 μl of 100 μM Z-Phe-Arg-*p*-nitroanilide in the same buffer was added. The residual activity was monitored as function of increasing absorbance at 410 nm with Perkin-Elmer Lambda 18 Spectrophotometer. The active concentration of saxiphilin was determined from a plot of residual activities against molar ratios of initial enzyme and inhibitor concentrations ($[I_o]/[E_o]$). The data were analyzed by computer fitting to the theoretical binding equation (33).

Titration of saxiphilin was performed in a similar way with cathepsin B (0.13 μM final concentration), using 50 mM sodium acetate buffer, pH 5.0, containing 0.1 M NaCl, 5 mM dithiothreitol, and 1 mM EDTA and with cathepsin L (2 nM final concentration) in 0.34 M sodium acetate buffer, pH 5.5, containing 5 mM dithiothreitol and 1 mM EDTA. Z-Phe-Arg-AMC (10 μM) was used as substrate for cathepsin L, and the release of the product was monitored at excitation and emission wavelengths of 370 and 460 nm, respectively, by a Perkin-Elmer LS50B spectrofluorimeter.

Kinetics of Inhibition of Cysteine Proteinases—In all kinetic experiments, papain and cathepsin B were assayed using 0.1 M phosphate buffer, pH 6.0, containing 5 mM dithiothreitol and 1 mM EDTA, whereas for cathepsin L, 0.34 M sodium acetate buffer, pH 5.5, containing 5 mM dithiothreitol and 1 mM EDTA was used. Saxiphilin (variable concentrations) and substrate (10 μM Z-Phe-Arg-AMC) were dissolved in 1.98 ml of the appropriate buffer. The reaction was started by the addition of 20 μl of activated papain (87 pM final concentration), cathepsin B (900 pM final concentration), or cathepsin L (160 pM final concentration). All experiments were performed under pseudo-first order, *i.e.* at molar ratio of the inhibitor to enzymes of 10:1. The progress curves were monitored as described under "Active-site Titrations" and fitted by nonlinear regression to the following integrated rate equation (34): $p = v_s t + (v_z - v_s)(1 - e^{-kt})/k$, where v_s is the steady state velocity, v_z is the initial velocity, and k is the pseudo-first order rate constant describing the pre-steady state reaction. The association rate constant (k_a) was determined from a plot of k *versus* $[I]$ according to equation $k = k_d + k_a[I]/(1 + [S]/K_m)$, where the dissociation rate constant, k_d, was calculated from each individual progress curve according to equation $k_d = k(v_s/v_z)$. K_i was obtained from k_d/k_a. Substrate K_m values of 65 μM for papain (35), 2 μM for cathepsin L (36), and 150 μM for cathepsin B (32) were used.

Effect of pH on k_a—The pH dependence of k_a for the inhibition of cathepsin B by saxiphilin was studied over the pH range of 3.5–7.5. Buffers of pH 3.5–5.5 contained 50 mM sodium acetate, and buffers covering the pH range 6.0–7.5 contained 50 mM sodium phosphate. All buffers also contained 100 mM NaCl and 1 mM EDTA. The activating solution in all experiments was 5 mM EDTA, 10 mM dithiothreitol, pH 6.0. Reactions were monitored with a DX 17MV stopped-flow apparatus (Applied Photophysics, UK). One syringe was filled with buffer, saxiphilin (200 nM final concentration), and substrate Z-Phe-Arg-AMC (20 μM), and the second was filled with preactivated cathepsin B (20 nM). 100 μl of solution from each syringe was used per run, and an average of 6–8 runs was performed for each determination of the constant. The emission of released products was observed at an excitation wavelength of 360 nm through the cutoff filter with ~50% transmission at 420 nm. The data were fit to the following equation (37): $k_a = k_{a(\mathrm{lim})}/(1 + [H^+]/K_1 + K_2/[H^+])$, where $k_{a(\mathrm{lim})}$ presents the limiting value of k_a.

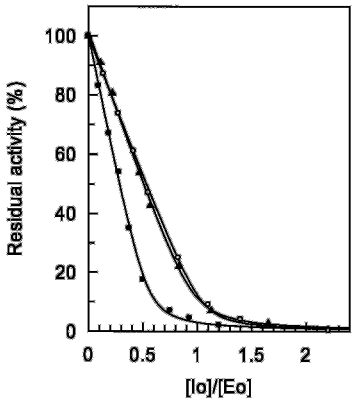

FIG. 1. **Inhibition of papain and human cathepsins B and L by increasing amounts of equistatin.** Active site titration of 100 nM papain (●), 136 nM cathepsin B (○), and 2 nM cathepsin L (▲) with increasing concentrations of recombinant saxiphilin. Residual activity is expressed as a percentage of a control samples with no saxiphilin. *Abscissa* units correspond to the mole ratio of proteinase to saxiphilin based on independent active-site titrations of enzymes and the protein concentration of saxiphilin. The data points were fit to the theoretical binding equation (32) by nonlinear regression.

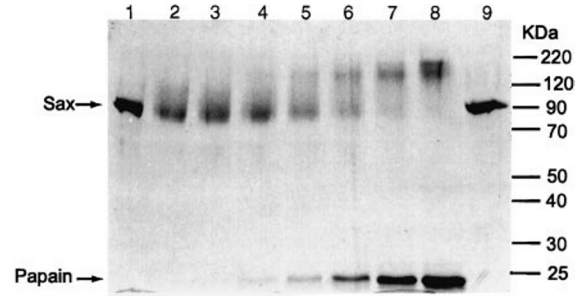

FIG. 2. **Demonstration of complex formation between saxiphilin and papain by glutaraldehyde cross-linking.** Saxiphilin (1 μM in 30 μl) was incubated in the presence of increasing amounts of iodoacetamide-inactivated papain: 0.125, 0.25, 0.5, 1, 2, 4, and 8 μM papain in *lanes 2–8*, respectively, for 10 min on ice. Glutaraldehyde (20 mM) was added, and after 20 min at 22 °C, the samples were subjected to SDS-PAGE (10% acrylamide). *Lanes 1* and *9* are control samples of saxiphilin not treated with glutaraldehyde. Protein bands visualized by staining with Coomassie Blue.

RESULTS

Recombinant saxiphilin was produced as a secreted protein in insect cells using a baculovirus expression vector (10) and purified to homogeneity from insect cell medium using [^{3}H]STX binding to monitor activity. The stoichiometry of binding interactions between saxiphilin and papain-related cysteine proteinases (papain and cathepsins B and L) was determined by titrating each enzyme with increasing amounts of saxiphilin and monitoring the loss of enzymatic activity. The concentration of each proteinase used in this experiment was determined by independent active-site titration (see "Experimental Procedures"). Fig. 1 shows that the residual activity of cathepsin B and cathepsin L plotted as a function of increasing molar ratios of saxiphilin/enzyme is consistent with a 1:1 stoichiometry for inhibition of both cathepsins. Specifically, 1.03 ± 0.01 and 0.96 ± 0.02 mol of saxiphilin were found to inhibit 1 mol of cathepsin B and cathepsin L, respectively, based on a fit of the titration data to a one-site binding reaction (33). In contrast, a lower molar ratio of inhibitor to enzyme was required for complete inhibition of papain by saxiphilin (Fig. 1). This analysis showed that 0.56 ± 0.03 mol saxiphilin was sufficient to inhibit 1 mol of papain, indicating that there are two functional binding sites for papain on each saxiphilin molecule.

Physical association of saxiphilin and papain was also investigated by chemical cross-linking and affinity adsorption. Fig. 2 shows results of an experiment in which a constant amount of saxiphilin was incubated with increasing amounts of papain that were inactivated by pretreatment with iodoacetamide. Complex formation between the two proteins was assessed by exposure to the cross-linking agent, glutaraldehyde, followed by SDS-PAGE. This assay detected a shift of the 91-kDa saxiphilin band to higher molecular weight as a function of increasing papain concentration. In control experiments, neither saxiphilin nor papain (23 kDa) produced such higher molecular weight bands when incubated alone and treated with glutaraldehyde, demonstrating that the band shift is due to formation of a saxiphilin-papain complex. The diffuse nature of the glutaraldehyde cross-linked bands on SDS-PAGE did not allow us to clearly resolve 1:1 and 1:2 saxiphilin/papain species, but the observed shift is consistent with complexes in this range. Since carboxymethylated papain coupled to Sepharose has been previously used to isolate cysteine proteinase inhibitors such as cystatin (38) from egg white and equistatin from an extract of sea anemone (17), we also tested whether such a papain affinity matrix is capable of binding saxiphilin. We found that a column of papain-Sepharose medium prepared as described (38) effectively adsorbs [^{3}H]STX binding activity from a crude protein sample such as insect medium containing recombinant saxiphilin. Elution of this washed column at high pH yielded a fraction containing purified saxiphilin (results not shown). Thus, both glutaraldehyde cross-linking and papain affinity chromatography provided evidence of specific physical association of saxiphilin and papain, as one would expect from the enzyme inhibition experiments of Fig. 1.

Since saxiphilin also binds STX, a small organic molecule, we performed several experiments to investigate possible ligand interactions between the binding of [^{3}H]STX and papain. In one type of experiment, a fixed amount of papain was titrated with increasing saxiphilin in the absence or presence of 200 nM STX, and papain activity was measured as in Fig. 1. We found that STX had no effect on the papain inhibition curve (not shown). Since STX binds to saxiphilin with a K_d of ~1 nM under these conditions (39), this result indicates that the affinity of papain for saxiphilin is not disturbed by the binding of STX. In a converse experiment, we also found that there is no effect of papain on binding of [^{3}H]STX to saxiphilin when studied at 0 °C. This latter experiment was performed with papain inactivated by the sulfhydryl reagent, methyl methanethiosufonate, in order to differentiate simple binding interactions from proteolytic effects. However, in the presence of a sulfhydryl-reducing agent (5 mM DTT), increasing papain concentration inhibited [^{3}H]STX binding by ~60% reduction of initial [^{3}H]STX-binding sites (data not shown). Further studies revealed that inhibition of STX binding by DTT-activated papain is due to combined effects of an irreversible loss in the total number of STX sites and a severalfold increase in the apparent K_d of remaining STX sites. The most likely cause of the loss of STX binding activity under these conditions is partial proteolysis of saxiphilin, resulting in modification and destruction of the STX-binding site. The effect of DTT is explained by the fact that this reagent re-activates papain that is thiomethylated at the active-site cysteine residue by methyl methanethiosufonate. In support of this conclusion, we observed proteolysis of saxiphilin exposed to DTT-activated papain, as monitored by disappearance of the 91-kDa band on SDS-PAGE (not shown). Thus, although saxiphilin is a potent inhibitor of papain, certain regions of the saxiphilin protein are susceptible to cleavage by active papain molecules.

The interaction kinetics of saxiphilin with cathepsins B and L were characterized under pseudo-first order conditions by continuous fluorometric assays using Z-Phe-Arg-AMC as a sub-

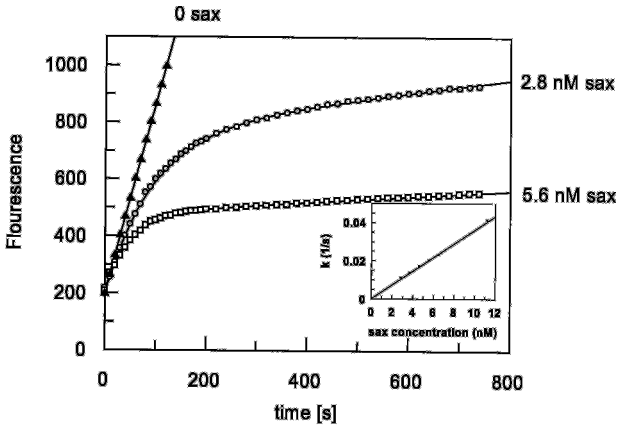

FIG. 3. **Progress curves for the inhibition of cathepsin L and saxiphilin under pseudo-first order conditions.** The concentration of saxiphilin (*Sax*) was zero (▲), 2.8 nM (○), and 5.6 nM (□). The *solid lines* were curves computed by fitting the experimental data to the theoretical rate equation (33). *Inset,* dependence of the derived pseudo-first order rate constant, *k,* on saxiphilin concentration for the interaction between cathepsin L and saxiphilin.

strate. Fig. 3 shows an example of typical biphasic progress curves observed when hydrolysis of a fluorescent substrate by cathepsin L is initiated in the presence of saxiphilin. The progress curves are well fit by a single exponential function as described under "Experimental Procedures." The pseudo-first order rate constant, *k,* derived from this experiment increases linearly with saxiphilin concentration (Fig. 3, *inset*), indicative of a simple bimolecular process for the association of saxiphilin and cathepsin L. Similar behavior was found for cathepsin B. The kinetic and equilibrium constants derived for the interaction of saxiphilin with cathepsin B and cathepsin L (Table I) show that saxiphilin binds tightly and rapidly to both enzymes but has ~80-fold higher affinity for cathepsin L *versus* B. The binding parameters for inhibition of these enzymes by saxiphilin are very similar to those previously reported for equistatin (17), which also has one active Thyr-1 domain that inhibits cathepsins B and L (20). The lower K_i value for the interaction of saxiphilin with cathepsin L *versus* cathepsin B, 0.020 ± 0.003 nM and 1.67 ± 0.43 nM, respectively, is primarily due to a 30-fold faster association rate for cathepsin L (Table I).

The rate of complex formation of saxiphilin and papain was monitored by the same assay used for the two cathepsins. The progress curve was also well fit by a single exponential function (not shown). Since one saxiphilin molecule is capable of inhibiting two papain molecules (Fig. 2), this kinetic behavior indicates either that the interaction of one of the Thyr-1 domains of saxiphilin dominates the reaction or that the association kinetics of two Thyr-1 domains of saxiphilin are indistinguishable. Since this reaction is carried out under pseudo-first order conditions, with saxiphilin concentration >10-fold higher than papain, the kinetics may simply reflect the activity of the most accessible Thyr-1-binding site. In the absence of data discriminating association kinetics at two inhibitory sites, we assumed a single apparent site in calculating the parameters reported in Table I for papain. With this assumption, the rate constants and K_i values derived for interaction of saxiphilin and papain (K_i = 1.72 nM) are similar to those found for cathepsin B (Table I).

The pH dependence of the association rate constant, k_a, of saxiphilin with cathepsin B was also measured with a stopped-flow apparatus under pseudo-first order conditions at constant saxiphilin concentration. The dependence of k_a on pH followed a narrow bell-shaped curve with a maximum between pH 5.0 and 5.5 (Fig. 4). In analyzing the data, we assumed that the fundamental kinetic mechanism of the association reaction

TABLE I

Kinetic data for the interaction of saxiphilin with papain, cathepsin B, and cathepsin L

The experimental conditions are described under "Experimental Procedures." K_i was calculated from the ratio k_d/k_a. Error estimates for K_i were calculated from the square root of the sum of the relative squared errors for k_a and k_d assuming that variance is additive.

Enzyme	$10^{-6} \times k_a$	$10^4 \times k_d$	K_i
	$M^{-1}\,s^{-1}$	s^{-1}	nM
Papain	1.8 ± 0.2	31 ± 5	1.72 ± 0.34
Cathepsin B	0.6 ± 0.1	10 ± 2	1.67 ± 0.43
Cathepsin L	18 ± 0.5	3.6 ± 0.6	0.020 ± 0.0034

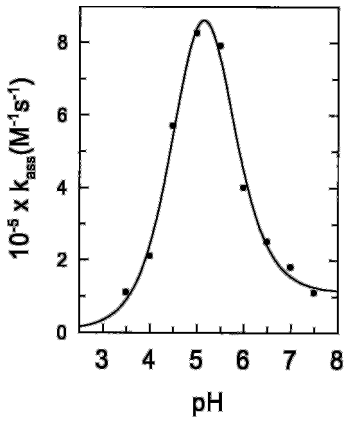

FIG. 4. **pH dependence of the second order rate constant, k_a, for the interaction between cathepsin B and saxiphilin.** Progress curves at 25 °C used to measure k_a were generated using a stopped-flow apparatus. The saxiphilin and cathepsin B concentrations were 200 and 20 nM, respectively. The data were fit to a model for activation and inhibition by H^+ at two independent titratable groups (32). The *solid line* is the theoretical curve corresponding to best fit with pK_a values given in the text.

elucidated at pH 6.0 does not change from pH 3.5 to 7.5 but that the magnitude of the k_a rate constant is altered by H^+ titration of the enzyme. The association rate was sufficiently high throughout the tested pH range, such that the effect of complex dissociation under the conditions used in the experiment is negligible. The observed dependence of k_a on pH was adequately fit by a function involving two acid dissociation constants (Fig. 5) as described under "Experimental Procedures." The two pK_a values derived from this analysis were pK_1 = 4.6 and pK_2 = 5.6. It has been previously shown that the active site of cathepsin B is highly complex in that a minimum of four dissociable groups influence cathepsin B activity toward Z-Phe-Arg-AMC substrates as follows: pK_1 = 3.51, pK_2 = 4.93, pK_3 = 7.58, and pK_4 = 7.82 (40). Our results indicate that saxiphilin binding is also complex and controlled by at least two ionizable groups that may be different from those controlling substrate hydrolysis.

DISCUSSION

This study shows that saxiphilin is a potent inhibitor of at least three cysteine proteinases of the papain family. Saxiphilin, a bullfrog plasma protein, was originally identified as a member of the transferrin family that does not bind Fe^{3+} but binds the microbial neurotoxin, STX (8, 9). Transferrins such as serum transferrin and lactoferrin are composed of two homologous structural domains called the N-lobe and the C-lobe that each bind a Fe^{3+} ion and roughly comprise the N- and C-terminal halves of the protein, respectively (7). Each of these transferrin lobes is further subdivided into two subdomains, N1/N2 and C1/C2, that form two independent binding clefts for Fe^{3+} and HCO_3^-. The insertion of two tandem Thyr-1 modules in saxiphilin occurs precisely at a hinge region between subdo-

FIG. 5. **Alignment of thyroglobulin type 1 repeats.** The comparison sequences are invariant chain fragment (*ii*) and both domains of saxiphilin (*sax_1* and *sax_2*). Identical amino acids in all sequences are shown in *black boxes*; residues in *dark gray boxes* are present in at least 2 of 3 sequences and conservative replacements are in *light gray boxes*. The positions of loops and disulfide bridges are marked *below* and *above* the alignment, respectively.

mains N1 and N2, as predicted from sequence homology and the crystal structures of transferrins (10, 41). On the basis of this location, one may speculate that the two Thyr-1 modules in saxiphilin fold as independent structural domains that engage in binding interactions with other proteins. This inference is consistent with the present findings which show that one saxiphilin molecule is capable of inhibiting two molecules of papain. In view of previous work (15–17) that has established the specific interaction between Thyr-1 modules and cysteine proteinases, the simplest interpretation of our results is that both Thyr-1 modules of saxiphilin can simultaneously bind papain, whereas only one of them can bind cathepsins B and L. The fact that the single STX-binding site of saxiphilin is located in the C-lobe domain of saxiphilin (10) and the two Thyr-1 domains are located in the N-lobe is also compatible with the absence of binding interactions between [^{3}H]STX and papain except under conditions in which papain is capable of proteolysis.

The finding that multiple Thyr-1 domains in the same protein can have different activity toward various proteinases is reminiscent of equistatin, a protein with three Thyr-1 modules. Only the N-terminal Thyr-1 domain of equistatin inhibits cysteine proteinases, while the combined second and third Thyr-1 domain has the ability to inhibit cathepsin D, an aspartic proteinase (20). When tested in a similar manner, saxiphilin itself did not exhibit inhibitory activity toward cathepsin D or other aspartic proteinases (data not shown). Such findings lead to the conclusion that Thyr-1 domains must possess a set of rather specific structural determinants that allow them to recognize different classes and subclasses of proteinases. Sequence comparison of fragment of p41 form of invariant chain with saxiphilin Thyr-1 domains (Fig. 5) has revealed that the major differences are two short insertions located within the first and third binding loop regions. Due to the lack of a reliable homology model of these regions, we were unable to predict the three-dimensional structure of these two loops, which presumably form additional interactions within the active site cleft of the proteinases. The first binding loop interacts with the residues around the S2-binding site, whereas the third loop builds interactions with the surface of the primed binding sites (21).

The binding constant of saxiphilin to cathepsin L is comparable to the constant of the p41 fragment, allowing a conclusion that the two insertions do not disrupt interactions within the active site cleft of cathepsin L. Similarly, also the strength of interactions with another endopeptidase, papain, was not affected (K_i = 1.4 nM for p41 compared to K_i = 1.7 nM for saxiphilin).

Cathepsin B is inhibited by saxiphilin and cystatin type inhibitors in approximately the same range, whereas p41 fragment does not inhibit cathepsin B. The active site cleft of cathepsin B differs from the other papain-like cysteine proteinases by an additional insertion of 20 amino acids, termed occluding loop, which partially occludes the active site, and by a different conformation of the His^{190}–Gly^{198} region (42). This region in cathepsin B embraces the S1′-binding site. However, in the related enzymes it embraces the S2-binding site. It was shown that the occluding loop interferes with cystatin-type inhibitor binding (43–46), by decreasing the association constant. The occluding loop residues are expected to interfere with the third binding loop of saxiphilin, similarly as the second hairpin loop of cystatins (47), by decreasing the strength of interactions and not by preventing the binding (see Table I). The His^{190}–Gly^{198} region was suggested to prevent binding of p41 fragment to cathepsin B due to clashes within the first selective region of the p41 fragment (21). As saxiphilin binds to cathepsin B, it can be suggested that this region does not disrupt interactions between the two molecules. This outlines an interesting problem for future research to understand the detailed nature of the molecular interactions that determine Thyr-1 specificity as an anti-proteinase domain.

Present findings suggest that the Thyr-1 domains of saxiphilin could play a role in regulating the degradation of saxiphilin if it is internalized by cells in a manner similar to transferrin and is later exposed to a lysosomal compartment.

The interaction with cysteine proteinases also provides a new avenue for exploring the structure and function of saxiphilin-related proteins. In particular, the demonstration of a tight complex formation with papain opens the possibility of using inactivated papain affinity chromatography for purification and isolation of saxiphilin and other variants of this protein expressed by diverse animal species (18).

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