

Inactivation of papain by antithrombin due to autolytic digestion: a model of serpin inactivation of cysteine proteinases

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Cross-class inhibition of cysteine proteinases by serpins differs from serpin inhibition of serine proteinases primarily in that no stable serpin–cysteine proteinase complex can be demonstrated. This difference in reaction mechanism was elucidated by studies of the inactivation of the cysteine proteinases, papain and cathepsin L, by the serpin antithrombin. The two proteinases were inactivated with second-order rate constants of $(1.6 \pm 0.1) \times 10^3$ and $(8.6 \pm 0.4) \times 10^2 \text{ M}^{-1} \cdot \text{s}^{-1}$ respectively. An antithrombin to papain inactivation stoichiometry of ~ 3 indicated extensive cleavage of the inhibitor concurrent with enzyme inactivation, a behaviour verified by SDS/PAGE. N-terminal sequence analyses showed cleavage predominantly at the P_2 – P_1 bond, but also at the P_2' – P_3' bond of antithrombin. The papain band in SDS/PAGE progressively disappeared on reaction of the enzyme with increasing amounts of antithrombin, but no band representing a

stable antithrombin–papain complex appeared. SDS/PAGE with ¹²⁵I-labelled papain showed that the disappearance of papain was caused by cleavage of the enzyme into small fragments. These results suggest a mechanism in which papain attacks a peptide bond in the reactive-bond loop of antithrombin adjacent to that involved in serine proteinase inhibition. The reaction proceeds, similarly to that between serpins and serine proteinases, to form an inactive acyl-intermediate complex, although with the substrate pathway dominating in the papain reaction. In this complex, papain is highly susceptible to proteolysis and is degraded by still active papain, which greatly decreases the lifetime of the complex and results in liberation of fragmented, inactive enzyme. This model may have relevance also for the inactivation of physiologically or pathologically important cysteine proteinases by serpins.

INTRODUCTION

The serpin superfamily of proteins comprises most plasma inhibitors of serine proteinases, such as α_1 -proteinase inhibitor, α_1 -antichymotrypsin, antithrombin, C1-inhibitor and α_2 -antiplasmin, as well as several non-inhibitory proteins [1]. Like low-molecular-mass protein inhibitors of proteinases, serpins inactivate their target serine proteinases by trapping them in stable, equimolar complexes in a reaction that involves the enzyme recognizing a reactive bond in an exposed loop of the inhibitor. However, in contrast to the low-molecular-mass inhibitors, the trapping is effected by a conformational change, which most likely involves insertion of part of the reactive-bond loop as a middle strand into the major β -sheet of the serpin, the A sheet. The resulting complex is kinetically stable, and slowly, with a half-life of several days, dissociates into a cleaved, inactive inhibitor and active enzyme. The complex does not dissociate under denaturing conditions, but can be cleaved by nucleophiles, and may thus be stabilized by an acyl bond. In many serpin–proteinase reactions, some enzyme molecules escape being trapped in the inhibition pathway but instead inactivate the serpin by cleaving the reactive-bond loop in a partly parallel, although usually minor, substrate pathway, leading to apparent stoichiometries of inhibition greater than one. The general mechanism of serpin inhibition of target enzymes thus resembles that of suicide inhibitors [1,2].

The classical serpin target enzymes are serine proteinases. However, certain proteins of the serpin superfamily, such as an α_1 -antichymotrypsin-like protein from the pituitary gland, the cowpox virus serpin, Crm A, and squamous cell carcinoma antigen, have recently been shown also to inactivate cysteine

proteinases [3–6]. In addition, the ability of the plasma serpin inhibitor of coagulation proteinases, antithrombin, to inactivate the plant cysteine proteinase, papain, and the bacterial cysteine proteinase, Arg-gingipain, has been reported [7,8]. The mechanism of this inhibition of cysteine proteinases by serpins, which has been termed cross-class inhibition [4], appears to differ from that of serpin inhibition of serine proteinases. In particular, no complex stable under denaturing conditions, analogous to that formed in the reaction with serine proteinases, has been demonstrated for reactions with cysteine proteinases [4,6,8]. Moreover, cleavage of the serpin has been shown to be the dominating event in the inhibition of several cysteine proteinases [4,8].

To elucidate further how serpins inactivate cysteine proteinases, we have characterized the mechanism of the antithrombin–papain reaction, based on recent knowledge regarding the serpin inhibitory mechanism. We propose that inactivation of papain by antithrombin involves formation of an acyl-intermediate complex between inhibitor and enzyme, in which the bound proteinase is highly sensitive to proteolysis and is degraded by free proteinase. This degradation promotes dissociation of the complex, resulting in the release of fragmented, inactive enzyme. The inactivation of more physiologically or pathologically important cysteine proteinases by serpins may occur by a similar mechanism.

EXPERIMENTAL

Serpins

Human α -antithrombin was purified by affinity chromatography on heparin-agarose [9]. Reactive-bond-cleaved antithrombin was

Abbreviations used: DTT, dithiothreitol; E-64, *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane.

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produced by reacting antithrombin with thrombin in the presence of heparin at 10.03 and was isolated by heparin affinity chromatography [10,11]. Human α_1 -proteinase inhibitor was purchased from Athens Research and Technology, Inc. (Athens, GA, U.S.A.). Recombinant human plasminogen activator inhibitor-1, expressed in *Escherichia coli*, was a gift from Dr. Daniel A. Lawrence (American Red Cross, Rockville, MD, U.S.A.). Human C1 inhibitor was isolated as detailed previously [12]. Concentrations of the proteins were determined from absorption measurements with the use of published absorption coefficients [9,12,13].

Enzymes

The purification and activation of the plant cysteine proteinases papain (EC 3.4.22.2) and actinidin (EC 3.4.22.14) have been reported elsewhere [14]. The preparations contained 0.9 ± 0.1 and 0.8 ± 0.1 mol of thiol group/mol of protein respectively, and were fully active in binding to the cysteine proteinase inhibitor cystatin C. Papain was inactivated with iodoacetamide to give *S*-(carbamoylmethyl)papain [14]. Active papain was labelled with ^{125}I by the Iodo-Gen procedure (Pierce, Rockford, IL, U.S.A.) to a specific activity of 4.8×10^4 Bq/ μg . The lysosomal cysteine proteinase, cathepsin L (EC 3.4.22.15), from sheep liver [15] was a gift from Dr. Clive Dennison (University of Natal, Pietermaritzburg, South Africa). The enzyme was activated with 1 mM dithiothreitol (DTT) before use. Concentrations of papain and actinidin were determined by absorption measurements from absorption coefficients reported previously [14]. The molar concentration of cathepsin L was determined by active-site titration with *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)-butane (E-64) [15].

Human α -thrombin, > 99% α -form and > 90% active by active-site titrations [9], was a gift from Dr. John Fenton (New York State Department of Health, Albany, NY, U.S.A.).

Heparins

Heparin with high affinity for antithrombin and with an average M_r of ~ 8000 and reduced polydispersity was isolated as described [9]. The antithrombin-binding heparin pentasaccharide [16] was a gift from Dr. M. Petitou (Sanofi Recherche, Toulouse, France).

Experimental conditions

All measurements were done in 20 mM sodium phosphate buffer, pH 6.5, containing 0.1 M NaCl, 100 μM EDTA, 20–200 μM DTT and 0.1% (w/v) poly(ethylene glycol) 4000. The pH of the experiments was that of optimum activity and stability of papain. The DTT was added to the serpin solutions immediately before the start of the experiments to minimize the risk of reduction of disulphide bonds. Control experiments showed that antithrombin activity against thrombin was unaffected over the time of the experiments by the DTT concentrations used.

Inactivation kinetics

The rate of inactivation of cysteine proteinases by serpins was measured under pseudo-first-order conditions by continuous monitoring of the loss of enzyme activity in the presence of a fluorogenic substrate [9]. Activated papain, actinidin or cathepsin L, at final concentrations of 10–80 nM, 50 nM and 20–80 nM respectively, were added to a cuvette at 25 ± 0.2 °C, containing the substrate, carbobenzoxy-L-arginyl-L-arginine 4-methylcoumaryl-7-amide (Peptide Institute, Osaka, Japan) at a final concentration of 20 μM . The rate of cleavage of the substrate by

the enzyme alone was monitored for a short time in a Hitachi (Tokyo, Japan) Model F-4000 spectrofluorimeter with excitation and emission wavelengths of 370 and 440 nm respectively. The serpin was then added in a small volume to a concentration of 1–30 μM , and the fluorescence increase with time due to the enzyme cleaving the substrate and concomitantly being inactivated by the serpin was recorded. The molar ratio of inhibitor to enzyme was $\geq 100:1$ and substrate hydrolysis was $\leq 5\%$ in all experiments. The comparably slow inactivation rates necessitated the use of an inefficient enzyme substrate. Pseudo-first-order rate constants for proteinase inactivation were derived by non-linear regression analyses of the progress curves [9]. Apparent second-order rate constants were obtained from the slopes of plots of these pseudo-first-order rate constants versus serpin concentration and were corrected for substrate competition [9] with the use of published K_m values for proteinase hydrolysis of the substrate [17,18].

The effect of heparin or the heparin pentasaccharide on the rate of inactivation of papain by antithrombin was assessed by a similar procedure. The papain and antithrombin concentrations were 40 nM and 4 μM respectively, and heparin or pentasaccharide was included at concentrations ranging from 0.01 to 10 μM . Pseudo-first-order rate constants were obtained from progress curves of substrate hydrolysis, as detailed above.

Dissociation kinetics

The rate of dissociation of a putative complex between papain and antithrombin was investigated by incubating 10 μM papain with 32–40 μM antithrombin for 15–60 min at 25 °C. The incubation mixture was then diluted 1000- to 40000-fold into a cuvette containing a 20–200 μM solution of the substrate, carbobenzoxy-L-phenylalanyl-L-arginine 4-methylcoumaryl-7-amide (Peptide Institute), the tightest-binding ($K_m \sim 60$ μM) fluorescent papain substrate available [17]. In some experiments, 10–100 nM thrombin and 50–200 nM heparin were also present in the cuvette. The fluorescence increase due to hydrolysis of the substrate was monitored continuously as in the measurements of association kinetics. Control experiments showed that thrombin at the concentrations used contributed insignificantly to substrate cleavage.

Apparent stoichiometry of inactivation

Two series of samples of papain, at constant final concentrations of 10 and 1 μM , were incubated for 15 and 150 min respectively at 25 °C with antithrombin at molar ratios to the enzyme ranging from 0.5 to 4. The incubation mixtures were then assayed for residual papain activity. The samples having a papain concentration of 10 μM were diluted 5-fold with a solution of the chromogenic substrate, *N*- α -benzoyl-L-arginine *p*-nitroanilide (final concentration 300 μM), and the initial rates of substrate cleavage were monitored by absorption measurements at 410 nm in a Cary 3 spectrophotometer (Varian Techtron, Mulgrave, Australia). The samples with 1 μM proteinase concentration were diluted 100-fold with a solution of carbobenzoxy-L-arginyl-L-arginine 4-methylcoumaryl-7-amide (final concentration 20 μM), and the initial cleavage rates were monitored by fluorescence measurements as in the analyses of the inactivation kinetics. For both series, the residual activity was plotted against the molar ratio of antithrombin to papain, and the apparent stoichiometry of inactivation was obtained by extrapolation of the linear portion of this plot to the abscissa.

SDS/PAGE

Serpin and papain (labelled with ^{125}I in some experiments) were incubated at concentrations of 15–225 μM and 15 μM respectively for 15 min at 25 °C. Hydroxylamine at concentrations of 0.1–1 M was also included in certain experiments with antithrombin. The reactions were stopped by addition of the cysteine proteinase inhibitors E-64 and iodoacetamide to concentrations of 50 μM and 1 mM respectively. After a further 5 min, SDS and mercaptoethanol (when required) were added to concentrations of 1% (w/v) and 0.35 M respectively. In some experiments with antithrombin, 1 M hydroxylamine or methylamine was also added, and these solutions were then incubated for 1 h and dialysed overnight against 20 mM sodium phosphate buffer, pH 7.5, containing 0.1% (w/v) SDS. All samples were boiled for 3 min and analysed by SDS/PAGE with the Tricine buffer system on a 16.5% T, 2.6% C separating gel, overlaid with a 10% T, 2.6% C spacer gel and a 4% T, 2.6% C stacking gel [19]. The gels were stained with Coomassie Brilliant Blue R250 and in some cases were subjected to autoradiography or were electroblotted to a poly(vinylidene difluoride) membrane (Qiabran PVDF; Qiagen, Chatsworth, CA, U.S.A.). For immunoblotting, the membrane was treated for 15 min with 0.5% (w/v) glutaraldehyde at pH 7.4 and was then allowed to react for 1 h with appropriate dilutions of antisera against papain (a gift from Daniel C. Tessier, Biotechnology Research Institute, Montreal, Canada) or human antithrombin (DAKO, Glostrup, Denmark). The binding of the primary antibodies was detected with horse radish peroxidase-conjugated anti-rabbit IgG in conjunction with the enhanced chemiluminescence (ECL; Amersham, Little Chalfont, U.K.) system. For analyses of N-terminal sequences, the membrane was stained with Coomassie Brilliant Blue R250, appropriate bands were excised and the peptides were sequenced directly in an Applied Biosystems (Foster City, CA, U.S.A.) 470A gas-phase sequencer connected on-line to a 120A PTH (phenylthiohydantoin) analyser.

PAGE under non-denaturing conditions

Serpin and papain were incubated and the reactions stopped with E-64 and iodoacetamide as for SDS/PAGE. The samples were then analysed by electrophoresis on 7.5% gels [20], which were stained with Coomassie Brilliant Blue R250 or electroblotted as described above.

In-gel digestion and peptide mapping

Samples for in-gel digestion were separated by SDS/PAGE under reducing conditions, as described above, but were reduced and alkylated before electrophoresis. Appropriate bands were excised from Coomassie-stained gels and were digested with endoproteinase Lys C, essentially following the protocol in [21]. Briefly, the gel pieces were washed extensively and completely dried. Endoproteinase Lys C (Wako Chemicals, Neuss, Germany) was allowed to absorb into the swelling gel, appropriate buffer was added and the sample was incubated at 30 °C overnight. Generated protein fragments were extracted and isolated by narrow-bore reverse-phase liquid chromatography on a $\mu\text{RPC C2/C18 SC 2.1/10}$ column, operated in the SMART System (Pharmacia Biotech, Uppsala, Sweden). Peptides were eluted by a linear gradient of acetonitrile in 0.06% (v/v) trifluoroacetic acid at a flow rate of 100 $\mu\text{l}/\text{min}$.

Computer simulations

Simulations of the kinetics of the proposed model for antithrombin inactivation of papain (see Scheme 1) and variants of

this model were done with the program Scientist (Micromath Scientific Software, Salt Lake City, UT, U.S.A.). The differential equations for a particular model were input into the program, and progress curves for reactants, intermediates and products were generated numerically for different starting concentrations of antithrombin and proteinase and different values of the rate constants for each step of the model. Concentrations and rate constants were chosen to best duplicate the conditions of the analyses of inactivation kinetics and stoichiometry.

RESULTS

Kinetics of inactivation of cysteine proteinases by antithrombin

Addition of antithrombin to papain resulted in a progressive decrease of papain activity, as demonstrated by continuous monitoring of this activity in the presence of substrate (Figure 1). The progress curves showed that a slow rate of substrate hydrolysis, corresponding to $\leq \sim 1\%$ of the initial enzyme activity, persisted at the end of the reaction. The curves were satisfactorily fitted by non-linear regression to the equation for a first-order reaction with a sloping end-point [9]. The pseudo-first-order rate constants evaluated in this manner increased linearly with antithrombin concentration (Figure 2) and gave a second-order association rate constant, corrected for substrate competition, of $(1.6 \pm 0.1) \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$. No decrease in papain activity was observed in the absence of antithrombin under the conditions of these analyses.

The mammalian lysosomal cysteine proteinase, cathepsin L, a member of the papain superfamily of proteinases with a structure and substrate specificity similar to those of papain, was also inactivated by antithrombin. The progress curves were similar to the curves observed for papain (not shown), although a larger proportion of the activity remained at the end of the reaction, ranging from $\sim 20\%$ at 2 μM antithrombin to $\sim 10\%$ at 8 μM antithrombin. The linear increase in the derived pseudo-first-order rate constants with antithrombin concentration (Figure 2) gave a corrected second-order association rate constant of $(8.6 \pm 0.4) \times 10^2 \text{ M}^{-1} \cdot \text{s}^{-1}$, i.e. about half that for the inactivation

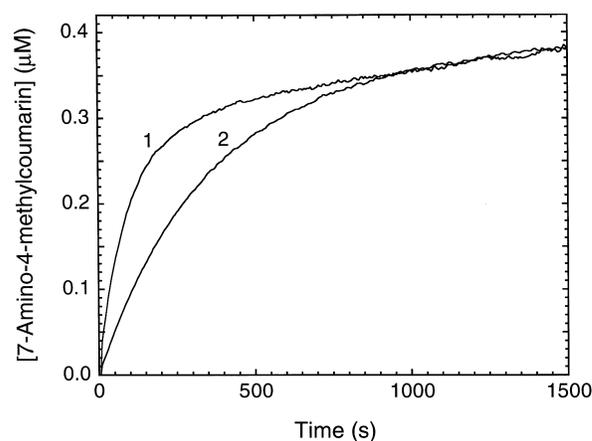


Figure 1 Progress curves of antithrombin inactivation of papain

Curve 1, 8 μM antithrombin and 80 nM papain; curve 2, 2 μM antithrombin and 20 nM papain. The analyses were done at 25 ± 0.2 °C in the presence of the papain substrate carbobenzoxy-L-arginyl-L-arginine 4-methylcoumarinyl-7-amide at a concentration of 20 μM . The curves were monitored by measurements of the fluorescence of 7-amino-4-methylcoumarin, the product of substrate hydrolysis, with excitation and emission wavelengths of 370 and 440 nm respectively.

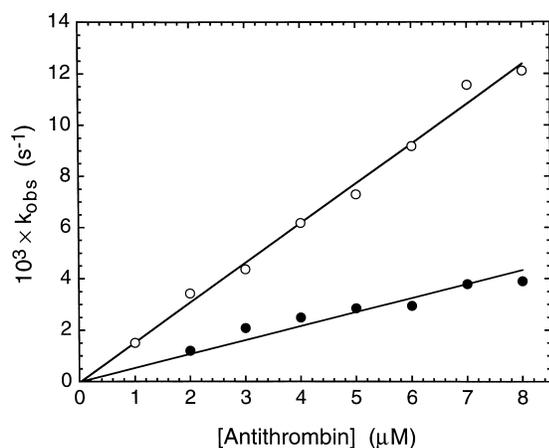


Figure 2 Observed pseudo-first-order rate constants, k_{obs} , for antithrombin inactivation of papain and cathepsin L as a function of antithrombin concentration

(○) Papain; (●) cathepsin L. The values of k_{obs} were derived by non-linear least-squares regression analyses [9] of the progress curves in Figure 1 and analogous curves. The molar ratio of antithrombin to proteinase was $\geq 100:1$ in all analyses.

of papain. Because of the limited amounts of cathepsin L available, no detailed studies of the inactivation of this enzyme could be made. In contrast to papain and cathepsin L, no inactivation of the plant cysteine proteinase, actinidin, by antithrombin could be demonstrated at inhibitor concentrations up to 30 μM .

Inactivation of papain by other serpins

No inactivation of papain by the serpins α_1 -proteinase inhibitor, plasminogen activator inhibitor-1 and C1 inhibitor, was observed up to inhibitor concentrations of 20–30 μM .

Heparin effect on antithrombin inactivation of papain

Heparin greatly accelerates the inhibition of thrombin and Factor Xa by antithrombin in a catalytic manner and also increases the rate of the reaction with other proteinases, albeit to a smaller extent [2]. In contrast, both heparin and the synthetic antithrombin-binding heparin pentasaccharide decreased the rate of the antithrombin–papain reaction, although the effect was small and high concentrations of the saccharides were required (results not shown). At heparin or pentasaccharide concentrations of 10 μM , sufficient to saturate antithrombin, the rate was reduced about 50%. This decrease was paralleled by a comparable effect of the two saccharides in reducing the rate of papain cleavage of the fluorogenic substrate used. While these observations are consistent with the binding of heparin or pentasaccharide to antithrombin reducing the reactivity of the inhibitor toward papain, a weak interaction of the saccharides with papain may also contribute to this reduced reactivity.

Dissociation of a putative antithrombin–papain complex

Attempts to demonstrate dissociation of a complex between antithrombin and papain similar to that formed with serine proteinases were done by allowing enzyme and inhibitor to react at high concentrations and then extensively diluting the reaction mixture into a solution of the most efficient fluorogenic papain substrate available [9]. Such a substrate would be expected

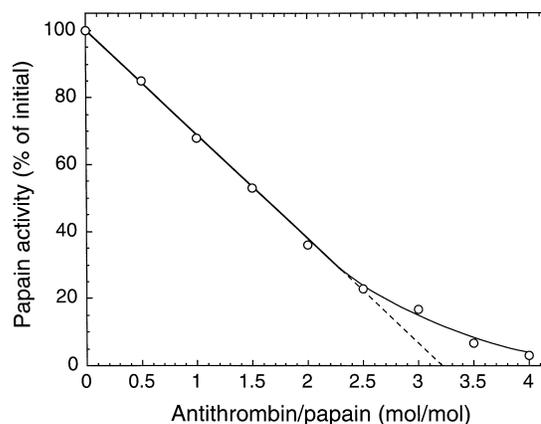


Figure 3 Titration of papain with antithrombin, monitored by the loss of papain activity

Papain (10 μM) was incubated with different concentrations of antithrombin for 15 min at 25 °C. Residual papain activity against the substrate *N*- α -benzoyl-L-arginine *p*-nitroanilide (300 μM) was measured by absorption at 410 nm after 5-fold dilution of the incubation mixtures. The dotted line represents an extrapolation by linear least-squares regression of the linear portion of the titration curve to the abscissa to give the apparent inactivation stoichiometry.

largely to prevent reassociation of liberated enzyme and inhibitor by binding to the enzyme. In some experiments, thrombin and heparin were present together with the substrate to minimize further such reassociation by trapping any antithrombin that had not reacted with papain or may have dissociated from an antithrombin–papain complex. However, no parabolic increase of fluorescent product with time, characteristic of release of active enzyme from a complex with an inhibitor [9], was observed under any condition investigated. No dissociation of an antithrombin–papain complex to give active papain during the time-scale of these experiments could thus be substantiated.

Apparent stoichiometry of antithrombin inactivation of papain

Titration, monitored by measurements of residual enzyme activity, of 10 μM papain with increasing antithrombin concentrations gave an apparent antithrombin to papain inactivation stoichiometry of 3.2 ± 0.1 (Figure 3). Similar titrations at a 10-fold lower papain concentration gave a stoichiometry of 3.5 ± 0.4 (results not shown), the larger error presumably being due to the longer incubation times required. Such apparent inhibition stoichiometries appreciably greater than one have been obtained for the heparin-catalysed antithrombin–thrombin reaction at low ionic strength and for reactions of certain recombinant serpin variants with serine proteinases [1,10,22–24], and have been shown to arise from the substrate pathway dominating in these cases. The apparent stoichiometry obtained is thus consistent with antithrombin being extensively cleaved and inactivated during the reaction with papain.

Analyses of antithrombin–papain reaction by PAGE

SDS/PAGE under reducing conditions of reaction mixtures of antithrombin and papain showed that a band with an apparent M_r of $\sim 120\,000$ appeared and increased in staining intensity with increasing concentrations of antithrombin (Figure 4). Concomitantly, the intensity of the papain band was greatly reduced, and a peptide band with an apparent M_r of $\sim 4\,500$ appeared and became stronger, as did a much weaker band with an apparent

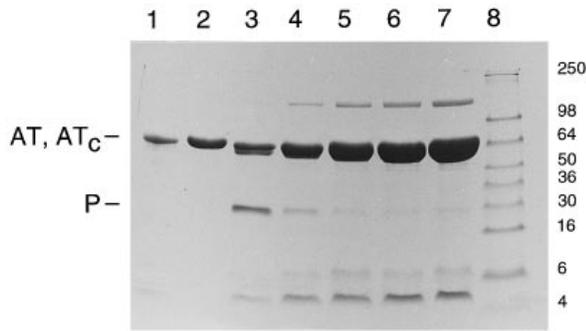


Figure 4 SDS/PAGE under reducing conditions of the reaction between antithrombin and papain analysed by staining

Lane 1, reactive-bond-cleaved antithrombin (15 μM); lane 2, intact antithrombin (15 μM); lanes 3–7, papain (15 μM) allowed to react with antithrombin for 15 min at 25 °C at molar ratios of inhibitor to enzyme of 1, 2, 3, 4 and 5 respectively; lane 8, standards with the molecular masses (in kDa) indicated. Sample volumes of 10 μl were applied to each lane. AT, antithrombin; AT_c, reactive-loop-cleaved antithrombin; P, papain.

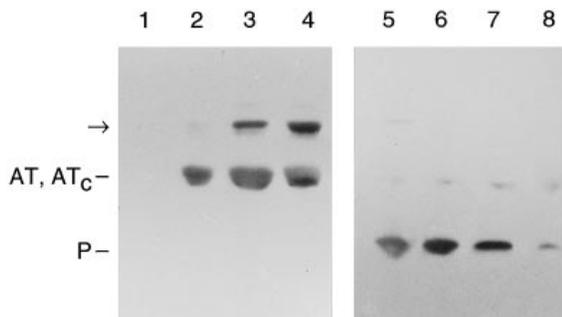


Figure 5 SDS/PAGE under reducing conditions of the reaction between antithrombin and papain analysed by immunoblotting

Lanes 1–4, antibodies against antithrombin; lanes 5–8, antibodies against papain. Lanes 1 and 5, papain (15 μM); lanes 2 and 6, 3 and 7, 4 and 8, papain (15 μM) allowed to react with antithrombin for 15 min at 25 °C at molar ratios of inhibitor to enzyme of 1, 3 and 5 respectively. Sample volumes of 10 μl were applied to each lane. The position of the putative antithrombin dimer (M_r 120000) band on the stained gel is indicated by an arrow. AT, antithrombin; AT_c, reactive-loop-cleaved antithrombin; P, papain.

M_r of ~ 6500 . Moreover, a band with a slightly lower apparent M_r than antithrombin or reactive-bond-cleaved antithrombin, which migrated indistinguishably under the conditions used, was evident. None of these features were seen when antithrombin was incubated with inactive *S*-(carbamoylmethyl)papain. Moreover, no decrease of the intensity of the papain band was observed when active papain was incubated alone.

A band analogous to that with an apparent M_r of ~ 120000 in SDS/PAGE, and of comparable intensity, was identified also by PAGE under non-denaturing conditions (results not shown). The two bands were initially considered to represent an antithrombin–papain complex. However, the apparent M_r of the band in SDS/PAGE was higher than that expected for such a complex, 81000. Also, the putative complex could not be dissociated by treatment with 1 M hydroxylamine or methylamine in SDS, as evident by SDS/PAGE under reducing conditions (results not shown), in contrast with analogous complexes between antithrombin and serine proteinases [25]. This treatment should also dissociate a complex between anti-

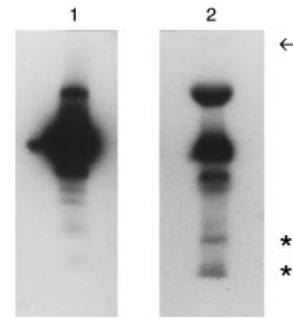


Figure 6 SDS/PAGE under reducing conditions of the reaction between antithrombin and ^{125}I -labelled papain analysed by autoradiography

Lane 1, ^{125}I -labelled papain (15 μM); lane 2, ^{125}I -labelled papain (15 μM) allowed to react with antithrombin for 15 min at 25 °C at a molar ratio of inhibitor to enzyme of 5. Sample volumes of 10 μl were applied to each lane. The overexposure of the autoradiograph revealed minor impurities in the papain preparation that were not apparent, or were only weakly apparent, on the stained gel. The position of the putative antithrombin dimer (M_r 120000) band on the stained gel is indicated by an arrow. The low-molecular-mass bands seen in the antithrombin–papain reaction mixture, but not in unreacted papain, are marked by asterisks.

thrombin and a cysteine proteinase expected to be held together by a thioester bond with lower stability than the corresponding ester bond in serine proteinase complexes. Moreover, N-terminal sequence analyses of both the band in SDS/PAGE and the analogous band in PAGE under non-denaturing conditions showed only the sequence of antithrombin, HGSPVDI-. Also, only antithrombin, and no papain, could be detected in the SDS/PAGE band in immunoblots with antibodies against antithrombin or papain (Figure 5), and no papain was identified in this band by autoradiography, even with long exposure times, of gels run with ^{125}I -labelled papain (Figure 6). Similarly, no papain was detected by immunoblotting in the band seen in PAGE under non-denaturing conditions (results not shown). It was thus concluded that the two bands contained only antithrombin and presumably represented the same molecular species, the apparent M_r being consistent with an antithrombin dimer. The amount of the band seen in SDS/PAGE under reducing conditions was appreciably reduced when antithrombin and papain were allowed to react in the presence of 0.1 M hydroxylamine, and the band disappeared completely at ≥ 0.5 M hydroxylamine.

In-gel digestion of the band having an apparent M_r of ~ 120000 in SDS/PAGE with endoproteinase Lys C and separation of generated peptides by reverse-phase chromatography gave a peptide pattern highly similar to that obtained by the same treatment of a band containing intact antithrombin with an apparent $M_r \sim 60000$ (Figure 7). This finding supports the conclusion that the band corresponding to an $M_r \sim 120000$ contained only antithrombin. However, in spite of extensive work, no peptides could be identified that unequivocally demonstrated the manner in which the two monomers of the putative antithrombin dimer could be linked together.

In agreement with the stained SDS/PAGE gels, the immunoblots showed that the amount of intact papain was considerably reduced with increasing antithrombin concentrations (Figure 5). However, these blots failed to demonstrate a corresponding increase in the amount of papain elsewhere in the gel. Only with the use of ^{125}I -labelled papain and autoradiography with long exposure times could labelled papain peptides with apparent $M_r \leq \sim 5000$, not present in the unreacted labelled papain, be detected (Figure 6). These results thus indicate that papain had been cleaved to small fragments during the reaction with anti-

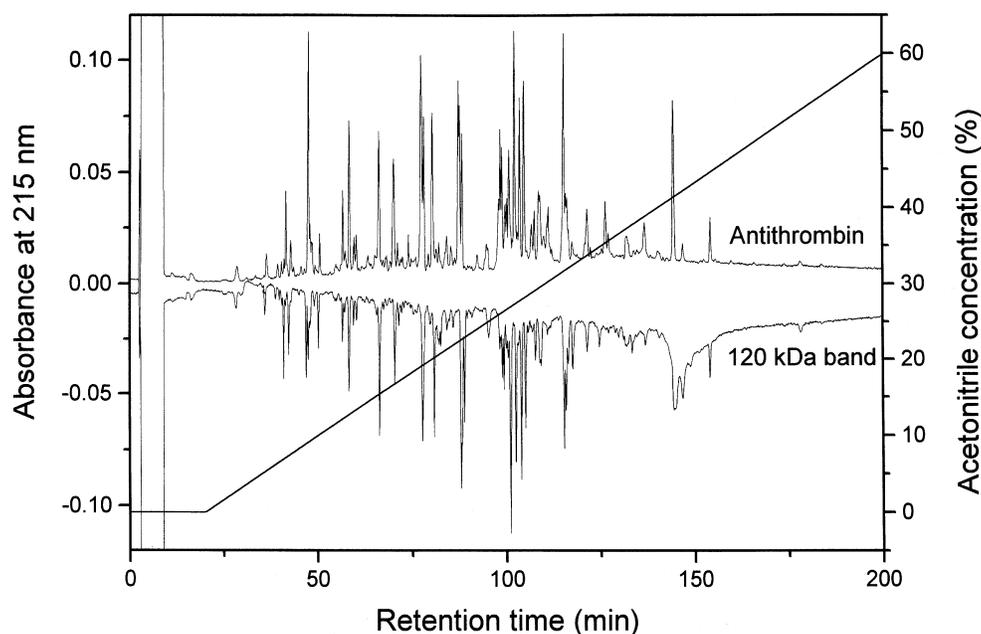


Figure 7 Separation by reverse-phase chromatography of fragments produced by in-gel endoproteinase Lys C digestion of intact antithrombin and the putative antithrombin dimer (120 kDa) band

Samples were digested as described in the Experimental section. Generated peptides were eluted by a linear gradient of acetonitrile (0–60%) in 0.06% (v/v) trifluoroacetic acid at a flow rate of 100 μ l/min from a 350 μ l μ RPC C2/C18 SC 2.1/10 column operated in the SMART System.

thrombin. The absence of larger-size fragments in these experiments presumably was due to the comparatively high proteinase concentrations used.

N-terminal sequence analyses of the peptide band with an apparent $M_r \sim 4500$ that was formed during the antithrombin–papain reaction gave results compatible with two sequences, RSLNP- and NPNRV-, in a proportion of $\sim 3:1$. Comparison with the sequence of antithrombin [26] indicated that these peptides had arisen by cleavages within the antithrombin reactive-bond loop, at the Gly-392–Arg-393 and Leu-395–Asn-396 bonds, i.e. the P_2 – P_1 and P_2' – P_3' bonds respectively. Analyses by PAGE under non-denaturing conditions and by SDS/PAGE under non-reducing conditions (results not shown), both of which separate intact and reactive-loop-cleaved antithrombin [10], in contrast to SDS/PAGE under reducing conditions (see Figure 4), supported this conclusion. Apart from a small amount of antithrombin transformed into the putative dimer band, the inhibitor was thus essentially completely converted into a form migrating as reactive-bond-cleaved antithrombin during the reaction with papain at molar ratios lower than that corresponding to the apparent stoichiometry of inactivation.

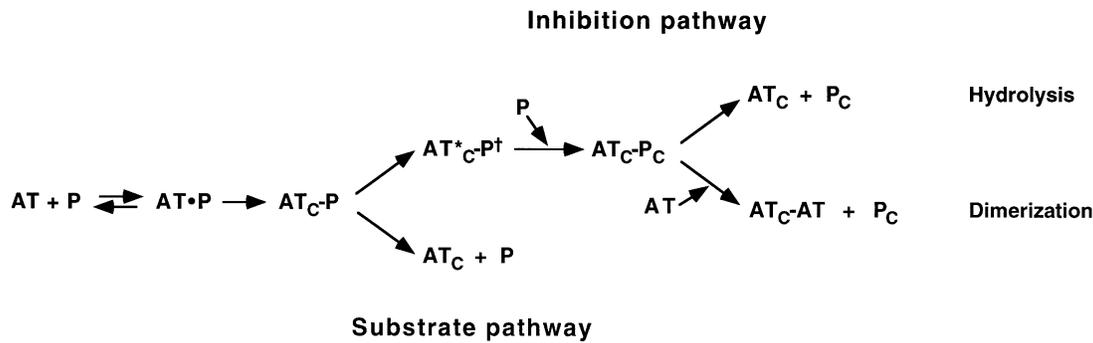
The band with a slightly lower apparent M_r than antithrombin or reactive-loop-cleaved antithrombin in SDS/PAGE under reducing conditions was isolated by prolonged electrophoresis and was found to have the N-terminal sequence ATNR-. This finding, together with the results presented above, indicated that this band represented reactive-loop-cleaved antithrombin that was also cleaved at the Glu-42–Ala-43 bond. This bond is located in an exposed loop that is undefined in the crystal structures of antithrombin [27,28] and presumably was easily accessible to attack by papain, both before and after the reactive-bond loop had been cleaved.

Analyses of reactions of other serpins with papain by PAGE

No band comparable to that with apparent $M_r \sim 120000$ seen with antithrombin could be detected in reactions of the serpins, α_1 -proteinase inhibitor, plasminogen activator inhibitor-1 and C1 inhibitor, with papain (results not shown). Moreover no decrease in the intensity of the papain band was observed, even at high serpin concentrations. However, all three serpins were cleaved by papain, as evident from a somewhat decreased apparent molecular mass of the major band, in agreement with previous results [29].

DISCUSSION

This work shows that the serpin antithrombin inactivates the cysteine proteinases papain and cathepsin L with second-order rate constants that are comparable to those by which antithrombin inhibits its physiological target enzymes thrombin and factor Xa [2]. However, heparin does not accelerate the inactivation of papain, in contrast with the inhibition of the clotting enzymes [2]. The inactivation of cysteine proteinases appears to be specific for antithrombin, as several other serpins had no effect. The results indicate that the mechanism of antithrombin inactivation of cysteine proteinases differs appreciably from that of serine proteinases. The decrease in papain activity and the concomitant disappearance of the papain band in SDS/PAGE are thus not due to formation of a stable complex with antithrombin but instead apparently involve proteolytic cleavage of papain into small fragments, presumably by still active papain. This conclusion is indicated by the fact that no complex-bound papain could be demonstrated by electrophoresis under non-denaturing conditions or by SDS/PAGE, whereas



Scheme 1 Model of the reaction of antithrombin with cysteine proteinases

AT, antithrombin; AT_c , reactive-loop-cleaved antithrombin; P, proteinase; P_c , proteolytically cleaved proteinase; $AT \cdot P$, initial antithrombin–proteinase complex; $AT_c \cdot P$, acyl-intermediate antithrombin–proteinase complex, in which the attacked bond in the reactive loop of antithrombin is cleaved but the resulting large N-terminal fragment of the inhibitor is still linked to the proteinase by an acyl (i.e. thioester) bond; $AT_c \cdot P^\dagger$, acyl-intermediate antithrombin–proteinase complex, in which both antithrombin and proteinase have undergone conformational changes, the changes in antithrombin involving insertion of the cleaved reactive loop into the A β -sheet and leading to trapping of the proteinase, and those in the proteinase resulting in greater susceptibility to proteolysis; $AT_c \cdot P_c$, acyl-intermediate antithrombin–proteinase complex, in which the proteinase moiety has been cleaved by free, active enzyme, and the conformational changes trapping the proteinase have been reversed; $AT_c \cdot AT$, antithrombin dimer, consisting of a cleaved and an intact inhibitor.

small fragments of papain were evident in SDS/PAGE. It is also consistent with the fact that no release of active papain from a putative antithrombin–papain complex, analogous to the enzyme release seen for antithrombin–serine proteinase complexes [9,13], could be verified. Nevertheless, the inactivation and accelerated cleavage of papain must be mediated by antithrombin, as no loss of activity, degradation or disappearance of papain was observed in the absence of the inhibitor, nor in the presence of the other serpins investigated. The inactivation of papain is accompanied by pronounced cleavage of antithrombin as a normal substrate at two bonds adjacent to the reactive bond involved in the inhibition of serine proteinases, and also by partial cleavage of an exposed bond in the N-terminal region of the inhibitor. Moreover, a substantial amount of antithrombin is converted into a product that appears to be an antithrombin dimer.

Together, these observations lead to the following model for the reaction of antithrombin with cysteine proteinases (Scheme 1). The proteinase attacks a peptide bond in the reactive loop of antithrombin, predominantly the P_2 – P_1 bond, but also the P_2' – P_3' bond. The reaction proceeds in a manner similar to that between serpins and serine proteinases, i.e. via an initial Michaelis complex to the acyl-intermediate, which is transformed in two different ways [1,2]. The major pathway in the reaction with cysteine proteinases is the substrate pathway, in which the thioester bond in the acyl-intermediate is hydrolysed by water, resulting in an inactive antithrombin cleaved at the attacked bond and free, intact proteinase. The predominance of this pathway, in comparison with that in the reaction with serine proteinases, is consistent with the greater rate of deacylation of cysteine proteinase acyl-enzymes than of serine proteinase acyl-enzymes [30]. The less favoured pathway in the cysteine proteinase reaction is the inhibitory pathway, in which the acyl-intermediate undergoes conformational changes, presumably analogous to those induced in the corresponding complexes between serpins and serine proteinases. These changes involve insertion of the reactive loop of the serpin into the A β -sheet, resulting in the trapping of the enzyme in a stable complex [1,11,22,27,28,31]. In this complex, the cysteine proteinase is extensively cleaved by still active enzyme. Such cleavage is supported by a number of previous observations showing that target serine proteinases of serpins are considerably more susceptible to proteolysis by still active proteinase when bound in a complex with the inhibitor than

when free [32–34], presumably due to a conformational change in the proteinase as a result of the binding. This susceptibility can lead to appreciable degradation of the complex-bound enzyme [32], similar to what was observed in this work, albeit at a slower rate.

Degradation of the cysteine proteinase in the acyl-intermediate complex presumably reverses the conformational changes trapping the enzyme and promotes nucleophilic attack on the thioester linkage, which is exposed as a result of this reversal. The fact that papain does not attack the P_1 – P_1' bond normally involved in stabilization of serpin–enzyme complexes, but only bonds in its vicinity, may contribute to such instability of the acyl-intermediate complex once the proteinase has been degraded. The cysteine proteinase complex is therefore considerably more short-lived than the analogous complexes with serine proteinases [1,13,35]. The nucleophilic attack can occur in two different ways. The predominant outcome is that the acyl-intermediate is hydrolysed by water, yielding cleaved antithrombin and fragmented, irreversibly inactivated enzyme. Alternatively, the acyl-intermediate is instead cleaved by a nucleophilic group of free antithrombin, which may be the α -amino group, a lysine amino group or a serine hydroxy group. The reaction results in displacement of the cleaved, inactive proteinase and in the formation of an antithrombin dimer, consisting of a cleaved inhibitor with intact antithrombin linked to the C-terminus of its heavy chain by an amide or acyl bond. This mechanism of dimer formation is supported by the disappearance of the dimer band when antithrombin and papain were allowed to react in the presence of the competing nucleophile hydroxylamine. The difficulties encountered in isolating a unique peptide that revealed how the antithrombin dimer is linked may be the consequence of different nucleophilic groups in antithrombin being involved in dimer formation, resulting in heterogeneity of the cross-linking. The greater susceptibility of papain than of serine proteinases in the acyl-intermediate to cleavage by free proteinase, together with the high reactivity with nucleophiles of the thioester linkage in the degraded complex, presumably is the reason why a corresponding dimer has not been observed in reactions of antithrombin with serine proteinases.

The finding that of several serpins tested, only the interaction of papain with antithrombin leads to inactivation and cleavage of the proteinase, is presumably due to the substrate pathway

being the exclusive pathway in the reaction of papain with other serpins. This behaviour probably results from the acyl-intermediate deacylating before undergoing the conformational changes that inactivate the proteinase and render it susceptible to attack by free enzyme. Such rapid deacylation could result from different sites of cleavage by papain in the reactive loop of other serpins producing a shorter residual loop fragment, the insertion of which into the A sheet is incapable of inducing these conformational changes. The specific conformation of the antithrombin loop, requiring activation by heparin to become fully reactive with target serine proteinases [1,2,27,28], may also contribute to promoting the conformational changes that result in inactivation and autoproteolysis of papain. The small effect of heparin in reducing the rate of inactivation of papain may be due to heparin decreasing the rate by which the complex-bound enzyme is cleaved, in analogy with the heparin-induced decrease in the rate of cleavage of a low-molecular-mass substrate by the enzyme.

Variants of the model in which the acyl-intermediate is hydrolysed after the conformational changes in antithrombin and cysteine proteinase have occurred, but before the proteinase in the intermediate has been cleaved, were ruled out by kinetic simulations. These simulations revealed that any regeneration of active proteinase before autoproteolysis of the enzyme trapped in the acyl-intermediate complex, a situation equivalent to temporary inhibition, would result in non-exponential progress curves and a marked dependence of the stoichiometry and kinetics of inactivation on proteinase concentration. The observed simple second-order kinetic behaviour and independence of the inactivation stoichiometry on proteinase concentration instead require that the conformational changes trapping the proteinase at the acyl-intermediate result in permanent inactivation. The enzyme must thus be inactivated at this stage regardless of its subsequent proteolysis in the complex, although such proteolysis is responsible for the fact that a stable serpin-enzyme complex cannot be observed. This requirement is satisfied by the proposed model. However, certain other variants of this model are consistent with the data. For instance, only one of the two bonds cleaved in antithrombin, P_2-P_1 and $P_2'-P_3'$, could be involved in formation of the acyl-intermediate that renders the proteinase susceptible to degradation by free enzyme, attack of the other bond merely leading to normal substrate cleavage of the inhibitor. Moreover, a nucleophilic group of proteolytically cleaved antithrombin formed by the substrate pathway may also cleave the acyl bond in the inhibition pathway, producing a dimer of two cleaved antithrombin molecules linked by a covalent bond. However, these possibilities, and the fact that the nature of the cross-linking of the antithrombin dimer could not be clarified, do not affect the main features of the model. These features are that inactivation of cysteine proteinases by antithrombin is due to formation of an acyl-intermediate, the stability of which is reduced by extensive cleavage of the complex-bound proteinase by still active enzyme.

Consideration of published results suggests that the proposed model may also have relevance for the inactivation by serpins of more physiologically or pathologically important cysteine proteinases. The best studied such reaction, that between the cowpox virus serpin, Crm A, and a member of the caspase family of cysteine proteinases, interleukin-1 β -converting enzyme [4], was thus accompanied by substantial cleavage of the serpin in a substrate pathway, as indicated by the high apparent stoichiometry of inhibition. Moreover, no stable serpin-proteinase complex could be identified by SDS/PAGE, although a band behaving as a complex was shown by electrophoresis under non-denaturing conditions. This band may have represented an acyl-

intermediate complex, as discussed above, although more long-lived than that formed in the antithrombin-papain reaction. Also, the possibility that proteolysis of the interleukin-1 β -converting enzyme accompanied the reaction with Crm A cannot be excluded by the published results. Reactions between Crm A and other caspases showed similar characteristics [6]. The only aspect of the interaction between Crm A and interleukin-1 β -converting enzyme in some discordance with the proposed mechanism is the high rate constant, about $10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$.

In many respects, the reaction between antithrombin and a trypsin-like enzyme from *Porphyromonas gingivalis* [8], later identified as a cysteine proteinase and named Arg-gingipain [36], behaved similarly to the antithrombin-papain reaction. Substantial cleavage of the Arg-393-Ser-394 reactive bond of antithrombin in a substrate pathway thus occurred concurrently with inactivation of the proteinase with a rate constant of $\sim 5 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$. Moreover, no SDS-stable complex could be identified. Interestingly, neither α_1 -proteinase inhibitor nor C1 inhibitor had any effect on the activity of Arg-gingipain, in analogy with their inability to inactivate papain. The other serpin-cysteine proteinase interactions reported have been investigated in less detail, but no features in apparent contradiction with the proposed mechanism are evident [3,5]. A band interpreted as an SDS-stable complex was identified in the reaction of an α_1 -antichymotrypsin-like serpin with prohormone thiol protease [3], but may have represented a serpin dimer analogous to that formed in the antithrombin-papain reaction. More detailed characterization of these other serpin-cysteine proteinase reactions may reveal whether their mechanism is akin to that proposed in this work for reactions of antithrombin with cysteine proteinases.

Note added in proof (received 22 September 1998)

In a recent paper [37] it was reported that a form of squamous cell carcinoma antigen inhibited cathepsin S with equimolar stoichiometry in a reaction involving formation of an SDS-stable complex. In contrast with other previously characterized serpin-cysteine proteinase reactions, the mechanism of this reaction thus appears more similar to that of serpin inhibition of serine proteinases.

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REFERENCES

- Gettins, P. G. W., Patston, P. A. and Olson, S. T. (1996) Serpins: Structure, Function and Biology, R. G. Landes, Austin
- Olson, S. T. and Björk, I. (1994) *Semin. Thromb. Hemostasis* **20**, 373-409
- Hook, V. Y. H., Purviance, R. T., Azaryan, A. V., Hubbard, G. and Krieger, T. J. (1993) *J. Biol. Chem.* **268**, 20570-20577
- Komiyama, T., Ray, C. A., Pickup, D. J., Howard, A. D., Thornberry, N. A., Peterson, E. P. and Salvesen, G. (1994) *J. Biol. Chem.* **269**, 19331-19337
- Takeda, A., Yamamoto, T., Nakamura, Y., Takahashi, T. and Hibino, T. (1995) *FEBS Lett.* **359**, 78-80
- Zhou, Q., Snipas, S., Orth, K., Muzio, M., Dixit, V. M. and Salvesen, G. S. (1997) *J. Biol. Chem.* **272**, 7797-7800
- Valeri, A. M., Wilson, S. M. and Feinman, R. D. (1980) *Biochim. Biophys. Acta* **614**, 526-533
- Curtis, M. A., Slaney, J. M., Carman, R. J. and Pemberton, P. A. (1993) *FEMS Microbiol. Lett.* **108**, 169-174
- Olson, S. T., Björk, I. and Shore, J. D. (1993) *Methods Enzymol.* **222**, 525-560
- Olson, S. T. (1985) *J. Biol. Chem.* **260**, 10153-10160
- Björk, I., Nordling, K. and Olson, S. T. (1993) *Biochemistry* **32**, 6501-6505

- 12 Olson, S. T., Sheffer, R. and Francis, A. M. (1993) *Biochemistry* **32**, 12136–12147
- 13 Olson, S. T., Bock, P. E., Kvassman, J., Shore, J. D., Lawrence, D. A., Ginsburg, D. and Björk, I. (1995) *J. Biol. Chem.* **270**, 30007–30017
- 14 Björk, I. and Ylinenjärvi, K. (1990) *Biochemistry* **29**, 1770–1776
- 15 Pike, R. N., Coetzer, T. H. and Dennison, C. (1992) *Arch. Biochem. Biophys.* **294**, 623–629
- 16 Choay, J., Petitou, M., Lormeau, J. C., Sinay, P., Casu, B. and Gatti, G. (1983) *Biochem. Biophys. Res. Commun.* **116**, 492–499
- 17 Tchoupé, J. R., Moreau, T., Gauthier, F. and Bieth, J. G. (1991) *Biochim. Biophys. Acta* **1076**, 149–151
- 18 Dalet-Fumeron, V., Guinec, N. and Pagano, M. (1991) *J. Chromatogr.* **568**, 55–68
- 19 Schägger, H. and von Jagov, G. (1987) *Anal. Biochem.* **166**, 368–379
- 20 Bryan, J. K. (1977) *Anal. Biochem.* **78**, 513–519
- 21 Hellman, U. (1997) in *Protein Structure Analysis. Preparation, Characterization, and Microsequencing* (Kamp, R. M., Choli-Papadopoulou, T. and Wittman-Liebold, B., eds.), pp. 97–104, Springer-Verlag, Heidelberg
- 22 Skriver, K., Wikoff, W. R., Patston, P. A., Tausk, F., Schapira, M., Kaplan, A. P. and Bock, S. C. (1991) *J. Biol. Chem.* **266**, 9216–9221
- 23 Cooperman, B. S., Stavridi, E., Nickbarg, E., Rescorla, E., Schechter, N. M. and Rubin, H. (1993) *J. Biol. Chem.* **268**, 23616–23625
- 24 Hood, D. B., Huntington, J. A. and Gettins, P. G. W. (1994) *Biochemistry* **33**, 8538–8547
- 25 Fish, W. W. and Björk, I. (1979) *Eur. J. Biochem.* **101**, 31–38
- 26 Bock, S. C., Wion, K. L., Vehar, G. A. and Lawn, R. M. (1982) *Nucleic Acids Res.* **10**, 8113–8125
- 27 Schreuder, H. A., De Boer, B., Dijkema, R., Mulders, J., Theunissen, H. J. M., Grootenhuys, P. D. J. and Hol, W. G. J. (1994) *Nature Struct. Biol.* **1**, 48–54
- 28 Carrell, R. W., Stein, P. E., Fermi, G. and Wardell, M. R. (1994) *Structure* **2**, 257–270
- 29 Chang, W. S. W., Wardell, M. R., Lomas, D. A. and Carrell, R. W. (1996) *Biochem. J.* **314**, 647–653
- 30 Brubacher, L. J. and Bender, M. L. (1966) *J. Am. Chem. Soc.* **88**, 5871–5880
- 31 Shore, J. D., Day, D. E., Francis-Chmura, A. M., Verhamme, I., Kvassman, J., Lawrence, D. A. and Ginsburg, D. (1995) *J. Biol. Chem.* **270**, 5395–5398
- 32 Fish, W. W., Orre, K. and Björk, I. (1979) *Eur. J. Biochem.* **101**, 39–44
- 33 Kaslik, G., Patthy, A., Bálint, M. and Gráf, L. (1995) *FEBS Lett.* **370**, 179–183
- 34 Stavridi, E. S., O'Malley, K., Lukacs, C. M., Moore, W. T., Lambris, J. D., Christianson, D. W., Rubin, H. and Cooperman, B. S. (1996) *Biochemistry* **35**, 10608–10615
- 35 Lawrence, D. A., Ginsburg, D., Day, D. E., Berkenpas, M. B., Verhamme, I. M., Kvassman, J. O. and Shore, J. D. (1995) *J. Biol. Chem.* **270**, 25309–25312
- 36 Kadowaki, T., Yoneda, M., Okamoto, K., Maeda, K. and Yamamoto, K. (1994) *J. Biol. Chem.* **269**, 21371–21378
- 37 Schick, C., Pemberton, P. A., Shi, G.-P., Kamachi, Y., Çataltepe, S., Bartuski, A. J., Gornstein, E. R., Brömme, D., Chapman, H. A. and Silverman, G. A. (1998) *Biochemistry* **37**, 5258–5266