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REACTION OF ANTITHROMBIN WITH PROTEASES

EVIDENCE FOR A SPECIFIC REACTION WITH PAPAIN

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Summary

Experiments were performed to determine if the sulfhydryl protease, papain (EC 3.4.22.2), reacts with the plasma protease inhibitor antithrombin (antithrombin III, heparin cofactor) in a specific manner analogous to the reaction of thrombin (EC 3.4.21.5) and other serine proteases with this inhibitor.

The esterolytic activity of papain is blocked by the addition of antithrombin, but not by antithrombin-thrombin complex or by protein substrates such as bovine serum albumin. Likewise, in the presence of papain, antithrombin was unable to displace the active site dye proflavine from thrombin, or to inhibit thrombin-catalysed hydrolysis of an anilide substrate. The reaction of antithrombin and papain was not accelerated by low concentrations of heparin. Approximately stoichiometric amounts of heparin completely inhibited the reaction of papain with antithrombin. The mutual inhibition indicates that plasma antithrombin does react with papain but the reaction differs from the interaction with coagulation factors, particularly in the heparin effect.

Introduction

The question of whether the plasma protease inhibitor antithrombin can react with the sulfhydryl protease papain (EC 3.4.22.2) is of interest for several reasons. First, antithrombin is one of many protein inhibitors the function of

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Abbreviations: Bz-Arg-OEt, benzoyl L-arginine ethyl ester; Cbz-Lys-ONP, *N*-carbobenzyloxy L-lysine *p*-nitrophenyl ester; S-2160, *N*-benzoyl L-phenylalanyl L-valyl L-arginyl *p*-nitroanilide.

which is to regulate proteolytic activity, in this case, the enzymes of the coagulation cascade (review Ref. 1). It has been proposed [2] that its mechanism of reaction is similar to that of inhibitors of the soybean trypsin inhibitor type [3] whereby a covalent bond is formed between the serine hydroxyl of trypsin and the carboxyl group of a specific susceptible peptide bond (reactive center) of the inhibitor. Laskowski and Sealock [3], in reviewing the specificity of protease inhibitors, have suggested that there exist separate classes of inhibitors for sulfhydryl and serine proteases. It is thus of interest to know whether a sulfhydryl enzyme can react with antithrombin.

A second question of interest in antithrombin chemistry is the biological specificity of the reaction, in particular the nature of the target for heparin, a glycosaminoglycan which is a powerful positive effector of the antithrombin-protease reaction. It is generally believed that antithrombin is the target for heparin and that the heparin-antithrombin complex reacts rapidly with proteases [1]. It has however, been claimed by some workers that the enzymes are the target for heparin [4,5]. It is, in fact, known that heparin binds to thrombin [4—6] but there is evidence that suggests that this binding of heparin to thrombin is actually inhibitory to the thrombin-antithrombin reaction [7—9]. It is thus important to understand the reaction of antithrombin with proteases which are not closely related, from a structural or evolutionary standpoint, to the coagulation enzymes.

Finally, it has been reported that there is substantial anti-papain activity in plasma [10], and recently Sasaki et al. [11] and Ryley [12] have isolated a thiol protease inhibitor that is immunologically and electrophoretically different from antithrombin. The physiologic importance of plasma antipapain activity is presumably in protection against infection and endogenous tissue proteases, or in control of thiol proteases involved in inflammation. The possibility that antithrombin is also capable of reaction with thiol enzymes and that this activity might be in competition with control of coagulation may be of clinical importance.

In this report we show that antithrombin, in fact, inactivates papain; concomitantly, the antithrombin activity against thrombin is lost. This constitutes evidence that a specific interaction takes place between these proteins.

Materials and Methods

Materials

Papain was obtained from Worthington (Freehold, NJ) as a twice recrystallized suspension. It was activated by a modification of the procedure of Kimmel and Smith [13] at the desired pH in the presence of 5 mM dithiothreitol/2 mM EDTA at 25°C for 45 min or in the presence of 0.5 mM dithiothreitol/2 mM EDTA at 30°C for 30 min. The concentration of active papain was determined by the method of Bender et al. [14] using benzoyl arginine ethyl ester (Bz-Arg-OEt). Routinely, a rate assay using Cbz-Lys-ONP (see below) was used with Bz-Arg-OEt assay as a primary standard.

Antithrombin preparation was previously described [15,16]. Human α-thrombin was a gift from Dr. John W. Fenton, II of the New York State Department of Health, Albany, NY. Its preparation and properties are described in the literature [16,17].

High affinity heparin was a generous gift from Dr. Erik Holmer of A.B. Kobi, Stockholm, Sweden. The approx. activity was 350 U/mg as compared to commercial standards. An approx. M_r of 11 000 was used for estimation of the range of molar concentration used.

All other reagents were commercial products of the highest available purity. Particular reagents were obtained as follows: bovine serum albumin, *N*-carbobenzyloxy L-lysine *p*-nitrophenyl ester and benzoyl arginine ethyl ester from Sigma (St. Louis); *N*-benzoyl L-phenylalanyl L-valyl L-arginyl *p*-nitroanilide (S-2160) from Ortho (Raritan, NJ).

Methods

The use of proflavine for measuring thrombin active sites has been described previously [6—8]. The papain-antithrombin reaction was carried out by mixing the proteins and sampling for remaining antithrombin or enzyme activity. A description of a typical series of experiments is as follows. Stock papain (40—60 μM) was activated with 0.5 mM dithiothreitol/2 mM EDTA in 0.05 M Tris-HCl/10 μM EDTA pH 7.4. Stock antithrombin (0.2—0.4 mM) was stored in 0.05 M sodium barbital/0.10 M NaCl, pH 8.3. Papain and antithrombin were allowed to react in a 1—10 μM concentration range at papain/antithrombin molar ratios from 1 : 5 to 10 : 1 for 45—120 min at 25° C. Residual papain activity was monitored at convenient time points by sampling small aliquots of the reaction mixture and measuring the hydrolysis of 30 μM carbobenzyloxy-L-lysine *p*-nitrophenyl ester in 0.05 M acetate buffer pH 5.2 containing 10 μM EDTA at 25° C. The reaction was monitored at 340 nm in a Beckman Acta III Spectrophotometer. Residual antithrombin activity was determined by measuring the amount of thrombin inactivated. Thrombin activity was determined by proflavine, clotting times or S-2160 hydrolysis as described previously [6—8,16].

Standard curves for papain activity were prepared from a rate assay using Cbz-Lys-ONP hydrolysis; the rate of nitrophenol appearance was plotted against papain concentration as determined for stock solutions using Bz-Arg-OEt [14]. This gave linear plots in the 1—10 nm range with an estimated 5% error.

Results

Antithrombin inhibits papain

The esterase activity of papain is lost when antithrombin is added to the enzyme. Fig. 1 shows the time course of the decrease in activity for two different ratios of antithrombin/papain. Equilibrium is reached after about 30 min; this is the approximate time for the reaction of antithrombin with thrombin [1,2,7,8]. No inactivation of papain was observed if preformed thrombin-antithrombin complex was added instead of antithrombin alone. It can be seen in Fig. 1 that the equilibrium point does not correspond to complete inactivation. It was found, in fact, that the extent of inactivation was dependent on the concentrations of papain and the concentration of antithrombin. Titration of a fixed concentration of papain with antithrombin is shown in Fig. 2. Antithrombin is known to form reversible complexes with proteases [18—21] and

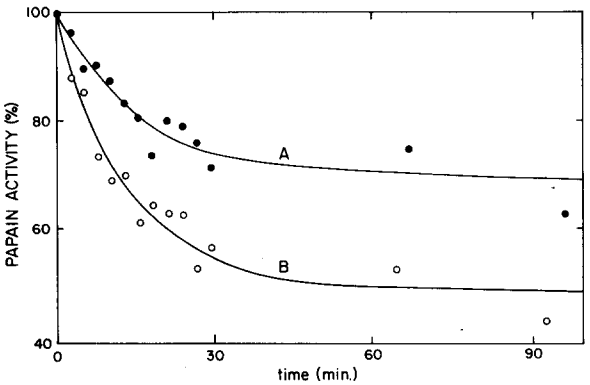

Fig. 1. Loss of papain activity in the presence of antithrombin. Reactions were carried out in 0.05 M Tris-HCl/10 μM EDTA, pH 7.4, at 25° C. Papain concentration was 2 μM. Antithrombin was added and, at indicated times, aliquots were removed and assayed for papain activity against Cbz-Lys-ONP as described in Methods. Antithrombin concentrations were A (●———●): 1 μM, B (○———○): 2 μM.

if a simple equilibrium were assumed for this reaction a dissociation constant of 0.8 μM can be calculated (inset to Fig. 2).

In order to test the possibility that proteolyzed fragments of antithrombin might be inhibitors of papain, antithrombin preparations were incubated with low concentrations of papain (less than 5%) for 2 h. This was found to be without effect on the subsequent ability of the antithrombin to inactivate stoichiometric concentrations of papain. Likewise, incubation of papain with bovine serum albumin did not produce any products capable of inhibiting the effect of antithrombin. The control incubation of papain alone showed only about 10% loss in activity due presumably to autolysis and oxidation of the sulfhydryl group of the active site.

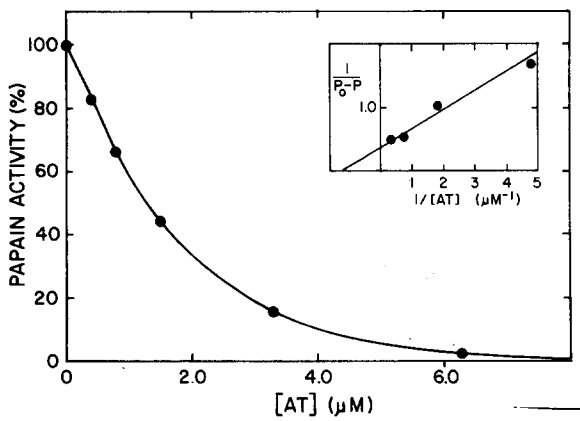

Fig. 2. Dependence of the extent of inhibition of papain on the concentration of added antithrombin. A fixed concentration (2 μM) of papain was titrated with antithrombin. Final enzyme concentrations (no further loss in activity) were determined, at 30—120 min depending on the particular concentration. Conditions were as described in the legend to Fig. 1. Points are means of 2—12 repetitions. Inset: double-reciprocal plot of remaining papain vs. antithrombin concentration.

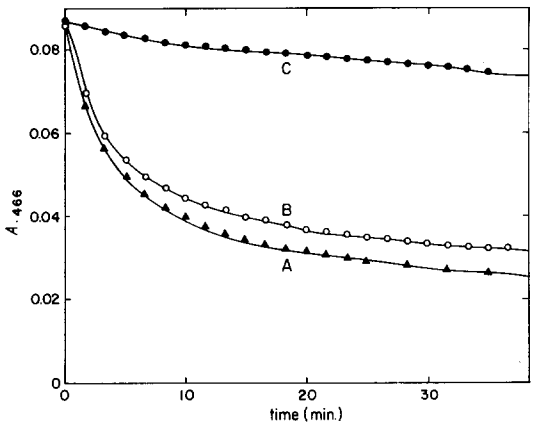

Fig. 3. Displacement of proflavine from thrombin by antithrombin in the presence and absence of papain. The reactions were carried out at 25°C in 0.05 M Tris-HCl, pH 7.4 and release of dye from thrombin was followed at 466 nm. [Proflavine] = 50 μM; [thrombin] = 2.8 μM. A (▲———▲): no additions. B (○———○): 3 μM papain which had not been activated. C (●———●): 3 μM papain activated with dithiothreitol and EDTA.

Papain inactivates antithrombin

To demonstrate that treatment of antithrombin with papain results in loss of the specific reactive site of the inhibitor, the ability of remaining papain-treated antithrombin to inhibit thrombin was measured. The reactions of thrombin can be observed spectrophotometrically using an active site dye indicator proflavine [6—8]. Fig. 3A shows that when antithrombin is added to a thrombin solution containing proflavine there is a decrease in absorbance at 466 nm. This spectral change has been shown to reflect loss in thrombin active sites, or, in this case, molar antithrombin activity [7,8,16]. When the antithrombin sample was preincubated with papain this loss of dye was inhibited (Fig. 3, curve C). If the antithrombin was incubated with a papain sample that had not been activated by the addition of dithiothreitol or EDTA, which are necessary for the generation of enzymatic activity, no inhibition of the thrombin reaction is seen (curve B of Fig. 3). Thus, an active sulfhydryl is required for papain inactivation of the ability of antithrombin to react with thrombin. Similar results were found for the inhibition of thrombin-catalysed hydrolysis of the synthetic substrate, S-2160.

The time course of disappearance of antithrombin is approximately the same as the loss of papain in the same reaction mixture; a half-time of about 15 min was found for equimolar concentrations of antithrombin and papain at 2.3 μM. The equilibrium extent of antithrombin inactivation depends on both antithrombin and papain concentration although somewhat greater loss of the inhibitor is observed. At concentrations of reactants which give 75% loss of papain, antithrombin activity is completely lost. This suggests that some non-specific proteolysis takes place. To test whether this was the major effect, we incubated antithrombin with catalytic amounts of papain as described above. This treatment did not destroy the ability of antithrombin to inactivate thrombin, or, as noted above, its ability to inactivate stoichiometric concentra-

tions of papain. An alternative explanation for the greater loss of antithrombin activity is that a modified inhibitor is formed in which the susceptible bond has been hydrolyzed (see Discussion).

Effect of heparin on the papain-antithrombin reaction

We investigated the effect of heparin on the inactivation of papain by antithrombin. At concentration ratios as high as 1 : 10 of heparin/antithrombin there was no effect on the reaction rate by addition of heparin. This ratio would greatly accelerate the thrombin reaction, for example. However, at high concentrations of heparin, in the equimolar range, the antithrombin-papain reaction was completely inhibited. Such inhibition is known in the thrombin system where it is due to the thrombin-heparin association but is usually masked by the much greater acceleration due to antithrombin-heparin [7—9]. An alternative explanation is that the antithrombin protein isomerization induced by heparin which caused increased rate of reaction with thrombin has the effect of causing reduced reaction with papain. It should be said that activation by heparin is not absolutely excluded because the sulfhydryl reagents which are required for papain activity are known to destroy the heparin-sensitivity of antithrombin (although unaccelerated anti-protease activity is retained) [22]. That reaction, however is much slower than any significantly accelerated antithrombin-papain reaction would be and therefore it is unlikely that any rate-enhancing activity was missed.

Discussion

When antithrombin is added to a papain solution there is a slow loss of hydrolytic activity towards lysine esters. Concomitantly, the antithrombin activity is lost. This observation indicates reaction of the active site of the enzyme with the reactive site of the inhibitor. In studying reactions of proteolytic enzymes, however, it is important to consider whether nonspecific proteolysis might account for the results. Autolysis of papain does not seem to contribute to loss of its activity since incubation of papain alone leads to only 10% loss in activity in a time period greater than the reaction time with antithrombin. Also, incubation of catalytic amounts of papain with antithrombin for 1 h does not destroy the ability of antithrombin to inactivate stoichiometric amounts of enzyme. It is relevant that Fisher et al. [10] found that the antipapain activity of plasma was, likewise, not destroyed by incubation with papain. Also, the observations that (1) papain-antithrombin mixtures do not inhibit fresh added papain or thrombin, and (2) incubating papain with bovine serum albumin does not cause inhibition of activity exclude the possibility that some nonspecific proteolysis product might inhibit the enzyme. Thus, generalized proteolysis is not a major factor in this system. Moreover, the fact that both proteins are inactivated in an approximately parallel manner points to a specific association. It should be further mentioned that the time for reaction is in the range found for antithrombin inhibition of other enzymes [1,7,16]. On the other hand, at least some proteolysis of antithrombin would be expected to have taken place. The fact that there is a generally greater loss in antithrombin activity as compared to enzyme inactivation might suggest

some nonspecific proteolysis of the inhibitor. An alternative explanation, however, is that a modified inhibitor is formed. Such modified inhibitors, formed by specific reactive site cleavage, are well known in the trypsin inhibitors of the soybean type; modified antithrombins have also been observed in the thrombin reaction [23]. The formation of such a derivative might, in fact, be especially favored with the papain system since if a covalent bond were formed between enzyme and inhibitor, it would be of the thiol ester type, which is less stable than the oxygen ester demonstrated with thrombin [24]. If this explanation holds then the proposed modified inhibitor would not be able to react with papain.

The fact that plasma antithrombin is capable of reaction with papain means that this inhibitor exhibits relatively low specificity with regard to complex formation, per se. Biological specificity resides, rather, in the ability to react more rapidly in the heparin-activated form, which was not observed with papain. The physiological significance of an antipapain activity is not apparent. The fact that the reaction is not enhanced by heparin strongly suggests that it has not evolved as a biologic thiol protease antagonist. The possible competition between antithrombin and the plasma inhibitors of sulfhydryl enzymes [11,12] may be of importance in interactions of inflammation and coagulation. Relative rate and equilibrium data will be necessary before it is possible to assess the relationship of the two anti-thiol proteases.

The work presented here also questions the generality of Laskowski's suggestion [3] that there may be separate classes of inhibitors for serine and sulfhydryl enzymes. On the other hand, the structure of antithrombin [25] shows few homologies with known trypsin inhibitors with the exception of plasma α_1-antitrypsin. Finally, it is interesting that papain is capable of stimulating platelets [26,27] although higher concentrations (compared to thrombin or trypsin) are needed to obtain maximal stimulation. It has been proposed [26, 27] that the platelet thrombin receptor, because it resembles both an agonist receptor and a substrate, is analogous to a protease inhibitor of the soybean or antithrombin type.

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