High-molecular-weight kininogen binds two molecules of cysteine proteinases with different rate constants

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Abstract Fluorescence titrations showed that high-molecular-weight kininogen binds two molecules of papain, cruzipain and cathepsin S with high affinity. The 2:1 binding stoichiometry was confirmed by stopped-flow kinetic measurements of papain binding, which also revealed that the two sites bind the enzyme with different association rate constants ($k_{ass,1} = 23.0 \times 10^6$ M^{-1} s^{-1} and $k_{ass,2} = 3.4 \times 10^6$ M^{-1} s^{-1}). As for low-molecular-weight kininogen, comparison of these kinetic constants with previous data for intact low- and high-molecular-weight kininogen and the separated domains indicated that the faster-binding site is also the tighter-binding site and is that of domain 3, whereas the slower-binding, lower-affinity site is on domain 2. The results further demonstrate that there is no appreciable steric interference between the two domains or by the kininogen light chain in the binding of proteinases. Similarly, the binding of kininogen via its light chain to a surface, as indicated by the binding to the model surface, heparin, did not affect the inhibitory properties of kininogen. The M_r of high-molecular-weight kininogen was determined to be 83 500 by sedimentation equilibrium measurements, in agreement with the value calculated from amino acid sequence and carbohydrate analysis.

Key words: Kininogen; Cathepsin; Cystatin; Cysteine proteinase; Kinetics

1. Introduction

High-molecular-weight kininogen (HK) and low-molecular-weight kininogen (LK) are large multifunctional glycoproteins in mammalian plasma [1,2]. In addition, a third form of kininogens, T-kininogen (TK), has been identified in the rat and was found to be the major accute-phase protein in this species [3,4]. Kininogens have long been known as precursors of vasoactive peptides, the kinins. In addition, HK plays an important role in the contact phase of blood coagulation [1,2], acts as an antagonist of thrombin-induced platelet aggregation [5], and has been shown to bind to neutrophils [6].

Both human HK and LK are products of the same gene as a result of alternative mRNA splicing [7,8]. The originally single-chain proteins are easily converted to two-chain forms, consisting of a heavy and a light chain connected by a single disulfide bond, by limited proteolysis by kallikreins, thereby releasing the kinin segment [2]. The heavy chains of HK and LK are identical, whereas the light chain of HK is substantially larger than that of LK [8].

About a decade ago, kininogens were shown to be potent inhibitors of papain-like cysteine proteinases [9,10]. Structural analyses demonstrated that the kininogen heavy chain consists of three domains, designated D1–D3, which share a high internal homology with low-molecular weight cystatins [2,11]. The kininogens therefore were classified as family 3 of the cystatin superfamily [12]. Isolated domains D2 and D3 were shown to inhibit papain-like cysteine proteinases (D2 and D3) and calpain (D2), whereas domain D1 was found to lack inhibitory activity [11,13].

We have recently shown that intact human LK simultaneously can bind two molecules of cysteine proteinases with high affinity [14]. In this work we demonstrate that human HK can also bind two molecules of cathepsin S, cruzipain and papain independently. Moreover, kinetic studies of papain binding showed that the two sites bound the proteinase with different rate constants.

2. Materials and methods

2.1. Materials

Z-Phe-Arg-AMC was purchased from Serva (Heidelberg, Germany). Stock solutions of the substrate were prepared in dimethylsulfoxide (Merck, Germany). Q Sepharose-Fast Flow and Superdex 200 HR 10/30 columns were obtained from Pharmacia Biotech (Uppsala, Sweden). Chicken cystatin, bovine cathepsin S and cruzipain were purified as described previously [15–17]. Heparin, a gift from Dr. Steven T. Olson, University of Illinois at Chicago, had an average M_r of ~8000 and was similar to material used in a previous study [18]. Papain (2×crystallized; Sigma, St. Louis, USA) was further purified by affinity chromatography [19]; the purified enzyme had a thiol content of 0.96 ± 0.03 mol/mol protein. Papain was inactivated with methylmethanethiol sulfonate as described elsewhere [20]. The concentration of HK was determined from a molar absorbance coefficient of 63 500, calculated from the amino acid sequence [8] according to the method of Pace et al. [21]. Concentrations of proteinases and of chicken cystatin were determined as described previously [14].

2.2. Experimental conditions

Unless otherwise stated, all experiments were performed at 25°C in 100 mM phosphate, 1.5 mM EDTA, pH 6.0.

2.3. Purification of HK from human plasma

HK and LK were purified from human plasma by a modification of a previous procedure [22]. Plasma (500 ml) was exposed to pH 11.0 for 30 min, followed by acidification to pH 6.5 with acetic acid. A precipitate was removed by centrifugation at 10 000×*g* for 15 min. The supernatant was applied to a carboxymethyl-papain-Sepharose

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Abbreviations: -AMC, aminomethyl coumaryl amide; HK, high-molecular-weight kininogen; LK, low-molecular-weight kininogen; Z-, benzoyloxycarbonyl

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4B column (200 ml [23]), equilibrated with 50 mM NH_4OAc buffer, pH 6.5, containing 0.5 M NaCl. Unbound proteins were washed out with the same buffer, and the bound material was eluted with 10 mM NaOH. The inhibitory fractions were immediately neutralized with 1 M NH_4OAc buffer, pH 5.5, and were then concentrated and dialyzed against 20 mM Bis-Tris buffer, pH 6.5. The material was applied to a Q-Sepharose Fast Flow column (40×2.0 cm), equilibrated with the same buffer, and the column was washed extensively. Bound proteins were eluted with a 0–0.5 M NaCl gradient (total volume 800 ml) in the same buffer, with a noninhibitory peak eluting at 0.1 M NaCl and LK and HK eluting at 0.25 and 0.32 M NaCl, respectively. The inhibitory fractions were pooled, concentrated and dialyzed against 0.1 M acetate buffer, pH 5.5, containing 0.3 M NaCl. Minor impurities in the HK and LK preparations were removed by further purification on a Superdex 200 HR 10/30 column, equilibrated in the same buffer. Final yields of 30 mg of LK and 15 mg of HK were obtained from 500 ml of plasma.

2.4. Fluorescence titrations

Fluorescence titrations of proteinases with chicken cystatin or HK were performed in an SLM 4800S spectrofluorimeter (SLM-Aminco, Urbana, IL, USA) as described previously [14]. The binding curves were analysed by non-linear least-squares computer fitting to the equilibrium binding equation [24].

2.5. Kinetic analyses

The kinetics of inhibition of papain by HK were analysed in an SX-17MV stopped-flow apparatus (Applied Photophysics, Leatherhead, UK). The reactions were monitored either by the decrease in tryptophan fluorescence emission accompanying the interaction or by the formation of fluorescent product released by the proteinase in experiments carried out in the presence of substrate, essentially as described previously [14]. The association rate constant determinined from experiments in the presence of substrate was corrected for substrate competition with the use of a K_m value of 65 μM [25].

2.6. Analytical ultracentrifugation

Sedimentation equilibrium measurements were conducted at 20°C in an MSE Centriscan analytical ultracentrifuge (MSE, Crawley, UK) and were monitored by photoelectric scanning [26]. The concentration of HK was 0.6 mg/ml. A partial specific volume of 0.695 ml/g was calculated for HK from the amino acid sequence and carbohydrate composition [8,27].

3. Results

3.1. Binding stoichiometry by fluorescence titrations

Titrations of fully active papain with chicken cystatin showed the inhibitor to be essentially 100% active in enzyme binding (inhibitor to enzyme binding stoichiometry 1.1). Cathepsin S and cruzipain were shown by similar titrations with chicken cystatin to be 80 and 70% active, respectively. The proteinases were then titrated with HK, demonstrating that 0.50–0.56 mol of HK were needed to saturate 1 mol of proteinase (Fig. 1). Computer fitting of the titration curves gave apparent proteinase to HK binding stoichiometries of 2.0, 1.9 and 1.8 for papain, cathepsin S and cruzipain, respectively. The value for papain was shown to be independent of proteinase concentration in the range 0.1–1 μM. The results thus indicate two binding sites for proteinases on each kininogen molecule.

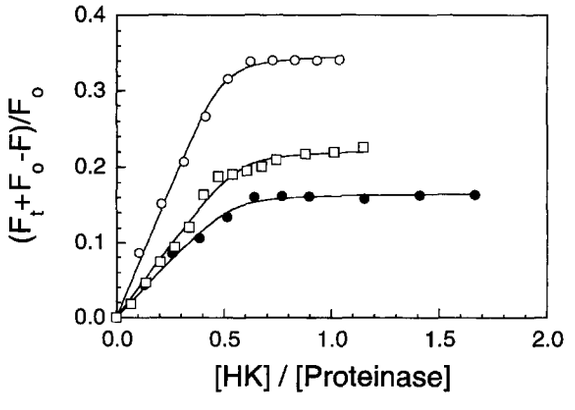

Fig. 1. Fluorescence titrations of papain (○), cathepsin S (●) and cruzipain (□) with HK. The proteinase concentrations were 500 nM. Other experimental conditions were as described in Section 2. F_t, fluorescence of added HK; F_o, fluorescence of proteinase; F, fluorescence of HK-proteinase mixture. Fitted curves were generated by nonlinear regression analysis [24].

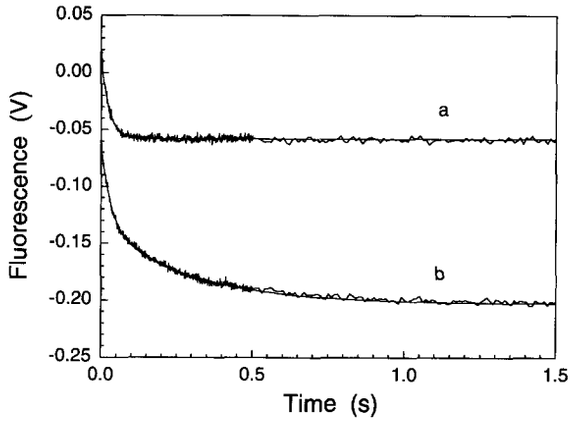

Fig. 2. Kinetics of binding of *S*-(methylthio)papain to HK. The decrease in tryptophan fluorescence accompanying the interaction was monitored as described in Section 2. (a) Single-exponential progress curve for the reaction between 1 μM HK and 50 nM *S*-(methylthio)papain. (b) Double-exponential progress curve for the reaction between 1 μM *S*-(methylthio)papain and 50 nM HK. Both curves are shown with the best fit generated by nonlinear regression analysis.

3.2. Kinetics of binding

The kinetics of binding of HK to papain were studied by two different methods. In one method, the reactions were monitored by the decrease in tryptophan fluorescence accompanying the binding. The experiments were performed under pseudo-first-order conditions with a 20-fold molar ratio of proteinase to HK, enabling detection of proteinase binding to both inhibitory sites. Papain was inactivated with methylmethanethiol sulfonate [20] before the analyses to prevent cleavage of HK by excess proteinase and to increase the fluorescence change [28]. This modification has been shown only minimally to affect the association rate constants for various cystatin-cysteine proteinase interactions [14,28]. The kinetics of papain binding to HK showed two phases and were best fitted to a double-exponential function (Fig. 2). The dependence of the observed pseudo-first-order rate constants for the two phases on the papain concentration was linear for both phases up to the highest concentration used (16 μM; Fig. 3a). Second-order association rate constants of $23.0 \pm 0.9 \times 10^6$ M^{-1} s^{-1} and $3.4 \pm 0.1 \times 10^6$ M^{-1} s^{-1} were calculated for the two phases from the slopes of these lines. The amplitude ratio of the two phases was 0.90 ± 0.07.

Since HK is known to bind to surfaces via its light chain (reviewed in [1]), we investigated the effect of such binding on the inhibitory properties of the protein, using heparin as a

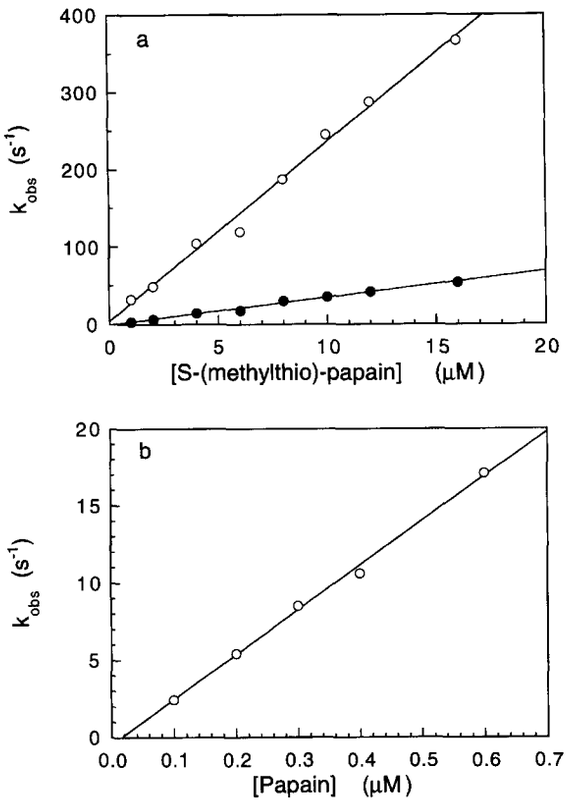

Fig. 3. (a) Dependence of the rate constants of the slow (●) and fast (○) phases on *S*-(methylthio)papain binding to HK (see Fig. 2) on proteinase concentration. (b) Dependence of the rate constant of HK binding to active papain on proteinase concentration in experiments monitored in the presence of substrate, as described in Section 2. Solid lines in (a,b) were generated by linear regression analysis.

model surface [29,30]. Heparin (final concentration 2 μM) was incubated with HK (final concentration 100 nM) for 20 min and the kinetics of papain binding were then analysed as described above. Identical kinetic behaviour was observed in the absence and presence of heparin.

In a further experiment, also employing analysis by tryptophan fluorescence, a 20-fold molar ratio of HK to *S*-(methylthio)papain was used instead. In this case, monophasic kinetics were observed (Fig. 2), in contrast with the biphasic behaviour evident at an excess of *S*-(methylthio)papain. However, both the observed pseudo-first-order rate constant (37.9 s^{-1}) and the amplitude (0.073 V) obtained in this manner at 1 μM HK were in good agreement with the corresponding values for the faster process determined at 1 μM *S*-(methylthio)papain with an excess of the latter (32.2 s^{-1}, 0.075 V). These results strongly suggest that at an excess of HK the proteinase binds predominantly to the faster-binding site, whereas the slower-binding site remains largely unoccupied.

In the second kinetic method, a fluorescent substrate was used to monitor papain activity. The experiments were carried out under pseudo-first-order conditions with a 10-fold molar ratio of HK to papain, which precluded determination of the association rate constant for the slower-binding site (see above). All progress curves had a final slope of zero, showing that the reactions were essentially irreversible at the inhibitor concentrations used, and were thus fitted to a simple exponential function [31]. The plot of the observed pseudo-first-order rate constant vs. HK concentration was linear and gave an association rate constant of $33.3 \pm 1.1 \times 10^6$ M^{-1} s^{-1} after correction for substrate competition. This value is similar to that determined for the binding of *S*-(methylthio)papain to the faster binding site of HK, in agreement with results from analogous studies of the LK/papain interaction [14]. The value also agrees well with values of $14–30 \times 10^6$ M^{-1} s^{-1} determined previously for the HK/papain interaction [32].

3.3. Molecular weight determination by ultracentrifugation

An M_r of $83\,500 \pm 4000$ was determined for HK by sedimentation equilibrium measurements. This value is in reasonable agreement with that of 91 000 calculated from the amino acid sequence [8] and carbohydrate analysis [27], but lower than the value of 110 000 estimated by SDS-PAGE (reviewed in [1,2]). This difference indicates that HK behaves anomalously in SDS-PAGE, probably due to the glycosylation of the molecule.

4. Discussion

We recently showed that intact LK simultaneously can bind two molecules of papain-like cysteine proteinases with high affinity [14]. As the light chain of HK is considerably larger than that of LK [8], it might be expected to interfere with proteinase binding to the HK heavy chain. However, we demonstrate here that human HK can also simultaneously bind two molecules of papain-like cysteine proteinases. This conclusion is apparent from fluorescence titrations of enzymes with carefully standardized concentrations and from stopped-flow kinetic analyses of the papain-HK interaction.

It has been shown previously that the free kininogen domains, isolated by proteinase digestion or expressed in bacteria, differ both in the kinetics and affinities of binding of target proteinases. Free domain 3 thus binds the enzymes 1–3 orders of magnitude faster and tighter than domain 2, depending on the proteinase [11,13,33,34]. Our previous work showed that the rate constants for proteinase binding to the individual domains of intact LK differ significantly, with the values ($0.8–1.4 \times 10^6$ M^{-1} s^{-1} for domain 2 and $11–24 \times 10^6$ M^{-1} s^{-1} for domain 3 [14]) being close to those determined for isolated domains 2 and 3. It was therefore concluded that the faster-binding site in intact LK is the site located in domain 3, whereas the slower-binding site is that of domain 2 [14]. The comparable kinetics of papain binding to HK as to LK shown here leads to the same assignment for the two proteinase-binding domains of HK. However, the rate constant for the slower reaction was ~2.5-fold higher for HK (3.4×10^6 M^{-1} s^{-1}) than for LK, indicating a small, possibly conformational, effect of the HK light chain on the binding of proteinases to domain 2. Nevertheless, the results show that there is no major steric hindrance for proteinase binding to individual domains of HK or LK, either by the adjacent domains or by the light chain. Moreover, the binding of the light chain of HK to a surface, as exemplified by binding to heparin in this work, similarly does not appear to affect the inhibitory properties of the individual domains of HK.

The kininogens are the major physiological plasma inhibitors of cysteine proteinases, due to their high concentration in plasma [35] and high affinity for such proteinases. Under nor-

mal conditions, target enzymes are inactivated mainly by binding to domain 3 of both HK, as shown here, and LK [14]. Under certain circumstances domain 2 may serve to enhance inhibitory capacity of both kininogens and also functions by binding calpain [11]. However, the possible functional significance of the tandem arrangement of inhibitory domains in the kininogen heavy chain remains unclear.

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