Inactivation of thrombin by a complex between rat mast-cell protease 1 and heparin proteoglycan

Gunnar PEJLER,* Kajsa SÖDERSTRÖM* and Anders KARLSTRÖM†

*Department of Veterinary Medical Chemistry, Biomedical Center, P.O. Box 575, S-751 23 Uppsala, Sweden

and †Pharmacia, BioScience Center, Department of Biochemistry, S-112 87 Stockholm, Sweden

Rat peritoneal mast cells were shown to inactivate thrombin rapidly. The thrombin-inactivating activity was purified to homogeneity by a combination of anion-exchange chromatography and h.p.l.c. on a Superdex 75 column. The purified thrombin inactivator had an apparent molecular mass of 29 kDa and an N-terminal amino acid sequence identical to rat mast-cell protease 1 (RMCP-1). After labelling of the mast cells *in vivo* with $^{35}\text{SO}_4^{2-}$, RMCP-1 was recovered in a macromolecular complex with ^{35}S heparin proteoglycans. Dissociation of RMCP-1 from the heparin proteoglycans by Superdex 75 chromatography in the presence of 2 M NaCl resulted in a marked loss of the thrombin-

inactivating activity displayed by the enzyme. When RMCP-1 was reconstituted with either endogenous ^{35}S heparin proteoglycans or standard pig mucosal heparin, the enzyme regained its thrombin-inactivating properties. Affinity chromatography of endogenous ^{35}S heparin on matrix-linked RMCP-1 demonstrated that all of the heparin molecules contained high-affinity binding sites for the mast-cell protease. In contrast, the endogenous mast-cell heparin showed low affinity for antithrombin, a protease inhibitor involved in the regulation of coagulation enzymes.

INTRODUCTION

Mast cells are well known for their importance in host defence mechanisms. In particular, mast cells have been shown to play a central role in immediate hypersensitivity. Moreover, several reports suggest that mast cells participate in various inflammatory conditions such as rheumatoid arthritis (Bromley et al., 1984), scleroderma (Hawkins et al., 1985), wound repair (Wichmann, 1955) and thioglycollate-induced inflammation (Qureshi and Jakschik, 1988). After activation by different mechanisms, such as cross-linking of IgE on the surface of the mast cells or exposure to anaphylatoxins C5a or C3a, the mast cells release their granular contents to the extracellular milieu. The mast-cell granules contain various mediators including histamine, proteoglycans, chemotactic factors, serine proteases and carboxypeptidases (Stevens and Austen, 1989). The exact composition of the granules varies in different animal species. Furthermore, different subpopulations of mast cells within the same species contain different mediators. For instance, the major serine protease expressed by rat mast cells isolated from the gastrointestinal mucosa (mucosal mast cells) is a chymotrypsin-like enzyme termed rat mast-cell protease 2 (RMCP-2) (Benfey et al., 1987), whereas the dominating serine protease synthesized by rat connective-tissue mast cells is a different chymotrypsin-like protease: rat mast-cell protease 1 (RMCP-1) (LeTrong et al., 1987). Moreover, mucosal mast cells synthesize predominantly chondroitin sulphate proteoglycans (Enerbäck et al., 1985), whereas heparin proteoglycan is the major proteoglycan species expressed by the connective-tissue mast cells (Yurt et al., 1977).

The mast-cell secretory granule proteases constitute a large portion of the total protein content of the cells, suggesting an important role for these enzymes in the inflammatory response. However, despite the identification of several substrates for the mast-cell proteases (Reilly et al., 1982, 1985; Schwartz et al., 1983, 1985; DuBuske et al., 1984; Kokkonen et al., 1986; Mizutani et al., 1991), the biological function of these enzymes

remains to be determined. Inflammatory reactions are often characterized by extravascular fibrin deposition due to activation of the coagulation cascade by macrophages (Geczy, 1983), and recent results indicate that mouse mast-cell proteases may participate in the down-regulation of the procoagulant activity expressed by macrophages: during our studies of macrophage prothrombinase activity (Lindahl et al., 1989), expressed in cultures of mouse adherent peritoneal cells, we observed that thrombin, generated by the prothrombinase, was inactivated in the cell cultures (Pejler and Seljelid, 1992). Subsequent purification of the thrombin-inactivating activity showed that the activity was associated with secretory granule chymase expressed by mast cells present in the cell cultures (Pejler and Karlström, 1993). In the present report the effect of rat peritoneal mast cells on thrombin was investigated. The mast cells were shown to rapidly inactivate thrombin. Purification of the thrombin-inactivating activity showed that the activity was associated with RMCP-1, complex-bound to heparin proteoglycan.

MATERIALS AND METHODS

Reagents

The chromogenic peptide substrates S-2238 and S-2586 were from Pharmacia (Stockholm, Sweden). *N*-Ethylmaleimide and pepstatin were from Sigma and EDTA was obtained from Merck. Quantigold reagent was obtained from Diversified Biotech (Newton Centre, MA, U.S.A.). DEAE-Sephacel, activated CH-Sepharose 4B, Sepharose CL-2B and the Superdex 75 and Superose 6 columns were from Pharmacia. $^{35}\text{SO}_4^{2-}$ (specific radioactivity 1200–1400 Ci/mmol) was purchased from NEN and Polybrene was obtained from Aldrich (Beerse, Belgium). Bovine antithrombin, α -thrombin, chymotrypsin and trypsin were kindly donated by Dr. I. Björk (Dept. of Veterinary Medical Chemistry, Biomedicum, Uppsala, Sweden), and Factor Xa was a gift from Dr. J. Stenflo (Dept. of Clinical Chemistry, Malmö General Hospital, Malmö, Sweden). Antithrombin-

Sephacel, standard pig mucosal heparin and [^3H]NAC-labelled pig mucosal heparin were generous gifts from Dr. Ulf Lindahl, Dept. of Medical and Physiological Chemistry, Uppsala, Sweden. Purified RMCP-1 (approx. 2 μg) was coupled to activated CH-Sepharose 4B (in the presence of 10 μg of *N*-acetylated pig mucosal heparin) according to the procedure described by the manufacturer. Metrizamide was from Nycomed AS (Oslo, Norway).

Animals and cells

Peritoneal cells from Sprague-Dawley rats (females, 10–15 weeks old) were collected by peritoneal washing with 0.025 M Tris/HCl, pH 7.6, containing 0.12 M NaCl and 0.01 M EDTA. Mast cells (connective-tissue type) of approx. 90% purity, as judged by staining with Toluidine Blue, were prepared by density-gradient centrifugation on metrizamide as described by Sterk and Ishizaka (1982). Cells were seeded in 16-mm-diam. wells in Costar plastic dishes (Costar, Broadway, Cambridge, MA, U.S.A.; 2×10^5 cells/well in 0.4 ml of medium). The mast cells were cultured in Iscove's medium (Flow Laboratories) supplemented with BSA (0.4 mg/ml), human transferrin (1 μg /ml), gentamicin (Flow Laboratories; 100 μg /ml) and amphotericin (Flow Laboratories; 2.5 μg /ml). Incubations were conducted in a humidified atmosphere of 5% CO_2 in air at 37 °C.

Enzymic assays

Assays for thrombin-inactivating activity in cell cultures were performed after addition of purified thrombin (0.5 μg) to the mast cells. Samples (50 μl) from spent media were collected at various time points. Aliquots (15 μl ; duplicates) of the samples were transferred to 96-well microtitre plates and mixed with 200 μl of PBS. After addition of 20 μl of a solution of S-2238 (2.5 mM in H_2O), a chromogenic thrombin substrate, remaining thrombin activity was monitored with a Titertek Multiskan (Flow Laboratories) plate-reader.

For assessment of thrombin-inactivating activity in cellular extracts or in fractions eluted at different chromatographic steps, samples were, if necessary, diluted with 0.1% Triton X-100 to yield a final NaCl concentration of 0.14 M. Aliquots (200 μl) of the diluted samples were transferred to 96-well microtitre plates. To each well was added 20 ng of thrombin. After incubation of the plates at 37 °C for appropriate times, 20 μl of the S-2238 solution (see above) was added to each well and residual thrombin activity was monitored. Thrombin-inactivating activities are expressed as units, where 1 unit is defined as the amount of activity that inactivates 1 μg of thrombin/h.

Chymotrypsin-like activity was measured after dilution of samples with 0.1% Triton X-100 to a final NaCl concentration of 0.15 M. Aliquots (200 μl) of the diluted samples were transferred to 96-well microtitre plates. To each well was added 20 μl of a solution of the chromogenic chymotrypsin substrate S-2586 (4 mM in H_2O). Chymotrypsin-like activity was monitored at 405 nm.

In some experiments, thrombin-inactivating and chymotrypsin-like activities of RMCP-1 were determined in the presence of various concentrations of standard pig mucosal heparin or endogenous mast-cell heparin. In these experiments, the purified enzyme was preincubated with heparin for 15 min before the addition of S-2586 or thrombin.

Purification of thrombin-inactivating activity

Peritoneal mast cells were purified from two rats. Cells were

pelleted by centrifugation at 300 *g* for 10 min, and were subsequently solubilized in 2 ml of 10 mM phosphate buffer, pH 7.5, containing 1% Triton X-100, 2 M NaCl and protease inhibitors (10 mM EDTA, 1 mM *N*-ethylmaleimide, 10 μg /ml pepstatin). The cellular extract was then diluted with 12 vol. of 0.5% Triton X-100 (yielding a final NaCl concentration of 0.15 M) containing protease inhibitors, and was applied to a column (4 ml) of DEAE-Sepharcel equilibrated with PBS/0.1% Triton X-100. The anion-exchange column was eluted at approx. 9 ml/h with a NaCl gradient extending from 0.15 M to 2 M (total volume 150 ml) in PBS/0.1% Triton X-100. Fractions (approx. 3 ml) were collected and analysed for thrombin-inactivating activity, chymotrypsin-like activity or ^{35}S radioactivity. ^{35}S radioactivity was determined by liquid-scintillation counting. Fractions containing thrombin-inactivating activity were pooled and were diluted with 0.1% Triton X-100, containing protease inhibitors to a final NaCl concentration of 0.3 M, before application to a small (0.5 ml) column of DEAE-Sepharcel equilibrated with PBS/0.1% Triton X-100/0.3 M NaCl. After washing with 5 ml of PBS/0.1% Triton X-100/0.5 M NaCl, the column was eluted with 6×0.5 ml of PBS/0.1% Triton X-100/2 M NaCl. Eluted fractions containing thrombin-inactivating activity were pooled and stored in aliquots at -70 °C. Further purification was achieved by h.p.l.c. on a Superdex 75 column (1.3 cm $\times$ 30 cm) equilibrated with PBS/0.1% Triton X-100/2 M NaCl. The column was eluted at 0.5 ml/min with equilibrating buffer and fractions (0.5 ml) were collected and analysed for thrombin-inactivating activity, chymotrypsin-like activity and for ^{35}S radioactivity.

For *in vivo*-labelling of mast cells with $^{35}\text{SO}_4^{2-}$, rats were injected intraperitoneally with carrier-free $^{35}\text{SO}_4^{2-}$ (2 mCi/rat). After 4 h the peritoneal cells were harvested, followed by purification of mast cells and subsequent purification of thrombin-inactivating activity as above.

The N-terminal sequence of the purified protein was determined by adsorptive, biphasic column technology using a HP G1005 A protein sequenator (Hewlett-Packard protein chemistry system, Palo Alto, CA, U.S.A.). SDS/PAGE was performed according to the method of Laemmli (1970). Briefly, samples were loaded on 20-cm-long gradient gels extending from 10 to 18% (w/v) polyacrylamide. Electrophoresis was performed at 12 mA for approx. 18 h. After electrophoresis, gels were silver stained according to Morrissey (1981).

Gel-filtration chromatography was carried out on a column (0.7 cm $\times$ 53 cm) of Sepharose CL-2B, equilibrated and eluted at approx. 1 ml/h with PBS/0.1% Triton X-100 containing 0.15 M or 2 M NaCl. Fractions (approx. 0.3 ml) were collected and were analysed for ^{35}S radioactivity and chymotrypsin-like activity. Affinity chromatography on an antithrombin-Sepharose column was carried out according to Thunberg et al. (1982) using a column (3 ml) eluted with a linear salt gradient. Free glycosaminoglycan chains were prepared by β -elimination of proteoglycans with 0.5 M NaOH for approx. 18 h (4 °C), followed by neutralization with 4 M HCl and dialysis against H_2O . HNO_2 degradation (pH 1.5) of glycosaminoglycans was carried out as previously described (Pejler and David, 1987).

Affinity chromatography on RMCP-1, coupled to activated CH-Sepharose 4B was performed on a column (approx. 1 ml) equilibrated with PBS/0.1% Triton X-100. Samples of endogenous ^{35}S -labelled heparin (approx. 500 c.p.m.) were applied to the column, followed by washing three times with 1 ml of equilibrating buffer. The column was then eluted stepwise with PBS/0.1% Triton X-100 containing 0.3 M, 0.5 M, 1 M, 2 M and 4 M NaCl, respectively (2 ml of each buffer). The eluted fractions were subsequently analysed for ^{35}S radioactivity.

RESULTS

Inactivation of thrombin in cell culture

Incubation of purified peritoneal mast cells with thrombin resulted in rapid inactivation of the enzyme (approx. 15 μg of thrombin inactivated/h per 1×10^6 cells) (Figure 1). Thrombin was not significantly inactivated in control incubations with cell culture medium (Figure 1). The activity showed specificity for thrombin as neither Factor Xa, trypsin nor chymotrypsin, three serine proteases exhibiting structural and functional similarities with thrombin, were inactivated (results not shown). Washing of the cells only marginally reduced the thrombin-inactivating activity expressed by the mast cells, indicating that the majority of the activity was cell-associated (results not shown). However, some of the activity appeared to be released from the cells as conditioned medium from the mast cells contained significant

Figure 1 Thrombin-inactivating activity of peritoneal mast cells

Thrombin (0.5 μg) was added to cultures of mast cells (2×10^6 cells/well; □), to conditioned medium from mast-cell cultures (●), or to mast cells that had been preincubated (5 min) with 10 μg of Polybrene (■). ○, control incubation of thrombin with cell culture medium. Samples (50 μl) from the media were collected at the time points indicated and were analysed for residual thrombin activity.

Figure 2 Anion-exchange chromatography of mast-cell extracts

Extracts from mast cells, purified from rats that had been injected intraperitoneally with ^{35}S (2 mCi/rat), were applied to a column of DEAE-Sephacel. Eluted fractions were analysed for thrombin-inactivating activity (○), chymotrypsin-like activity (●) and for ^{35}S radioactivity (■). ---, NaCl concentration.

levels of thrombin-inactivating activity (approx. 4 μg of thrombin inactivated/h per 1×10^6 cells; medium conditioned for 1 h by the mast cells) (Figure 1). Ultracentrifugation (100 000 g , 1 h) of the thrombin-inactivating activity recovered in the conditioned medium reduced the activity by approx. 80 %, indicating that the thrombin-inactivating activity was released from the mast cells in a particulate form (not shown). Incubation of the mast cells (Figure 1) or the conditioned medium (results not shown) with the polycation Polybrene (10 μg) completely abolished the thrombin-inactivating activity. Addition of ^{125}I -thrombin to the mast-cell cultures, followed by SDS/PAGE and autoradiographic analysis of spent media recovered after various times showed that thrombin was degraded into distinct proteolytic fragments with molecular masses of approx. 27 kDa and 15 kDa respectively (G. Pejler and K. Söderström, unpublished work).

Purification of the thrombin-inactivating activity

Initial experiments showed that the thrombin-inactivating activity could be recovered after treatment of the mast cells with Triton X-100. However, to obtain solubilization of the activity, the buffers had to be supplemented with NaCl at concentrations above 0.5 M (results not shown). For purification of the thrombin-inactivating activity, mast cells were solubilized with PBS supplemented with 1 % Triton X-100 and 2 M NaCl.

The inhibition of the thrombin-inactivating activity by the polycation Polybrene (Figure 1) suggested that the activity exhibited a negative net charge. Therefore, the mast-cell extracts were applied to a DEAE-Sephacel anion-exchange column eluted with a salt gradient. The thrombin-inactivating activity bound strongly to the column and was eluted at a NaCl concentration of approx. 0.8 M (Figure 2). For further purification, the thrombin-inactivating activity was applied to a column of Superdex 75 and eluted with PBS/0.1 % Triton X-100/2 M NaCl. The thrombin-inactivating activity was eluted from the column as one major peak along with a minor, slightly retarded, peak (Figure 3b). To detect thrombin-inactivating activity, 0.1 $\mu\text{g}/\text{ml}$ heparin was added to the fractions which were eluted from the column (see below). Fractions were pooled as indicated in the figure by bars and were analysed by SDS/PAGE. Fractions containing thrombin-inactivating activity demonstrated the presence of a single band of molecular mass 29 kDa. The 29 kDa band was absent in fractions devoid of activity (Figure 3c). After Superdex 75 chromatography the thrombin-inactivating activity had been purified approx. 25-fold from the mast-cell extracts, with a yield of approx. 20 %.

The purified thrombin-inactivating activity was subjected to N-terminal sequence analysis. The obtained sequence, Ile-Ile-Gly-Gly-Val-Glu-Ser-Arg-Pro-His-Ser-Arg-Pro-Tyr-Met-Ala-His-Leu-Glu-Ile-, demonstrated 100 % identity with RMCP-1 (LeTrong et al., 1987). Incubation of the purified thrombin-inactivating activity with [^3H]di-isopropyl fluorophosphate, followed by SDS/PAGE and autoradiography showed incorporation of radioactivity into the 29 kDa band (results not shown). Incubation of the purified protease with thrombin resulted in degradation of thrombin into proteolytic fragments with molecular masses of 27 kDa and 15 kDa respectively (G. Pejler, A. Karlström and L. Berg, unpublished work).

RMCP-1 has been shown to display chymotrypsin-like substrate specificity and is consequently termed a 'chymase'. Experiments were thus conducted to assess if the thrombin-inactivating activity co-eluted with chymotrypsin-like activity. Chymotrypsin-like activity was measured utilizing the chromogenic chymotrypsin substrate S-2586. The thrombin-inactivating activity and the chymotrypsin-like activity were indeed shown to

Figure 3 H.p.l.c. of mast-cell extracts

Thrombin-inactivating activity (approx. 5 units), purified by anion-exchange chromatography, was applied to a Superdex 75 column eluted with PBS/0.1% Triton X-100 (a) or with PBS/0.1% Triton X-100/2 M NaCl (b). Eluted fractions were analysed for thrombin-inactivating activity (○, □), chymotrypsin-like activity (●) and for ^{35}S radioactivity (■). After high-salt chromatography (b) the thrombin-inactivating activity was assayed in the absence (□) or the presence (○) of 0.1 $\mu\text{g}/\text{ml}$ of pig mucosal heparin. Fractions were pooled as indicated by bars in the figure. (c) Pooled fractions were analysed by SDS/PAGE followed by silver staining. The numbering of the lanes corresponds to the numbering of the pooled fractions from (b).

co-purify both on anion-exchange (Figure 2) and on Superdex 75 (Figure 3) chromatography.

Interaction of RMCP-1 with heparin

The mast-cell proteases are stored in the secretory granules together with negatively charged, sulphated heparin or chondroitin sulphate proteoglycans. To determine if RMCP-1 was recovered as a complex with proteoglycans, rats were injected intraperitoneally with $^{35}\text{SO}_4^{2-}$, followed by purification of RMCP-1 as described above. The ^{35}S radioactivity showed co-distribution with both thrombin-inactivating activity and chymotrypsin-like activity on anion-exchange chromatography (Figure 2). When the material, after purification by anion-exchange chromatography, was analysed by Superdex 75 chromatography in low salt conditions (PBS/0.1% Triton X-100), the thrombin-inactivating activity, chymotrypsin-like activity and ^{35}S radioactivity showed co-distribution (Figure 3a).

Figure 4 Gel filtration of RMCP-1

A sample of RMCP-1 (corresponding to approx. 5 units of thrombin-inactivating activity), purified by anion-exchange chromatography from mast cells that were labelled *in vivo* with $^{35}\text{SO}_4^{2-}$ (see Figure 2), was applied to a column of Sepharose CL-2B. The column was equilibrated and eluted with either PBS/0.1% Triton X-100 (a) or PBS/0.1% Triton X-100/2 M NaCl (b). Fractions (approx. 0.3 ml) were collected and analysed for chymotrypsin-like activity (●) and for ^{35}S radioactivity (○).

In contrast, when the column was eluted in high salt conditions (PBS/0.1% Triton X-100/2 M NaCl), the ^{35}S radioactivity was completely separated from the thrombin-inactivating activity and chymotrypsin-like activity (Figure 3b).

To determine the nature of the recovered ^{35}S -labelled material, ^{35}S radioactivity obtained after high-salt chromatography (see Figure 3b) was pooled, dialysed against H_2O , evaporated to dryness and treated with HNO_3 at pH 1.5. The HNO_3 treatment achieves degradation of heparin-like polysaccharides, whereas chondroitin sulphate is not affected. Superdex 75 chromatography of the HNO_3 -treated ^{35}S radioactivity showed that approx. 95% of the material was degraded into, predominantly, disaccharides, indicating that it was heparin (results not shown). To determine whether the mast-cell heparin was present as proteoglycans or as free polysaccharide chains, the ^{35}S -labelled material was treated with alkali (see the Materials and methods section) followed by gel filtration on a Superose 6 column. The alkali treatment, known to release glycosaminoglycan chains from the protein core of proteoglycans, yielded a marked decrease in size of the ^{35}S -labelled material, demonstrating that the mast-cell heparin was present as a proteoglycan (results not shown). The [^{35}S]heparin chains were considerably larger in size than standard pig mucosal heparin (not shown).

To obtain evidence for complex formation between the heparin proteoglycan and RMCP-1, material purified by anion-exchange chromatography was subjected to gel filtration on a Sepharose CL-2B column. On chromatography in low salt (PBS/0.1% Triton X-100), RMCP-1 was eluted as a single peak in the void volume. The [^{35}S]heparin proteoglycans eluted as two peaks: one

Figure 5 Activation of RMCP-1 by heparin

Purified RMCP-1 was incubated with pig mucosal heparin (a, b) or endogenous, ³⁵S-labelled mast-cell heparin (c, d) at the concentrations indicated. A sample (10 μl) of endogenous [³⁵S]heparin corresponds to approx. 1000 c.p.m. After 15 min preincubation of RMCP-1 with heparin, the thrombin-inactivating activity (a, c) and chymotrypsin-like activity (b, d) of the enzyme was determined.

Figure 6 Thrombin-inactivating activities of RMCP-1, antithrombin and chymotrypsin

Thrombin (20 ng) was incubated with a 2-fold molar excess of antithrombin (□, ■), RMCP-1 (○, ●) or chymotrypsin (▲) in the absence (open symbols) or presence (filled symbols) of 0.1 μg/ml of heparin. Residual thrombin activity was determined at the times indicated.

sharp peak showing co-distribution with RMCP-1, and one broad peak with a K_{av} value of approx. 0.5 (Figure 4a). When the column was eluted with 2 M NaCl, the void volume peak (both RMCP-1 and [³⁵S]heparin proteoglycans) was abolished, and RMCP-1 was eluted close to the total volume (Figure 4b). These results indicate that RMCP-1 is indeed recovered as a macromolecular complex with heparin proteoglycan.

Activation of RMCP-1 by heparin

Initial experiments showed that most of the thrombin-inactivating activity was lost after chromatography on Superdex

Table 1 Affinity chromatography of mast-cell heparin on RMCP-1-Sepharose

Samples (approx. 500 c.p.m.) of endogenous [³⁵S]heparin proteoglycans or free [³⁵S]heparin chains obtained by alkali treatment of the heparin proteoglycans were applied to a column of RMCP-1-Sepharose. The column was eluted with PBS/0.1% Triton X-100 supplemented with NaCl at the concentrations indicated. Eluted fractions were analysed for ³⁵S radioactivity.

NaCl concn. (M)	³⁵ S radioactivity eluted from the column (c.p.m.)	
	Proteoglycans	Free chains
0.15	8	13
0.3	22	26
0.5	74	70
1.0	211	191
2.0	29	10
4.0	8	4

75 in 2 M NaCl, but not when the chromatography was performed at 0.15 M NaCl (Figure 3). As RMCP-1 is dissociated from heparin proteoglycans at 2 M NaCl (Figure 3b), experiments were conducted to determine whether heparin was important for the activity of the enzyme. Purified RMCP-1 was incubated with either standard pig mucosal heparin or endogenous ³⁵S-labelled mast-cell heparin proteoglycans (recovered after Superdex 75 chromatography in 2 M NaCl; see Figure 3b), followed by assessment of the thrombin-inactivating activity of the enzyme. As a comparison, the effect of heparin on the direct cleavage of a chromogenic chymotrypsin substrate (S-2586) by RMCP-1 (chymotrypsin-like activity) was determined. Both pig mucosal (Figure 5a) and endogenous heparin (Figure 5c) strongly potentiated the thrombin-inactivating activity of RMCP-1. Optimal stimulation (up to 9-fold) of the thrombin-inactivating activity occurred at approx. 0.1 μg/ml of standard heparin. At higher heparin concentrations, a marked decrease in the potentiation was observed. In contrast, the S-2586-cleaving activity of RMCP-1 showed only approx. 2-fold maximal stimulation by standard (Figure 5b) or endogenous (Figure 5d) heparin. Control experiments showed that heparin had no effect on thrombin in the absence of RMCP-1 (results not shown).

Experiments were conducted to compare the thrombin-inactivating activities of RMCP-1 and antithrombin (Figure 6). In the absence of heparin both RMCP-1 and antithrombin showed only slow inactivation of thrombin. Heparin induced a marked potentiation of the thrombin-inactivation activities displayed by both RMCP-1 and antithrombin. Incubation of thrombin with chymotrypsin did not result in any detectable inactivation of the coagulation enzyme.

Affinity chromatography

For further studies of the interaction between RMCP-1 and heparin, purified RMCP-1 was coupled to activated CH-Sepharose 4B. ³⁵S-labelled endogenous heparin proteoglycans or free [³⁵S]heparin chains, released by alkali treatment of proteoglycans, were applied to the column, followed by stepwise elution of the column with increasing NaCl concentrations. The results showed that essentially all of the proteoglycans and the heparin chains bound strongly to the column, and that most of the material was eluted at NaCl concentrations between 0.5 and 1 M NaCl (Table 1). In contrast, affinity chromatography of the ³⁵S-labelled heparin proteoglycans (not shown) or free [³⁵S]heparin chains (Figure 7) on antithrombin-Sepharose showed that the

Figure 7 Affinity chromatography of heparin on antithrombin-Sepharose

Endogenous, ^{35}S -labelled mast-cell heparin chains (approx. 10 000 c.p.m.), obtained by alkali treatment of heparin proteoglycans, were mixed with standard ^3H -labelled pig mucosal heparin (approx. 40 000 c.p.m.). The mixture was applied to a column (3 ml) of antithrombin-Sepharose that was eluted with a linear salt gradient. Fractions were analysed for ^{35}S - (●) and for ^3H -radioactivity (○). The elution positions of standard heparin with high (HA) and low (LA) affinity for antithrombin are indicated by arrows.

endogenous heparin displayed low affinity for the protease inhibitor.

DISCUSSION

Mast cells express a variety of proteases that are stored in the secretory granules. Human mast cells contain both a trypsin-like (tryptase; Miller et al., 1989) and a chymotrypsin-like serine protease (chymase; Schechter et al., 1986) as well as a carboxypeptidase (Reynolds et al., 1989). Mouse mast cells express, in addition to a carboxypeptidase (Serafin et al., 1987), seven different secretory granule serine proteases termed murine mast-cell proteases 1–7 (MMCP-1–7) (Reynolds et al., 1990; Huang et al., 1991; McNeil et al., 1992) of which MMCP-1–5 are chymases and MMCP-6 and 7 are tryptases. In rats, two different mast-cell chymases, RMCP-1 and RMCP-2 have been identified (Benfey et al., 1987; LeTrong et al., 1987) along with a tryptase (Kido et al., 1985) and a carboxypeptidase (Everitt and Neurath, 1980). The specific expression of the various proteases appears to be tightly coupled to the cellular differentiation process. Thus, whereas mouse mucosal mast cells show expression of MMCP-1 and MMCP-2, mature connective-tissue mast cells express MMCP-3–6. MMCP-7 appears only to be expressed transiently in immature mast cells. In rats, the mucosal mast cells selectively express RMCP-2, whereas the connective-tissue mast cells show selective expression of RMCP-1. Despite the identification of several substrates for the mast-cell proteases (see the Introduction for references), the biological function of these enzymes has not yet been determined. In the present report, a new activity is attributed to the mast-cell proteases. Rat peritoneal mast cells were shown to inactivate thrombin efficiently. Purification of the thrombin-inactivating activity showed that the activity was associated with RMCP-1.

Inflammatory reactions are usually characterized by activation of the coagulation cascade resulting in extravascular fibrin deposition. The coagulation is thought to be initiated by macrophages which, after activation by various inflammatory stimuli,

express different procoagulants including prothrombinase activity (Geczy, 1983; Lindahl et al., 1989). Thrombin, generated by the macrophage prothrombinase, displays, in addition to its procoagulant activity, a wide spectrum of activities of potential importance in an inflammatory reaction. Thrombin is a potent chemotactic factor for leucocytes (Bar-Shavit et al., 1983), induces proliferation of various cell types (Carney and Cunningham, 1978; Clohisy et al., 1990), elicits degranulation of mast cells (Razin et al., 1985) and induces the expression of neutrophil adhesion molecules on the surface of endothelial cells (Hattori et al., 1989). Thus, the inactivation of thrombin by RMCP-1 may be of physiological significance by down-regulating the thrombin that is generated by the macrophage prothrombinase, thereby restricting the procoagulant and pro-inflammatory activities of the coagulation enzyme. In a recent report we showed that mouse mast-cell chymases (MMCP-3 or -4) also had thrombin-inactivating properties (Pejler and Karlström, 1993). RMCP-1 shows a high degree of structural similarity with MMCP-4 (88 % identity; Huang et al., 1991) and is thought to be the rat counterpart of the mouse protease. Apparently, RMCP-1 and the corresponding mouse enzymes also share certain functional properties.

The mast-cell proteases generally carry net positive charges (Huang et al., 1991) and are packaged in the secretory granules together with negatively charged chondroitin sulphate or heparin proteoglycans. In the present investigation, after *in vivo*-labelling of the peritoneal mast cells with $^{35}\text{SO}_4^{2-}$, RMCP-1 was recovered as a macromolecular complex with [^{35}S]heparin proteoglycans. The protease–heparin complex apparently displayed a net negative charge since it bound strongly to anion-exchange columns. After anion-exchange chromatography, the RMCP-1–heparin complex was essentially pure with only trace amounts of visible contaminating bands on SDS/PAGE. After h.p.l.c. on a Superdex 75 column eluted with high salt (2 M NaCl) the [^{35}S]heparin proteoglycan and RMCP-1 were completely separated, yielding purification of RMCP-1 to apparent homogeneity. The present paper thus presents a new, simple two-step method for the purification of RMCP-1. Previously published protocols for purification of RMCP-1 involve different purification steps (Yurt and Austen, 1977; Woodbury et al., 1981; Gibson and Miller, 1986).

After dissociation from the heparin proteoglycan, RMCP-1 showed a marked loss of thrombin-inactivating activity. However, when RMCP-1 was reconstituted with either endogenous mast-cell [^{35}S]heparin proteoglycans or with standard pig mucosal heparin, RMCP-1 regained its ability to inactivate thrombin. The contribution of endogenous heparin in the inactivation of thrombin by the RMCP-1–heparin complex is further supported by the finding that Polybrene, a well-known cationic heparin antagonist, was a potent inhibitor of thrombin inactivation. The mechanism by which heparin stimulates thrombin inactivation is unclear. However, as both thrombin (see Olson et al., 1991) and RMCP-1 interact with heparin, it is possible that heparin, by binding both to the chymase and to thrombin, mediates contact between the two molecules. Another possibility is that heparin induces a conformational change in RMCP-1 that increases its thrombin-inactivating activity. In contrast, the direct cleavage of the chromogenic chymotrypsin substrate S-2586 by RMCP-1 was only marginally stimulated by heparin. Possibly, the cleavage of S-2586 by RMCP-1 does not require interaction of the chromogenic substrate with heparin.

Several other investigators have reported various, partly contradictory, effects of heparin on mast-cell protease function. Yurt and Austen (1977) reported that heparin partly masked the active site of rat chymase. Heparin was later demonstrated to

stabilize human lung mast-cell tryptase into an active tetramer (Schwartz and Bradford, 1986), and was shown to alter the substrate specificity of the tryptase (Schwartz et al., 1983). Sayama et al. (1987) reported that heparin decreased the activity of human chymase by sterically hindering large substrates from entering the active site. Further, heparin has been shown to be important for the fibrinogenolytic activity of human tryptase (Schwartz et al., 1985) and for the degradation of anaphylatoxin C3a by rat mast-cell chymase (Gervasoni et al., 1986). It is clear that more work is required to understand fully the functional significance of the interaction between heparin and the mast-cell proteases.

In an attempt to characterize further the interaction between heparin and RMCP-1, endogenous ^{35}S -labelled mast-cell heparin was subjected to affinity chromatography on matrix-linked RMCP-1. The results showed that virtually all of the heparin proteoglycans and all of the free heparin chains, released by alkali treatment of intact proteoglycans, contained high-affinity binding sites for RMCP-1. Further work will be required to determine if heparin is bound to RMCP-1 through unspecific electrostatic interactions, or if the binding is mediated by a specific saccharide sequence. Such specific interactions have been identified for the interaction between heparin and the protease inhibitor antithrombin (Bourin and Lindahl, 1993), and in the binding of heparan sulphate (a heparin-like glycosaminoglycan) to basic fibroblast growth factor (Habuchi et al., 1992).

Heparin is well known for its anticoagulant activity, mediated by antithrombin, which in the presence of heparin inactivates various proteases of the coagulation cascade (e.g. Factor Xa, thrombin; Bourin and Lindahl, 1993). Affinity chromatography of heparin on antithrombin-Sepharose has shown that commercial heparin preparations can be separated into two populations: heparin with low affinity for antithrombin showing low anticoagulant activity and heparin with high affinity for antithrombin displaying high anticoagulant activity (Höök et al., 1976). In the present investigation it was shown that most of the heparin isolated after *in vivo*-labelling of the peritoneal mast cells with $^{35}\text{SO}_4^{2-}$, showed low affinity for antithrombin, suggesting that it is not involved in the regulation of coagulation enzymes. Instead, the tight association of heparin with RMCP-1, with consequent activation of the enzyme, suggests that the physiological function of rat peritoneal mast-cell heparin involves interaction with the mast-cell proteases.

This work was supported by grants from the Swedish Medical Research Council (grant no. 9913), Crafoords Fund and from King Gustaf V's 80th Anniversary Fund. We are grateful to Dr. Lena Kjellén for critical reading of the manuscript.

REFERENCES

- Bar-Shavit, R., Kahn, A., Fenton, J. W., II and Wilner, G. D. (1983) *J. Cell Biol.* **96**, 282–285
- Bentley, P. N., Yin, F. H. and Leder, P. (1987) *J. Biol. Chem.* **262**, 5377–5384
- Bourin, M.-C. and Lindahl, U. (1993) *Biochem. J.* **289**, 313–330
- Bromley, M., Fisher, W. D. and Woolley, D. E. (1984) *Ann. Rheum. Dis.* **43**, 76–79
- Carney, D. H. and Cunningham, D. D. (1978) *Cell* **15**, 1341–1349
- Clohisy, D. R., Erdmann, J. M. and Wilner, G. D. (1990) *J. Biol. Chem.* **265**, 7729–7732
- DuBuske, L., Austen, K. F., Czop, J. and Stevens, R. L. (1984) *J. Immunol.* **133**, 1535–1541
- Enerbäck, L., Kolset, S. O., Kusche, M., Hjerpe, A. and Lindahl, U. (1985) *Biochem. J.* **227**, 661–668
- Everitt, M. T. and Neurath, H. (1980) *FEBS Lett.* **110**, 292–296
- Geczy, C. L. (1983) *Lymphokines* **8**, 201–247
- Gervasoni, J. E., Jr, Conrad, D. H., Hugli, T. E., Schwartz, L. B. and Ruddy, S. (1986) *J. Immunol.* **136**, 285–292
- Gibson, S. and Miller, H. R. P. (1986) *Immunology* **58**, 101–104
- Habuchi, H., Suzuki, S., Saito, T., Tamura, T., Harada, T., Yoshida, K., and Kimata, K. (1992) *Biochem. J.* **285**, 805–813
- Hattori, R., Hamilton, K. K., Fugate, R. D., McEver, R. P. and Sims, P. J. (1989) *J. Biol. Chem.* **264**, 7768–7771
- Hawkins, R. A., Claman, H. N., Clark, R. A. F. and Steingerwald, J. C. (1985) *Ann. Intern. Med.* **102**, 182–186
- Höök, M., Björk, I., Hopwood, J. and Lindahl, U. (1976) *FEBS Lett.* **66**, 90–93
- Huang, R., Blom, T. and Hellman, L. (1991) *Eur. J. Immunol.* **21**, 1611–1621
- Kido, H., Fukusen, N. and Katunuma, N. (1985) *Arch. Biochem. Biophys.* **239**, 436–443
- Kokkonen, J. O., Vartiainen, M. and Kovanen, P. T. (1986) *J. Biol. Chem.* **261**, 16067–16072
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- LeTrong, H., Parmelee, D. C., Walsh, K. A., Neurath, H. and Woodbury, R. G. (1987) *Biochemistry* **26**, 6988–6994
- Lindahl, U., Pejler, G., Bögwald, J. and Seljelid, R. (1989) *Arch. Biochem. Biophys.* **273**, 180–188
- McNeil, H. P., Reynolds, D. S., Schiller, V., Ghildyal, N., Gurley, D. S., Austen, K. F. and Stevens, R. L. (1992) *Proc. Natl. Acad. Sci. U.S.A.* **89**, 11174–11178
- Miller, J. S., Westin, E. H. and Schwartz, L. B. (1989) *J. Clin. Invest.* **84**, 1188–1195
- Mizutani, H., Schechter, N., Lazarus, G., Black, R. A. and Kupper, T. S. (1991) *J. Exp. Med.* **174**, 821–825
- Morrissey, J. H. (1981) *Anal. Biochem.* **117**, 307–310
- Olson, S. T., Halvorsen, H. R. and Björk, I. (1991) *J. Biol. Chem.* **266**, 6342–6352
- Pejler, G. and David, G. (1987) *Biochem. J.* **248**, 69–77
- Pejler, G. and Karlström, A. (1993) *J. Biol. Chem.* **268**, 11817–11822
- Pejler, G. and Seljelid, R. (1992) *J. Biol. Chem.* **267**, 3136–3142
- Razin, E., Baranes, D. and Marx, G. (1985) *Exp. Cell Res.* **160**, 380–386
- Qureshi, R. and Jakschik, B. A. (1988) *J. Immunol.* **141**, 2090–2096
- Reilly, C. F., Tewksbury, D. A., Schechter, N. M. and Travis, J. (1982) *J. Biol. Chem.* **257**, 8619–8622
- Reilly, C. F., Schechter, N. M. and Travis, J. (1985) *Biochem. Biophys. Res. Commun.* **127**, 443–449
- Reynolds, D. S., Gurley, D. S., Stevens, R. L., Sugarbaker, D. J., Austen, K. F. and Serafin, W. E. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 9480–9484
- Reynolds, D. S., Stevens, R. L., Lane, W. S., Carr, M. H., Austen, K. F. and Serafin, W. E. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 3230–3234
- Sayama, S., Iozzo, R. V., Lazarus, G. S. and Schechter, N. M. (1987) *J. Biol. Chem.* **262**, 6808–6815
- Schechter, N. M., Choi, J. K., Slavin, D. A., Deresienski, D. T., Sayama, S., Dong, G., Lavker, R. M., Proud, D. and Lazarus, G. S. (1986) *J. Immunol.* **137**, 962–970
- Schwartz, L. B. and Bradford, T. R. (1986) *J. Biol. Chem.* **261**, 7372–7379
- Schwartz, L. B., Kawahara, M. S., Hugli, T. E., Vik, D., Fearon, D. T. and Austen, K. F. (1983) *J. Immunol.* **130**, 1891–1895
- Schwartz, L. B., Bradford, T. R., Littman, B. H. and Wintroub, B. U. (1985) *J. Immunol.* **135**, 2762–2767
- Serafin, W. E., Dayton, E. T., Gravallese, R. M., Austen, K. F. and Stevens, R. L. (1987) *J. Immunol.* **139**, 3771–3776
- Sterk, A. R. and Ishizaka, T. (1982) *J. Immunol.* **128**, 838–843
- Stevens, R. L. and Austen, K. F. (1989) *Immunol. Today* **10**, 381–386
- Thunberg, L., Bäckström, G. and Lindahl, U. (1982) *Carbohydr. Res.* **100**, 393–410
- Wichmann, B. E. (1955) *Acta Pathol. Microbiol. Scand.* **108**, 5–35
- Woodbury, R. G., Everitt, M. and Neurath, H. (1981) *Methods Enzymol.* **80**, 588–609
- Yurt, R. W. and Austen, K. F. (1977) *J. Exp. Med.* **146**, 1405–1419
- Yurt, R. W., Leid, R. W., Austen, K. F. and Silbert, J. E. (1977) *J. Biol. Chem.* **252**, 518–521