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Chemical modification of the carboxyl groups of protein substrates enhances their thrombin susceptibility

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Native or denatured protein substrates which are hardly digested by thrombin can become much more efficiently cleaved by the enzyme after chemical modification of their carboxyl groups. Five antibody κ-chains were used to demonstrate this effect. The selective cleavage sites were determined by quantitative N-terminal analysis and N-terminal sequencing. All five κ-chains share the same cleavage sites at Arg-Thr (residues 108–109), Arg-Glu (residues 142–143, Glu side chain modified with glycine amide), Lys-Ser (residues 207–208) and Arg-Gly (residues 211–212). One of the major cleavage sites (Arg-Thr) is located at the joint of the variable/constant region. The amino acids adjacent to these cleavage sites underline the proposed structural requirements for a potential thrombin substrate [(1985) Eur. J. Biochem. 151, 217–224]. This approach can facilitate the application of thrombin in generating large polypeptide fragments of proteins.

Proteolysis; Thrombin specificity; N-terminal analysis

1. INTRODUCTION

In analysis of the structure and function of proteins, it is often desirable to generate large polypeptide fragments. These large polypeptides are not only more suitable for sequencing by modern automatic instruments [1–3] but also generally more useful for delineation of the functional domain [4–6]. Chemical methods which cleave selectively at the carboxyl end of methionine [7] and tryptophan [8,9] have been widely used. However, no enzymatic method with similarly predicted specificity is available for routine production of large polypeptide fragments.

Thrombin is a serine proteinase best known for its selective cleavage of two Arg–Gly bonds during fibrinogen-fibrin conversion [10–12]. Thrombin also cleaves a large number of non-fibrinogen polypeptides with highly restricted specificities [13–17], and the amino acid sequences surrounding those selective cleavage sites were found to exhibit common structures (see [15] and section 4). These data suggested that selective cleavage by thrombin can be reasonably predicted [15]. We have indeed applied this predictability to isolate a pure variable region and a pure constant region from an antibody light chain [16]. Nonetheless, we have also found that not all Arg/Lys–X bonds with optimized structures can be effectively cleaved by thrombin. Here, we demonstrate that chemical modification of carboxyl groups of non-fibrinogen substrates can enhance their thrombin susceptibility.

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Abbreviations: DABITC, dimethylaminoazobenzene isothiocyanate; DABTH, dimethylaminoazobenzene thiohydantoin; DABS-Cl, dimethylaminoazobenzene sulfonyl chloride; HPLC, high-performance liquid chromatography

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2. MATERIALS AND METHODS

Five human antibody $\varkappa$-chains were kindly provided by Dr N. Hilschmann [18,19]. The sources of DABITC, DABS-Cl, as well as other chemicals used in both N-terminal sequence and amino acid analyses were described in [20,21]. Bovine thrombin was purchased from Serva.

Proteins were oxidized using performic acid. Carboxyl groups were modified with glycine amide [22] and desalted by gel filtration (G-25) with 1 M acetic acid. The extent of modification was evaluated by amino acid analysis using the DABS-Cl pre-column derivatization method [23]. The ratios mol incorporated glycine amide/mol carboxyl group in the five $\varkappa$-chains were found to be between 0.87 and 1.2, indicating quantitative modifications. Thrombin digestions were carried out under the conditions of 1 nmol substrate/0.5 NIH unit thrombin in 35 μl of 50 mM ammonium bicarbonate at 25°C. The digestions were stopped by freeze-drying. The cleavage sites were determined by sequence analysis using the DABITC/PITC method [20]. The relative cleavage rates at each site were determined by quantitative N-terminal analysis [24] with the recovery of the N-terminus Asp as 100%.

3. RESULTS

The extent of thrombin cleavage was first assessed with the technique of quantitative N-terminal analysis (fig.2). The selective cleavage sites were then determined by 4–8 steps of N-terminal sequencing. For each protein, an 8 h and a 24 h digest were sequenced. This approach is suitable for analysing protein substrates with known amino acid sequences. However, with peptide mixtures as complex as those indicated in fig.2, there is a limit to which one can confidently assign all the minor cleavage sites, and it is likely that some minor cleavage sites which amounted to less than 10% of the major cleavage site may have been overlooked. The native $\varkappa$-chains (fig.1) are hardly digested by thrombin. None of the Arg/Lys–Xaa bonds in all five $\varkappa$-chains yield more than 30% of cleavage after 48 h of thrombin incubation (figs 2–4). Denaturation (by oxidation) does not significantly improve their thrombin susceptibility (figs 2–4). However, when oxidized chains were modified with glycine amide, several Arg/Lys–Xaa bonds were efficiently cleaved by thrombin (figs 2–4). For instance, when oxidized, glycine amide modified BI was digested by thrombin for 8 h (fig.2), four new N-termini, namely Glu (side chain

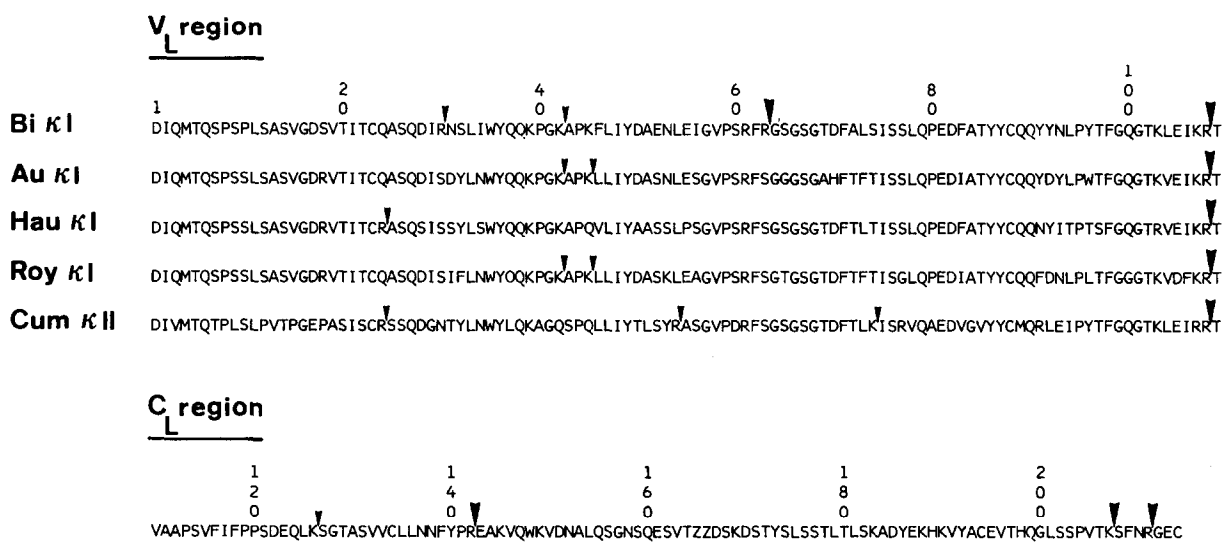

Fig.1. Selective cleavage sites of antibody $\varkappa$-chains by thrombin. The $\varkappa$-chains were oxidized and their carboxyl groups modified with glycine amide. The major cleavage sites are indicated by large arrows and the minor cleavage sites by small arrows. V_L, variable region of the light chain; C_L, constant region of the light chain.

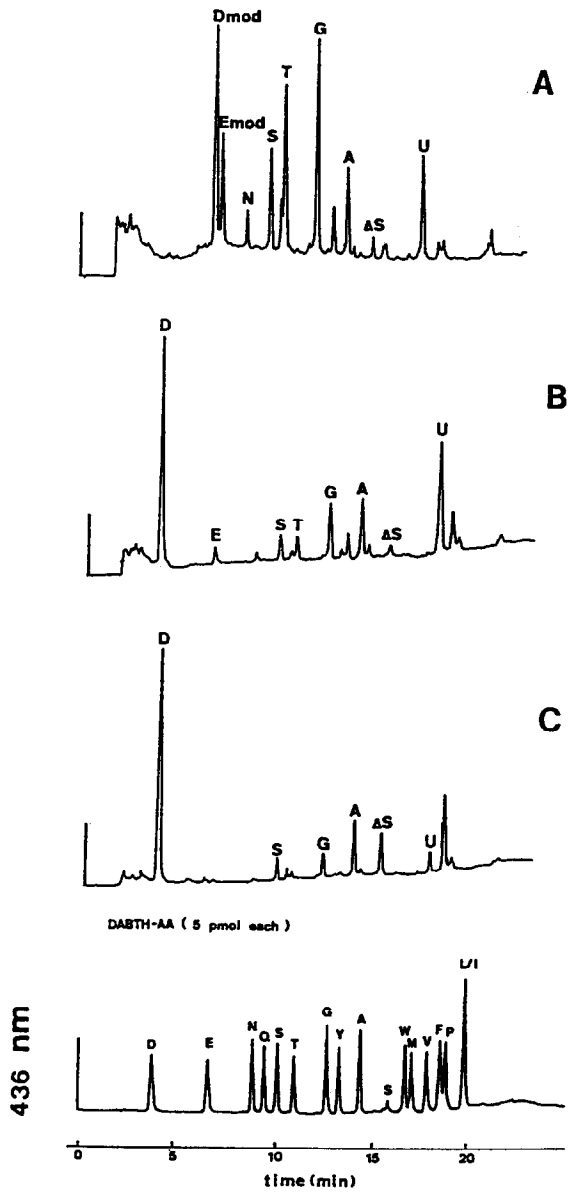

Fig.2. Quantitative N-terminal analysis (by the DABITC method) of antibody $\varkappa$-chain BI digested by thrombin for 8 h. (A) Oxidized and glycine amide modified. (B) Oxidized. (C) Native. A standard mixture of DABTH-amino acids is shown in the bottom panel. The released DABTH-amino acids were analysed by HPLC [20], and are symbolized by the one-letter code of their corresponding amino acids. S^d, DABTH-dehydroserine; Dmod and Emod, DABTH-Asp and DABTH-Glu with their side chain carboxyl groups modified with glycine amide; U, thiourea by-product.

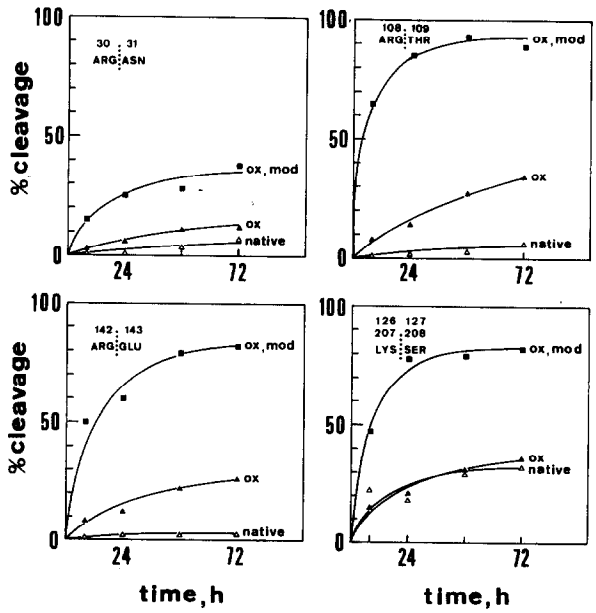

Fig.3. Selective thrombin cleavages of native, oxidized, oxidized plus modified BI at various Arg/Lys–Xaa bonds. The cleavage positions (see also fig.1) are indicated at the top left of each panel. Percentage cleavages were calculated based on the recovery of N-terminal DABTH-Asp as 100%.

carboxyl group modified), Ser, Thr and Gly, appeared. Sequence analysis indicates that Glu was derived from cleavage of Phe Tyr Pro Arg–Glu (142–143), Ser from that of Pro Val Thr Lys–Ser (207–208), Thr from that of Glu* Ile Lys Arg–Thr (108–109) (* glycine amide modified) and Gly from the combined cleavages of Ser Arg Phe Arg–Gly (63–64) and Ser Phe Asn Arg–Gly (211–212). This enhanced thrombin susceptibility was also found with modified AU, HAU, ROY and CUM, and the same cleavage sites were found within their constant regions (fig.1). One interesting major cleavage site detected within all five $\varkappa$-chains was Arg-Thr (108–109). This bond is located at the joint of the variable region and constant region. Since no major cleavage sites were identified within the variable regions of AU, HAU, ROY and CUM, the selective cleavage at Arg-Thr may be used to prepare the intact variable regions for some light chains. We have not investigated this enhanced effect on the carboxymethylated $\varkappa$-chains. But in a separate study [16], it has been shown that both oxidized and carboxy-

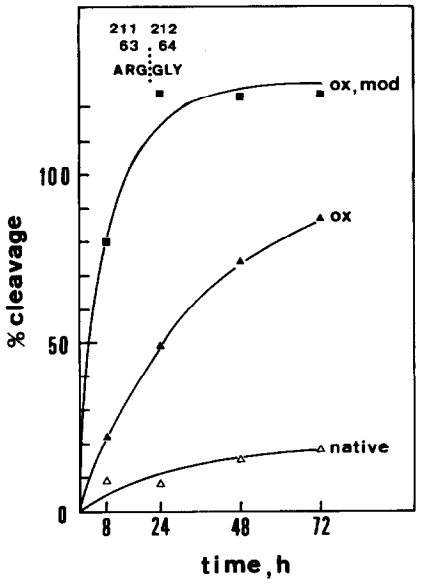

Fig.4. Selective thrombin cleavages of native, oxidized, oxidized plus modified BI at two Arg–Gly bonds. Percentage cleavages were calculated based on the recovery of N-terminal DABTH-Asp as 100%.

methylated $\varkappa$-chains were cleaved by thrombin with indistinguishable specificities and efficiencies.

4. DISCUSSION

Although thrombin cleaves both fibrinogen and non-fibrinogen substrates with high specificity, it does so with vastly different efficiencies. Even the best polypeptide hormone substrate (gastrin-releasing peptide) [15] has a K_{cat}/K_m value one to two orders of magnitude lower than that of fibrinogen Aα chain. Studies employing chemically modified thrombin [25] and autolysed thrombin [17,26,27] have revealed that mechanisms for the selective cleavages of fibrinogen and non-fibrinogen substrates are governed at two different levels [15,17]. Selective cleavage of non-fibrinogen substrate requires the intact active site of thrombin only. Selective cleavage of fibrinogen requires in addition to the active site an independent recognition site which is made up by clusters of basic amino acids [11,13,17,30]. This recognition site can be disrupted without major effect on its active site. Thus, a thrombin derivative devoid of clotting activity but still retaining almost full activity toward non-fibrinogen substrates can be prepared

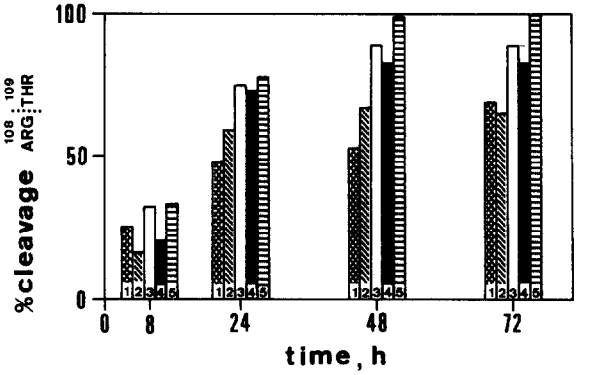

Fig.5. Selective thrombin cleavage at the Arg-Thr (positions 108–109) of five antibody $\varkappa$-chains (all oxidized and glycine amide modified). 1, CUM. 2, AU. 3, BI. 4, HAU. 5, ROY.

by chemical modification [25] or autolysis [17,26,27].

Documented thrombin-susceptible bonds (P4-P3-P2-Arg/Lys-P1′-P2′) of non-fibrinogen substrates can be grouped based on the structures adjacent to the cleavage sites, and this information can be applied to predict, at least qualitatively, the potential thrombin cleavage site in polypeptides with known sequences [15]. (i) The most abundant cases have Pro at the P2 position, hydrophobic amino acids at positions P3–P4 and non-acidic amino acids at positions P1′ or P2′. (ii) A Ser or Thr at position P1′ will enhance thrombin cleavage of those with optimized P2–P4 structures described above. (iii) Those with Gly at positions P1′ or P2 are also preferential thrombin substrates. These unique proteolytic specificities have been attributed to the constrained catalytic site and a hydrophobic binding pocket adjacent to it [13,15,17,28,29].

The thrombin cleavage sites identified within the chemically modified $\varkappa$-chains have surrounding sequences which comply with the above-mentioned structural requirements (fig.1). Among the five major cleavage sites detected within BI (with 50% cleavage time of approx. 4–8 h), two have Gly at position P1′, two have Ser or Thr at position P1′ and the other has Pro and hydrophobic amino acids at positions P2–P4. The Pro Arg–Glu was digested by thrombin only after Glu had been modified with glycine amide. Most minor cleavage sites identified within variable regions also have

Pro, Gly or hydrophobic residues at the P2 position. However, those minor cleavages (with 50% cleavage time on average longer than 72 h under described conditions) are insignificant as compared to most documented thrombin cleavage sites [11,15].

It is likely that chemical modification at carboxyl groups with glycine amide could further denature the oxidized proteins. However, we believe that this enhanced susceptibility underlines the importance of the hydrophobic binding pocket in thrombin [17,28,29]. Modification of carboxyl groups could in general facilitate the interactions of the polypeptide substrates with the hydrophobic binding site in thrombin.

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