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Direct kinetics of bait region cleavage of α-2-macroglobulin by a rapid quenching method

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The rate of bait region cleavage of human α-2-macroglobulin by chymotrypsin was determined by a rapid quenching method under conditions where the bimolecular encounter between the two reactants was not rate-limiting. α_2M was first mixed with a 30 molar excess of chymotrypsin in a sequential stopped-flow apparatus and after programmed time intervals the activity of chymotrypsin was quenched with 1 N HCl. The fraction of uncleaved subunits was quantitated by SDS-PAGE under reducing conditions. The result indicated that the bait region cleavage proceeded following a two-exponential decay curve with respective rate constants of $k_1 = 40\ s^{-1}$ and $k_2 = 2\ s^{-1}$.

α-2-Macroglobulin; Bait region cleavage; Rapid quenching method

1. INTRODUCTION

α-2-Macroglobulin (α_2M) is a tetrameric serum glycoprotein that can inhibit a large variety of proteinases not directly by blocking their active sites but by trapping them in its presumed molecular cavity and isolating them from the high molecular weight substrates [1]. Such a mechanism for α_2M was first proposed by Barrett and Starkey as the 'trap hypothesis' and has since been elaborated by many others in detail [2–4]. According to the accepted idea of the trap mechanism of today, a stretch of about 30 amino acid residues around 670 to 700 within a total of 1,451 is highly susceptible to proteolytic cleavage. Once a peptide bond in this so-called 'bait region' is hydrolyzed by encountering proteinases, the native conformation of α_2M collapses around the proteinase and traps it as if the entire structure of α_2M is designed as a molecular scale mouse trap. Such a mechanism is well suited for catching a large variety of proteinases and inhibiting their activities regardless of their active site chemistry. We also know that somewhere in the above reaction sequence, events like spontaneous thiolester cleavage and the exposure of the receptor binding sites take place [2].

The working principle of α_2M thus seems to be one of the triggered release of preset mechanical contraptions and is unique among proteins. From the body of accumulated knowledge on the trap mechanism, we know that (1) one molecule of tetrameric human α_2M can trap up to two molecules of small proteinases such as chymotrypsin or trypsin, (2) when two mol of chymotrypsin are trapped per mol of α_2M under molar abundance of the former, all four bait regions of tetrameric α_2M are cleaved. The structural changes of α_2M and its homologues that takes place after bait region cleavage can be detected by a variety of physical and biochemical methods including electron microscopy [5,6], X-ray solution scattering [7], circular dichroism [8], electrophoresis [9], gel chromatography [10], fluorescence spectroscopy and sedimentation velocity [11]. We have little information, however, on the kinetic aspects of the reaction such as (1) how fast the bait regions are cleaved after the encounter of α_2M with proteinases, (2) how many bait regions must be cleaved before the proteinases are trapped, (3) how closely connected in time are the bait region cleavage and the structural change? This work aims to clarify some of these questions by directly determining the kinetic parameters of the bait region cleavage.

2. MATERIALS AND METHODS

2.1. α_2M

α_2M was purified from the plasma of healthy Japanese males as described previously [12]. The purity of the sample was checked by gel electrophoresis.

2.2. *Rapid quenching method*

A DX17MV sequential stopped-flow apparatus (Applied Photophysics Limited, UK) was used to perform a rapid quenching experiment. The initial mixing of α_2M with chymotrypsin (Sigma, St. Louis, MO) was carried out in the first mixer within 1 ms. The concentration of α_2M was usually 5.8×10^{-6} M (4.2 mg/ml) and that of the enzyme

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Abbreviations: SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; α_2M, α-2-macroglobulin.

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1.7×10^{-4} M. The effective bimolecular encounter of the two reactants was completed within the initial mixing time, since the bimolecular rate constant between α_2M and chymotrypsin has been reported to be 1.2×10^7 s^{-1} [13]. The reaction mixture was kept in the Teflon tubing of the apparatus for specified time intervals before the activity of chymotrypsin was destroyed with 1 N HCl in the second mixer. It was confirmed on the same apparatus that mixing with 1 N HCl could completely quench the activity of chymotrypsin within 10 ms or less. Experiments were done at 25°C and 5°C.

2.3. *Electrophoresis*

The quenched reaction mixture was recovered from the stop syringe of the stopped-flow apparatus and neutralized with 1.5 M Tris-HCl buffer prior to 10% SDS-PAGE. Gels were stained with Coomassie brilliant blue R-250 for proteins after electrophoresis. The intensity of each band was measured with a CS 9000 dual wave length flying-spot scanner (Shimadzu, Kyoto, Japan) and normalized as percentage of the total intensity for α_2M-derived peptides in each lane. The linear dependence of staining intensity on the amount of applied protein was established prior to the experiment.

3. RESULTS

Fig. 1 is a typical example of SDS-PAGE of the quenched reaction mixture after specified time intervals from the initial mixing of α_2M with chymotrypsin. The reaction was carried out at 25°C. The 180 kDa band represents those after bait region cleavage. It is apparent that there is a steady decrease in the intensity of 180 kDa band. In a separate experiment with a manual operation we confirmed that all the bait regions were completely cleaved one minute after the initial mixing. The fractional band intensity of the 180 kDa band designated as S(180kDa) was plotted vs. time (s) in Fig. 2. The decrease of 180 kDa band showed a clear biphasic change. The reaction curve was fitted to a function having two exponential terms and the best fitting curve was,

$$S(180kDa) = 0.55 \exp(-39\ t) + 0.45 \exp(-2.1\ t)$$

Fig. 3 shows a similar kinetic result obtained at 5°C and the best fitting curve in this case was,

$$S(180kDa) = 0.5 \exp(-29\ t) + 0.5 \exp(-0.42\ t)$$

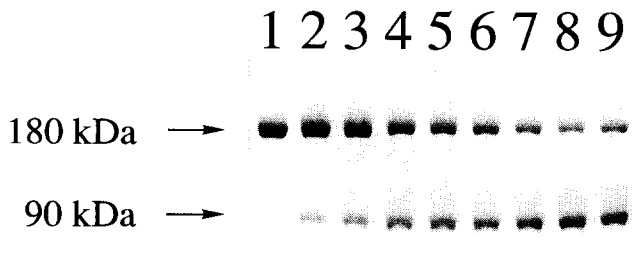

Fig. 1. A typical example of SDS-PAGE of the quenched reaction mixtures at 25°C. Lanes 1, α_2M; 2, 19.1 ms after mixing with chymotrypsin; 3, 21.6 ms; 4, 30.0 ms; 5, 43.0 ms; 6, 59.3 ms; 7, 102.3 ms; 8, 188.2 ms; 9, 78.2 ms. Similar experiments were repeated at least five times at each temperature.

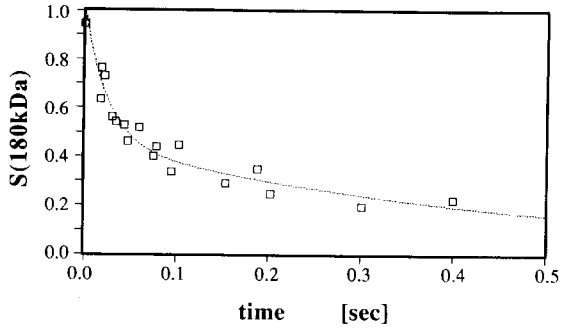

Fig. 2. Time course of bait region cleavage by chymotrypsin at 25°C. S(180kDa) on the ordinate represents the fraction of residual subunits with uncleaved bait region. (□) experimental; (····) best fit curve.

From the results given above, we concluded that at both temperatures about 50% of the bait region was cleaved in the faster phase and the rest in the slower phase.

4. DISCUSSION

It was most surprising that the result from direct observation of the bait region cleavage revealed that the reaction took place in two well separated time ranges. Both at 25°C and 5°C, approximately 50% of the bait region was cleaved in the faster and the rest in the slower reaction. Since under the experimental conditions of the present study the initial bimolecular encounter should be completed within the mixing time, all the rate constants must be assigned to the processes taking place after the formation of initial encounter complexes. From this point of view the following major possibilities can be considered.

(1) There are two populations of α_2M, one with a faster and the other with a slower rate constant for bait region cleavage.

(2) The first chymotrypsin to make an effective encounter with α_2M cleaves two bait regions with a faster and the second one with a slower rate constant.

(3) Two chymotrypsin molecules attack α_2M almost simultaneously and both cleave the first bait regions with a faster and the second bait regions with a slower rate constant.

Although at present there is no definite reason to give preference to one hypothesis over another, the first possibility given above may safely be discarded on the basis of the established homogeneity of human α_2M at the amino acid sequence level. Experiments to distinguish the second and the third alternatives are in progress in our laboratory.

It is noteworthy that the faster rate constant of bait region cleavage is about the same order of magnitude as the fastest of the three rate constants obtained by Larsson et al. from fluorescence stopped-flow kinetics [14], and the slower is in the same order of magnitude

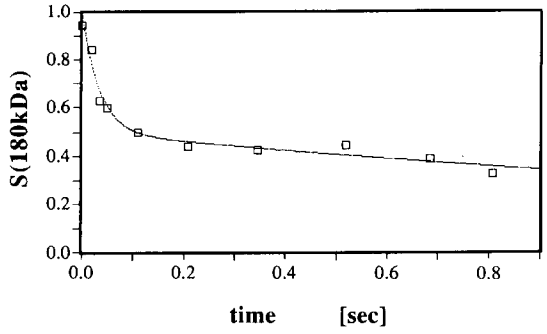

Fig. 3. Time course of bait region cleavage by chymotrypsin at 5°C. Symbols are the same as in Fig. 2.

as the rate constant for the structural change determined in our laboratory using X-ray small-angle scattering (to be published). Integration of the present data in a unified model of trap mechanism will be published elsewhere.

Finally, we must address the relation between acid quenching of the peptide bond hydrolysis and the results of SDS-PAGE. If the amount of proteinase is much lower than that of the substrate, the result of SDS-PAGE accurately represents the amount of residual substrate because almost all the substrates are either pre- or post-reaction and only a small fraction is in the process of hydrolysis. Under our experimental conditions of 30 molar excess of enzyme, however, a significant fraction of the bait region could be caught in the process of enzymatic hydrolysis when acid quenching took place. In such cases we consider it most reasonable to assume that those peptide bonds that are at least in the acylation step or further are counted as cleaved [15].

Despite such experimental ambiguities and significant experimental errors expected in our quantitation of the extent of reaction using SDS-PAGE, the present work is the first to show that the bait region cleavage proceeds at widely different time ranges.

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