THE NATURE OF THE RENNIN - SENSITIVE BOND IN CASEIN AND ITS POSSIBLE RELATION TO SENSITIVE BONDS IN OTHER PROTEINS

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Casein is similar to a number of enzyme precursors and to other proteins such as fibrinogen in possessing a bond that is hydrolysed rapidly by a particular enzyme, resulting in the removal of a peptide fragment from the molecule. The action of the enzyme is very largely limited to the attack on the sensitive bond - the phenomenon described as 'limited proteolysis' by Lindestrøm-Lang and Otteson (1949). The removal of the peptide fragment from a zymogen is a step in the formation of an active enzyme; for casein and fibrinogen its removal is a prerequisite for the polymerisation of the altered protein. The purpose of this communication is to report the preparation of a pentapeptide substrate for rennin which has some of the features of the region in casein around the rennin-sensitive bond, and to suggest a reason for the sensitivity of this bond and of similar ones in other proteins.

Although something is known of the specificity of rennin (EC 3.4.4.3.) as a proteolytic enzyme (Fish 1957), no satisfactory explanation has been given for the very rapid attack by rennin - and some other enzymes (Dennis and Wake 1965) - upon the sensitive bond in casein. This bond has been identified as that between a phenyalanine and methionine pair in the κ-casein fraction (Delfour et al 1965). The phe-met bond, however, does not appear to be intrinsically sensitive to rennin as I have found that the dipeptide phe-met, its methyl ester and its N-carbobenzoxy derivative are not hydrolysed by rennin. The reason

for this difference in behaviour has been sought by studying the action of rennin on a number of peptides synthesised to mimic part of the amino-acid sequence in the region of the phe-met bond in κ-casein. This sequence, from work by Jollès et al (1962) and in our own laboratory (Beeby, personal communication), is ser-leu-phe-met-ala. The action of rennin on the dipeptide esters phe-met-, phe-met-ala-, leu-phe-met-ala- and ser-leu-phe-met-ala-ome was studied at pH 3.7, near the proteolytic optimum for rennin (Foltmann, 1966), and at pH 4.7 and 5.9. Rates of attack were measured by assaying the amine groups released, using the trinitrobenzenesulfonic acid method of Harmeyer et al (1968), and the products of proteolysis were identified by thin layer chromatography. The rates of attack were negligibly small except for that on the penta-peptide ser-leu-phe-met-ala-ome. With a substrate concentration of 2×10^{-3} M and a molar ratio of enzyme to substrate of 1 to 2000, this peptide was split at the phe-met bond moderately rapidly at pH 3.7 and 4.7, and more slowly at pH 5.9, the respective times for half-reaction being 4 and 12 hr. The site of attack was determined by identifying the new C-terminal amino acid (viz phenylalanine) released by carboxy-peptidase A (EC 3.4.2.1.) from the rennin-treated peptide. The attack by rennin was limited to the phe-met bond, although all bonds were split by leucine aminopeptidase (EC 3.4.1.1.) and pronase. (A more complete account of the preparation and testing of these peptides and a number of other possible substrates for rennin will be published later.)

From the foregoing evidence it seems probable that the attack by rennin on the phe-met bond is accelerated by the nearby serine residue. A catalytic, rather than a binding, role for the serine is indicated because of the strong tendency for rennin to hydrolyse bonds in proteins adjacent to hydrophobic side chains (Fish 1957). These chains are therefore the likely binding sites for the enzyme. The

attack on the pentapeptide substrate is, however, slower by two to three orders of magnitude than that on the sensitive bond in κ-casein. This special sensitivity is lost when κ-casein is photo-oxidised, and this effect is related to the destruction of histidine and does not occur when other photo-oxidisable side chains are modified by specific chemical treatments (Hill and Laing 1965). In another fraction of casein rich in sialic acid and containing the rennin-sensitive bond (Beeby 1965) I have observed a similar loss of sensitivity toward rennin following photo-oxidation. It is therefore suggested that the enzymic attack upon this phe-met bond in κ-casein is catalysed by nearby serine and histidine side chains. A catalytic role has of course been postulated for these side chains in the active centres of enzymes; in casein however it is suggested that these catalytic agents may be so situated in the substrate itself that they render a particular bond more susceptible to enzyme action. Such an arrangement could also result in the susceptibility of this bond to high temperatures reported by Alais et al (1967).

In view of the correspondences outlined in the opening paragraph, it seemed possible that similar structures might exist around the sensitive bonds in other proteins. To test this possibility, bovine fibrinogen (3 mg/ml) was photo-oxidised with methylene blue as sensitizer in conditions similar to those used for κ-casein (Hill and Laing 1965). Following photo-oxidation, the methylene blue was removed and the control and photo-oxidised fibrinogens were treated with human thrombin (EC 3.4.4.13.) at 37°C. The control gelled after 3 m, while the fibrinogen that had been photo-oxidised to the extent of 10-12 moles uptake of O_2 per 10^5 g protein did not gel at all. To determine whether this effect was caused by an interference with the polymerisation of fibrin, or whether there was an inhibition of the primary attack by thrombin on the fibrinogen, samples similar to

the above were incubated with thrombin for 15 m. and were tested for the presence of the peptides normally released by thrombin. According to Osbahr et al (1964), these peptides contain a C-terminal arginine residue. The samples were therefore treated with 4% TCA to precipitate protein, and the protein-free supernatants were tested for arginine using the Sakaguchi test (MacPherson, 1946). The results of these tests, and also of a separate experiment in which the TCA soluble peptides were separated by gel chromatography on G25 Sephadex, showed that the amount of peptide released from the photo-oxidised fibrinogen was only 60% of that from the control. The photo-oxidation had therefore resulted in a loss of sensitivity of the fibrinogen to the primary attack by thrombin, even though the residues at the actual site of proteolysis (arginine-glycine) Laki et al (1960) are not themselves affected by photo-oxidation. According to the amino-acid sequences determined by Blombäck et al (1967), the photo-oxidisable residue nearest to the bond cleaved by thrombin is histidine. The behaviour of photo-oxidised fibrinogen is thus quite similar to that of photo-oxidised casein, and gives grounds for suggesting that not only may the phe-met bond in κ-casein be rendered sensitive to enzymic action by nearby catalytic side chains, but that similar structures may also exist around the sensitive bonds in other proteins.

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