Proteolytic enzymes as probes of the secondary structure of fibrous proteins

We have recently observed that the tryptic hydrolysis of myosin and the proteolysis of ichthyocol collagen by collagenase exhibit similar over-all kinetics. In these studies, peptide-bond cleavage was followed using a pH-stat, and, in part, a colorimetric ninhydrin method. Proteolysis was also followed by changes in optical rotation or non-protein nitrogen**. In both protein systems, kinetic analysis reveals the presence of two independent and parallel reactions, both first order in substrate (potentially cleavable bonds) concentration over several half-times. Under most conditions the rate constant for the fast reaction is about 10 times that for the slow reaction. Since both of these enzymes are highly specific[1,2], catalyzing the hydrolysis of only one or two types of peptide bonds, large differences in rate should not be expected to arise from *chemical* differences in the bonds attacked. The results suggest that in these two fibrous proteins, the proteolytic enzymes attack simultaneously and independently peptide bonds in the "ordered" and the "disordered" regions of the macromolecule, the rate of cleavage in the "disordered" or "amorphous" segments being about 10 times faster than in the "crystalline" or "folded" areas. This suggestion is given further support by temperature studies on myosin and gelatin. On raising the temperature of myosin solutions to 41° (which partially heat-denatures the molecule, as judged by changes in optical rotation), a significant fraction of the bonds split in the slow reaction are transferred to the fast reaction (Table I). At 37°, where ichthyocol gelatin approaches an open, randomly coiled structure[4,5], proteolysis follows simple first-order kinetics as measured in the pH-stat. On lowering the temperature, the kinetics become more complex as a second, slower enzymic reaction, also first-order in substrate concentration, makes its appearance concurrently with the onset of the large changes in optical rotation characteristic of the gelatin $\longrightarrow$ collagen-fold structural transition[6,7]. The fraction of the total bonds split by the slow reaction increases rapidly to 90% at 10°, indicating the extent of the folded collagen-type structure at this temperature (see Table I).

Activation energies (ΔE_a^*) derived from linear Arrhenius plots (log k versus 1/T) are presented in Table I. ΔE_a^* for the fast reaction in gelatin (about 15 kcal/mole) is

TABLE I

Protein	*Temp. range(°)*	*Bonds split (%)§*		*ΔE_a^* (kcal/mole)*	
		Fast reaction	*Slow reaction*	*Fast reaction*	*Slow reaction*
Collagen	10–20	35	65	40	50
Gelatin	10–20	Increases from 10 to 26	Decreases from 90 to 74		29
Gelatin	28–37	100	0	15	—
Myosin	6–25	21	79	11	13
Myosin	41	50	50		

§ A total of about 300 moles bonds/420,000 g protein are split in myosin; about 180 moles bonds/100,000 g protein in gelatin or collagen[2].

** SEIFTER, GALLOP AND MEILMAN[3] have demonstrated that the apparent kinetics of the collagen-collagenase reaction measured by viscosity differ markedly from those measured by optical rotation.

closely similar to values obtained in studies on the proteolysis of denatured proteins and synthetic substrates (1). In view of the structure of gelatin at 37°, this reaction may be considered as a model for the attack of a proteolytic enzyme on an unfolded polypeptide chain. On the other hand, the slow reaction which appears when the temperature is lowered into the range of the gelatin ⟶ collagen-fold transition, has a significantly larger ΔE_a^*, (about 29 kcal/mole). This value of ΔE_a^* should be further compared with the approx. 40 and 50 kcal/mole found in the "amorphous" and "crystalline" regions of the native collagen macromolecule. The difference between the values of ΔE_a^* found for the fast and the slow reactions when trypsin attacks myosin are much smaller than the differences found in collagen, and it will be interesting, in future studies, to see whether other systems containing the α-helix also show such small energy of activation differences between the "amorphous" and the "folded" regions.

It should be quite clear that in these studies we are measuring the distribution of *susceptible bonds* throughout the macromolecule. To the extent that these bonds are randomly distributed, the ratio of bonds cleaved in the fast and slow reaction should give a valid indication of the extent of "crystalline" structure. It seems that the use of proteolytic enzymes to thus distinguish "ordered" and "disordered" regions should allow one to establish the precise location of these regions. For example, the enzyme reaction could, in principle, be stopped at an early stage when cleavage is largely confined to the "amorphous" regions, the cleaved bonds could be labeled (*e.g.*, with dinitrofluorobenzene) and thus identified in terms of amino acid sequence. Moreover, it seems possible that structurally distinct regions in other fibrous systems (*e.g.*, the nucleic acids) might also be examined in this way.

Detailed discussions of both the myosin-trypsin and the collagen-gelatin-collagenase systems, correlating rates of peptide-bond cleavage with changes in optical rotation, viscosity, light-scattering, sedimentation and non-protein nitrogen, are in preparation.

The authors wish to thank Drs. PAUL GALLOP and SAM SEIFTER for the collagenase and the ichthyocol used in these studies, and for the many stimulating discussions.

National Heart Institute, National Institutes of Health, Bethesda, Md. (U.S.A.) WILLIAM F. HARRINGTON

Naval Medical Research Institute, Bethesda, Md. (U.S.A.) PETER H. VON HIPPEL*

National Heart Institute, National Institutes of Health, Bethesda, Md. (U.S.A.) ELEMER MIHALYI

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Received October 28th, 1958

* The opinions expressed in this article are those of the authors, and do not necessarily reflect the opinions of the Navy Department or the Naval Service at large. (Statement added in accordance with U.S. Navy regulations.)