SC 2168

Action of rennin on casein: Heat of reaction

The nature of the initiation of gel formation in cows' milk has been the subject of much study for a number of years. It has been suggested[1] that the hydrolysis of a peptide bond by the enzyme is initially responsible for the sequence of events that culminates in the formation of a gel, although experiment has indicated that only one bond is cleaved in casein per molecular weight of $12 \cdot 10^5$. Nevertheless the evidence for enzymic hydrolysis is in part substantiated by the finding that a non-protein nitrogen fraction is released prior to gel formation when rennin acts on casein[2]. Much of the non-protein nitrogen is obtained from a calcium-insensitive fraction, κ-casein, which forms about 15 % of the total casein in cows' milk and which is believed to be the major component concerned in casein micelle stabilization[3].

The enthalpy change, ΔH, accompanying the hydrolysis of peptide bonds in simple synthetic peptides has been found to be in the range -1240 to -2250 cal/mole[4]. Although similar data are not available for the hydrolysis of peptide bonds in native proteins, it may be assumed that the actual hydrolysis of such bonds involves reaction heats of a similar order of magnitude. However, in the case of proteins overall hydrolysis heats might well be expected to include significant contributions, either exo- or endothermic, from conformational changes and inter- or intramolecular interactions. Such effects have been observed[5] in the action of thrombin on fibrinogen; here a relatively large heat of reaction was attributed to intermolecular hydrogen bonding following the proteolysis of fibrinogen to fibrin monomer.

In spite of the uncertainty necessarily involved in the interpretation of apparent heats of hydrolysis of native proteins, it is of interest to determine the heat effect accompanying rennin action. An attempt to measure the heat effect in the action of rennin on milk was made by PHIPPS[6], but the sensitivity of his calorimetric apparatus was relatively low and he was unable to observe any significant heat changes. In this paper we report measurements on casein made with the same calorimetric apparatus[7] as used in previous experiments with synthetic peptides.

The substrates used were sodium caseinate in 0.04 M Tris buffer (pH 6.6) containing 0.1 M NaCl and 0.001 M merthiolate, or κ-casein, prepared by the method described by CHEESEMAN[8], in the same buffer, and also in 0.02 M phosphate buffer (pH 6.6) containing 0.1 M NaCl. Substrate concentration of 10–55 mg/ml were employed. In each experiment equal volumes of substrate solution and of a solution of α-rennin dissolved in the same buffers were mixed in the calorimeter after establishment of thermal equilibrium at $25.00 \pm 0.05°$. The α-rennin was obtained from peak A in the fractionation method described by JIRGENSONS *et al.*[9]. The amount of enzyme used, 0.5–1.0 μg/ml, was selected to give a convenient rate of reaction. The heat evolution followed apparent first-order kinetics to at least 75 % completion of the reaction. The rate of the reaction with κ-casein in phosphate buffer was also measured by micro-Kjeldahl determination of the release of non-protein nitrogen soluble in 2 % trichloroacetic acid.

The results obtained are summarized in Table I. As indicated in the table, the heat effects in cal/mole were calculated from the observed heat effects on the assumptions that the sodium caseinate and the κ-casein contained respectively, 15 and 90 % of active κ-casein, and that the molecular weight of κ-casein is 26000 (ref. 10). For unknown reasons, the reproducibility in these experiments was unusually poor, as

indicated by the large average deviations from the mean given in the table. It is seen that the rate of heat evolution in the case of κ-casein in phosphate buffer is approximately the same as the rate of release of non-protein nitrogen, suggesting that the two methods observe the same overall process. Although the heat quantities observed are of reasonable magnitude for a process involving the hydrolysis of one peptide bond, as explained above there is no way to ascertain that other processes do not make substantial thermal contributions.

TABLE I

HEAT OF REACTION AND APPARENT FIRST-ORDER RATE CONSTANTS FOR THE ACTION OF RENNIN ON CASEIN AT 25° (pH 6.6)

Protein	*Buffer*	*Number of determinations*	$-\Delta H$ *(cal/mg × 10³)*	$-\Delta H$ *(cal/mole)*	*Apparent first-order rate constant (min⁻¹)*	
					Calorimetry	*Non-protein nitrogen release*
Sodium caseinate	Tris	6	1.9 ± 0.5	3200 ± 900*	0.092 ± 0.024	—
κ-Casein	Tris	6	9.0 ± 4.0	2300 ± 1000**	0.15 ± 0.05	—
κ-Casein	Phosphate	8	6.7 ± 1.2	1800 ± 300**	0.11 ± 0.02	0.13 ± 0.05

* Calculated assuming 15% κ-casein, molecular weight of κ-casein = 26000.
** Calculated assuming 90% κ-casein, molecular weight of κ-casein = 26000.

It has recently been suggested by GARNIER *et al.*[11] that rennin specifically attacks in κ-casein an ester linkage which involves a carboxyl group in the paracasein portion and an hydroxyl group of a serine, threonine or carbohydrate residue in the glycomacropeptide. At the pH of our experiments the hydrolysis of an ester bond is expected to liberate a proton while that of a peptide bond is not. Titration of κ-casein at pH 6.6 shows that the protein has inadequate buffering power to absorb completely a liberated proton and hydrolysis experiments in unbuffered solutions failed to indicate a significant proton release. A further indication that there is no proton release is afforded by the fact that the heat effects observed for κ-casein are essentially the same in Tris and phosphate buffers, in spite of the much larger heat of ionization of Tris[12]. These considerations do not confirm the ester hypothesis.

This work was supported by grants from the National Institute of Health, U.S. Public Health Service (RG-4725) and the National Science Foundation (G-9625).

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Received August 14th, 1962

Biochim. Biophys. Acta, 69 (1963) 169–171

SC 2183

The action of *S*-methylisothiourea on corticotropin

α-Melanocyte-stimulating hormone isolated from mammalian pituitary glands has been shown to be an *N*-acetyl tridecapeptide[1] amide in which the sequence of amino acids is identical to that of the N-terminal tridecapeptide portion of corticotropin[2]. However, in spite of this remarkable similarity in chemical structure corticotropin possesses less than 1 % of the melanocyte-stimulating potency of α-MSH.

Although corticotropin consists of 39 amino acid residues, its melanocyte-stimulating activity is undoubtedly associated with the sequence of amino acids which it possesses in common with the melanocyte-stimulating hormones (α-MSH and β-MSH)[3,4]. Within this common sequence the most obvious chemical difference between the two hormone molecules is that the α-NH_2 group of the N-terminal serine occurs as the *N*-acetyl derivative in α-MSH. Thus in corticotropin the $\begin{matrix}CH_2\text{–}CH\text{–}\\ |\quad\quad |\\ OH\quad NH_2\end{matrix}$ structure which is known to be essential for ACTH activity[5] may at the same time have an inhibitory effect on its potential melanocyte-stimulating activity.

In order to investigate this possibility, it was necessary to achieve the selective acetylation of the α-NH_2 group of the N-terminal serine in corticotropin. A possible approach to this problem would be to protect the ε-NH_2 groups of its four lysine residues by reaction with *S*-methylisothiourea and then to acetylate the resulting guanidyl derivative with acetic anhydride. Earlier studies with chymotrypsinogen[6], RNAase[7] and growth hormone[8] had indicated that under strongly alkaline conditions (pH 10.5–11.0), *O*-methylisourea reacts selectively with ε-NH_2 groups of lysine residues, or δ-NH_2 groups of ornithine residues[9], and that α-NH_2 groups do not react to any significant extent under these conditions.

When corticotropin was allowed to react with *S*-methylisothiourea the resulting guanidyl derivative was found not to possess a free α-NH_2 group, indicating that both α- and ε-NH_2 groups had reacted with the reagent. Although this unexpected development was clearly inimical to the original purpose, it was decided to study the reaction and to investigate the chemical structure of the resulting product. In this communication evidence is presented to demonstrate that the free α- and ε-NH_2 groups had reacted.

Corticotropin A_1 (5 mg) was dissolved in 0.5 ml ammonium hydroxide (pH 10.5) and allowed to react with excess of *S*-methylisothiourea (25 mg) at room temperature for 48 h. The pH was maintained at this value by intermittent additions of small