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The reactivation of rennin inhibited by dansyl chloride

It has been reported by HILL AND LAING[1,2] that the reaction of crystalline calf rennin with a 2-fold molar excess of dansyl chloride at pH 8.0 and 5° for 4 h was accompanied by an 80% loss in rennet activity and by the incorporation of 1.5 moles of dansyl per mole of protein. After the dansylated protein had been degraded enzymatically at pH 9 and 50° for 16 h, a dansyl conjugate was isolated and characterized by thin-layer chromatography. Based on the comparison of the mobility of this compound with that of several synthetic dansyl derivatives, it was identified as ε-dansyl lysine. Consequently, the authors suggested that dansyl chloride inhibited rennet activity by coupling with either a specific lysine or histidine residue which was presumed necessary for enzymatic activity[2]. Since the experimental conditions were chosen to minimize the possibility of a transfer of the dansyl moiety, they concluded that a lysine residue, not histidine, was the initial site of the reaction. Although this conclusion has been generally accepted and widely quoted[3-5], additional information in the present report suggests that dansylation of a group other than lysine could be responsible for the loss in enzymatic activity.

Crystalline rennin, a generous gift from Chr. Hansens Laboratorium A/S, was treated with dansyl chloride, and the dansylated enzyme isolated by gel filtration by the method of HILL AND LAING[2]. The absorption spectrum of the dansylated protein was recorded, and a determination in duplicate of the amino acid composition was made after a 24-h acid hydrolysis in sealed tubes at 110°. An aliquot of the dansylated rennin solution was also treated with an equal volume of 4 M NH_2OH solution, pH 6.25, at room temperature for 1 h. After separation of the protein from the reagent by gel filtration, the proteolytic activity, absorption spectra and amino acid composition of the pooled protein fraction were determined.

The results of the reaction of rennin with dansyl chloride were similar to those reported by HILL AND LAING[2]; namely, a 70-75% loss in proteolytic activity and the incorporation of 1.40-1.43 moles of dansyl per mole of rennin (Table I). In addition, exposure of the dansylated protein to NH_2OH resulted in a pronounced increase in enzymatic activity and the liberation of about one mole of the bound dansyl per mole of enzyme. When polylysine, which had been dansylated to the extent of 2.1 moles per mole, was exposed to 2 M NH_2OH, no release of bound dansyl was detected either by ion-exchange chromatography using DEAE-Sephadex or by difference spectroscopy. Therefore, it seemed unlikely that a dansyl moiety bound to an ε-NH_2 group would be displaced by NH_2OH under these conditions.

Since ε-dansyl lysine is stable to acid hydrolysis[9], the reaction of dansyl chloride with a lysine residue in rennin should have resulted in a decrease of about one mole per mole of protein in the amount of lysine as determined by amino acid analysis. Although the decrease in lysine content of acid hydrolysates of dansyl polylysine was in accord with the amount of bound dansyl determined spectroscopically, the decrease observed in the case of dansyl rennin was only about one-fifth of that expected assuming that one lysine out of the 7.5 had reacted (Table I). This discrepancy between the spectroscopic and analytical data implies that ε-dansyl lysine was not the major product of the reaction of rennin with dansyl chloride.

TABLE I

THE PROPERTIES OF DANSYLATED RENNIN AND DANSYLATED POLYLYSINE

Estimations of activity were made using acid-denatured hemoglobin as substrate[6]. The enzyme concentration was established from the absorbance of solutions at 278 nm after correction for the absorbance of bound dansyl. Bound dansyl was determined from the absorbance at pH 6.25, of rennin solutions at 335 nm and polylysine solutions at 340 nm, assuming a molar extinction coefficient of $4.4 \cdot 10^3$ $M^{-1} \cdot cm^{-1}$ (ref. 7). The lysine content was obtained by amino acid analysis of duplicate samples. Values for native and dansylated rennin were based on a glutamic acid content of 28.9 moles per mole[8]. In the case of the polylysine sample, a 1.5 mM solution of polylysine hydrobromide of molecular weight 1500 was treated at pH 9.0 and 37° with a 2.5 molar excess of dansyl chloride in acetone. After completion of the reaction, the dansylated polylysine was isolated by DEAE-Sephadex chromatography, eluting with 0.01 M borate buffer, pH 9.0.

Sample	*Activity (% of control)*		*Bound Chromophore (moles/mole)*		*Lysine content (moles/mole)*		
	Without NH_2OH	*With NH_2OH*	*Without NH_2OH*	*With NH_2OH*	*Native*	*Dansylated*	*Difference*
1	25	78	1.43	0.22	7.50	7.40	0.10
2	31	80	1.40	0.66	7.50	7.20	0.30
Polylysine			2.10	2.10	7.00	4.90	2.10

The facile liberation of approximately 60% of the bound dansyl from dansyl rennin upon treatment with NH_2OH with the concomitant increase of about 2.5-fold in proteolytic activity suggests that the modification of a residue other than lysine was responsible for the loss in enzymatic activity but does not eliminate a possible involvement of a lysine residue in the catalytic mechanism. Although the residue which reacts with dansyl chloride has not, as yet, been isolated or characterized, it could well be the histidine, which was shown by HILL AND LAING[10] to be necessary for rennet activity. Dansyl histidine also meets the necessary requirement for lability since it has been reported to be unstable below pH 4 and above pH 6 (ref. 9). Such a labile adduct could also account for the 18% increase in rennet activity which was observed after several weeks storage of dansylated rennin[2].

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