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Specific reaction of dansyl chloride with one lysine residue in rennin

Recently we have shown that rennin (EC 3.4.4.3) may be inactivated by photo-oxidation with methylene blue and concluded that this effect is associated with the destruction of histidine[1], which may therefore be part of the active centre of the enzyme. Rennin is not inactivated by diisopropylfluorophosphate (DFP) (refs. 2, 3) nor by toluene sulphonyl chloride[4], from which it could be inferred[5,6] that rennin does not possess an enzymatically active serine residue. We found, however, that rennin could be inactivated by dimethylaminonaphthalene sulphonyl chloride (dansyl chloride) and further that this was probably caused by modification of a single residue on the rennin. A preliminary report of this work was published earlier[4]. This paper contains a more detailed account, including evidence that the residue concerned is lysine.

The rennin used in these experiments was prepared from Benger's powdered rennet by three-fold precipitation with saturated NaCl at pH 5.4, followed by chromatography on DEAE-cellulose in 0.1 M sodium acetate at pH 5.7 and 3° in a linear gradient of 0–1 M NaCl (*cf.* ref. 7). The purified rennin had an activity of 58 000 rennin units per g, one unit being the activity required to clot 10 ml of a standard substrate[8] in 1 min at 30°. 12 ml of solution containing 24 mg rennin, approximately 0.4 M in NaCl and 0.025 M phosphate buffer at pH 8 and 5° was treated with dansyl chloride in acetone (6.5 mg/ml). To a control solution acetone only was added. The solutions were stirred and at intervals 0.1 ml of each was withdrawn and assayed for rennin activity. When the inactivation was sufficient the samples were adjusted to pH 6.25 and the protein separated from reagents by chromatography on Sephadex G-25 in 0.03 M sodium acetate at pH 6.25. Portions of each eluted solution were adjusted to the same rennin concentration (0.25 mg/ml) and the amount of dansyl bound to the rennin was estimated by ultraviolet difference spectroscopy, assuming that the absorption of the bound dansyl was the same as that of dansyl chloride in isopropanol (ε = 16 000 at 245 mμ). The remainder of the eluted protein was used in the chromatographic tests described later.

The results in Table I show that treatment of the rennin with relatively small amounts of dansyl chloride at pH 8 and 5° causes considerable inactivation. Controls were stable in these conditions. In 4 experiments (Preps. 3–6) the extents of inactivation and of reaction with dansyl chloride are in good agreement, which would be the case if the dansyl chloride reacted solely with residues essential for enzyme action. When the amount of dansyl chloride was increased the correspondence between extent of inactivation and uptake of dansyl was not as close, suggesting that in these conditions it had also reacted with unessential residues (Preps. 6–8). Therefore, in preparing samples for tests to determine the nature of the critical residues, the conditions of Preps. 3–6 were used.

When the treated rennin was kept at pH 6.25 and 3° for 1–3 weeks, increases in activity of 14–18% were observed. Rennin was not inactivated by treatment with dansyl chloride at pH 6.5, nor by fluorodinitrobenzene at pH 6.5 although slow inactivation was caused at pH's of 7.5 and 8.2 by a 60-fold molar excess of 1-fluoro-2,4-dinitrobenzene (FDNB).

TABLE I

REACTION OF RENNIN WITH DANSYL CHLORIDE AT 5°

Prepn. Number	*pH*	*Time (h, min)*	*Moles dansyl/mole enzyme*		*Inactivation (%)*
			added	*bound*	
1	8.2	4.10	0.95	–*	53
2	8.0	3.45	1.5	0.65	71
3	8.0	3.45	1.5	0.69	70
4	8.0	3,30	1.5	0.66	69
5	8.0	2,20	2.1	0.73	73
6	8.0	3,50	2.1	1.40	84
7	8.0	3,30	3.1	0.93	74
8	8.2	2,55	5.0	1.2	62

* Not determined.

For identification of the dansylated residue, 8 mg of dansyl-treated rennin was hydrolysed with 0.08 mg pronase at pH 9 and 40° for 16 h, and the solution freeze-dried. Fluorescent material was isolated by chromatography in butanol–acetic acid–water (4:1:1, by vol.) on a small silica gel column. When analysed by thin-layer chromatography on silica gel in the same solvent system, the greater part of the fluorescence (and in two cases all of it) occurred in a spot which was also ninhydrin-positive. This material showed the same R_F (0.34) as ε-dansyl lysine* on 20-cm thin-layer chromatography slides, and when the unknown and ε-dansyl lysine were chromatographed together they ran as a single spot also of R_F 0.34. Acid hydrolysis of the unknown (6 N HCl, 110°, 16 h) did not alter this result.

Some caution is required in identifying a lysine side chain as the primary site of reaction, as it is possible that the dansyl chloride reacts first with another residue (*e.g.*, imidazole) and is transferred to the lysine during analysis. We tried to minimise this possibility by hydrolysing the rennin with pronase, rather than with HCl at elevated temperatures and by separating the fluorescent material in the hydrolysate as quickly as possible. It is also possible that the loss of activity on reaction with dansyl chloride is associated with a change of conformation or a masking effect of the bulky dansyl substituent, but the fact that the dansyl chloride-treated rennin slowly regains some activity (*cf.* DFP-inactivated cholinesterase[11]) suggests that reaction occurs initially at an enzymatically active site. Dansyl chloride has been shown to inactivate chymotrypsin by reacting with serine[12]. For rennin, however, the lack of reaction with DFP (refs. 2, 3) and tosyl chloride[4] and the fact that it is inactivated by FDNB are in accord with the chromatographic evidence that the residue affected is a lysine side chain.

Lysine is involved in the action of a number of enzymes such as ribonuclease[13], aldolases[14] and glyceraldehyde-3-phosphate dehydrogenase[15]. In aldolases its function is the binding of the enzyme to the substrate through an azo-methene bond[14]. The

* Four amino acid derivatives with dansylated side chains were prepared using the copper complex technique[9] (tyrosine and lysine) or *o*-nitrophenyl sulphenyl chloride[10] (histidine and serine) to protect the α-NH_2 groups. The desired derivatives were separated from side products by chromatography on silica gel in butanol–acetic acid–water (4:1:1, by vol.).

relatively great reactivity toward dansyl chloride of the lysine residue in rennin suggests an alternative function, *viz.*, that it may be part of a positively charged site which binds to an anionic part of the substrate, as proposed for lysine in ribonuclease[16]. More definite knowledge on this point may be gained from a study of the amino-acid sequence near the dansylated lysine.

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Purification of two alkaline phosphatases from *Aspergillus nidulans*

It has been shown previously that under conditions of limiting phosphate, *Aspergillus nidulans* produces two electrophoretically distinct alkaline phosphatases (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1). The activity of phosphatase I is under the control of at least nine genetic loci whereas no mutations have ever been isolated which affect the activity of phosphatase II (refs. 1,2). Kinetic growth studies have suggested that the synthesis of phosphatase I is repressible by high levels of inorganic phosphate while phosphatase II appears to be a constitutive enzyme[3]. The genetic and growth studies indicate strongly that the two phosphatases are distinct enzymes; however, very little is known about the biochemical nature and physical structure of these enzymes. This paper describes a procedure for chemically separating the two phosphatases. Electrophoretically homogeneous preparations of phosphatase I have been obtained. Evidence is presented that the two enzymes have different pH optima and molecular weights.

Phosphatase activity was measured by observing the absorbance change at