CHEMICAL MODIFICATION OF ONE CARBOXYL-GROUP OF PAPAIN ABOLISHES THE CATALYTIC ACTIVITY OF THE ENZYME

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1. Introduction

The participation of a carboxyl-group in the catalytic mechanism of papain was postulated for the first time by Smith in 1954 [1]. The papain mechanism proposed by Smith was based mainly on kinetic data. Later investigators have refused the function of a carboxyl-group in the catalytic process and have given experimental evidence for the implication of an imidazole residue in the catalytic cycle [2,3]; this concept seemed to be supported by the results of X-ray analysis of the enzyme [3]. Nevertheless there are some objections to the involvement of an imidazole residue in the catalytic process concerning especially the pK-values [4,5].

The pH-profile of the catalysis has led to the postulation that the apparent pK of the respective imidazolyl group must be abnormally high, being as high as 9.8–10 [3] or as low as 4 [2]. Our modification experiments of the enzyme with diazo-1-H-tetrazole [6] as well as the results of photo-oxidation [4] do not give any indications on an imidazolyl-group with an abnormal pK-value. We therefore have undertaken a renewed investigation of the role of the carboxyl-groups of papain using chemical modification with a nucleophil and carbodiimid [7]. In the present communication we describe the results of these experiments which demonstrate the loss of catalytic activity after chemical modification of only one carboxyl-group of the enzyme.

2. Materials and methods

Papain (30 IU/mg) was a gift of Boehringer, Mannheim. [1-^{14}C]Glycinethylester (GEE) (spec. activ. 8.8 μCi/mM) and Aquasol were obtained from NEN Chemicals, Frankfurt. *N*-Ethyl-*N'*-(3-dimethyl-amino)-propyl-carbodiimide (EDC), α*N*-benzoyl-DL-arginin-4-nitroanilid (BAPA), buffer substances and other chemicals were purchased from Merck, Darmstadt. Benzoyl-glycin-ethylester (BGEE) was prepared following loc. cit. [8].

Radioactivity was measured by liquid scintillation counting in a Packard TRI CARB Model 3003.

Details of the modification experiments are given in the legend of fig. 1. The reaction was stopped in definite time intervals by mixing with 1 ml 2 M acetate buffer pH 4.5 and dialyzed against distilled water. One ml of the dialyzed probe was mixed with 15 ml Aquasol and counted in a Tri Carb. Extend of modification was calculated from protein concentra-

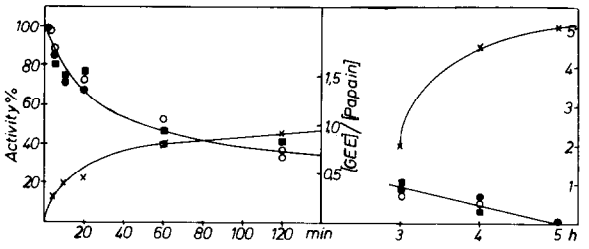

Fig. 1. Time dependence of the modification of the carboxyl-groups of papain with EDC/[1-^{14}C]glycin-ethylester. Reaction conditions: 20 mg enzyme in 10 ml 5.5 M urea and 0.23 M [^{14}C]glycin-ethylester were mixed with 1 g EDC (solid). EDC-concentration at the start of the reaction was 0.12 M. After 2 hr additional 1.5 g EDC were added to the reaction mixture. (X——X) extend of modification, (●——●) activity with DL-BAPA; (○——○) with BGEE; (■——■) with casein as substrate.

tion, radioactivity measurements and spec. activ. of the nucleophil. Papain concentrations were determined by absorption measurements at 280 nm using a molar extinction coefficient of 58 500 ($M^{-1} \times cm^{-1}$).

3. Results and discussion

Chemical modification of the carboxyl-groups of proteins is most conveniently performed by reaction with labelled glycin-ethylester or -amide and a water soluble carbodiimide [6]. We have applied this reaction to papain to gain further information on the function of a carboxyl-group in the catalytic process of the enzyme. The time dependence of the extent of modification and the parallel loss of activity during incubation of papain with glycinethylester and the water-soluble carbodiimide EDC is shown in fig. 1. After a reaction time of 60 min one gets a decrease in activity of about 60% following an introduction of 0.8 glycin-ethylester pro mol papain.

A better insight into the relation between extend of modification and loss of activity gives fig. 2. From this figure it can be seen, that a linear relation exists between the remaining activity and the rate of modification. Extrapolation of the linear plot to zero activity shows that modification of about 1.5 carboxyl-groups out of 13 leads to a complete inactivation of the enzyme.

Because of the low solubility of papain the modification reaction was performed in 5.5 M urea; in urea free solution papain could not be dissolved in the required concentration. As was shown by Sluyterman [9] urea is without any effect on the conformation of the enzyme. The apparent diminution of the catalytic activity in 8 M urea is only caused by impairment of the binding of the substrate as is shown in fig. 3. V_{max} is not influenced even by high concentrations of urea.

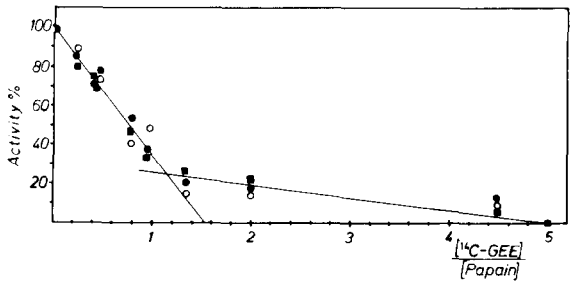

Fig. 2. Relation between activity and extend of modification. For further details see fig. 1.

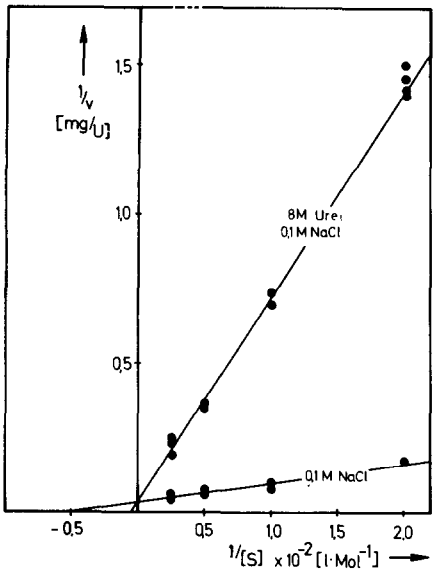

Fig. 3. Kinetics of papein in the presence and without urea at pH 6.5 and 25°C; substrate benzoylarginin-ethylester, $K_m = 1.8 \times 10^{-2}$ M; K_m with urea 0.2 M.

Our experiments demonstrate that urea does also not level the reactivity of the carboxyl-groups of the enzyme, as is often observed as a consequence of impairment of the active conformation of proteins. Even in 5 M urea one carboxyl-group from 13 present in the enzyme exhibits a preferential reactivity with carbodiimide. Blocking of this one residue completely abolishes the catalytic activity.

We assume, that this is the carboxyl-group of the aspartic acid 158, which has a distance from the essential SH group of 7.5 Å [3]. This carboxyl-group has been proposed to be responsible for the preferential binding of basic substrates by papain [10] or to promote the removal of the acyl-moiety of the substrate which contains an anionic carboxylate group [11]. There are two objections against this function of the carboxyl-group in the active center; first of all the course of inactivation of the enzyme is equal with neutral (BGEE) and with basic (DL-BAPA) substrates; this should not be the case if the carboxyl-group has something to do with an electrostatic attraction of the

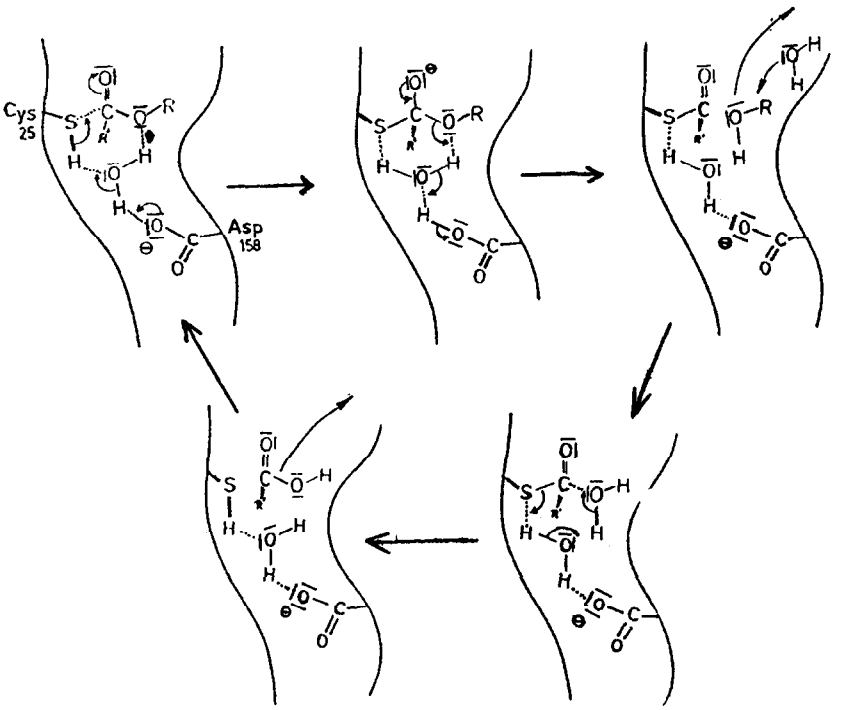

Fig. 4. Proposal for a catalytic mechanism of papain involving a carboxylgroup.

basic substrate; secondly the exclusive function of the carboxyl-group in promoting the removal of the product of deacylation would not require a negative group with exceptional reactivity.

We therefore suggest, that the respective carboxyl-group participates in the catalytic process. The distance between the essential SH-group and the carboxyl-group may be bridged by a water molecule. The bond breaking and bond forming steps in the catalytic cycle can be formulated as is shown in fig. 4.

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